"Green Gold: Sustainable Biodiesel Production and Bioactive Compound Extraction from Microalgae"

Abstract

Microalgae have attracted significant interest from both scientific and industrial sectors as a potential source of high-lipid feedstock for biodiesel production. Current research has mainly concentrated on the production of biodiesel from algal biomass, with a focus on collecting, isolating, screening, and characterizing suitable algal species. In this regard, a total of sixty-seven microalgal strains were isolated from both freshwater and marine sources across various regions. The screening for high-lipid microalgae strains was conducted using the Nile Red method to identify neutral lipid droplets.

The screening for high-lipid microalgae strains was performed using the Nile Red method to detect neutral lipid droplets. Out of the sixty-seven strains, four promising biodiesel-producing strains *Chlorococcum aquaticum, Scenedesmus obliquus, Nannochloropsis oculata, and Chlorella pyrenoidosa* were selected based on their high lipid and biomass accumulation, along with a favorable FAME profile containing palmitic (16:0), palmitoleic (C16:1), stearic (C18:0), and oleic (C18:1) acids. These characteristics suggest that these strains are suitable candidates for biodiesel production.

Keywords: Biodiesel, FAME, Lipid, Microalgae, Nile Red

1. Introduction

The increasing levels of carbon dioxide (CO2) and the excessive consumption of fossil fuels have had a significant negative impact on the global climate, contributing to issues such as the energy crisis and the greenhouse effect [1-5] These environmental challenges have raised concerns about the sustainability of current energy systems and highlighted the urgent need for alternative energy solutions [6-10]Additionally, the fluctuating prices of crude oil, the anticipated decline in petroleum reserves, and the growing global demand for energy have all further emphasized the necessity of exploring alternative biofuels as viable options. Among the various biofuels, biodiesel stands out as a renewable, biodegradable, and clean-burning fuel with substantial potential to replace conventional fossil fuels [11-14] Biodiesel is typically produced through the transesterification process, which involves converting triglycerides into biodiesel using alkali acids, bases, or enzymatic methods. This process reduces the viscosity of oils, making them more suitable for use as fuel [15-17]

However, one of the key challenges in biodiesel production is the high cost of feedstocks, which accounts for approximately 70% of the total production cost [50] This makes biodiesel less economically viable and undermines its potential as a widespread alternative to conventional fuels. [51] Various feedstocks, including vegetable oils, Jatropha curcas, non-edible oils, and animal lipids, have been investigated for biodiesel production [51] Despite their potential, several limitations hinder the widespread adoption of these feedstocks, such as their limited availability, competition with food production (leading to the food vs. fuel debate), and the need for large areas of land to cultivate the necessary crops. These factors collectively pose significant barriers to the large-scale application of biodiesel as a sustainable and cost-effective alternative to fossil fuels [51]

Microalgal biodiesel has emerged as one of the most promising renewable biofuels, offering significant potential as an ideal feedstock for biodiesel production [**19]** Compared to traditional biofuel feedstocks, microalgae present several distinct advantages that make them an attractive option. One of the primary benefits of microalgae is their exceptionally high lipid yield, which is crucial for biodiesel production [19-20] Additionally, microalgae are not affected by seasonal fluctuations, unlike many terrestrial crops, which ensures a more consistent and reliable production of biomass throughout the year. This feature makes microalgae a more stable and predictable source of biodiesel [20-22]

Another important advantage of microalgae is that they require significantly less water and land compared to conventional feedstocks such as crops like soybeans or palm oil. Microalgae can be cultivated in non-arable land, and they have the ability to grow in water bodies that are not suitable for agriculture, thereby reducing competition for land and freshwater resources that are vital for food production [23] Furthermore, microalgae have an exceptionally high photosynthetic rate, which means they can efficiently convert sunlight into biomass [24] This makes them an ecologically beneficial alternative to traditional feedstocks, as they can grow quickly and absorb carbon dioxide, potentially helping mitigate greenhouse gas emissions [25] These characteristics collectively position microalgal biodiesel as a more sustainable and environmentally friendly option for the future of biofuel production [26]

Additionally Microalgae provide important foods and nutrients such as proteins, lipids, and bioactive substances with various bioactivities. Protein is essential to human health . For many reasons, the utilisation of microalgae as a protein source has been highlighted **[62]** Indian tropical environment is also ideal for nurturing and growth of algal species that serves as an ultimate benefit over other countries. India being a country with rich microbial species diversity with an approximate 841 species of marine algae, numerous microalgal species, with diverse characteristics, have been researched in India for their ability to serve as viable

feedstocks for biodiesel production [**27-28**]. To develop a viable biodiesel production process, selection of promising biodiesel producing strains and optimization of growth factors are keys steps [**29**]. For this reason several programs worldwide aimed to screen a big number of newly isolated strains adapted to the culture conditions of local conditions [**30-32**]. India has rich biodiversity and abundant algal resources that could be explored for biofuel production. The aim of this study was to isolate, identify, and assess microalgae from diverse agro-climatic environments to determine those with the highest lipid content, making them suitable candidates for biodiesel production.

2. MATERIAL AND METHODS

2.1 Sample collection

Algal samples were carefully collected from a variety of freshwater and marine water bodies across different regions in India, including Haryana, Punjab, Rajasthan, Uttarakhand, and Maharashtra. The samples were collected in sterilized, clean plastic bottles to avoid any contamination and ensure the integrity of the algae for further analysis. (Table 1)

2.2 Isolation of algal strains

The process of isolating microalgae was carried out using two widely recognized techniques: the standard plate streaking method and the micropipette method, both of which utilized BG11 medium for cultivating the algae. Both methods are designed to ensure that the microalgae grow in a controlled environment with the appropriate nutrients, leading to the successful isolation of pure algal cultures for further screening and analysis. The BG11 medium provides essential nutrients, such as nitrogen, phosphorus, and trace elements, that support the growth of a wide range of freshwater and marine algae, making it an ideal medium for this isolation process.Concentrations of nutrients in media (gL^{-1}) were as follows: ($NaNO_3$, 1.5); (K_2HPO_4, 0.04); ($MgSO_4 \cdot 7H_2O$- 0.075); ($CaCl_2 \cdot 2H_2O$- 0.036); (citric

acid- 0.006); (ferric ammonium citrate, 0.006); (EDTA disodium salt- 0.001); ($Na2CO_3$, 0.02) and 1 mL trace element solution (composition in gL^{-1}: (H_3BO_3-2.86); ($MnCl_2 \cdot 4H_2O$-1.81); ($ZnSO_4 \cdot 7H_2O$-0.222); ($NaMoO_4 \cdot 2H_2O$ - 0.39); ($CuSO_4 \cdot 5H_2O$ - 0.079); ($Co(NO_3)_2 \cdot 6H_2O$ - 0.0494) at pH 7.0±1. The grown algal colonies were initially enriched in autoclaved sterilized BG-11 nutrient broth in conical flask (250 mL) at 27±1°C in phototrophic condition for 10 days. After that serially (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) diluted enriched algal cultures were spread on BG-11 agar plates and kept in shaking cum incubator at 25±2 °C for 7 days. These individual colonies were then transferred onto new BG-11 agar plates for further purification. The streaking process was repeated multiple times to ensure the isolation of pure axenic cultures. Schematic protocol of isolation and purification is shown in supplementary **Fig.1**. Purified and isolated algal cultures were maintained in culture room under cool fluorescence light at 25±2°C in liquid BG-11 medium.

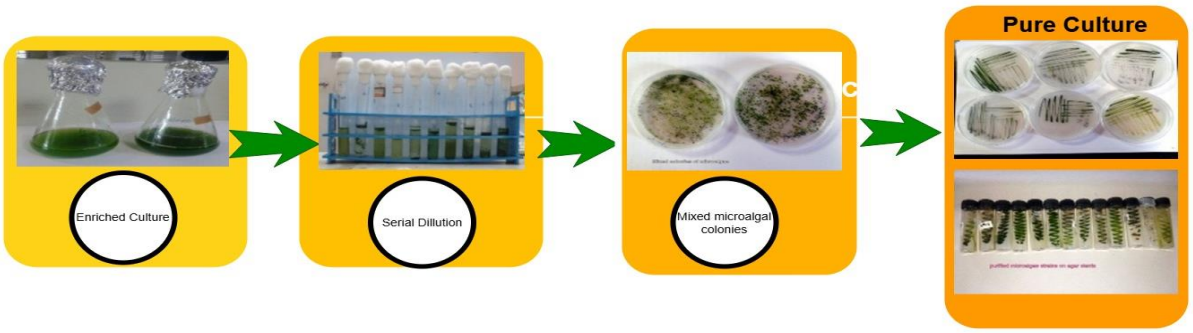


Fig. 1: Schematic protocol for isolation and purification of algal strains

2.3 Microscopic observation and identification of algal strains

The isolated microalgal strains were examined for their morphological characteristics and cell structure details using an Olympus binocular microscope, with identification aided by an algal identification guide. To maintain the purity of the cultures, regular subculturing was carried out, and the cultures were microscopically observed at regular intervals to ensure their consistency and purity.

2.4 Molecular identification and phylogenetic relationship of screened algal species

Genomic DNA extraction was carried out using a modified CTAB (cetyl trimethyl ammonium bromide) method [33]. Following the extraction, DNA quantification was performed using a UV-spectrophotometer at a wavelength of 280 nm. The most promising algal strains for biodiesel research were identified through a combination of morphological examination and molecular characterization, specifically the 18S rRNA approach [34].

Comparison was made between nucleotide sequences by submiting in NCBI nucleotide BLAST program. Phylogenetic tree was constructed from 18S rRNA gene sequence obtained from the organisms by Mega 6.1 version of EBI by using neighbour joining method.

2.5 Screening of indigenous biodiesel producing algal strains

For the purpose of screening, the algal strains were evaluated through both qualitative and quantitative methods. The process of identifying high-potential microalgal strains and optimizing the conditions that promote their growth is crucial for biodiesel production studies. Initially, the selected microalgal strains underwent preliminary screening using Nile Red dye, a fluorescent stain that specifically targets lipids, allowing for the visualization of lipid accumulation within the cells. Following this, the strains were further evaluated based on their lipid content and the amount of cellular biomass they accumulated. These characteristics were essential for determining which strains held the most promise for biodiesel production. The detailed procedure for screening the strains is illustrated in the flowchart shown in Fig 2, which outlines the systematic approach followed for strain selection and optimization.

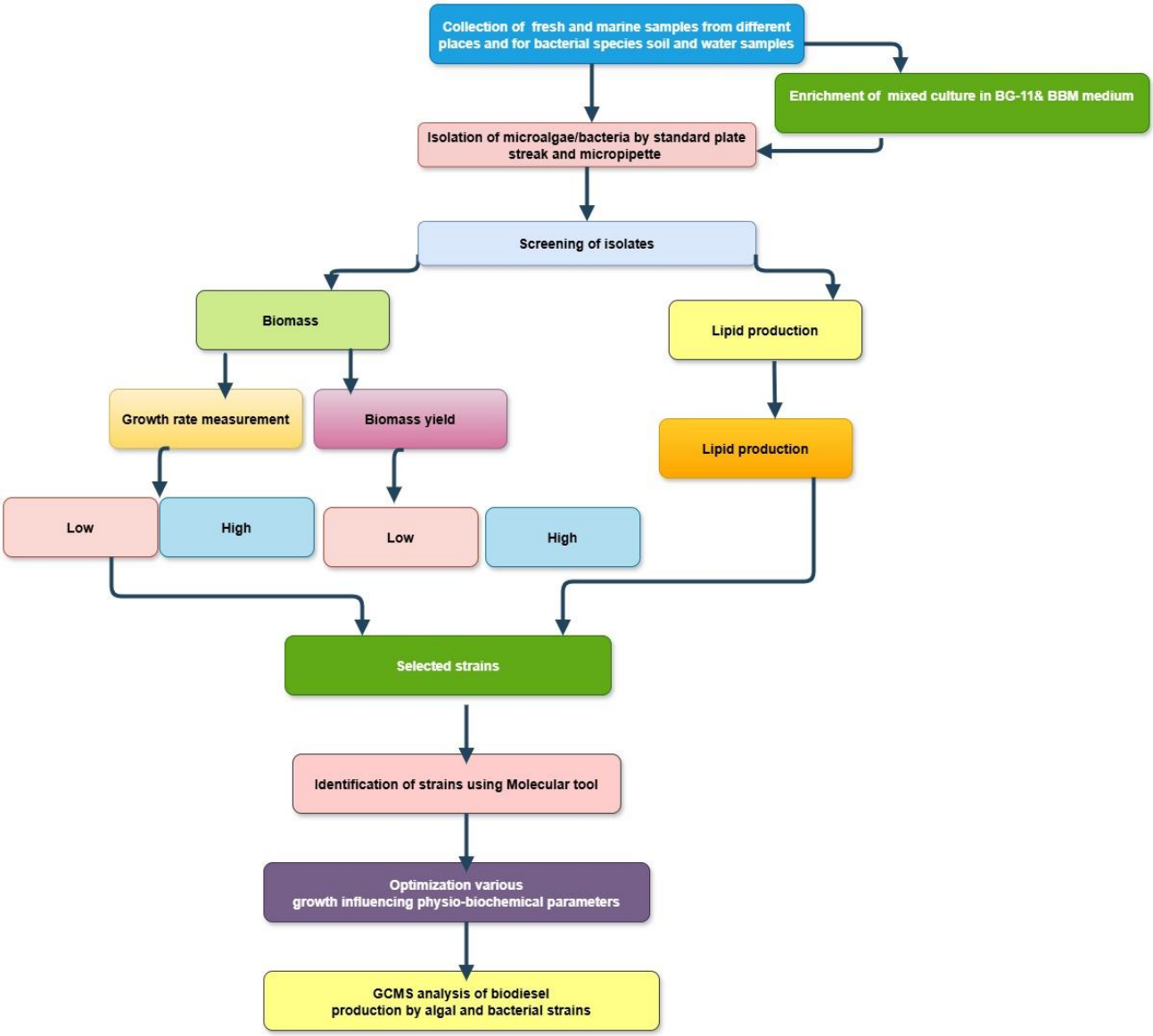


Fig 2. A flowchart showing screening process of promising biodiesel producing microalgae

2.6 Nile Red staining for preliminary screening

Based on improved Nile red staining, a specific volume of algal culture was centrifuged at 5000 rpm for 10 minutes, followed by washing with distilled water and then with a saline (NaCl) solution. The algal pellets were subsequently immersed in Nile Red solution and incubated in the dark for 15 minutes. The fluorescence was then observed and measured using an Olympus Magnus microscope [35].

Analytical Methods for bio-actives compounds extraction

2.7 Total lipid estimation

The total lipid content (Dry Cellular Weight, DCW) of the algal strains was determined using a modified Bligh and Dyer method. For this process, the algal culture was centrifuged and then mixed with a methanol-chloroform solution (in a 2:1.5 v/v ratio). The formula used for calculating the oil extraction yield (%w/w) is based on the method outlined in [36].

$$\text{Oil extraction yield (dcw \%)} = \frac{\text{Weight of extracted oil}}{\text{Weight of biomass}} x100$$

2.8 Total Chlorophyll estimations

The estimation of photosynthetic pigments was carried out using a modified version of the MacKinney method (1941). Algal cultures were first centrifuged and then mixed thoroughly with a known volume of methanol. The mixture was centrifuged again at 5000 rpm for 10 minutes. The chlorophyll content in the collected supernatants was measured spectrophotometrically [37] using the following formula.

$$\text{Total chlorophyll (mgmL}^{-1}) = 2.55 \times 10^{-2}\ E650 + 0.4 \times 10^{-2}\ E665 \times 10^{3}$$

2.9 Total carbohydrate and protein estimation

The total glucose content in the centrifuged algal samples was determined using a modified Anthrone reagent method, with spectrophotometric measurements taken at 625 nm [38]. For total protein estimation, the modified Lowry method was employed. A standard calibration curve was prepared using BSA (Bovine Serum Albumin), with concentrations expressed in $mgmL^{-1}$.

$y = 0.1097x - 0.0005$, $R^2 = 0.9989$ [**39**]

2.10 Transesterification process for extraction of free fatty acids and GCMS analysis

An amount of 500 mg of lyophilized algal biomass was placed in a reagent bottle, to which 10 ml of hexane was added and thoroughly mixed. The mixture was then heated in a hot water bath at 50°C for 1 hour. After heating, it was transferred into a separating funnel, as shown in Fig. 3. The supernatant was collected by centrifuging the algal samples at 10,000 rpm for 10 minutes and then subjected to GC-MS analysis [40].

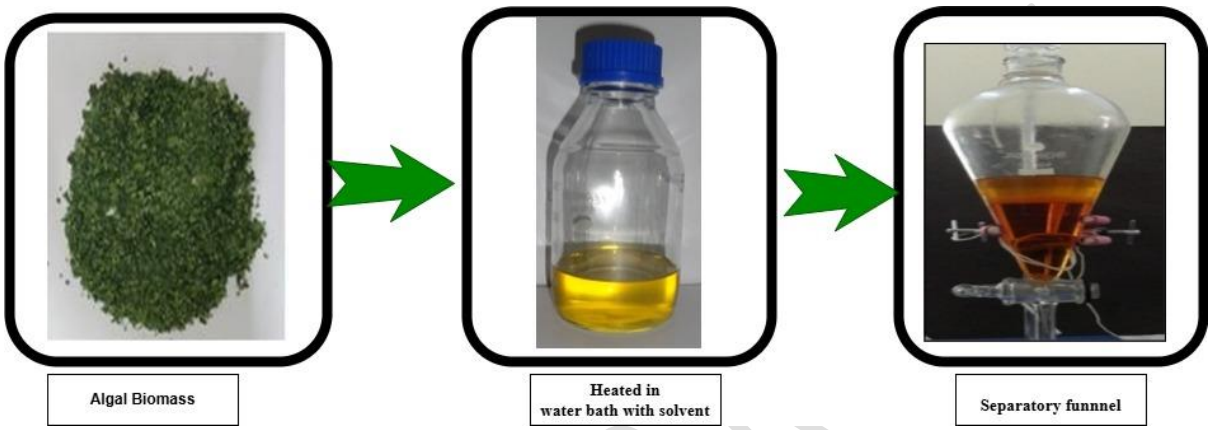


Fig. 3: Schematic protocol for tranesterification process for lipid extraction

2.11 Statistical analysis

Statistical comparison was analyzed between different groups by multi factors one-way (ANOVA) and Duncan's multiple-range test (SPSS version 21.0.). The *p*-values that were less than 0.05 were considered significant.

3. Results and Discussion

3.1 Collection of samples having algal growth

A total of fifteen samples were collected from various water sources, including both freshwater and marine environments, as detailed in Table 1. Microalgae provide a diverse spectrum of biodiversity in a number of environmental conditions, including freshwater, brackish, lacustrine, and hypersaline environments [**52**]. Previous research on oleaginous

microalgae from various locations shown that the sampling environment is critical in determining strain selection as well as strain survivability [**52,53**] .

Table 1: Sites of water samples having algal growth from Haryana, Punjab, Rajasthan, Uttarakhand, Maharashtra

S.No	Nameof samples	No. samples	Collection sites	Latitude Longitude
1.	Fresh water	1	Sangha village(Mansa)	29.983306°N, 75.383294°E
2.	Fresh water	1	Rajrana village(Mansa)	29.983306 N, 75.383294°E
3.	Fresh water	1	Shahidawaali (Sirsa)	29.4752° N, 74.9947°E
4.	Fresh water	1	Otto (Sirsa)	29.5008193°N, 74.889816°E
5.	Fresh water	1	Shahpur Begu Village (Sirsa)	29.45853°N, 75.06675°E
6.	Fresh water	1	Santnagar village (Sirsa)	29.13085 ° N, 75.62005°E
7.	Fresh water	1	Banni village (Sirsa)	29.6007403°N,74.6210115°E
8.	Fresh water	1	Aulakpur village (Mukthsar)	30°28'48.1512°N,74.518204°E
9.	Fresh water	1	Chattergarh patti village (Sirsa)	29.5352856°N, 75.032854°E
10.	Fresh water	1	Sahuwaala village (Sirsa)	29.24471°N, 76.04186°E
11.	Fresh water	1	Karndi village (Mansa)	29.72482°N, 75.28589°E
12.	Fresh water	1	34 GG village (Ganganagar)	29.8218° N, 73.7390°E
13.	Fresh water	1	Chickenwaas village (Hisar)	29.3168°N, 75.6273°E
14.	Marine water	1	Mumbai Coastal area (Maharashtra)	19°433.9240N,72°5238.7336° E
15.	Fresh water	1	Dehradun, Shestradhara	30.331073°N,78.066173° E

3.2 Isolation of algal strains

For the isolation procedure, the algal samples were initially diluted.To plate these diluted samples, sterilised plastic petri dishes containing about 50 mL of agarized media were utilised. One millilitre of the diluted sample was placed uniformly across the surface of a media plate. Standard plating procedures involving plate streak and micropipette were employed to separate algal colonies in order to isolate single microalgal species from field water samples. Isolation of algal strains was carried out using BG-11 medium placed under continuous light of 3000 lux at 25±2°C in shaking cum incubator. This streaking process was continued until axenic unialgal cultures were isolated. Sixty seven strains were isolated from fresh water bodies and six strains were isolated from marine water sources. The morphological features of the culture and the microscopic cellular appearance of the isolated colonies were used to classify all of the isolates. The isolated microalgae strains varied in size from unicellular to filamentous. All the isolated and purified culture were transferred to freshly prepared BG-11 medium for growth and maintained from time to time under controlled conditions of temperature and light in culture room.

3.3 Identification of isolated and purified microalgal strains

On the basis of cell morphology, habitat and lipid detection, preliminary identification of purified fifteen microalgal strains was performed by Olympus (CX41) light microscope equipped with digital camera coupled with algal identification manual. The majority of our isolated isolates were recognized at the genus level using microscopic morphological inspection. Based on the cellular appearance of each separated strain, several distinct microalgae strains were identified. The isolated microalgae strains varied in size from

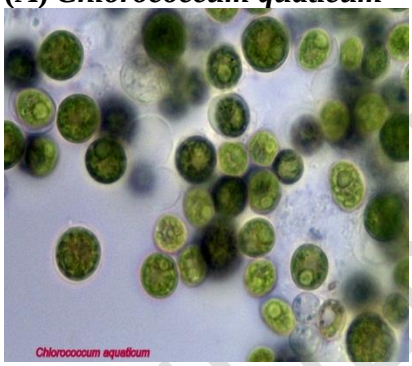
unicellular to filamentous. (**Fig. 4**) depicts microscopic images of a few selected microalgae. The description of identified strains are listed in Table 2.

Table 2 : List of preliminary identified microalgal strains

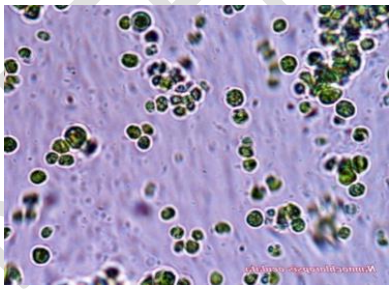
Strains	Morphological features	Strains	Morphological features
KB1-Chlorococcum aquaticum	Fresh water, grass-green, ovoid, mucilaginous envelope, size from 8 to 25 µm	KB9-*Tetraselmis sp.*	Green fresh water unicellular , motile, flagellates with elliptical or almost spherical, slightly flattened cells,belonging to family *Chlorodendraceae*, phylum chlorophyta, 10 µm long x 14 µm wide.
KB2-*Nannochloropsis oculata.*	Marine water algae, dark green spherical, with nonmotile spheres belonging to family Monodosidaoe, genus-*Nannochloropsis*, size from 2 to 3 µm	KB10-*Oocystis sp.*	Planktonic genus, fresh water green alga, round in shape belonging to family *Oocystaceae*. Cell size 6 µm long x 11 µm.
KB3-*Scenedesmus obliquus*	Fresh water Green algae,small spherical belonging to family *Scenedesmaceae*, genus *Scenedesmus* Size 20 µm	KB11-*Chlorococcus limneticus*	planktonic coccoid genus, fresh water green algae, Family Chlorophycae, size 20 µm
KB4-*Chlorella pyrenoidosa*	Single celled non motile green Unicellular cells, cup- to girdle shaped chloroplast seen in some cells, pyrenoids presentalgae belonging to family *Chlorellaceae*,genus *Chlorophyta*, Size 5-10 µm	KB12-*Scenedesmus dimophus*	Fresh water, green algae belonging to class *Chlorophyceae*, family *Scenedesmaceae* Small, spherical green algae that has a *size* of 5-10µm, consisting of four or eight elogated cells,central pyrenoid,
KB5-Haematococcus spp	freshwater species of Chlorophyta from the family *Heamatococcaceae*,Size 10-20 µm, celled appreance red due to astaxanthin	KB13-*Chlorella vulgaris*	Small, spherical algae belonging to chlorellaceae, genus *Chlorophyta*, Size 5-10 µm.
KB6-Chlamydomonas subtilis	Unicellular dark green alga belonging to famiy Phylum Chlorophyta family-*Chlamydomonadaceae* ,pyriform shaped chloroplast cup shaped,	KB14-*Bracteacoccus sp.*	Fresh coccoid green algae, belonging to family *Chlorococcaceae*, Cell size 20 µm

	size 10 µm, in length, 3 µm in width		
KB7-Desmodesmus sp.	Fresh water green microalage belonging to *Chlorophyta* phyllum, family *Scenedesmaceae,8-10* µm in long and 3-7 µm width.	KB-15-*Botrydiopsis alpine*	Green microalgae,spherical or ellipsoidal , *Botrydiopsis* Family size 10-23 µm, small disc-shaped chloroplasts
KB-8Apatococcus sp.	Green fresh water microalage, belonging to family *Chlorellaceae*, cells globular consisting of 2 or 3 planes to form irregular cuboidal packets,paritetal chloroplast without pyrenoids, cell size 20 µm		

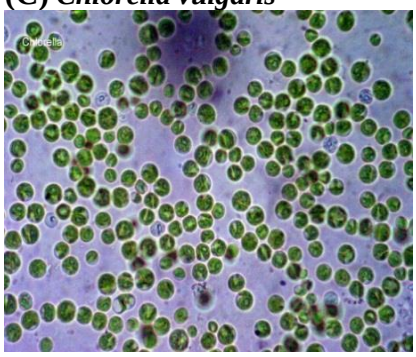
(A) *Chlorococcum quaticum*



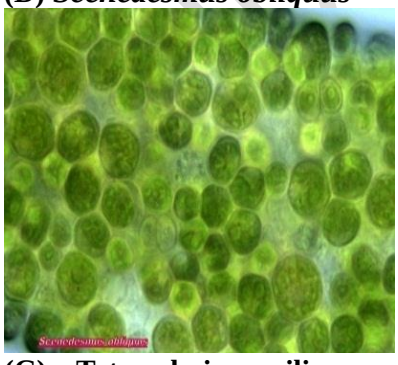
(B) *Nannochloropsis oculata*



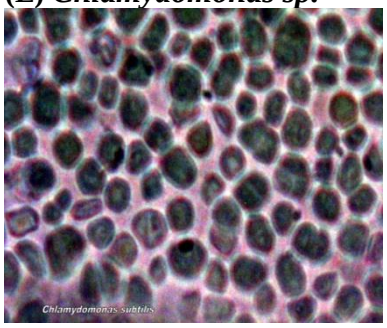
(C) *Chlorella vulgaris*



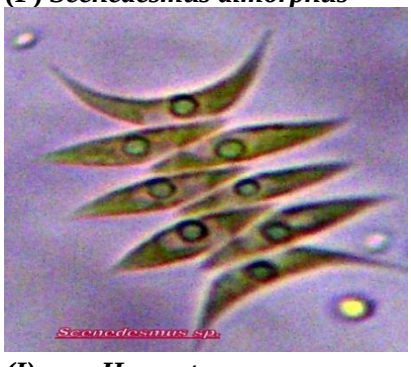
(D) *Scenedesmus obliquus*



(E) *Chlamydomonas sp.*



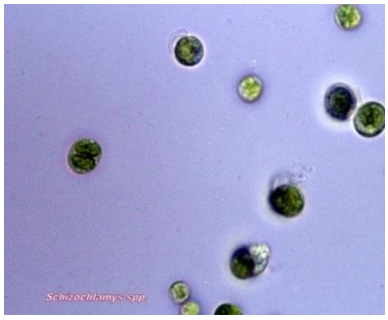
(F) *Scenedesmus dimorphus*



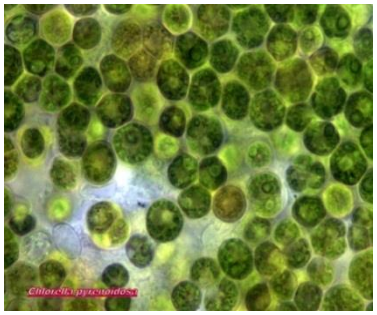
(G) Tetraselmis gracilis

(H) *Chlorella pyrenoidosa*

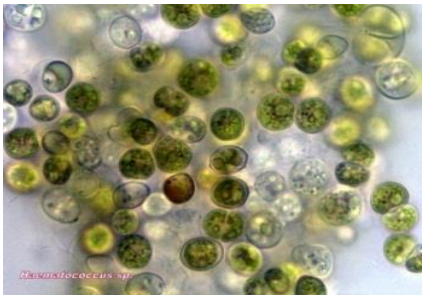
(I) Heamatococcus sp.



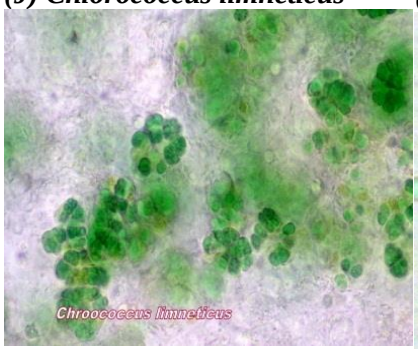
(J) Chlorococcus limneticus



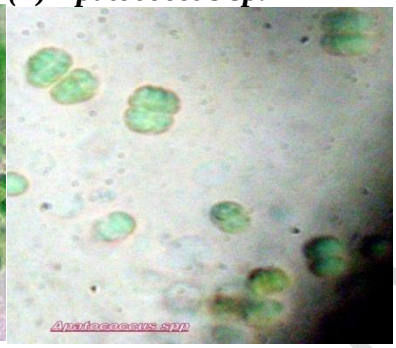
(K) Apatococcus sp.



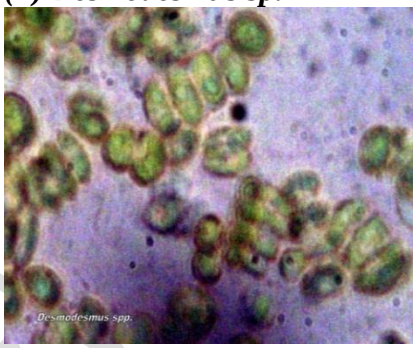
(L) Desmodesmus sp.



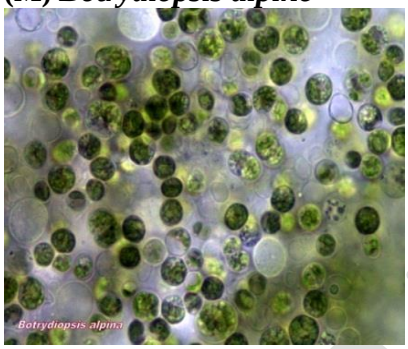
(M) *Botrydiopsis alpine*

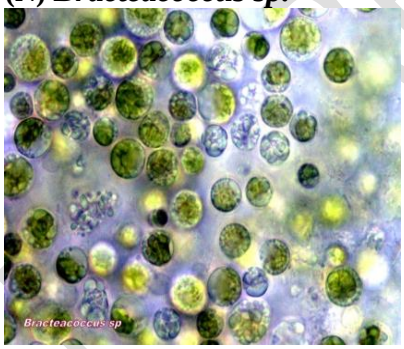


(N) *Bracteacoccus sp.*



(O) *Oocystis sp.*





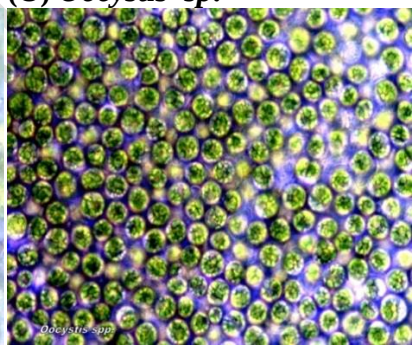


Fig. 4. Microscopic images of (A) *Chlorococcum sp.* (B) *Nannochloropsis oculata* (C) *Chlorella vulgaris* (D) *Scenedesmus obliquus* (E) *Chlamydomonas subtilis* (F) *Scenedesmus dimorphus* (G) *Tetraselmis gracilis* (H) *Chlorella pyrenoidosa* (I) *Haematococcus sp.* (J) *Chroococcus limneticus* (K) *Apatococcus* sp. (L) *Desmodesmus sp.* (M) *Botrydiopsis alpine* (N) *Bracteacoccus sp.* (O) *Oocystis sp.*

3.4 Screening of potential biodiesel producing microalgal strains

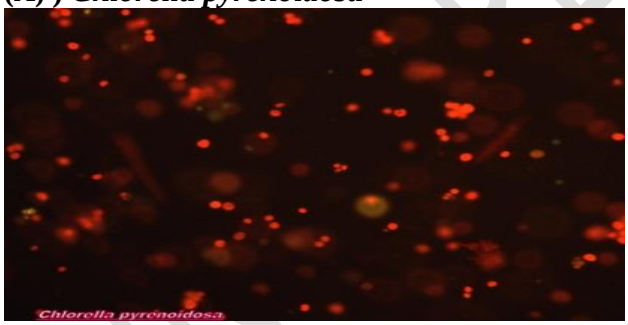
3.4.1 *Nile Red staining for intracellular lipid*

Screening of identified and purified species was done with the help of Olympus binocular fluorescent microscope by lipid staining fluorescent Nile Red dye and cellular neutral lipid droplets were detected with the help of fluorescent microscope. For pre-screening purpose, Nile red staining is generally used for identification and confirmation of lipid droplets in intracellular membranes of oleaginous organisms. The existence of a

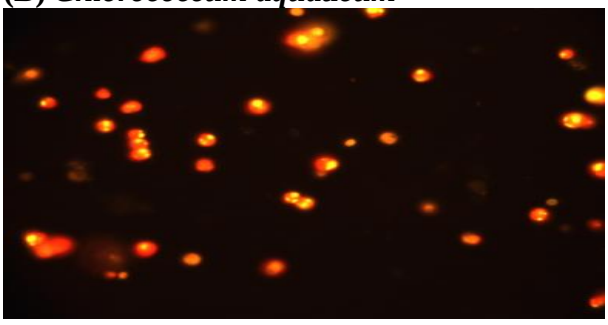
substantial amount of lipids accumulated in the microalgal cells was verified by the intensity of the Nile red fluorescence [**52**]. For qualitative screening, out of the fifteen microalgal strains, thirteen species were fresh water and two were marine water strains. Neutral lipid droplets were noticeably visible only in eight algal strains namely: KB1: *Chlorococcum aquaticum*, KB2: *Nannochloropsis oculata*, KB3: *Scenedesmus obliquus*, KB4: *Chlorella pyrenoidosa*, KB6: *Chlamydomonas subtilis, KB12: Scenedesmus sp.,* KB9: *Schizochlamys sp.* and KB 13: *Chlorella vulgaris*.

Under fluorescence microscope with excitation 420 nm and emission 580 nm wavelength, in screened algal strains neutral lipid or triglycerides appeared as predominately yellow in colour while chlorophyll and polar lipid-stained orange red color cells by Nile red dye. Fluorescence microscopic images are represented in **Fig.5**. Similar findings have been reported by many researchers for lipid staining by using Nile Red dye for intracellular lipid identification [**41-43, 52,54**].

(A)) *Chlorella pyrenoidosa*

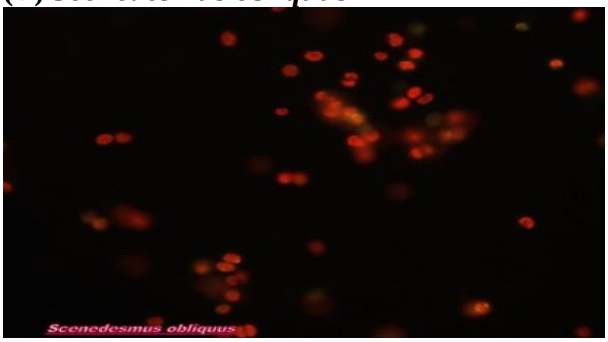


(B) *Chlorococcum aquaticum*



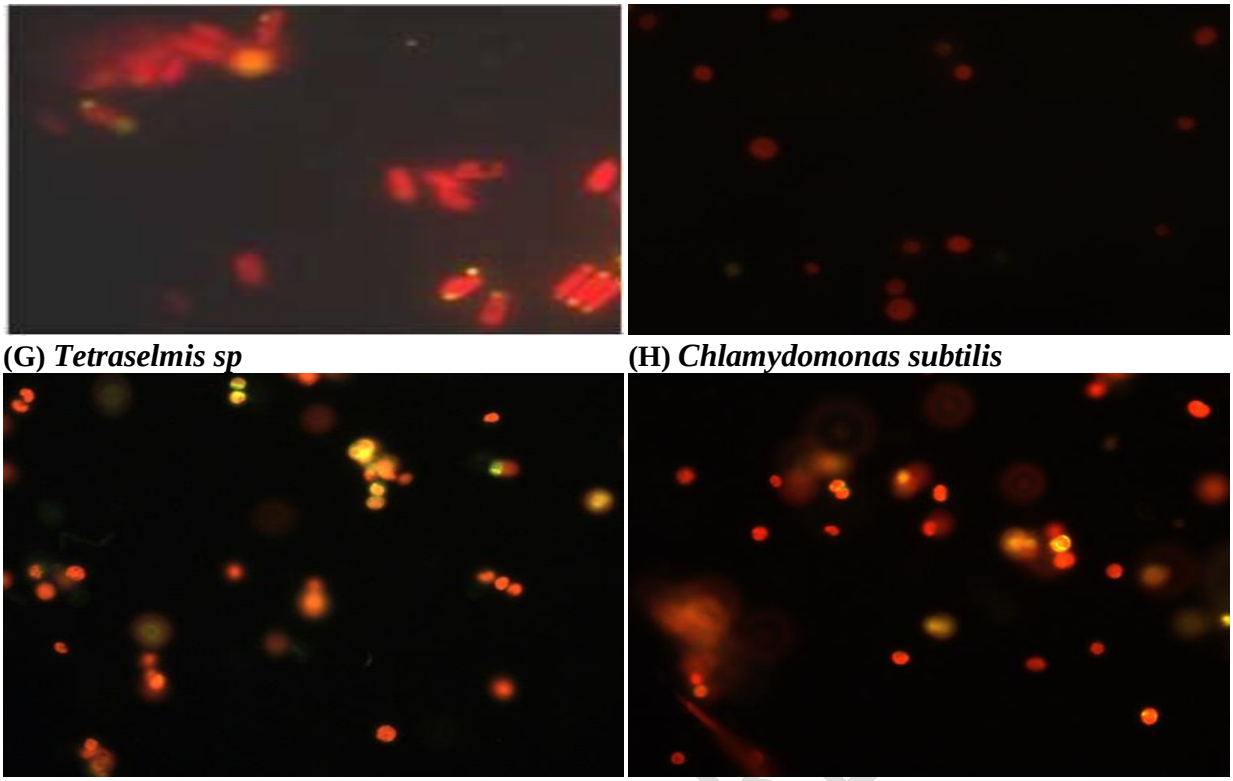
(C) *Nannochloropsis oculata*

(D) *Scenedesmus obliquus*



(E) *Scenedesmus dimorphus*

(F) *Chlorella vulgaris*



(G) *Tetraselmis sp* **(H) *Chlamydomonas subtilis***

Fig. 5. Microscopic Fluorescence images of (A) *Chlorella pyrenoidosa.* (B) *Chlorococcum aquaticum* (C) *Nannochloropsis oculata* (D) *Scenedesmus obliquus* (E) *Scenedesmus dimorphus* (F) *Chlorella vulgaris* (G) *Tetraselmis sp.* (H) *Chlamydomonas subtilis*

3.3.2 Biomass and lipid analysis of screened algal strains

Further algal species were screened out quantitatively on the basis of biomass yield and lipid production. Growth rate and biomass concentration of each microalgal species were analyzed by standard analytical methods. Strains those having high lipid contents and high biomass yield were selected for further study. The indigenous microalgal strains that were screened were further validated through molecular techniques to confirm their identity. The biomass yield, along with the contents of lipids, proteins, carbohydrates, and total

chlorophyll, were measured in the various pre-screened algal strains. The detailed results of these analyses are provided in **Table 3**.

Table 3: Physio-biochemical components of prescreened algal strains

Pre-screened algal strains	Biomass yield (gl^{-1})	Lipid (%dcw)	Protein ($mgmL^{-1}$),	Total carbohydrates ($mgmL^{-1}$)	Total chlorophyll (μgmL^{-1})
KB1 (Chlorococcum sp.)	0.95 ± 0.012^{D}	11.32 ± 0.034^{E}	0.053 ± 0.002^{C}	0.46 ± 0.024^{C}	15.02 ± 0.041^{A}
KB2 (Scenedesmus obliquus)	1.32 ± 0.023^{A}	13.55 ± 0.028^{C}	0.051 ± 0.007^{C}	0.75 ± 0.031^{B}	14.32 ± 0.034^{C}
KB3 (Nannochloropsis sp)	1.13 ± 0.010^{B}	14.81 ± 0.015^{B}	0.066 ± 0.001^{B}	0.71 ± 0.014^{B}	12.84 ± 0.025^{D}
KB4 (Chlorella pyrenoidosa.)	1.08 ± 0.025^{C}	15.27 ± 0.022^{A}	0.070 ± 0.003^{A}	0.83 ± 0.021^{A}	14.57 ± 0.018^{B}
KB9 (Schizochlamys sp.)	0.62 ± 0.017^{F}	9.85 ± 0.030^{G}	0.023 ± 0.005^{F}	0.37 ± 0.030^{D}	8.75 ± 0.029^{G}
KB13(Chlorella vulgaris	0.53 ± 0.020^{G}	13.02 ± 0.021^{D}	0.036 ± 0.002^{E}	0.29 ± 0.024^{E}	8.55 ± 0.017^{H}
KB12 (Scenedesmus dimorphus)	0.88 ± 0.024^{E}	10.86 ± 0.019^{F}	0.044 ± 0.005^{D}	0.32 ± 0.020^{D}	9.02 ± 0.033^{F}
KB6 (Chlamydomonas subtilis)	0.43 ± 0.011^{H}	9.71 ± 0.013^{H}	0.025 ± 0.007^{F}	0.25 ± 0.015^{E}	9.25 ± 0.011^{E}

Means with unlike superscript in row and column differ significantly ($p\leq0.05$)

The lipid, biomass and other cellular contents of several algal strains were calculated and compared in (**Table 3)**. Finally four potent biodiesel producing strains namely *Chlorococcum aquaticum, Scenedesmus obliquus, Nannochloropsis sp. Chlorella pyrenoidosa* were selected for further study were selected on the basis of higher physio-biochemical parameters for further study. Among various prescreened algal strains, *Scenedesmus obliquus* possessed highest biomass (1.32±0.023 g/L). Lipid was extracted from various microalgal strain using modified Bligh and dyer method as shown in supplentary **Fig. 6**. *Chlorella pyrenoidosa* contained significantly higher lipid percentage of 15.27 % (**Table 3**). Moreover many studies have been carried out for screening microalgae on the basis of lipid content using solvent extraction method [**48,52,54**]. Similarly, lipid content in *Scenedesmus quadricauda* was found to be 6.12% by using Bligh and Dyer [**24**]

Fig.6. Extracted lipid content from screened microalgal strains

3.3.3 Protein content of screened algal strains

Microalgae, *Nannochloropsis sp.* (0.066±0.001 $mgmL^{-1}$) and *Chlorella* (0.070±0.003 $mgmL^{-1}$) accumulated their dry biomass in proteins (**Table 3**). The protein content and amino acid composition of microalgae are highly dependent on the species as well as the production process **[61]** Many key aquaculture species, such as mollusks, shrimps, and fish, eat algae as a natural food source [**57**]. In another recent study, green microalgae *Scenedesmus* sp. was cultivated outdoor and utilized as stable rich protein food source in Denmark [**58**].

3.3.4 Carbohydrate content of screened algal strains

Under the conditions used in this study, the *Chlorella pyrenoidosa* and *Chlorococcum* strains of our collection seemed to accumulate carbohydrates (**Table 3**). Microalgae accumulate starch as the main carbohydrate source in their cellulose-based cell walls, some species such as *Chlorella, Scenedesmus Chlamydomonas,* and *Dunaliella* have been reported to accumulate more than 50% carbohydrate based on their dry cell weight, microalgae are considered a promising feedstock for bioethanol production [**55**]. In another recent study, deoiled algal biomass residue of *Scenedesmus obliquus* could be used as an alternative energy source for bio-ethanol synthesis using various heterogeneous catalysts [**56**]

3.3.5 Total chlorophyll content of screened algal strains

Among various reported species, *Chlorococcum sp*. accumulated higest chlorophyll (15.02±0.041 µgmL^{-1}) content as shown in Table 2. Microalgae biomass is recognised as a spectacular source of co-products in addition to being used as a food and feed source. Microalgae coproducts include chlorophyll, polysaccharides, fucoidans, phycocyannin, β-carotene, β-1,3-glucan, , agar, phycobiliprotein, lutein, alginates, etc. also gaining importance day by day [**59**]

3.4 PCR amplification, DNA sequencing and Blast homology search for screened algal strains

The most promising algal strains for biodiesel study were identified by using 18S rRNA approach. Random Amplified Polymorphic DNA (RAPD-PCR), a modified fingerprinting technique was used to study an unknown organism. RAPD-PCR was performed on all DNA samples extracted from four different algal strains. A number of amplified products were observed as shown in **Fig. 7.** Minimum two bands were cut down from the agarose gel for further DNA elution. Eluted DNA was used for the sequencing. 18SrRNA gene of screened algal strains were amplified from its genomic DNA using a pair of RAPD primers.

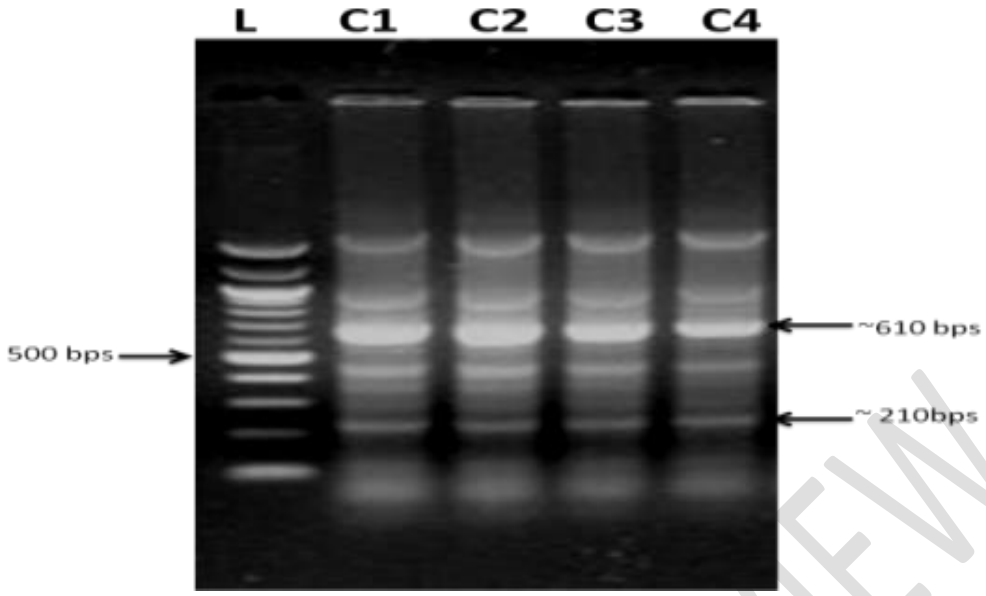


Fig. 7. Genomic DNA samples showing good quality and quantity of DNA in different wells. Lane 1: DNA Ladder; Lane 2: KB1 strain; Lane 3: KB2 strain: Lane 4: KB3; Lane 5: KB4. RAPD-PCR for detecting genomic markers for screened algal strains.

A number of amplifications were observed. Amplified products which were cloned and sequenced were marked differently.The purified PCR products were sequenced by the Amnion Biosciences Pvt. Ltd. (Bengaluru). Using the Blast tool, the resulting sequences were compared to the GenBank nucleotide database [**60**]. Sequence alignment outcomes revealed that screened algal strains were exhibiting 100% homology with *Chlorococcum aquaticum* (KB1) (Accession No. KT961379), *Scenedesmus obliquus* (KB2) (Accession No. KT983434), *Nannochloropsis oculata* (KB3) (Accession No. KU160538), *Chlorella pyrenoidosa* (KB4) (Accession No. KU236002)

3.5 GC-MS ANALYSIS

The FAME percentage of total esters obtained from extracted algal oil after the transesterification process (with respect to dry biomass) of fresh and marine water algal strains (Table 4) The FAME profile determined from peak areas of GCMS chromatograph of four microalgal strains *Scenedesmus obliquus*, *Chlorella pyrenoidosa*, *Nannochloropsis oculata* and *Chlorococcum aqauticum* possesed Palmitic, Palmitoleic, Stearic acid, Oleic acid. For excellent low temperature operability and oxidative stability, quality biodiesel should have relatively low amounts of both long chain saturated FAME and polyunsaturated FAME [**52**]

Chlorella pyrenoidosa comprises of both saturated and unsaturated fatty acids profile mainly short, medium and long chain fatty acids. Furthermore, polyunsaturated FA (4 double bond) was absent in *Chlorella* strains. Short chain free fatty acids were predominately present in *Chlorella pyrenoidosa* than compared to long chain hydrocarbons. The saturated fatty acids were identified mainly as palmitic acid methyl ester (C16:0, 35.811%). Like wise Sharma and his co-workers have found that microalage *Chlorella sp* has possessed maximum percentage of linolenic acid (14.20 %) [**46**]

In *Chlorococcum aquaticum*, hexanoic acid (C6:0, 7.903%) , Octanoic (C8:0, 7.9%) acid, Undecanoic acid (C11:0, 9.97%), (Palmitic-C16:0, 27.08%), (Palmitoleic -C16:1, 21.44%) (Stearic acid-C:18, 9.86%) (Oleic acid-C:18:1, 20.64%), linoleic acid (C18:2, 7.91%) were detected. Similar FAME profile in *Chlorococcum humicola* which makes it suitable feedstock for biodiesel production [**47**]. Another report on microalgal strain *Chlorococcum sp. RAP13* has consisted of suitable fatty acids profile which make it promising source for food and petrochemical industry [**48**]

Table 4 : Analysis of free fatty acids (FFAs) of screened algal strains by GCMS

					Screened Microalgal strains			
S. No	Common Name	IUPAC Name	Abbreviation	Molecular formula	*Chlorococcum aquaticum*	*Scenedesmus obliquus*	*Nannochloropsis oculata*	*Chlorella Pyrenoidosa*
1.	Caproic acid	Hexaonic acid	C6:0	$C_6H_{12}O_2$	7.903	0.21	0.5	ND
2.	Caprylic acids	Octanoic acid	C8:0	$C_8H_{16}O$	ND	ND	ND	ND
3.	Undecanoic acid	Undecanoic acid	C11:0	$C_{11}H_{22}O_2$	0.02	ND	0.04	6.27
4.	Lauric acid	Dodecanoic acid	C12:0	$C_{12}H_{24}O_2$	ND	1.21	ND	2.53
5.	Lauric acid,	Dodecanoic acid	C12:1	$C_{13}H_{26}O_2$	1.3	ND	1.33	1.26
6.	Myristic acid	Tetradecanoic acid	C14:0	$C_{14}H_{28}O_2$	0.99	1.23	ND	0.33
7.	Pentadecanoic acid	Pentadecanoic acid	C15:0	$C_{15}H_{30}O_2$	0.68	ND	1.61	1.55
8.	Pentadecenoic acid	Cis-10-heptadecenoic acid	C15:1	$C_{15}H_{28}O_2$	ND	ND	4.5	ND
9.	Palmitic acids	Hexadecanoic acid	C16:0	$C_{16}H_{32}O_2$	27.082	52.10	41.21	35.81
10.	Palmitoleic acids	Cis-9- Cis-10-heptadecenoic acid	C16:1	$C_{16}H_{30}O_2$	21.44	2.661	12.429	10.05
11.	Margaric acids	Heptadeconic acids	C17:0	$C_{17}H_{34}O_2$	ND	ND	1.967	1.15
12.	Cis-10-heptadecenoic acid	Cis-10-heptadecenoic acid	C17:1	$C_{17}H_{32}O_2$	ND	10.34	6.67	ND
13.	Stearic acid	Octadecanoic acid	C18:0	$C_{18}H_{36}O_2$	9.86	17.21	23.04	1.782
14.	Oleic acid	Cis-9-octadecanoic acid	C18:1	$C_{18}H_{34}O_2$	20.64	12.602	2.719	19.72
15.	Linoleic acid	Cis-9,12,octadecadienoic acid	C18:2	$C_{18}H_{32}O_2$	7.91	10.01	10.780	9.29
16.	Linolenic acid	Cis- 9,12,15-octatetradecoic acids	C18:3	$C_{18}H_{30}O_2$	4.181	12.7	1.21	12.62
1	Nonadeca	Nonadecyclic	C19:0	$C_{19}H_{88}O$	ND	0.1	0.3	0.483

7.	noic acid	acid		$_2$				
1 8.	Arachidic acid	Eicosanoic acid	C20:0	$C_{20}H_{40}O_2$	2.7	4.76	ND	6.395
1 9.	Behenic acid	Docosenoic acid	C22:0	$C_{22}H_{44}O_2$	ND	ND	1.73	ND
2 0.	Erucic acid	Cis-13-docosenoic acid	C22:1	$C_{22}H_{42}O_2$	ND	ND	ND	ND
2 1.	Heptacosylic acid	Heptacosanoic acid	C27:0	$C_{27}H_{56}O_2$	ND	ND	ND	0.166
Relative percentage of free acids detected by various transesterification methods				SFAs%	45.83	69.31	66.21	46.37
				MUFA %	42.08	25.60	21.80	31.77
				PUFA %	10.76	22.71	11.99	21.96

SFA-Saturated fatty acids, Mono unsaturated fatty acids, PUFA- polyunsaturated fatty acids, ND-Not detected

Scenedesmus obliquus also possessed both saturated and unsaturated fatty acids. Percentage of saturated fatty acids was found to be higher i.e. 69.31%. FAME profile mainly consisted of fatty acids (C16:0, 52.10%), (C18:0, 17.21%), (C18:1, 12.60%), (C18:2, 10.01%), and (C18:3, 12.70%). These results indicated that *Scenedesmus obliquus* has a favorable $FAME_S$ profile that can be successfully utilised for biodiesel production. Likewise, Arif et al. [**49**] has explored four potential microalgal strains viz. *Chlorella* sp., *T. dimorphus, C. sorokiniana* and *T. obliquus* for production of biofuels mainly : biodiesel and bioethanol. According to Abomohra et al.[**2**], *Scenedesmus obliquus* was found be the highest biomass producing species and showed maximum fatty acid 10 % and lipid content 19% DCW as we incorporated in our study.

Similarly, *Nannochloropsis oculata* has consisted of Palmitic- 41.21%, Palmitoleic, 12.42%, Stearic acid 23.04%, Oleic acid, 2.719%, linoleic, 10.78%, linoleic, 1.21%. It demonstrated that *Scenedesmus obliquus*, *Nannochloropsis oculata* contained (69.31% and 66.21%) mainly saturated fatty acids (respectively of total acyl methyl esters) which confirmed that its high oxidative stability. In previous study reported by Duong et al. [**14**] *Chlorella* and

Scenedesmus dimorphus strains has saturated fatty acids and unsaturated fatty acids ranged from 67.42 to 72.95% DCW. It consisted of predominately FAME profile C16:0, C18:1, C18:2, and C18:3. Therefore, *Scenedesmus obliquus* and *Nannochloropsis oculata*, *Chlorella pyrenoidosa* could be considered as ideal candidates for biodiesel production [**23,33**]. As per Piligaev et al. [**54**] *Micractinium sp.* IC-76 acquired a 36.29 % lipid content, with a total of 71.9 % saturated and monounsaturated fatty acids. This is very close to our findings. From the above results and discussion it has been found that all screened algal species have characteristics of diesel hydrocarbons that make them potent strains for biodiesel production.

4. Conclusion

One of the most important limiting elements in deciding which microalgal strains can be cultivated fast in an established area is the weather. Despite its high lipid content, the microalgal species cannot be utilised to produce biodiesel if it does not develop well at the planned area. Selection of the right species is crucial step algal based biofuel production. For this purpose, some essential criteria needs to be considered which include high biomass and lipid, rapid growth, easy cultivation with adapation of local environment. A total of 15 samples were collected from different water bodies. Isolation and purification of algal strains was done by standard isolation techniques. A total of 67 strains were isolated from fresh water samples sources and out of these six strains were isolated from marine water sample sources. Preliminary identification of all the isolated and purified strains was done with microscopic examination and fifteen strains were identified and confirmed on the basis of cellular details and habitat. Pre-screening (qualitative) from preliminary identified algal strains was done with the help of fluorescent microscope by using lipid staining fluorescent dye Nile Red and polar lipid were detected clearly only in eight microalgal strains. Lipid content was achieved significantly higher in *Chlorella pyrenoidosa, Nannochloropsis oculata* followed by *Scenedesmus obliquus, Chlorococcum aquaticum. Scenedesmus obliquus* was

found to be highest biomass producing species among various screened species and it has the potential for biodiesel feedstock on a large scale as a result of this research. Hence these species can be successfully use for mass production biodiesel. For the future of biodiesel, research should prioritize co-culturing algae with bacteria to increase biomass yield. Additionally, genetic engineering can be a powerful tool for enhancing lipid content, growth rates, and stress tolerance. In photobioreactor systems, improving reactor efficiency, scalability, and cost-effectiveness will be crucial. Emerging trends also suggest that valorizing algal biomass and implementing microalgae-based carbon capture systems for industrial emissions could help scale up biomass production.

Statements & Declarations

Data Availabilty Not applicable

Declarations

Ethics approval Not applicable

Consent to participant Not applicable

Consent to publish Not applicable

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