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Short Communication

Stress-induced lipids are unsuitable as a direct biodiesel feedstock: A case study with *Chlorella pyrenoidosa*

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HIGHLIGHTS

- pH 8–10 (nitrate sufficient, indoor) yielded best lipid as a biodiesel feedstock.
- C16:0, C18:1, C18:2 and C18:3 were found to be the major FAMEs.
- Stress caused enhanced PUFA rich lipid accumulation.
- Biodiesel fuel properties were determined using FAME profile.
- Biodiesel fuel of stress-induced lipid did not comply with worldwide standards.

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ABSTRACT

The effects of various stresses on the suitability of lipid synthesized by *Chlorella pyrenoidosa* for biodiesel production were investigated. Lipids were characterized for detailed fatty acid methyl ester profiling and biodiesel properties like cetane number (CN), iodine value, cold filter plugging point (CFPP). Maximum biomass productivity (106.63 mg L^{-1} d^{-1}) and lipid content (29.68%) were obtained at indoor cultivation (nitrate sufficient, pH 8–10, 24 h illumination). However, compared to this condition, other nitrate sufficient cultures [pH 6–8 and 10–12 (24 h illumination), and at ambient CO_2 and 16:8 h light:dark photoperiod (pH unadjusted)] showed ~12–14% lower lipid productivity. Upon 50% nitrate depletion (at indoor and outdoor; pH unadjusted) lipid content has increased by 7.62% and 17%, respectively. Though stress conditions helped enhancing lipid accumulation, there was two-fold increase in PUFA content compared to that observed at pH 8–10. This resulted in fuel properties which did not comply with the biodiesel standards.

1. Introduction

Microalgae are an attractive choice for a sustainable and environmentally-friendly alternative to petrodiesel. Usually, high lipid content of the microalgae is considered as the major screening criteria for selecting the species for biodiesel production (Francisco et al., 2010). This has led to an upsurge in research for enhancing microalgal lipid production through manipulation of cultivation conditions. Many a researcher investigated the effect of nutritional stress, photo-oxidative stress, and other undesirable environmental stress on microalgal lipid production (Dayananda et al., 2007; Converti et al., 2009 Nigam et al., 2011; Ruangsomboon, 2012). However, unlike thorough characterization of fuel properties (cetane number, oxidative stability, iodine number and cold filter plugging point) of lipids obtained from the plant and vegetable sources (Santoria et al., 2012; Ramos et al., 2009; Knothe, 2009), studies on fuel characterization of microalgal lipid are scarce (Francisco et al., 2010). Surprisingly, while the concept of employing various stresses on microalgae for enhanced production of lipid-rich feedstock for biodiesel has been explored continuously, the investigations on the suitability of lipid synthesized at various cultivation conditions (including stress conditions) as a biodiesel feedstock have been grossly overlooked. Fundamental question such as whether stress induced lipids are suitable as biodiesel feedstock still remains unanswered and hence the need to find the most suitable cultivation which compromises neither fuel quality nor quantity is pertinent. Therefore, we investigated for the first

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time, the effect of various cultivation conditions on the suitability of microalgae synthesized-lipid for biodiesel production. *Chlorella pyrenoidosa* (*C. pyrenoidosa*) was taken as a model microalga to study biodiesel quality at different nutritional, environmental, and physiological cultivation conditions. Important fuel properties of synthesized-lipids were characterized through detail fatty acid methyl esters (FAMEs) profiling.

2. Methods

2.1. Microalgal strain

The microalgal specie *C. pyrenoidosa* was procured from National Collection of Industrial Microorganisms (NCIM), CSIR-National Chemical Laboratories (NCL), Pune; Maharashtra, India.

2.2. Culture media and growth conditions and biomass quantification

Bold's Basal Medium (Dayananda et al., 2007) was used to subculture, maintain and acclimatize the microalgal species to laboratory conditions prior to using it for experimental purpose. Sodium bicarbonate (4.5 g L^{-1}) was used as the sole source of inorganic carbon except for the cultures grown at ambient CO_2 concentration (0.03%). The culture medium was seeded with 20% inoculums (exponential phase culture) and incubated for 16 days. For indoor cultivation, cultures were incubated in laboratory with controlled temperature (28 ± 1 °C) and light intensity of 50 μmol m^{-2} s^{-1}. For outdoor cultivation studies, the cultures were kept outside the laboratory windows exposed to natural conditions of temperature (38.5 °C) and light (250 μmol m^{-2} s^{-1}). All the experiments were carried out in triplicates. For biomass quantification; the standard calibration curve of optical density (O.D.) at 680 nm verses concentration of cells (mg L^{-1}) was plotted. Growth rate was monitored by taking O.D. (UV–Visible spectrophotometer, Cintra 6, Australia).

2.3. Effect of pH, 0.03% CO_2 and 16:8 h light:dark (L:D) photoperiod

To study the effect of pH on biomass productivity and lipid content, microalgal cultures were grown at three different pH ranges viz at pH 6–8, pH 8–10, and pH 10–12. For ambient CO_2 concentration (0.03%), cultures were incubated as open flasks. The light:dark photoperiod cycle of 16:8 was maintained to study the effect of photoperiod. All these experiments were carried out in triplicates.

2.4. Effect of nitrate depletion at indoor and outdoor cultivation

Microalga was initially inoculated in BBM supplemented with Sodium Nitrate (0.25 g $NaNO_3$/L BBM) as a source of nitrate (It is defined as the nitrate sufficient condition). After exponential phase, the biomass was harvested, washed twice with distilled water and re-suspended in modified BBM with concentration of nitrate source being half depleted (0.125 g $NaNO_3$/L BBM, nitrate depleted condition). The cultures were then re-incubated for 6 days.

2.5. C, H, N, S analysis and carbon dioxide (CO_2) biofixation rate

The dried samples (5–10 mg) were analyzed for carbon (C), hydrogen (H), nitrogen (N), sulfur (S), and C/N ratio using CHNSO analyzer (VARIO EL III Elementar, Germany). Carbon dioxide biofixation rate [*Rco*2 (g L^{-1} d^{-1})] was calculated according to equation derived by Tang et al. (2009).

2.6. Total lipid extraction and quantification

Slightly modified Bligh and Dyer method was used for total lipid extraction. Lyophilized microalgal biomass was homogenized with chloroform: methanol (2:1) using a mortar and pestle. After three cycles of homogenization, the homogenate was filtered through sodium sulfate to remove moisture. Filtrate was collected in a round bottom flask and the solvent was evaporated using rotary evaporator (Buchi, Germany). Lipid was re-dissolved in hexane (10 ml) in a dried and pre-weighed glass test tube. Hexane was completely evaporated by nitrogen sparging (Turbovap LV concentrator, Caliper life sciences, USA). The lipid content was measured gravimetrically and expressed as percentage of dry cell weight.

2.7. Trans-esterification of lipids to fatty acid methyl esters (FAMEs)

Lipid sample (300 μl) was saponified using 3 ml of saponification reagent [(15% NaOH in Methanol: Water (1:1) mixture] into a screw cap boiling test tube by boiling in water bath for 30 min. 6 ml of methylation reagent [325 ml 6 N HCl + 275 ml methanol] was added to the cooled saponified mixture and again heated at 70 °C in boiling water bath for 20 min. Methylated mixture was cooled at room temperature. To it, added 3.75 ml extraction [(Hexane: Anhydrous diethyl ether (1:1)] solvent and mixed by end to end rotation for 1 min. FAMEs were recovered in upper solvent phase and used for GC–MS analysis.

2.8. Determination of FAMEs composition by GC–MS

FAME Composition was analysed using GC–MS (Clarus 680; Perkin Elmer, Germany) equipped with quadrupole mass analyzer. 1 μl sample was injected for analysis in a 100 m capillary column (SP-2560, Supelco). Helium was used as a carrier gas (flow rate, 1.21 ml min^{-1}). An injector, interline and ion source temperature were set at 250 °C, 260 °C and 250 °C, respectively. Oven ramping was started with 140 °C for 5 min; then increased to 240 °C at 4 °C min^{-1} and was held at 240 °C for 15 min. Unknown FAMEs were identified by comparison of their retention times with those of standard FAMEs (Supelco) and comparing mass spectra from NIST library.

2.9. Determination of quality characteristics of biodiesel

Biodiesel fuel characteristics such as saponification value (SV), iodine value (IV), degree of unsaturation (DU), long-chain saturated factor (LCSF) and cold filter plugging point (CFPP) were determined by empirical equations (Francisco et al., 2010). SV, IV, CN, and DU were calculated using Eqs. (1)–(4).

$$SV = \sum \frac{560N}{M} \tag{1}$$

$$IV = \sum \frac{254DN}{M} \tag{2}$$

$$CN = 46.3 + \frac{5458}{SV} - 0.225IV \tag{3}$$

where *SV*, *IV* and *CN* are the saponification value, iodine value and cetane number respectively. *D*, *M* and *N* denotes the number of double bonds, molecular mass and percentage (%) of each fatty acid component. Degree of unsaturation, *DU* (%) was determined as follows-

$$DU(\%) = MUFA + (2 \times PUFA) \tag{4}$$

where *MUFA* is the monounsaturated fatty acids content (wt.%) and *PUFA* is the polyunsaturated fatty acids content (wt.%).