



Effects of various abiotic factors on biomass growth and lipid yield of *Chlorella minutissima* for sustainable biodiesel production

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Abstract

In this study, effects of different abiotic factors were studied on biomass and lipid yield of green microalga *Chlorella minutissima* (*C. minutissima*). Various concentrations of abiotic factors like nitrogen, phosphorus, glucose, iron, zinc, different values of pH, temperature, light intensity and different photoperiods were observed on the biomass growth and lipid yield of *C. minutissima* cultivated with modified CHU-13 medium. Initially, three cultivation media namely, Bold's basal medium (BBM), modified CHU-13 and blue-green-11 (BG-11) were used to culture *C. minutissima* in batch mode. Microalga cultivated with modified CHU-13 medium resulted in maximum biomass and lipid yield of 970 ± 0.21 and 356.63 ± 0.51 mg/L, respectively. To maximize biomass and lipid yield of microalga further, it was cultivated with modified CHU-13 medium and variation of above mentioned abiotic factors was done. Different biomass and lipid yields were achieved for different abiotic factors varied. Highest biomass of 1840.49 ± 0.62 mg/L was achieved with 12 g of glucose containing medium and highest lipid yield of 579.86 ± 0.76 mg/L was achieved with 0.3 g of nitrogen containing medium. GC-MS analysis of biodiesel obtained from *C. minutissima* biomass cultivated with modified CHU-13 medium shown the presence of C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C20:0, C20:1 and C22:0. Properties of biodiesel obtained from *C. minutissima* were found in compliance with ASTM-6751-02 and European biodiesel standards EN14214. These results suggest that *C. minutissima* can be used as a potential biodiesel feedstock for microalgal biodiesel production.

Keywords *Chlorella minutissima* · Nutrients · Biomass · Lipid yield · Light intensity · Photoperiod · Temperature · pH

Highlights

- Initially modified CHU-13 medium produced highest biomass and lipid yield in *C. minutissima*.
- Highest biomass and lipid yields of 1840.49 ± 0.62 and 579.86 ± 0.76 mg/L were achieved with 12 g of glucose and 3 g of nitrogen containing CHU-13 medium.
- Cultivation temperature of 27 ± 2 °C and pH 8 were found optimal for higher biomass yield.
- Zinc concentration beyond 100 mg/L resulted in reduced biomass growth.
- Biodiesel quality was found in compliance with international fuel standards.

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Introduction

Globally, high energy demands are pushing toward fast depletion of fossil fuels. Rapid industrialization and transportation emissions are continuously contributing in global warming. Due to future fuel needs, 50% use of plant resources will increase by 2050 (Perlack et al. 2005). Currently biofuels have become viable option to compensate future energy needs. Previously biofuel production was mainly based on edible agri-resources like corn, sugarcane, soybeans and vegetable oil, etc., but it could not be propagated commercially because of criticism due to food supply issues and non availability of arable lands worldwide (Patel et al. 2015; Searchinger et al. 2015). High demands of fossil fuels worldwide are compelling scientific community toward exploration and research on the renewable energy production (Chisti 2007a). Development of new technologies for renewable energy production can replace use of fossil fuels (Patel et al. 2014). Biodiesel gained popularity in recent years due to its renewable potential. Collectively, methyl esters of fatty acids are called as

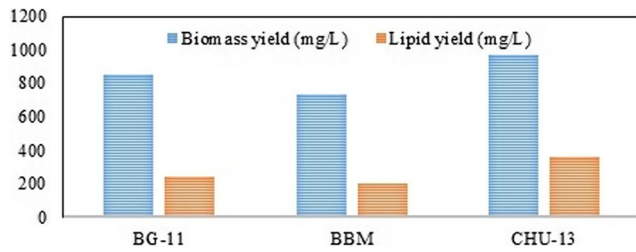


Fig. 1 Biomass and lipid yield of *C. minutissima* obtained with three different cultivation media

biodiesel. Generally, biodiesel is produced by transesterification of fats/lipids in presence of a suitable catalyst. Lipids can be obtained from animal tissues, plant seeds, and other biomass materials. (Chisti 2007b). Recently, microalgae have been extensively explored for biodiesel production. Microalgae can grow easily anywhere simply, like in ponds, photobioreactors, etc. in presence of light, carbon, nitrogen, and phosphorus source (Ji et al. 2014; Abomohra et al. 2017). Microalgae have 15–80% of lipid content and high growth rates (Liu et al. 2008; Smith et al. 2009). However, microalgal biodiesel industrial production is still facing many constraints due to the expensive downstream processes in biodiesel production. Sometimes, harvesting of microalgae involves 30–40% of total capital cost of biodiesel production. So, the selection of microalgal strain, culture medium, and other growing conditions become important for reasonable cost of biodiesel production. High growth of microalgae can be achieved by varying the chemical and physical parameters in culture environment. Temperature, pH, light duration, and nutrients (N, P, and carbon source) greatly affect the growth and lipid synthesis in microalgae (Oh et al. 2009; Pandit et al. 2017). In spite of all these constraints, currently, microalgae are the potential and best-suited bio-resource for biodiesel generation (Weissman and Goebel 1987). Lipid content and biomass production can be increased by altering cultivation medium components and other growth conditions (Mandotra et al. 2014). This will help in production of cost-effective industrial microalgal biofuel production up to an extent. Lipid content can be enhanced by altering culture media ingredients for a particular microalgal strain (Cheirsilp and Torpee 2012). Thus, by optimizing growth conditions, biomass, and lipid productivity, we may achieve economic biodiesel production (Mandotra et al. 2014).

Materials and methods

Microalgal culture

Culture of *C. minutissima* was procured from the Division of Microbiology, Indian Agricultural Research Institute (IARI),

New Delhi, India. The culture was maintained in BBM medium at 25 ± 2 °C under continuous illumination of 2500 lux.

Screening of most efficient growth medium

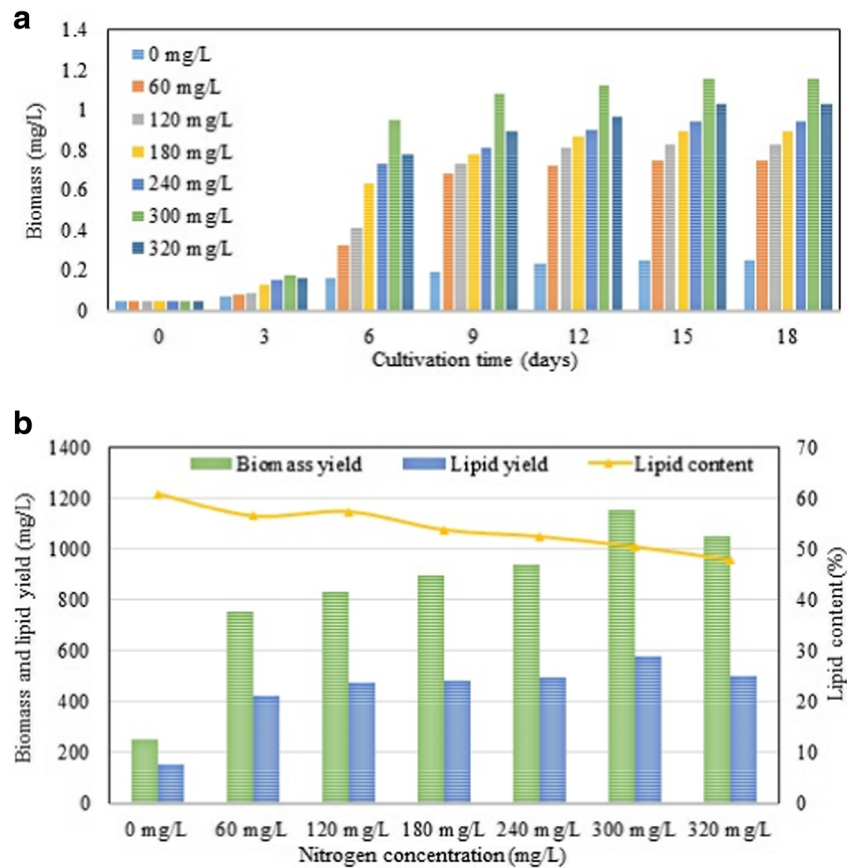
Initially, 5 mL samples of *C. minutissima* from maintained culture were inoculated in different 500 mL conical flasks having each of 300 mL of BG-11, modified CHU-13 and BBM medium at 25 ± 2 °C, 2500 lux under 12:12 h light/dark cycle for 18 days. Light was supplied using cool white fluorescent light tubes and light intensity was measured using digital lux meter.

Harvested microalgal biomass obtained from three different cultivation media was used for lipid extraction as per protocol described by Folch et al. (1957). Highest microalgal biomass and lipid yield was obtained from the culture cultivated with modified CHU-13 medium. So, in further sets of experiments, modified CHU-13 medium was used for cultivation of microalga. Compositions of three different media used in this study are presented in Table 1. In all the experiments, only one parameter was variable at a time and others were constant.

Table 1 Composition of three media used for the culture of *C. minutissima*

Components (mg/L)	Culture medium		
	Modified CHU-13	BG-11	BBM
KNO ₃	400	Nil	Nil
NaNO ₃	Nil	1500	250
K ₂ HPO ₄	80	40	75
KH ₂ PO ₄	Nil	Nil	175
NaCl	Nil	Nil	25
Na ₂ CO ₃	Nil	20	Nil
MgSO ₄ ·7H ₂ O	200	75	75
CaCl ₂ ·2H ₂ O	107	36	25
C ₁₀ H ₁₆ N ₂ O ₈	Nil	1	50
FeSO ₄	Nil	Nil	4.98
C ₆ H ₈ FeNO ₇	Nil	6	Nil
C ₆ H ₅ FeO ₇	20	Nil	Nil
C ₆ H ₈ O ₇	100	6	Nil
CoCl ₂ ·2H ₂ O	0.02	Nil	Nil
H ₃ BO ₃	5.72	2.86	11.42
MnCl ₂ ·4H ₂ O	3.62	1.81	1.44
ZnSO ₄ ·7H ₂ O	0.44	0.22	8.82
CuSO ₄ ·5H ₂ O	0.16	0.079	1.57
Na ₂ MoO ₄ ·2H ₂ O	0.084	0.39	Nil
Co(NO ₃) ₂ ·6H ₂ O	Nil	0.049	0.49
MoO ₃	Nil	Nil	0.79

Fig. 2 **a** Growth pattern of *C. minutissima* under different concentrations of nitrogen (KNO_3) using modified CHU-13 medium. **b** Biomass yield, lipid yield and lipid content of *C. minutissima* under different concentrations of nitrogen using modified CHU-13 medium



Abiotic factors tested for maximum biomass and lipid yield of *C. minutissima*

Following nitrogen (KNO_3 as nitrogen source) concentrations of 0, 60, 120, 180, 240, 300, and 360 mg/L, phosphorus (K_2HPO_4 as phosphorus source) concentrations of 10, 20, 50, 100, 150, 250, and 350 mg/L, iron (FeSO_4 as iron source) concentrations of 10, 20, 30, 40, 45, and 50 mg/L, zinc (ZnSO_4 as zinc source) concentrations of 20, 30, 40, 50, 100, and 200 mg/L and glucose concentrations of 3000, 5000, 7000, 10,000, and 12,000 mg/L were used to see the effect on biomass and lipid yield of *C. minutissima*. To see the effect of pH it was tested for the values of 5, 6, 7, 8 and 9. Temperature and light intensity were varied in the range of 17 ± 2 to 33 ± 2 °C and 3500 to 10,000 lux, respectively, while different photoperiods (light/dark) cycles of 14:10, 16:08, 18:06 and 24:00 h were applied.

Biomass growth and dry cell weight determination

Biomass productivity (g/L/day) was measured gravimetrically on dry cell weight basis at an interval of 3 days. Microalgal suspensions of 10 mL each were centrifuged at 5000 rpm for 10 min, washed with double distilled water, and dried at 65 °C for 24 h until attainment of

constant weight, cooled in a desiccator, and weighed. The biomass productivity was calculated as follows:

$$\text{Biomass productivity (BP)} = (C_2 - C_1) / (T_2 - T_1) \quad (1)$$

where C_1 and C_2 are biomass concentrations (g/L) and T_1 and T_2 are initial and final sampling times.

Lipid extraction and estimation

Lipid extraction was performed according to protocol described by Folch et al. (1957). In short, freeze-dried algal biomass was mixed with chloroform and methanol in the ratio of 2:1 (v:v) and the mixture was agitated for 20 min. The resulting mixture was centrifuged at 7000 rpm and 4 °C for 7 min to discard impurities from supernatant. Supernatant was washed with 0.9% NaCl (0.2 volumes) and vortexed.

After 5 min of 3000 rpm centrifugation, the upper phase was removed and the lower chloroform phase containing lipids was evaporated under vacuum using a rotary evaporator in pre-weighed weighing bottles. The lipid productivity was calculated by the formula given by Griffiths and Harrison (2009).

$$\text{Lipid productivity (LP)} = \text{BP} \times \text{lipid content (\% dry cell weight basis)} \quad (2)$$

Transesterification of lipids

Extracted lipids were transesterified using 10% H₂SO₄ in the molar ratio of alcohol:lipid as 6:1 at 100 °C for 5 h with constant stirring (Mandotra et al. 2014). Direct transesterification was performed by taking dry algal biomass and methanol in ratio of 1:3 (w/v) at 100 °C under continuous stirring for 5 h in presence of H₂SO₄ (20% w/w of dry algal biomass) as catalyst (D’Oca et al. 2011). The remaining alcohol was removed through a rotatory evaporator and hexane was added to the mixture. The mixture was filtered and dried using anhydrous Na₂SO₄. Finally, hexane was removed using a rotatory evaporator to get fatty acid methyl esters (FAMES).

Fatty acid methyl esters (FAMES) and GC-MS analysis

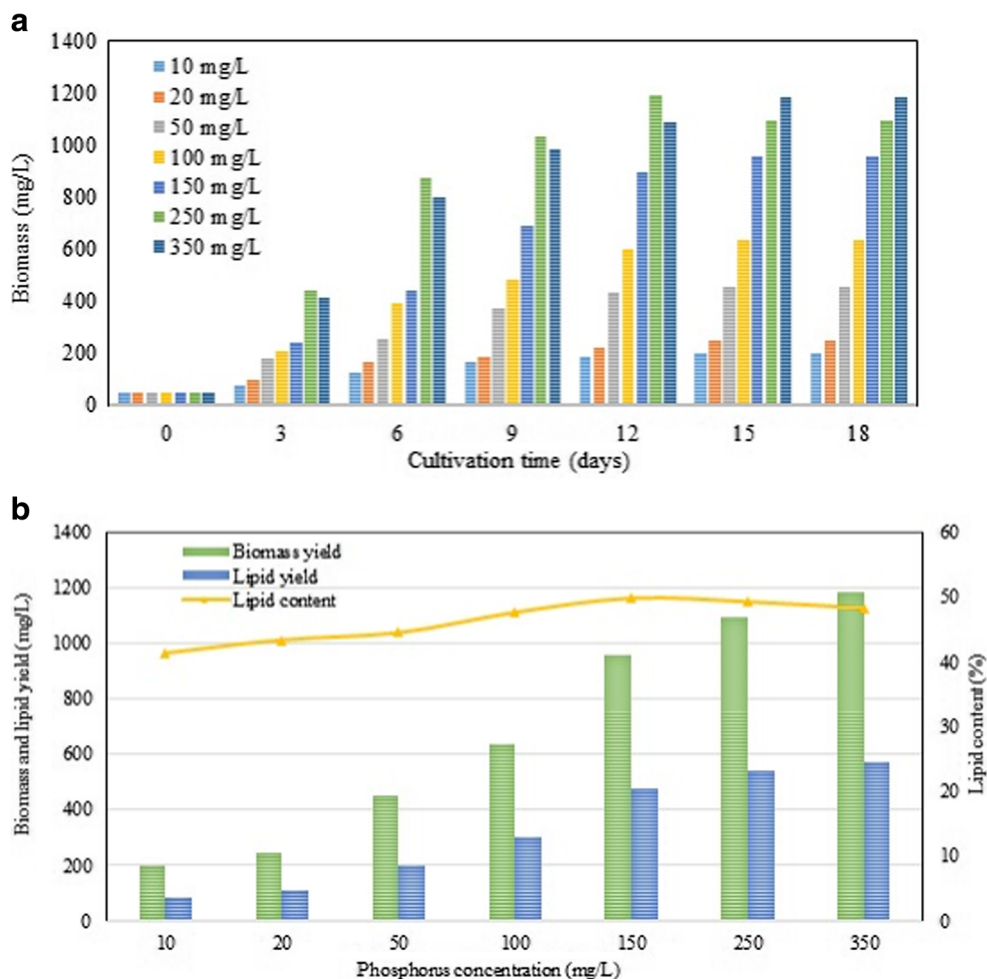
GC-MS (Clarus 500, Perkin Elmer) analysis was done to see FAMES profile according to protocol described by Härtig (2008). Splitless injection mode at 250 °C was selected, and injection volume was kept at 1 µL. Helium was used as carrier gas. Initially, column temperature was set at 50 °C for 1.5 min, later on, for 1 min, hold temperature was raised up to 180 °C

(25 °C min⁻¹). A further increment in temperature of up to 220 °C (10 °C min⁻¹) was done and held for 1 min. The maximum increment in the temperature was made up to 250 °C (15 °C min⁻¹) and held for 3 min. Line of mass transfer and ion source were set at 250 and 200 °C, respectively. Scan mode (50–600 m/z) with electron ionization potential of 70 eV was used to detect FAME profile.

Attenuated total reflectance-Fourier transform infrared and thermogravimetric analyses

Attenuated total reflectance (ATR)-Fourier transform infrared (FTIR) spectra of biodiesel was obtained using Perkin Elmer FTIR Spectrum Two (USA) machine. The FTIR was equipped with an ATR sampling accessory. All spectra were collected at 20 (1 °C using an average of 32 scans and with a spectral resolution of 2 cm⁻¹). Thermogravimetric analysis (TGA) was performed using Netzsch TG 209F3 TGA analyzer to measure the thermal behavior of biodiesel. Samples of 10 µL biodiesel were heated at a constant heating rate of 10 °C min⁻¹ in inert atmosphere using nitrogen gas. The temperature range was kept at 25 to 500 °C.

Fig. 3 **a** Growth pattern of *C. minutissima* under different concentrations of phosphorus using modified CHU-13 medium. **b** Biomass yield, lipid yield and lipid content of *C. minutissima* under different concentrations of phosphorus using modified CHU-13 medium



Statistical analysis

Data were analyzed by applying one-way analysis of variance (ANOVA) at a confidence level of 5%. Experiments were performed in triplicate.

Results and discussion

During screening for the most efficient growth medium for *C. minutissima*, modified CHU-13 medium resulted in a maximum biomass and lipid yield of 970 ± 0.21 and 356.63 ± 0.51 mg L⁻¹, respectively, followed by BG-11 medium which resulted in a biomass and lipid yield of 850 ± 0.12 and 243.65 ± 0.30 mg/L⁻¹, respectively. The lowest biomass and lipid yields of 730 ± 0.42 and 196.83 ± 0.43 mg/L⁻¹ were achieved with BBM medium, respectively. Figure 1 depicts various values of biomass and lipid yield of *C. minutissima* obtained with three different cultivation media.

Effects of different abiotic factors on biomass and lipid yield of *C. minutissima*

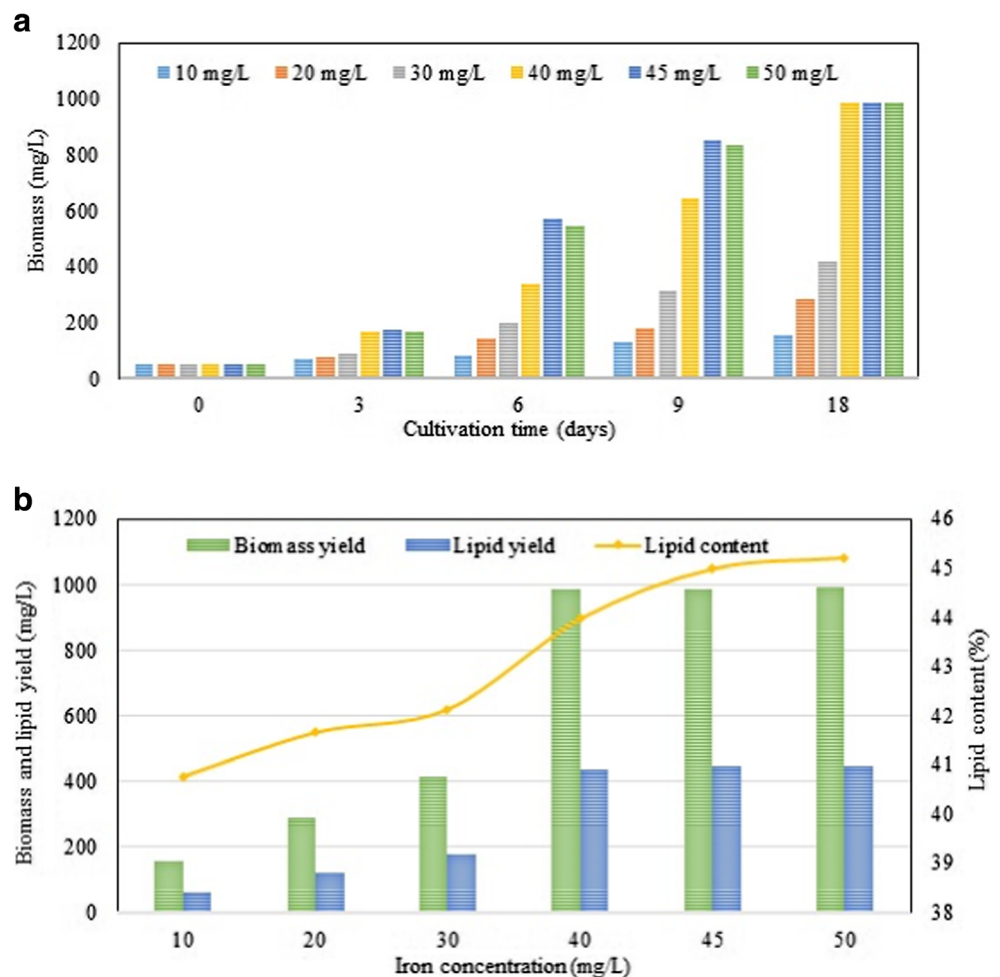
Effect of nitrogen

Nitrogen is the basic element for the formation of proteins and nucleic acids in cell. It constitutes 7–20% of cell dry weight. Nitrogen deficiency boosts protein translation and lipid synthesis (Ruangsomboon 2012). The highest biomass concentration of 1150.52 ± 0.44 mg/L, lipid yield of 579.86 ± 0.076 mg/L, lipid content of $50.39 \pm 0.018\%$ and lipid productivity of 48.32 ± 0.0063 mg/L/day were obtained, respectively by cultivating microalga with nitrogen concentration of 300 mg/L (Fig. 2a, b).

Effect of phosphorus

Phosphorus is an important abiotic macronutrient for algae growth. Its requirement is necessary for protein synthesis, nucleic acids, and cellular framework development. Phosphorus deficiency can lead to chlorophyll death and poor protein content (Healey 1982). The effects of initial phosphorus concentration are shown in Fig. 3a, b. Biomass and lipid

Fig. 4 **a** Growth pattern of *C. minutissima* under different concentrations of iron using modified CHU-13 medium. **b** Biomass yield, lipid yield and lipid content of *C. minutissima* under different concentrations of iron using modified CHU-13 medium



yield of microalga increases with increasing phosphorus concentration from 10 to 350 mg/L. The highest biomass of 1185.71 ± 0.81 mg/L with lipid yield of 572.78 ± 0.45 mg/L, lipid content of $48.30 \pm 0.023\%$, and lipid productivity of 44.81 ± 0.023 mg/L/day was achieved with 350 mg/L of phosphorus concentration while phosphorus concentration of 250 mg/L resulted in biomass yield of 1091 ± 0.64 mg/L with lipid yield of 537.79 ± 0.27 mg/L and lipid content of $49.31 \pm 0.024\%$. Thus, higher concentration of phosphorus resulted in higher biomass but lower lipid content. This may be due the inefficient utilization of higher concentrations of phosphorus by microalga.

Ruangsoomboon (2012) has demonstrated that the phosphorus concentration from 22 to 444 mg/L resulted in 7.3 times more biomass growth with a decrease in lipid content of *Botryococcus braunii* KMITL 2.

Effect of iron

Iron-deficient medium can cause retarded microalgal growth. However, excess of iron can lead toward oxidative stress and physiological changes in microalgae (Hu 2004; Yeesang and Cheirsilp 2011). Various effects of iron concentration on

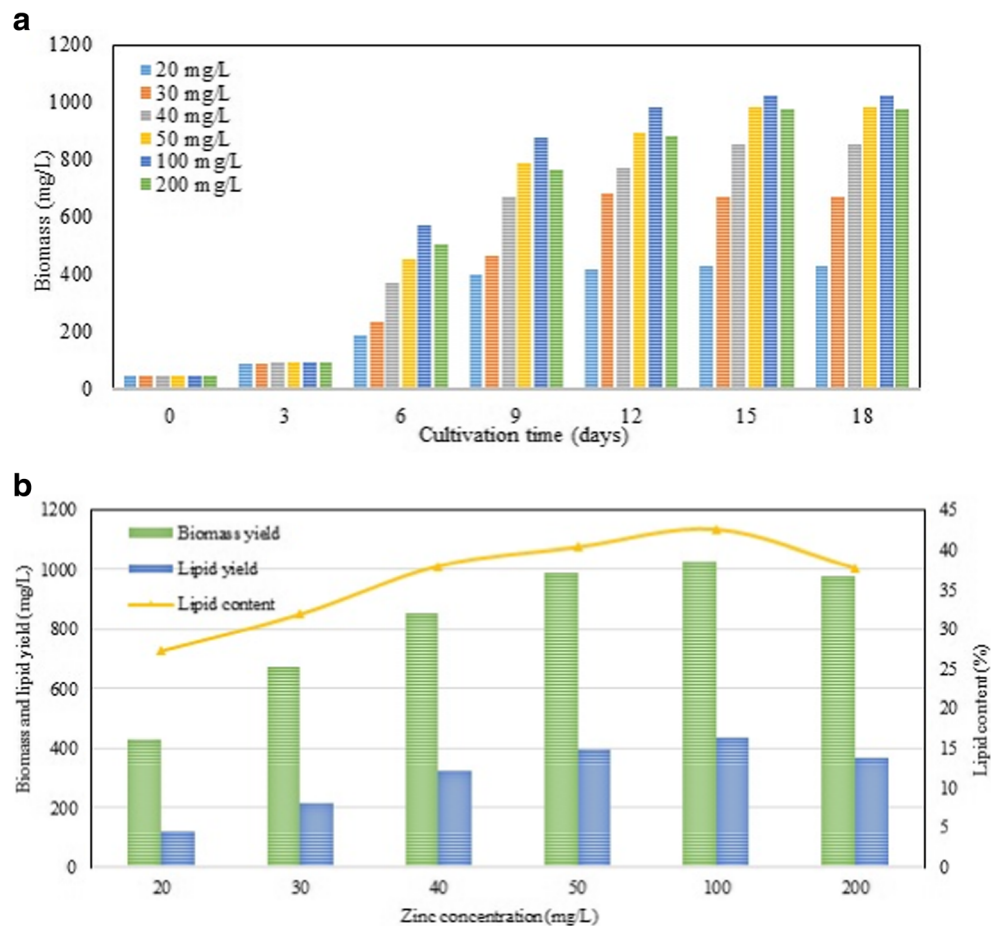
C. minutissima biomass, lipid content, and lipid yield are depicted in Fig. 4a, b. Biomass yield, lipid yield and lipid content of microalga increase with increasing iron concentration from 10 to 50 mg/L. The highest biomass of 988.68 ± 0.77 mg/L, lipid yield of 446.9 ± 0.42 mg/L, lipid content of $45.2 \pm 0.077\%$, and lipid productivity of 37.24 ± 0.035 mg/L/day were achieved with iron concentration of 50 mg/L. There was a very little difference in biomass yield (986.76 ± 0.33 mg/L), lipid yield (443.86 ± 0.62 mg/L), and lipid content of ($44.98 \pm 0.074\%$) microalga obtained with 45 mg/L of iron concentration in comparison with 50 mg/L iron concentration.

Maximum lipid content of $34.93 \pm 1.89\%$ and lipid yield of 0.08 g/L were reported with iron concentration of 27 mg/L in *Botryococcus braunii* KMITL 2 (Ruangsoomboon 2012).

Effect of zinc

Zinc is an essential micronutrient for microalgal growth. Zinc deficient environment leads to retarded growth (Shrotri et al. 1981). The effects of initial zinc concentration are presented in Fig. 5a, b. Biomass yield, lipid yield, and lipid content of microalga increase with increasing zinc concentration from

Fig. 5 a Growth pattern of *C. minutissima* under different concentrations of zinc using modified CHU-13 medium. **b** Biomass yield, lipid yield and lipid content of *C. minutissima* under different concentrations of zinc using modified CHU-13 medium



20 to 100 mg/L. The highest biomass of 1026.82 ± 0.51 mg/L, lipid yield of 437.04 ± 0.19 mg/L, lipid content of $42.56 \pm 0.0066\%$, and lipid productivity of 36.42 ± 0.016 mg/L/day were obtained by cultivation with the zinc concentration of 100 mg/L. Beyond this concentration (200 mg/L), decreased biomass yield, lipid yield, lipid content, and lipid productivity of 976.6 ± 0.77 mg/L, 368.19 ± 0.53 mg/L, $37.70 \pm 0.057\%$, and 30.68 ± 0.044 mg/L/day were achieved, respectively.

Travieso et al. (1999) have reported that *Chlorella vulgaris* showed 600 mg/L of zinc tolerance limit but microscopic observation of cells shows deformity in cell morphology.

Effect of pH

pH is one of the crucial parameters that control the microalgal biomass growth. However, it has been cumbersome to conclude a generalization for optimum pH. It depends on the selected species and its growth environment. Moreover, it can be derived on the basis of previous studies that in controlled pH environment improved microalgal growth can be achieved. Wang et al. 2010 reported improved biomass growth and lipid yield of *Chlorella vulgaris* at pH 7 to 8.5. Cho et al.

(2015) reported that pH 7 is most suitable for *Chlorella vulgaris* in a study conducted by him. The effects of different pH values on biomass and lipid yield of *C. minutissima* are shown in Fig. 6a, b. The highest biomass of 1023.17 ± 0.23 mg/L, lipid yield of 418.90 ± 0.73 mg/L, lipid content of $40.94 \pm 0.071\%$, and lipid productivity of 34.91 ± 0.061 mg/L/day were obtained at pH 8. Biomass and lipid yields of 1007.09 ± 0.13 and 382.91 ± 0.52 mg/L were achieved at pH 8.

Effect of temperature

Temperature is most important abiotic environmental factor that directly affects microalgal growth and metabolic activities of cell. Its role in microalgae metabolism and lipid synthesis has been established by many authors (Sushchik et al. 2003; Amit et al. 2017). The effects of temperature variation on microalgal biomass and lipid yield are shown in Fig. 7a, b. The highest biomass yield of 997.99 ± 0.064 mg/L, lipid yield of 378.32 ± 0.44 mg/L, lipid content of $37.91 \pm 0.041\%$, and lipid productivity of 31.52 ± 0.036 mg/L/day were obtained when microalga was cultivated at 27 ± 2 °C.

Fig. 6 a Growth pattern of *C. minutissima* under different pH using modified CHU-13 medium. b Biomass yield, lipid yield and lipid content of *C. minutissima* under different pH using modified CHU-13 medium

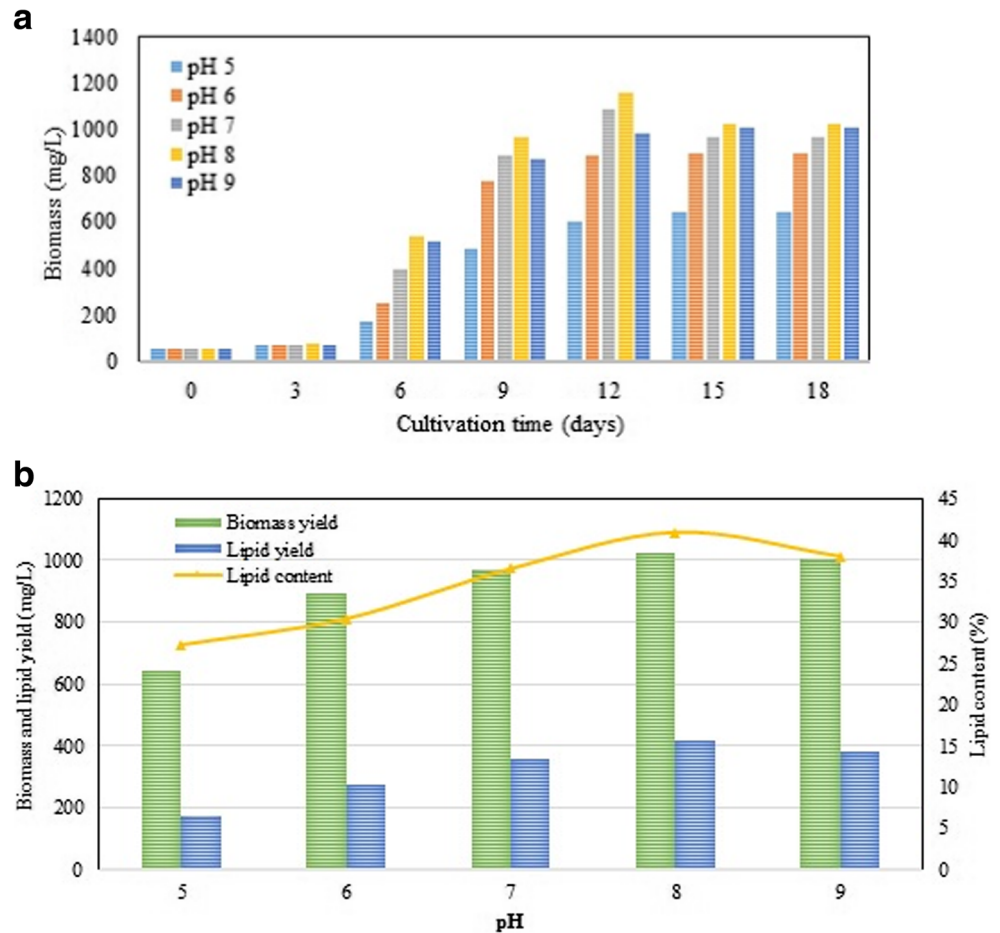
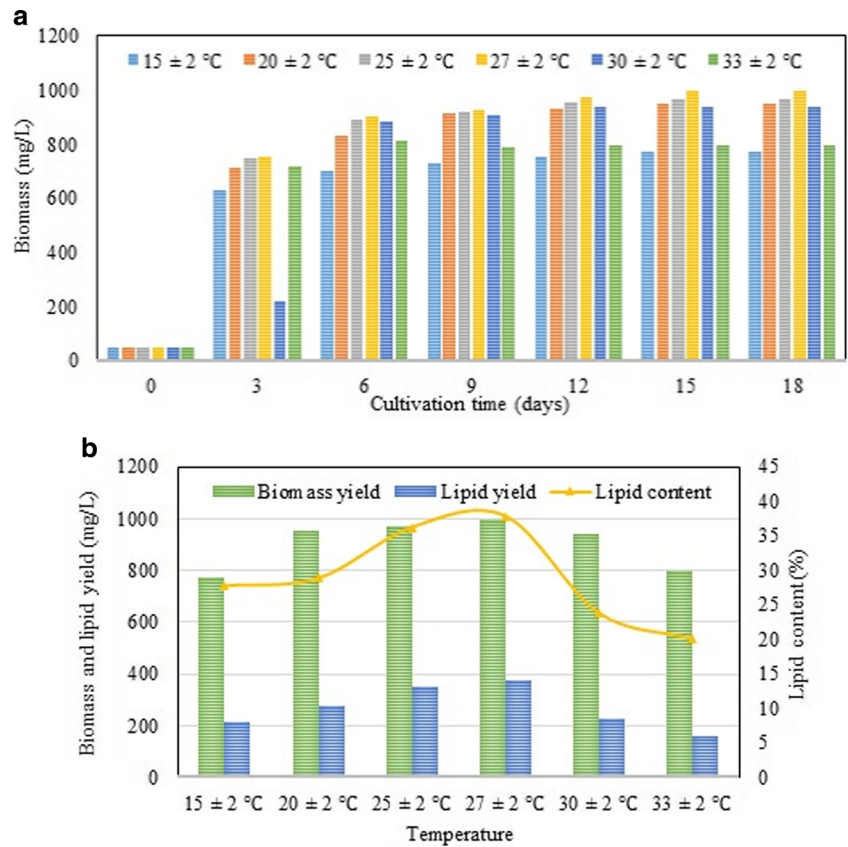


Fig. 7 **a** Growth pattern of *C. minutissima* under different temperatures cultivated using modified CHU-13 medium. **b** Biomass yield, lipid yield and lipid content of *C. minutissima* under different temperatures cultivated using modified CHU-13 medium



Effect of glucose

Altering cultivation conditions to improve the biomass and lipid yield of microalgae is well known (Borowitzka 1999). High biomass growth and lipid yield can be achieved by the heterotrophic cultivation of microalgae (Minowa et al. 1995; Miao and Wu 2006). Cheirsilp and Torpee (2012) reported that the *Chlorella* sp. and *Nannochloropsis* sp. high biomass and lipid yield glucose supplemented medium by using fed batch cultivation system. A biomass yield of 2.01 g/L was achieved for *Phaeodactylum tricornerutum* UTEX#640 with a glucose concentration of 5.0 g/L (Garcia et al. 2005). The effects of glucose concentration are shown in Fig. 8a, b. Biomass and lipid yield of microalga increased with increasing glucose concentration up to 12,000 mg/L. The highest biomass of 1840.49 ± 0.62 mg/L, lipid yield of 405.36 ± 0.96 mg/L, lipid content of $22.02 \pm 0.060\%$, and lipid productivity of 33.78 ± 0.081 mg/L/day were obtained at glucose concentration of 12,000 mg/L.

Effect of photoperiod

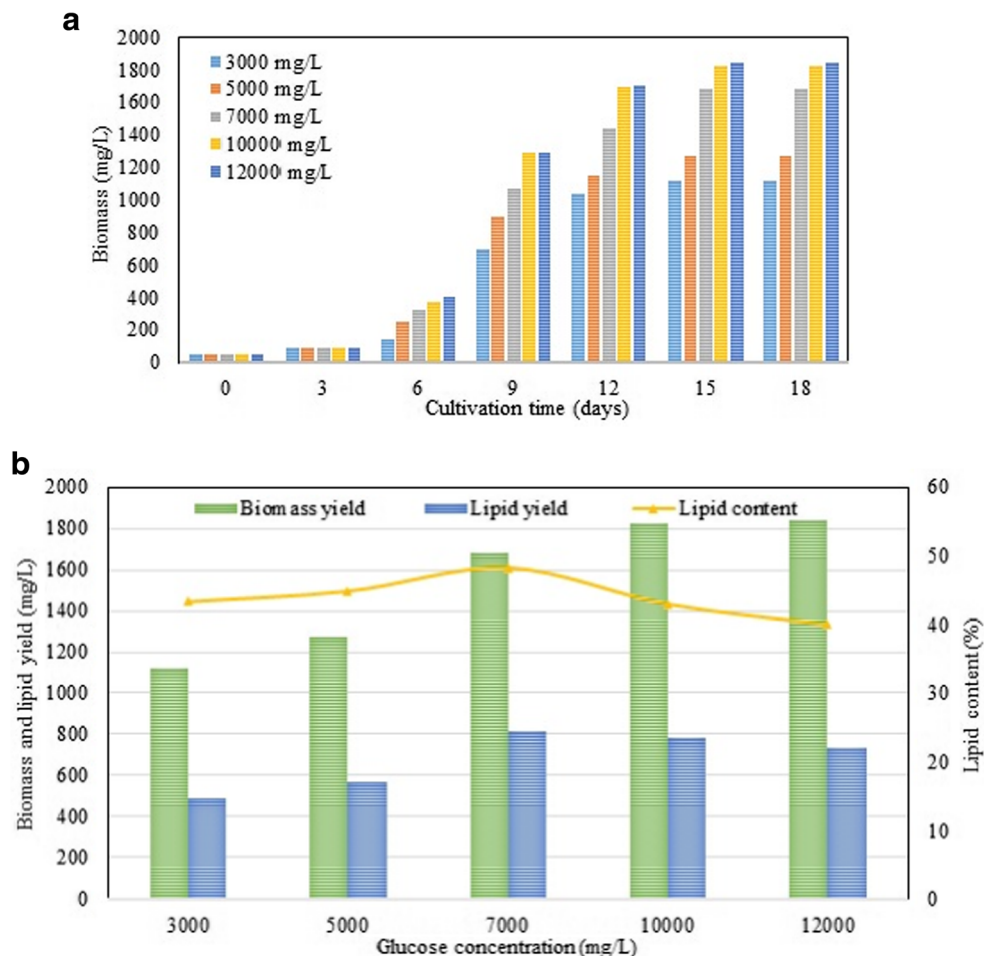
The effects of various selected photoperiods (light/dark) cycles are shown in Fig. 9a, b. *C. minutissima* accumulated a

biomass yield of 1041.18 ± 0.17 mg/L, lipid yield of 382.38 ± 0.41 mg/L, lipid content of $36.73 \pm 0.042\%$, and lipid productivity of 31.86 ± 0.033 mg/L/day under a photoperiod of 24:00 hours. Matos et al. (2017)) reported a maximum biomass growth of 1.25 g/L in *Nannochloropsis gadianta* with a photoperiod of 16:8 h. Ruangsomboon (2012) reported a maximum biomass with a 24:00 h photoperiod in *Botryococcus braunii* KMITL 2.

Effect of light intensity

Light is one of the most crucial and mandatory abiotic factors which affect biomass growth and biosynthesis of many vital molecules in cell. Ruangsomboon (2012) obtained a lipid yield of 0.45 g/L in *Botryococcus braunii* KMITL 2 at $538 \mu\text{E}/\text{m}^2/\text{s}$. Kojima and Zhang (1999) reported that the optimized light intensities can enhance microalgal lipid yield. The effects of light intensity variation are shown in Fig. 10a, b. The highest biomass yield of 1165 ± 0.26 mg/L, lipid yield of 491.73 ± 0.24 mg/L, lipid content of $42.21 \pm 0.015\%$, and lipid productivity of 40.97 ± 0.02 mg/L/day were obtained by cultivating microalga at light intensity of 9000 lux at 25 ± 2 °C. Decreased biomass and lipid yields of 1011.29 ± 0.50 and 387.04 ± 0.12 mg/L were obtained, respectively, at 10,000 lux at 27 ± 2 °C. These results are in compliance with

Fig. 8 **a** Growth pattern of *C. minutissima* under different concentrations of glucose cultivated using modified CHU-13 medium. **b** Biomass yield, lipid yield and lipid content of *C. minutissima* under different glucose concentrations cultivated using modified CHU-13 medium



work done by Cheirsilp and Torpee (2012) where the growth of marine *Chlorella* sp. increased up to 8000 lux light intensity, and beyond it, with 10,000 lux, biomass growth was decreased. This can be attributed to photoinhibition. However, in *Botryococcus braunii*, maximum growth was reported under continuous irradiance (Dumrattana and Tansakul 2006).

FAME profile and biodiesel characteristics

As shown in Fig. 11, the FAME profile of *C. minutissima* was found to be mainly composed of myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), arachidic acid (C20:0), gondoic acid (C20:1), and behenic acid (C22:0) which account to 3, 21.78, 9.69, 3, 9.38, 24.23, 13.45, 10.33, 1 and 1%, respectively, of total fatty acids.

Table 2 presents various biodiesel properties obtained in this study. Various physical properties of biodiesel like cetane

number (CN), degree of unsaturation (DU), kinematic viscosity (kV), density (D), and oxidative stability (OS), which control the vehicular quality of biodiesel determined by empirical formulas (Francisco et al. 2010) as follows:

$$\text{CN} = 46.3 + 5458/\text{SV} - (0.255 * \text{IV}) \quad (3)$$

$$\text{DU} (\%) = \text{MUFA} + (2 * \text{PUFA}) \quad (4)$$

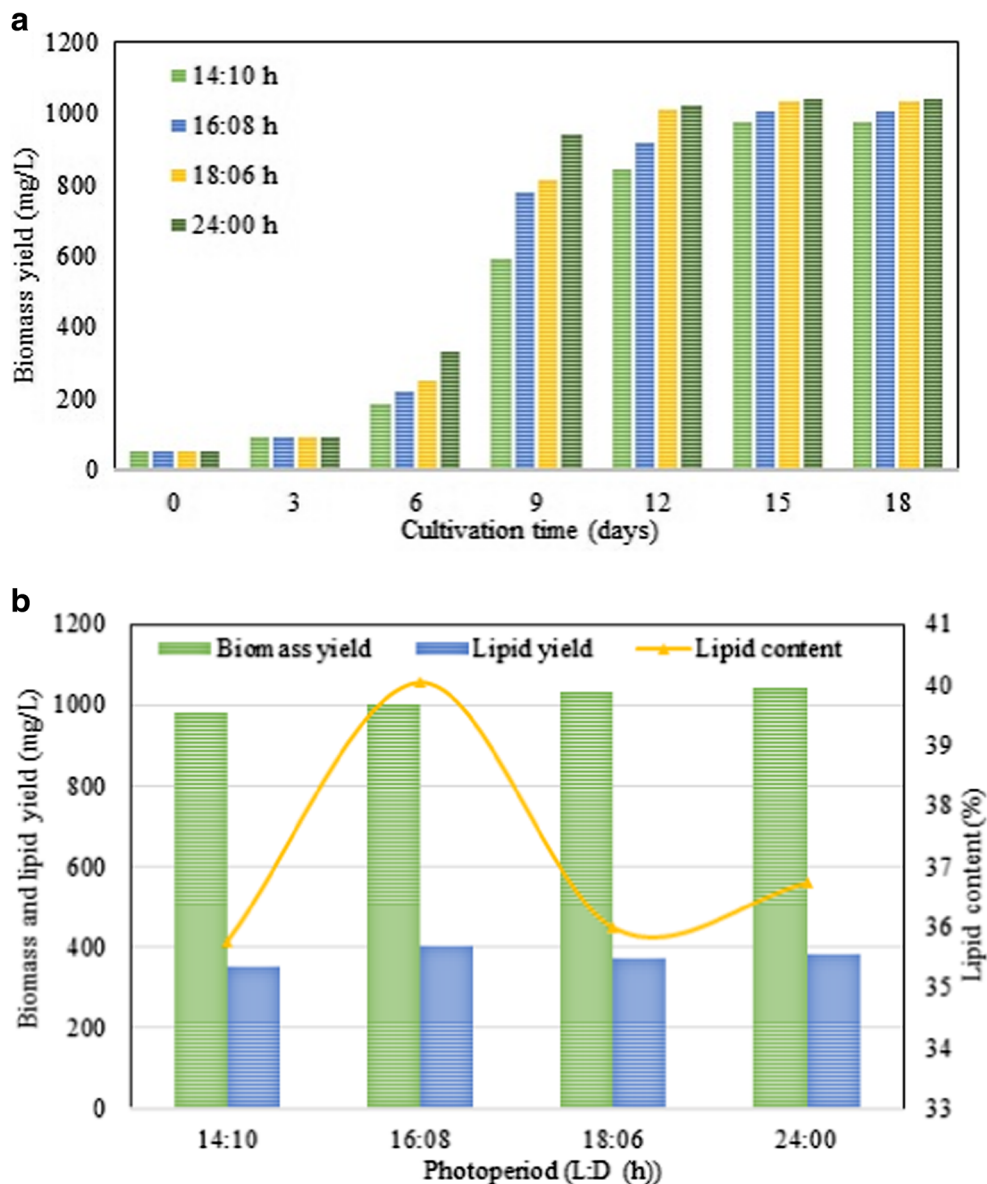
$$\ln(\text{kV}) = -12.503 + 2.496 * \ln(\Sigma\text{M}) - 0.178 * \Sigma\text{DB} \quad (5)$$

$$\text{Density} = 0.8463 + 0.49/\Sigma\text{M} + 0.0118 * \Sigma\text{DB} \quad (6)$$

$$\text{OS} = 117.9295 / (\text{wt}\% \text{C18:2} + \text{wt}\% \text{C18:3}) + 2.5905 \quad (7)$$

where M is the molecular mass of each fatty acid component, DB is the number of double bonds, FC is the percentage of each fatty acid component, MUFA is the weight percentage of monounsaturated fatty acids, and PUFA is the weight percentage of polyunsaturated fatty acid. Degree of unsaturation (DU) is the sum of the molar masses of unsaturated fatty acids. DU is an important property that affects the OS of biodiesel (Francisco et al.

Fig. 9 **a** Growth pattern of *C. minutissima* under different photoperiods cultivated using modified CHU-13 medium. **b** Biomass yield, lipid yield and lipid content of *C. minutissima* under different photoperiods cultivated using modified CHU-13 medium



2010). The presence of polyunsaturated fatty acids (PUFAs) in excess deteriorates the OS of the biodiesel as double and triple bonds carbon sites are prone toward free radical attacks. FAME profile of *C. minutissima* obtained shows 59.18% of saturated and monounsaturated fatty acids which is an indicator of good biodiesel quality.

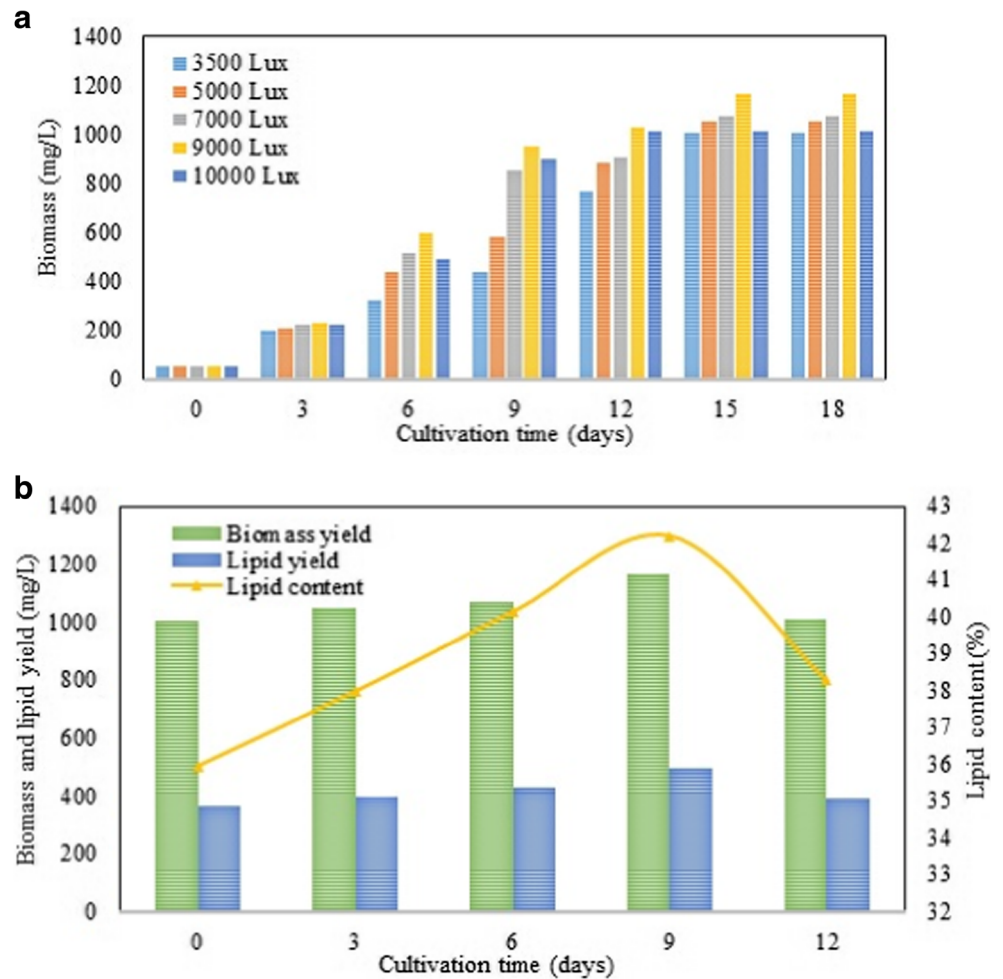
According to Gouveia and Oliveira (2009) and Pereira et al. (2013), linolenic acid (C18:3) and PUFAs with P4 double bonds are important for good quality biodiesel if they are present in the range of 12 and 1%, respectively, as per European standards EN14214. In the present study, the linolenic acid contributes 10.33% of the FAME, whereas, polyunsaturated FA with P4 double bond were completely absent. Cetane number (CN) is one of the important fuel properties of biodiesel which is highly influenced by the fatty acid profile (Table 2). High cetane

value is the indicator of better combustion, low nitrous oxide (NO_x) emission, less occurrence of knocking, and easier start-up of engine (Knothe 2012; Predojević et al. 2012). Diesel fuel with large quantities of saturated and monounsaturated FAMES have high value of CN.

Attenuated total reflection-Fourier transform infrared spectroscopy and thermogravimetric analysis

The ATR-FTIR analysis showed biodiesel spectra of 4000–500 cm⁻¹, as shown in Fig. 12. In the spectrum biodiesel, the bands in the 2800–3000 cm⁻¹ region are attributed to the symmetric CH₂ and asymmetric CH₃ and CH₂ stretching (Mahamuni and Adewuyi 2009). The characteristic bands for CO stretching of all samples are assigned in the 1800–1700 cm⁻¹ region (Mahamuni and

Fig. 10 **a** Growth pattern of *C. minutissima* under different light intensities cultivated using modified CHU-13 medium. **b** Biomass yield, lipid yield and lipid content of *C. minutissima* under different light intensities cultivated using modified CHU-13 medium



Adewuyi 2009). The major bands that allow structural distinction of FAMES are allocated in the 1500–1000 cm^{-1} region.

The appearance of two major bands in the spectra of biodiesel at 1436 and 1198 cm^{-1} corresponding to CH_3

asymmetric bending and $\text{O}-\text{CH}_3$ stretching, respectively, are characteristic bands for FAMES (Mahamuni and Adewuyi 2009).

Figure 13 represents the TGA plots of biodiesel sample. The reduction in the mass of the biodiesel starts

Fig. 11 FAME profile of *C. minutissima*

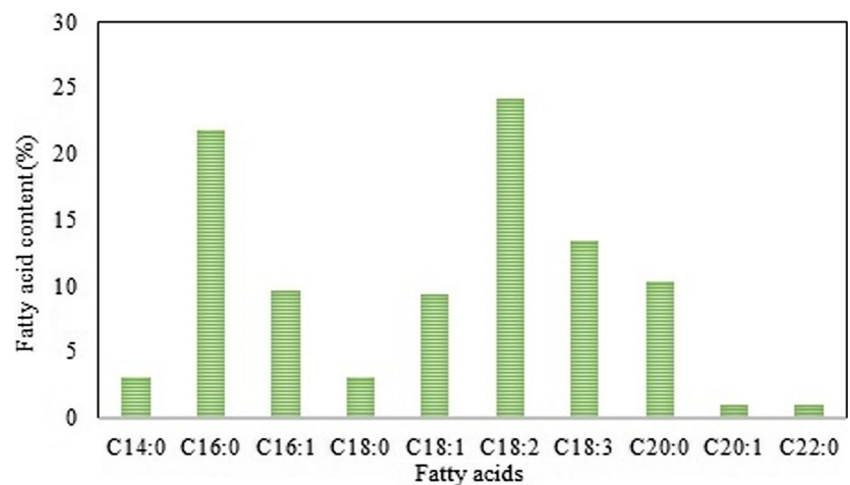


Table 2 Biodiesel properties of *C. minutissima*

Physical properties of biodiesel	International standards		Present study
	ASTM-6751-02	EN14214	
Cetane number	47	51	53.35
Degree of unsaturation (%)	Not reported	Not reported	77.34
High heating value (MJ kg ⁻¹)	Not reported	Not reported	39
Kinematic viscosity (mm ² s ⁻¹)	1.9–6.0	3.5–5.0	5.4
Density (g cm ⁻³)	Not reported	0.86–0.90	0.92
Oxidative stability (h)	Not reported	≥ 6	9

Fig. 12 ATR-FTIR spectrum of biodiesel

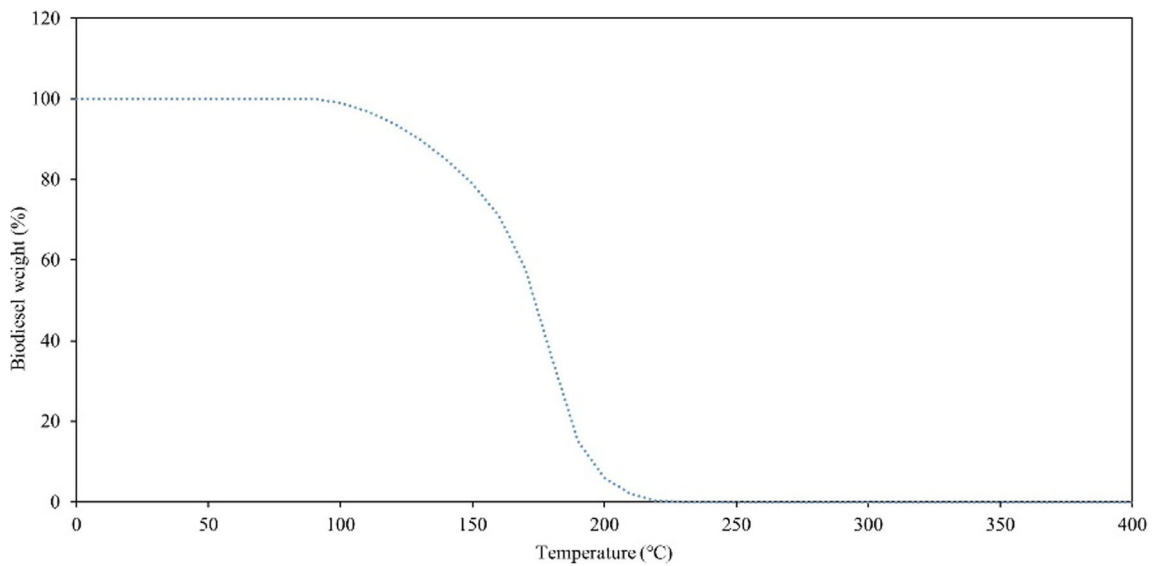
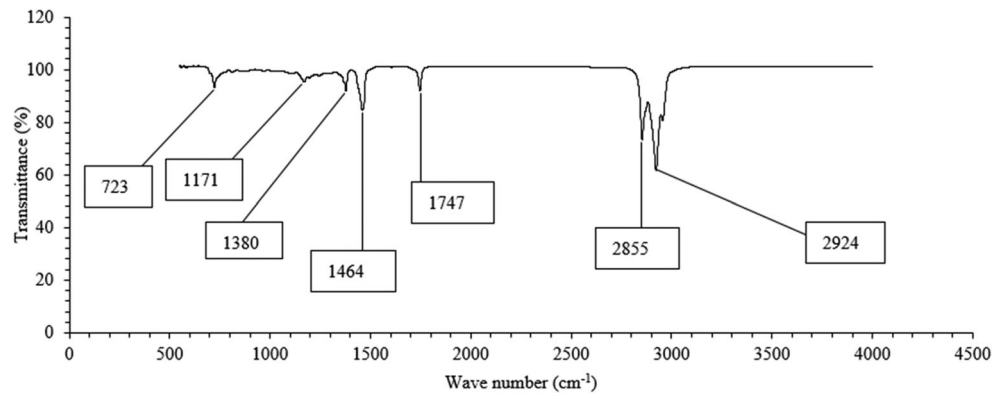


Fig. 13 TGA plot of biodiesel

between 100 and 150 °C and continues to decrease till the moment that all the biodiesel sample is vaporized.

It was assumed that the amount of biodiesel present in the sample is equal to the average weight percentage of over the

range where weight starts to drop in between 100 and 150 °C. Beyond 200 °C, all the biodiesel is vaporized. The loss in mass of biodiesel in the range of 100–150 °C correlates to the mass percentage of biodiesel present in the sample.

Conclusions

The results of the present work indicate that the microalga *C. minutissima* is a good renewable bio-resource for biodiesel production. It showed a significant increase in biomass and lipid yields by varying different abiotic factors when cultivated with modified CHU-13 medium. The biodiesel quality of *C. minutissima* met the criteria of ASTM-6751-02 and European biodiesel standard EN14214.

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