



Lipid Production Capacity of a Newly Characterized Cyanobacterial Strain *Synechocystis* sp. MH01: A Comparative Performance Evaluation of Cyanobacterial Lipid-Based Biodiesel

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Background: Cyanobacteria have been the focus of extensive researches because of their high potential for the development of new generations of useful natural compounds with vast applications. For the entire last ten years, a lot of attention has been dedicated to the cyanobacterial lipids as a main source of valuable materials for clean energy production.

Objectives: As there is a direct relationship between biofuel properties and compositional characteristics of fatty acids, a selected lipid-producing cyanobacterial strain was examined and analyzed in terms of fatty acid composition. The biodiesel quality parameters were carefully examined as well.

Materials and Methods: A cyanobacterial strain was isolated from waterfalls in the northern part of Iran and identified as *Synechocystis* sp. MH01. The fatty acids profile of the selected strain, as tested in various culture conditions, was analyzed by gas chromatography (GC) and compared with control subjects to further validate the biodiesel quality parameters.

Results: The autotrophic cultivation of *Synechocystis* sp. MH01 resulted in biomass and lipid productivity of 109 mg.L⁻¹ day⁻¹ and 22.89 mg.L⁻¹ day⁻¹, respectively. The mixotrophic cultivation of MH01 strain in sucrose-containing medium led to an approximately 1.8 and 1.22 fold increase in biomass and lipid productivity compared with the autotrophic condition. The addition of glycine to BG11 medium caused up to ~1.3 and ~1.18 fold increase in biomass and lipid productivity compared with control subjects. The analysis of qualitative parameters of the biodiesel, as derived from the lipids, indicated that *Synechocystis* sp. MH01 has a high ability for lipid production under optimal culture conditions.

Conclusions: It seems feasible to evolve the *Synechocystis* sp. MH01 further particularly for more lipid production as a promising primary raw material for biofuel production through fine-tuning of medium composition.

Keywords: Biodiesel, Biodiesel parameters, Gas chromatography, Lipid, MH01, *Synechocystis* sp

1. Background

Due to the non-renewable nature of fossil fuel resources, on the one hand, and the increasing deal of greenhouse gas emissions caused by their widespread utilization, on the other hand, there is, therefore, an urgent need to replace them with renewable and more eco-friendly alternatives (1).

Numerous studies have emphasized the use of cyanobacterial lipids as a valuable primary raw

material for the production of clean fuels (2, 3). The cyanobacterial fatty acid composition is influenced by its growing conditions, such as medium components, temperature, light intensity, light/dark cycle times, and aeration (3-5). As the amount of biomass and lipid composition is affected by the components of the medium, the proportion of the chemical composition of the culture medium is, therefore, very important to achieve higher lipid production, subsequently

influencing the quality of biodiesel made from the lipids (6). The growth of cyanobacteria requires the presence of various elements including, nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), sulfur (S), iron (Fe), lead (Pb), manganese (Mn), and zinc (Zn) (7). In addition, lipid accumulation in cyanobacteria has been reported to vary in different salinity environments (8). Microalgae represent a practical feedstock as having huge potential for future renewable energy supply (9, 10). The main parameters related to the quality of biodiesel, including the iodine value, cetane number, oxidative stability, lubricity, viscosity, and flow properties, are dependent on the composition of fatty acids from which it can be made. Besides, two factors are crucial to determining the physicochemical properties of biodiesel as follows: the number of unsaturated bonds and their distributions in the fatty acid chain (11). However, the presence of a high level of polyunsaturated fatty acids in the lipid composition has deleterious effects on biodiesel quality, reducing its oxidative stability (12). The viscosity of liquid fuel has been considered as a key parameter, indicating its resistance to deformation under certain conditions (13). The iodine number is a factor that correlates with the biodiesel stability toward oxidation. The iodine value, represented as the amount of iodine (in grams), is being spent by 100 ml/100 grams of a fuel/substance. The cloud flow performances of the fuels can be characterized by cloud point (CP), the pour point (PP), the cold filter plugging point (CFPP), and viscosity. The cloud point (CP) is the temperature at which crystals first start forming in the fuel. The biodiesel cloud point is typically higher than the cloud point of conventional diesel. The cold filter plugging point (CFPP) is the lowest temperature at which 20 ml of fuel passes through a filter within 60 s by applying a vacuum of 2 kPa (14, 15).

2. Objectives

In pursuit of understanding the effect of various physicochemical factors on cyanobacterial growth and its capacity for lipid production, as well as the parameter values of produced biodiesel, several experiments were designed and carried out to achieve the goal as follows: 1) Isolation and identification of a native cyanobacterial strain with a high capacity for lipid production, 2) study of the effect of several physical and chemical factors on cyanobacterial growth, and its capacity for lipid production, 3) analysis of the fatty acid composition of the lipids produced by selected cyanobacterial strain using gas chromatography (GC) analytical technique, 4) evaluation of quality parameters of the biodiesel, as

can possibly be derived from cyanobacterial lipids.

3. Materials and Methods

3.1. Cyanobacteria Isolation, Growth Medium, And Identification

Cyanobacteria strains were isolated from a sample taken from a waterfall located in the Northern region of Iran. Briefly, 5 mL aliquots of the samples were transferred into 100 mL BG-11 medium composed of [NaNO₃ (1.5 g), NaCl (1 g), MgSO₄·7H₂O (0.075 g), CaCl₂·2H₂O (0.036 g), citric acid (6 mg), H₃BO₃ (2.86 g), MnCl₂·4H₂O (1.81 g), ZnSO₄·7H₂O (0.22 g), Na₂MOO₄·2H₂O (0.39 g), CuSO₄·5H₂O (0.079 g), Co(NO₃)₂·6H₂O (0.049 g), ferric ammonium citrate (6 mg), Vitamin B₁₂ solution (100 mL), Na₂CO₃ (0.02 g), K₂HPO₄ (0.04 g), and Na₂-EDTA (1 mg)]. The flasks containing culture medium were then aerated with filtered air and incubated at 27 °C under continuous illumination for 15 days. Several passages were performed from the culture medium to obtain an axenic culture of the selected cyanobacterial strain, and the morphological features of the isolate were examined by light microscopy ((at a magnification of ×40 & 100). For molecular identification, DNA was extracted from cyanobacterial isolate, and the amplification of the 16S rRNA gene performed by PCR technique with universal primers of 27F and 1492R, as the forward and reverse primers. (16). The purification of the PCR products was carried out through a QIAquick gel extraction kit and subjected to sequence analysis. The multiple sequence alignments were achieved through the available almost-complete sequence of type strains of genus *Synechocystis* as well as the corresponding sequences of the representative cyanobacteria species (17). Maximum-Likelihood and Neighbor-joining (NJ) methods were employed to create phylogenetic trees from the collected data using MEGA5 (18).

3.2. Screening of the Cyanobacterial Strains Based on Lipid Production Capacity

The total lipid content of the cyanobacterial isolates was considered as a criterion for strain selection. The cyanobacterial strains were cultivated in BG-11 medium with some modifications. All the experiments were conducted under the following conditions: temperature: 27 °C, initial OD: 0.1, initial pH: 7.1, light intensity: 500 lux, and aeration rates of 1.5 vvm. The cellular weight (mg.L⁻¹), biomass productivity (mg.d⁻¹), lipid content (%), lipid production (mg.L⁻¹) and lipid productivity (mg.L⁻¹.d⁻¹) were evaluated as well. Lastly, in line with the results obtained from lipid productivity

and growth rate, a cyanobacterial strain, with the best specifications, was nominated for further examination.

3.3. Growth Pattern and Lipid Production by Cyanobacterial Isolate

Cyanobacterial cells were collected at the stationary growth phase by centrifugation at $6000\times g$ for 10 min at 4 °C. Then, the separated cells were washed with distilled water for three times, and the fresh weights of the cell pellets were quantified. Dry weights were recorded after drying the pellets at 60 °C until constant weight was achieved. All the experiments were carried out in triplicate. The total lipid content was evaluated gravimetrically. Cyanobacterial cells were collected by centrifugation ($4500\times g$, 4 °C, 10 min), after that frozen at -80 °C, and lyophilized. Total lipid content was extracted from lyophilized cyanobacterial cells based on the Bligh–Dyer approach (19), with a slight modification. For this purpose, 100 mg of the dried cell mass was mixed with 5 mL chloroform/methanol (2:1) and sonicated for 30 s. The mixture was warmed to 65 °C for 1 h, methanol, and NaCl solution (1 % w/v) is then added to a final volume percentage of 1: 1: 0.9 (chloroform/methanol/NaCl solution).

Cellular weight ($\text{mg}\cdot\text{L}^{-1}$), biomass productivity ($\text{mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$), lipid content (%), lipid production ($\text{mg}\cdot\text{L}^{-1}$), lipid productivity ($\text{mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$) were measured for each experiment.

3.4. The Effects of Different Carbohydrates and Amino Acids on Growth and Accumulation of Lipids by Selected Cyanobacterial Strain

The mixotrophic cultivation of the cyanobacterial strain was performed in BG11 medium comprising of various carbon sources, including glucose, galactose, fructose, lactose, sucrose, maltose, mannitol, starch, dextran, arabinose, and xylose, to evaluate the growth and lipids production, as compared with autotrophic culture condition. The effect of addition of various amino acids to BG11 medium was considered to examine the changes in cyanobacterial cell growth and lipid production.

3.5. Effect of Physicochemical and Nutritional Parameters on Biomass and Lipid Production

3.5.1. Sodium Thiosulfate Pentahydrate

As sodium thiosulfate pentahydrate is an agent capable of rapidly neutralizing reactive oxygen intermediates, it might be associated with the scavenging of free radicals caused by photosynthesis (20). Therefore, different concentrations of sodium thiosulfate pentahydrate (1, 2.5, and 5 mM) was added to the BG-11 medium,

evaluating its effect on growth and lipid production by selected cyanobacterial strain.

3.5.2. Initial pH

The pH of the culture medium has been considered an important factor influencing the cyanobacterial growth and its capacity for lipid production (21). In this regard, the effect of the initial pH of culture medium ranging from 7 to 9.5 on the growth, and lipid production by the selected cyanobacterial strain was studied.

3.5.3. NaNO_3 Concentration

The effect of NaNO_3 , as an important inorganic nitrogen source, on the lipid production capacity of cyanobacterial strain was examined (22). To this end, cyanobacteria cells were grown in BG-11 medium containing different concentrations of NaNO_3 , 0.5 (starvation), 1.5, 3, 5, and 10 $\text{g}\cdot\text{L}^{-1}$. BG-11 culture medium without NaNO_3 was considered as control.

3.5.4. NaCl

As the amount of salt and osmotic pressure in the external environment increases, cyanobacteria can accumulate small osmoregulatory substances, such as glycerol, mannitol, glucosylglycerol, etc. It has been revealed that an increase in salinity as abiotic stress may result in a slight increase in the total lipid content of cyanobacteria (23). Thus, to assess the effect of salinity on growth and lipid productivity of the selected cyanobacterial strain, it was cultivated in the BG-11 medium with various sodium chloride concentrations (0.1%, 0.5%, 1% (as control), 1.5%, 2%, and 3%).

3.5.5. Temperature & Light/ Dark Cycle Time

The selected cyanobacterial strain was grown in BG-11 medium under a varying temperature of 17, 20, 25, 27 (control), 29, 30, and 35 °C. Various light/dark cycle times were designed to evaluate the effect of light cycle length on growth and lipid production by the selected cyanobacterial isolate, as follows: 0-24/ 12-12/ 14-10/16-8/ 24-0 (23).

3.5.6. Effect of Vitamins

The effect of various vitamins, especially vitamin B12 on cell metabolism is of great importance. In this study, several vitamins including A, D, E, K, C, B1, B2, B3, B6, B7, B8, B9, and B12) (Sigma, USA) at a concentration of 0.1 mg (designated as 1 x concentrations) were added to BG11 medium to study their effect on biomass and lipid production. Vitamin solutions were filter-sterilized before they were added to the autoclaved media. Effect of supplementing BG11 medium with different

vitamin on biomass production and lipid productivity of cyanobacterial cells was examined.

3.6. Compositional Analysis of Fatty Acids by GC Analytical Technique

The fatty acid methyl esters (FAMES) were prepared through transesterification of the extracted lipids with potassium hydroxide solution, (2 N), in methanol. Further extraction of the FAMES was performed by n-hexane for being prepared for GC analysis (Varian CP-3800), as equipped with a CP-Sil 18 Varian column (60 m×0.25 mm) and flame ionization detector. The column fixed temperature was 175 °C for isothermal analysis. The injector and detector temperatures were 280 and 300 °C, respectively. The standard fatty acid samples, including C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C20:0, C20:1 (Merck & Co., Inc.), were firstly injected to attain the exact retention time for each compound. All samples were injected and analyzed three times, and means were reported as well (24, 25).

3.7. Biodiesel Parameters

The biodiesel quality properties including, Saturated Fatty Acid (%), Mono Unsaturated Fatty Acid (%), Poly-

unsaturated Fatty Acid (%), Degree of Unsaturation, Saponification Value ($\text{mg}\cdot\text{g}^{-1}$), Cetane number, Long Chain Saturated Factor, Cold Filter Plugging Point (°C), Cloud Point (°C), Pour Point (°C), Allylic Position Equivalent, Bis-Allylic Position Equivalent, Oxidation Stability (h), Higher Heating Value, Kinematic Viscosity ($\text{mm}^2\cdot\text{s}^{-1}$), Density ($\text{g}\cdot\text{cm}^3\cdot\text{s}^{-1}$) were evaluated based on the fatty acid composition of cyanobacterial lipids by the BiodieselAnalyzer software ver. 2.2 (26).

4. Results

4.1. Characterization of the Cyanobacterial Strain

Among isolates, a cyanobacterial isolate with the highest lipid productivity value of $22.89 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$, was selected for further analysis. The morphological examination of the cyanobacterial isolate demonstrated that it has a sphere-shaped form with an average size of 12 μm . The results obtained from 16S rRNA gene sequencing revealed that the selected cyanobacterial isolate has a $\geq 99\%$ similarity with the reference sequences of the members of the *Synechocystis* genus (**Fig. 1, 2**). The 16S rRNA gene sequence of cyanobacterial isolate, tentatively labeled as *Synechocystis* sp. MH01 was submitted to NCBI through the accession number of MG742357.

4.2. Effect of Nutritional and Physicochemical Factors on Lipid Production

As shown in **Table 1**, while *Synechocystis* sp. showed improved lipid production yield when grown in the presence of a particular sugar, some sugars added to the culture medium led to reduced growth and lipid productivity. The addition of sucrose (1%) mannitol (1%) to BG11 medium caused the highest and lowest lipid productivity, respectively, as compared to the control medium (without carbohydrate).

The results confirmed that, while glutamine had the lowest effect, glycine showed the highest effect on lipid productivity among different amino acids tested. (**Table 2**).

The addition of the low concentrations of sodium thiosulfate pentahydrate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) (1 mM) to the culture medium has led to the highest lipid productivity, and the lowest value was recorded at $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ concentration of 5 mM. The results indicated that the cyanobacterial growth and lipid content were slightly affected by the pH of the medium. More specifically, as the initial pH increased, biomass productivity and lipid productivity is decreased. The highest biomass productivity and lipid productivity was achieved at pH 7 (BP: $109 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$, LP: $22.89 \text{ mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$). However,

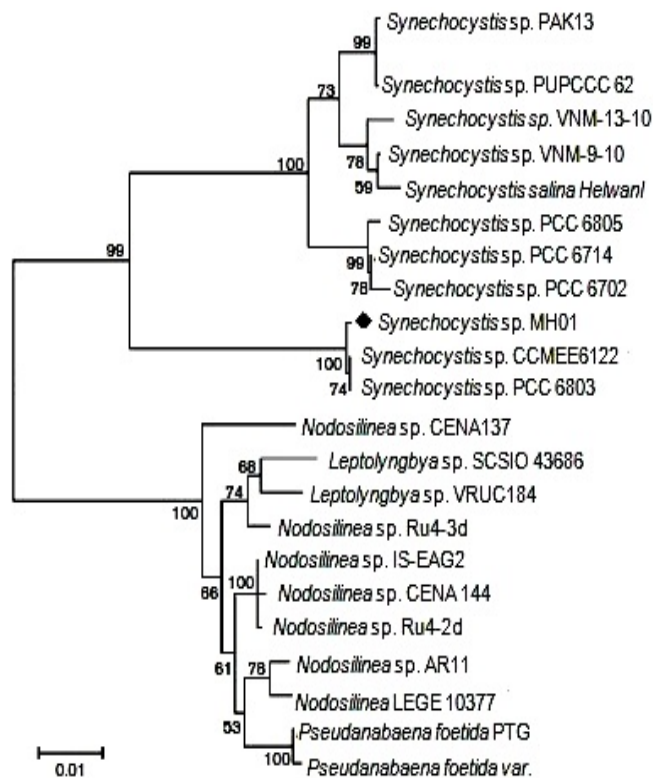


Figure 1. Phylogenetic analysis of *Synechocystis* sp. MH01 based on nucleotide sequences of 16S rRNA genes.

Table 1. Biomass and lipid productivity of *Synechocystis* sp. MH01 grown in BG11 medium supplemented with different carbohydrates.

Carbohydrate (%)	Final OD	Cellular Weight (mg.L ⁻¹)	Biomass productivity (mg.L ⁻¹ day ⁻¹)	Lipid content (%)	Lipid production (mgL ⁻¹)	Lipid productivity (mg.L ⁻¹ day ⁻¹)
Control	1.68±0.3	763±0.4	109±0.7	21±0.4	160±0.3	22.89±0.2
Glucose	1.71±0.6	433±0.3	61±0.5	42±0.2	181±0.4	25.98±0.5
Galactose	1.73±0.6	786±0.5	112±0.2	20±0.9	157±0.6	22.45±0.2
Fructose	1.83±0.9	898±0.3	128±0.2	30±0.1	269±0.4	38.48±0.6
Lactose	0.5±0.6	322±0.5	46±0.5	22±0.3	70±0.1	10.12±0.1
Sucrose	2.11±0.7	1361±0.8	194±0.4	37±0.6	503±0.8	71.93±0.3
Maltose	1.54±0.8	587±0.3	55±0.1	18±0.7	69±0.2	9.95±0.2
Manitol	1.65±0.3	208±0.2	29±0.7	22±0.3	45±0.2	6.53±0.5
Starch	1.42±0.2	467±0.6	66±0.5	22±0.5	102±0.3	14.67±0.9
Dextran	1.32±0.5	498±0.2	71±0.2	25±0.4	124±0.5	17.78±0.1
Aabinose	1.87±0.4	715±0.8	102±0.4	20±0.9	143±0.4	20.42±0.2
Xylose	1.65±0.5	423±0.5	60±0.4	25±0.1	105±0.4	15.10±0.1

Synechocystis sp. MH01 grows well over a wide range of pH values, showing good tolerance to pH 9.

Five different NaNO₃ concentrations (0.5, 1.5, 3, 5, and 10 g.L⁻¹) were taken into account to investigate their impacts on cell growth and lipid accumulation of the *Synechocystis* sp. MH01. The biomass productivity decreased when NaNO₃ concentration is increased. Lipid productivity decreased from 23.28% to 2.28%, with an increase in NaNO₃ concentration. It seems that nitrogen shortage can lead to a higher accumulation of lipids in cyanobacterial cells as affecting adversely on the protein synthesis process (27). The optimum NaNO₃ concentration stood at 0.5 g.L⁻¹ for both biomass and total lipid contents. The study of the effect of different salinities (0.1, 0.5, 1, 1.5, 2, and 3% NaCl) disclosed that with an increase in salinity, the growth rate and lipid production capacity of the MH01 strain is decreased. The initial increase of NaCl concentration from 0.1 to 1% increased the lipid productivity from 10.28 to 22.98%. The maximum lipid production was achieved at 1.5% NaCl and subsequently decreased with an increase in NaCl concentration, which might be due to the accumulation of lipid content under stress conditions. This finding is also in agreement with a prior study conducted by Takagi and Toshiomi (28).

The results from the impact of culture temperature (17, 20, 25, 27, 28, 29, 30 and 35 °C) on biomass and lipid productivity showed that the most favorable lipid productivity was achieved at 30 °C ((BP: 111 mgL⁻¹d⁻¹, LP: 33.42 mg.L⁻¹.d⁻¹).

Previous studies have shown that photo-period could

affect cyanobacterial growth and its capacity for lipid production (29, 30). Here, the MH01 strain produced the highest rate of biomass and lipid productivity under a light/dark cycle time of 24:0 h. This was because, under the light condition (24:0-h cycle), the cyanobacterial cells perform O₂ photoreduction, absorbing energy from light to store it through energy-carrying molecules such as ATP and NADPH. These energy-pool molecules could then be used for the synthesis of biomolecules, promoting the growth of the cyanobacterial cells (33). The biomass and lipid productivity of the MH01 strain at different light-dark period (12-12, 16-8, 14-10, 24-0, and 0-24) were as follows: 12-12 (BP: 25 mg.L⁻¹.day, LP: 6.85 mg.L⁻¹.d⁻¹), 16-8 (BP: 57 mg.L⁻¹.d⁻¹, LP: 18.28 mg.L⁻¹.d⁻¹), 14-10 (BP: 31 mg.L⁻¹.d⁻¹, LP: 7.17 mg.L⁻¹.d⁻¹), 24-0 (BP: 75 mg.L⁻¹.d⁻¹, LP: 24 mg.L⁻¹.d⁻¹), 0-24 (BP: 4 mg.L⁻¹.d⁻¹, LP: 2.31 mg.L⁻¹.d⁻¹).

In some microalgae species, the alteration of culture conditions could be a promising way to increased lipid contents. In contrast to the most microalgal strains that produce low-level of lipids, the lipid production by *Synechocystis* sp. MH01 grown in BG11 medium, as supplemented with different vitamins, was quite considerable. The lipid productivity (LP) of 71.93 mg.L⁻¹.d⁻¹ was achieved in the cells grown in BG11 medium supplemented with myo-inositol (B8). The addition of vitamin B8 to the medium led to higher lipid productivity even more than vitamin B12.

The cyanobacterial cells grown in the presence of biotin (B7) and niacin (B3) showed, respectively, lipid productivity of 40 mg.L⁻¹.d⁻¹ and 38.43 mg.L⁻¹.d⁻¹ in

Table 2. Biomass and lipid productivity of *Synechocystis* sp. MH01 grown in culture media supplemented with different amino acids.

Amino Acids (0.1%)	Final OD	Cellular Weight (mg.L ⁻¹)	Biomass productivity (mg.L ⁻¹ day ⁻¹)	Lipid content (%)	Lipid production (mg.L ⁻¹)	Lipid productivity (mg.L ⁻¹ day ⁻¹)
Control (0g/l)	1.68±0.4	763±0.7	109±0.3	21±0.2	160±0.1	22.89±0.2
Phenylalanine	0.5±0.2	392±0.3	56±0.6	12±0.3	47±0.6	6.71±0.1
Asparagine	1.3±0.7	450±0.8	64±0.2	19±0.4	85±0.4	12.41±0.8
Cysteine	0.4±0.5	613±0.1	87±0.5	10±0.4	61±0.4	8.71±0.7
Arginine	0.3±0.5	154±0.4	22±0.4	13±0.3	20±0.9	2.85±0.5
Threonine	1.3±0.8	556±0.6	79±0.6	25±0.2	139±0.2	19.85±0.4
Methionine	0.5±0.3	238±0.1	34±0.6	15±0.3	35±0.3	5.0±0.3
Lysine	0.4±0.2	735±0.3	105±0.8	19±0.5	139±0.3	19.85±0.1
Glycine	1.42±0.4	913±0.2	130±0.3	22±0.9	200±0.4	40±0.4
Glutamine	0.4±0.2	267±0.7	38±0.3	8±0.5	21±0.7	4.2±0.3
Leucine	0.6±0.9	280±0.8	40±0.1	17±0.3	47±0.4	6.71±0.7

comparison to control subjects (29.89 mg.L⁻¹.d⁻¹). Lipid production under these particular conditions was much higher than many reported in the literature so far.

4.3. The Compositional Analysis of Fatty Acids Under Different Culture Conditions Using Gas Chromatography (GC) and Biodiesel Properties

While the lowest proportion of saturated fatty acids (SFA) (33.73%) achieved in sucrose-containing BG-11 medium, the highest percentage of SFA (40.69%) obtained from glycerin-grown cells (**Table 3**). The highest ratio of MUFA (52.71%) was observed in a medium containing sodium thiosulfate pentahydrate. The results also confirmed that PUFA had the highest proportion (18.33%) when medium supplemented with biotin. However, the lowest SFAs (33.73%) and PUFAs (5.97%) were determined in sucrose supplemented medium and control condition, respectively.

The low amounts of PUFAs show a significantly

weakened interaction between p orbitals attributable to the special geometries of their chains, with weak intermolecular (Van der Waals) forces, hindering the packing of the molecules (31, 32). Moreover, the highest and lowest degree of unsaturation values of 79.48 and 60.91 were recorded, respectively, for the lipids extracted from the cyanobacterial cells grown in medium supplemented with biotin and control conditions. The highest and lowest amount of saponification values (209.15 and 196.64) were defined, respectively, for the lipid samples extracted from growing cells in media containing glycine and biotin.

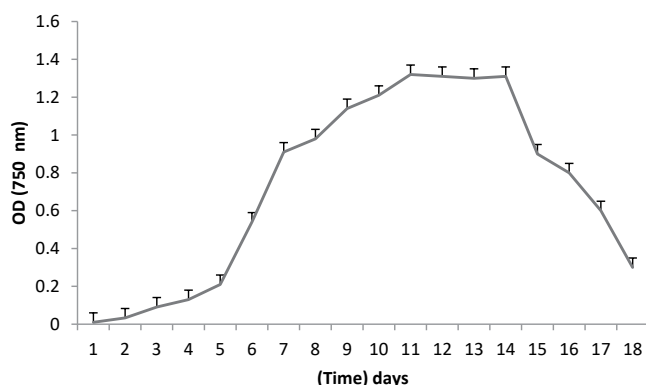
For fuel quality assessment (Table 4), different parameters including, cetane number, long-chain saturated factor (LCSF), cold filter plugging point (CFPP), cloud point (CP), allylic position equivalents (APE), bis-allylic position equivalents (BAPE), oxidation stability (OS), higher heating value, kinematic viscosity, density and pour point (DPP) were carefully

Table 3. The percentage of fatty acid composition in *Synechocystis* sp. MH01 cells grown in BG-11 medium under different culture conditions.

Fatty Acid methyl ester Yield (%)	Control	Sodium thiosulphate pentahydrate (1mM)	pH:7	NaNO3 (0.5 g.L ⁻¹)	NaCl (%0.5)	Glycine	Carbohydrate Sucrose	Vitamin Biotin	30 °C	Light/dark 24-0
(C14)	0.3864	1.0713	0.4616	0.4189	1.4759	1.0111	0.718	2.6132	4.2785	1.0141
(C16:0)	34.9824	29.3409	34.6489	33.5542	31.862	36.320	27.274	26.766	29.66	29.658
(C16:1)	29.4161	8.9117	26.4319	19.8926	13.422	20.770	8.2024	3.1281	5.2327	11.587
(C18:0)	2.9446	4.4231	2.6699	3.4007	4.5451	3.3661	5.7464	4.8836	4.8855	5.1482
(C18:1)	31.5084	43.8025	21.3235	30.8927	34.666	28.894	43.7667	39.679	39.9624	35.750
(C18:2)	5.9758	11.1416	7.7302	6.4377	11.325	8.877	7.6626	14.02	13.3787	18.337
(C18:3)	0	0	0.159	0	0	0	0	0	0	0

Table 4. The evaluation of the quality parameters of biodiesel derived from cyanobacterial lipids, in selected culture conditions, with the highest lipid productivity, through biodiesel analyzer version 2.2.

Biodiesel properties	Control	Sodium thiosulphate pentahydrate (1mM)	pH:7	NaNO ₃ (0.5g.L ⁻¹)	NaCl (%0.5)	Glycin	Sucrose	Biotin	30°C	Light/dark cycling (24-0)
Saturated Fatty Acid(%)	38.313	34.835	37.78	37.374	37.884	40.697	33.738	34.263	38.824	35.821
Mono Unsaturated Fatty Acid(%)	48.967	52.714	47.755	50.785	48.089	49.665	51.969	42.807	45.195	47.338
Poly Unsaturated Fatty Acid(%)	5.976	11.142	7.889	6.438	11.325	8.877	12.253	18.337	13.379	14.89
Degree of Unsaturation(mg/g)	60.918	74.997	63.534	63.661	70.739	67.419	76.475	79.482	71.953	77.118
Saponification Value	198.586	204.118	198.27	198.89	203.04	209.157	201.929	196.645	202.345	203.509
Iodine Value	57.774	68.468	60.002	59.302	65.089	62.8	69.741	72.02	65.394	70.689
Cetane number	60.785	57.634	60.328	60.399	58.536	58.265	57.638	57.851	58.56	57.214
Long Chain Saturated Factor	4.971	5.146	4.8	5.056	5.459	5.315	5.601	5.118	5.409	5.54
Cold Filter Plugging Point(°C)	-0.861	-0.311	-1.397	-0.593	0.673	0.221	1.118	-0.397	0.516	0.928
Cloud Point(°C)	13.409	10.441	13.233	12.658	11.768	14.112	9.354	9.087	10.609	10.608
Pour Point(°C)	7.735	4.514	7.545	6.919	5.954	8.499	3.333	3.043	4.696	4.695
Allylic Position Equivalent	31.502	66.086	37.102	43.768	57.317	46.649	68.273	76.354	66.72	65.531
Bis-Allylic Position Equivalent	5.976	11.142	8.048	6.438	11.325	8.877	12.253	18.337	13.379	14.89
Oxidation Stability(h)	22.325	13.175	17.539	20.909	13.003	15.875	12.215	9.022	11.405	10.511
Higher Heating Value	36.585	38.889	36.668	37.189	38.289	38.997	38.618	37.602	38.355	38.607
Kinematic Viscosity(mm ² /s)	3.334	3.774	3.353	3.486	3.646	3.68	3.753	3.597	3.686	3.692
Density(g/cm ³)	0.814	0.862	0.816	0.825	0.85	0.866	0.856	0.834	0.85	0.857

**Figure 2.** The growth profile of *Synechocystis* sp. MH01 in BG11 medium versus time

defined (33). The ability of eleven cyanobacterial strains for biodiesel production was evaluated in a preceding study conducted by Anahas and Muralitharan, (34). They employed the equations to predict the cetane numbers (typically between 42 and 65) for biodiesel derived from, which most of them demonstrating values ~ 50 . These data directly corroborate the levels of saturation of the constituent esters (34). In the current work, the highest and lowest iodine values of 72.02 and 57.774 g I₂ 100 g⁻¹ were defined for the 24-0 light/dark cycle and control subjects, respectively.

A high range of iodine values could be related to the high level of unsaturated esters. The methyl linoleate (18.377%), with two bisallylic positions in its structure, demonstrates the ease of removal of two hydrogen atoms resulted from the low dissociation energy, as compared with the allylic positions. The iodine index values, as defined in the present work, were consistent with the prior published reports (35, 36). For example, Song *et al.* (2013) reported the ability of 10 algae species for biodiesel production through the determination of their fatty acid composition, biodiesel characteristics along with biomass and lipid productivity. Among these strains, *Phaeodactylum tricornutum* and *Isochrysis sphacrica* with a higher capacity for lipid production and better biodiesel properties were nominated for further analysis. *P. tricornutum*, as the best potential candidate, showed a lipid content of $61.43 \pm 0.95\%$, lipid productivity of 26.75 mg.L⁻¹.d⁻¹, and proper fatty acid composition of C16–C18 (74.50%), C14:0 (11.68%) and C16:1 (22.34%). The analysis of quality parameters of the lipid-derived biodiesel indicated a higher cetane number (55.10), lower iodine number (99.2 g I₂/100 g), and a rather low cloud point (4.47 °C) (37). The iodine index is an important conventional method used to define the degree of unsaturation of oils, fats, and biodiesel, indicating their propensity to oxidize and form deposits and cause motor problems. It is worth

noting that this parameter is not specified by the ANP (38), but the European Union standard for biodiesel (EN 14214) defined a maximum iodine value of 120 mg I₂100g⁻¹. The long-chain saturated factor (LCSF) was achieved in the range of 4.8 (for pH 7) to 5.6 (for 24-0 light/dark cycle condition). Kinematic viscosity is a critical biodiesel quality parameter as it affects the injection systems of motors and could compromise fuel atomization. All tested conditions in the present study exhibited a viscosity value within a limit defined by ANP (3.6 mm²s⁻¹). The cold filter plugging point (CFPP) of biodiesel is another important criterion related to the long saturated alkyl chains, having the greatest impact on crystallization at low temperatures and restricting the circulation of fuels through the filters of motors. It was defined in the range of -1.39 °C (pH 7) - 1.11 °C (sucrose-grown cells). The other cold flow criterion, such as cloud point (CP), was also found to have a close association with the FAME composition, including a varied range of 14.112 (glycine-grown cells) to 9.078 (biotin-supplemented medium). The maximum and minimum pour point (PP) temperatures were determined as 8.49 °C (glycine-grown cells), and 3.04 °C (biotin supplementation). The measurement of both allylic (APE) and bis-allylic position equivalent (BAPE) indicated that the specimens prepared from cyanobacterial cells grown in the presence of biotin had APE and BAPE values of 76.354 and 18.337, respectively. The assessment of oxidation stability of produced biodiesel showed that the specimens prepared from the cells grown in standard culture medium (control) had higher oxidation stability compared to those of other conditions examined. While the lowest oxidation activity value of 9.022 achieved through the extracted lipids of cells grown in the presence of biotin, the highest value of 22.32 achieved for control subjects (**Table 4**).

The lowest level of polyunsaturated esters was obtained from MH01 cells grown in standard condition (control) in addition to NaNO₃-grown cells. It seems that the biodiesel, originated from all operating conditions examined here, to meet the fuel specifications in terms of their densities, as having almost essentially similar values (0.8 kg.L⁻¹). As carbon chains of an alkyl ester get longer, the density is usually going to be better. This means that the longer alkyl ester chain caused a higher corresponding biofuel density and vice versa.

However, the density is decreased with a higher degree of unsaturation. Since microalgae show distinct mixtures of long-chain and polyunsaturated esters, their density values tend to remain constant (39).

5. Discussion

The fine-tuning of medium composition is of considerable importance since it is expected to have a direct impact on cyanobacterial growth and its capacity for lipid production. This needs to be further taken into account so as to use cyanobacterial lipids as valuable primary raw materials for the production of clean energy. As a common approach, different parameters have been considered for fuel quality assessment (40). Here, a newly identified native cyanobacterial strain, *Synechocystis* sp. MH01 was scrutinized for its ability for optimal lipid production through tuning of medium composition. In an earlier study, the co-culture of microalgal strains *Chlorella vulgaris*, *Pseudokirchneriella subcapitata*, and *Microcystis aeruginosa* with the cyanobacterium *Synechocystis salina* led to an increase in biomass and lipid productivity compared to single cultures (41). In another study, an algal polyculture, as comprised of *cyanobacterium aponinum*, *Parachlorella kessleri*, with some halotolerant bacterial species, was cultivated in an aqueous-based medium with different salt concentrations (from 15 to 60 g total dissolved solids (TDS)/L) (42). The results demonstrated a higher growth and lipid productivity in a medium with a salinity level of 60 g TDS/L. Besides, the composition of fatty acids was mainly composed of palmitic (C16:0) and stearic (C18:0) acid, which is suitable for biodiesel production. The findings of this study had practical implications, in which a mixed culture of microalgae and other organisms (of a particular region) might be a favorable approach for cultivation in hypersaline aqueous media, as aimed at biofuel generation (42). The effect of the fatty acid composition of ten refined vegetable oils has previously been studied in association with the biodiesel properties (35). The analysis of several parameters related to biodiesel quality including, oxidation stability, cetane number, iodine value, and cold filter plugging point, showed a direct relationship between them and the degree of unsaturation and long saturation chain of fatty acids (35). In another similar study, the physical characteristics of biodiesel produced by six microalgal strains (three cyanobacteria, two green algae, and one diatom) were screened in terms of their capacity for lipid production and fuel quality (43). Among them, *Chlorella vulgaris* was selected as the best strain aimed to use as a primary raw material for biodiesel production (43). Recent studies have mainly been directed toward the determination of the distinctive nature of the key players involved in enhanced lipid production by microalgal cells through the manipulation of fatty acid biosynthesis metabolic pathways (44). Despite the complexity of the metabolic

pathways of microalgal cells, these studies also point out a new direction in the development of effective lipid production by these photosynthetic organisms (45). Considering that the adjustment of culture medium composition has a direct impact on lipid productivity by microalgal cells, it could also be a simple and cost-effective strategy to achieve this goal.

6. Conclusion

In the current study, for the first time, the effect of water-soluble vitamins, in addition to different physicochemical factors, on growth and lipid production capacity of a native cyanobacterium *Synechocystis sp.* MH01 was investigated. Further, the qualitative parameters of the biodiesel, as derived from the lipids, were analyzed in detail.

Although the precise details of the changes that occurred in the level of lipid production by cyanobacteria under different environmental conditions remain unclear, it appears there are several parallel reports, where the fatty acids composition shows a significant effect on biodiesel quality parameters. All things considering, the cyanobacterium *Synechocystis sp.* MH01 was shown to have a high ability for lipid production under optimal culture conditions, aiming to use for the possible clean fuel production. Currently, research is underway in our laboratory to divulge the exact role of the mixed culture of *Synechocystis sp.* MH01 with other microalgal strains concerning its effect on cell growth and lipid productivity in a deliberately optimized culture medium.

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Conflict of Interest Statement

The authors declare that they have no conflict of interests.

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