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Scenedesmus obliquus and *Chlorella vulgaris* – A Prospective Algal Fuel Source

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ABSTRACT

In recent years, the prospective use of algae as an alternate fuel source for petroleum-based fuels has increased drastically. It has been researched extensively and proven that it can be used as a sustainable feedstock for producing green energy considering environmental safety. This article focused on the economically viable algal feedstock for the production of lipid content for its use as a feedstock for biodiesel production. For this purpose, the algal species *Scenedesmus obliquus* and *Chlorella vulgaris* were selected, and it was grown under lab and open ambient conditions with two Blue green Medium (BG-11) and Bold Basal medium (BBM). Upon the yield, it was noticed that the BG-11 medium gave optimum lipid yield for both species. Hence, it was determined that through this medium higher lipid yield can be expected, and based on the GC-MS result it was notified that it can be a viable source of alternate fuel.

INTRODUCTION

Fuel price increase in India demands the presence of an alternate fuel that can neutralize the burden of a common person in terms of economy. Not just the economy but also global warming which increases the greenhouse gas (GHG) emissions in the atmosphere. This is of greater concern considering the environmental conditions (Hariram et al. 2017a, 2017b).

Moreover, in India, industrialization increases due to economic growth, and the population also increases day by day, both influences the annual emission of carbon dioxide which acts as a major emission source in global warming. For decades, researchers have been working on biodiesel that can be produced from algae which actively supports its use in terms of performance and emission characteristics through better combustion (Godwin et al. 2017).

Species of blue-green and green algae were isolated and the selected strain chlorella vulgaris under certain optimized conditions was cultured to produce the biomass. Biomass was then converted to oils and its calorific value was determined to possess closer fuel values. This shows that this energy source can be utilized as an alternate form while it also mitigates greenhouse gases (Ghayal et al. 2013). Algae-based biofuel showed great potential in replacing petroleum-based fuels. One of the greater influences was that its carbon-neutral character helps in environment-cleaning applications. The focus was made to have a bio-refinery feedstock which was found to be economically possible (Trivedi et al. 2015). Microalgal refinery design can be made with certain optimal production conditions. *Scenedesmus obliquus* was taken as the base model organism and the research detailed that a good refinery can be made with optimal development like nutrient conditions so that the biomass can be improved. From 100 grams of *Scenedesmus obliquus* biomass, the following products were yielded namely, 0.06 gram of βcarotene, 38 grams of biodiesel, 2 grams of omega-3 fatty acids, 3 grams of glycerol and 17 grams of ethanol (Reeza et al. 2015). The research study was done to develop the *Scenedesmus obliquus* in various conditions for lipid and biomass production. The study was to implement the application also for wastewater treatment. The mixotrophic condition was found suitable for this application. It was noted that the palmitoleic acid-rich *Scenedesmus obliquus* strain developed in mixotrophic conditions can be utilized for wastewater treatment along with good algal biomass production and better algal oil yield (Yanan et al. 2021). The research was carried out to develop Levulinic acid from microalgae wherein methyl-sulphonic acid mediated conversion method was utilized. The study proved that the microalgae Scenedesmus obliquus was a promising feedstock (Gwi-Taek & Sung-Koo 2021). Researchers did a study on the development of strategies involving Mixotrophic cultivation to improve biomass production in freshwater microalga *Scenedesmus obliquus* by using sodium acetate in an appropriate concentration. Both autotrophic and mixotrophic lab scale study was done in which mixotrophic condition favored the situation. Also, the sodium acetate used should have an appropriate concentration to enhance the photosynthetic reaction and for better biomass production (Jie et al. 2021). From the microalgae *S. obliquus*, the production of bioethanol and biodiesel was studied. During this study, the growth was incorporated with the waste glycerol taken from biodiesel production as a nutrient supplement. It was also noticed that increased bioethanol occurred after sequential fermentation after lipid extraction (Shannan et al. 2019). An energy study was carried out by authors wherein the centrifugation and flocculation methods were undergone to compare the yield. Lipid recovery was found to be 17.4% for centrifugation and 20.7% for flocculation, but the bio-oil yield was a little lesser. The energy output in biodiesel production from centrifugation and flocculation was 0.87 and 0.68 GJ per ton. From the comparison, it was concluded that flocculation would be the optimized method for biodiesel production (Shuang et al. 2019). A study on harvesting and drying methods was done for the microalgae *Scenedesmus obliquus*. The method for harvesting was flocculation and centrifugation, while freeze drying, freezing, drying, and hot-oven dehydrating were incorporated for testing. It was determined that for polyunsaturated acid yield freeze drying and biodiesel production from biomass freezing would be more appropriate. Moreover, it was found that centrifugation was the best method (Carlos et al. 2020). Among various Scenedesmus species, it was also determined that S. Obliquus yielded with highest biomass productivity. It was also noted that the Cetane number and viscous value of *S.obliquus* FAME met the international standard (Mostafa et al. 2018).

From these studies, it was noted that *Chlorella vulgaris* and *Scenedesmus obliquus* showed promising results. Henceforth, an analysis was undergone to study both these microalgae for potential fuel sources.

Scope of Both the Species

Chlorella vulgaris acts as an encouraging bioenergy source. It is viewed as a good substitute comparing the current biodiesel crops like corn, rapeseed, or soybean, as it supplements increased productivity and is not competing with edible food crops. It can yield enormous quantities of bio-lipids up to 15 times greater than the feedstocks benchmarked for biodiesel production. The literature revealed that *Chlorella vulgaris* also contains a notable quantity of starch which could be a value-added feedstock for bio-ethanol production. However, biofuel yielded from microalgae has a competitive gap with conventional fossil fuel due to its controversial sustainability and higher production cost.

Bio-Oil Extraction

Although Scenedesmus algal family can produce numerous varieties of bioenergy such as biodiesel, bioethanol, bio-hydrogen, and drop-in fuels, all-encompassing investigations have been carried out towards the production of biodiesel. Similar to the algal bioenergy ecosystem, operational challenges in the cohesive biodiesel production from the Scenedesmus family are encountered with implementation commercially. A few of the key encounters include gas and fluid transfer, recycling, nutrient supply, cultural integrity, PAR (Photosynthetically Active Radiation) delivery, water-land availability, environmental control, and genetic and metabolic engineering harvesting (Godwin et al. 2018).

Bio-Diesel Production

Literature confirms that the Scenedesmus microalga family is capable of producing a notable quantity of lipids and a higher concentration of biomass when compared with other blue-green and green algae with a limited supply of micro and macronutrients. This was also showcased by many researchers in the production of biodiesel. The Scenedesmus microalga family has proved to yield increased lipid and biomass concentration at optimized environmental conditions in heterotrophic surroundings than in autotrophic circumstances. Variable and optimized supply of micro-macro nutrients in nitrogen-carbon-di-oxide deficient conditions enhanced the production of biomass and lipids significantly. Recent studies reported that Scenedesmus retain maximum lipid and biomass at its complete growth cycle at around 62% in dry cell weight. Assimilation of carbon-di-oxide was also found to be better in Scenedesmus algae in comparison with other blue-green algae. The growth environment with nitrogen deficit conditions promoted the concentration of lipids significantly. Despite notable lipid content, its separation using chemical methods with alcohols and its recovery is a challenging factor as its increases the production cost greatly.

Bioethanol

Chlorella vulgaris and *Scenedesmus obliquus* were found to possess a higher concentration of carbohydrates (more than

47% by dry weight) making them competitive feedstock in the production of bio-ethanol. An investigation reported that *Scenedesmus obliquus* at nitrate deficient condition produced higher biomass and lipid with a notable increase in carbohydrates. Hydrolysis of carbohydrates with sulphuric acid underwent a fermentation reaction for four hours yielding 8.45 grams of bio-ethanol in the ratio of 0.214-gram ethanol per gram of biomass.

Wastewater Management

Scenedesmus obliquus and *Chlorella vulgaris* were also found to be very effective in removing the phosphorus and ammonia content from the waste waters of the agro-industrial sector. *Scenedesmus obliquus* reacted with the wastewater effectively for the removal of ammonia in a cylindrical bio-reactor whereas phosphorous removal was found to be similar with bot both species. In the presence of Algal Turf Scrubber (ATS), *Scenedesmus obliquus* and *Chlorella vulgaris* were very active and operative in the removal of phosphorus and ammonia from the polluted waters. Literature reported that the metallic phosphorous removal cost was brought down to 24$ from 77$ when the system is employed with *Scenedesmus obliquus*. On the other hand, the substrates used of *Scenedesmus obliquus* growth could also be used as an organic fertilizer, cattle feed, and paper industry as a value-added feedstock.

The above-mentioned are all the scope and needs which can be fulfilled by using these two algal species. In this article, the lipid extraction from these two species will be studied for the effective usage of these lipids as fuel sources.

MATERIALS AND METHODS

Chlorella Vulgaris

Chlorella vulgaris, under the classification of genus *Chlorella*, is a green eukaryotic microalga. The species name was derived from the Greek-Latin language, Chloros (□□□□ó□) referring to its size which is a microscopic – single-celled organism in nature. Martinus Willam Beijerinck discovered *Chlorella vulgaris* in 1890 possessing a prominent nucleus. In the early 1990s, German researchers identified a spike protein in *Chlorella vulgaris* which make it suitable for edible purpose also. Due to its enhanced medicinal value, protein-rich content, and enriched dietary supplements, Japan consumes it largely across the globe. *Chlorella vulgaris* is a eukaryotic, unicellular green alga existed on the earth for more than 2.4 billion years. Its natural higher protein level and higher mineral content transformed it into a major multivitamin supplement and hydration enhancer. Nowadays, due to its significant concentration in lipids under certain growth environments, it played a vital role in biodiesel production. It was estimated that *C. vulgaris* possess lipid at about 42.5% which was 22% greater than sugarcane, corn, or soybean making it a viable and promising feedstock for biodiesel production. Fig. 1 shows the microscopic view of *Chlorella vulgaris*.

Scenedesmus Obliquus

Scenedesmus obliquus is a flat plate, unicellular green algae belonging to the genus of Chlorophyceae. It a

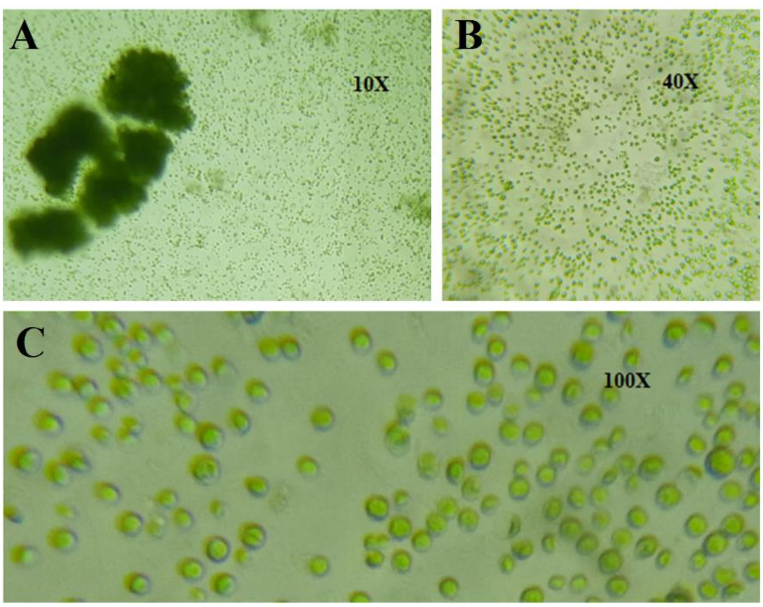

Fig. 1: Microscopic view of *Chlorella vulgaris* in various lenses at 10X (A), 40X (B), and 100X (C).

microscopically small, colonial in nature, and non-motile. The microscopic view distinguishes the chloroplast and a pyrenoid which is mono-parietal visible. Its view under a scanning electron microscope at 10X magnification released the cell wall membrane is not flat but with reticulations and bumps. Vast studies were reported on *Scenedesmus obliquus* with emphasis on potential nutrient value. Recent literature showcased it to be a promising aquatic organism with produces variable proportions of carbohydrates and lipids enhancing its value addition. The latest technologies transform and promote the usefulness of *Scenedesmus obliquus* more towards bioenergy-producing fuel feedstocks and supporting green energy. Numerous investigations were carried out in NREL (National Renewable Energy Laboratory, India) to understand its oil production capacity and it was evidenced that it could yield 19 to 21 times more bio-oil than oil-producing crops.

This investigational study focuses on the oil production capability of *Scenedesmus obliquus* on a laboratory scale with optimized environmental conditions. The advantages of using *Scenedesmus obliquus* are easy accessibility, eco-friendly nature, and renewability. The algal reproduction was through the conventional photosynthesis process providing luminescence and a pre-determined quantity of micro-macro nutrients along with carbon-di-oxide. Upon achieving exponential growth upto 10^4 to 10^6 cells.cm^{-1}, the harvesting of biomass was carried out. Centrifugation and soxhlet extraction process expelled the lipids from *Scenedesmus obliquus*. The microscopic view of *Scenedesmus Obliquus* is shown in Fig. 2.

Morphology of *Chlorella Vulgaris* and *Scenedesmus obliquus*

Table 1 signifies the algal class and division of *Scenedesmus obliquus* and *Chlorella vulgaris*. *Chlorella vulgaris*, is a spherical and small unicellular green algae sizing between 5 and 10 µm. is small, spherical algae that have a size of 5-10µm. The microscopic structure of *Chlorella vulgaris* showed mixtures of 16 chromosomes between 0.97 Mb and 3.8 Mb which may be due to the isolated geographical locations of the strains. *Scenedesmus obliquus* was microscopically seen with spikes and cell patterns. A few cells were also seen with pointed ends which lack spikes. Few cells were also arranged in colonies with the oblong-ovate arrangement and even the absence of spikes. The abundance of unicellular *Scenedesmus obliquus* was identified in rows with the presence of spikes

Table 1: Micro algae division information.

Parameters	*Chlorella vulgaris*	*Scenedesmus obliquus*
Hierarchy	Taxonomic (Beijerinck 1890) & (IT IS – Taxonomic Serial No: 5815)	Taxonomic: (Turpin) (Kützing 1833)
Kingdom	Plantae	Plantae
Sub-kingdom	Viridiplantae	Viridiplantae
Division	Cholorphyta	Cholorphyta
Subdivision	Cholorphytina	Cholorphytina
Class	Trebouxiophyceae	Chlorophyceae
Genus	Chlorella	Scenedesmus
Family	Oocystaceae	Scenedesmaceae
Order	Chlorellales	Sphaeropleales

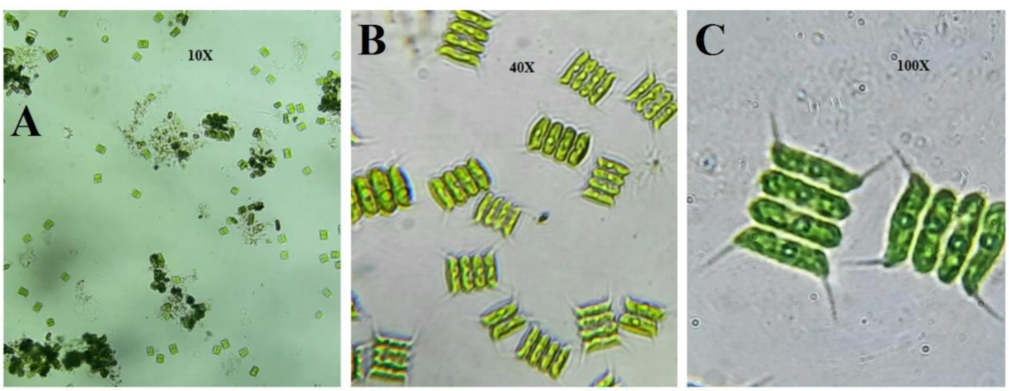

Fig. 2: Microscopic view of *Scenedesmus obliquus* at 10X (A), 40X (B), and 100X (C).

between 3 and 33 with attachments in the outer periphery of the cell wall membrane. The morphologies of *Scenedesmus obliquus* showcased unicellular and multicellular cells influenced by surrounding abiotic and biotic factors which will affect its growth significantly.

Cell Wall

Chlorella vulgaris is composed of carbohydrates inhibiting cellulose up to 82% in its cell wall membrane. As a result, it was very rigid and integrated with the other surrounding membranes thus serving as a protecting layer. *Scenedesmus obliquus* was seen as unicellular and multicellular as well and found in colonies with 2 to 8 cells covered with the parental cell wall membrane. The concentration of unicellular organisms was found to be higher in the amalgamated medium but proper illumination and elevated surrounding temperature pronounced unicellular growth significantly in a non-colonial manner.

Reproduction

Chlorella vulgaris assimilates natural sunlight through the chloroplast along with a minimal quantity of micro and macro nutrient for its reproduction. Further, it in-digests carbon-di-oxide to perform the process of photosynthesis in fresh clean water in an aseptic condition. As the strains of *Chlorella vulgaris* start multiplying on the water surface, it blocks the natural sunlight from the top surface thereby deteriorating the growth of *Chlorella vulgaris* and eventually dying underneath. This could be avoided by supplying proper aeration and artificial luminance for their growth.

Light Conditions

Since *Chlorella vulgaris* and *Scenedesmus obliquus* are autotrophic, the photosynthetic process gains importance greatly as it promotes algal growth. In the open pond culture system, the biomass production cost was brought down significantly due to the availability of natural sunlight. On the other hand, open pond cultivation deteriorates the algal growth in a few instances of lack of proper care, the mother algae become a parasite to the other living organism which could affect the growth of *Chlorella vulgaris* and *Scenedesmus obliquus* largely.

Natural sunlight compresses numerous components based on its wavelength, visibility, x-ray, gamma ray, and others. The photosynthesis process uses only natural sunlight between the wavelength of 370 nm and 740 nm for promoting algal growth. Filtering the light rays from the amalgamation was difficult and also leads to a negative impact on the algal biomass concentration. The climatic condition in the area of the open pond system selected for the algal growth also plays a vital role in its growth.

Growth Medium

The two main media used in the biomass cultivation of, *Chlorella vulgaris* are Bold's Basal medium BG11. These two media are often used which successfully supports growth without denaturing the cells and there nurture the cells by supplying them with essential nutrients and other compounds. The vital role of the medium is to support the growth of the cells but here the medium not only supports the growth of the cells but also nurtures them and helps to keep the constituents and compounds present together without any distortion. Each medium will have a composition different from one another which marks the uniqueness of each medium. Thus, the medium used here is unique as they contribute to the growth of the microalgae as well as supports it by maintaining the micro algal cells throughout their growth without causing damage to the cells and their components.

RESULTS AND DISCUSSION

In this section, the methods for optimization of the algal growth towards lipid production are detailed for both the species *Chlorella vulgaris* and *Scenedesmus obliquus*.

Microalgal Cultivation

To optimize the process, both the microalgae species were inoculated to two different mediums (BBM & BG-11) after collecting 3ml of each species sample, later which was cultured in a plant tissue culture lab and made up to 250 mL of each species in a sterile conical flask and maintained in a different environment for the optimization process. The Bold Basal Medium (BBM) and Blue-Green medium (BG-11) were used to cultivate microalgae of two different species in different environments to analyze the growth of algae and production of lipids, and toidentify the best medium for the fast growth of algae and high lipid production.

Ambient Condition Control

Three different types of environmental temperatures (16°C, 25°C, and 32°C) were selected and cultured for both the microalgae species to identify a suitable environmental temperature to get a high specific growth rate and biomass productivity of algae.

At 16°C, the sample was placed in a plant tissue culture lab under a fixed artificial light provided by a fluorescent tube and the effects of light on the growth of the algae were calculated by the total cell count. At 25°C, the sample was

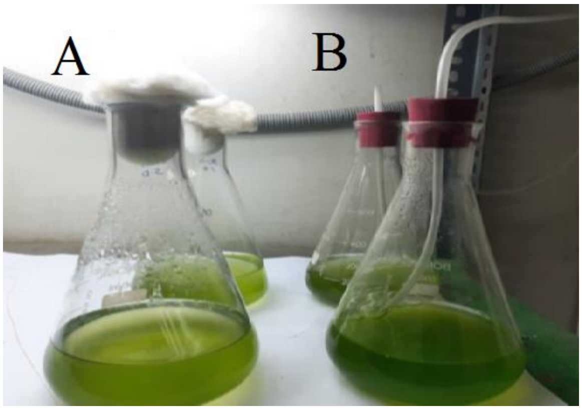

Fig. 3: Non-aerated (A) and aerated culture (B).

placed in a window side of the lab receiving partial sunlight and at room temperature with manual agitates for every one-hour interval gap to enhance the growth and mixing of the medium and culture.

At 32°C, the sample was placed in a window side of the lab receiving partial sunlight and at room temperature with manual agitates for every one-hour interval gap to enhance the growth and mixing of the medium and culture. In the culture lab, the sample was also tested with aeration, and without aeration, aeration was supplied through an aerating pump. The flow rate of the pump was 3L.min^{-1}, which mixes the medium and sample for 24 h and enhances the growth as shown in Fig. 3. The growth results were then compared with the non-aerated. For non-aeration, the supply was added to the culture medium, it was cultured in a plant tissue culture lab and agitated manually for a 1h interval gap to avoid the sedimentation of the culture and to enhance the mixing of the culture with the medium. The aeration can be seen as shown in Fig. 3.

Chlorella Vulgaris **and** ***Scenedesmus obliquus*** **Morphology**

The morphological studies of the two microalgae species *Chlorella vulgaris* and *Scenedesmus obliquus* were performed to observe and study the growth of the cells from the young stage to the mature stage. Hence, microscopic studies were done on the 7th day after inoculation of the sample into the medium and it was carried out for one month with seven days of interval gap. The sample for the morphological studies is collected from different environments maintained for the optimization process. The growth rate is detailed in the form of a graphical representation shown below in Figs. 4, 5, 6, 7, 8 and 9.

The growth determination of samples 1 (BBM) & 2 (BG-11) of microalgae *Chlorella vulgaris* and samples 1 (BBM) & 2 (BG-11) of microalgae *Scenedesmus obliquus* were performed by taking the growth density readings with the help of colorimeter at three respective nanometers (620 nm, 680 nm & 700 nm) at the different time intervals (9 am, 12 pm & 3 pm). The maximum growth of the two microalgae species was observed at the absorbance of 620nm. The total cell count of the microalgae was studied to identify the growth rate of algae in the interval gap of seven days for four weeks where the hemocytometer was used for the total cell count. The result of the phytochemical screening is shown below in Table 2.

The preliminary phytochemical screening of the microalgae of *Chlorella vulgaris* and *Scenedesmus obliquus* showed the methanolic extracts containing alkaloids, amino acids and proteins, carbohydrates, fixed oils and fats, and phenols and tannins. The phytochemical screening demonstrated the presence of different types of phytocompounds which could be responsible for the various pharmacological &

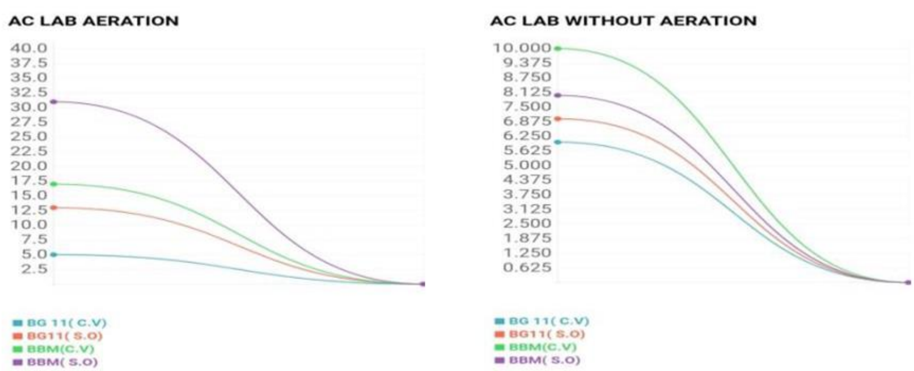

Fig. 4: Growth rate study with and without aeration at 620 nm.

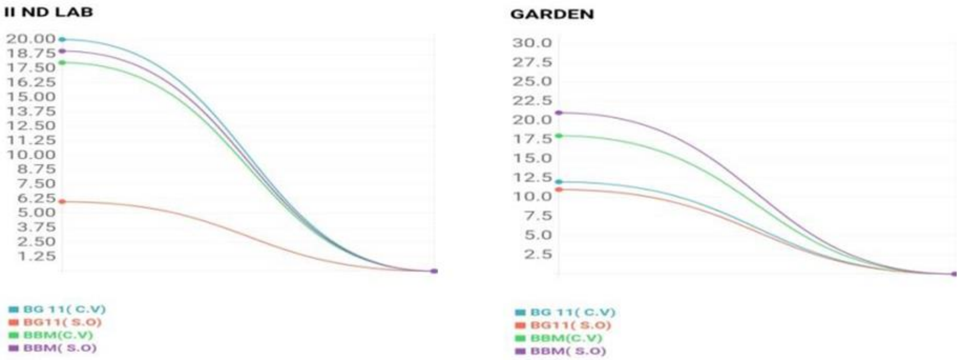

Fig. 5: Growth rate study in lab and garden at 620 nm.

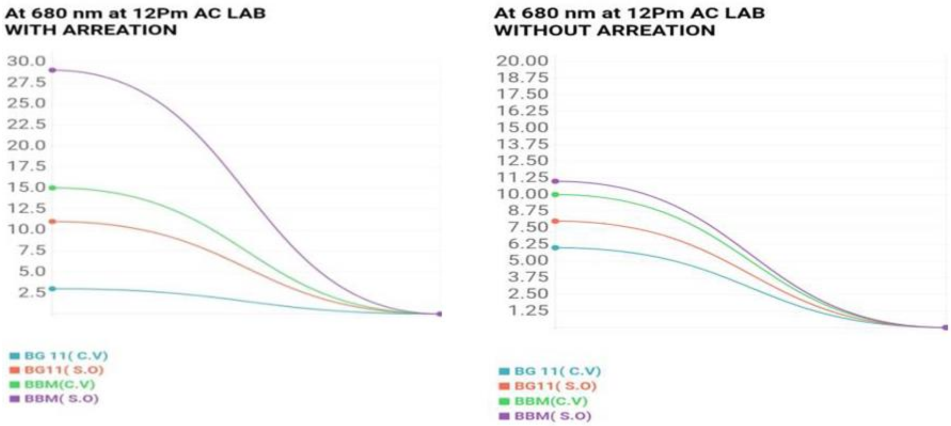

Fig. 6: Growth rate study with and without aeration at 680 nm.

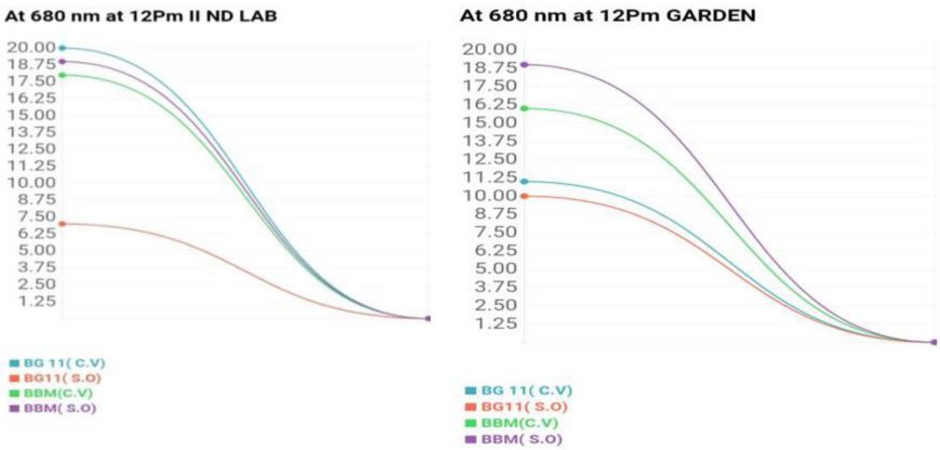

Fig. 7: Growth rate study in lab and garden at 680 nm.

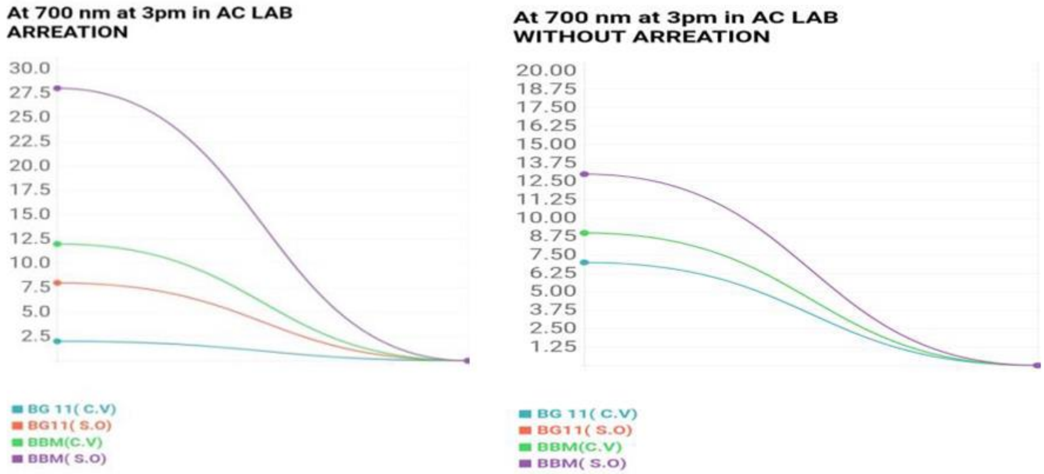

Fig. 8: Growth rate study with and without aeration at 700 nm.

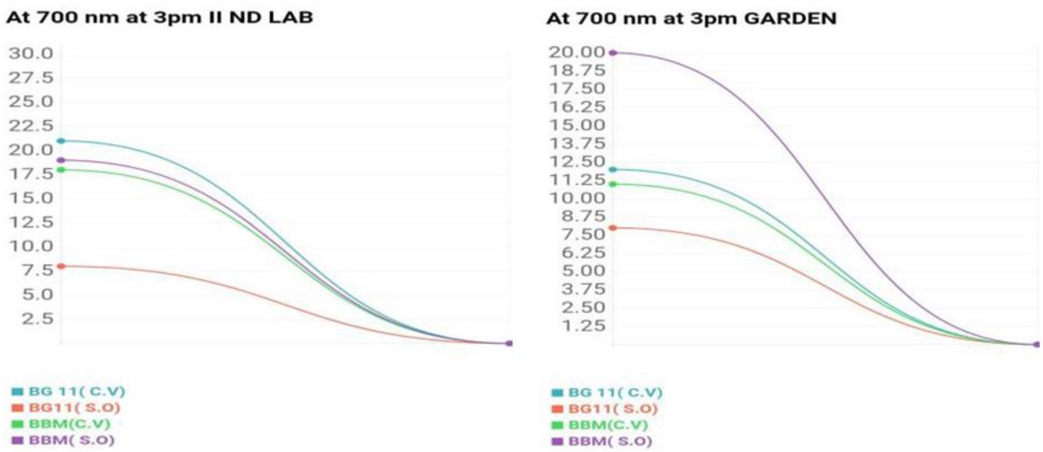

Fig. 9: Growth rate study in lab and garden at 700 nm.

Table 2: Phytochemical screening.

Phytochemical Test	Reagents used (Test performed)	Result for (*C.vulgaris*) (BBM)/(BG)		Result for (*S.obliquus*) (BBM)/(BG)	
Alkaloid test	Mayer's test	+	+	+	+
Amino acids & proteins	Ninhydrin test	-	+	+	+
Carbohydrates	Benedict's test	+	+	+	+
Fixed oils & fats	Saponification test	+	+	+	+
Phenolic compounds &tannins	Ferric chloride testLead acetate test	+ +	+ +	+ +	+ +

Table 3: Lipid Content in *Chlorella Vulgaris* and *Scenedesmus Obliquus.*

Algae	*Chlorella vulgaris*		*Scenedesmus obliquus*	
Medium	BG11	BBM	BG11	BBM
Lipid Content [%]	17.630 ± 0.002	11.560 ± 0.002	26.320 ± 0.003	19.760 ± 0.003

Table 4: Major chemical composition determined from GCMS of *Chlorella vulgaris* and *Scenedesmus obliquus.*

S.No.	*Chlorella vulgaris*	*Scenedesmus obliquus*
1	Heptaldehyde	3-hexadecyloxycarbonyl-5-(2-hydroxyl)-4-methylimidazolium
2	Octadecenoic acid, methyl ester	dodecanoic acid 3-hydroxy3.32%,
3	Hexadecanoic acid	9-octadecenoic acid (Z)41.59%,
4	3-Decyn-2-Ol	9,12,15-octadecadienoic acid2.42%,
5	(E)- 3,7,11,15-tetramethylhexadec-2-ene	2-hexadecenal 5.11%,
6	Heptadecane-1,2,3,4,5-pentol	quercetin 7,30,40-trimethoxy 13.48%,
7	Docosane, 4-methyl	octasiloxane

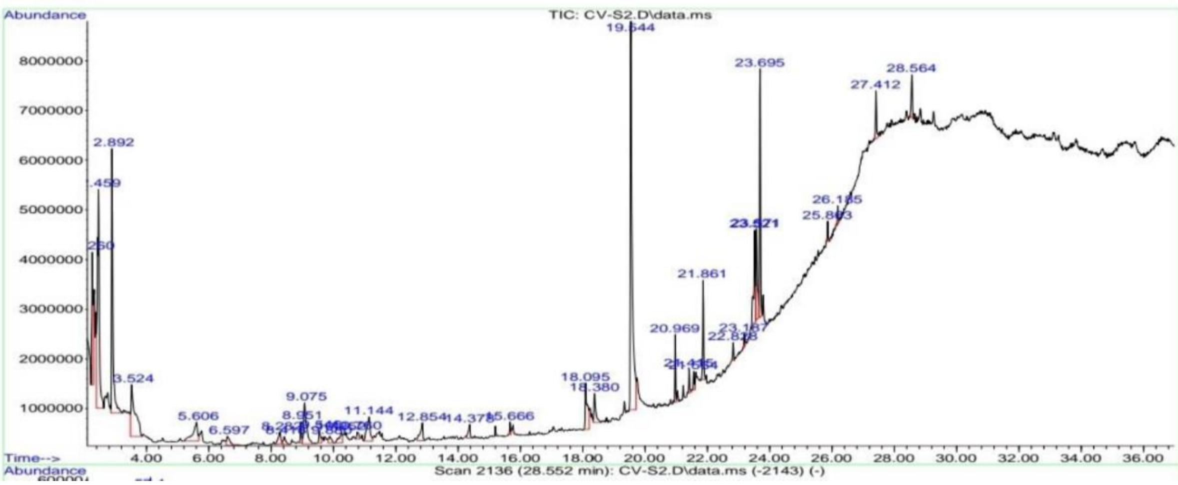

Fig. 10: GCMS of *Chlorella vulgaris* in BBM.

medicinal properties. Among these compounds, fixed oils and fats are important. Secondary metabolites are responsible principles for the biofuel properties of the microalgae.

Lipid Extraction

To extract the lipid content, Folch's extraction procedure was used which is one of the most popular methods for isolating lipidsfrom biological samples. In this method, 200 mL of the sample was taken and mixed with 1.5 mL of methanol, then vortexed with 3 ml of chloroform and then incubated for 1 h at room temperature. After which, 1.25 mL of water content was added and left for 10 min. Later, the centrifugation was performed for 10 min at 10 rpm which extracted the lipid at the lower phase which was collected for testing. From the extracted lipid, it was noted that BG11 medium supported better yield in both species which can be noted in Table 3.

GC-MS

The GC-MS analysis of the microalgae *C.vulgaris* and *S.obliquus* revealed the presence of various compounds. The major chemical compounds present in *Chlorella vulgaris* and Scenedesmus *obliquus* were confirmed based on the peak area and retention time which is shown in Table 4. The identification of compounds on the mass spectrum of GC-MS was done using the database NIST - National Institute of Standard and Technology, which details that it can be used as a biofuel. The graph obtained with its peak was shown below in Figs.10, 11, 12, and 13. Similar GC-MS testing was also done by Supriya et al. (2012).

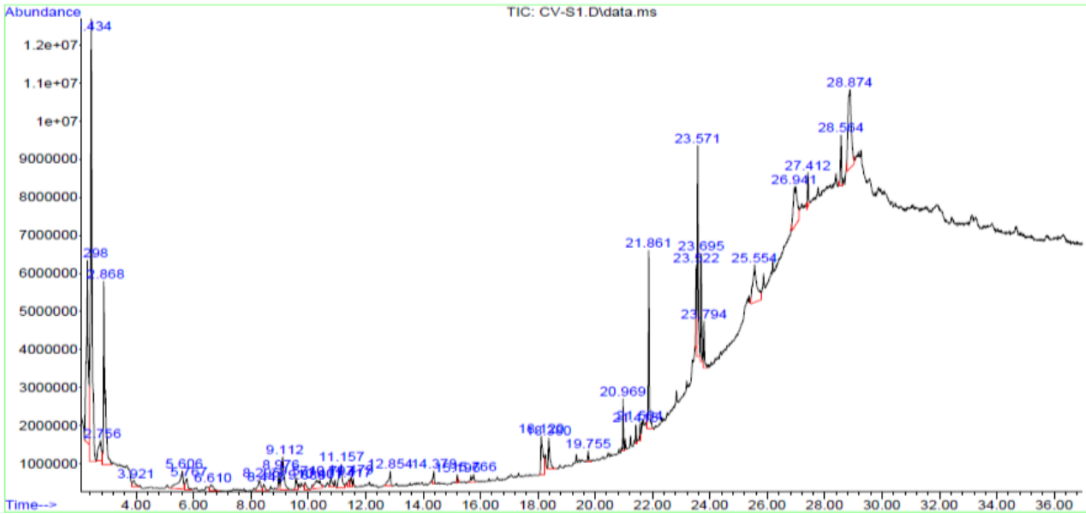

Fig. 11: GCMS of *Chlorella vulgaris* in BG11 medium.

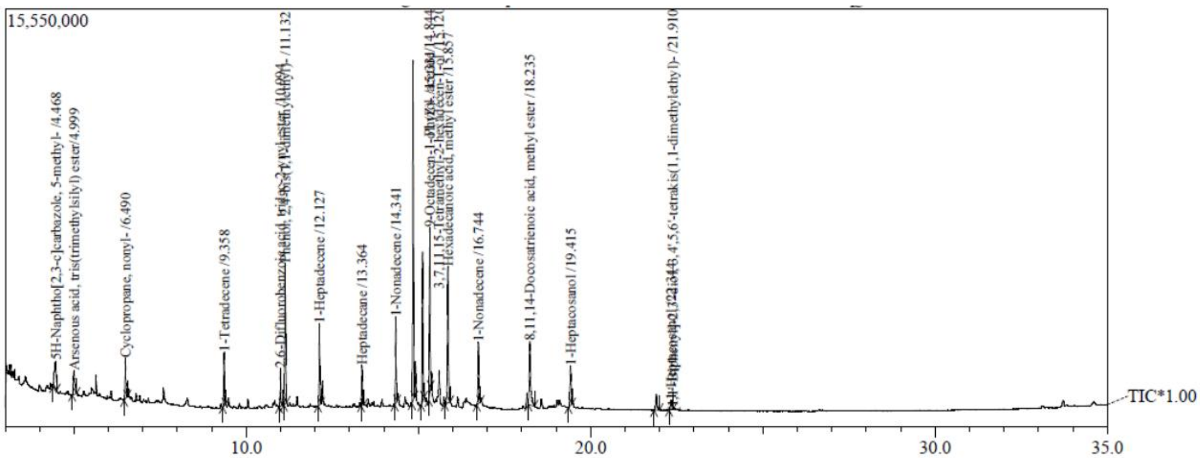

Fig. 12: GCMS of Scenedesmus Obliquus in BBM.

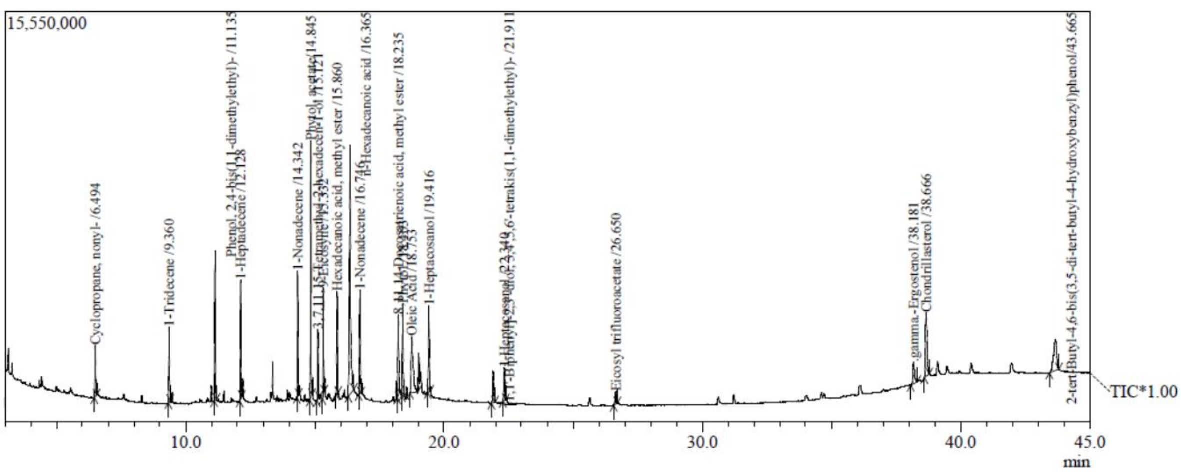

Fig. 13: GCMS of *Scenedesmus obliquus* in BG11 medium.

CONCLUSION

This study mainly focused on the optimization and comparative analysis of the *Chlorella vulgaris* and *Scenedesmus obliquus* microalgae growth and high lipid productions. The growth determination of the microalgae revealed the time period of the algae growth which was observed at 620nm. The phytochemical analysis was performed for the microalgae species to check the phytoconstituents. It was revealed that the concentration was more in theBG-11 medium from both samples. The GC-MS analysis of the microalgae *C.vulgaris* and *S.obliquus* revealed the presence of various compounds. The presence of major compounds in *C.vulgaris* and *S.obliquus* was confirmed based on the peak area and retention time, which concludes that these extracts can be utilized as a biofuel. From the analysis, it can also be concluded that the *Chlorella vulgaris* and *Scenedesmus obliquus* growth is more in the BG-11 medium than in the BBM medium.

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