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SCREENING OF INDIGENOUS MICROALGAL STRAINS FOR LIPID PRODUCTIVITY AND BIODIESEL POTENTIAL

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ABSTRACT

Microalgae have attracted significant attention from scientists and industry because they can produce large amounts of lipid, which can be used to make biodiesel. Most recent studies have focused on how to grow algae, select the best types, and characterise their properties to identify the most suitable for biodiesel production. This study explores the isolation, characterisation, and biodiesel potential of indigenous freshwater microalgal strains obtained from various ponds in Muzaffarpur, Bihar. Fourteen distinct strains were identified using morphological and molecular techniques, with *Botryococcus braunii (Lab Strain-MA10)* and *Scenedesmus obliquus (Lab Strain-MA5)* emerging as the most promising candidates for biofuel production. Detailed biochemical analyses revealed that *B. braunii* exhibited exceptionally high lipid content (22% dry cell weight) and optimal fatty acid profiles dominated by saturated and monounsaturated fatty acids, resulting in superior biodiesel stability and yield. In parallel, *S. obliquus* demonstrated the highest biomass yield (1.32g/L) and significant carbohydrate accumulation (0.75mg/mL), indicating suitability for integrated biofuel applications such as biodiesel and bioethanol. *C. vulgaris* (Lab Strain-MA1) demonstrated a lipid content of 16.98% DCW, and *Coelastrum sp. (Lab Strain-MA2) Lipid percentage DCW is* 18.57% when analysed using the Bligh and Dyer method. GC-MS profiling confirmed that selected strains produced key long-chain fatty acids (C16–C18), essential for high-quality fatty acid methyl ester (FAME) synthesis. This research supports future scaling-up, process optimisation, and bioengineering efforts aimed at large-scale biofuel production under Indian tropical conditions.

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Keywords: Microalgae, Biodiesel, Biomass productivity, Fatty acid methyl ester (FAME), Lipid content, GC-MS.

INTRODUCTION

The continuous rise in atmospheric carbon dioxide (CO_2) levels, coupled with the overexploitation of fossil fuels, has significantly affected the global climate (Omemen and Mona, 2025). The consequences are evident in pressing challenges such as the ongoing energy crisis and the heightened greenhouse effect. Such environmental concerns have brought the sustainability of present-day energy systems into question and underscored the pressing need for alternative, eco-friendly energy solutions. Furthermore, the unpredictable nature of crude oil prices, the projected exhaustion of petroleum reserves, and the ever-increasing global energy demand have collectively highlighted the urgency of identifying viable alternative fuels (Hanieh, 2024).

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Among these alternatives, biodiesel has attracted considerable attention due to its renewable, biodegradable, and cleaner-burning properties, making it a strong contender to replace conventional fossil fuels (Balwan and Kour, 2021). Biodiesel is most commonly synthesised via transesterification (Najeeb et al., 2021), a chemical process where triglycerides are converted into esters (biodiesel) using acid, base, or enzyme catalysts (Rozina et al., 2025). This reaction reduces the viscosity of the oil, enhancing its suitability as a fuel. The major obstacle to expanding biodiesel production lies in the costly feedstocks, accounting for nearly 70% of overall production expenses (Pydimalla et al., 2023). This economic challenge limits the commercial feasibility of biodiesel as a mainstream alternative to petroleum fuels (Sarwer et al., 2022). Although various feedstocks such as vegetable oils, *Jatropha curcas*, non-edible oils, and animal fats have been explored for this purpose, their broader application faces several obstacles (Riayatsyah et al., 2022). These include limited availability, land-use competition with food crops (raising the food vs. fuel debate), and the extensive land requirements for cultivation (Gheewala et al., 2025). These challenges significantly restrict the scalability of biodiesel as a sustainable and cost-effective energy source.

In contrast, microalgae-based biodiesel has emerged as a promising renewable energy source, offering numerous advantages over traditional biofuel feedstocks (Thanigaivel et al., 2022). Microalgae are particularly attractive due to their high lipid productivity, a critical factor for biodiesel yield (Thanigaivel et al., 2022). Unlike many terrestrial crops, microalgae are not subject to seasonal limitations (Holdmann and Hirth, 2019), enabling year-round biomass production and a more stable, consistent supply (Ahamefule et al., 2025). Another key advantage is that microalgae cultivation demands significantly less land and water (D bowski et al., 2020). It can even be conducted on non-arable land using water sources unsuitable for agriculture (Tahir et al., 2024). This reduces competition for essential agricultural resources. Additionally, microalgae exhibit a high photosynthetic efficiency, allowing them to rapidly convert sunlight into biomass (Kumar et al., 2021) while also capturing carbon dioxide, thereby offering the potential to mitigate greenhouse gas emissions (Ma et al., 2022). These features make microalgae a sustainable, eco-friendly, and scalable feedstock for biodiesel production.

Beyond energy applications, microalgae are also a valuable source of nutrients, including proteins, lipids, and bioactive compounds with diverse health benefits (Ampofo and Abbey, 2022). Protein, in particular, is essential for human nutrition, and the use of microalgae as an alternative protein source has received increasing attention (Barkia et al., 2019). India's tropical climate provides an ideal environment for the cultivation and proliferation of diverse algal species, giving the country a comparative advantage (Zuo et al., 2025). With a rich biodiversity that includes approximately 841 species of marine algae, India has been the focus of extensive research into microalgal strains suitable for biodiesel production (Aravindh et al., 2023)

Developing an efficient biodiesel production process involves two key steps: selecting high-performing strains and optimising their growth conditions (Li et al., 2020). In this context, numerous international efforts have been undertaken to screen large numbers of newly isolated microalgal strains tailored to specific local environmental conditions (Chiellini et al., 2020). Given its rich algal diversity, India holds great potential for advancing biofuel technologies (Maheshwari et al., 2020). Therefore, the present study aims to isolate, identify, and evaluate microalgal species from various areas of Muzaffarpur, Bihar ponds in order to identify strains with the highest lipid content, making them promising candidates for sustainable biodiesel production.

1. MATERIAL AND METHODS

1.1 Sample collection

Freshwater algal samples were carefully gathered from various sites in Muzaffarpur, Bihar, situated at a latitude of 25.0960°N and a longitude is 85.31311940. Including K1RQ ((Lat 26.108881°N Long 85.376921°E) Reader Quarter BU Campus B.R.A Bihar University Muzaffarpur , K2LF((Lat 26.108881°N Long 85.376921°E) Lecturers Flat B.U Campus, B.R.A Bihar university , K3BR (Lat 26.122672°N Long 85.372128°E) Butler Rd, Near radhe krishna mandir, tilak nagar, Muzaffarpur Bihar, K4SP (Lat 26.1229°N, Long 85.3930°E) Sahu Pokhar, Muhammadpur Kazi, Musahri Muzaffarpur, K5MM (Lat 26.0911°N, 85.4627°E) Manika Man bihar and Motipur pond Muzaffarpur. The collected freshwater samples were transported to the University Department of Botany laboratory, B.R.A. Bihar University Muzaffarpur, in ice-filled coolers to preserve sample integrity. Once in

the lab, they were filtered using Chemlab GF5 grade glass microfiber filters with a 0.7 m pore size. The filtered material was then subjected to an enrichment process by suspending it in liquid BG-11. These cultures were maintained at room temperature (27 ± 1°C) under continuous illumination using cool white fluorescent light for two weeks, with aeration supplied by air enriched with approximately 1.5% CO . Following enrichment, aliquots were streaked onto BG-11 agar plates and incubated under the same environmental conditions for at least four weeks, allowing for the development of distinct green colonies. Subculturing was performed repeatedly to obtain pure isolates, and their purity was validated through microscopic examination

2.2 Isolation of microalgae

The isolation of microalgae was carried out through two widely accepted methods, namely the plate streaking technique and the micropipette method (Indrayani, 2017). Both approaches utilised BG-11 medium, which is specifically formulated to support the growth of a broad range of freshwater and marine algae. These methods provided a nutrient-rich and controlled environment that facilitated the successful isolation of pure algal cultures, which were then used for further screening and analysis.

BG-11 medium contains essential macronutrients and micronutrients necessary for algal growth. The key components of the medium include sodium nitrate ($NaNO_3$) at 1.5 g/L, dipotassium hydrogen phosphate (K HPO) at 0.04 g/L, magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) at 0.075 g/L, and calcium chloride dihydrate ($CaCl_3 \cdot 2H_3O$) at 0.036 g/L. It also includes citric acid (0.006 g/L), ferric ammonium citrate (0.006 g/L), EDTA disodium salt (0.001 g/L), and sodium carbonate (Na_2CO_3) at 0.02 g/L. Additionally, 1 mL of trace element solution is added, which contains boric acid (H_3BO_3) at 2.86 g/L, manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$) at 1.81 g/L, zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) at 0.222 g/L, sodium molybdate dihydrate ($NaMoO_4 \cdot 2H_2O$) at 0.39 g/L, copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) at 0.079 g/L, and cobalt nitrate hexahydrate ($Co(NO_3)_2 \cdot 6H_2O$) at 0.0494 g/L. The medium is adjusted to a pH of 7.0 ± 1.

Initially, the algal colonies were enriched in sterilised BG-11 nutrient broth in 250 mL conical flasks and incubated under phototrophic conditions at 27 ± 1°C for 10 days. After enrichment, the cultures were serially diluted (10^{-1} to 10^{-10}) and spread onto BG-11 agar plates. These plates were incubated in a shaking cum incubator at 25 ± 2°C for 7 days. Selected colonies were carefully inoculated onto fresh BG-11 agar plates, with repeated streaking performed to ensure the establishment of a pure axenic culture.

The detailed stepwise protocol of the isolation and purification process is shown in Fig. 1. The purified microalgal cultures were then maintained in a culture room at 25 ± 2°C under cool white fluorescent light in liquid BG-11 medium for further experimentation.

Figure 1: Isolation and purification process of microalgae cultures.

2.3 Morphological observation and identification of microalgae strains

Morphological characteristics and cellular structures of the isolated microalgal strains were analysed using an Olympus binocular microscope at 100× magnification, with the observations summarised in Table 2. Identification was carried out with the aid of a standard algal identification manual. To maintain the purity of the cultures, regular subculturing was performed under sterile conditions. Microscopic observations were conducted at regular intervals to monitor culture consistency and confirm the absence of contamination.

2.4 Microalgae cultures

Four potential freshwater microalgal strains, MA5-Scenedesmus obliquus, MA10-Botryococcus braunii, MA2-Coelastrum sp and MA1-Chlorella vulgaris were

obtained as pure cultures through an initial isolation process. These strains originated from environmental samples collected at the designated sampling site. For cultivation, BG-11 medium was used, with all media freshly prepared and sterilised by autoclaving to ensure aseptic conditions. To initiate liquid cultures, 5 mL of inoculum derived from previously grown plate cultures was transferred into 50 mL of sterile medium in 250 mL Erlenmeyer flasks. The cultures were maintained at a constant room temperature of 27 °C, illuminated continuously using cool white fluorescent lighting, and aerated with ambient air supplemented with approximately 1.5% CO_2 to promote growth

2.5 Genomic identification and analysis of phylogenetic relationships among selected microalgae species

Genomic DNA was isolated using a modified CTAB (Cetyl trimethyl ammonium bromide) protocol (Kunyalung et al., 2021). The purity and concentration of the extracted DNA were assessed with a UV spectrophotometer at 280 nm. Algal strains with the highest potential for biodiesel production were selected through a combination of morphological observation and molecular analysis targeting the 18S rRNA gene. The obtained nucleotide sequences were compared using the NCBI BLAST tool. Subsequently, a phylogenetic tree was constructed based on the 18S rRNA gene sequences employing the Neighbour-Joining method in MEGA version 11 (EBI platform).

Table 1: Sites of water samples having algal growth from Muzaffarpur ponds.

SNo	Site Name	Location	Latitude	Longitude	Type of water	pH	Temperature	Turbidity (NTU)	Light Penetration(cm)	Algal Growth	Macrophytes Present
1	K1RQ	BU Campus, BRA Bihar University Muzaffarpur	26.108881°N	85.378921°E	Freshwater	7.2	28.5	12.4	45	Yes	Yes
2	K2JF	BU Campus, BRA Bihar University Muzaffarpur	26.108881°N	85.378921°E	Freshwater	7.5	29.0	10.8	50	Yes	Yes
3	K3ER	Butler Rd, radhe krishna mandir, tilak nagar, Muzaffarpur Bihar	26.122672°N	85.372128°E	Freshwater	6.9	30.2	15.2	30	Yes	Yes
4	K4SP	Shiv Pokhar, Mohammadpur Kazi, Mushri Muzaffarpur	26.1229°N	85.3980°E	Freshwater	7.8	27.5	9.1	55	Yes	Yes
5	K5VM	Manika Mandir and Motipur pond Muzaffarpur	26.0911°N	85.4627°E	Freshwater	7.1	28.8	13.7	40	Yes	Yes

Table 2: Preliminary identification of Microalgae strains and their morphological features.

Strain ID	Species Name	Habitat	Morphological Characteristics	Size	Family / Phylum	References
MA1	*Chlorella vulgaris*	Freshwater	Small, spherical, non-motile; thick, rigid cell wall; single cup-shaped chloroplast with chlorophyll a and b; pyrenoids present.	2–10 μm	Chlorellaceae / Chlorophyta	Arora and Sahoo, (2015)
MA2	*Coelastrum sp.*	Freshwater	Colonies hollow, spherical (4–64 cells); individual cells spherical to polygonal; parietal chloroplast with pyrenoid; non-motile.	2–30 μm	Scenedesmaceae / Chlorophyta	Minhas et al., (2023)
MA3	*Tetraselmis sp.*	Freshwater	Motile, with four equal flagella; elliptical to slightly flattened cells; single large cup-shaped chloroplast with four lobes; prominent pyrenoid; eyespot present	10–25 μm	Chlorodendraceae / Chlorophyta	Janßen, (2021)
MA4	*Pediastrum*	Freshwater	Flat, disc/star-shaped colonies (4–128 cells); fixed number of cells; cells in distinct symmetrical arrangement; peripheral horn-like projections; ornamented cell wall	20–200 μm (colony diameter)	Hydrodictyaceae / Chlorophyta	Chellappa et al., 2021
MA5	*Scenedesmus sp.*	Freshwater	Colonies of 2–32 cylindrical/elongate cells, often in rows; outer cells with long spines; single parietal chloroplast with pyrenoid; non-motile	3–78 μm	Scenedesmaceae / Chlorophyta	Alam et al., (2019)
MA6	*Characium sp.*	Freshwater	Solitary, attached via basal pad or stalk; cell shape variable (cylindrical, fusiform, ovoid); single parietal chloroplast with central pyrenoid	11–45 μm	Characiaceae / Chlorophyta	Aziz, (2020)
MA7	*Oedogonium sp.*	Freshwater	Unbranched filaments, attached by holdfast cell; cylindrical vegetative cells; cell ends with ring-like caps; thick multilayered wall; reticulate chloroplast, pyrenoids	Variable	Oedogoniaceae / Chlorophyta	Büdel and Friedl, (2024)
MA8	*Hydrodictyon*	Freshwater	Large net-like, hollow cylindrical colonies (pentagonal/hexagonal mesh); multinucleate cylindrical cells; parietal reticulate chloroplast; many pyrenoids	Colonies 20–80 cm; cells 20–50 μm	Hydrodictyaceae / Chlorophyta	Zhang and Luo, (2022)
MA9	*Zygnema sp.*	Freshwater	Unbranched filaments; vegetative cells with two stellate (star-shaped) chloroplasts; conjugation by scalariform or lateral means	20–35 μm wide, 30–50 μm long	Zygnemataceae / Charophyta	Feng et al., (2021)
MA10	*Botryococcus braunii*	Freshwater	Colonies of cells embedded in mucilaginous matrix; irregular clusters; cells obovoid; parietal chloroplast; hydrocarbon production	Colonies up to 100 μm; cells 6–14 μm	Botryococcaceae / Chlorophyta	Weiss et al., (2012)
MA10	*Botryococcus braunii*	Freshwater	Colonies of cells embedded in mucilaginous matrix; irregular clusters; cells obovoid; parietal chloroplast; hydrocarbon production	Colonies up to 100 μm; cells 6–14 μm	Botryococcaceae / Chlorophyta	Weiss et al., (2012)
MA11	*Chlorococcum sp.*	Freshwater	Solitary/small group, ellipsoidal to spherical; smooth wall; parietal chloroplast with one/more pyrenoids; zoospore motility (with 2 flagella in motile phase)	5–20 μm	Chlorococcaceae / Chlorophyta	Temraleeva and Moslalenko, 2019
MA12	*Pithophora sp.*	Freshwater	Slender, cylindrical filaments; thick, non-layered wall; reticulate chloroplast with many pyrenoids; terminal cell conical/rounded; prominent akinetes	Length 1,100–1,450 μm; width 50–120 μm	Pithophoraceae / Chlorophyta	HARITHA, (2024)
MA13	*Spirogyra sp.*	Freshwater	Unbranched filamentous; mucilaginous sheath; ribbon-shaped spiral chloroplasts; multiple pyrenoids; central nucleus, large vacuole	Width 10–100 μm; length up to several cm	Zygnemataceae / Charophyta	Jana, (2024)
MA14	*Ulothrix sp.*	Freshwater	Unbranched filaments, cylindrical/barrel-shaped cells; basal holdfast; girdle-shaped parietal chloroplast with pyrenoid; thickens with age, some mucilage	Variable	Ulotrichaceae / Chlorophyta	Pichrtová, (2015)

Table 3: Preliminary identification of Microalgae strains and their morphological features.

Strain ID	Species Name	Biomass Yield (g/L)	Lipid (% DCW)	Lipid Extract(g)	Protein (mg/mL)	Carbohydrate	Total Chlorophyll (mg/mL)
MA11	Chlorococcum sp.	0.95	11.32	0.1075 g	0.053 ± 0.002	0.46 ± 0.024	11.02 ± 0.041
MA5	Scenedesmus obliquus	1.32	13.64	0.1792 g	0.051 ± 0.007	0.75 ± 0.031	14.32 ± 0.034
MA10	Botryococcus braunii	~1.00	22	0.22	~0.020 ± 0.010	~0.40 ± 0.10	~5.00 ± 1.00
MA2	Coelastrum sp.	~0.70	18.57	0.13g	~0.050 ± 0.010	~0.60 ± 0.10	~11.00 ± 2.00
MA3	Tetraselmis sp.	~0.90	~11.00	0.099 g	~0.040 ± 0.010	~0.50 ± 0.10	~10.00 ± 2.00
MA4	Pediastrum sp.	~0.70	~10.00	0.07 g	~0.030 ± 0.010	~0.40 ± 0.10	~8.00 ± 1.00
MA13	Spirogyra sp.	~0.70	~7.00	0.049 g	~0.030 ± 0.010	~0.30 ± 0.10	~7.00 ± 1.00
MA1	Chlorella vulgaris	0.53	16.98	0.090 g	0.036 ± 0.002	0.25 ± 0.024	8.55 ± 0.017
MA14	Ulothrix sp.	~0.70	~7.00	0.049 g	~0.030 ± 0.010	~0.30 ± 0.10	~7.00 ± 1.00

Table 4: Comprehensive Evaluation of Physio-Biochemical and Biodiesel-Related Parameters of Selected High-Lipid Microalgae Strains.

Strain ID	Species Name	Biomass Yield (g/L)	Lipid (% DCW)	Lipid Productivity (g/L/day)	Protein (mg/mL)	Carbohydrate (mg/mL)	Total Chlorophyll (μg/mL)	FAME C16–C18 (%)	Saponifiable Lipid (%)	Unsaponifiable Matter (%)	Transesterification Efficiency (%)	Biodiesel Obtained
MA10	*Botryococcus braunii*	~1.00	22%	~0.22	~0.02	~0.40	~5.00	80–90	70–90	10–20	85%	0.187g
MA2	*Coelastrum* sp.	~0.70	18.57%	~0.13	~0.05	~0.60	~11.00	70–85	70–80	10–15	85%	0.1105
MA5	*Scenedesmus obliquus*	1.32	13.64%	0.179	0.051	0.75	14.32	65–80	65–80	15–20	85%	0.153
MA1	*Chlorella vulgaris*	0.53	16.98%	~0.09	0.036	0.29	8.55	60–78	60–75	15–22	85%	0.0765

Analytical Methods for bio-actives compounds extraction

3.1 Total lipid estimation

The total lipid content, expressed as a percentage of Dry Cell Weight (DCW), was estimated using a modified version of the Bligh and Dyer extraction method (Alam et al., 2019). Algal biomass was first harvested through centrifugation, followed by treatment with a methanol–chloroform mixture in a 2:1.5 (v/v) ratio to extract lipids. The organic phase containing the extracted oil was then separated, dried, and weighed to determine the lipid yield. The oil extraction efficiency (w/w) was calculated using the method outlined by Mwaurah et al. (2020).

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

This method enabled the quantification of lipid content, providing a crucial parameter for assessing the biodiesel potential of each algal strain.

3.2 Total Chlorophyll estimations

Photosynthetic pigment estimation was performed using a modified MacKinney (1941) method (Market, 1972). Initially, the algal cultures were centrifuged to collect the biomass, which was then homogenised in a known volume of methanol to extract the pigments. The mixture was centrifuged at 5000 rpm for 10 minutes to separate the supernatant, after which the chlorophyll content was quantified spectrophotometrically. The total chlorophyll concentration (mg/mL) was calculated using the following formula:

$$\textit{Total chlorophyll (mg/mL)} = (2.55 \times 10^{-2} \times E_{650}) + (0.4 \times 10^{-2} \times E_{665}) \times 10^{3}$$

where E_{650} and E_{665} represent the absorbance values at 650 nm and 665 nm, respectively. This method allowed for the precise quantification of chlorophyll, which is a critical parameter for evaluating algal growth and photosynthetic efficiency.

3.3 Total carbohydrate and protein estimation

The glucose concentration of the centrifuged algal biomass was determined through a modified Anthrone reagent method, with absorbance readings taken at 625 nm using a spectrophotometer. For protein quantification, a modified Lowry method was utilised. Bovine Serum Albumin (BSA) was used to generate a standard curve, and the protein content was expressed in milligrams per millilitre (mg/mL). The equation for the standard curve was:

$$y = 0.1097x - 0.0005, \text{ with } R^2 = 0.9989$$

where y represents the absorbance and x the protein concentration (mg/mL). This high correlation coefficient indicates excellent linearity and accuracy of the protein estimation method.

3.4 Extraction of fatty acid methyl esters (FAME) through transesterification and their analysis using GC-MS

A total of 500 mg of lyophilised algal biomass was transferred into a reagent bottle, followed by the addition of 10 mL of hexane and thorough mixing. The mixture was then incubated in a water bath at 50 °C for 1 hour to promote lipid extraction. Following heating, the mixture was transferred to a separating funnel. The supernatant obtained after centrifugation at 10,000 rpm for 10 minutes was collected, and the extracted lipids were subjected to GC–MS analysis.

***3.5* Statistical analysis**

Statistical analysis was performed using one-way ANOVA with multiple factors, followed by Duncan's multiple range test to determine significant differences between groups. The analysis was conducted using SPSS software (version 21.0). Differences were considered statistically significant at $p < 0.05$.

4. RESULTS AND DISCUSSION

4.1 Collection of samples having microalgae growth

A total of fourteen freshwater samples were obtained from different aquatic sources in Muzaffarpur, Bihar, as outlined in Table 1. Microalgae display extensive biodiversity and are capable of thriving in diverse habitats such as freshwater, brackish waters, lakes, and hypersaline environments. Previous studies on lipid-producing microalgae from diverse locations have shown that the sampling environment plays a crucial role in both strain selection and the ability of strains to survive. For isolation, the collected algal samples were initially diluted. Sterile petri dishes containing approximately 50 mL of solidified agar media were used to plate the diluted samples. One millilitre of each diluted sample was evenly spread over the surface of the agar media. Standard microbiological techniques, such as streaking and micropipette plating, were applied to isolate individual algal colonies from the environmental samples. The isolation process was carried out using BG-11 medium, with cultures incubated under continuous illumination at 3000 lux and maintained at a temperature of 25±2°C in a shaking cum-incubator. This streaking was repeated until axenic unialgal cultures were successfully established. In total, 14 algal strains were isolated from five distinct freshwater sites. Among them, three strains were derived from the site K1RQ, while site K2LF yielded two strains. The site K3BR contributed one strain. Notably, the highest diversity was recorded at the site K4SP and site K5MM locations, with each providing four distinct strains. This distribution underscores the significant variation in algal richness and presence across the surveyed freshwater ecosystems. All isolates were identified based on their morphological traits and microscopic cellular structures. The recovered strains ranged from unicellular to filamentous forms. The purified cultures were transferred to freshly prepared BG-11 medium and routinely maintained under controlled light and temperature conditions in the culture room.

4.2 Identification of isolated and purified microalgal strains

Fourteen purified microalgal strains were identified at the initial stage by observing their cell shape, natural habitat, and lipid content. The observations were done using a Magnus (CH20i) binocular microscope with a digital camera, along with the help of an algal identification manual. The majority of the isolates were identified at the genus level based on microscopic examination of their morphological characteristics. Distinct microalgal strains, ranging from unicellular to filamentous forms, were recognised as outlined in Table 2. Subsequent quantitative screening was performed using biomass yield and lipid content as selection criteria. Growth

rate and biomass concentration of each strain were evaluated using standard analytical methods, and those with higher lipid content and biomass yield were chosen for further study. The selected indigenous strains were then validated through molecular techniques to confirm their identity. In addition, biomass yield along with lipid, protein, carbohydrate, and total chlorophyll contents were measured in the pre-screened strains, with detailed results summarized in Table 3. The lipid, biomass, and other cellular components of several algal strains were estimated and compared, as presented in Table 3. Based on superior physiobiochemical characteristics, four promising biodiesel-producing strains were selected for further study: strain-MA5 (*Scenedesmus obliquus*), strain-MA10 (*Botryococcus braunii*), strain-MA2 (*Coelastrum* sp.), and strain-MA1 (*Chlorella vulgaris*). Among the pre-screened isolates, *S. obliquus* (strain-MA5) exhibited the highest biomass yield (1.32). Lipid extraction was performed using a modified Bligh and Dyer method (Supplementary Fig. 2). Notably, *B. braunii* (Strain-MA10) showed the highest lipid content, ranging between 22% DCW (Table 3). Several previous studies have similarly employed solvent-based extraction methods for lipid screening in microalgae. In the present study, *C. vulgaris* (Strain-MA1) demonstrated a lipid content of 16.98% DCW, and *Coelastrum sp. (Strain-MA2) Lipid percentage DCW is* 18.57% when analysed using the Bligh and Dyer method.

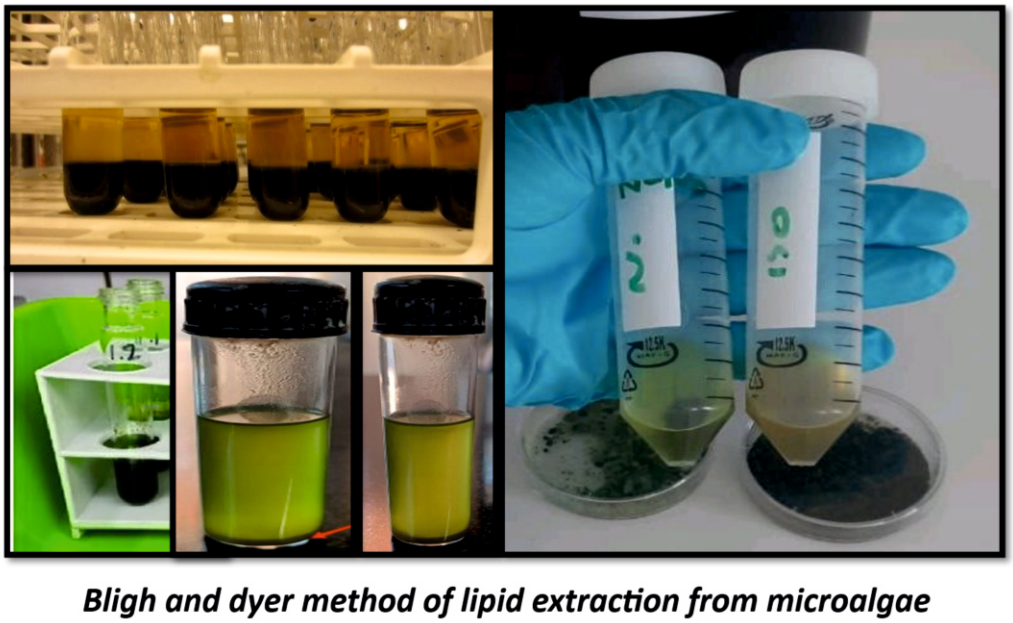

Fig 2.: Extracted lipids from selected microalgae by Bligh and dyer method.

4.3 Protein, Carbohydrate and total chlorophyll content of screened microalgae strains

Among the reported strains, *Scenedesmus obliquus* (MA5) exhibited the highest chlorophyll content (14.32 μg/mL), as shown in Table 3. Microalgal biomass is widely recognised not only as a sustainable food and feed source but also as a rich reservoir of valuable co-products. These include chlorophyll, polysaccharides, fucoidans, phycocyanin, β-carotene, β-1,3-glucan, agar, phycobiliproteins, lutein, and alginates, all of which are gaining increasing industrial and commercial importance. Under the experimental conditions of this study, *S. obliquus* (MA5) also recorded the highest carbohydrate content (0.75 mg/mL) among the analysed strains (Table 3), whereas *Chlorella vulgaris* (MA1) exhibited the lowest carbohydrate content (Table 4). Carbohydrate accumulation in microalgae is highly dependent on cultivation conditions, with nutrient or nitrogen limitation often stimulating increased carbohydrate reserves. Microalgae primarily accumulate starch as their main carbohydrate reserve within cellulose-based cell walls.

***4.4* PCR amplification, DNA sequencing and Blast homology search for screened microalgae strains**

The most promising algal strains for biodiesel studies were identified using the 18S rRNA approach. To analyse genetic variation, Random Amplified Polymorphic DNA (RAPD-PCR), a modified

fingerprinting technique, was used to analyse DNA samples extracted from four algal strains. Multiple amplified products were obtained, and at least 2 bands were excised from the agarose gel for DNA elution. The eluted DNA was subsequently used for sequencing. The 18S rRNA gene of the screened strains was amplified from genomic DNA using a set of RAPD primers. Several amplification products were obtained, and those selected for cloning and sequencing were distinctly marked. The purified PCR products were sequenced at Dextrose Technologies Lab (Bengaluru). The resulting sequences were analysed using the BLAST tool and compared against the GenBank nucleotide database. The culture, which was labelled as Lab Strain-MA10, was found to be *Botryococcus braunii* strain OIT-844 18s rRNA gene (GeneBank Accession Number: *LC702211.1).* Lab strain-MA2 was found to be *Coelastrum sp. Strain SEG-3* 18s rRNA gene (GeneBank Accession No. MF401431.1), Lab strain-MA5 was found to be *Scenedesmus sp. Strain* AS-6-1 18s rRNA gene (GeneBank Accession No. HE717102.1) and Lab strain-MA1, was found to be *Chlorella pyrenoidosa* strain (KB4) 18s rRNA gene (GeneBank Accession No. KU236002), based on nucleotide homology and phylogenetic analysis

4.5 GC-MS ANALYSIS

The GC-MS analysis of fatty acid methyl esters (FAMEs) extracted from *Botryococcus braunii*, *Coelastrum* sp., *Scenedesmus obliquus*, and *Chlorella vulgaris* revealed the presence of a diverse range of saturated (SFAs), monounsaturated (MUFAs), and polyunsaturated fatty acids (PUFAs), with notable variation among species. Palmitic acid (C16:0) was the most abundant saturated fatty acid detected across all four strains, with *Coelastrum* sp. showing the highest relative content (52.10%), followed by *Scenedesmus obliquus* (41.21%) and Chlorella vulgaris (35.81%). Among the monounsaturated fatty acids, oleic acid (C18:1) and palmitoleic acid (C16:1) were predominant, with notably high proportions in *Botryococcus braunii* (20.64% and 21.44%, respectively), indicating strong potential for biodiesel quality enhancement due to their oxidative stability. In contrast, *Coelastrum* sp. displayed significant levels of linolenic acid (C18:3) and heptadecenoic acid (C17:1), contributing to its relatively high PUFA content (22.71%).

The presence of long-chain fatty acids such as arachidic acid (C20:0) and behenic acid (C22:0) further supports the diverse lipid profile of these strains. Notably, certain fatty acids like caprylic acid (C8:0) and erucic acid (C22:1) were not detected (ND) in any of the species, indicating their absence or presence below detection limits. Overall, the variation in fatty acid composition suggests species-specific metabolic pathways and highlights *Botryococcus braunii* and *Coelastrum* sp. as promising candidates for biodiesel production due to their favourable SFA and MUFA profiles. *Chlorella vulgaris* is a viable but not optimal microalgal species for biodiesel due to its moderate MUFA content and relatively high PUFA levels, which limit fuel stability and quality compared to more suitable strains like *Botryococcus braunii*.

Table 5: GC-MS Analysis of Microalgal Strain.

S.No	Common Name	IUPAC Name	Abbreviation	Molecular Formula	Botryococcus braunii	Coelastrum sp.	Scenedesmus obliquus	Chlorella vulgaris
1	Caproic acid	Hexanoic acid	C6:0	C6H12O2	7.903	0.21	0.5	ND
2	Caprylic acid	Octanoic acid	C8:0	C8H16O2	ND	ND	ND	ND
3	Undecanoic acid	Undecanoic acid	C11:0	C11H22O2	0.02	ND	0.04	6.27
4	Lauric acid	Dodecanoic acid	C12:0	C12H24O2	ND	1.21	ND	2.53
5	Lauric acid (unsat.)	Dodecanoic acid	C12:1	C13H26O2	1.3	ND	1.33	1.26
6	Myristic acid	Tetradecanoic acid	C14:0	C14H28O2	0.99	1.23	ND	0.33
7	Pentadecanoic acid	Pentadecanoic acid	C15:0	C15H30O2	0.68	ND	1.61	1.55
8	Pentadecenoic acid	cis-10-Pentad ecenoic acid	C15:1	C15H28O2	ND	ND	4.5	ND

9	Palmitic acid	Hexadecanoic acid	C16:0	C16H32O2	27.082	52.10	41.21	35.81
10	Palmitoleic acid	cis-9-Hexad-ecenoic acid	C16:1	C16H30O2	21.44	2.661	12.429	10.05
11	Margaric acid	Heptadecanoic acid	C17:0	C17H34O2	ND	ND	1.967	1.15
12	Heptadecenoic acid	cis-10-Heptad-ecenoic acid	C17:1	C17H32O2	ND	10.34	6.67	ND
13	Stearic acid	Octadecanoic acid	C18:0	C18H36O2	9.86	17.21	23.04	1.782
14	Oleic acid	cis-9-Octad-ecenoic acid	C18:1	C18H34O2	20.64	12.602	2.719	19.72
15	Linoleic acid	cis-9,12-Octadecadienoic acid	C18:2	C18H32O2	7.91	10.01	10.780	9.29
16	Linolenic acid	cis-9,12,15-Octadecatrienoic acid	C18:3	C18H30O2	4.181	12.7	1.21	12.62
17	Nonadecanoic acid	Nonadecanoic acid	C19:0	C19H38O2	ND	0.1	0.3	0.483
18	Arachidic acid	Eicosanoic acid	C20:0	C20H40O2	2.7	4.76	ND	6.395
19	Behenic acid	Docosanoic acid	C22:0	C22H44O2	ND	ND	1.73	ND
20	Erucic acid	cis-13-Docosenoic acid	C22:1	C22H42O2	ND	ND	ND	ND
21	Heptacosylic acid	Heptacosanoic acid	C27:0	C27H56O2	ND	ND	ND	0.166

Table 6: Comparison of Fatty Acid Composition in Selected Microalgae Species.

Fatty Acid Type	Botryococcus braunii	Coelastrum sp.	Scenedesmus obliquus	Chlorella vulgaris
SFAs %	45.83	69.31	66.21	46.37
MUFAs %	42.08	25.60	21.80	31.77
PUFAs %	10.76	22.71	11.99	21.96

5. CONCLUSION

The present study successfully isolated, cultured, and characterised a diverse array of freshwater microalgal strains from Muzaffarpur, Bihar, to identify high-performing candidates for biodiesel production. Among the strains studied, Botryococcus braunii (MA10) emerged as the most promising for biodiesel applications, exhibiting exceptionally high lipid content (22% DCW) and biodiesel yield (0.187 g), substantial lipid productivity (~0.22 g/L/day), and favorable fatty acid profiles predominantly comprising saturated (45.83%) and monounsaturated fatty acids (42.08%), which collectively ensure high oxidative stability and good cold flow properties of the resulting biodiesel. In addition, *Scenedesmus obliquus* (MA5) demonstrated the highest biomass yield (1.32g/L) and a remarkable carbohydrate content (0.75mg/mL) and biodiesel yield (0.153 g), suggesting its dual potential in integrated biofuel strategies, including both biodiesel and bioethanol production. Lipid productivity and carbohydrate content varied among strains, indicating different metabolic profiles. GC-MS analysis further confirmed that these strains produce C16–C18 and long-chain fatty acids, such as palmitic, stearic, and oleic acids, which are desirable for FAME synthesis. The molecular identification via

18S rRNA sequencing and RAPD-PCR validated the taxonomic positioning of these strains, ensuring genetic authenticity and reproducibility. While other species, such as *Coelastrum sp*. and *Chlorella vulgaris,* exhibited moderate lipid content and biochemical traits, their relatively high PUFA levels may limit biodiesel fuel quality when compared to SFA and MUFA-dominant strains. Overall, this study underscores the untapped potential of indigenous microalgae as valuable bioresources for third-generation biofuels. The findings also pave the way for future work focused on optimising cultivation parameters, enhancing lipid induction through stress modulation or genetic engineering, and scaling up production systems to develop economically viable and environmentally sustainable algal biofuel technologies tailored to tropical regions like India.

DECLARATION OF COMPETING INTEREST

The authors declare no competing financial interests or personal relationships that could have influenced the work presented in this paper.

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