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Life cycle assessment of *Chlorella* species producing biodiesel and remediating wastewater

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Constantly rising energy demands, finite fossil fuel reserves and deteriorating environmental conditions have invoked worldwide interest to explore the sustainable sources of renewable biofuels. Locally adapted photosynthetic oleaginous microalgae with rapid growth on variable temperatures could be an ideal way for bioremediating the wastewater (WW) while producing the feedstock for biodiesel. To test this notion, an unknown strain was isolated from a sewage fed lake (Neela-Hauz). It was discerned as *Chlorella sorokiniana*-I using the 16S rDNA and 18S rDNA barcodes. The culture conditions such as pH, illumination, different temperature ranges and growth medium were cohesively optimized prior to the assessment of *C. sorokiniana*-I's efficacy to remediate the WW and biodiesel production. The strain has thrived well up to 40°C when continuously grown for 15 days. The highest lipid accumulation and biomass productivity were recorded in 100% WW. Fatty acid methyl ester (FAME) content was observed to be more than twice in WW (47%), compared to control synthetic media, TAP (20%) and BG11 (10%), which indicate the importance of this new isolate for producing economically viable biodiesel. Moreover, it is highly efficient in removing the total nitrogen (77%), total phosphorous (81%), iron (67%) and calcium (42%) from the WW. The quality of WW was considerably improved by reducing the overall chemical oxygen demand (48%), biological oxygen demand (47%) and alkalinity (15%). Thus, *C. sorokiniana*-I could be an ideal alga for the tropical countries in the remediation of WW while producing feedstock for biodiesel in a cost-effective manner.

Keywords. Biomass; green algae; mixotrophic algae; thermotolerant; wastewater; water pollution

Abbreviations: BG11, blue green medium; TAP, Tris-acetate phosphate; WW, wastewater; COD, chemical oxygen demand; BOD, biological oxygen demand; TOC, total organic carbon; TIC, total inorganic carbon; TN, total nitrogen; TP, total phosphorous; FAME, fatty acid methyl ester

1. Introduction

Algae-based biofuel is considered one of the best renewable resources that hold an answer to the global energy crisis (Chisti 2007; Lei *et al.* 2012). Currently, bioprospecting for photosynthetic microalgae with rapid growth, higher lipid accumulation and biomass productivity is of special interest as a feedstock for biodiesel production (Hu *et al.* 2008; Pereira *et al.* 2011). The quality and quantity of algal feedstock vary with the lipid, carbohydrate and protein content present in the microorganism (Chia *et al.* 2017). The variation in the culture conditions, such as pH, irradiance, temperature and nutrient availability greatly influence the lipid accumulation and composition in microalgae (Ngangkham *et al.* 2012). Nevertheless, the lipid content of algae may also vary when laboratory acclimatized cultures are subjected to outdoor cultivation in a large volume due to the inadaptability to environmental conditions of the particular area. Thus, the prime focus of this research is to identify a locally adapted strain to generate biomass that may withstand the changing environmental conditions (Arumugam *et al.* 2013).

Only 3% of the Earth's surface water is considered as fresh water that is being polluted at an alarming rate in tropical countries due to anthropological activities. The recycling of polluted water via bioremediation will be very

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essential to fulfill the needs of global population to avoid potable water crises. Microalgae are vital for wastewater (WW) treatment because of (a) cost-effectiveness, (b) ecologically favorable, chemical-free process and (c) simultaneous removal of nitrogen and phosphorous nutrients by photosynthetic assimilation (Wang *et al.* 2010). Thus, bioremediation properties of microalgae can be synergized to produce a sustainable feedstock for biofuel using WW resources.

Chlorella sorokiniana is a non-motile, unicellular microalga with a tough cell wall (Eckardt 2010). It grows well under mixotrophic conditions (Lizzul *et al.* 2014). The biomass obtained from *C. sorokiniana* can be enhanced using a simple carbon source as acetate (Qiao *et al.* 2009) or glucose (Wan *et al.* 2012). It is suitable for producing lipid, polysaccharides and other higher value commodity products (Lu *et al.* 2012). Worldwide *C. sorokiniana* has been known as a robust species and successfully scaled-up in tubular reactors (Lee *et al.* 1996) for various commercial applications. For a short span of time (intermittently), some reported strains of *C. sorokiniana* may tolerate higher temperature between 35 and 40°C. (De-Bashan *et al.* 2008). Its growth has been explored in different liquid wastes, like diluted final effluent from municipal WW treatment plant, bubbled with 12% CO_2 to generate biomass as feedstock for biofuel (Lizzul *et al.* 2014). Cultivation was also demonstrated using the 10% diluted cattle manure anaerobic digester effluent (Kobayashi *et al.* 2013).

In the present study, we have isolated an indigenous robust fast-growing *Chlorella* species from aboriginal sewage fed urban lake and its feasibility was assessed for the generation of biomass for biofuel feedstock while remediating WW. The microalga isolated from WW lake was characterized using various parameters and named as *C. sorokiniana*-I. Its *in-vitro* growth was comprehensively optimized by varying the pH, light intensity, temperature and culture media. Biomass productivity, lipid accumulation and fatty acid methyl esters (FAME) were studied using both autotrophic and mixotrophic conditions. The thermal tolerance was tested in the temperature range of 25–40°C. The nutrient removal efficiency of *C. sorokiniana*-I from undiluted (100%) WW was determined and FAME was assessed in the complacency of international standards. Life cycle assessment (LCA) of biodiesel production from *C. sorokiniana*-I was studied at the piolet scale using photobioreactors.

2. Materials and methods

2.1 *Sample collection, isolation and growth conditions*

The algal sample was collected from Neela-Hauz Lake, situated at 28.528950°N latitude and 77.170910°E longitude in Delhi, India. The environmental sample (1 mL) was enriched in sterilized 100 mL BG11 medium (Himedia, India) with 1% trace metal solution. The trace metal solution consists of: H_3BO_3 2.86 g/L; $MnCl_2{\cdot}4H_2O$ 1.81 g/L; $ZnSO_4{\cdot}7H_2O$ 0.22 g/L; $Na_2MoO_4{\cdot}2H_2O$ 0.39 g/L; $CuSO4{\cdot}5H_2O$ 0.079 g/L and $Co(NO_3)_2{\cdot}6H_2O$ 49.4 mg/L. The algal cells were purified by serial dilutions followed by inoculation onto Petri plates containing BG11 medium and 1.5% (w/v) of agar. The repetitive streaking of alga culture on solidified-BG11 plates helped in the purification of isolates from WW. Later, purified single colonies were also cultivated in the liquid medium. After completing the purification steps, culture was maintained at 25°C with the exposure to 16 h light (6000 lux irradiances) and 8 h dark photoperiod.

2.2 *Molecular identification and phylogenetic analysis*

Total genomic DNA was isolated from 500 mg of wet weight of algal culture following cetyl trimethyl ammonium bromide i.e. the CTAB method (Doyle 1987). The 18S and 16S rDNA conserved regions were amplified with the polymerase chain reaction (PCR) and commonly used primers as mentioned,

18SF: 5′GTAGTCATATGCTTGTCTC3′; *18SR*: 5′GCATCACAGACCTGTTATTGCCTC3′; *16SF*: 5′ATTTTCATGGAGAGTTTGATCCTGGCTCAGG3′; *16SR*: 5′AGAGTGCTTTCGCCTTTGGTGTTCCTCC3′.

The PCR amplification was carried out in a 20 μL reaction containing 10 mM dNTP, 5 picomoles of each primer, 1× *Taq* buffer, 0.2 U of *Taq DNA* polymerase (Real Biotech Corporation, Delhi) and 50 ng template DNA. The conditions for PCR amplification were: an initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation for 30 s at 95°C, primer annealing for 30 s and extension for 30 s at 72°C.

The PCR-amplified product was sequenced (Invitrogen Bioservices India Pvt. Ltd., Gurgaon, India). The resultant sequences were compared with the known sequences of the NCBI Database with the help of basic local alignment search tool (BLAST) algorithm (Zhang *et al.* 2000; Morgulis *et al.* 2008). The search results were aligned using Clustal W (Thompson *et al.* 1994). A phylogenetic tree was constructed using the neighbor-joining (NJ) method (Saitou and Nei 1987) on Molecular Genetics Analysis (MEGA) 6.0 software (Tamura *et al.* 2013) under default parameters. Bootstrap resampling analysis was performed to estimate the confidence of phylogenetic relationship (1000 replicates). *Elliptochloris bilobata* (Accession No. KT253169) and *Auxenochlorella protothecoides* (Accession No. KC843975.1) were used as an outgroup to root the phylogenetic tree using 18S rDNA and 16S rDNA markers, respectively.

2.3 *Optimization of cultivation conditions for isolated-algal species*

To find the optimal cultivation conditions, the following four major parameters were studied using 1L culture volume in three replicates:

(1) *pH*: The culture was inoculated in BG11 medium supplemented with 1% trace metals solution with varying pH values 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. The pH of growth medium was adjusted using 1 M NaOH and 1 N HCl as applicable.
(2) *Light intensity*: The inoculated cultures were exposed to the following range of light intensities of 110, 2200, 6000, 12,000 and 16,500 lux.
(3) *Temperature*: Using BG11 medium, algal growth was tested at 25, 30, 35, 37 and 40°C to find out the optimum temperature yielding the best biomass and lipid content.
(4) *Medium*: To assess the growth of isolated strain in different media, tris-acetate-phosphate (TAP) medium as defined by Gorman and Levine (1965) and 100% WW were also explored while using BG11 as a control medium. WW was sterilized by passing through a 0.2 μm filtration unit (Corning Incorporated, USA).

All the cultures were inoculated with 5% (v/v) of exponentially growing primary culture at 25°C in BG11 medium and pH 8.0 unless mentioned otherwise.

2.4 *Specific growth rate and doubling time (DT)*

The growth of microalga was monitored at an optical density (OD) of 750 nm with a UV-Visible spectrophotometer (Amersham Biosciences, Ultrospec 3100 pro) using 1 mL culture. The growth rate (μ) and DT of the culture were calculated using the equation as follows (Duong *et al.* 2015): Growth rate $(\mu) = [\ln(N1) - \ln(N0)]/(T1–T0)$, where $N0$ and $N1$ represent the OD at the beginning ($T0$) and end ($T1$) of time interval (in days). Each recorded OD was corrected by the corresponding blank sample. The DT was calculated using the formula as follows: $DT = \ln(2)/\mu$.

2.5 *Intracellular lipid estimation*

2.5.1 *Fluorescence microscopy of Nile red-stained cells:* To visualize the intracellular lipid bodies, Nile red (9-diethylamino-5H-benzo[alpha]phenoxazine-5-one) was used to stain the algal cells. The algal cells (1 mL) were briefly centrifuged (3000 rpm × 3 min in Eppendorf centrifuge 5424R) and the pellet was suspended in 1 mL of 20% dimethyl sulphoxide (DMSO). The mixture was agitated gently by vortexing, and briefly centrifuged (3000 rpm × 3 min). The pellet was resuspended in 1 mL of water. The Nile red stock solution (5 μL of 1 mg/mL), prepared in DMSO, was added to the algal cells and incubated in the dark for 10 min. The Nile red-stained cells were visualized under a fluorescent microscope (Nikon Model 50i) at 530 nm excitation and 575 emission wavelengths (Chen *et al.* 2009).

2.5.2 *Nile red cell assay using spectroscopy:* Fresh algal culture (200 μL at OD_{750} 0.3–1.0) aliquots were added into a 96-well microtiter plate, followed by 50 μL of 15 μg/mL of Nile red in DMSO. The working solution of 15 μg/mL of Nile red was prepared by diluting 5 mg/mL stock solution of Nile red in DMSO (Higgins *et al.* 2014). The microtiter plate was incubated at room temperature in the dark for 10 min, and then the fluorescence was read at 530 nm (excitation), 575 nm (emission) and 570 nm cut-off using a Spectramax M3 multimode spectrophotometer (Molecular Devices, USA). The fluorescence was obtained by subtracting the value of the unstained cell from that of stained cells, and normalized by dividing with OD of culture at 750 nm.

2.6 *Determination of dry-cell weight (DCW) and lipid extraction*

The DCW of the microalgal culture was routinely measured each day. Wet biomass was centrifuged (6000 rpm for 5 min), washed twice with distilled water and dried at 60°C in pre-weighed glass-vials (Rai *et al.* 1991). The weight of glass-vials was recorded at periodic intervals till the weight reached a constant value. The DCW was determined by subtracting the weight of the empty vial from that of the vial containing the biomass.

The lipid was extracted from the dried algal biomass by a reported method (Bligh and Dyer 1959) with some modifications as follows. The dried algal biomass was ground in 2:1 chloroform/methanol and then 0.9 mL of distilled water was added. After overnight shaking, an additional 1 mL chloroform and 0.9 mL distilled water was further added to the mixture and shaken for about 4 h. The layers were allowed to separate. The bottom chloroform layer was aspirated and filtered through Whatman No. 1 filter paper into a pre-weighed glass-vial. Chloroform was evaporated and the total lipid was calculated by subtracting the weight of the empty vial from that of the vial containing the lipid.

2.7 *FAME analysis*

The lipid transesterified into FAME was analyzed by gas chromatography (GC). For transesterification, 10 mg lipid was mixed with 2 mL of hexane and 200 μL of 2 M methanolic KOH was used as a catalyst (Ahmad *et al.* 2013). The mixture was vigorously agitated by using a vortexer for 5 min. The upper clear supernatant (hexane) was collected for FAME analysis. Quantification of FAME was carried out using a gas chromatograph (Agilent GC) equipped with an Omega Wax 250 column (30 m × 0.25 mm × 0.25 μm)

and a flame ionization detector. The operating conditions are as follows: split ratio 1:10, injection volume 1 μL, nitrogen carrier gas with constant linear velocity 33.9 cm/s, H_2 at 40 mL/min, air at 400 mL/min, makeup gas (nitrogen) at 30 mL/min, injector temperature of 270°C, detector temperature of 280°C, oven temperature started at 140°C for 5 min and increased at the rate of 4°C/min to 240°C and hold time of 20 min at 240°C. Heptadecanoic acid methyl ester (C17:0) was used as an internal standard.

2.8 *Assessment of biodiesel properties*

Critical biodiesel properties were calculated using predictive equations based on fatty acid composition. The kinematic viscosity and density of biodiesel were calculated from the FAME composition according to Ramírez-Verduzco *et al.* (2011). Saponification and iodine values, cetane number (CN) and higher heating values of biodiesel were calculated according to the models described by Amirsadeghi *et al.* (2015).

2.9 *Nutrient removal analysis of WW*

The nutrient content of WW was analyzed at the beginning (0 day) and at the end of the experiment (15 days). These include the chemical properties of WW such as chemical oxygen demand (COD), biological oxygen demand (BOD), total organic carbon (TOC), total inorganic carbon (TIC), total nitrogen (TN) and total phosphorous (TP) content, and also, iron, magnesium, calcium and alkalinity of the WW were analyzed. These parameters were studied following the standard method of American Public Health Association (Singh *et al*. 2017b). On day 0, the WW was filtered through a 0.2 μm filtration unit (Corning Incorporated, USA) and the filtrate was analyzed (C_0). On day 15, the microalgal culture was harvested by centrifugation (3000 rpm for 10 min), the supernatant was passed through 0.2 μm filters (Corning Incorporated, USA) and the filtrate was analyzed (C_{15}).

The percentage nutrient removal was calculated with the equation:

$$\text{Nutrient removal efficiency}\,(\%) = [(C_0 - C_{15}/C_0)] \times 100.$$

2.10 *LCA of microalga-based biodiesel*

Based on the experimental data on the cultivation of *C. sorokiniana*-I in urban WW, a scale-up was proposed using 100% WW. Here a LCA study was used to describe the sustainability of the process. The projected values were estimated based on the empirical *in-vitro* results. Energy inputs and economic viability were calculated at each step of the process, viz. cultivation, harvesting/dewatering, drying and transesterification. The process, however, takes a few assumptions into consideration as follows:

- *C. sorokiniana*-I was used on account of its high-growth rate and adaptability to elevated temperatures.
- Cultivation system was mixotrophic – utilizing 100% WW and light.
- Wet biomass was harvested and dewatered by centrifugation after reaching the highest probable lipid content and then dried.
- While lipid extraction, the solvents used were assumed to be fully recycled back into the process.
- Biodiesel was produced through transesterification of microalgal lipids with methanol in the presence of strong alkali as a catalyst.
- The electricity required for the operation of the process was supplied from the state electricity board but later would be contributed in part or full by the assembled solar panels.

The process started with culturing the microalgae in a photobioreactor with the capacity of 1000 L used to generate biomass. Undiluted urban WW was used as cultivation medium, known to have essential nutrients (C, N and P) for microalgal growth. The culture required constant gentle agitation by a propeller. Here in this case mixing by propeller at a mild pace has been considered. Harvesting and dewatering were carried out by using a centrifugal device running for about 3 h. The water was removed and thick algal biomass slurry was obtained. The biomass was dried in a hot air circulated oven for lipid extraction requiring around 5 h. Chloroform and methanol were used in the extraction of lipid from the algal biomass. The lipid extracted was transesterified with methanol and strong alkali and dissolved in hexane to be used as biodiesel. The final step of transportation and usage of algal biodiesel has not been included in this LCA as there is not sufficient data to support.

2.11 *Statistical analysis*

All experiments were conducted in triplicates and the data are reported as the mean ± standard deviation (±SD). The results were compared across treatments using ANOVA and Duncan's Multiple Range Test. The statistical level of significance was fixed at $p < 0.05$.

3. Results

3.1 *Phylogenetic analysis of unknown microalgal strain*

The microalga isolated from Neela-Hauz lake was genetically characterized using conserved-DNA sequences of the 18S rDNA and 16S rDNA regions. Using a pair of reverse and forward primers, 18SF-18SR and 16SF-16SR, 18S

rDNA and 16S rDNA regions yielded about 816 bp and 719 bp amplicons, respectively. The 18S rDNA sequence of isolated strain was observed closest to *C. sorokiniana* with 99% similarity, using the BLAST algorithm (*https://www.ncbi.nlm.nih.gov*). Further, phylogenetic relationships of 18S rDNA were inferred using the NJ method (figure 1A). Similarly, the phylogenetic relationship of the unknown microalgal isolate was deduced using the 16S rDNA marker and the NJ tree was generated. The 16S rDNA-based sequence also had maximum similarity (99%) with *C. sorokiniana,* studied using BLAST and supported by 81% bootstrap value (figure 1B). Hence, the new algal isolate was named as *C. sorokiniana*-I, where I denote the ICGEB. The accession numbers of 16S and 18S rDNA sequences were submitted in GenBank as MK177540 and MK177541.

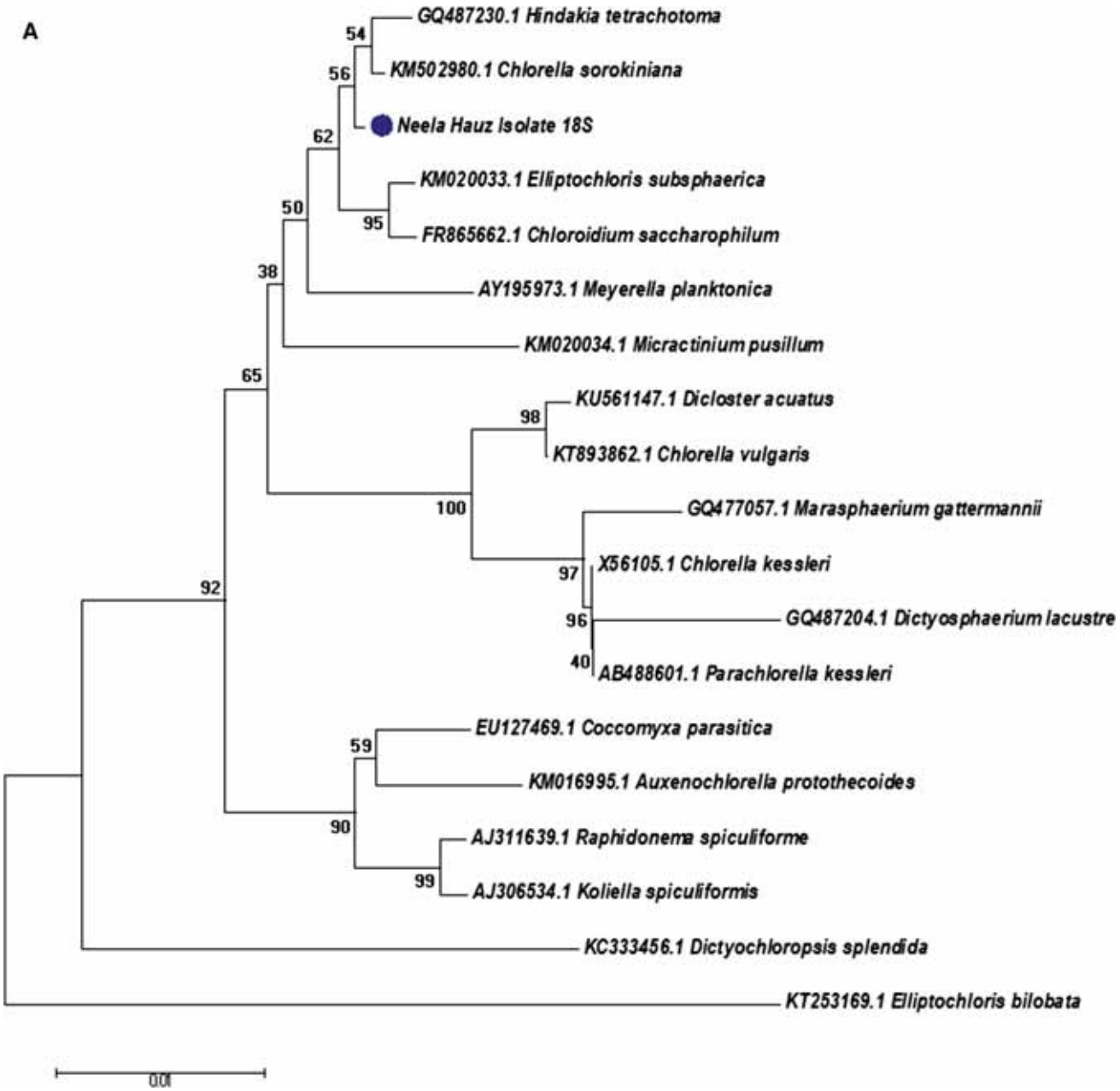

Figure 1. (**A**) Genetic identification of unknown species based on phylogenetic relationships of 18S rDNA sequences inferred using the NJ method. The BLAST results show the maximum similarity (99%) to *C. sorokiniana* with a bootstrap value of 56%. *E. bilobata* (family Ulvophyceae) was used as an outgroup to root the tree. The scale bar indicates nucleotide substitution per site. (**B**) Phylogenetic relationship of unknown microalgal isolate based on the 16S rDNA marker elucidated using the NJ method. The maximum similarity (99%) was observed with *C. sorokiniana* on BLAST search supported by the 81% bootstrap value. *A. protothecoides* is used as an outgroup to root the tree. The scale bar indicates nucleotides substitution per site.

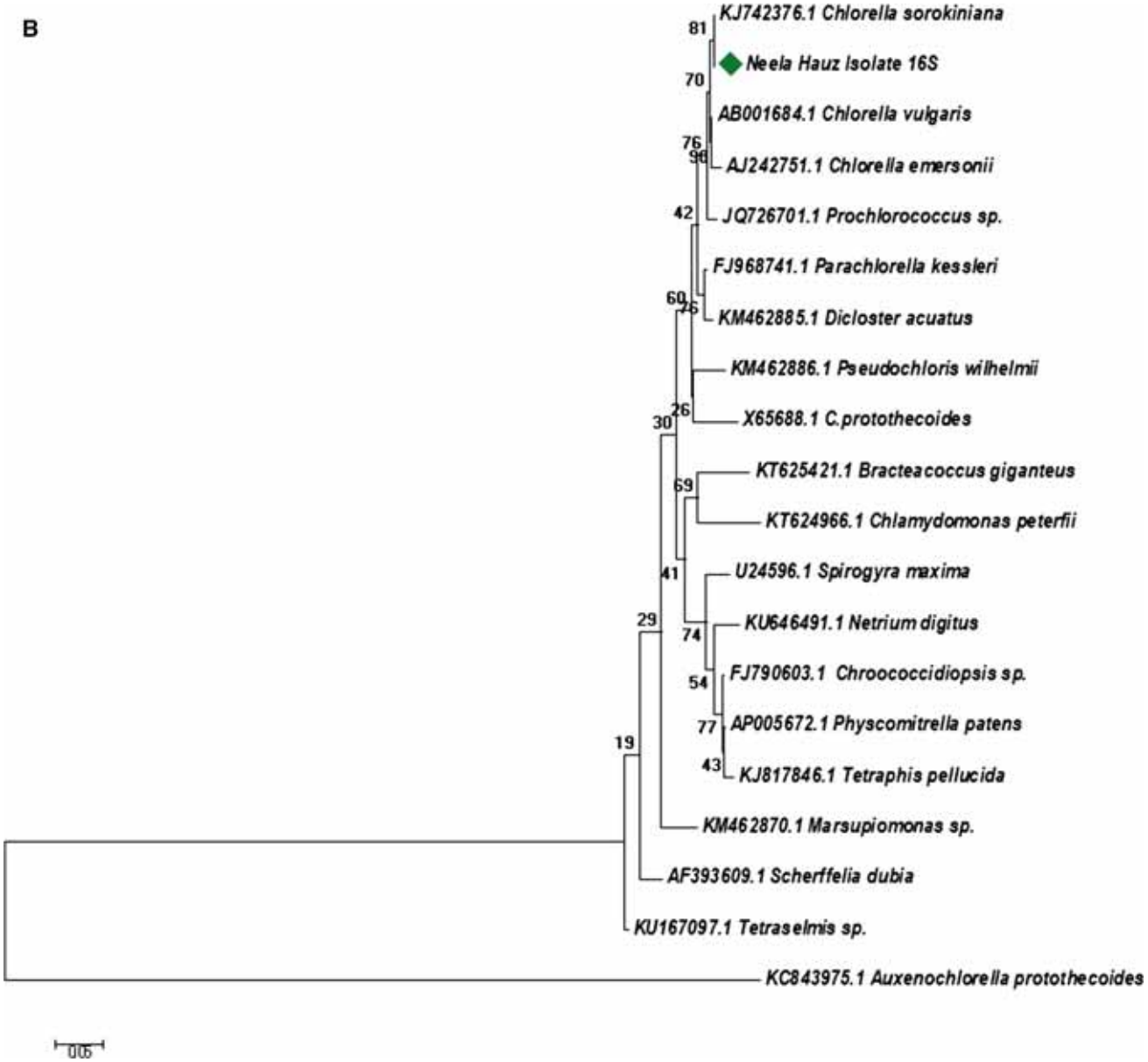

Figure 1. continued

3.2 *Impact of pH and light on microalgal growth and intracellular lipid accumulation*

The pH and light intensity are primarily essential factors that affect the microalgae growth. We have tested the growth of *C. sorokiniana*-I using autotrophic BG11 medium at varying pH levels 6.0, 7.0, 8.0 and 9.0 (supplementary figure 1). The maximum specific growth rate ($\mu = 0.58$ per day) was observed at pH 8.0.

The maximum specific growth rate ($\mu = 0.59$ per day) in *C. sorokiniana*-I culture was observed under 6000 lux light intensities, pH 8.0 using the BG11 medium. However, higher light intensities above 6000 lux were ineffective in elevating the μ value (supplementary figure 2). Therefore, 6000 lux was selected as an optimum light intensity for the *in-vitro* growth of *C. sorokiniana*-I.

The Nile red cell assay as reported (Higgins *et al.* 2014) was used to selectively stain and determine relative intracellular neutral lipids. The fluorescence value (Arbitrary units A.U.) of the stained algal cells was observed to decline in the lag and early exponential growth phase (day 6). However, the fluorescence of neutral lipid was increased steadily in the late exponential growth phase (day 11). The relative lipid content (seen as yellow golden lipid globules increased in size and number) was observed maximum on the 15th day (figure 2). Further on 16th day onwards, Nile red fluorescence began to decrease in *C. sorokiniana*-I culture. Therefore, cultures were harvested on the 15th day

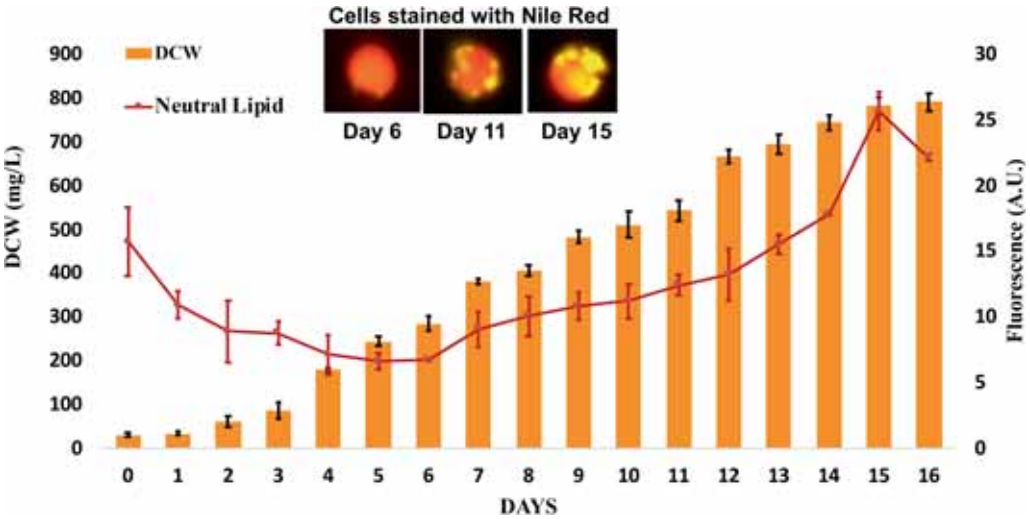

Figure 2. Determination of biomass and intracellular lipid content of *C. sorokiniana*-I by staining cells with Nile red fluorescent dye, cultured for 16 days using the BG11 medium. The biomass cell weight (DCW) increases through the exponential growth phase and reached the stationary phase on the 13th day. Lipid droplets were observed as golden globules in cells, which keep on increasing both in numbers and size during the exponential phase (day 6) through late stationary phase (day 15). The maximum relative lipid content was observed on day 15. Data are an average of three experiments ± S.D (n = 3).

(figure 2), to retrieve the maximum accumulated lipid for the FAME.

3.3 *Impact of temperatures on microalgal biomass, lipid and FAME*

The growth pattern of *C. sorokiniana*-I was studied using an autotrophic BG11 medium and the temperature range of 25–42°C (figure 3A). With an increase in temperature to 25, 30, 35, 37 and 40°C, algal-biomass (DCW) was observed to gradually decrease to 898, 881, 793, 574 and 495 mg/L, respectively. The biomass was almost reduced to half when *C. sorokiniana*-I cultures were maintained for two weeks at an uninterrupted 40°C (figure 3A; table 1). The higher temperature (45°C) was observed lethal for the growth of *C. sorokiniana*-I and led to complete cessation of algal growth after 5 days.

The maximum growth of *C. sorokiniana*-I (without any significant loss in the DCW) was observed in the range of 25 to 35°C (figure 3A). Additionally, *C. sorokiniana*-I cultures were able to thrive well (under autotrophic conditions) continuously for 15 days when cultures were constantly grown at a higher temperature of 40°C. This distinguishes *C. sorokiniana*-I from the previously reported *C. sorokiniana* strains, which could sustain the high temperature for a brief intermittent period only (De-Bashan *et al.* 2008).

The higher temperature not only impacted the growth but also total lipid accumulation in *C. sorokiniana*-I. Using a BG11 medium, by a gradual increase of temperature to 25, 30, 35, 37 and 40°C, total lipid accumulation also declined gradually to 108.6, 98, 99, 59 and 24 mg/L, respectively (table 1). Though the amount of total lipid accumulated at

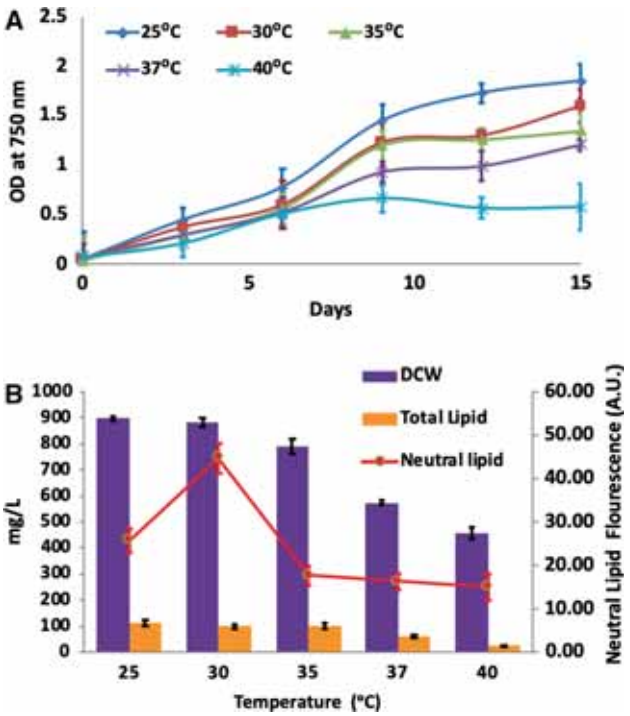

Figure 3. (**A** and **B**). Impact of varying temperatures on biomass and lipid content of *C. sorokiniana*-I. (**A**) The growth of alga at different temperatures. (**B**) Biomass (DCW) and lipid content at different temperatures on the 15th day. The maximum accumulation of intracellular neutral lipid and the highest FAME recorded at 30°C using Nile red stain on day 15. Data are an average value of three experiments ± S.D (n = 3).

temperatures 25, 30 and 35°C was more or less the same (108–99 mg/L), the FAME content was significantly higher at 30°C (10.65% of total lipid) in BG11 medium (table 1).

Table 1. Comparison of specific growth rate, DT and growth parameters of *C. sorokiniana*-I, using BG11 medium at varying temperatures (25–40°C)

Temperature (°C)	Specific growth rate (day^{-1})	Doubling time (h)	DCW (mg/L)	Total lipid (mg/L)	Lipid content (% DCW)	FAME (%)
25	0.59 ± 0.008^{a}	18.63 ± 0.831^{d}	898 ± 20.1^{a}	109.5 ± 9.0^{a}	12.19 ± 0.846^{a}	6.03 ± 0.840^{c}
30	$0.55 \pm 0.008^{a,b}$	20.08 ± 0.701^{c}	881 ± 10.7^{a}	98 ± 1.4^{b}	11.12 ± 0.241^{a}	10.65 ± 0.96^{a}
35	$0.53 \pm 0.090^{a,b}$	20.80 ± 0.454^{c}	793 ± 20.9^{b}	99 ± 1.7^{b}	12.48 ± 2.603^{a}	4.030 ± 0.41^{d}
37	0.50 ± 0.030^{b}	31.22 ± 0.031^{a}	574 ± 10.0^{c}	59 ± 0.7^{c}	10.27 ± 1.040^{a}	7.86 ± 0.74^{b}
40	0.47 ± 0.003^{b}	29.59 ± 0.710^{b}	459 ± 23.1^{c}	24 ± 0.2^{d}	5.22 ± 0.174^{b}	11.44 ± 0.16^{a}

Cultures were harvested on the 15th day.

Data are an average value of three experiments ± S.D ($n = 3$).

[a–d] Significantly ($P < 0.05$) different from each other (Duncan's new multiple range tests).

Table 2. FAME (%) of *C. sorokiniana*-I cultured in the BG11 Medium at varying temperatures (25–40°C) on the 15th day

	Temperature range				
Fatty acid methyl ester (carbon chain length)	25°C	30°C	35°C	37°C	40°C
Palmitic acid, methyl ester (C16:0)	45.92 ± 1.46^{c}	64.83 ± 0.94^{a}	64.20 ± 1.14^{a}	63.37 ± 1.76^{a}	55.34 ± 1.28^{b}
9-Hexadecenoic acid, methyl ester (C16:1)	2.22 ± 0.56^{a}	1.81 ± 0.27^{a}	1.76 ± 0.05^{a}		
7,10-Hexadecadienoic acid, methyl ester(C16:2)	3.51 ± 0.47^{b}	4.61 ± 0.15^{a}	2.69 ± 0.08^{c}	1.73 ± 0.63^{d}	4.61 ± 0.32^{a}
7,1013-Hexadecatrienoic acid, methyl ester (C16:3)	5.58 ± 0.86^{a}	$1.56 \pm 0.17^{b,c}$	1.94 ± 0.45^{b}	1.08 ± 0.05^{c}	
Stearic acid, methyl ester (C18:0)	6.23 ± 1.06^{b}	3.44 ± 0.72^{c}	7.97 ± 0.86^{b}	18.56 ± 1.96^{a}	7.55 ± 0.39^{b}
Oleic/elaidic acid, methyl ester (C18:1)	10.22 ± 0.67^{b}	10.73 ± 0.06^{b}	7.79 ± 0.96^{c}	7.59 ± 0.06^{c}	12.23 ± 0.11^{a}
Linoleic acid, methyl ester (C18:2)	15.59 ± 2.00^{a}	$8.85 \pm 1.04^{b,c}$	10.81 ± 1.50^{b}	7.67 ± 1.06^{c}	15.72 ± 0.62^{a}
Linolenic acid, methyl ester (C18:3)	10.73 ± 1.42^{a}	$4.17 \pm 0.64^{b,c}$	2.84 ± 0.04^{c}		4.54 ± 0.61^{b}
SFA	52.15 ± 2.51^{d}	68.27 ± 1.66^{b}	72.17 ± 2.00^{b}	81.92 ± 3.71^{a}	62.89 ± 1.66^{c}
MUFA	12.44 ± 1.23^{a}	12.54 ± 0.34^{a}	9.55 ± 1.01^{b}	7.59 ± 0.06^{c}	12.23 ± 0.11^{a}
PUFA	35.41 ± 4.75^{a}	19.19 ± 2.00^{c}	18.28 ± 3.07^{c}	10.49 ± 1.74^{d}	24.88 ± 1.55^{b}

Data are an average value of three experiments ± S.D ($n = 3$).

[a–d] Significantly ($P < 0.05$) different from each other (Duncan's new multiple range test).

The same results were reaffirmed by the Nile red assay wherein the intracellular neutral lipid content with the maximum fluorescence was observed at 30°C (figure 3B), in comparison with 25 and 35°C (supplementary figure 3).

The FAME profiles of *C. sorokiniana*-I majorly contained saturated fatty acid (SFA) such as palmitic acid (C16:0) and polyunsaturated fatty acids (PUFAs) such as oleic acid (C18:1) and linoleic acid (C18:2). SFA increased with an increase in temperature from 25 to 37°C. However, this increase was in contrast to that of PUFA, which gradually decreased with an increase of temperature from 25 to 37°C (table 2). The highest amount of monounsaturated fatty acid (MUFA; 12.54%) was recorded at 30°C (table 2). 30°C was considered as an optimum temperature for yielding the highest FAME content.

3.4 *Impact of culture media on biomass, lipid and FAME*

Initially, two different media (BG11 and TAP) were randomly tested on an unknown microalgal strain to optimize the growth. Later, after genetic characterization of *C. sorokiniana*-I, TAP and 100% WW were tested for assessing the biomass yield on day 15 at 30°C (figure 4A and B). Under mixotrophic growth conditions, WW was explored as a cost-effective alternative to a commercially available TAP medium. Autotrophic BG11 medium was used as a control for relative assessment of the growth parameters. Both TAP and 100% WW produced higher biomass (1028 and 1019 mg/L, respectively) compared to BG11 medium (881 mg/L) at 30°C (table 3). Remarkably, the FAME content was observed to be twice (47.04%) in 100% WW medium when compared to that of synthetic media TAP (20.48%) and BG11 (10.65%) (table 3), though the total lipid accumulation was slightly higher in 100% WW (106.8 mg/L) than TAP medium (99 mg/L) and BG11 (98 mg/L).

Intracellular lipid (determined by Nile red cell assay) was observed to be the highest in WW-grown *C. sorokiniana*-I compared to TAP and BG11 (supplementary figure 4). The FAME composition has the highest amount of SFA (68.27%) in BG11, the highest PUFA (58.98%) in TAP medium while the highest MUFA (14.02%) was observed in WW. The major fatty acids like palmitic acid/C16:0 followed by stearic

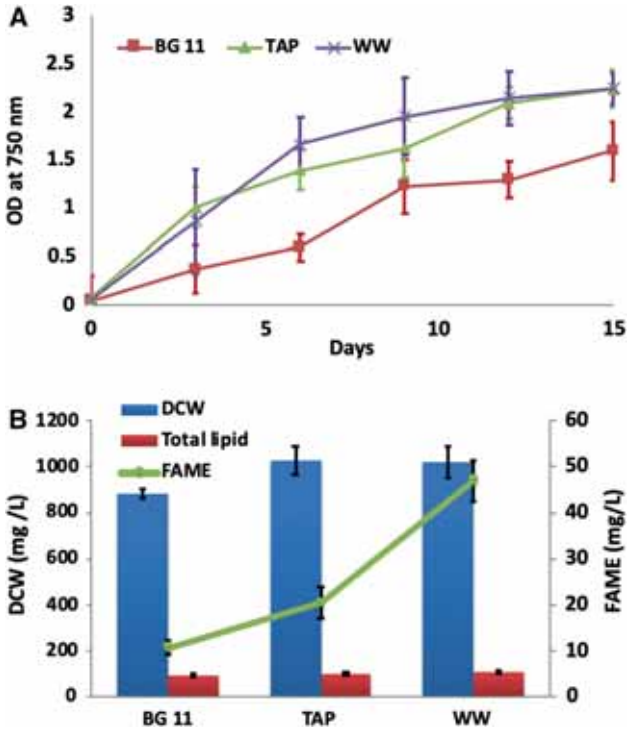

Figure 4. (**A** and **B**) Effect of culture media on growth parameters of *C. sorokiniana*-I. (**A**) The maximum growth noticed in WW, comparable to that of heterotrophic TAP medium. (**B**) The maximum total lipid content and FAME were observed in WW used as a growth medium. Data are an average value of three experiments ± S.D (n = 3).

acid/ C18:0 and oleic acid/C18:1) were observed in different proportions in all the three-tested media (table 3).

3.5 *Biodiesel (FAME) properties*

The quality of *C. sorokiniana*-I's biodiesel (cultivated in different media) was studied in compliance with the quality standards of European (EN) and American Society for Testing and Materials (ASTM) (table 4). The physical properties of biodiesel such as density, kinematic viscosity, CN, iodine value, saponification value and higher heating value were calculated based on the FAME content. CN, iodine value and higher heating values of FAMEs produced by *C. sorokiniana*-I in all tested media were observed in the same range as specified by EN and ASTM. The kinematic viscosity of the FAME produced using BG11 and TAP medium was observed as per the ASTM standard. The density of FAME produced in BG11 grown cells was slightly lower than the specified value. However, WW grown *C. sorokiniana*-I has produced FAME with properties in perfect compliance with the international standards (see table 4).

3.6 *Nutrients removal from WW by C. sorokiniana-I*

Using an integrated approach, the remediation properties of *C. sorokiniana*-I were investigated using 100% WW at 30°C. The percentage of nutrient removal was analyzed after harvesting the culture on day 15. The nutrient removal was observed as, TN ∼77%, ammoniacal nitrogen ∼86%,

Table 3. Comparison of different growth media TAP and 100% WW and BG11 and FAME (%) of *C. sorokiniana*-I at 30°C (optimized temperature)

Growth parameters	BG11	TAP	WW
Biomass (mg/L)	881 ± 10.7^{b}	1028 ± 7.7^{a}	1019 ± 4.0^{a}
Total lipid (mg/L)	98 ± 1.4^{b}	99 ± 1.5^{b}	106.8 ± 5.5^{a}
Lipid content (% DCW)	11.12 ± 0.241^{a}	9.63 ± 1.67^{a}	10.49 ± 0.70^{a}
FAME (% total lipid)	10.65 ± 0.96^{c}	20.48 ± 4.7^{b}	47.04 ± 4.27^{a}
Relative FAME composition (%)			
Hexadecanoic acid, methyl ester (C16:0)	64.83 ± 0.94^{a}	11.95 ± 1.07^{c}	46.94 ± 2.55^{b}
9-Hexadecenoic acid, methyl ester (C16:1)	1.81 ± 0.27^{b}	7.62 ± 1.32^{a}	2.76 ± 0.14^{b}
7,10-Hexadecadienoic acid, methyl ester (C16:2)	4.61 ± 0.15^{c}	5.66 ± 0.27^{b}	6.97 ± 0.29^{a}
4,7,10-Hexadecatrienoic acid, methyl ester (C16:3)	1.56 ± 0.17^{b}	12.03 ± 2.05^{a}	1.38 ± 0.1.32^{b}
Stearic acid, methyl ester (C18:0)	3.44 ± 0.72^{c}	20.54 ± 2.51^{a}	11.68 ± 0.55^{b}
Oleic/elaidic acid, methyl ester (C18:1)	10.73 ± 0.06^{a}	4.92 ± 0.72^{b}	11.26 ± 0.28^{a}
Linoleic acid, methyl ester (C18:2)	8.85 ± 1.04^{b}	19.11 ± 1.21^{a}	5.10 ± 6.01^{b}
Linolenic acid, methyl ester (C18:3)	4.17 ± 0.64^{c}	18.18 ± 2.33^{a}	12.30 ± 0.63^{b}
SFA	68.27 ± 1.66^{a}	32.49 ± 1.51^{c}	58.62 ± 3.10^{b}
MUFA	12.54 ± 0.34^{b}	12.52 ± 0.25^{b}	14.02 ± 0.42^{a}
PUFA	19.19 ± 2.00^{b}	58.98 ± 1.99^{a}	25.75 ± 8.26^{b}

BG11 used as a control medium. Cultures were harvested on day 15.

Data are an average value of three experiments ± S.D (n = 3).

$^{a–d}$ Significantly ($P < 0.05$) different from each other (Duncan's new multiple range test).

Table 4. FAME (biodiesel) properties of *C. sorokiniana*-I grown in BG11, TAP and WW (100%) in compliance with international standards (EN/ASTM)

Physical properties	BG11	TAP	WW	EN 14214:2008	ASTM D6751
Cetane number	65.85	53.03	62.30	≥51	≥47
Iodine value (g iodine/100 g oil)	47.33	104.08	66.25	<120	–
Saponification value (mg KOH/g oil)	218.63	202.77	211.02	–	–
Higher heating value (MJ/Kg)	45.66	45.03	45.48	–	≥35
Kinematic viscosity (mm^2/s)	2.34	3.14	4.26	3.5–5.0	1.9–6.0
Density (g/cm^3)	0.75	0.89	0.86	0.860–0.900	0.875–0.900

Data are an average value of three experiments ± S.D (n = 3).

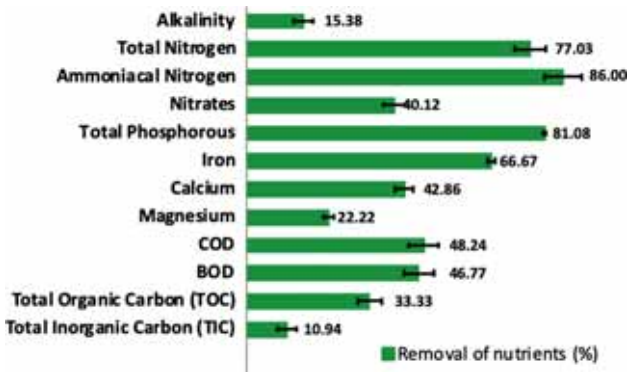

Figure 5. Removal of nutrients (%) by *C. sorokiniana*-I from WW. Cultures were harvested on the 15th day. Data are an average value of three experiments ± S.D (n = 3).

nitrates ∼40%, TP ∼81%, TOC ∼33% and TIC ∼11%. However, COD and BOD were reduced by 48 and 47%, respectively. The metal ions such as iron, magnesium, calcium and potassium were reduced by 61, 43, 22 and 18%, respectively. The alkalinity of WW was reduced by 15% i.e. 88 ppm from 104 ppm of $CaCO_3$ (figure 5). Thus, *C. sorokiniana*-I has an excellent nutrient removing efficiency while improving the overall COD/BOD of water. This makes it an ideal strain for the remediation of WW and generating biomass in cost-effective *modus operandi* compared to artificial media.

3.7 *LCA for biodiesel production using up-scale study of C. sorokiniana-I*

Biodiesel production from microalgae is a multilevel process comprising of different stages – cultivation, harvesting/dewatering, drying and transesterification (lipid extraction and processing). Each stage has different requirements with respect to energy inputs and outputs. Due to insufficiency of reported industrial-scale data, a full model construction of microalgal biodiesel production plant was not feasible for *C. sorokiniana*-I. Hence projected values of large scale processing are estimated based on the empirical *in-vitro* results and assumptions based on the reported literature.

The process starts with the culturing stage, wherein electricity is required for continuous agitation of the culture by a propeller and/or sparging air/CO_2 and various sensors to measure the physical parameters such as pH and light. Typical growth and harvest cycles are considered to be 15 days based on the *in vitro* study. According to the available empirical data on *C. sorokiniana*-I, 1 kg of algal biomass (DCW) can be obtained from ∼1000 L of microalgal culture in 100% WW. For the culturing purpose, about 2593 MJ energy would be required for the function of propellers and sensors in the photobioreactor (PBR) for a period of 15 days. Harvesting and dewatering are carried out by centrifugation that would consume 9.648 MJ energy for about 3 h of operation. The removed water was used for gardening and/or agricultural purposes, which has low-metal ion concentration and alkalinity. The biomass is dried in a hot-air circulated oven for lipid extraction that requires 15.12 MJ energy for 5 h. The final stage of the process is transesterification using organic solvents. The entire process utilizes 12.6 MJ of energy required for heating the mixture of organic solvents. The growth, harvesting, drying and transesterification were performed in the same premises. The electricity utilized in the entire operation was supplied from the solar panels installed over the photobioreactor premises facility.

4. Discussion

The selection and identification of algal strain with rapid growth and adaptability to diverse habitats are vital for the production of biomass to be used as a sustainable biofuel feedstock. We have isolated a fast-growing robust microalga from a WW that thrived well in varied temperatures, media, pH and environmental conditions. This strain was characterized as *C. sorokiniana*-I using DNA barcode studies. Axenic culture of *C. sorokiniana*-I under a microscope was observed as a single-celled, non-motile spherical microalga (diameter of 3–5 μm) with a distinct cup-shaped chloroplast, similar to the previously reported description for *C. sorokiniana* species (Lizzul *et al.* 2014).

The pH is an important factor for microalgae growth, which regulates the uptake of inorganic carbon from growth media (Azov *et al.* 1982). Microalgae have a specific

characteristic to release some acidic extracellular metabolites that help in reducing the surrounding pH and aid their survival under an alkaline environment (Mishra *et al.* 2018). In our study, *C. sorokiniana*-I flourished well in a wide range of pH 6.0 to 9.0 (supplementary figure 1), making it suitable for cultivation under diverse outdoor conditions.

In all photosynthetic organisms, light and dark periods are essential for productive photosynthesis. In general, microalgae's exposure to low light reduces the photosynthetic efficiency while intense light causes photoinhibition and reduced-photosynthetic productivity (Han *et al.* 2000). However, *C. sorokiniana*-I is highly adapted to grow in a broad range of illumination from 6000 to 12,500 lux (supplementary figure 2), without displaying any adverse impact on the biomass or photosynthetic productivity. This characteristic of *C. sorokiniana*-I is quite similar to the previous report of *C. sorokiniana* UTEX 2805, which has been reported to grew well in a higher range of irradiances (De-Bashan *et al.* 2008).

In the course of microalgal growth, a dynamic carbon partitioning occurs between starch and lipid accumulation. In general, after a decline of starch, lipid accumulation enhances in the later phase due to the depletion of nutrients from medium, which enables the algal cell to survive during the unfavorable drastic conditions. The starch levels in microalgal cells increase rapidly during an exponential growth (Log phase) and decline steadily thereafter (Li *et al.* 2015). Thus, the lipid profile of the microalgae changes with the phases of cell growth. We have used the Nile red stain to relatively estimate the intracellular neutral lipids of *C. sorokiniana*-I under varied environmental conditions. The fluorescence was high on day 0 due to intracellular lipids present in older cells that were used as an initial inoculum. Lipids are storage molecules in algae, which was low in *C. sorokiniana*-I during the log phase. In the log phase, the cells were actively dividing and hence required more phospholipids to be incorporated into the membranes. Hence the amount of neutral lipids found was less (see figure 2, on day 6). The neutral lipid gradually increases when the cells approached the stationary phase (on day 11). In the stationary phase, the cell division of algal cells almost ceased and accumulation/secretion of secondary metabolites began. As the culture reached the late stationary phase, the maximum fluorescence (neutral lipid accumulation) was observed on day 15, this phenomenon is similar to the previous reports in other algal species (Ahmad *et al.* 2013). Thereafter after 16th day onwards, Nile red fluorescence was steeply declined (figure 2). This indicates that a period of 15 days was the optimized cultivation period or maturation cycle for *C. sorokiniana*-I to obtain the highest lipid and biomass productivity.

The temperature determines the activity of intracellular enzymes, thereby influencing photosynthesis, respiration intensity and growth of microalga (Han *et al.* 2000). India and tropical countries get high iridescences and elevated temperatures during the summer. Usually, the optimum temperature for *in vitro* growth of microalgal species is reported between 20 and 25°, while *Chlorella* species can grow in a broad range of temperatures (Ras *et al.* 2013). Among *Chlorella* species, *C. sorokiniana* is reported as most tolerant to a high-temperature and high-light intensity (Duong *et al.* 2015). This makes the *C. sorokiniana* species more suitable for outdoor cultivation in the tropical countries where the temperature increases above 30°C during the summer. In our study, *C. sorokiniana*-I flourished well in the range of 25–37°C. Additionally, it grew well continuously for 15 days at a constant temperature of 40°C. This can be an additional advantage of *C. sorokiniana*-I for the reduction in the energy consumption and cost for cooling of photobioreactors (Li *et al.* 2013).

The mortality in *C. sorokiniana*-I cells was observed after 4–5 days when a constant temperature was maintained at 45°C. This result is similar to the study reported by De-Bashan *et al.* (2008), wherein the temperature was increased in two steps from 30 to 35°C and 35 to 40°C for 30 min each and the cultures were maintained for 5 h at 40°C. In our literature survey, most of the previous reports on *C. sorokiniana* pertaining to high temperatures are either restricted to a few hours per day or an intermittent increase of high temperature. Thus, our isolate *C. sorokiniana*-I from WW is a robust strain and well adaptable to grow at elevated temperatures and high-diurnal irradiance conditions.

Temperature is also a crucial factor in altering the FAME composition of *C. sorokiniana* (Patterson 1970). SFAs are dominant at elevated temperatures while polyunsaturated fatty acids are abundant at lower temperatures (Aussant *et al.* 2018), which indicate that the lipid profile in microalgae species alters over a range of temperature. We have observed the maximum biomass productivity and lipid accumulation at 25 and 30°C, respectively, under autotrophic conditions (supplementary figure 3). These results agree with the previous report (Li *et al.* 2013), where C. *sorokiniana* produced the maximum biomass at 25°C and accumulated the highest lipid at 30°C, though their study was conducted under the mixotrophic conditions using glucose. Interestingly, we have noticed an abrupt increase in the FAME content of *C. sorokiniana*-I at 30°C, which may be due to an increase in the enzyme activity involved in the neutral lipid production. In *C. sorokiniana*-I, PUFA decreased gradually with an increase of temperatures from 25 to 37°C, which is a common observation in other microalgal species too since at lower temperatures, unsaturated fatty acids are needed by cells to maintain membrane fluidity and function (Li *et al.* 2013). Temperature also impacts the degree of fatty acid saturation and affects the vital properties of biodiesel, like ignition and oxidative stability (Knothe 2008). Thus, the temperature is the most important factor in improving the biomass productivity and lipid quality pertaining to biodiesel production.

Different media with varying nutrient compositions considerably change the quantity of biomass produced during algal cultivation. Microalgae are photoautotrophs i.e. they

biosynthesize their own food (organic molecules) from inorganic carbon sources (CO_2 and HCO_3) for their growth (Oswald 1988b). Some microalgal species utilize organic acids, sugars, acetate or glycerol as alternative organic carbon sources (Fogg 1975; Kawaguchi 1980; Borowitzka 1998; Wood *et al.* 1999; Ogbonna *et al*. 2000). The deficiency of nutrients tends to cause retardation in algal cell growth, resulting in lower CO_2 fixation and higher accumulation of fatty acids due to stress conditions (Zhu *et al.* 2016). Zhang *et al.* (2018) reported increased lipid productivity in *C. sorokiniana* SDEC 18 under a salinity stress of 20g NaCl/L. Among different modes of cultivation, autotrophy is known to accumulate less biomass in comparison with heterotrophy and mixotrophy.

In our study, *C. sorokiniana*-I under mixotrophic conditions (100% WW containing organic carbon) showed biomass at par with TAP medium (containing acetate) and significantly higher amounts of FAME compared to synthetic media (BG11 and TAP). Thus, WW used as a nutrient supply has helped in achieving a sufficient amount of biomass (DCW) and the maximum FAME content in *C. sorokiniana*-I. The high-FAME content in *C. sorokiniana*-I seems to be due to comparatively lower amounts of nutrients (N and P) in the WW (supplementary table 6). The fatty acids accumulated in *C. sorokiniana*-I using WW majorly constituted of palmitic acid, linolenic acid, stearic acid and oleic acids. Higher concentrations of both palmitic and oleic acid are essential for producing good quality of biodiesel that provides a better ignition quality, higher oxidative stability and good lubrication properties (Durrett *et al.* 2008; Knothe 2008; Hu *et al.* 2015). Higher biomass and FAME produced in WW are also vital for the economic scale-up of *C. sorokiniana*-I if growth medium (WW) is considered as zero cost. The use of 100% WW for *C. sorokiniana*-I cultivation will also reduce a load on clean water repositories.

A systematic analysis of the FAME composition and fuel properties, in compliance with the international standards, is important for the selection of appropriate microalgal species for biodiesel production. CN is a measure of the fuel's ignition characteristics and it decreases with an increase in unsaturation in FAMEs (Saxena *et al.* 2013). The CN of FAME produced by *C. sorokiniana*-I is well above the number specified by EN and ASTM standards. The iodine value (IV) which is a measure of the degree of unsaturation in biodiesel is used to determine its oxidative rancidity and chemical stability. Usually, fuels with lower IV are considered better fuels (Ismail and Ali 2015). In the case of *C. sorokiniana*-I, FAMEs derived from all the three-tested media fall below the value approved by EN (120 g iodine/100 g oil). The saponification value has not changed significantly using BG11, TAP and WW i.e. similar molecular weights of the biodiesel produced. Higher heating values of FAME indicate the energy content of a fuel (Amirsadeghi *et al.* 2015), which was observed in compliance with the ASTM standard (35 MJ/Kg) in *C. sorokiniana*-I using all the three media. The viscosity affects the behavior of fuel injection and high viscosity can lead to overall poorer combustion of fuel (Saxena *et al.* 2013), and also fall within the range defined by the ASTM tested for the three media. The density of FAMEs produced by *C. sorokiniana*-I in TAP and WW was found to be within the specified range of both EN and ASTM. However, the density of FAME using BG11 was slightly lower than the specified range by both EN and ASTM. This may be due to the presence of a relatively higher amount of palmitic acid (C16:0) in the FAME produced by *C. sorokiniana*-I using BG11 medium. In brief, overall biodiesel produced by *C. sorokiniana*-I, using WW, has excellent balanced properties with respect to International standards, making this strain amenable for future production of biodiesel on a commercial scale using an integrated approach.

Many microalgal species have shown potential for remediation of WW and have been extensively studied in past as summarized in table 5. Microalgal species like *Botryococcus braunii* are known for high-lipid accumulation but cells grew slow and take a month to complete the growth cycle and relatively produce lesser biomass (Kim *et al.* 2007). *Chlorella vulgaris* is known to accumulate high amounts of lipids in domestic WW (Mulbry *et al.* 2008). However, a study conducted by Singh *et al.* (2017a) on the same strain showed high-biomass productively but low-lipid accumulation, lesser than *C. sorokiniana*-I. The biomass and lipid accumulation in microalgae depends on the amount of nutrients present in the WW, which tends to change with external factors such as rain, inflow effluents, etc. Autotrophs carry out WW remediation through the photosynthetic process. It poses advantages such as cost-effectiveness, nutrient retrieval from WW and sequestering CO_2 in the form of bicarbonates (Whitton *et al.* 2015). The excess amount of nitrogen and phosphorous present in WW is the major cause of eutrophication (Mahapatra *et al.* 2013), which is contributed largely by the urban and agricultural waste dumped into the downstream running water (Salim *et al.* 2013). We have investigated the phycoremediation and nutrient removal efficiency of *C. sorokiniana*-I using 100% WW. It has efficiently removed the 86% ammoniacal nitrogen (figure 5), a preferred form of nitrogen assimilated by *C. sorokiniana* species (Lizzul *et al.* 2014). It has remarkably decreased 81% TP and 77% TN, indicating its potential to prevent the eutrophication in the open WW system that contained excess nitrogen and phosphorous (Abdel-Raouf *et al.* 2012). The excess COD/BOD values also indicate higher organic and biological pollution in the water bodies, which was effectively reduced by *C. sorokiniana*-I. The COD and BOD values of WW were reduced to 48 and 47%, respectively, which should be useful in reducing the biological activity of organisms. The isolated strain, *C. sorokiniana*-I lowered the concentration of metal ions (Fe, Mg, Ca and K) and reduced the alkalinity of WW by 15%. These excellent characteristics make *C. sorokiniana*-I an ideal strain to be used on a commercial scale for

Table 5. Comparison of different microalgal species biomass (DCW) and lipid (%) yield cultivated in different sources of WW

WW type	Microalgae species	Biomass (DCW) productivity (mg/L/day)	Lipid (% DCW)	Lipid productivity (mg/L/day)	Reference
Agricultural (fermented swine urine)	*Scenedesmus* sp.	6[a]	0.9[b]	0.54[b]	Ip *et al*. (1982)
Agricultural (swine effluent, maximum manure loading rate)	*R. hieroglyphicum*	10.7 g m^{-2} day^{-1}	0.7[b]	72[b] mg m^{-2} day^{-1}	Martinez *et al*. (2000)
Industrial (carpet mill, untreated)	*B. braunii*	34	13.20	4.5[c]	Kim *et al*. (2007)
Industrial (carpet mill, untreated)	*C. saccharophila*	23	18.10	4.2[c]	Kim *et al*. (2007)
Industrial (carpet mill, untreated)	*Dunaliella tertiolecta*	28	15.20	4.3[c]	Kim *et al*. (2007)
Industrial (carpet mill, untreated)	*Pleurochrysis carterae*	33	12.00	4.0[c]	Kim *et al*. (2007)
Domestic WW	*C. vulgaris*	40.9	32.7	13.4[c]	Mulbry *et al*. (2008)
Agricultural (digested dairy manure, 20× dilution)	*Chlorella* sp.	81.4[d]	13.6[b]	11[b]	Wang *et al*. (2010)
Influent WW	*Botryococcus braunii IBL C116*	59.35	Nd	8.31[c]	Chinnasamy *et al*. (2010)
Final effluent (1:10 diluted)	*C. sorokiniana*	55	Nd	4[c]	Lizzul *et al*. (2014)
Centrate (1:10 diluted)	*C. sorokiniana*	42.5	Nd	3[c]	Lizzul *et al*. (2014)
Urban WW	*C. vulgaris*	161.4[e]	8.31	1.91[e]	Singh *et al*. (2017a)
Undiluted urban WW	*C. sorokiniana*-I	67.93[f]	17.61	3.26[b]	Present study

nd – not determined; DW – dry weight.

[a] Estimated from biomass value of 197 mg L^{-1} after 31 days.

[b] Fatty acid content (FAME) and productivity determined rather than total lipid.

[c] Total lipid productivity values in mg L^{-1} per day.

[d] Estimated from the biomass value of 1.71 mg L^{-1} after 21 days.

[e] Estimated after 1.13G/L biomass after 7 days of growth; Adapted in part from Pittman *et al*. (2011).

[f] Estimated from the biomass value of 1019 mg L^{-1} after 15 days.

remediating the WW while generating the biomass for biodiesel feedstock.

The main motive in using microalgae for biofuels is to reduce land use, incorporate waste materials in the process and reduce the environmental impact of transportation fuels. LCA is a tool for systematic management of environmental factors associated with a process throughout its life cycle or stages. In the process of producing biodiesel from *C. sorokiniana*-I, the total energy required for the complete process is 2697.5 MJ; the majority of which is utilized in the culturing of biomass in the PBR. The total energy requirement is still considerably less than that reported by Sander and Murthy (2010), who has calculated approximate costs of microalgae production using a raceway pond. The most energy-intensive step in the raceway pond-based system appears to be harvesting and dewatering (Soomro *et al.* 2016) which in the case of PBRs can be streamlined for better efficiency. Another factor that contributes greatly to reduce the environmental impact of biodiesel production from WW is that it serves twin purpose i.e. remediation of a water body and reduction in operational costs pertaining to culture media. The greenhouse gas emissions from biodiesel are far lesser than the conventional diesel (Sander and Murthy 2010). Other factors pertaining to microalgae-based biodiesel, that it has a lower impact on the environment when compared to petroleum diesel include land use, marine eutrophication, terrestrial and freshwater ecotoxicity (Collet *et al.* 2014). Also, the global warming potential, calculated in kg CO_2 Eq. for microalgal biodiesel is lesser than that of diesel (Sieira *et al.* 2015). This can further be reduced by using flue gas from industrial exhaust pipes to bubble through the microalgal culture.

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