Microalgal potential for sustainable biodiesel production and other bioactives compounds

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Abstract

Microalgae have been gaining wide attention from scientific and industrial point of view as a source of high-lipid feedstock to produce biodiesel. Current research work was focused primarily on biodiesel production using algal biomass with the objectives of collection, isolation, screening and characterization of appropriate algal species. In this context, sixty seven microalgal strains were isolated including fresh and marine water sources from five regions of India. Screening of high lipid microalgae strains was carried out using Nile Red method for detection of neutral lipid droplets. Out of sixty seven, four potent biodiesel producing strains namely *Chlorococcum aquaticum*, *Scenedesmus obliquus, Nannochloropsis oculata* and *Chlorella pyrenoidosa* were selected on the basis of high lipid and biomass accumulation and having desirable FAME profile of palmitic (16:0), palmitoleic (C16:1), stearic (C-18:0) and oleic acids (C-18:1), respectively, which implies that these strains can be successfully used for biodiesel production.

1. Introduction

Rising level of CO_2 and excessive consumption of fossil fuels has adversly impacted on global climate that lead to energy crisis and green house effects [1–5]. Furthermore, dwindling crude oil prices, projected shrinkage of petroleum reserves and increasing energy demand have triggered to look an alternative biofuels [6–10]. Among biofuels, biodiesel is a renewable, biodegradable and clean burning fuel that has potential to replace conventional fuel [11–14]. Biodiesel is produced by transesterification of triglycerides by alkali acids, bases or enzymatic methods that reduces the viscosity of the oils [15–17]. For biodiesel production, feedstocks incur higher cost (around 70%) that forfeit the purpose of biodiesel production [50] Feedstocks such as vegetable oils, *Jatropha curcas*, non-edible oils and animal lipids have been studied. However, succinct supply, dilemma over food vs fuel and requirement of acreage are major factors that hindered the wide applicability of these substrates [51]

Microalgal biodiesel appeared to be most promising renewable biofuel that has the potential to be used as a best feedstock for biodiesel production [19–22]. Microalgae has several advantages over other feedstocks, including high lipid yield, denuded of seasonal variation, less competitive for water and land with high photosynthetic rate which make it more ecological important counterpart than the conventional feedstocks [23–26].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, has explored only less than 100 species for biodiesel extraction [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 objective of this study was to isolate, identify, and screen out the potential microalgae from varied agro-cimatic conditions having high lipid content that suits for biodiesel production"

2. Material And Methods

2.1 Sample collection

Algal samples were collected from different fresh and marine water bodies in sterilized, clean plastic bottles from various locations in Haryana, Punjab, Rajasthan, Uttarakhand and Maharashtra.

2.2 Isolation of algal strains

Microalgal isolation process was done by using standard plate streaking and micropipette method using BG11 medium. 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. After incubation, algal colonies were appeared on the agar plates. Individual colonies were streaked on BG-11 agar plates for further purification and streaking repeated several times for obtaining pure axenic culture. Schematic protocol of isolation and purification is shown in **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.

2.3 Microscopic observation and identification of algal strains

Olympus binocular microscope was used for morphological and different cell structure details and identified the isolated microalgal strains with the help of algal identification guide. For maintaining purity of cultures regular sub culturing as well as microscopic observation was undertaken by microscope at regular interval of time.

2.4 Molecular identification and phylogenetic relationship of screened algal species

Extraction of genomic DNA was performed by modified CTAB (cetyl trimethyl ammonium bromide) method [33]. After DNA extraction, quantification of DNA samples was carried out using UV-spectrophotometer at 280 nm. The most promising algal strains for biodiesel study were identified based on morphological and molecular characterization (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 screening purpose, algal strains were characterized both qualitatively and quantitatively. Screening of potent micro-algal strains and optimization of algal growth inducing conditions for biodiesel study is prerequisite. Preliminary screening of identified micro-algal strains were carried out using lipid staining fluorescent Nile Red dye. Further these strains were screened out on the basis of lipid content and higher cellular biomass as shown in detail flowchart of screening procedure in Fig. 2 .

2.6 Nile Red staining for preliminary screening

Based on improved Nile red staining, a known volume of algal culture were centrifuged at 5000 rpm for 10 min followed by washing with distilled water then washed with saline (NaCl) solution. Further algal pellets were soaked in Nile red solution and incubated in dark place for 15 minutes, fluorescence effect was measured with Olympus Magnus microscope [35]

Analytical Methods

2.7 Total lipid estimation

Total lipid content (Dry Cellular weight)DCW of algal strains was calculated by using modified Bligh and dyer method. For this purpose, algal culture centrifuged and mix it with methanol chloroform (2:1.5 v/v) and formula used for estimation of oil extraction yield (%w/w): [36]

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

2.8 Total Chlorophyll estimations

Estimation of photosynthetic pigment was performed by modified method of MacKinney, 1941. Algal cultures were centrifuged and mixed well in known volume of methanol and centrifuged again at 5000 rpm for 10 minutes. Chollophyll content of collected supernatants was estimated spectrophotometrically [37] by using the following formula:

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

2.9 Total carbohydrate and protein estimation

Total glucose content of centrifuged algal samples was estimated by modified Anthrone reagent method spectro-photometerically at 625 nm [38]. Modified Lowry method was used for total protein estimation. For this purpose, standard calibration curve was prepared using BSA (Bovine serum albumin expressed in terms of $mgmL^{-1}$.

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

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

500 mg of lypholized algal biomass was taken in reagent bottle and to it 10 ml hexane was added and mixed thoroughly followed by heated in hot water bath at 50^oC for 1 hour then transferred in to separating funnel as shown in **Fig. 3.** Supernatant was collected by centrifuging the algal samples at 10,000 rpm for 10 minutes further subjected to GCMS analysis [40]

2.11 Statistical analysis

Statistical comparison was analysed 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

Collectively Fifteen samples were collected from different water bodies including freshwater and marine water sources as shown 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 isolation procedure, algal samples were first 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 recognised 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, size 10 µm, in length, 3 µm in width	KB14-*Bracteacoccus sp.*	Fresh coccoid green algae, belonging to family *Chlorococcaceae*, Cell size 20 µm
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		

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 colour 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].

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. These screened indigenous microalgal strains were also confirmed using molecular techniques. Biomass yield, lipid, protein, carbohydrate and total chlorophyll contents in various pre-screened algal strains are presented in Table 3.

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]

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].

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 ($p \leq 0.05$)					

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 coproducts 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.

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%). Likewise Sharma and his co-workers have found that *Chlorella sp* has possesed 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]

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.

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

S. No	Common Name	IUPAC Name	Abbreviation	Molecular formula	Screened Microalgal strains			
					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	$C_8H_{16}O_2$	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
17.	Nonadecanoic acid	Nonadecyclic acid	C19:0	$C_{19}H_{88}O_2$	ND	0.1	0.3	0.483
18.	Arachidic acid	Eicosanoic acid	C20:0	$C_{20}H_{40}O_2$	2.7	4.76	ND	6.395
19.	Behenic acid	Docosenoic acid	C22:0	$C_{22}H_{44}O_2$	ND	ND	1.73	ND
20.	Erucic acid	Cis-13-docosenoic acid	C22:1	$C_{22}H_{42}O_2$	ND	ND	ND	ND
21.	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								

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 future prospective of biodiesel, more research should be focused on co-culturing, namely algae and bacteria in order to obtain larger biomass.

Declarations

Authors Contribution

Corresponding author and writing—original draft; Kulvinder Bajwa, The research direction of the project; Narsi R. Bishnoi guides ;. conceived the idea and edited the MS meticulously; Muhammad Yousuf Jat Baloch; supports the data analysis, Silambrasan Tamil Selven; S.P. supports the experimental design and data analysis, Jeevan Kumar; meticulously edited the MS Anita Singh .

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Figures

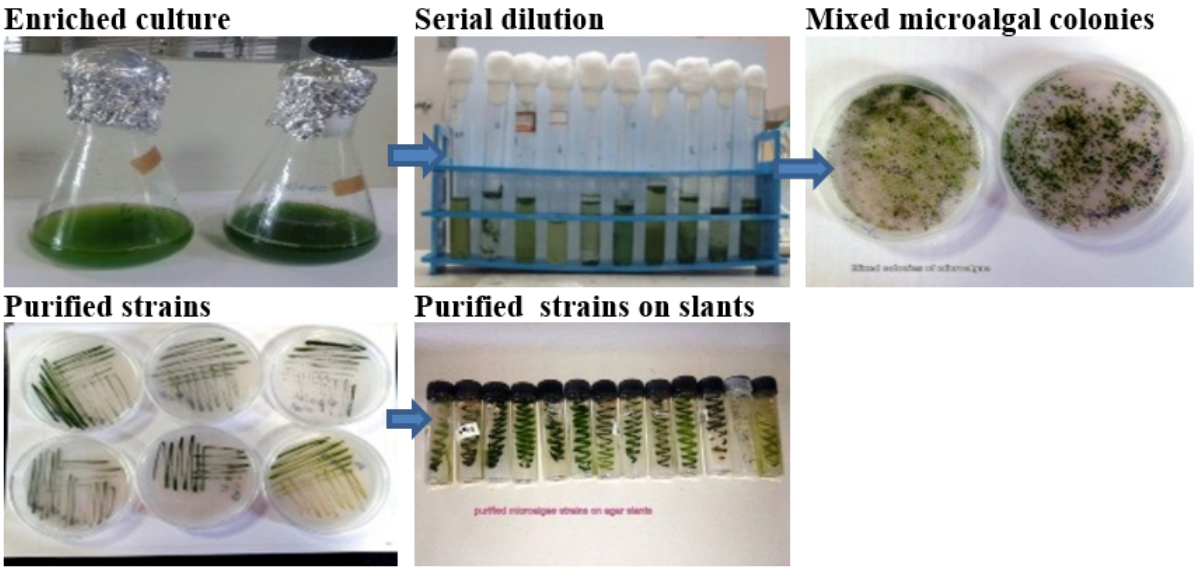

Figure 1

Schematic protocol for isolation and purification of algal strains

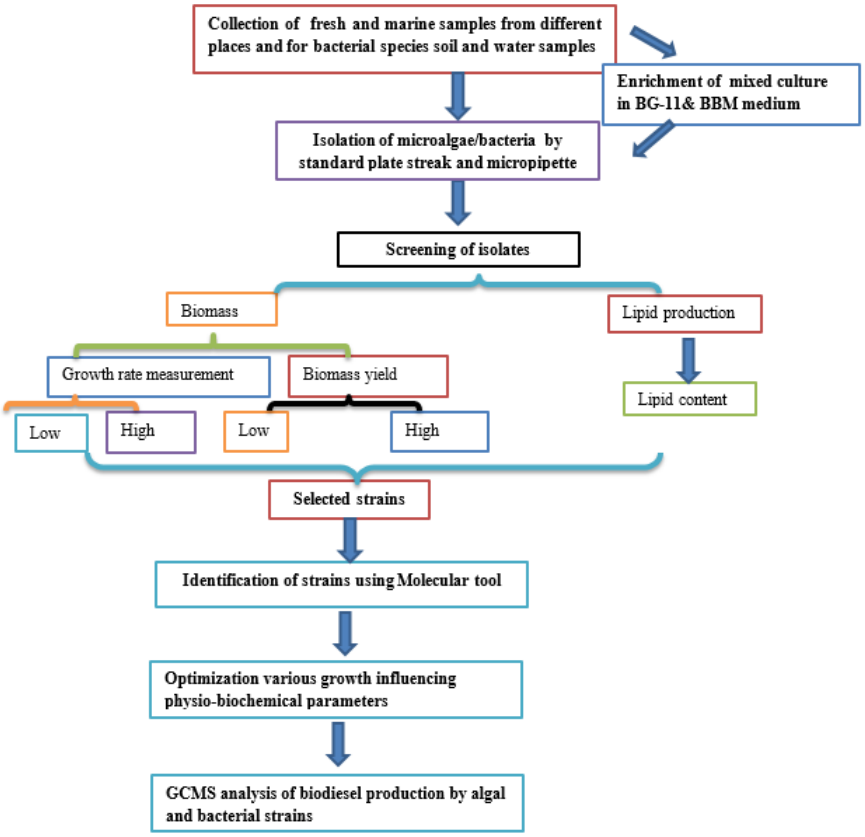

Figure 2

A flowchart showing screening process of promising biodiesel producing microalgae

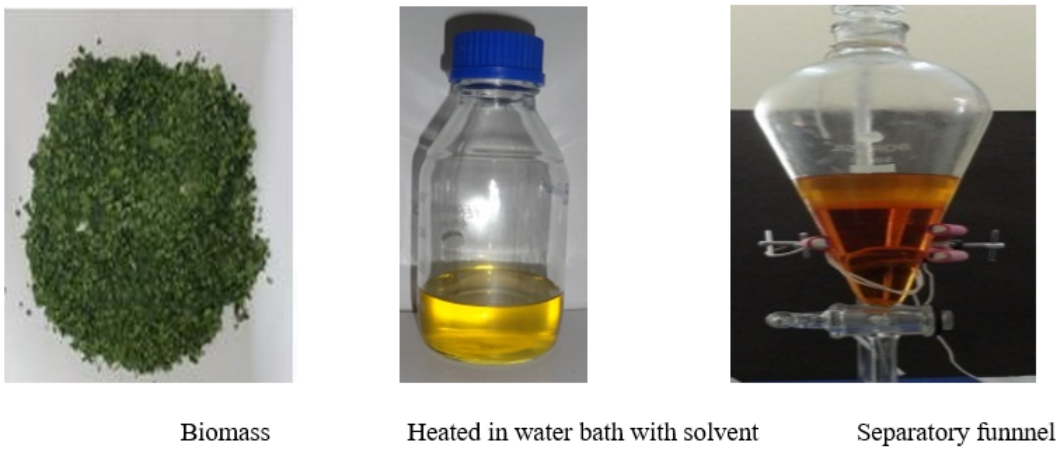

Figure 3

Schematic protocol for tranesterification process for lipid extraction

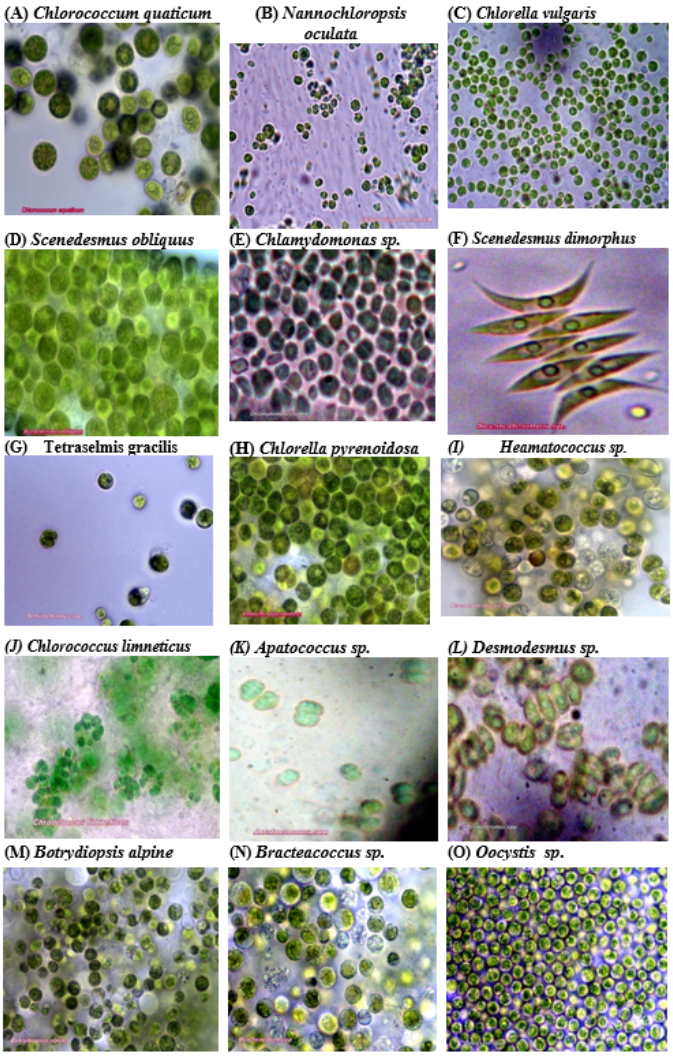

Figure 4

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.*

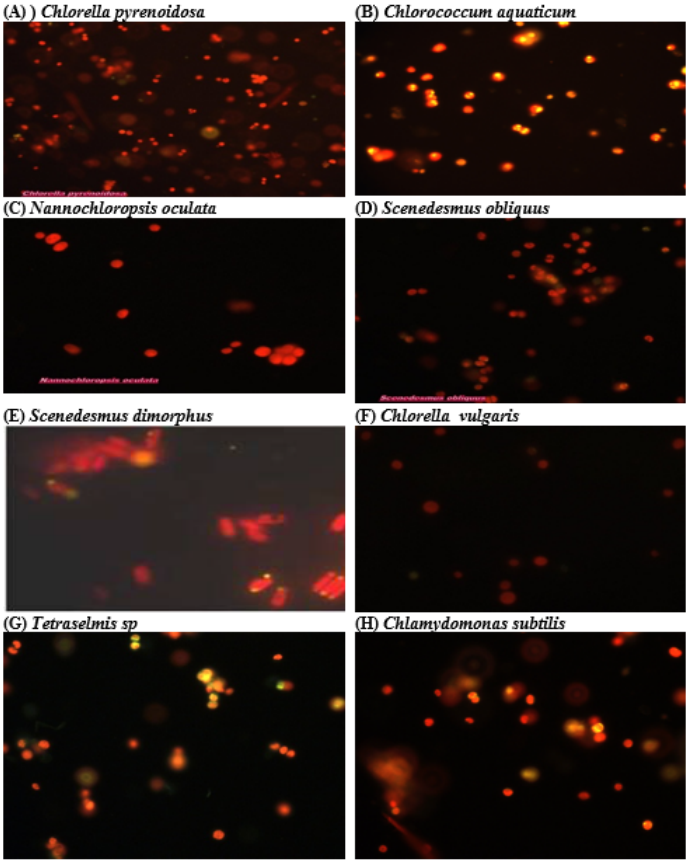

Figure 5

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*

Figure 6

Extracted lipid content from screened microalgal strains

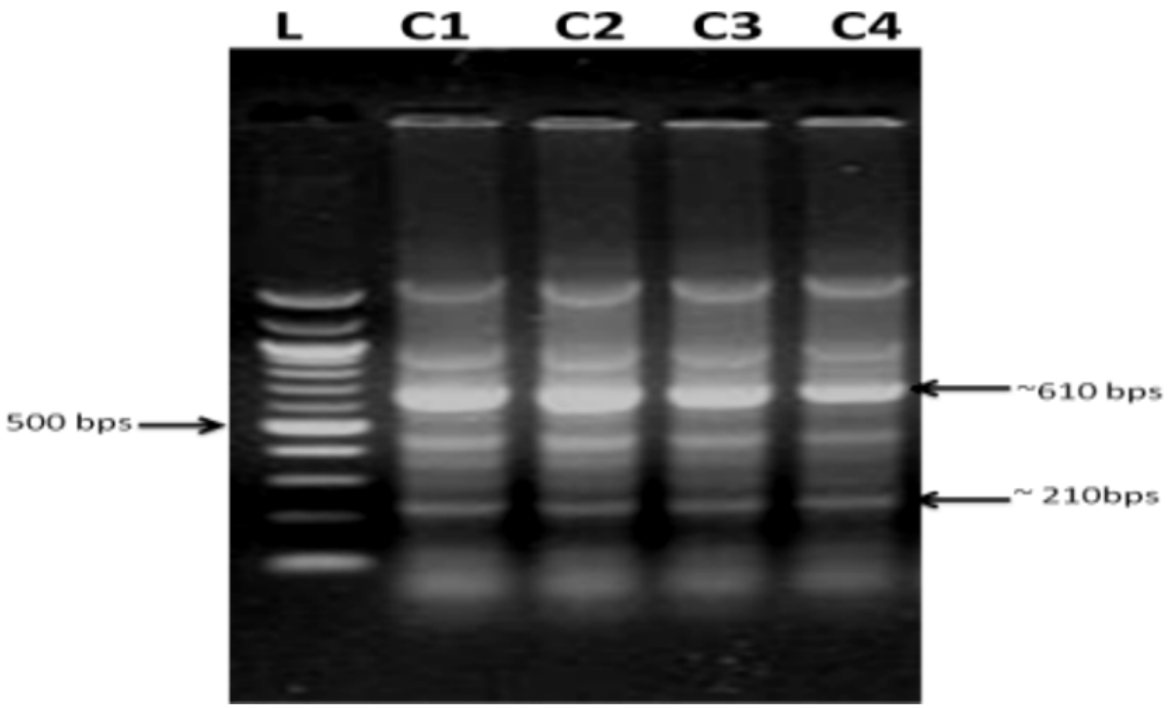

Figure 7

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.