Indian Journal of Chemical Technology
Vol. 28, March 2021, pp. 150-162

Molecular characterization of blue-green algae (*Anabaena constricta*) and comparative studies of biodiesel production from other species

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Received 7 August 2020; accepted 2 March 2021

Biodiesel can substitute fossil fuels adequately and thus, considered 'future fuel'. Thus, identifying a promising algal strain for efficient biodiesel production is a very important endeavor. In the present investigation, the blue green algae, *Anabaena constricta* could be grown aseptically attaining exponential phase, 3 days post inoculation, responds to phototropism and amenable to both autotrophic and heterotrophic mode. Phylogenetic analysis reveal that *Anabaena constricta* grouped separately from other algal strains used in the study, making it unique in nature. Further, comparative studies of selected algal and blue green algal strains based on the oil percentage and fatty acid profile indicate potential biodiesel properties. The total lipid content of *Anabaena constricta* is estimated to 14% of the dry weight, and fatty acids profiling indicate presence of 11 fatty acid methyl esters (FAMEs), principally palmitic acid (16:0), oleic acids (18:1), linoleic acid (18:2), palmitoleic acid (16:1). The algal species, *Hydrodictyon* registered highest percentage of unsaturated fatty acids (59.5%), while highest content of saturated fatty acid was found in *Anabaena constricta* (51.5%). Cetane Number of seven algal and blue green algal biodiesel varied from 54.77 to 58.2 and Saponification Value varied from 205.6 to 211.39. PROMETHEE & GAIA analyses indicate both the *Rhizoclonium* and *Tolypothrix* species outranked while *Hydrodictyon* and *Anabaena constricta* are the least suitable species in seven algal and blue-green algal species studied for biodiesel production. Well-characterized strains offer promise for biodiesel production at a cost-effective level. The present study focus on the renewable fuel, which is useful for reducing the carbon footprint with potential to impact self-sustainability in fuel sector, without modification in current infrastructure.

Keywords: Blue green algae, *Anabaena constricta*, Biodiesel, 16S rRNA, ITS, FAME

Presently, fossil fuels are getting used for a plethora of daily activities like automobiles, power generation, industrial uses, household activities, including cooking *etc*. Fossil fuels are a non-renewable energy source and shall be limited in times to come[1]. Currently, India consumes 213.22 MMT (Million Metric Tons) of petroleum products, while 226.50 MMT of crude oil was imported to meet domestic demand (2018-19 Provisional)[2]. The increasing import figures for petroleum products will affect the economy of India in a bigger way, besides hovering with the danger of depletion[2]. As per the conservative estimate from British Petroleum, at today's level of extraction and production rates, reserves of fossil fuel coal, natural gas and oil reserve would be exhausted by the year 2169, 2068, and 2066, respectively[3].

Fossil fuels also generate high levels of pollution that have an adverse effect on human health and ecology as a whole[4]. Thus, self-reliance in fuel production and other energy needs and exploration for better substitutes of fossil fuels, like biofuels, which may fulfil current and future energy demand without adversely affecting the environment, are vital for an independent nation. Though agro-based food products may be used as an alternate to bio-fuel[5], however, this might now and then be compromising with food security. It's prudent to look for living entity, with potential for biofuel generation/production, which are freely available in nature and may be cultured well in laboratory conditions. The thought of using algae as a source of fuel isn't new[6-8], however research interests renewed, as a result of escalating prices of fossil fuels and associated pollution[9].

Specific characteristics that promote algae as an energy source include lesser water requirements, generation of more biomass, and amenability to grow in various water characteristics like saltwater, sewage water (wastewater), etc.[10]. All these characteristics bolster the utilization of algae as an energy source. Further, growing phototrophic algae also reduce

environmental pollution[11-13]; because it consumes CO_2 and releases O_2 within the atmosphere, during its growth stages.

Algae can provide several different varieties of renewable biofuels *viz*. methane produced by anaerobic digestion of the algal biomass[14,15]; biodiesel derived from microalgal oil[8,13,16-19] and photo-biologically produced bio-hydrogen[20-25]. Researchers select algal strain for biodiesel production based on characteristics like a high percentage of oil in total biomass, maintenance of a high percentage of oil even under stress conditions, and compatibility with the local atmospheric conditions[26,27].

In our effort to identify potential algal strain indigenous to Indo-Gangetic plains of India, we report screening a set of seven algal/blue-algal strain based on FAME profile, Iodine Value, and Cetane number. Based on these fuel properties, we compared the biodiesel produced by these seven algal and blue green algal strains by PROMETHEE and GAIA software

Further, an indigenously available blue green algae, *Anabaena constricta*, was characterized based on the growth pattern, trophic nature, colony characteristics, fatty acid profile, and diversity analysis based on 16S rRNA and ITS sequence information.

Experimental Section

Collection of algae

Anabaena constricta (NAIMCC-C-00029), blue green algae collected from ICAR-National Bureau of Agriculturally Important Microorganisms (NBAIM), Mau, Uttar Pradesh. Comparative studies were done based on lipid content. The strain *Anabaena constricta* was selected for further studies because, it is indigenous species and details study was not previously reported. A slant culture was procured and sub-cultured in BG-11 media[28] for further studied in the Division of Plant Biotechnology, ICAR-Indian Institute of Pulses Research, Kanpur. Comparative analysis was done between *Anabaena constricta* and six algal and blue green algal strains from reported literature[29] (Table 1).

Growth kinetics, biomass and growth curve

Anabaena constricta strain was grown in 5 L flat bottom flask containing 2 L BG-11 medium at ~25°C under the light regime of (10000 lux) alternating with 12 h darkness/ 12 h light. Cultures were aerated with filtered atmospheric air at a maximum rate of 3 L/min throughout the experiment. The optical density of the algal culture was measured once in 48 h employing a spectrophotometer (BIORAD, USA) at 540 nm to estimate the growth of species. Average optical density values were calculated based on seven replications. Simultaneously, dry weight (biomass) of the culture was measured after oven drying (Thermex Industrial & Laboratory Instrument Co., Mumbai) of 10 mL of culture for 4 h, thrice per week employing a precision weighing balance (Anamed Precision Balance, Mumbai). The graph was derived by plotting optical density (OD) against time period (days) and corresponding dry weight (g) against time period (days).

Table 1 — Oil percentage in *Anabaena constricta* from this work and six other algal and blue green algal strains from literature from Indo-Gangetic plain of India

S. No.	Strain	Oil percentage	References
1.	*Anabaena constricta*	14	-
2.	*Tolypothrix*	12.78	[29]
3.	*Pithophora*	10.37	[29]
4.	*Spirogyra*	14.82	[29]
5.	*Hydrodictyon*	13.58	[29]
6.	*Rhizoclonium*	11.64	[29]
7.	*Cladophora*	11.76	[29]

Colony characteristics and trophic nature

To ascertain the colony characteristics, 15 days old algal culture grow in liquid BG-11 media. Sterilized loop dipped in algal mass was used for streaking culture plates (BG-11). Streaked plates were incubated at 25°C for 3 weeks, to record colony characteristics.

To ascertain the nature of the *Anabaena* cultures were streaked on Petri dish having two different media combinations, C-1: BG-11 media, C-2: BG-11 media containing 10 g/L glucose[30], glucose was used as a carbon source. Streaked plates were inoculated at 25°C for 3 weeks with a light regime of 12 h light and 12 h dark, to analyze the trophic nature of *Anabaena constricta*.

DNA extraction, PCR amplification, and sequencing

Total genomic DNA of *Anabaena constricta* was extracted employing the CTAB method as described earlier[31]. Isolated DNA was quantified employing a spectrophotometer (Eppendorf Biophotometer Plus, Germany) and all the aliquots were set at 100 ng/mL. The amplification of 16S rRNA and ITS gene were performed using the universal primers (16S F: CCTGGTTGATCCTGCCAG; 16SR: TTGATCCTTCTGCAGGTTCA; ITS4:

TCCTCCGCTTATTGATATGC; ITS5: GGAAGTAAAAGTCGTAACAAGG)[32]. PCR reactions were performed with a final volume of 20 µL, containing PCR Buffer (Mg^{+2}), dNTP mix (2 mM), 100 ng template DNA, 10 pM of corresponding forward and reverse primer, remaining volume adjusted with dH_2O and, 1U of *Taq* Polymerase (Thermo Fisher Scientific, USA). The amplification was performed in a Thermal-cycler (G-Storm, UK) with the thermal profile as follows: Initial denaturation: 94°C for 2 min., followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s (16S primer)/51°C for 30 s (ITS primer), extension at 72°C 60 s, final extension at 72 °C for 5 min. and finally reduced to 4°C for storage. All amplifications were done in triplicates to ensure the reproducibility of results. The amplification products were resolved on 1% agarose gel and stained with ethidium bromide dye (1 µg/mL), and all single amplification product were gel eluted (PCR Cleanup Gel Elution Kit, Nucleo-pore India). Single amplification eluted product was outsourced for Sanger sequencing (Xcleris Labs Ltd, Ahmedabad). The high quality chromatograms obtained was retrieved as nucleotide bases and consensus sequence obtained.

Construction of phylogenetic tree

To confirm the results obtained using universal primers based on 16S rRNA and ITS region, sequence alignment of high-quality reads was done, to get a consensus sequence. The NCBI-BLAST program[33,34] was used to determine the sequences showing maximum homology to the aligned sequence. A total of 20 sequences (Supplementary File 1) of 16S of algae and blue green algae and 21 sequences (supplementary file 2) of ITS of algae and blue green algae were retrieved from NCBI[35] for the construction of the phylogenetic tree. Multiple sequence alignment was done using CLUSTAL W program[36]. For 16S and ITS matrices, the number of base substitutions per site from between sequences and standard error estimate was recorded. Analyses were conducted using the Maximum Composite Likelihood model[37]. Codon positions included were $1^{st}+2^{nd}+3^{rd}$ and all ambiguous positions were removed for each sequence pair. Evolutionary analyses and subsequence bootstrap analysis were conducted in MEGA 5(Ref. 38).

Lipid content and FAME analysis

Total lipid content was estimated using the Soxhlet method[39], as a percentage of the total biomass (in % dry weight). The algal strain was grown aseptically for 15 days as described earlier and pelleted employing a centrifuge (Sorvall RC5C, USA) at 10°C and 4722g for 10 min[40]. Algae pellet was dried at 80°C for 4 h in hot air oven (Thermex Industrial & Laboratory Instrument Co., Mumbai)[41]. The Soxhlet unit (Borosil, India) was filled with the solvent mixture containing Chloroform: Methanol (2:1)[42], within the heating mantle (Perfit India) of the unit, followed by heating the solvent mixture to extract the lipid out of the dried algal sample from a thimble, which is subsequently collected within the bottom flask. The solvent was then separated from the lipid content by the distillation process. The mixture was heated to the boiling point of the solvent that selectively evaporate the solvent, leaving behind the lipid content. The lipid content was measured based on the weight of lipid extract compare to the dry weight of algal biomass.

The isolated lipid was converted into biodiesel by transesterification method using ethanol and lipid (6:1 ratio) in presence of base catalysis (NaOH)[43,44]. The resultant product was analyzed by Gas Chromatograph-Mass Spectrometry (GC-MS, model 7890/5977B, Agilent Technologies, USA)[45] equipped with an autosampler, split-splitless injector, flame ionization detector (FID) and a 60 m BPX70 capillary column (SGE, Ringwood, Australia) with an internal diameter of 0.25 mm and film thickness of 0.25 µm. Samples of 1 µL were injected splitless at 60°C, where the temperature was held for 3 min before it was raised by 40°C/min to 150°C followed by 1.5°C/min to 230°C. Helium gas was used as a carrier gas in constant flow mode with an estimated average velocity at the injection of 30 cm/s. Injector and detector temperatures were maintained at 250°C and 300°C, respectively. The fatty acid profile was recorded using Mass Hunter workstation software version B.04.07 (Agilent Technologies USA).

Estimation of biodiesel fuel properties based on fame profiles

Fatty acid methyl ester (FAME) profiles are commonly used indicator of algal biodiesel content and may vary according to chain size of carbon and the position and/or amount of double bonds[46,47]. The main parameters of biodiesel quality like Iodine Value (IV), Cetane Number (CN), the oxidation stability,

and the Cold Filter Plugging Point (CFPP) etc. were influenced by intrinsic molecular structures[47-50].

The CN, SV, IV, DU, LCSF and CFPP can be calculated with the following equations[51].

$$CN = 46.3 + \frac{5458}{SV} - (0.225 \times IV) \quad \ldots(1)$$

$$SV = \sum \frac{(560 \times N)}{M} \quad \ldots(2)$$

$$IV = \sum \left(\frac{254 \times D \times N}{M} \right) \quad \ldots(3)$$

Here D is that the number of double bonds, N is that the percentage of each fatty acid component and M is that the Molecular mass.

$$DU = MUFA + (2 \times PUFA) \quad \ldots(4)$$

Where MUFA is that the weight percentage of Monounsaturated fatty acids and PUFA is that the weight percentage of Polyunsaturated fatty acids.

The Long Chain Saturation Factor (LCSF) was measured by equation no. 5. LCSF was correlated with Cold Filter Plugging Point (CFPP) by using equation no. 6[52].

$$LCSF = (0.1 \times C16) + (0.5 \times C18) + (1 \times C20) + (1.5 \times C22)(2 \times C24) \quad \ldots(5)$$

$$CFPP = (3.1417 \times LCSF) - 16.477 \quad \ldots(6)$$

Where C16, C18, C20, C22, C24 are the weight percentage of each fatty acids (wt%).

FAs/FAMEs that impart a favorable Cetane Number on a fuel (low degree of unsaturation and long-chain) impart poor cold-flow properties and viscosities[53,54]. It is, therefore, necessary to achieve a balance between cold-flow properties and Cetane Number. Fatty Acid Methyl Esters that attain this balance are the monounsaturated methyl hexadecenoate (C16:1) and methyl octadecenoate (C18:1)[55,56]. Fuels rich in these FAMEs will have adequate viscosities, CNs, and cold-flow parameters. The ideal biodiesel feedstock would be composed entirely of C16:1 and C18:1 MUFAs[55], so in practice, a biodiesel feedstock should have higher concentrations of C16:1 and C18:1 fatty acids. However, feedstocks will have mixed fatty acid compositions and will contain non-ideal fatty acids. It is, therefore, necessary to quantify the maximum allowable levels of other fatty acids (not MUFAs) in biodiesel and to do this model that predicts the fuel properties like CN, viscosity, and CFPP from fatty acid composition should be used.

Selection of suitable algal species for biodiesel

The selection of best algal species done by using Preference Ranking Organisation Method for Enrichment Evaluation (PROMETHEE) and Graphical Analysis for Interactive Assistance (GAIA) analysis[57,58] with the selected criteria oil yield, IV, CN, SV, CFPP. Visual PROMETHEE Software 1.4.0.0 Edition was employed for comparative analysis of algal and blue green algal species. A graph was generated using PROMETHEE-GAIA software with all important multiple criteria by giving equal weightage to all criteria. This graph showed a comparative analysis and showed the phi value of all 7 species. Based on phi value the most suitable species for biodiesel production could be identified.

Results

Growth characteristics of *A. constricta*

Culture of *Anabaena constricta* could be grown in BG-11 media in controlled conditions, as described earlier. In liquid BG-11 medium, the growth pattern of algae was recorded based on OD values at 540 nm and subsequently on a dry weight basis. OD value of the cultures increased exponentially third day onwards, until eighteen days, followed by a plateauing effect, nineteenth day onwards (Fig. 1a and 1b). When average OD values of the culture were plotted against time (days), a near sigmoidal curve

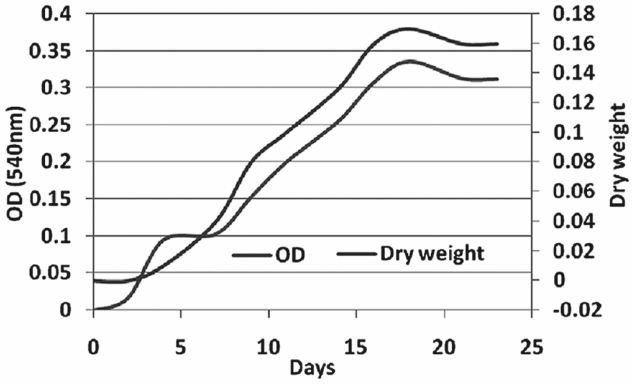

Fig. 1 — Growth of *A. Constricta* vis change in dry weight (g/ 10mL) & Optical Density (540nm)

was obtained. An identical pattern was observed when dry weight was measured against time (days) The algal culture exhibited following growth curves - lag or induction phase (up to 2 days), log or exponential phase (3-18 days) and stationary phase (19 days onwards) (Fig. 1a & 1b).

In BG-11 agar plates, *Anabaena* colonies appeared as a microscopic, mucilaginous, amorphous, green colored mass with moistly spreading type growth (Fig. 2). The growth of the culture was found to be augmented in the presence of light, making it photoautotrophic in nature. Further, in the presence of glucose as a carbon source in BG-11 media, chlorophyll disappeared (Fig. 2), indicating it can adopt a heterotrophic mode in the presence of carbon source (glucose). The *Anabaena* can grow in both autotrophic and heterotrophic mode, based on environmental conditions like the presence of carbon source.

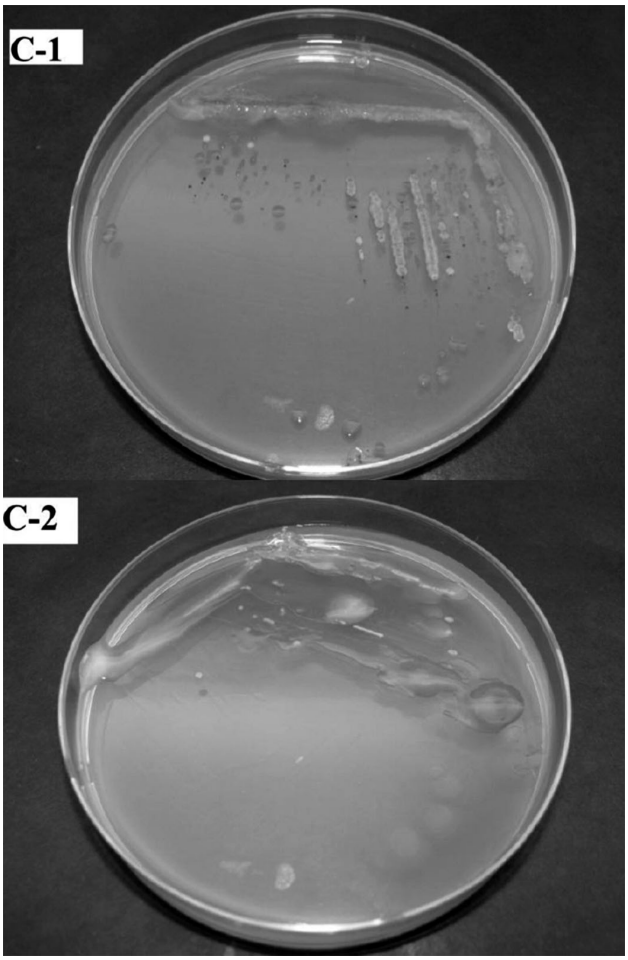

Fig. 2 — Colony characteristics of *Anabaena Constricta* in various media concentration. C-1: BG11; C-2: BG11 + 10 g/L glucose

Lipid content and FAME Analysis

Amongst the seven algal and blue green algal species of Indo-Gangetic plain of India, *Spirogyra* exhibited highest percentage of oil (14.82%) as compared to other algal and blue green algal strains, while *Anabaena constricta* species have 14.0% of oil. The *Pithophora* contains a minimum oil percentage (10.37%) in comparison to other strains.

Based on the percentage of oil, the most effective and potential strain for biodiesel production, could not be decided. The fuel properties of biodiesel produced from algae and blue green algae were dependent on fatty acid profiling as per biodiesel standards or guidelines[45,59,60].

Seven algal and blue green algal cultures were compared in terms of lipid content. Based on the Soxhlet method, *Anabaena constricta* culture was estimated to contain approximately 14% lipid on a dry weight basis. The lipid content obtained by the Soxhlet method was converted into esters of fatty acids (biodiesel) by standard transesterification reaction, and the resultant product was analyzed by GC-MS. Fatty acid methyl ester (FAME) analysis led to the identification of constituent of the trans-esterified product or biofuel, principally containing, palmitic acid (16:0), oleic acids (18:1), linoleic acid (18:2), palmitoleic acid (16:1), in the majority (Table 2).

DNA extraction, PCR amplification, and sequencing

Total genomic DNA of *Anabaena constricta* was isolated using the CTAB method and quantified as 100 ng/μL by spectrophotometer. PCR amplification employing primers specific to 16S and ITS region amplified product of ~1500 bp and ~750 bp respectively (Fig. 3). The amplified products were outsourced for Sanger sequencing and the retrieved assembled sequence based on high-quality chromatogram were obtained.

Phylogenetic analysis

To understand the evolutionary relationship of *Anabaena constricta* with other algal and blue green algal species, twenty 16S rRNA regions were retrieved from the NCBI database. Phylogenetic analysis indicated that *Anabaena constricta* grouped separately into a unique cluster comprising of *Pyrobaculum aerophilum* (L07510.1) (Fig. 4). The high bootstrap value (100) indicated the robustness of the tree constructed and also implied the confidence of a new species (Fig. 4). The estimate

Table 2 — Fatty acid profiles (saturated and unsaturated fatty acids) of algal and blue green algal Strains

Fatty acids	*Anabaena constricta*	*Tolypothrix*	*Pithophora*	*Spirogyra*	*Hydrodictyon*	*Rhizoclonium*	*Cladophora*
Saturated Fatty acids							
C 12:0 (Lauric acid)	-	0.7	1.3	1.1	0.9	1.4	1.2
C 13:0	-	0.2	0.4	0.3	0.1	0.3	0.7
C 14:0 (Myristic acid)	-	5.8	5.5	6.4	7.0	6.2	9.7
C 15:0 (Pentadecyclic acid)	-	-	0.8	0.6	0.4	0.6	-
C 16:0 (Palmitic icid)	42.6	31.8	29.7	25.2	26.9	30.4	27.5
C 17:0 (Margaric Acid)	1.4	2.8	0.4	0.3	0.4	1.7	0.8
C 18:0 (Stearic acid)	4.6	2.7	3.9	4.5	2.6	6.1	4.3
C 20:0 (Arachidic Acid)	1.2	1.4	1.2	1.3	1.1	1.9	1.6
C 21:0 (Heneicosylic Acid)	-	0.6	0.1	-	0.2	-	0.8
C 22:0 (Behenic Acid)	-	0.9	1.6	1.4	0.9	1.2	1.3
C 24:0 (Lignoseric Acid)	1.7	-	-	-	-	-	-
Total SFA	51.5	46.9	44.9	41.1	40.5	49.8	47.9
Mono unsaturated fatty acids (MUFA)							
C 14:1	-	1.9	0.9	1.1	0.3	2.1	1.2
C 16:1 (Palmitoleic acid)	3.3	4.7	6.2	5.4	3.8	4.8	5.4
C 17:1							
C 18:1 (Oleic Acid)	20.6	23.4	28.4	33.3	34.8	18.9	22.9
C 20 : 1 (Eicosenoic acid)	1.1	0.6	0.9	0.7	1.2	0.4	0.8
C 22 : 1 (Docosenoic Acid)	-	0.1	-	0.2	0.1	-	-
C 24:1	-	0.3	0.2	0.6	0.5	-	0.1
Total MUFAs	25.0	31.0	36.6	41.3	40.7	26.2	30.4
Poly unsaturated Fatty acids (PUFA)							
C 16:2 (Hexadecadieneic acid)	-	2.4	3.6	3.8	2.8	1.6	2.3
C 16:3 (Hexaidecatrienoic acid)	-	-	-	-	-	-	-
C 16:4 (Hexadecatetraenoic acid)	-	-	-	-	-	-	-
C 18:2 (Linoleic icid)	19.3	8.6	11.2	10.8	9.3	9.7	8.6
C 18:3 (Linolenic acid)	0.5	8.4	0.4	0.7	2.5	9.6	6.1
C 18:4 (Octadec itctracnoic acid)	-	-	-	-	-	-	-
C 20: 2 (Eicosadienoic acid)	3.7	0.4	0.1	-	0.2	-	0.3
C 20: 3 (Eicosatrienoic acid)	-	0.6	1.2	0.6	2.1	0.6	0.6
C 20:4	-	0.1	-	0.1	-	-	-
C 20:5 (Eicosapentaenoic acid)	-	-	-	-	-	-	0.2
C 22:2 (Docosadienoic acid)	-	-	-	-	-	-	-
C 22:3	-	0.2	0.3	0.2	0.4	0.6	0.4
C 22:3	-	1.4	1.7	1.4	1.5	1.9	3.2
Total PUFA	23.5	22.1	18.5	17.6	18.8	24	21.7
Total unsaturated Fattyacids	48.5	53.1	55.1	58.9	59.5	50.2	52.1

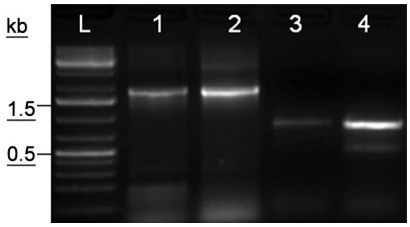

Fig. 3 — PCR amplification for 16S rRNA and ITS region of *Anabaena Constricta*. Lane L- 1 Kb DNA Ladder, Lane 1 & 2: PCR amplification product using 16SF & R primers, Lane 3 & 4: PCR amplification product using ITSF & R primers

of evolutionary divergence between the analyzed sequences also support our claim.

Similarly, in the case of ITS tree, *Anabaena constricta* clustered with uncultured *Pseudomonas* sp. (JF905929.1) with high bootstrap values (Fig. 5) among 21 ITS sequences employed in the study.

Estimation of Biodiesel Fuel Properties Based on FAME Profiles

Based on FAME profiles of algal and blue green algal species, the total saturated fatty acids,

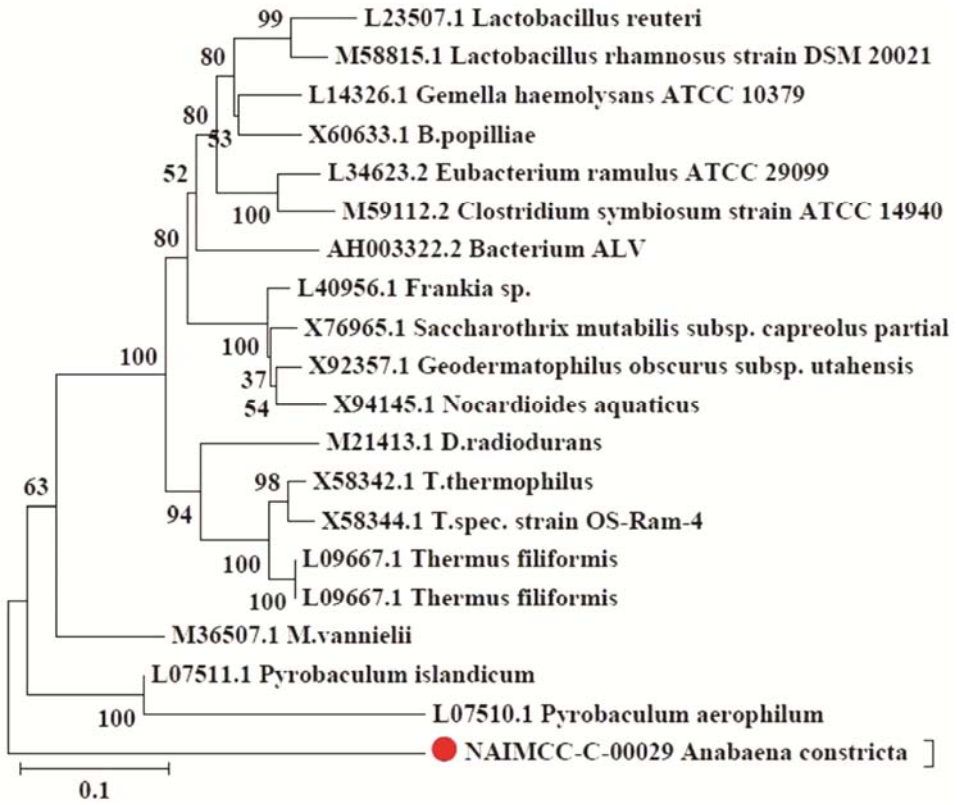

Fig. 4 — 16S rRNA Phylogenetic tree constructed using MEGA 5.0 software

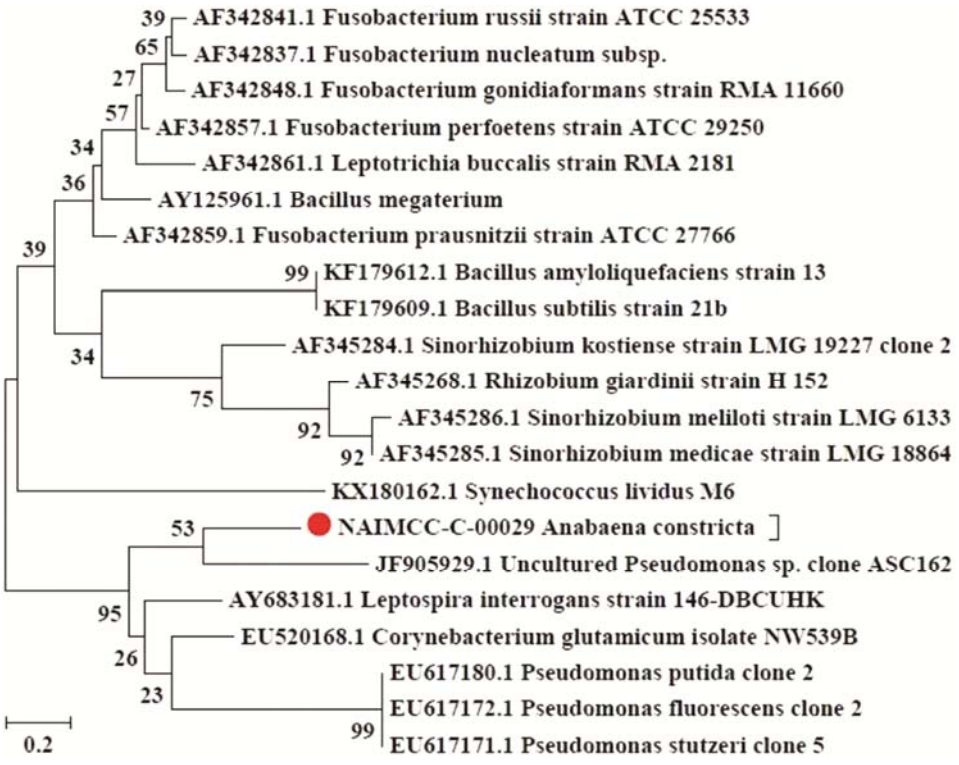

Fig. 5 — ITS Phylogenetic tree constructed using MEGA 5.0 software

monounsaturated fatty acids, and polyunsaturated fatty acids of algal and blue green algal species were estimated (Table 3). Based on the equations no. 1 to 6, IV, CN, SV, LCSF, DU, and CFPP values were documented (Table 4).

Selection of Suitable Algal Species for Biodiesel

To be a perfect source of sustainable biodiesel, the selected algal and blue green algal strains should contain a sufficient amount of lipid with suitable fatty acids for high-quality biodiesel properties. A multi-criteria decision method (MCDM) software PORMETHEE-GAIA was used to make objective selections for large-scale production of biodiesel.

The selection of suitable strains was done based on oil percentage and above-calculated biodiesel properties (IV, CN, SV, LCSF, DU, and CFPP) with the help of PROMETHEE-GAIA software.

GAIA plot of all the selected strains based on different biodiesel properties and the decision vector was derived (Fig. 6). Values of IV, CN, SV, LCSF, DU, CFPP as well as total lipid contents were given equal weightage. The decision vector that is long and not orthogonal (at the right angle) to the GAIA plane is preferred for decision making[61].The decision vector indicates the most suitable strain, *i.e.*, those that align with the direction of this vector, and the outermost criteria in the direction of the decision vector are the most preferable[62].

In contrast, C18:3 Db ≥ 4, and SFAs, were highly variable criteria (Table 2) and that they had a strong effect on the decision vector. Based on calculated outranking flows, the most suitable species ~~are~~ *Rhizoclonium* and *Tolypothrix* with the highest phi value could be derived (Fig. 6, Table 5). *Anabaena constricta* and *Hydrodictyon* with low phi value and are the least suitable species for biodiesel production in compared to above-mentioned species.

Discussion

The algal species isolated from other countries/continents might not perform better in Asia because of the remarkable variations within the regional atmospheric conditions. Therefore, indigenous species must be isolated to serve the aim and locally isolated species may have adapted to the actual weather conditions over their evolutionary period[63]. The present study pertains to comparative studies of a set of indigenous algal/blue-green algal strains for identification of potential strain for biodiesel production. We also characterized a native blue-green algae, *Anabaena constricta* originally collected from Varanasi region (Geographical coordinate 25.20° N 82.96° E) of Uttar Pradesh, India.

Earlier studies reported the growth of algal species in BG-11 media[32,64]. BG-11 media with different concentrations of $NaNO_3$ at 1:6 (V/V) dilution were

Table 3 — Total lipids, and percentage of saturated, monounsaturated and polyunsaturated FA in algal strains

S. No.	Species Name	Total lipid (%)	Saturated fatty acids	Monounsaturated fatty acids (MUFA)	Polyunsaturated fatty acids (PUFA)
1.	*Anabaena constricta*	14	41.8	20.3	19.1
2.	*Tolypothrix*	12.78	46.9	31.0	22.1
3.	*Pithophora*	10.37	44.9	36.6	18.5
4.	*Spirogyra*	14.82	41.1	41.3	17.6
5.	*Hydrodictyon*	13.58	40.5	40.7	18.8
6.	*Rhizoclonium*	11.64	49.8	26.2	24
7.	*Cladophora*	11.76	47.9	30.4	21.7

Table 4 — Fuel properties of Biodiesel from algal and blue green algal oil

Species Name	Cetane number (CN)	Saponification value (SV)	Iodine value (IV)	Degree of unsaturation (wt %) (DU)	Long chain saturation factor (wt %) (LCSF)	Cold filter plugging point (°C) (CFPP)
Anabaena constricta	58.2	205.6	65.13	72	11.16	18.58
Tolypothrix	54.77	209.48	78.13	75.2	9.11	12.14
Pithophora	56.64	209.32	69.92	73.6	8.52	10.29
Spirogyra	55.99	210.21	72.29	76.5	8.17	9.19
Hydrodictyon	55.42	208.17	76.02	78.3	6.44	3.76
Rhizoclonium	54.58	210.01	78.71	74.2	9.79	14.28
Cladophora	55.04	211.39	75.93	73.7	8.45	10.07

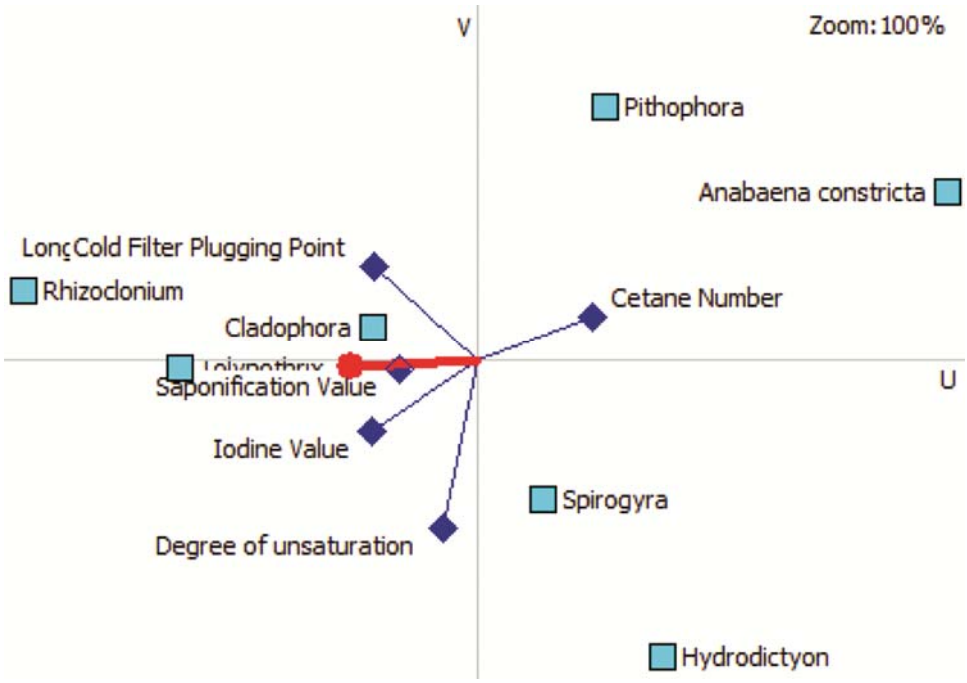

Fig. 6 — GAIA plot of one blue green algal species from the current study and six algal and blue green algal strains from different literature as shown in Table no. 1, based on different biodiesel properties from table no. 4 and the decision vector

Table 5 — Corresponding outranking flow

Rank	Species	Phi
1	*Rhizoclonium*	0.3889
2	*Tolypothrix*	0.2778
3	*Spirogyra*	0.1111
4	*Cladophora*	0.0556
5	*Pithophora*	-0.0556
6	*Hydrodictyon*	-0.2222
7	*Anabaena constricta*	-0.5556

used for growing microalgae *M. afer* HSO-3-1. The culture was maintained at 25 ± 1 °C with the infusion of air bubbles comprising air and 2% (V/V) Carbon dioxide. Continuous artificial illumination at 270± 20 µmol PAR photons/m^2/s was provided by fluorescent tubes[32]. Similarly, symbiotic algae were grown in BG-11 liquid media cultured in a light incubator at 20°C under 12:12 light-dark regime with a light intensity of 25 µmol/m^2/s[64]. In the present study, *Anabaena constricta* may be grown aseptically in BG-11 media in laboratory conditions with hermetically designed photo-bioreactor with controlled temperature and filtered atmospheric air supply.

Earlier sigmoidal curve of OD and DW vs time period are obtained[64] in *Chlorococcum* sp. cultured in BG-11 media. On day 22 of cultivation, the *Chlorococcum* sp. grew into a stable phase. *Chlorella sp.* was cultivated in different growth media at varying temperatures (25°C to 28°C) under 24 hour light illumination of (1900 Lux and 2700 Lux) for 20 days under neutral pH, the growth pattern of *Chlorella Sp.* was analyzed to be optimum on the seventeenth day of incubation[65]. Similar sigmoidal growth pattern of *Anabaena constricta* was obtained by measuring the optical density (OD) and dry weight (DW) vs time periods (days) in present study.

If glucose was provided to *Anabaena constricta* in BG-11 media, the *Anabaena* colony appears white. Glucose acts as a carbon source for *Anabaena*, so there's no need for the synthesis of carbon source by photosynthesis, possibly leading to disappearance chlorophyll in *Anabaena*. It was presumed that *Anabaena* can grow in both autotrophic and heterotrophic mode, based on culture conditions and availability of carbon source.

Anabaena constricta culture was estimated to contain approximately 14% lipid on a dry weight basis. However, the growth assumes the log phase in just over 2 and half weeks (18 days) of culture in controlled conditions. The present study also showed that among 7 algal and blue green algal species the lipid % on dry weight varies between 10.37 to 14.82 %, in these strains more than 14 % lipid on a dry weight basis found in *Spirogyra* 14.82 %[29].

In the present study, *Anabaena Constricta* was produced principally containing, C 16:0 (up to

42.6%). *Anabaena* is also rich in oleic acids (18:1), linoleic acid (18:2), palmitoleic acid (16:1) are suitable for biodiesel production. It's widely accepted that, *Anabaena* rich in MUFA (particularly, palmitoleic acid (16:1) and oleic acid (18:1) and SFAs are suitable for biodiesel production[66].

Phylogenetic study and homology relationships confirmed the uniqueness and novelty of the chosen species. The finding of novel strain is due to the fact that the species cluster separately from other species, and this is the first attempt to identify and characterize species from this region using molecular methods, and defined growth rates. Moreover, total biomass characterization unveiled potential constituents of the total biomass production. Similar phylogenetic analysis was done previously in *Ulva*[67], the sequences of *Ulva* were aligned using Clustal X, and further manually adjusted using BioEdit. The sequences were aligned to look at resemblance and to investigate the differences using the software DNAMAN with default parameters. With the multi-alignment analysis, we applied the program MEGA 3.1 with Kimura's two-parameter model. Identified *Ulva* by ITS and 18S sequence and also determined phylogenetic relationships among taxa; similarly, Chlorophyceae identified, based on 18S r DNA[68]. They determined 18S rDNA sequences were analyzed by a similarity search on the NCBI GenBank database using BLAST to compare with the related sequences. The Clustal X (1.8), windows interface for the Clustal W, was used to obtain multiple alignments of nucleotides for 18S rRNA genes. Phylogenetic and molecular evolutionary analyses were constructed using MEGA 5.0.

In the search of a renewable source of energy, algae, which are present in nature, may be used as a fuel source. Algae can even help tackle the matter of environmental pollution, by consuming atmospheric carbon dioxide. The growth rate of algae is drastically high compared to other crops. The cultivation of algae is comparatively simple, and also the yield of oil from algae is higher than other crops[69,70]. He analyzed that microalgae (70% oil (by wt) in biomass) could produce 136900 L/ha, for that only 2 M ha area needed for cultivation, while microalgae (30% oil (by wt) in biomass) could produce 58,700 L/ ha for this 4.5 M ha land needed, but in case of crop oil yield is very much low, and for the cultivation of those crops a vast land area is required.

In this study, ITS and 16S primers (universal green algal primers) are chosen because, these are highly conserved flanking sequences, easy to detect even from small quantities of DNA due to their high copy number[32,71,72]. Phylogenetic analysis indicates that *A. Constricta* belongs to the distinct class and different from other species reported earlier, making it unique.

The selection of species also depends on parameters like higher growth rates and high lipid contents for cost-effective biofuel production. Earlier reports nine microalgal species were cultivated in different growth media and compare total lipid, total fatty acid content, FAME analysis, fuel properties of nine microalgal species from their study, and twelve microalgal species from references[59]. They analyzed by Graphical Analysis for Interactive Assistance (GAIA). A large number of oleaginous species have already been substituted a fraction of petroleum-based fuels in some countries[73]. In the present study, we also compare the FAME of *Anabaena constricta* and six other algal and blue green algal strains found in Indo Gangetic plain of India, with different literature. FAME analysis shows that highest palmitic acid (C 16:0) was found in *Anabaena constricta* and highest total saturated fatty acid was also found in *Anabaena constricta*, highest Oleic acid (C 18:1) was found in *Hydrodictyon* and highest total monounsaturated fatty acid were found in *Spirogyra*, highest linoleic acid (C 18:2) was found in *Anabaena constricta*, and highest linolenic acid (C 18:3) was found in *Rhizoclonium*, and total polyunsaturated fatty acids were found in *Rhizoclonium* and total highest total unsaturated fatty acids were also found in *Hydrodictyon.* A statement was given that, algae rich in MUFAs (particularly, palmitoleic acid (16:1), oleic acid (18:1), and SFAs are suitable for biodiesel production[66].

Several kinds of lipids, like phospholipids, glycolipids, mono-, di-, and triglycerides, among others are produced by algae and blue green algae and also their ratio rely upon each species and the growing environmental conditions applied[74]. The present study also showed that the estimated CN for algal and blue green algal biodiesel varied between 54.77 to 58.2. The Indian Standards shows that the CN value is equal to or more than 51. Within the present study, all strains CN have more than 51. The Saponification Value estimated for biodiesels from this research varied between 205.6 to 211.39. *Tolypothrix, Pithophora, Spirogyra, Hydrodictyon, Rhizoclonium, Cladophora* SV are within the same range observed

for vegetable oils (196–202 for palm oil, 189–195 for soybean oil, and 188–194 for sunflower oil) [75]. This properties, however, is extremely variable because it's also directly associated with the technology used for biodiesel production. The Iodine Value (IV) is a parameter represents the Degree of Unsaturation (DU), involving the weighted sum of the masses of MUFA and PUFA. It's significant for biodiesel oxidative stability. High unsaturation levels may result in polymerization of glycerides and formation of deposits[51]. As compared to biodiesel from vegetable oils[75] all of the estimated IV for the biodiesels from the algal and blue green algal strains (Table 4) were lower than for soybean oil (120–141) and sunflower oil (110–143). Saturated fatty acids have higher melting points compared to unsaturated fatty acids compounds. When most saturated molecules of fatty acid esters are present in biodiesel, the crystallization process may occur at temperatures within the conventional engine operation range[51]. Biodiesel rich in palmitic and stearic acid methyl esters tend to present a poor Cold Filter Plugging Point (equivalent to a higher temperature of plugging point) because when liquid biodiesel is cooled, this FAME is the first to precipitate[74]. Within the present research, the levels of Stearic acid C 18:0 (Table 2) were generally very low (below 4%), except for *Spirogyra, Rhizoclonium,* and *Cladophora* (4.5, 6.3 and 4.3 %, respectively). These low values of stearic acid may have contributed to the lower temperatures of CFPP for the majority of the studied species. The CFPP values estimated for biodiesel from the strains focused within the present work ranged from 3.76 (*Hydrodictyon*) to 18.58 (*Anabaena constricta*). An equal parameter weighted PROMETHEE analyses established that the *Rhizoclonium* and *Tolypothrix* outranked, while *Hydrodictyon* and *Anabaena constricta* are the least suitable species in above mentioned seven species found in Indo Gangetic plain of India for biodiesel production.

Conclusion and future prospects

In the present study the molecular studies characterization of *Anabaena constricta* was done and we also compared 6 algal and blue green algal strains in respect to their fatty acid profiles and estimate biodiesel properties (CN, SV, IV, DU, LCSF and CFPP). An equal parameter weighted PROMETHEE analyses established that the *Rhizoclonium* and *Tolypothrix* outranked other strains used in the study.

Among various energy sources, biofuels alone provide liquid transportation fuels. Although, oil productivity of algae compared to other conventional energy crops is 300 folds, yet there are technical hindrances for its commercial applications. Therefore, it's necessary to isolate new species producing higher lipid contents with high growth rates. This study comprises identification, characterization, and lipid analysis for new indigenous species. As the physio-chemical properties of biodiesel are estimated by the molecular structures of the constituents fatty acid methyl esters, this research work suggests that the adequate mixture of fatty acids of algae and blue green algal oil must be priority criteria for strain selection, to form viable the algal-based biodiesel industry. According to fatty acid profiles, seven algae and blue green algae have the potential to produce biodiesel within most of the biodiesel standards. However, none of the above investigated species would naturally produce a lipid capable of fulfilling all the necessities for biodiesel production of the highest quality grade. On the other hand, almost of them contained one or more of the main fuel properties describing such high quality, it is suggested that good quality biodiesel could also be achieved employing a mixture of the distinct oil extracts, obtained from different species.

Taking into consideration the dependence of biodiesel fuel properties on the structure of FAME and the scarce information on the qualitative composition of algal oil, this research provides a vital contribution for further bio-prospection associated with algae and blue green algae for biodiesel production.

It is also suggested that the present work open the new challenges for future researchers to analyze the relationship between cell cycle and triacylglycerol production on molecular level, genes encoding enzymes for lipid productivity and their regulatory sequences for enhancing the lipid production.

Acknowledgement

Authors would like to thank the faculty members, and supporting staff of the Department of Oil Technology, HBTU for handling instruments used in the study. The authors are also thankful to Director, ICAR-IIPR for permitting me to work at Division of Plant Biotechnology, IIPR, Kanpur. Sincere thanks to Director, ICAR-NBAIM, Mau for providing algal strain.

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