Morpho-taxonomic, genetic, and biochemical characterization of freshwater microalgae as potential biodiesel feedstock

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Abstract

In the present study, seven axenic fresh water microchlorophytes were isolated and identified as *Tetradesmus dimorphus* (NEIST BT-1), *Chlorella sorokiniana* (NEIST BT-2), *Desmodesmus* sp. (NEIST BT-10), *Selenastrum* sp. (NEIST BT-A6), *Tetradesmus obliquus* (NEIST BT-A1), *Tetradesmus* sp. (NEIST BT-A10), and *Asterarcys* sp. (NEIST BT-A15) based on morphological and molecular characterization. Their potential to be used as biodiesel feedstock was evaluated depending on their growth characteristics and lipid profiles. Among the seven isolates, NEIST BT-2 was found to be the most promising candidate owing to its high biomass yield ($2.09 \pm 0.037 \text{ g L}^{-1}$) and lipid productivity ($107.60 \pm 10.175 \text{ mg L}^{-1} \text{ day}^{-1}$). The gas chromatography analysis confirmed the presence of significant amounts of palmitic acid, linoleic acid, linolenic acid, and oleic acid in the isolate which are some of the major constituents of any biodiesel. The predictive models showed that the biodiesel from this isolate has ideal fuel properties which comply with the ASTM D6751 and EN 14214 specifications. These findings demonstrate that NEIST BT-2 can be used as a prospective candidate for consideration of large-scale biodiesel production.

Keywords Biodiesel properties · Confocal microscopy · Fatty acid profiling · Lipid · Phylogeny

Abbreviations

ASTM	American society for testing and materials
BP	Biomass productivity
CFPP	Cold filter plugging point
CN	Cetane number
CP	Cloud point
DCM	Dry cell mass
DMSO	Dimethyl sulfoxide
DU	Degree of unsaturation
EN	European Standards

FAME	Fatty acid methyl ester
FID	Flame ionization detector
GCV	Gross calorific value
IV	Iodine value
LC	Lipid content
LCSF	Long-chain saturated factor
LP	Lipid productivity
MUFA	Monounsaturated fatty acid
NCV	Net calorific value
OD	Optical density
PUFA	Polyunsaturated fatty acid
SEM	Scanning electron microscope
SFA	Saturated fatty acid
SV	Saponification value

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Introduction

Increasing population has coupled itself with increased consumption of fossil fuel, which questions the future sustainability of these resources. A report released by British Petroleum (BP) on Statistical Review of World Energy, 2016 documented that world's petroleum demand has increased by

2% with diesel alone showing an increase of 1.2% from 2014 to 2015. This demand is further expected to increase up to 40% by 2050 (British Petroleum 2017). It has been estimated that, if the demand increases continuously at this staggering rate, the available oil reserves will be exhausted within 50 years. Apart from sustainability, environmental toxicity is another concern associated with the use of conventional fuels. Recent studies also have reported that the combustion of oil resulted in a 1.6% higher emissions of carbon dioxide in 2015 (British Petroleum 2017). To overcome these issues, scientists have come up with the idea of using microalgae as a renewable source of green energy (biodiesel), that can fulfill the global energy demand.

Microalgae are one of the most diverse groups of organisms, existing on the earth's crust for more than 3 billion years. The high adaptability potential of microalgae to withstand any adverse condition gives it an advantage to occupy almost all the types of ecosystems, providing us wide opportunities to screen them for different metabolites and bioactive compounds (Wu et al. 2015; Dantas et al. 2019). Being a high lipid accumulator, microalgae are considered potential feedstock for production of biodiesel. Microalgae are reported to accumulate an average of approximately 25% of lipid as storage lipid, which can further be enhanced to 55% on exposure to certain environmental stress (Yu et al. 2011; Jia et al. 2014). Other than the lipid content, the fatty acid composition of microalgae is considered promising for conversion to biodiesel as they contain high amount of saturated and monounsaturated fatty acids, which influence various properties of a biodiesel, like density, cetane number, kinematic viscosity, higher heating value, iodine value, cold flow plugging point, and oxidative stability (Hoekman et al. 2012; Talebi et al. 2013). Another advantage of using microalgae is its high biomass production ability per unit area of land compared to any traditional energy-plant sources (Mata et al. 2010; Ghosh et al. 2016). However, these properties are not consistent among all the species of microalgae, due to the influence of their surroundings and occurrence of evolutionary pressure. It has been estimated that more than 50,000 species of microalgae are present on earth, out of which 35,000 have been reported till date and only 15,000 are made in use (Borowitzka 2013; Marcel Martinez-Porchas 2017), although algal culture collections are considered as a good source for microalgae studies, but out of 50,000 species, expected to exist on earth, whereas only a few thousands are stored (Mondal et al. 2017). Thus, isolation, identification, and characterization of microalgae from different habitats should be a continuous effort.

In spite of various advantages associated with the use of microalgae as a biodiesel feedstock, major constrain remains in commercializing biofuel from microalgae to provide competitive cost with the other petroleum-based fuel or any other biofuel (Hannon et al. 2010; Mallick et al.

2016; Naghshbandi et al. 2019). Thus, to use algae as an efficient alternative energy source for biodiesel production, the challenge remains to increase both lipid productivity and biomass concentration. Meanwhile, there have been other efforts to increase biomass production as well as the lipid content of a microalgae through various genomic and proteomic approaches that will uncover the biological implications and pave the future tailored manipulation of microalgae for broader industrial applications (Anand et al. 2017; Banerjee et al. 2017; Li et al. 2019). However, for any kind of modifications to be made at the molecular level, the first step is to screen microalgae strains from different habitats and examine them for their inherent properties to be used as an ideal strain for biodiesel production (Jagadevan et al. 2018).

Microalgal classification and their taxonomic positioning have always been a challenging task. For a long time, the identification of microalgae has been done based on their morphological and cytological features of vegetative stages during their life cycle (Dariencko et al. 2015). However, due to a limited set of morphological features and phenotypic plasticity existing among species, these characters are not reliable enough and can result in misinterpretation when samples from two different environments are examined. To overcome this challenge, genetic identification of microalgae based on the evaluation of some of the specific sites on their genome has been considered as an important molecular tool. 18S rDNA and internal transcribed spacer (ITS) region of nuclear genome are two of the most commonly used regions for taxonomically differentiating microalgal species from one another. Other than 18S rDNA, 16S rDNA and *rbcL* region has also been used effectively in some studies, due to their conserved nature and high sensitivity of detection (Ahmad et al. 2013; Sun et al. 2017; Wang et al. 2016a). Hadi et al. (2016) suggested, *nuITS1* or *nuITS2* as suitable markers for DNA barcoding of freshwater green algae, with *nuITS2* having distinct advantages over *nuITS1*, due to the larger accessibility of analytical tools and a priori defined reference barcodes deposited at databases for this marker (Bérard et al. 2005).

The present study aimed at identifying, characterizing, and evaluating different freshwater microchlorophytes from the Eastern Himalayan biodiversity region of India as biodiesel feedstock. A total of seven axenic cultures were evaluated for their growth behavior, lipid profiling, and fatty acid composition. The biodiesel obtained from these isolates was also investigated for some of the very important fuel properties, like cetane number, iodine value, cloud point, etc., using the predefined predictive models. Moreover, the study gives a baseline data on microalgal identification using morphological and genetic (*rbcL* and ITS) characterization. Thus, the present research carries both fundamental and applied importance in the field of green energy.

Materials and methods

Isolation and identification of microalgae

The microalgae strains were collected from different fresh-water bodies of Upper Brahmaputra valley region of Assam, India. The collected algae cultures were grown in BG-11 liquid media (C3061 SIGMA) and then transferred to BG-11 agar plate-containing antibiotics (ampicillin – 100 µg mL⁻¹, chloramphenicol – 25 µg mL⁻¹ and amphotericin B – 2.5 µg mL⁻¹) to avoid bacterial and fungal contamination. The colonies grew on the agar plates were then transferred to BG-11 agar plates with no antibiotics. Single algal colonies were picked from the agar plates using sterilized micro-tips and were then inoculated to 1 mL BG-11 liquid media. When the microalgae started to form homogenous culture, they were allowed to grow in 250 mL conical flask-containing 100 mL BG-11 liquid media. The cultures were grown for 1–3 weeks at 25 ± 1 °C under a light intensity of 80 µmol s on 16:8 h (light: dark) photoperiod. The axenic cultures were stored in BG-11 agar slants and sub-cultured every 30 days.

Morphological characterization

A light microscope (Leica DM750, Wetzlar, Germany) was used to determine the primary morphological appearance of the isolates. For scanning electron microscopic (SEM) analysis, a drop of the algal sample was placed on a coverslip and air-dried. It was then fixed for a period of 24 h using 4% (w/v) glutaraldehyde at 4 °C, rinsed in distilled water thrice, and dehydrated in a graded series of ethanol (30%, 50%, 75%, 85%, 95%, and 100%) for 3 min followed by air drying under vacuum. The air-dried film was gold coated and loaded for SEM analysis (Sigma, Carl Zeiss, Jena, Germany) (Sadiq et al. 2017).

Molecular characterization

Genomic DNA of the microalgae species was extracted using DNeasy plant mini kit (Qiagen, Hilden, Germany) as per the manufacturer's instruction. The purity and quantity of the DNA were checked, and then used as a template for amplification of the respective genes. For identification of the strain using *rbcL* gene primers, 5'-GGTACTTGACAAACWGTW TGGAC-3' as forward and 5'-GAAACGGTCTCKCCARCG CAT-3' as reverse (Hadi et al. 2016) were used in combination with an initial denaturation of 1 min at 94 °C followed by 37 cycles of 50 s at 92 °C denaturation step, 50 s at 57 °C annealing step, and 50 s at 72 °C elongation. For amplification of the ITS region, eukaryotic primers ITS1 5'-AGGAGAAGTCGTAACAAGGT-3' and ITS4 5'-TCC

TCCGCTTATTGATATGC-3' (Hadi et al. 2016) were used. PCR conditions used for ITS gene amplification were: an initial denaturation of 2 min at 94 °C followed by 35 cycles of 1 min at 94 °C denaturation step, 1 min at 52 °C annealing step and 1 min 72 °C elongation step, and a final 10 min at 72 °C elongation step. The PCR products were visualized by running the PCR product in 1.2% agarose gel and then sequenced. The sequences were analyzed using BioEdit, and BLAST for homologous analysis, according to similarities by the ClustalW program.

Cultivation and microalgal growth analysis

Growth experiment was carried out in 250 mL Erlenmeyer flask-containing 100 mL of BG-11 media. The flasks were inoculated with 10% (v/v) of 0.2 OD culture of respective isolate and were incubated in a growth chamber at 23 ± 1 °C with humidity of 70% under a light intensity of 80 µmol with 16:8 h (light : dark) cycle. The optical density (OD) was measured every alternate day at 685 nm via UV–Vis spectrophotometer (Shimadzu UV-2600, North America) for 15 days. Dry cell biomass (DCM) was measured gravimetrically using glass filter paper and was expressed in gram per liter. The data were expressed as means and standard deviations from three replicates each. The growth parameters used for comparing the strains include the following:

Specific growth rate (μ) of the isolate was calculated using the formula (Wood et al. 2005):

$$\mu \text{ (day}^{-1}\text{)} = \frac{(\ln C_t - \ln C_0)}{(t - t_0)}, \quad (1)$$

where C_t is the OD at time t and C_0 is the OD at the start of exponential phase.

Doubling time (T_d) was calculated using the following:

$$T_d \text{ (days)} = \frac{\ln 2}{\mu}. \quad (2)$$

Biomass productivity (BP) was calculated using the following:

$$\text{BP (g L}^{-1} \text{ day}^{-1}\text{)} = \text{DCM} \times \mu. \quad (3)$$

Lipid estimation

Neutral lipid quantification using fluorescent spectroscopy

Nile red (9-diethylamino-5*H*-benzo[a]-phenoxazine-5-one)-based quantification of intracellular TAGs was done using the protocol described by Muthuraj et al. (2014), with slight modifications (Muthuraj et al. 2014). The cells with an OD₆₈₅ of 0.7 were treated with 25% DMSO (Hi-media) followed by a short period of vortex and centrifugation. The cells were then

suspended in $1 \times$ phosphate buffer saline (PBS) and vortexed for the second time. To the suspension, Nile red stain at a final concentration of $4 \mu\text{g mL}^{-1}$ was added and incubated at 50°C . A further incubation in the dark for 10 min was carried out at room temperature. Fluorescence of each sample was detected using microplate reader (Horiba Fluorolog®-3, Kyoto Prefecture, Japan) at excitation and an emission wavelength of 493 nm and 573 nm, respectively.

Neutral lipid imaging using confocal microscopy

For imaging, the intracellular neutral lipid bodies within the algal cells, $5 \mu\text{g mL}^{-1}$ of Nile red (9-diethylamino-5*H*-benzo[*a*]-phenoxazine-5-one) (Hi-media) in acetone (Merck, Germany) were used. The same staining procedure was followed as in fluorescence spectroscopy. In addition, the samples were washed three times with 1X PBS to remove the excess dye. Stained cells were visualized under a confocal microscope at excitation 488 nm and emission 573 nm. Autofluorescence of chlorophyll was observed between 676 and 696 nm.

Lipid extraction

For lipid extraction, 3 mL chloroform: methanol 1:2 (v/v) was added to 15 mg of the dried microalgae samples and kept overnight in a 10 mL glass centrifuge tube. The tubes were then vortex and sonicated for 15 min. 2.5 mL of distilled water was added in all the tubes to separate the phase between chloroform and methanol. The tubes were vortexed and sonicated for the second time, followed by centrifugation at $1200 \times g$ for 5 min. The entire chloroform layer was collected using a glass Pasteur pipette in a pre-weighed glass tube. Chloroform (1 mL) was again added to the samples, and the chloroform phase was collected and pooled with the first collected layer. Chloroform from the tubes was evaporated at 60°C and was kept in the desiccator for 3 h. The tubes containing the extracted lipid were weighed (Bligh and Dyer 1959). The dried lipid samples were measured gravimetrically and expressed as lipid % dry cell basis and lipid productivity (LP) ($\text{mg L}^{-1} \text{ day}^{-1}$):

$$\text{LC (gg}^{-1}\text{)} = \frac{(W2 - W1)}{B}, \quad (4)$$

where LC = lipid content, W1 = weight of empty beaker (g), W2 = weight of beaker with dry lipid (g), and B = weight of dry biomass;

$$\text{LP (g L}^{-1} \text{ day}^{-1}\text{)} = \text{LC} \times \text{BP}. \quad (5)$$

Fatty acid methyl ester profiling

The extracted lipid from the isolated microalgae was subjected to transesterification using 3% methanolic H_2SO_4 at

65°C for 4 h in a reflux system. The obtained mixture was then washed with distilled water and further dried over anhydrous sodium sulfate. The remaining solvent was removed under pressure in a rotary evaporator. The dried mixture was dissolved in hexane and analyzed using GC equipped with Flame ionization detector (FID) using DB 225 capillary column ($30 \text{ m} \times 250 \mu\text{m} \times 0.25 \mu\text{m}$). The initial column temperature was set at 160°C for 2 min, followed by a $5^\circ\text{C}/\text{min}$ ramp up to 230°C , and was maintained for 20 min. The injector and FID temperature was set at 250°C and 270°C , respectively. The FAME peaks were identified by comparing against the peaks obtained from Supelco 37 component FAME mix (Sigma-Aldrich, Missouri, United States) and quantified using the area normalization method.

Properties of biodiesel

The properties of the biodiesel obtained from the microalgae were calculated using the predictive models which are based on the fatty acid composition of the oil. The IV, SV, CN, CP, and DU were calculated using the following equations (Talebi et al. 2013).

Saponification value (SV) was calculated using the following:

$$\text{SV} = \sum \frac{(560 \times N)}{M}, \quad (6)$$

where N = percentage of each fatty acid, M = molecular mass of fatty acid, and iodine value (IV) was calculated using the following:

$$\text{IV} = \sum \left(\frac{254 \times DN}{M} \right), \quad (7)$$

where D = number of double bond, cetane number (CN) was calculated using the following:

$$\text{CN} = 46.3 + \left(\frac{5.458}{\text{SV}} \right) - (0.225 \times \text{IV}). \quad (8)$$

The degree of unsaturation (DU) was calculated using the following:

$$\text{DU} = \text{MUFA} + (2 \times \text{PUFA}), \quad (9)$$

where MUFA = monounsaturated fatty acid, PUFA = polyunsaturated fatty acid, and cloud point (CP) was calculated using the following:

$$\text{CP (}^\circ\text{C)} = (0.526 \times C_{16}) - 4.992. \quad (10)$$

Statistical analysis

All the data were mean of three replicates and were subjected to the analysis of variance (ANOVA) to see the significance difference among the individuals. The test of significance was analyzed by applying Duncan's multiple range

test (DMRT) and significance was compared at a p value less than or equal to 0.05 and 0.01, using Indostate version 8.2.

Results

Isolation and identification of microalgae

Morphological characterization

Microalgal samples were collected from some of the freshwater ponds of Jorhat, Shivasagar and Golaghat district of Assam, India (location: 26.75°N, 94.18°E; 26.75°N, 94.20°E; 26.73°N, 94.15°E; 26.30°N, 93.58°E; 26.23°N, 94.05°E; 26.40°N, 93.51°E; 26.57°N, 94.37°E) (Supplementary Table 1). A total of seven axenic microalgae cultures were obtained and were designated as NEIST BT-1, NEIST BT-2, NEIST BT-10, NEIST BT-A1, NEIST BT-A6, NEIST BT-A10, and NEIST BT-A15. The isolates were then identified based on their arrangement, shape, size, and pattern by

microscopic examination using light and scanning electron microscope (Fig. 1) (Supplementary Table 2). Morphologically, NEIST BT-1 were spindle-shaped, spineless, elongated cells with a smooth cell surface and tapered obtuse ends; arranged in flat or curved coenobia. The coenobium composed of 4–8 individual cells lacking any mucilaginous sheath. The cell size ranged approximately $16.07 \pm 0.64 \mu\text{m}$ in length with each cell consisting of a single conspicuous pyrenoid and a large parietal chloroplast which spans more than half of the cell. Examination of the morphology of NEIST BT-1 indicates its resemblance with that of genus *Tetradesmus*. On the other hand, NEIST BT-2 cells were coccoid cells existing solitarily as well as in groups showing a size of $2.43 \pm 0.29 \mu\text{m}$. The cells consisted of a single pyrenoid, a large vacuole, and a parietal cup-shaped chloroplast which are typical of the genus *Chlorella*. The ultra-morphological study using SEM revealed that NEIST BT-2 cells have a relatively smooth cell wall which is surrounded by a mucilaginous sheath. NEIST BT-10 were spine-bearing, ovoid-shaped cells with size ranging between 3.5 and 5 μm .

Fig. 1 Field-emission scanning electron microscopic and light microscopic images of the seven isolates

Similar to NEIST BT-2, NEIST BT-10 also consisted of a single pyrenoid, a parietal cup-shaped chloroplast. The cell wall of NEIST BT-10 consisted of strong, distinct rib, and wart aggregates along with some cilia-like structures on one side of the cell surface. They were found as single cells as well as in irregularly arranged colonies. The characteristic features observed in NEIST BT-10 are common to genus *Desmodesmus* (An et al. 1999). The morphological and colonial characteristics of NEIST BT-A1 showed resemblance with that of NEIST BT-A10. They were elongated cells with pointed ends, existing as segregates and also as amalgamates of 4, 8, 16, and 32 cells. SEM images depicted the presence of ridges and lateral ribs on the surface of both the microalga cells, characteristics of the group *Tetradesmus*. Microscopic observation of NEIST BT-A6 described them as irregularly arranged, small crescent-shaped microalga, lacking any sheath around it. The presence of limited phenotypic characters could not identify the alga to genus level; however, its morphological characters and cell distribution pattern were representative of the family *Selenastraceae*. NEIST BT-A15 cells on the other hand were coccoid in shape and varied significantly in size between 4 and 10 μm . Each cell consisted of a single prominent pyrenoid. Both light microscopic and SEM images confirmed the presence of a thin mucilaginous sheath over a rough outer surface. The identity of the strain could not be made up to the genus level based on the morphological characters of the strain alone. However, it was tentatively identified as *Asterarcys* sp. based on molecular studies and then comparing the morphological characters of NEIST BT-A15 with features of earlier reported strains of genus *Asterarcys* (Hong et al. 2012; Ghosh et al. 2017). The phenotypic characteristics of the isolates showed that all the isolates were green algae of class *Chlorophyceae* with an exception of *Chlorella sorokiniana* NEIST BT-2, which belonged to the class of *Trebouxiophyceae*.

PCR amplification and sequence analysis

Morphological heterogeneity among the isolates made it difficult to identify them based only on their appearance. Therefore, to confirm the identity of the isolates, *rbcl* gene of chloroplast genome and ITS region of their nuclear genome were amplified and NCBI nucleotide BLAST was run to study their sequence similarity with species already reported in the GenBank database. The length of the amplicons, their accession number, and the nearest identifiable match are shown in Table 1 and their taxonomic positions are described in Fig. 2. For construction of the phylogram for both *rbcl* and ITS sequences, *Elliptochloris bilobata* was used as outgroup. In case of *rbcl*, it was observed that NEIST BT-1 formed a group with two isolates of *Tetradesmus dimorphus*; KT777984 and KT999956, with a bootstrap value 99%. NEIST BT-A1 on the other hand, formed

a monophyletic clade with the other members of the family *Scenedesmaceae*. NEIST BT-2 group together with the members of genus *Chlorella* shows a similarity of 100% with *Chlorella sorokiniana* during nucleotide BLAST analysis. NEIST BT-A6 formed clade with strains *Selenastrum* sp. JQ315488 and KT308017 of the order *Sphaeropleales*.

ITS sequence analysis showed similar relationships among the isolates. NEIST BT-1, NEIST BT-A1, and NEIST BT-A10 nested together with the other strains belonging to genera *Tetradesmus* (Smith 2018), *Acutodesmus*, and *Scenedesmus* (Hegewald and Wolf 2003) (bootstrap value 95%). NEIST BT10 grouped with the *Desmodesmus* strains forming a well-supported clade with bootstrap value 100%. NEIST BT-A15 formed sister clade with *Asterarcys quadricellulare* JQ043184 (bootstrap value 94%). The primers used in this study for the amplification of ITS region of the isolates could not amplify in some of the isolates. Therefore, the identity of these isolates was made based of the *rbcl* gene sequence homology. Both these regions are used as the barcode regions and considered as an excellent choice for identifying microalgae.

Growth evaluation of the microalgal isolates

Growth pattern of all the seven isolates showed sigmoidal curve with an initial lag phase within 2–3 days (Fig. 3). The growth kinetics of the isolates are described in Table 2. Significantly, the highest specific growth rate with the lowest doubling time was obtained in NEIST BT-A10; followed by NEIST BT-A15 > NEIST BT-2 > NEIST BT-A1 > NEIST BT-A6 > NEIST BT-10 > NEIST BT-1. Biomass, on the other hand, showed an entirely different trend, with a significant variation among the isolates, ranging from 0.68 g L^{-1} to 2.09 g L^{-1} . NEIST BT-2 which was found to be the best biomass producer also showed a significantly high biomass productivity (0.57 $\text{g L}^{-1} \text{ day}^{-1}$) in terms of comprehensive consideration of the biomass and growth rate.

Lipid content and lipid productivities

Under the given set of conditions, it was found that, on the 15th day of growth, the total lipid content of the microalgal isolates ranged from 19.11 to 31.56% dry cell weight (Table 2). The isolate NEIST BT-A6 was found to be the highest lipid accumulator (31.56%), followed by NEIST BT-1 (26.00%). NEIST BT-2, and NEIST BT-10 contained the lowest amount of lipid, i.e., less than 20% dry cell weight. Despite having a low lipid content, NEIST BT-2 had the highest lipid productivity value of 107.60 $\text{mg L}^{-1} \text{ day}^{-1}$ as a result of high biomass and a high specific growth rate. NEIST BT-A10, which had the highest specific growth rate, showed the second highest lipid

Table 1 Accession number, amplicon length, and the similarity between amplified sequences and the closest relative for the microalgal isolates

Microalgal isolates	Family	Class	rbcL sequence comparison			ITS sequence comparison				
			Accession num-ber	Length (bp)	Closest relative	% Similarity	Accession num-ber	Length (bp)	Closest relative	% Similarity
<i>Tetradismus dimorphus</i> NEIST BT-1	Scenedesmaceae	Sphaeropleales	Under process	352	<i>T. dimorphus</i> KT777984	100	MF600429	614	<i>T. dimorphus</i> KT778101	99
<i>Chlorella sorokiniana</i> NEIST BT-2	Chlorellaceae	Chlorellales	MF600427	349	<i>C. sorokiniana</i> KT308016	100	No amplification			
<i>Desmodesmus</i> sp. NEIST BT-10	Scenedesmaceae	Sphaeropleales	No amplification				MF600430	576	<i>Desmodesmus</i> sp. KX818841	100
<i>Tetradismus obliquus</i> NEIST BT-A1	Scenedesmaceae	Sphaeropleales	Under process	361	<i>T. obliquus</i> KT777962	98	MF682415	618	<i>T. obliquus</i> MG022741	97
<i>Selenastrum</i> sp. NEIST BT-A6	Selenastraceae	Sphaeropleales	Under process	368	<i>S. capricornutum</i> JQ315488	92	No amplification			
<i>Tetradismus</i> sp. NEIST BT-A10	Scenedesmaceae	Sphaeropleales	No amplification				MG799371	618	<i>T. obliquus</i> KY303738	99
<i>Asterarcys</i> sp. NEISTBT-A15	Scenedesmaceae	Sphaeropleales	No amplification				MG799375	618	<i>A. quadricellulare</i> JQ043184	100

Fig. 2 Phylogenetic tree of the seven isolates. **a** *rbcL* and **b** ITS region. The taxonomic position of the seven isolates was inferred by neighbor-joining method using MEGA7. The value at each branching shows the bootstrap value as percentage based on 1000 replicates. The isolates studied are marked with black circle

productivity ($80.68 \text{ mg}^{-1} \text{ L}^{-1} \text{ day}^{-1}$). Least lipid productivity was observed in NEIST BT-10.

The fluorescent spectroscopic analysis was performed on 15-day-old cultures to estimate the neutral lipid content of the isolates. It was found that NEIST BT-A6 contained the highest amount of neutral lipid showing a fluorescence intensity of 260.29 a.u. NEIST BT-A6 was followed by NEIST BT-A10, NEIST BT-1, NEIST BT-A1, and NEIST BT-2 with fluorescent intensity ranging between 33.55 and 41.45 a.u. NEIST BT-10 and NEIST BT-A15 accumulated low neutral lipid showing an intensity of 23.23 and 26.27 a.u., respectively. These results were further established by visualizing the microalgae cells for neutral lipid accumulation, using confocal microscopy (Fig. 4). In the confocal images, the green spots indicated the intracellular neutral lipid droplets, whereas red was the autofluorescence from chlorophyll. The analysis of the lipid accumulation pattern of the microalgal isolates suggested NEIST BT-A6 as prospective biodiesel producer considering its high neutral lipid content.

Fatty acid compositional profile

The fatty acid composition of seven strains of microalgae is described in Table 3. The amount of saturated fatty acid ranged from 22.70 to 50.20%, whereas monounsaturated fatty acid ranged from 10.50 to 31.00%; polyunsaturated fatty acid on the other hand varied from 37.30 to 53.6%. Among the fatty acids detected palmitic acid (16:0) was found most abundantly in all the isolates; with an exception of NEIST BT-A1 and NEIST BT-A10 which were rich in linolenic acid (C18:3) and oleic acid (18:1), respectively. NEIST BT10 possessed highest amount of monounsaturated fatty acid with C16:1 and C18:1 accounting for 4.4% and 22.6%, respectively. Apart from the mid-chain and long-chain fatty acids, some short-chain (C14) fatty acids were also found in significant amount in all the microalgal isolates, ranging from 0.6 to 7.8%. The dominant fatty acids present in all the seven strains were the mid-chain fatty acids; C16 and C18.

Properties of biodiesel from the lipids of the isolates

To further confirm the applicability of the isolates as biodiesel feedstock, the standard fuel properties of the biodiesel derived from these algae were predicted based on their constituent fatty acid chain length and presence of the double

bond. As shown in Table 4, NEIST BT-2 was found to be the most ideal strain for producing biodiesel, considering the iodine value (101.99), cetane number (49.59), and degree of unsaturation (89.10). NEIST BT-10 also showed an IV of 119.08, which is in accordance with the standard value established by the European Committee for Standardization (CEN) ($\leq 120 \text{ g I}_2/100 \text{ g biodiesel}$). CN of the biodiesel obtained from NEIST BT-10 was 45.78 which was near to that of ASTM D675112 standard ≥ 47 . Flow property of a biodiesel is reflected by its cloud point (CP) and cold filter plugging point value (CFPP). In this study, the cloud point was found in the range of 5.63°C to 10.47°C ; whereas the cold filter plugging point varied from -5.76°C to 12.14°C . NEIST BT-A1 (-5.76°C) and NEIST BT-A10 (-5.73°C) were found to have CPPF values within the described standard range of -5.00°C to -13.00°C .

Discussion

Microalgae have the ability to adapt to various environmental conditions which result in their enormous diversity and also a varied lipid and fatty acid profile (Kalaiselvi Thangavel et al. 2018). Taking advantage of this feature of microalgae, researchers around the globe have come up with an idea of bioprospecting for microalgae to be used as biodiesel feedstock. In the present study, we have explored few freshwater ponds of Upper Brahmaputra valley region of Assam, India, for identifying a microalgae strain, which may prove to be a promising source for biodiesel production. As mentioned earlier, altogether 16 microalgae samples were collected from Jorhat, Shivsagar, and Golaghat districts of Assam, out of which only seven axenic cultures, belonging to the taxonomic group of Chlorophyceae, were obtained. These were identified as *Tetradesmus dimorphus*. (NEIST BT-1), *Chlorella sorokiniana* (NEIST BT-2), *Desmodesmus* sp. (NEIST BT-10), *Tetradesmus* sp. (NEIST BT-A1), *Sele-nastrum* sp. (NEIST BT-A6), *Tetradesmus obliquus* (NEIST BT-A10), and *Asterarcys* sp. (NEIST BT-A15), based on their morphology, *rbcL*, and ITS sequence analysis. Taking into account the earlier reports on microalgal diversity from this region of North-East India, the *Asterarcys* genus has been reported for the first time in the present study (Kaur et al. 2012; Kemprai 2013). The literature survey shows that this genus has mostly been documented from parts of north-east and southern Asia and inhabits both freshwater and soil ecosystem (Hong et al. 2012; Chaudhary et al. 2014; Ghosh et al. 2017; Prachi Varshney et al. 2018).

Morpho-taxonomic identification of microalgae is a tedious task due to the presence of morphotypic plasticity among some of the species that obstructs the conclusive identification of a strain (Krienitz and Bock 2012). Therefore, to avoid any misidentification of the isolates, molecular

Fig. 3 Dynamic growth profile of the seven isolates. The growth curve of the seven isolates was obtained using the optical density value at 685 nm every 2 days. The observations are the mean of three replicates

identification by PCR was performed along with phenotypic characterization. Identification of microalgae is considered complex using a single marker. Although many DNA markers have been suggested including chloroplast (*rbcL*, *tufA*, and *Cp23S*), mitochondrial (*COI*), and nuclear (*18S rDNA*, *nuITS1*, and *nuITS2*), but none of these are considered as an ideal marker to be used across all the lineages (Hadi et al. 2016). As per the literature, ITS and *rbcL* are two of the most commonly used phylogenetic markers for microalgal identification because of availability of many sequences in database for these two marker genes. In addition, *rbcL* gene has been reported as promising marker for green algae identification (Hall et al. 2010).

NEIST BT-1, NEIST BT-A1, and NEIST BT-A10 share common phenotypic characters with that of genus *Tetradismus* of family *Scenedesmaceae*, which includes coenobium of 2–32 cells arranged in one or two rows, elongated cells with acute to truncated cell poles and the absence of spines (Smith 2018). However, phylogenetic analysis using *rbcL* and ITS gene sequences distinguishes them from one another by placing them in different clades in the phylogram, suggesting their evolutionary divergence. Similar to that of *Tetradismus*, the morphology of coccoid microalgae does not replicate its phylogenetic position. In this study, the coccoid microalgae isolated were NEIST BT-2, NEIST BT-10, and NEIST BT-A15 which showed distinct variation in their morphological features when viewed under scanning electron microscope. The surface of NEIST BT-2 was smooth showing an absence of wall ornamentation, in contrast to NEIST BT-10, which was found to contain ribs, warts, and spines on their cell surface (Fig. 1); the characteristic

features of the genus *Desmodesmus* (Hegewald and Brand 2017). *Desmodesmus* was formerly known as *Scenedesmus*; however, it was later separated as a new genus based on molecular analysis. The non-spiny forms retained the name *Scenedesmus*, while the spiny forms were called as *Desmodesmus* (An et al. 1999). NEIST BT-2 consisted of a separate mucilaginous sheath around its cell wall (Fig. 1). The presence of this sheath in the cells of *Chlorella sorokiniana* was also detected by Watanabe et al. (2006) (Watanabe et al. 2006). In resemblance to the earlier report on *Chlorella* sp. by Kunrunmi and his team (Kunrunmi et al. 2017), NEIST BT-2 was found to contain inward depressions on the surface of a few of the cells. Identical to NEIST BT-2, NEIST BT-A15 also displayed the presence of this sheath and depression on its surface. NEIST BT-2 and NEIST BT-A15 showed lot of similarity in their morphology, but NEIST BT-A15 was found to have a rough surface due to the presence of irregular network of ribs. Evaluation of *rbcL* and ITS gene sequences identified NEIST BT-2 as *Chlorella sorokiniana* and NEIST BT-A15 as *Asterarcys* sp. Morphological characterization of NEIST BT-A6 which includes curved cells and the absence of a mucilaginous sheath shows its resemblance to the genus *Selenastrum* of family *Selenastreae* (Bellinger and Sige 2010; Yamagishi et al. 2017). The morphological identity was further confirmed with the molecular characterization using *rbcL* gene showing 92% similarity index with strain of the same genus.

To consider any strain to be a commercially applicable biodiesel feedstock, the strain need to have first, a high specific growth rate (μ); second, it should be a high biomass producer; third, it should be a high lipid accumulator; and

Table 2 Comparison of growth characteristics, biomass, and lipid productivity of the microalgal isolates

Microalgal isolates	Specific growth rate (μ day ⁻¹)	Doubling time (days)	Biomass (g L ⁻¹)	Biomass productivity (g L ⁻¹ day ⁻¹)	Total lipid (% dry cell weight)	Lipid productivity (mg L ⁻¹ day ⁻¹)	References
<i>Tetradismus dimorphus</i> NEIST-BT1	0.25 ± 0.003	2.72 ± 0.031	1.01 ± 0.021	0.26 ± 0.008	26.00 ± 0.384	66.84 ± 1.148	Present study
<i>Chlorella sorokiniana</i> NEIST-BT2	0.27 ± 0.005	2.56 ± 0.047	2.09 ± 0.037	0.57 ± 0.020	19.17 ± 2.520	107.60 ± 10.175	Present study
<i>Desmodesmus</i> sp. NEIST-BT10	0.26 ± 0.005	2.69 ± 0.055	0.83 ± 0.044	0.22 ± 0.016	19.11 ± 3.206	40.20 ± 3.645	Present study
<i>Tetradismus obliquus</i> NEIST BT-A1	0.27 ± 0.004	2.59 ± 0.039	0.68 ± 0.044	0.18 ± 0.014	23.33 ± 1.389	42.31 ± 1.461	Present study
<i>Selenastrum</i> sp. NEIST BT-A6	0.27 ± 0.006	2.61 ± 0.059	0.84 ± 0.008	0.22 ± 0.007	31.56 ± 2.561	70.30 ± 3.716	Present study
<i>Tetradismus</i> sp. NEIST BT-A10	0.35 ± 0.003	1.96 ± 0.015	1.07 ± 0.067	0.38 ± 0.022	21.33 ± 1.389	80.68 ± 9.114	Present study
<i>Asterarcys</i> sp. NEIST BT-A15	0.32 ± 0.009	2.18 ± 0.063	0.95 ± 0.058	0.30 ± 0.010	21.66 ± 0.333	65.31 ± 1.254	Present study
<i>Tetradismus</i> sp.	na	na	na	0.01	13.50 ± 1.200	3.0	Cobos et al. (2017)
<i>Chlorella sorokiniana</i> (HS)	0.27	2.50	0.37	0.10	13.55 ± 2.770	11.51	Minhas et al. (2016)
<i>Selenastrum capricornutum</i>	0.20	3.47	0.10	0.02	35.44 ± 0.420	6.94	Song et al. (2013)
<i>Asterarcys quadricellulare</i> FKN45	0.43	1.61	1.68	0.72	21.00 ± 1.400	355.00	Chaudhary et al. (2014)
Confidence distribution (CD)	0.018	0.049	0.045	0.015	6.284	17.159	

± 1.0 = standard error of the observed values

The significant difference among the observed value within the column were compared at $p < 0.1$ and $p < 0.5$

last but not the least, it should possess an appropriate fatty acid profile, which would impart good fuel property to the diesel produced (Knothe 2006, 2009; Song et al. 2013). All these aspects, however, depend on various biochemical as well as physiological factors like carbon source, nitrogen source, salinity, temperature, light, pH, etc. These factors significantly alter the microalgal metabolism by changing the carbon flux (Dong et al. 2013; Misra et al. 2013; Josephine et al. 2015; Tan and Lee 2016; Wang et al. 2016b) which results in considerable biochemical as well as morphological variation among the isolates from different habitat. In the present study, NEIST BT-10 and NEIST BT-A1 had the highest specific growth rate; however, the biomass produced by them was significantly low compared to the other isolates. *Chlorella sorokiniana* (NEIST BT-2) was found to be the chief biomass producer giving a biomass of 2.09 g L⁻¹ at the

end of 15th day, which was much higher than few of the earlier reported strains of *Chlorella*, like *Chlorella ellipsoidea* YSR03 (1.48 g L⁻¹) (Abou-Shanab et al. 2011) and *Chlorella sorokiniana* (0.093 g L⁻¹) (Junttila et al. 2015). Biomass productivity of NEIST BT-2 was 3.17 times higher than the lowest biomass producer, NEIST BT-A1. Although NEIST BT-2 yielded a high biomass, only a moderate amount of total lipid, i.e., 19.17% of dry cell weight was accumulated by it. Low lipid content of 4.65% and 19.47% w/w in *Chlorella sorokiniana* was also reported by Sinha et al. (2016) and Eladel et al. (2018), respectively. The highest amount of total, as well as neutral lipid (31.56% and, 260.29 a.u., respectively), was accumulated by NEIST BT-A6. This amount of total lipid was approximately 1.17 times higher than the freshwater water microalgae reported from China, *S. capricornutum* (Song et al. 2013). The microalgae isolates

Fig. 4 Representative confocal microscopic images showing the intracellular neutral lipid content of the isolates stained with Nile red. Green spots represent the intracellular neutral lipid content, whereas red is the autofluorescence from chlorophyll. Neutral lipid was visual-

ized at excitation 488 nm and emission collected within 563–579 nm. Autofluorescence of chlorophyll was collected between 676 and 696 nm

examined in the present study have been reported as lipid accumulators in some of the earlier studies done on these microphytes (Table 2). The lipid content of a microalgae can further be increased by altering the growth conditions including nutrients (Sánchez-García et al. 2013; Dhup et al. 2017), salinity (Pandit et al. 2017), temperature (Venkata et al. 2014), light (Wahidin et al. 2013), pH (Sakarika and Kornaros 2016), etc. Rosenberg et al. (2014) reported an increase in lipid content in *Chlorella sorokiniana* UTEX 1230 from 18 to 39% when grown heterotrophically. Apart from *Chlorella sorokiniana*; other species of *Chlorella* like *Chlorella vulgaris* and *Chlorella pyrenoidosa* have been suggested as potential biodiesel producer due to their high lipid content and fast growth rate (Yan et al. 2014). *Tetradesmus obliquus* was also shown to accumulate lipid under nitrogen-depleted conditions by Ismagulova et al. (2017). *Asterarcys quadricellulare* is another green algae which accumulates lipid when grown autotrophically (Hong et al. 2012; Chaudhary et al. 2014). A detailed study was also done on *Selenastrium capricornutum* by McLarnon-Riches et al. (1998), to test the effect of different environmental factors and metals on its lipid content. Lipid productivity is another attribute which is considered important to decide on whether a strain could be used economically for producing biodiesel. This is due to the fact that it takes into account both lipid content

and biomass produced by a strain. Considering this, despite having a high lipid content, the lipid productivity of NEIST BT-A6, i.e., $70.30 \text{ mg}^{-1} \text{ L}^{-1} \text{ day}$ was quite low compared to NEIST BT-2 ($107.60 \text{ mg}^{-1} \text{ L}^{-1} \text{ day}$) as a result of low biomass accumulation. Lipid profile of NEIST BT-1 showed that apart from total lipid, it also contained good amount of neutral lipid within the cells as depicted in the confocal microscopic images.

Physical and chemical properties of biodiesel depend on its constituent fatty acids. The fatty acid profile of all the seven strains of microalgae depicts the presence of palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) as the dominant fatty acids. All these fatty acids had been reported as the common fatty acids present in most of the feedstocks used for biodiesel production (Moser 2009). Both acyl chain length and degree of unsaturation play a major role in determining fuel properties of the biodiesel produced. Oxidation stability and cetane number of any biodiesel are directly linked to the number and position of double bonds of its constituent fatty acids. Both oxidation stability and cetane number are inversely proportional to the amount of unsaturated fatty acids. Higher the number of double bonds, more prone will it be to oxidation, and longer will be the ignition delay period (Knothe 2013). Considering the above fact, it was concluded that NEIST BT-2

Table 3 Fatty acid composition of the microalgal isolates

Fatty acid composition (wt %)											
	<i>Tetradasmus dimorphus</i> NEIST-BT1	<i>Chlorella sorokiniana</i> NEIST-BT2	<i>Desmodesmus</i> sp. NEIST-BT10	<i>Tetradasmus obliquus</i> NEIST BT-A1	<i>Selenestrum</i> sp. NEIST BT-A6	<i>Tetradasmus</i> sp. NEIST BT-A10	<i>Asterarcys</i> sp. NEIST BT-A15	Soybean (Song et al. 2013)	Yeast (<i>Rhodospiridium toruloides</i>) (Patel et al. 2016)	Bacteria (<i>Rhodococcus opacus</i>) (Archanaa et al. 2018)	Fungi (<i>Fusarium oxysporum</i>) (El-haj et al. 2015)
C14:0	3.7	7.8	1	1	1	0.6	0.9	na	1.9	2.9	0.01
C15:0	0.5	0.8	0	0	0	0	0	na	na	9.5	na
C16:0	20.4	25.1	29.4	20.2	24.9	21.1	25.5	11	21.6	25.92	22.31
C16:1	1.9	2.5	4.4	3.4	2.4	3.4	6.3	na	nd	7.6	0.98
C16:2	1.2	0.3	4.5	3.5	4	3.8	4.3	na	na	na	na
C16:3	6.1	3.2	3.4	1.8	0.6	5.6	0.7	na	na	na	na
C16:4	0	0	3.3	10.9	9.3	7.5	9.4	na	na	na	na
C17:0	1.7	7.1	0	0	0	0	0	na	na	15.3	na
C17:1	–	–	–	–	–	–	–	na	na	15.2	na
C18:0	8.6	7.5	1.2	0.9	0.3	1.1	0.6	4	5.8	4.2	5.98
C18:1	15.4	5.4	22.6	20	15	21.5	24.2	24	51.6	12.98	34.32
C18:2	17.3	14.7	13.3	9.3	11.7	14.2	5	53	17.7	na	34.84
C18:3	19.6	19.3	11.6	24.2	21.4	17.8	19.6	8	nd	na	0.04
C18:4	0	0	1.2	3.9	6.6	2.4	2.3	na	na	na	na
C19:0	–	–	–	–	–	–	–	na	na	1.1	na
C19:1	–	–	–	–	–	–	–	na	na	1.5	na
C20:0	0.4	0.8	0.4	0.1	0.1	0.1	0.1	na	nd	0.3	na
C20:1	0.1	0.1	1.2	0.3	0.1	0.5	0.5	na	na	na	na
C20:2	0.5	1.8	0	0	0	0	0	na	na	na	na
C20:3	0.2	0	0	0	0	0	0	na	na	na	na
C20:4	–	–	–	–	–	–	–	na	na	na	0.25
C22:0	0.7	0.3	1.2	0.3	0.2	0.3	0.3	na	nd	1.0	na
C22:1	0.8	1.6	0.3	0	0	0	0	na	na	na	na
C23:0	–	–	–	–	–	–	–	na	na	1.4	na
C24:0	0.5	0.8	1	0.2	2.4	0.1	0.3	na	na	na	na
C24:1	0.4	0.9	0	0	0	0	0	na	na	na	na
SFA	36.5	50.2	34.2	22.7	28.9	23.3	27.7	15	29.3	61.62	28.3
MUFA	18.6	10.5	28.5	23.7	17.5	25.4	31	24	51.6	37.28	35.3
PUFA	44.9	39.3	37.3	53.6	53.6	51.3	41.3	61	17.7	na	35.13

na data not available, nd not detected

Table 4 Predicted properties of the biodiesel derived from the microalgal isolates

Microalgal isolates	Properties of the biodiesel							
	SV	IV	CN	DU	LCSF	CFPP	CP	References
<i>Tetrademus dimorphus</i> NEIST-BT1	206.22	123.98	44.87	108.40	8.79	11.14	5.74	Present study
<i>Chlorella sorokiniana</i> NEIST-BT2	208.05	101.99	49.59	89.10	9.11	12.14	8.21	Present study
<i>Desmodesmus</i> sp. NEIST-BT10	207.73	119.08	45.78	103.10	7.74	7.84	10.47	Present study
<i>Tetrademus obliquus</i> NEIST BT-A1	208.50	176.15	32.84	130.90	3.42	−5.73	5.63	Present study
<i>Selenestrum</i> sp. NEIST BT-A6	207.74	167.91	34.79	124.70	7.84	8.15	8.11	Present study
<i>Tetrademus</i> sp. NEIST BT-A10	208.44	161.77	36.09	128.00	3.41	−5.76	6.11	Present study
<i>Asterarcys</i> sp. NEIST BT-A15	209.32	148.85	38.88	113.6	4.00	−3.91	8.42	Present study
Yeast (<i>Rhodospiridium toruloides</i>)	191.43	74.86	55.72	87	5.06	0.81	NA	Patel et al. (2016)
Bacteria (<i>Rhodococcus opacus</i>)	na	39.02	69.12	na	na	na	na	Archanaa et al. (2018)
Fungi (<i>Fusarium oxysporum</i>)	196.71	119.95	52.44	na	na	na	na	El-haj et al. (2015)
Soybean ^a	na	139.48	na	122.00	3.1	−6.74	0.79	Song et al. (2013)

SV saponification value, IV iodine value, CN cetane number, DU degree unsaturation, LCSF long-chain saturation factor, CFPP cold filter plugging point, CP cloud point, na data not available

^aSoybean is the major feedstock for biodiesel production in the United States holding a share of 52% in 2017 compared to the other feedstocks (EIA, November, 2018)

and NEIST BT-10 can act as a suitable source for producing oxidation resistant biodiesel with reduced ignition delay, due to the presence of high amount of saturated (50.2%, 34.2%, respectively) and monounsaturated (10.5%, 28.5%, respectively) fatty acid. Cetane number for NEIST BT-2 was calculated to be 49.59, a value accepted by ASTM D6751, and 45.78 for NEIST BT-10. One of the very important advantages of biodiesel over the conventional diesel is its capacity to emit a minor amount of harmful gases on its combustion and is displayed by its iodine value. According to EN 14214 biodiesel fuel standard, the maximum range for iodine value is 120 g I₂/100 g. Iodine value increases with decrease in fatty acid chain length and increase in number of double bonds, which in turn results in increased NO_x emission. Iodine value for NEIST BT-2 (101.99) and NEIST BT-10 (119.08) was proved suitable for producing an environment safe biodiesel. Iodine value of commercially available biodiesel (Soybean) is higher than the defined standards; therefore, they are generally used in blends with petrodiesel. The cloud point for the biodiesel from all the seven strains was quite high compared to that of petroleum diesel (4 °C) and biodiesel from soybean oil (0.79 °C) (Table 4). These data indicate that such fuels would result in poor performance at low temperatures, as they will lose their flow property and start to gel, and, thus, hamper the engine's function. However, they would be suited in temperate regions, where the fluidity of the oil would be maintained. A comparison of the biodiesel produced in the present study and other reported potential sources is given in Table 4. Furthermore, considering the fact that a high content of unsaturated fatty acids accounts for high lubricity of a biodiesel (Martin et al.

2013), the oil from the strains NEIST BT-1, NEIST BT-A1, NEIST BT-A6, NEIST BT-A10, and NEIST BT-A15 can be used as fuel additives.

Conclusions

In the present study, we have isolated seven freshwater strains of microalgae and screened them for their ability to produce biodiesel based on their growth characteristics, lipid content, and fatty acid composition. Out of the seven isolates, *Chlorella sorokiniana* NEIST BT-2 was found to be the most promising strain, showing highest lipid productivity (107.60 mg L^{−1} day^{−1}) with a biomass content of 2.09 g L^{−1} and lipid content 19.17%. Fatty acid profiling of the microalgal oil predicted that the biodiesel produced from NEIST BT-2 will have a high cetane number (49.59) and low iodine value (101.99), and will be resistant to oxidation. Overall, our experimental results suggest NEIST BT-2 as a potential candidate for further scale up of biodiesel production, owing to its robustness, high lipid productivity, and an appropriate fatty acid content. The potentiality of the strain can further be enhanced by increasing its lipid content, using media optimization and genetic alterations to modify the lipid metabolic pathway.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

- Abou-Shanab RAI, Hwang JH, Cho Y, Min B, Jeon B (2011) Characterization of microalgal species isolated from fresh water bodies as a potential source for biodiesel production. *Appl Energy* 88:3300–3306. <https://doi.org/10.1016/j.apenergy.2011.01.060>
- Ahmad I, Fatma Z, Yazdani SS, Kumar S (2013) DNA barcode and lipid analysis of new marine algae potential for biofuel. *Algal Res* 2:1–2. <https://doi.org/10.1016/j.algal.2012.10.003>
- An SS, Friedel T, Hegewald E (1999) Phylogenetic relationships of *Scenedesmus* and *Scenedesmus*-like coccoid green algae as inferred from ITS-2 rDNA sequence comparison. *Plant Biol* 1:418–428. <https://doi.org/10.1111/j.1438-8677.1999.tb00724.x>
- Anand V, Singh PK, Banerjee C, Shukla P (2017) Proteomic approaches in microalgae: perspectives and applications. *3 Biotech* 7:197. <https://doi.org/10.1007/s13205-017-0831-5>
- Archana S, Jose S, Mukherjee A, Suraishkumar GK (2018) Sustainable diesel feedstock: a comparison of oleaginous bacterial and microalgal model systems. *Bioenergy Res*. <https://doi.org/10.1007/s12155-018-9948-6>
- Banerjee A, Banerjee C, Negi S, Chang JS, Shukla P (2017) Improvements in algal lipid production: a systems biology and gene editing approach. *Crit Rev Biotechnol* 38:369–385. <https://doi.org/10.1080/07388551.2017.1356803>
- Bérard A, Dorigo U, Humbert JF, Martin-Laurent F (2005) Microalgae community structure analysis based on 18S rDNA amplification from DNA extracted directly from soil as a potential soil bioindicator. *Agron Sustain Dev* 25:285–291
- Bellinger EG, Sigee DC (2010) *Freshwater Algae: identification and Use as Bioindicators*. Wiley-Blackwell, Chichester, UK, pp 236–238
- Bligh EG, Dyer WJ (1959) A rapid method of lipid extraction and purification. *Can J Biochem Physiol* 37:911–917. <https://doi.org/10.1139/cjm2014-0700>
- Borowitzka MA (2013) Species and strain selection. In: Michael A, Borowitzka NRM (eds) *Algae for biofuels and energy*. Springer, Dordrecht, pp 77–89
- British Petroleum (2017) BP Statistical Review of World Energy 2017. British Petroleum 1–52. <http://www.bp.com/content/dam/bp/en/corporate/pdf/energy-economics/statistical-review-2017/bp-statistical-review-of-world-energy-2017-full-report.pdf>. Accessed 15 July 2018
- Chaudhary R, Khattar J, Singh D (2014) Microalgae as feedstock for biofuel: biomass yield, lipid content and fatty acid composition as selection criteria. *Int J Power Renew Energy Syst* 1:62–71
- Cobos M, Paredes JD, Maddox D, Vargas-Arana G, Flores L, Aguilar CP, Marapara JL, Castro JC (2017) Isolation and characterization of native microalgae from the peruvian amazon with potential for biodiesel production. *Energies* 10:1–16. <https://doi.org/10.3390/en10020224>
- Dantas DMDM, Oliveira YBDC, Costa RMPB, das Graças Carneiro-da-Cunha M, Gálvez M, de Souza Bezerra R (2019) Evaluation of antioxidant and antibacterial capacity of green microalgae *Scenedesmus subspicatus*. *Food Sci Technol Int*. <https://doi.org/10.1177/1082013218825024>
- Darienko T, Gustavs L, Eggert A, Wolf W, Proschold T (2015) Evaluating the species boundaries of green microalgae (Coccomyxa, Trebouxiophyceae, Chlorophyta) using integrative taxonomy and DNA barcoding with further implications for the species identification in environmental samples. *PLoS One* 10:1–31. <https://doi.org/10.1371/journal.pone.0127838>
- Dhup S, Kannan DC, Dhawan V (2017) Growth, lipid productivity and cellular mechanism of lipid accumulation in microalgae *Monoraphidium* sp. following different phosphorous concentrations for biofuel production. *Curr Sci* 112:539–548. <https://doi.org/10.18520/cs/v112/i03/539-548>
- Dong HP, Williams E, Wang DZ, Xie ZX, Hsia RC, Jenck A, Halden R, Li J, Chen F, Place AR (2013) Responses of *Nannochloropsis oceanica* IMET1 to Long-Term Nitrogen Starvation and Recovery. *Plant Physiol* 162:1110–1126. <https://doi.org/10.1104/pp.113.214320>
- Eladel H, Abomohra AEF, Battah M, Mohammed S, Radwan A, Abdelrahim H (2018) Evaluation of *Chlorella sorokiniana* isolated from local municipal wastewater for dual application in nutrient removal and biodiesel production. *Bioprocess Biosyst Eng*. <https://doi.org/10.1007/s00449-018-2046-5>
- El-haj M, Olama Z, Holail H (2015) Single cell oil of oleaginous fungi from lebanese habitats as a potential feed stock for biodiesel. *IJCMAS* 4:11–34
- Ghosh A, Khanra S, Mondal M, Halder G, Tiwari ON, Saini S, Bhowmick TK, Gayen K (2016) Progress toward isolation of strains and genetically engineered strains of microalgae for production of biofuel and other value added chemicals: a review. *Energy Convers Manag* 113:104–118. <https://doi.org/10.1016/j.enconman.2016.01.050>
- Ghosh A, Khanra S, Mondal M, Halder G, Tiwari ON, Saini S, Bhowmick TK, Gayen K (2017) Effect of macronutrient supplements on growth and biochemical compositions in photoautotrophic cultivation of isolated *Asterarcys* sp. (BTA9034). *Energy Convers Manag* 149:39–51. <https://doi.org/10.1016/j.enconman.2017.07.015>
- Hadi SIIA, Santana H, Brunale PPM, Gomes TG, Oliveira MD, Matthiensen A, Oliveira MEC, Silva FCP, Brasil BSAF (2016) DNA barcoding green microalgae isolated from neotropical inland waters. *PLoS One* 11:1–18. <https://doi.org/10.1371/journal.pone.0149284>
- Hall JD, Fucikova K, Lo C, Lewis LA, Karol KG (2010) An assessment of proposed DNA barcodes in freshwater green algae. *Cryptogam Algal* 31:529–555
- Hannon M, Gimpel J, Tran M, Rasala B, Mayfield S (2010) Biofuels from algae: challenges and potential. *Biofuels* 5:763–784
- Hegewald E, Braband A (2017) A taxonomic revision of desmodesmus serie desmodesmus (Sphaeropleales, Scenedesmaceae). *Fottea* 17:191–208. <https://doi.org/10.5507/ft.2017.001>
- Hegewald E, Wolf M (2003) Phylogenetic relationships of *Scenedesmus* and *Acutodesmus* (Chlorophyta, Chlorophyceae) as inferred from 18S rDNA and ITS-2 sequence comparisons. *Plant Syst Evol* 241:185–191. <https://doi.org/10.1007/s00606-003-0061-7>
- Hoekman SK, Broch A, Robbins C, Cenicerros E, Natarajan M (2012) Review of biodiesel composition, properties, and specifications. *Renew Sustain Energy Rev* 16:143–169. <https://doi.org/10.1016/j.rser.2011.07.143>
- Hong JW, Kim SA, Chang J, Yi J, Jeong J, Kim S, Kim SH, Yoon HS (2012) Isolation and description of a Korean microalga, *Asterarcys quadricellulare* KNUA020, and analysis of its biotechnological potential. *Algal* 27:197–203. <https://doi.org/10.4490/algal.2012.27.3.197>
- Ismagulova T, Chekanov K, Gorelova O, Baulina O, Semenova L, Selyakh I, Solovchenko A (2017) A new subarctic strain of *Tetradasmus obliquus*—part I: identification and fatty acid profiling. *J Appl Psychol* 30:2737–2750. <https://doi.org/10.1007/s10811-017-1313-1>
- Jagadevan S, Banerjee A, Banerjee C, Guria C, Tiwari R, Baweja M, Shukla P (2018) Recent developments in synthetic biology and metabolic engineering in microalgae towards biofuel production.

- Biotechnol Biofuels 11:185. <https://doi.org/10.1186/s13068-018-1181-1>
- Jia Z, Liu Y, Daroch M et al (2014) Screening, growth medium optimisation and heterotrophic cultivation of microalgae for biodiesel production. Appl Biochem Biotechnol 173:1667–1679. <https://doi.org/10.1007/s12010-014-0954-7>
- Josephine A, Niveditha C, Radhika A, Shali AB, Kumar TS, Dharamani G, Kirubakaran R (2015) Analytical evaluation of different carbon sources and growth stimulators on the biomass and lipid production of *Chlorella vulgaris*—implications for biofuels. Biomass Bioenergy 75:170–179. <https://doi.org/10.1016/j.biombioe.2015.02.016>
- Junttila DJ, Bautista MA, Monotilla W (2015) Biomass and lipid production of a local isolate *Chlorella sorokiniana* under mixotrophic growth conditions. Bioresour Technol 191:395–398. <https://doi.org/10.1016/j.biortech.2015.03.098>
- Kalaiselvi Thangavel PR, Krishna S, Nagaiah S, Kuppasamy S, Chinnaasamy JS, Rajadorai, Gopal Nellaiappan Olaganathan BD (2018) Growth and metabolic characteristics of oleaginous microalgal isolates from Nilgiri biosphere Reserve of India. BMC Microbiol 18:1–17. <https://doi.org/10.1186/s12866-017-1144-x>
- Kaur S, Sarkar M, Srivastava RB, Gogoi HK, Kalita MC (2012) Fatty acid profiling and molecular characterization of some freshwater microalgae from India with potential for biodiesel production. New Biotechnol 29:332–344. <https://doi.org/10.1016/j.nbt.2011.10.009>
- Kemprai R (2013) Biodiversity of cyno-bacteria in soils of greater haflong and their nitrogen fixing capacity. Int J Adv Res 1:31–41
- Knothe G (2006) Analyzing biodiesel: Standards and other methods. JAOCS. J Am Oil Chem Soc 83:823–833. <https://doi.org/10.1007/s11746-006-5033-y>
- Knothe G (2009) Improving biodiesel fuel properties by modifying fatty ester composition. Energy Environ Sci 2:759. <https://doi.org/10.1039/b903941d>
- Knothe G (2013) Production and properties of biodiesel from algal oils. In: Borowitzka MA, Moheimani NR (eds) Algae for biofuels and energy. Springer, Dordrecht, pp 207–221
- Krienitz L, Bock C (2012) Present state of the systematics of planktonic coccoid green algae of inland waters. Hydrobiologia 698:295–326. <https://doi.org/10.1007/s10750-012-1079-z>
- Kunrunmi O, Adesalu T, Kumar S (2017) Genetic identification of new microalgal species from Epe Lagoon of West Africa accumulating high lipids. Algal Res 22:68–78. <https://doi.org/10.1016/j.algal.2016.12.009>
- Li N, Zhang Y, Meng H, Li S, Wang S, Xiao Z, Chang P, Zhang X, Li Q, Guo L, Igarashi Y, Luo F (2019) Characterization of fatty acid exporters involved in fatty acid transport for oil accumulation in the green alga *Chlamydomonas reinhardtii*. Biotechnol Biofuels. <https://doi.org/10.1186/s13068-018-1332-4>
- Mallick N, Bagchi SK, Koley S, Singh AK (2016) Progress and challenges in microalgal biodiesel production. Front Microbiol 7:1019. <https://doi.org/10.3389/fmicb.2016.01019>
- Marcel Martineez-Porchas FV-A (2017) Microbial metagenomics in aquaculture: a potential tool for a deeper insight into the activity. Rev Aquac 9:42–56. <https://doi.org/10.1111/raq.12102>
- Martin JM, Matta C, Bouchet MIDB, Forest C (2013) Mechanism of friction reduction of unsaturated fatty acids as additives in diesel fuels. Friction 1:252–258. <https://doi.org/10.1007/s40544-013-0022-2>
- Mata TM, Martins AA, Caetano NS (2010) Microalgae for biodiesel production and other applications: a review. Renew Sustain Energy Rev 14:217–232. <https://doi.org/10.1016/j.rser.2009.07.020>
- McLarnon-Riches CJ, Rolph CE, Greenway DL, Robinson PK (1998) Effects of environmental factors and metals on *Selenastrum capricornutum* lipids. Phytochemistry 49:1241–1247. [https://doi.org/10.1016/s0031-9422\(98\)00095-8](https://doi.org/10.1016/s0031-9422(98)00095-8)
- Minhas AK, Hodgson P, Barrow CJ, Sashidhar B, Adholeya A (2016) The isolation and identification of new microalgal strains producing oil and carotenoid simultaneously with biofuel potential. Bioresour Technol 211:556–565. <https://doi.org/10.1016/j.biortech.2016.03.121>
- Misra N, Panda PK, Parida BK (2013) Agrigenomics for microalgal biofuel production: an overview of various bioinformatics resources and recent studies to link OMICS to bioenergy and bioeconomy. Omi A J Integr Biol 17:537–549. <https://doi.org/10.1089/omi.2013.0025>
- Mondal M, Goswami S, Ghosh A, Ghosh A, Oinam G, Tiwari ON, Das P, Gayen K, Mandal MK, Halder GN (2017) Production of biodiesel from microalgae through biological carbon capture: a review. 3 Biotech 7:1–21. <https://doi.org/10.1007/s13205-017-0727-4>
- Moser BR (2009) Biodiesel production, properties, and feedstocks. Vitro Cell Dev Biol Plant 45:229–266. <https://doi.org/10.1007/s11627-009-9204-z>
- Muthuraj M, Kumar V, Palabhanvi B, Das D (2014) Evaluation of indigenous microalgal isolate *Chlorella* sp. FC2 IITG as a cell factory for biodiesel production and scale up in outdoor conditions. J Ind Microbiol Biotechnol 41:499–511. <https://doi.org/10.1007/s10295-013-1397-9>
- Naghsbandi MP, Tabatabaei M, Aghbashlo M, Aftab MN, Iqbal I (2019) Metabolic engineering of microalgae for biofuel production. Methods Mol Biol. https://doi.org/10.1007/978-1-4939-9205-2_205
- Pandit PR, Fulekar MH, Karuna MSL (2017) Effect of salinity stress on growth, lipid productivity, fatty acid composition, and biodiesel properties in *Acutodesmus obliquus* and *Chlorella vulgaris*. Environ Sci Pollut Res 24:13437–13451. <https://doi.org/10.1007/s11356-017-8875-y>
- Patel A, Arora N, Sartaj K, Pruthi V, Pruthi PA (2016) Sustainable biodiesel production from oleaginous yeasts utilizing hydrolysates of various non-edible lignocellulosic biomasses. Renew Sust Energ Rev 62:836–855. <https://doi.org/10.1016/j.rser.2016.05.014>
- Prachi Varshney J, Beardalle S, Bhattacharya PPW (2018) Isolation and biochemical characterisation of two thermophilic green algal species—*Asterarcys quadricellulare* and *Chlorella sorokiniana*, which are tolerant to high levels of carbon dioxide and nitric oxide. Algal Res 30:28–37. <https://doi.org/10.1016/j.algal.2017.12.006>
- Rosenberg JN, Kobayashi N, Barnes A, Noel EA, Betenbaugh MJ, Oyler GA (2014) Comparative analyses of three *Chlorella* species in response to light and sugar reveal distinctive lipid accumulation patterns in the microalga *C. sorokiniana*. PLoS One 9(4):e92460. <https://doi.org/10.1371/journal.pone.0092460>
- Sadiq IM, Dalai S, Chandrasekaran N, Mukherjee A (2017) Corrigendum: “Ecotoxicity study of titania (TiO₂) NPs on two microalgae species: *Scenedesmus* sp. and *Chlorella* sp” [Ecotoxicol. Environ. Saf. 74/5 (2011) 1180–1187], (S0147651311000728), (10.1016/j.ecoenv.2011.03.006) Ecotoxicol Environ Saf 142:597. <https://doi.org/10.1016/j.ecoenv.2017.04.029>
- Sakarika M, Kornaros M (2016) Effect of pH on growth and lipid accumulation kinetics of the microalga *Chlorella vulgaris* grown heterotrophically under sulfur limitation. Bioresour Technol 219:694–701. <https://doi.org/10.1016/j.biortech.2016.08.033>
- Sánchez-García D, Resendiz-Isidro A, Villegas-Garrido T, Flores-Ortiz C, Chávez-Gómez B, Cristiani-Urbina E (2013) Effect of nitrate on lipid production by *T. suecica*, *M. contortum*, and *C. minutissima*. Open Life Sci 8:578–590. <https://doi.org/10.2478/s11535-013-0173-6>
- Sinha SK, Gupta A, Bharalee R (2016) Production of biodiesel from freshwater microalgae and evaluation of fuel properties based on fatty acid methyl ester profile. Biofuels 7:69–78. <https://doi.org/10.1080/17597269.2015.1118781>

- Smith G (2018) *Tetrademus*, a new four-celled coenobitic alga. *Bull Torrey Bot club* 40:75–87
- Song M, Pei H, Hu W, Ma G (2013) Evaluation of the potential of 10 microalgal strains for biodiesel production. *Bioresour Technol* 141:245–251. <https://doi.org/10.1016/j.biortech.2013.02.024>
- Sun Z, Fang X, Li X, Zhou Z (2017) Oleaginous microalgae from dairy farm wastewater for biodiesel production: isolation, characterization and mass cultivation. *Appl Biochem Biotechnol*. <https://doi.org/10.1007/s12010-017-2564-7>
- Talebi AF, Mohtashami SK, Tabatabaei M, Tohidfar M, Bagheri A, Zeinalabedini M, Mirzaei HH, Mirzajanzadeh M, Shafaroudi SM, Bakhtiari S (2013) Fatty acids profiling: a selective criterion for screening microalgae strains for biodiesel production. *Algal Res* 2:258–267. <https://doi.org/10.1016/j.algal.2013.04.003>
- Tan KWM, Lee YK (2016) The dilemma for lipid productivity in green microalgae: Importance of substrate provision in improving oil yield without sacrificing growth. *Biotechnol Biofuels* 9:1–14. <https://doi.org/10.1186/s13068-016-0671-2>
- Venkata SG, Rohit MV, Devi MP, Swamy YV, Venkata SM (2014) Temperature induced stress influence on biodiesel productivity during mixotrophic microalgae cultivation with wastewater. *Bioresour Technol* 169:789–793. <https://doi.org/10.1016/j.biortech.2014.07.019>
- Wahidin S, Idris A, Shaleh SRM (2013) The influence of light intensity and photoperiod on the growth and lipid content of microalgae *Nannochloropsis* sp. *Bioresour Technol* 129:7–11. <https://doi.org/10.1016/j.biortech.2012.11.032>
- Wang C, Zou S, Fei C, Wang C, Gao Z, He M, Wang C (2016a) How DNA barcoding can be more effective in microalgae identification: a case of cryptic diversity revelation in *Scenedesmus* (Chlorophyceae). *Sci Rep* 6:1–13. <https://doi.org/10.1038/srep36822>
- Wang Y, He B, Sun Z, Chen YF (2016b) Chemically enhanced lipid production from microalgae under low sub-optimal temperature. *Algal Res* 16:20–27. <https://doi.org/10.1016/j.algal.2016.02.022>
- Watanabe K, Imase M, Sasaki K, Ohmura N, Saiki H, Tanaka H (2006) Composition of the sheath produced by the green alga *Chlorella sorokiniana*. *Lett Appl Microbiol* 42:538–543. <https://doi.org/10.1111/j.1472-765X.2006.01886.x>
- Wood AM, Everroad RC, Wingard LM (2005) Measuring growth rates in microalgal cultures. In: Andersen RA (ed) *Algal culturing techniques*. Elsevier Academic Press, San Diego, pp 269–285
- Wu L, Xu L, Hu C (2015) Screening and characterization of Oleaginous microalgal species from Northern Xinjiang. *J Microbiol Biotechnol* 25:910–917. <https://doi.org/10.4014/jmb.1411.11075>
- Yamagishi T, Yamaguchi H, Suzuki S, Horie Y, Tatarazako N (2017) Cell reproductive patterns in the green alga *Pseudokirchneriella subcapitata* (= *Selenastrum capricornutum*) and their variations under exposure to the typical toxicants potassium dichromate and 3,5-DCP. *PLoS One* 12:1–12. <https://doi.org/10.1371/journal.pone.0171259>
- Yan Y, Li X, Wang G, Gui X, Li G, Su F, Wang X, Liu T (2014) Biotechnological preparation of biodiesel and its high-valued derivatives: a review. *Appl Energy* 113:1614–1631. <https://doi.org/10.1016/j.apenergy.2013.09.029>
- Yu W-L, Ansari W, Schoepp N, Hannon MJ, Mayfield SP, Burkart MD (2011) Modifications of the metabolic pathways of lipid and triglyceride production in microalgae. *Microb Cell Fact* 10:91. <https://doi.org/10.1186/1475-2859-10-91>