Growth kinetic and fuel quality parameters as selective criterion for screening biodiesel producing cyanobacterial strains

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Abstract

The efficiency of cyanobacterial strains as biodiesel feedstock varies with the dwelling habitat. Fourteen indigenous heterocystous cyanobacterial strains from rice field ecosystem were screened based on growth kinetic and fuel parameters. The highest biomass productivity was obtained in *Nostoc punctiforme* MBDU 621 (19.22 mg $L^{-1}d^{-1}$) followed by *Calothrix* sp. MBDU 701 (13.43 mg $L^{-1}d^{-1}$). While Lipid productivity and lipid content was high in *Nostoc spongiaeforme* MBDU 704 (4.45 mg $L^{-1}d^{-1}$ and 22.5 % dwt) followed by *Calothrix* sp. MBDU 701 (1.54 mg $L^{-1}d^{-1}$ and 10.75 % dwt). Among the tested strains, *Nostoc spongiaeforme* MBDU 704 and *Nostoc punctiforme* MBDU 621 were selected as promising strains for good quality biodiesel production by Preference Ranking Organization Method for Enrichment Evaluation (PROMETHEE) and Graphical Analysis for Interactive Assistance (GAIA) analysis.

Keywords: Cyanobacteria, Biodiesel, FAME, Fuel quality parameters, Growth kinetics, PROMETHEE-GAIA

1. Introduction

Fast depletion of fossil fuels with exploding population mandates global energy agendas towards the development of renewable sources of fuel. Among different generations of renewable feedstock, photosynthetic microalgae serve as a promising biomass for a diverse number of products such as fine chemicals, nutraceuticals, aquaculture, feed and cosmetics in addition to biofuels (Gerardo et al., 2015). Despite much research drive for the past two decades, microalgal biofuel has not yet been a commercial reality. For the past two years, collapse in the oil prices imposes large economic pressure on biofuel production (Cate and Ball, 2016). In order to make microalgal biofuels commercially feasible and practically viable, microalgal biomass must be processed similar to petroleum refinery for extracting multiple products in addition to biofuel, in a biorefinery concept (Maurya et al., 2016). Many up- and downstream processes have successfully been integrated during the conversion of microalgal biomass. For example, integrating the upstream microalgal cultivation with wastewater treatment reduces overall residual waste component and favors sustainable economy (Mohan et al., 2016). During downstream processing, high volumes of products such as proteins and carbohydrates, and low volume high value products such as astaxanthin, β-carotene, and polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid have also been co-extracted from microalgal biomass, and have significant market demand (Gerardo et al., 2015). Therefore, a biorefinery should be able to produce a gamut of marketable products and energy in a sustainable fashion (Gravitis, 2008). Utilisation of additional products help to subsidize the overall fuel costs (Chuck et al., 2015).

Owing to its simple growth requirements, increased growth rate and ease of genetic engineering with developed molecular tools, cyanobacteria serve as an attractive candidate over

eukaryotic microalgae in terms of biomass feedstock utilization. Until recently, many researchers have successfully co-produced various valuable products in metabolically engineered cyanobacteria (Angermayr et al., 2015). However, only little attempt is made in the exploration of natural cyanobacterial species with the potency of producing different commercially important products. Knowledge of these properties is an important criteria in the selection of most suitable strains which can be exploited successfully at commercial level. With this in view, the present study is carried out to explore the lipid productivity, lipid content and biodiesel properties of phytohormone producing cyanobacterial strains.

2. Methods

2.1. Cultivation of cyanobacterial strains

The fourteen cyanobacterial strains, *Scytonema bohneri* MBDU104, *Calothrix* sp. MBDU901, *Nostoc spongiaeforme* MBDU704, *Nostoc commune* MBDU101, *Nostoc muscorum* MBDU702, *Nostoc* sp MBDU804, *Anabaena spiroides* MBDU903, *Nostoc Punctiforme* MBDU621, *Calothrix* sp MBDU701, *Aphanothece stagnina* MBDU803, *Anabaena variabilis* MBDU103, *Nostoc sp* MBDU001, *Nostoc commune* MBDU703, and *Nostoc microscopicum* MBDU102, characterised previously for phytohormone production (Gayathri et al., 2017) were grown in BG-11_o medium in 250 mL Erlenmeyer flask at 28 ± 1°C under continuous light (50 μE m^{-2} s^{-1}) (Rippka et al., 1979).

2.2. Growth kinetic parameters

The growth kinetic parameters of fourteen strains were determined after harvesting the cells in their stationary growth phase. All the measurements were performed in triplicates. The parameters analyzed included:

- **Biomass productivity (Pb)** indicates the amount of dry biomass produced (g L^{-1} day^{-1}). For Pb determination, algal suspensions were centrifuged at 3000 g for 10min at room temperature and the resulting pellets were washed with deionized water, lyophilized at −40 °C for 48 h and their dry weights were determined gravimetrically.
- **Total lipid content (Lc)**, reported as percentage of the total biomass (% dwt), and determined based on the method by Folch et al., (1957).
- **Volumetric lipid productivity (Lp,** mg L^{-1} day^{-1}), was calculated according to the following equation (Liu et al., 2011b).

$$Lp = Pb \times Lc \quad (1)$$

2.3. Total lipid extraction

The total lipid from the tested strains was extracted according to Folch et al., (1957). 40 mg of freeze-dried biomass was extracted with 10 mL of chloroform:methanol (2:1) using pestle and mortar. The extract was filtered through Whatman No. 1 filter paper. To the filtrate three volumes of distilled water was added. The filtrate was then vortexed for 5 mins, and allowed to undergo phase separation for 15 mins. Lower phase containing essentially all extracted lipids were transferred into a weighed, clean glass vials and allowed to dry in a rotary evaporator to remove solvent mixture. The dried lipid was quantified and expressed as percent on dry weight basis.

2.4. FAME analysis by GC

Fatty acid profile was analysed by preparation of fatty acids methyl ester (FAME) and Gas Chromatography–Mass Spectrometry analysis. FAME was prepared directly using the transesterification method described by Indarti et al. (2005), with minor modification. Dried

115 algae samples (about 30 mg) were weighed onto clean glass vials and allowed to react directly
116 with 10 mL mixture of methanol, concentrated sulfuric acid and chloroform (4.25:0.75:5).
117 Transesterification was carried out in a 90°C water bath for 90 min. On completion of the
118 reaction, the vials were cooled down to room temperature and then, 1 mL of distilled water was
119 added into the mixture and thoroughly vortexed for 5 min. After the formation of two phases, the
120 lower phase containing FAME was transferred to a clean glass vial and dried. The samples were
121 analyzed via GC (Shimadzu, QP 2010, Japan) with FID detector. The oven temperature was set
122 at 80 °C, and held for 5 min, then raised to 290 °C at a rate of 4 °C/min, and held at 290 °C for 5
123 min, while the injector and detector temperature were set at 270 °C and 280 °C, respectively. The
124 SP-2560 column (Supelco, USA) (100 m × 0.25 mm I.D. × 0.20 μm film thickness) was used for
125 the analysis of FAME. The carrier gas (helium) was controlled at 2 mL/ min. Concentrations of
126 individual FAMEs were determined by comparing sample peak areas with C-8 to C-24 FAME
127 mixture from Supelco Analytical (Bellefonte, PA).

128 *2.5. Calculation of fuel properties*

129 To screen the suitable indigenous cyanobacterial strains for biodiesel production based on FAME
130 profile, the following 15 biodiesel properties were calculated: i) iodine value (IV)- Equation (2)
131 ii) saponification value (SV) – Equation (3) iii) cloud point (CP)- Equation (4) iv) pour point
132 (PP)- Equation (5) v) cetane number (CN)- Equation (6) vi) Degree of unsaturation (DU)-
133 Equation (7) vii) long chain saturation factor (LCSF)- Equation (8) viii) cold filter plugging point
134 (CFPP)- Equation (9) ix) allylic position equivalent (APE)- Equation (10) x) bisallylic position
135 equivalent (BAPE)- Equation (11) xi) kinematic viscosity (υ)- Equation (12) xii) density (ρ)-
136 Equation (13) xiii) high heating value (HHV)- Equation (14) (Anahas and Muralitharan, 2015).

xiv) Oxidative stability (OS) - Equation (15) (Wang et al., 2012) xv) flash point temperature (FP) - Equation (16) (Agarwal et al., 2010).

$$IV = \Sigma (254 \times DN)/M \quad (2)$$

D is the number of double bonds, *M* is the molecular weight and *N* is the percentage of each fatty acid.

$$SV = \Sigma (560 \times N)/M \quad (3)$$

$$CP = (0.526 \times C16) - 4.992 \quad (4)$$

$$PP = (0.571 \times C16) - 12.240 \quad (5)$$

$$CN = 46.30 + (5458/SV) - (0.225 \times IV) \quad (6)$$

$$DU = MUFA + (2 \times PUFA) \quad (7)$$

MUFA – monounsaturated fatty acid, PUFA-polyunsaturated fatty acid (in WT %)

$$LCSF = (0.1 \times C16) + (0{:}5 \times C18) + 1 \times C20) + (1.5 \times C22) + 2 \times C24) \quad (8)$$

$$CFPP = (3.1417 \times LCSF) - 16.477 \quad (9)$$

$$APE = \Sigma (apn \times Acn) \quad (10)$$

160 $BAPE = \Sigma (bpn \times Acn)$ (11)

161 where apn and bpn are the numbers of allylic and bisallylic positions in a specific FA,

162 respectively, and Acn is the amount (mass percent) of each FA in the mixture.

163

164 $\ln(\upsilon_i) = -12.503 + 2.496 \times \ln(M_i) - 0.178 \times N$ (12)

165 $\rho_i = 0.8463 + 4.9/M_i + 0.0118 \times N$ (13)

166 $HHV_i = 46.19 - 1794/M_i - 0.21 \times N$ (14)

167 where v_i is the kinematic viscosity of at 40 °C in mm^2/s; ρ_i is the density at 20 °C in g/cm^3; and

168 HHV_i is the higher heating value in MJ/kg of *i*th FAME.

169 $OS = -0.03844 \times DU + 7.770$ (15)

170

171 $FP = 205.226 + 0.083 \times C16{:}0 - 1.723 \times C18{:}0$

172 $-0.5717 \times C18{:}1 - 0.3557 \times C18{:}2 - 0.467 \times C18{:}3 - 0.2287 \times C22$ (16)

173 *2.6. Biochemical analysis*

174 Freeze-dried biomass (5mg) was suspended by vortexing in 0.2ml of 24% (w/v) TCA and

175 incubated 95°C for 15 mins in a screw capped micro-centrifuge tubes and allowed to cool at

176 room temperature. TCA precipitation was carried out by adding 0.6 mL of distilled water and

177 cooling the suspension at room temperature. After centrifugation at 15,000 rpm for 20 mins at

178 4°C, the supernatant was discarded and the precipitate was re-suspended in 0.5mL of Lowry

179 reagent 'D'. This alkaline suspension was incubated at 55°C for 3 hr followed by centrifugation

180 at 15,000 rpm for 15 mins at room temperature. The pellet was discarded and the supernatant

181 was used for protein and carbohydrate estimation (Slocombe et al., 2013).

The carbohydrate content, including reducing sugar and total carbohydrates was determined using a dintiro salicylic acid method (Miller, 1959) and Anthrone method (Hedge and Hofreiter, 1962), respectively. Protein estimation was carried out by Lowry method against BSA as a standard (Lowry et al., 1951). Data for all experiments represent the average of three replicates.

2.7. Selection of suitable strains for biodiesel production using MCDA-PROMETHEE

Among the fourteen cyanobacterial strains, the highest lipid yielding strain was selected based on the Preference Ranking Organization Method for Enrichment Evaluation (PROMETHEE) analysis by choosing the linear preference and appropriate threshold value for both p-preference threshold (smallest difference enough to generate a full preference) and q-indifference threshold (largest difference that is considered negligible by the decision maker and it is enough to generate a full preference) (Brans and Mareschal, 2005) for the criteria. The criteria taken for initial screening was biomass productivity, lipid productivity and lipid content. The selected best five strains were further analyzed for FAME yield and biodiesel properties by gas chromatography. Based on the FAME yield, fifteen biodiesel properties were included as criteria and calculated along with saturated fatty acid (SFA), poly unsaturated fatty acid (PUFA), and mono unsaturated fatty acid (MUFA) to select a best strain. The threshold values were set as per Table. 4. by giving equal weight to all biodiesel quality parameters. This MCDA – PROMETHEE and strain selection was well reported for biodiesel producing microbes (Islam et al., 2013; Anahas and Muralitharan, 2015).

3. Results and discussion

The tested cyanobacterial strains belonging to five different genera ie. *Nostoc*, *Calothrix*, *Scytonema*, *Anabaena*, and *Aphanothece* were isolated and identified at morphological and

molecular level in our previous study (Gayathri et al., 2017). To evaluate whether an algal strain is suitable for biodiesel production, the key criteria such as lipid content, lipid productivity, TAG content in total lipid and suitable fatty acid composition (Talebi et al., 2013) were analysed. Typically, lipid content was reported as percentage dry weight (% DCW). Lipid productivity was influenced by both biomass accumulation and lipid content (Hoekman et al., 2012). For all the tested cyanobacterial strains, biomass productivity varied from 19.22 to 2.9 mg/L/ day, lipid productivity from 4.45 to 0.1 mg/L/ day and lipid content from 22.5 to 1.49 in terms of % dwt (Fig. 1). The highest biomass productivity was shown by *Nostoc punctiforme* MBDU 621 (19.22 mg/L/day) followed by *Calothrix* sp. MBDU 701 (13.427 mg/L/day), *Scytonema bohneri* MBDU 104 (12.62 mg/L/day), *Nostoc spongiaeforme* MBDU 704 (11.9) and *Nostoc* sp. MBDU001 (11.48 mg/L/day). With the exception of *Nostoc punctiforme* MBDU 621, the leading biomass producing cyanobacterial strains showed high lipid productivity and lipid content. For example *Nostoc spongiaeforme* MBDU 704 showed a lipid productivity and lipid content of 4.452 mg/L/day and 22.5 % dwt, respectively, followed by *Calothrix* sp. MBDU 701 and *Scytonema bohneri* MBDU 104. Similar to our results the lipid productivity of 4.39-7.13 mg/L/day was reported for single cultures of tested cyanobacterial and algal strains and reported an increase in lipid productivity during dual-species cultures (Gonçalves et al., 2016). Compared to the lipid content of previously reported heterocystous cyanobacterial strains (4.68 to 18.65 % dwt) (Anahas and Muralitharan, 2015), the tested cyanobacterial strains in this study showed higher lipid content of 1.495 to 22.5 % dwt. Similarly, our tested strains showed an increased lipid content under normal extraction method (chloroform:methanol; 2:1 v/v) than the cyclohexance:methanol (2:1, v/v) extraction reported as best solvent system for *Microcystis aeruginosa* (Ashokkumar, 2014). Lipid content is a key criterion for choosing oleaginous

228 microalga species, and the basal lipid content of microalga are usually limited to not higher than
229 20% or 30% DCW under standard conditions (Hu et al., 2008). The lipid content reported in
230 literature for most microalga was very variable and dependent on the environmental and
231 cultivation conditions. For example, when the microalga cells became old or were exposed to
232 stress conditions, an extraordinary increment in the lipid content could be observed (Hu et al.,
233 2008).

234 The multi criterion decision analysis was performed based on the growth kinetics to select
235 the prominent strains among the fourteen tested cyanobacterial strains for FAME analysis (Fig.
236 2). PROMETHEE displays the decision axis towards the *Calothrix* sp. MBDU 701 which was
237 the most promising strains in the tested parameters viz. biomass and lipid productivity, lipid
238 content. Though *Nostoc spongiaeforme* MBDU 704 showed high lipid productivity, it was
239 averaged among others in biomass productivity. *Calothrix* sp. MBDU 701, *Scytonema bohneri*
240 MBDU 104 and *Calothrix* sp. MBDU 901 were the promising strains in these three parameters
241 analysed and were located along with decision axis. The criteria of lipid productivity and lipid
242 content were directed adjacent to *Nostoc spongiaeforme* MBDU 704. *Nostoc punctiforme*
243 MBDU 621 was positioned orthogonal to the decision axis. This was due to the fact that it is was
244 good in biomass productivity but least in lipid productivity and lipid content (Fig. 2). The phi
245 score displays the rank of these tested cyanobacterial strains and *Nostoc spongiaeforme* MBDU
246 704 was listed as best since it tops the two of three parameters analysed (Table 1). It was
247 followed by *Nostoc punctiforme* MBDU 621, *Scytonema bohneri* MBDU 104, *Calothrix* sp.
248 MBDU 901 and *Calothrix* sp. MBDU 701. In our previous study, we reported that Biowet
249 Extract (BWE) 10 % and 1 % of *Nostoc spongiaeforme* MBDU 704 and *Nostoc punctiforme*
250 MBDU 621, respectively increased the radicle length of *Pisum sativum* seedlings and ranked at

third and first place among other tested cyanobacterial strains (Gayathri et al., 2017). The top listed strains were further analysed by GC to study the FAME yield and biodiesel properties.

3.1. Fatty acid composition

In addition to screening based on biomass productivity, lipid content and lipid productivity, FA profiles of the selected strains were further examined and are considered important for assessing the quality of the biodiesel produced. The quality depends mainly on the unsaturation ratio because unsaturated fatty acids (UFA) enhance cold-flow properties whereas saturated FAs maintain good oxidative stability (Wu and Miao, 2014). Knothe, (2008) reported that Palmitic (C16:0), stearic (C18:0), oleic (C18:1), and linolenic acid (C18:2) as the most common fatty acids contained in biodiesel. In particular, oils with high oleic acid content have been reported to have a reasonable balance of fuel properties. The fatty acid compositions of selected five strains were listed in Table.2. C16:0 (palmitic acid) was the predominant fatty acid group in all strains and it was high in *Scytonema bohneri* MBDU 104 (37.39%) followed by *Calothrix* sp. MBDU 901 (25.34%), *Calothrix* sp. MBDU 701 (23.47%), *Nostoc punctiforme* MBDU 621 (21.84%) and *Nostoc spongiaeforme* MBDU 704 (14.39%). The other fatty acid group C16:1 (palmitoleic acid), C18:0 (stearic acid), C18:1 (oleic acid and elaidic acid) were also important for good quality of biofuel since these FAs provide a good balance between cold flow property and oxidative stability (Hoekman et al., 2012). While comparing these groups of fatty acids, C16:1 was not detected in *Scytonema bohneri* MBDU 104 and was low in *Calothrix* sp. MBDU 901 (1.46%). Whereas, *Calothrix* sp. MBDU 701 showed 22.22% of C16:1, 3.75% of C18:1, 3.93% of C18: 2 (linoleic and linoleaidic acid) and 3.15% of C18:3 (α and γ linolenic acid) fatty acids. The other strains *Nostoc punctiforme* MBDU 621 and *Nostoc spongiaeforme* MBDU 704 showed 6.13 % and 5.34% respectively of C16:1.

The cyanobacterial strains having the high amount of C16:0 fatty acid showed minimum quantity or absence of other fatty acid group like C18:0, C18:1, C18.2 and C18:3 (Fig. 3). Though dominance of C16:0 makes good feedstock for biodiesel production, presence of C16:1 and C18:1 also most common and suitable for biodiesel production. C18:0 was high in *Nostoc spongiaeforme* MBDU 704 (10.93%) and *Scytonema bohneri* MBDU 104 (4.8%) while the other strains have limited amount of C18:0 fatty acid. The ratio of C18:1 and C18:2 were not significantly varied among the tested five strains and the high quantity was shown in *Nostoc punctiforme* MBDU 621 (7.22%). C18:3 was detected only in *Nostoc punctiforme* MBDU 621 (2.25%) and *Calothrix* sp. MBDU 701 (3.15%). The saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) contents of tested cyanobacterial strains were represented in Fig. 4. All the strains showed high amount of SFAs which varied from 36.92% to 73.31%; compared to MUFAs (12.18 to 32.79%) and PUFAs (4.85% to 8.64%). SFAs was high in *Calothrix* sp. MBDU 901 (73.31%), followed by *Scytonema bohneri* MBDU 104 (61.12%), *Nostoc spongiaeforme* MBDU 704 (56.23%), *Calothrix* sp. MBDU 701 (37.13%) and *Nostoc punctiforme* MBDU 621 (36.92%). Likewise a higher amount of SFA than unsaturated fatty acids was reported in *Synechocystis* PCC 6803 (Velmurugan and Incharoenskadi, 2016).

Next to SFAs, MUFAs was high in *Calothrix* sp. MBDU 701 (32.79%), followed by *Nostoc punctiforme* MBDU 621 (21.51%), *Nostoc spongiaeforme* MBDU 704 (16.2%), *Calothrix* sp. MBDU 901 (15.64) and *Scytonema bohneri* MBDU 104 (12.18%). When the proportion of polyunsaturated FAs (PUFAs) increased, the biodiesel can be easily oxidized and thus reducing the overall CN. Therefore, mono-unsaturated FAs (MUFAs) such as C18:1, which is dominant in high quality feed stock such as canola oil, is generally important and preferred compared to

297 saturated FAs or PUFAs for increasing the quality of biodiesel because they provide balance
298 between cold flow, oxidative stability and combustion properties (Knothe, 2014).

299 *3.2. Biochemical composition*

300 Biochemical composition was also evaluated in terms of total and reducing sugar, protein in
301 dried biomass (Fig. 5). Carbohydrates are the major products derived from photosynthesis and
302 the carbon fixation metabolism (i.e., the Calvin cycle) (Ho et al., 2011). These carbohydrates are
303 either accumulated as starch, or component of cell walls as cellulose, pectin, and sulfated
304 polysaccharides. In microalgae, the composition and metabolism of carbohydrates differ
305 significantly from species to species (Rismani-Yazdi et al., 2011) and it is of great importance in
306 biofuel production with high carbohydrate productivity. The polysaccharides, mainly comprising
307 of cellulose, can be hydrolyzed to obtain reducing sugars for further application in bioethanol
308 fermentation (Sun and Cheng, 2002). A number of studies (Ho et al., 2012; Siaut et al., 2011)
309 have demonstrated that nitrogen-depletion leads to a sharp increase in the lipid or carbohydrate
310 content of microalgae, because this forces them to transform protein or peptides to lipids or
311 carbohydrates. Carbohydrate content was in the range of 0.005-0.072 mg/mL as reducing sugar,
312 0.036 (Fig. 5c) - 0.816 mg/mL as total sugars (Fig. 5b).

313 Protein was in the range of 0.08-0.344 mg/%dwt (Fig.5a) within the limit of already reported
314 literature that ranged between 11.1% and 19.1% (DW) as low quantity which is favorable to
315 microalgal biofuel production since the high protein content means a high proportion of nitrogen
316 in the bio-oil produced.

317

318 *3.3. Fuel properties*

319 The analysis of fatty acid composition can provide useful information to determine the quality of
320 biodiesel parameters like IV, SV, CN, CFPP, LCSF, CP, PP, DU, APE, BAPE, viscosity and
321 density (Table.3). The difference in iodine values are related to fatty acid composition. The
322 European standard defines a maximum value of 120 g I_2 100 g^{-1}, which may be necessary
323 because heating higher unsaturated fatty acids results in the polymerization of glycerides, which
324 leads to the formation of deposits or to the deterioration of the lubricating oil and it may increase
325 due to double bonds in the FA. So limited unsaturated fatty acids may decrease the iodine
326 number (Francisco et al., 2010). In this study, all tested strains showed low iodine value than the
327 maximum accepted standards and proved them as a good candidate for biofuel synthesis.
328 Biodiesel standards did not specify the limit of Saponification value (SV). Table 3 shows the SV
329 of tested cyanobacterial strains and it was in the range of 145.32–217.52. Our results were in
330 consistence with the already reported SV range of 203.18-214.38 (Mandotra et al., 2016).
331 During cold climate, CP and PP are considered important for fuel quality. The CP is the
332 temperature at which a cloud of wax crystals first appear when the fuel is cooled, whereas the PP
333 is the temperature at which the wax formed fuel can flow. Higher proportions of SFAs indicate
334 the higher PP of biodiesel, usually biodiesel has higher CP and PP than diesel fuel (Torres-
335 Jimenez et al., 2011). Biodiesel fuels derived from fats or oils with significant amounts of
336 saturated fatty compounds will display higher CPs and PPs. ASTM D6751 specified CP range of
337 -3 to 12°C and PP of -15 to 20°C. The tested five strains exhibited CP values ranged between
338 2.58 to 17.4°C and PP values of -4.01 to 9.1°C that corroborated with the standard. The CP of
339 *Nostoc punctiforme* MBDU 621 (17.4) and *Scytonema bohneri* MBDU 104 (14.6) was exceeding
340 the standard while the PP of all strains were within the standard limit.

Higher CN improves the combustion properties of fuel and easier engine start-up, less occurrence of knocking and low nitrous oxide emission (Arias- Peñarands et al., 2013). Fatty acid profile with higher SFA and MUFA content has higher value of CN. The minimum value of CN specified by EN 14214 and IS 15607 was 51, whereas, in ASTM D6751-08 it was 47 (Mandotra et al., 2014). All the tested cyanobacterial strains showed the CN in the range of 65.95 to 75.71.

Degree of unsaturation (DU) influences the oxidative stability of biodiesel and it is the sum of the masses of MUFA and PUFA (Francisco et al., 2010). The DU was in the range of 25.18- 49.07. The DU of *Scenedesmus abundans* at various culture conditions was shown to be in the range of 26.57- 110.04 and was already proven to have good biodiesel properties.

The CFPP, which indicates the flow performance of biodiesel at low temperature, is related to the amounts of unsaturated fatty acid in biodiesel (Kwak et al., 2016). All the tested strains met the standard values except *Calothrix* sp. MBDU 901. LCSF is a critical parameter for oxidative stability and determining the cold response of biodiesel. There was no specification for LCSF in the standards and the highest LCSF value was recorded in *Nostoc spongiaeforme* MBDU 704 (25.93).

The APE and BAPE value in FAME are significant in predicting oxidation stability of the biodiesel (Knothe, 2012). The tested isolates showed APE and BPE range of 11.8-24.9 and 5.8-14.74, respectively. Kinematic viscosity (v) is the resistance of liquid to flow and depends on the thickness of the oil. The higher viscosity caused insufficient fuel atomization leading to the formation of soot occurs and gets deposited in engine deposits (Shu et al., 2007) while lower viscosity is easier to pump and achieve final droplets to injector (Refaat, 2009). Therefore appropriate kinematic viscosity (v) of biodiesel ensures adequate fuel supply at different

364 operating temperatures (Ramirez- Veruzco et al., 2012). The ASTM 6751-02, EN 14214, IS
365 15607 has set kinematic viscosity limits to 1.9-6.0 $mm^2 s^{-1}$, 3.5-5.0 $mm^2 s^{-1}$ and 2.5-6.0 $mm^2 s^{-1}$.
366 The range of all strains was between 2.53-4.46 $mm^2 s^{-1}$ and meeting the mentioned standards.
367 The fuel injection system supplies fuel by volume not by mass which means denser
368 biodiesel will be injected with greater mass in to the combustion chamber consequently affecting
369 the stoicheometric ratio of air and fuel (Ng et al., 2012). Therefore, density (ρ), for which a
370 standard value has been set at 0.86–0.90 g cm^{-3} according to EN 14214, ASTM D6751-02 and IS
371 15607 is another important parameter for biodiesel quality. FAME profile-derived ρ-values of
372 tested cyanobacterial strains were within this range (0.88).
373 Although, HHV not specified in either ASTM D6751 or EN 14214, heat of combustion
374 impacts fuel efficiency and consumption. In addition, the European heating oil standard, EN
375 14213, specifies that the energy content of FAMEs must be at or above 35 MJ kg^{-1} (Knothe
376 2010). The HHVs for all samples were relatively similar, with values ranging from 40.15 to
377 42.93 MJ kg^{-1}.
378 Oxidation stability (OS) is the resistance of fuel degradation due to oxidation during
379 long-term storage. Biodiesels show less oxidative stability compared with petroleum diesel due
380 to their different chemical composition, and this is one of the major issues that limits the wide
381 spread use of biodiesel as a fuel in automobile engines. OS was high in *Nostoc spongiaeforme*
382 MBDU 704 (7.67), while all other cyanobacterial strain met the EN 14214 standard.
383 The flash point (FP) is the lowest temperature at which the fuel will begin to vaporize to
384 form an ignitable mixture when it comes in contacts with the air. Australian and European
385 biodiesel specification required flash point temperature of at least 120 °C, whereas in the US the
386 minimum requirement level is 93 °C. Tested cyanobacterial strains showed FP temperature in the

range of 184.21 and 199.22°C that was higher than the specified biodiesel standards (Jahirul, 2015).

3.4. Preference ranking of cyanobacterial strains

To produce a profitable biodiesel over diesel fuel, cyanobacteria should have suitable chemical content to establish the concurrence with various biodiesel standards. To figure out the suitability, 15 fuel properties IV, SV, CP, PP, CN, DU, LCSF, CFPP, APE, BAPE, viscosity, density, HHV, SFA, MUFA, PUFA, OS and FP was taken as multiple criteria and analyzed through PROMETHEE-GAIA since it provides logical decision towards the solution compared to other tools (Islam et al., 2013) (Fig. 6a). In GAIA plane, the criteria near to (±45°) were correlated, while those in the other side (135°–225°) were not related and those in orthogonal have no or less impact (Espinasse et al., 1997). For example, *Nostoc spongiaeforme* MBDU 704 was correlated since it positioned along with decision axis. The preference function was set to maximum or minimum (lower/higher values preferred for quality based biodiesel) which influenced the orientation of criteria.

The direction and length of the criteria influence the decision axis (Islam et al., 2013). For example parameters like OS, viscosity, PUFA, HHV, FP, IV have little effect on the decision vector. The decision axis pointed *Nostoc spongiaeforme* MBDU 704 as best since it was located along with decision axis and *Nostoc punctiforme* MBDU 621 as second since it located adjacent to decision axis and *Calothrix* sp. MBDU 701 followed by other strains (Fig. 6a). Fig. 6b. showed the overall ranking of cyanobacterial strains based on the fuel properties. The Phi value is the net flow score that could be negative or positive depending upon the angular distance from the decision vector and the distance from the centre (Jahirul et al., 2015). Based on the phi score, *Nostoc spongiaeforme* MBDU 704 was the most suitable strain in parameters like PP, CP, OS,

viscosity and density. *Nostoc punctiforme* MBDU 621 was preferred in CFPP, MUFA, HHV, PUFA and CFPP. These two strains lie closer to decision axis and met most of the criteria than the other strains tested. The GAIA plane from the analysis has a quality level of 88.5% which is reliable as it was above 70% quality significance level (Ahmad et al., 2015).

4. Conclusion

Here, we report on the ability of heterocystous cyanobacterial strains for biodiesel production. The method of robust strain selection based on FAME profiling and fuel quality parameters using PROMETHEE-GAIA analysis were reported. Based on our study, *Nostoc spongiaeforme* MBDU 704 and *Nostoc punctiforme* MBDU 621 were selected as the promising strains for biodiesel production. These strains were already shown to produce plant growth promoting substances in our earlier work. The cost of biodiesel can greatly be reduced if co-products of commercial value are looked for. Our study highlights this important aspect of multi-potency of cyanobacterial strains for future commercial utilization.

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570 **Figure legends**

571 **Fig. 1.** The performance of (a) Lipid productivity (mg/L/day) (b) biomass productivity
572 (mg/L/day) and lipid content (%dwt) of fourteen cyanobacterial strains. The bar diagram
573 represent the biomass productivity and lipid content and line art represent the lipid productivity.
574 Data values are means (± SE) of three replicates.

575

576 **Fig. 2.** PROMETHEE- GAIA algorithm showing the (a) fourteen cyanobacterial strains
577 indicated in Fuschia dots, pink lines indicated the biomass prodcuctivity (BP), lipid productivity
578 (LP) and lipid content (LC) as criteria, redline is the decision axis

579

580 **Fig. 3.** The major fatty acid content in five cyanobacterial strains appropriate for suitable
581 biodiesel expressed in percentage. The bar diagram represent the C18:0 (stearic), C18:1 (oleic),
582 C18:2 (linoleic) and C18:3 (linolenic) fatty acids. The line art represent the C16:0 (palmitic)
583 fatty acid.

584

585 **Fig. 4.** Fatty acid of five cyanobacterial strains based on the classes represented in percentage.
586 SFA- saturated fatty acid; PUFA- polyunsaturated fatty acid; MUFA- mono unsaturated fatty
587 acid.

588 **Fig. 5.** Biochemical composition of fourteen cyanobacterial strains (a) protein expressed in dwt
589 % (b) carbohydrate estimation by Anthrone method expressed in mg/ml (c) reducing sugar
590 estimation in DNS method expressed in mg/ml.

591

Fig. 6. PROMETHEE- GAIA algorithm showing (a) five cyanobacterial strains indicated in Fuschia dots, blue lines indicated the fifteen biodiesel properties along with SFA, PUFA and MUFA as criteria, redline is the decision axis (b) PROMETHEE table displays the phi score of five cyanobacterial strains based on the rank obtained through biodiesel properties.