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Article

Screening and Evaluation of Some Green Algal Strains *(Chlorophyceae)* Isolated from Freshwater and Soda Lakes for Biofuel Production

Ramganesh Selvarajan [1,2,3,*], Tamás Felföldi [2], Tamás Tauber [2], Elumalai Sanniyasi [3], Timothy Sibanda [1] and Memory Tekere [1]

[1] Department of Environmental Sciences, University of South Africa, Florida 1710, South Africa; E-Mails: sibant@unisa.ac.za (T.S.); Tekerm@unisa.ac.za (M.T.)

[2] Department of Microbiology, Eötvös Loránd University, Pázmány Péter stny. 1/c., H-1117 Budapest, Hungary; E-Mails: tamas.felfoldi@gmail.com (T.F.); ekkaton@gmail.com (T.T.)

[3] Department of Plant Biology and Plant Biotechnology, Presidency College, University of Madras, Chennai 600005, Tamil Nadu, India; E-Mail: emalaibiolab@gmail.com

* Author to whom correspondence should be addressed; E-Mail: selvar@unisa.ac.za; Tel.: +27-116-709-722; Fax: +27-114-712-866.

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Abstract: Microalgae are photosynthetic microorganisms that can produce lipids, proteins and carbohydrates in large amounts and within short periods of time and these can be processed into both biofuels and other useful commercial products. Due to this reason microalgae are considered as a potential source of renewable energy; and one of the most important decisions in obtaining oil from microalgae is the choice of species. In this study, the potential of *Chlorophyceae* species isolated from freshwater and soda lakes in Hungary and Romania (Central Europe) were characterized and evaluated by determining their biomass accumulation, lipid productivity, fatty acid profiles, and biodiesel properties besides protein and carbohydrate productivity. Out of nine strains tested, three accumulated more than 40% dry weight of protein, four accumulated more than 30% dry weight of carbohydrate and the strain *Chlorella vulgaris* LC8 accumulated high lipid content (42.1% ± 2.6%) with a favorable C16-C18 fatty acid profile (77.4%) as well as suitable biodiesel properties of high cetane number (57.3), low viscosity (4.7 mm^2/s), lower iodine number (75.18 g I_2/100 g), relative cloud point (8.8 °C) and negative cold filter plugging point (−6.5 °C). Hence the

new strain, *Chlorella vulgaris* LC8 has potential as a feedstock for the production of excellent quality biodiesel.

Keywords: microalgae; biofuel; fresh water; soda lake; fatty acids

1. Introduction

Global consumption of crude oil is predicted to grow continuously. This explains why despite improvements in the recovery of traditional fossil fuels, more attention needs to be paid to the search for clean and viable alternative renewable energy resources with the prospect of minimizing increases in atmospheric CO_2 by recycling carbon from the atmosphere [1–3].

Algae are a large and highly diverse group of organisms which can be found in almost all ecosystems [4]. Microalgae are a promising feedstock for biofuel production [5,6] and different applications, such as wastewater purification [7–10], biogas production [11,12], and extraction of value added compounds for food and pharmaceutical products [13]. An important aspect of biodiesel production is the selection of a suitable algal species [14]. Selected strains should have two important key characteristics: high biomass productivity as well as adaptation to regional climatic conditions [15,16].

Many microalgae have the ability to produce amounts of triacylglycerols (TAG) of up to 50% dry cell weight as a storage lipid under photo-oxidative stress or other adverse environmental conditions [17]. Recent studies [18–20] prove that algae have inherent advantageous qualities like rapid biomass formation, high lipid content, tolerance for extreme environments, and thus they have generated significantly increased interest as potential feedstocks for biodiesel.

One of the potential advantages in the development and utilization of algae-derived biofuels is the greater production efficiency of the required oils compared to other fuel crops [1]. A suitable microalgae candidate for biodiesel production requires not only high lipid productivity, but also suitable fatty acid (FA) composition, as this composition can significantly influence biodiesel fuel properties such as kinematic viscosity, specific gravity, cetane number (CN), cloud point, iodine value (IV), long-chain saturated factor (LCFF) and Cold Filter Plugging Point (CFPP) [21,22]. However, inadequacies in research and development, policies and strategies for all the stages of biofuel production chain are still a limiting factor to the full exploitation of algal bioresources [14].

The isolation and characterization of oleaginous microalgae from freshwater and soda lakes from Hungary and Romania for biodiesel production is described here and this represents the first description of isolation and characterization of microalgal strains from these habitats for biofuel production.

2. Results and Discussion

2.1. Sampling, Isolation and Morphological Identification of Microalgae

The sampling sites sampled in this study were all slightly alkaline, with Lake Velence having the highest pH at 9.1. Since sampling was performed in winter in this case, the Lacul Ciucas site had the lowest temperature (4.1 °C), while the others had water temperatures between 12 and 16 °C, as listed in Table 1. Microalgae represent a large variety of species living under a wide range of environmental

conditions, including freshwater, lacustrine, brackish, marine and hyper-saline sites [23,24]. Previous investigations on oleaginous microalgae from different sites showed that the sampling environment plays a pivotal role in the determination of strain selection as well as strain viability [17,25,26]. For example, site specific factors, such as salinity and temperature have roles in determining lipid accumulation [27]. Microscopic observation of the nine new algal isolates confirmed their purity and allowed preliminary identification of isolates, which resulted in their classification into the class *Chlorophyceae* based on the morphological characters represented in Table 1.

2.2. Molecular Identification and Phylogenetic Analysis

The 18S rRNA gene is commonly used for the molecular identification of microeukaryotes [26,28]. Polymerase chain reaction (PCR) amplification of the genomic DNA of the algal isolates with the selected primers revealed efficient amplification. The PCR products of small sub unit (SSU) rRNA with a size of ~980 bp were recorded for all isolates. Sequencing results revealed that all isolated strains are chlorophytes *(Chlorellaceae*, *Chlorophyta)* belonging to three different genera: *Chlorella*, *Dictyosphaerium* and *Micractinium* (Table 2).

Phylogenetic analysis of the 18S rRNA gene sequences (Figure 1) affiliated the isolates LF5, LC2, LC8 and LC9 (Lake Feneketlen (LF); Lacul Ciucas (LC)) clearly to *Chlorella vulgaris*, with the closest similarity to microalgal strain *C. vulgaris* SAG 211-11b. Strains RP1 and IL2 (Lake Velence-Reed Pond (RP); Inner Lake (IL) Tihany) were identified as *Chlorella sorokiniana* and *Dictyosphaerium ehrenbergianum*, with the closest similarities to *C. sorokiniana* CCAP 211/8K and *D. ehrenbergianum* CCAP 222/10, respectively. The other sequences from isolates IL3, LC3, and LC11 were related to *Micractinium* strains having pairwise nucleotide sequence similarities ranging between 99.89% and 99.66%. Members of the division *Chlorophyta* are considered to be promising for biofuel production, due to their high growth rates and ease of cultivation, however different enrichment and gating procedures are required in order to isolate strains from other taxa [28,29].

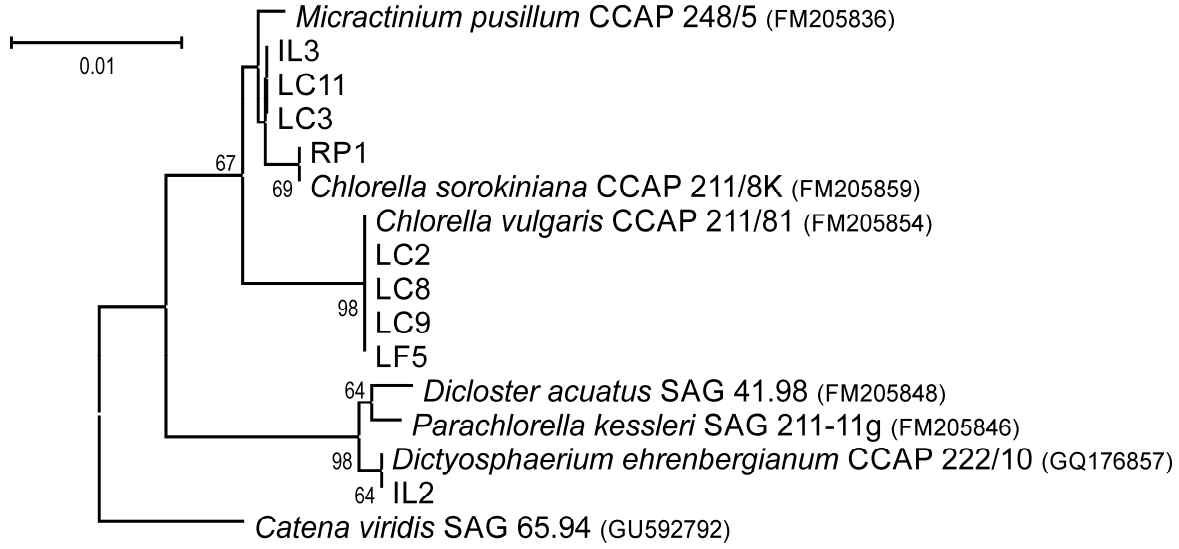


Figure 1. Phylogenic tree showing the relationships among the screened microalgal stains and the most similar sequences based on the 18S rRNA gene (neighbor joining method, Kimura 2-parameter nucleotide substitution model, 1000 rounds of bootstrap resampling).

Table 1. Sampling sites description and morphological characterization of isolated *Chlorophyceae* strains.

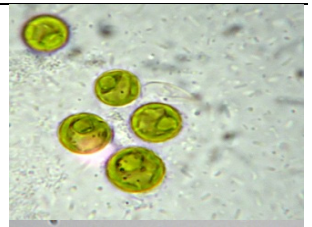
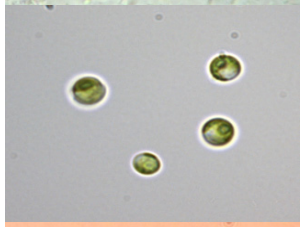
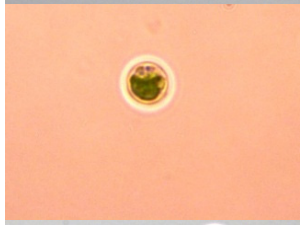
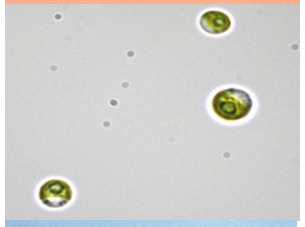
Genus	Sampling Site Characteristics	Strain No.	Species	Morphological Characters	Cell Dimensions	Microscopic Image
Chlorella	Lake Feneketlen (LF) 47°28′36″ N; 19°2′29″ E; Temperature: 12.3 °C; pH: 8.6; Electrical Conductivity: 325 µS/cm	LF5	*Chlorella vulgaris*	Cells unicellular, circular, pyrenoids present, cup shaped chloroplast present, mucilagenous sheath absent	4.7 µm (length) × 4.2 µm (width)	
	Lacul Ciucas (LC) 46°08′44.7″ N; 25°51′25.0″ E; Temperature: 4.1 °C; pH: 8.1; Electrical Conductivity: 804 µS/cm	LC2	*Chlorella vulgaris*	Unicellular, circular shaped, pyrenoids present, cup shaped chloroplast, mucilagenous sheath absent	3.4 µm (length) × 3.0 µm (width)	
	Lacul Ciucas (LC) see above	LC8	*Chlorella vulgaris*	Unicellular cells, cup- to girdle shaped chloroplast seen in some cells, pyrenoids present	4.0 µm (length) × 4.2 µm (width)	
	Lacul Ciucas (LC) see above	LC9	*Chlorella vulgaris*	Cells unicellular, circular, pyrenoids present, cup shaped chloroplast present, no mucilagenous sheath observed	5.4 µm (length) × 4.9 µm (width)	
	Lake Velence-Reed Pond (RP) 47°12′40.6″ N; 18°34′10.5″ E; Temperature: 15.0 °C; pH: 9.1; Electrical Conductivity: 1845 µS/cm	RP1	*Chlorella sorokiniana*	Cells unicellular, circular, pyrenoids present, cup shaped chloroplast present	5.6 µm (length) × 6.1 µm (width)	

Table 1. *Cont.*

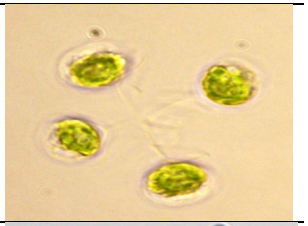
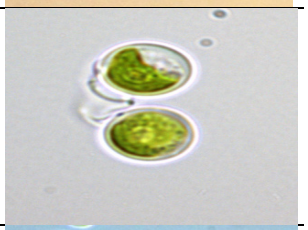
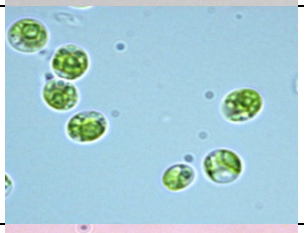
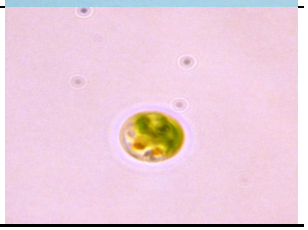
Genus	Sampling Site Characteristics	Strain No.	Species	Morphological Characters	Cell Dimensions	Microscopic Image
Dictyosphaerium	Inner Lake, (IL) Tihany 46°54′33.9″ N; 17°53′09.7″ E; Temperature: 16.1 °C; pH: 8.4; Electrical Conductivity: 860 µS/cm	IL2	*Dictyosphaerium ehrenbergianum*	Unicellular cells perpendicular to colony surface, attached to the ends of thin stalks emerging from centre of colony and branching tetrachotomously, spines are absent	4.9 µm	
Micractinium	Inner Lake (IL) Tihany see above	IL3	*Micractinium* sp.	Solitary cells without mucilaginous sheath, planktonic, spherical, Chloroplast single parietal, cup shaped with an ellipsoidal pyrenoid	6.5 µm (length) × 5.8 µm (width)	
	Lacul Ciucas (LC) see above	LC3	*Micractinium* sp.	Cells are solitary without mucilaginous sheath, spherical, pyrenoid present within the chloroplast	3.9 µm (length) × 4.0 µm (width)	
	Lacul Ciucas (LC) see above	LC11	*Micractinium* sp.	Cells are solitary without mucilaginous sheath, spherical, cup-shaped chloroplast with an ellipsoidal pyrenoid	6.0 µm (length) × 6.2 µm (width)	

Table 2. Taxonomic identification of screened microalgal strains based on partial 18S rRNA gene sequence analysis.

Strain Code	Sequence Length (nt)	Closest Relative	Similarity (%)	Accession Number
LF5	925	*Chlorella vulgaris SAG 211-11b*	100	KF569724
LC2	926	*Chlorella vulgaris SAG 211-11b*	100	KF569728
LC8	907	*Chlorella vulgaris SAG 211-11b*	100	KF569734
LC9	924	*Chlorella vulgaris SAG 211-11b*	100	KF569735
RP1	919	*Chlorella sorokiniana CCAP 211/8K*	100	KF569750
IL2	926	*Dictyosphaerium ehrenbergianum CCAP 222/10*	100	KF569743
IL3	924	*Micractinium* sp.	99.89	KF569744
LC3	925	*Micractinium pusillum CCAP 248/3*	99.78	KF569729
LC11	879	*Micractinium pusillum CCAP 248/3 (and 248/1)*	99.66	KF569742

2.3. Nile Red Staining Observations

Neutral lipids including hydrocarbons and triglycerides were stained in yellow, while polar lipids were stained in red [30]. In our observations, a large number of algal cells emitted yellow fluorescence indicating the presence of neutral lipids including hydrocarbons and triglycerides as shown in Figure 2 [31,32]. The intensity of the Nile red fluorescence confirmed the presence of a large amount of lipids accumulated in the microalgal cells. The use of glycerol increases fluorescence intensity without inhibiting cell growth, thereby allowing stained cells to be isolated and cultured [33].

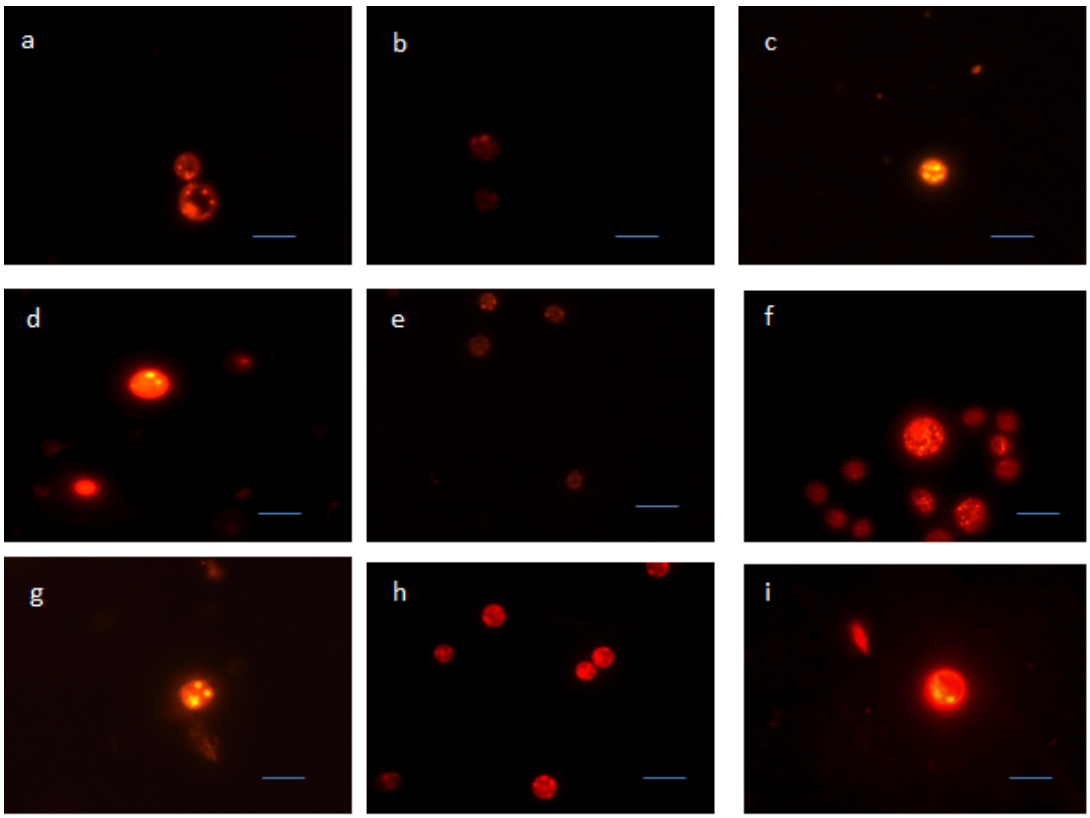


Figure 2. Microscopic photographs of Nile red-stained screened microalgae: (**a**) LF5; (**b**) LC2; (**c**) LC8; (**d**) LC9; (**e**) RP1; (**f**) IL2; (**g**) IL3; (**h**) LC3; (**i**) LC11.

2.4. Biomass and Lipid Content of Screened Microalgae Strains

The biomass and lipid content results are presented in Table 3. In the present study *Chlorella vulgaris* LC2 was found to be the best biomass producer (615.8 ± 10.5 mg/L). In the species *Micractinum* sp. LC3, the lowest biomass productivity was measured (294.3 ± 13.4 mg/L) as compared to the other strains where it was higher, varying between 396.0 ± 24.1 and 571.5 ± 56.0 mg/L.

Many microalgal strains naturally have high lipid contents (20%–50% dry weight). Lipid accumulation refers to increased concentration of lipids within the algal cells without consideration of the overall biomass production [34,35]. In this work however the best biomass producers did not correspond to the best lipid producers. Results shown in Table 3 reveal the notable ability of the microalgae to accumulate lipids, surpassing the total average lipid content (% of dwt) of 20% previously reported for *Chlorella* species [25,36–38] under the same conditions. The highest amount of lipid content was observed in *Chlorella vulgaris* LC8 (42.1% ± 2.6% dwt). In contrast, the best biomass producer, *Chlorella vulgaris* LC2, showed the lowest lipid level (8.9% ± 1.2% dwt) while in the other strains this parameter varied between 10.1 ± 1.0 and 34.1% ± 2.5% dwt. It must be noted that this study was carried out in a CO_2-limited environment which could have led to reduced biomass productivity. However, there is evidence of an inverse relationship in biomass and lipid productivity for microalgae [39]. An assessment of lipid production as a dry weight percentage of the whole biomass by the diatoms *Navicula pelliculosa*, *Navicula saprophila* and *Phaeodactylum tricornutum* [37] showed that they all produced 28%, 26% and 22% lipid dwt, respectively, which compares well with the microalgal strains in this study whose lipid production by dry weight ranged from 8.9% ± 1.2% to 42.1% ± 2.6 %. However, for a credible basis of comparison to be achieved, cultivation and production may need to be done under the same conditions.

Table 3. Biomass composition of screened microalgal strains.

Strain Code	Cell Dry wt (mg/L)	Protein (% dwt)	Carbohydrate (% dwt)	Lipid (% dwt)
LF5	443.8 ± 15.5	40.2 ± 8.2	19.3 ± 1.2	16.5 ± 0.1
LC2	615.8 ± 10.5	42.6 ± 5.8	24.4 ± 0.9	8.9 ± 1.2
LC8	396.0 ± 24.1	28.7 ± 1.3	20.1 ± 1.5	42.1 ± 2.6
LC9	549.7 ± 17.2	22.3 ± 0.6	43.6 ± 2.3	14.2 ± 0.1
RP1	571.5 ± 56.0	43.6 ± 3.5	10.0 ± 0.4	10.1 ± 1.0
IL2	403.3 ± 49.7	17.3 ± 2.5	40.3 ± 6.1	34.1 ± 2.5
IL3	414.3 ± 49.7	14.3 ± 3.5	41.5 ± 8.1	28.1 ± 2.5
LC3	294.3 ± 13.4	27.5 ± 2.8	38.2 ± 2.3	23.6 ± 1.8
LC11	428.1 ± 1.0	21.3 ± 1.5	13.9 ± 0.9	32.3 ± 6.7

2.5. Carbohydrate Composition

In this study, it became evident that the strains *C. vulgaris* LC9, *D. ehrenbergianum* IL2 and *Micractinum* sp. IL3 of our collection appeared to accumulate carbohydrates at up to 40% of their dry biomass (Table 3) under the conditions used. Some species such as *Chlorella*, *Dunaliella*, *Chlamydomonas* and *Scenedesmus* have been reported to accumulate more than 50% carbohydrate based on their dry cell weight; therefore microalgae are considered a promising feedstock for bioethanol

production because they accumulate starch as the main carbohydrate source in their cellulose-based cell walls. Both starch and cell wall polysaccharides can be converted into fermentable sugars for subsequent bioethanol production via microbial fermentation [40]. According to the recently proposed bioconversion process of solid by-products with oleaginous microorganisms [41], they could be recycled into the lipid production process, otherwise these strains might be suitable feedstocks for bioethanol or biogas production [34]. Biobutanol can also be produced from carbohydrate-based microalgae as this alternative fuel contains more energy and is less corrosive and water soluble [42].

2.6. Protein Composition

In this work, strains LF5, LC2 and RP1 accumulated proteins up to 43% of their dry biomass under the given conditions (Table 3). Algae are natural food sources of many important aquaculture species such as molluscs, shrimps and fish [34]. According to other previous data, production of *Chlorella*, which has nutritional value and high protein content, is 2000 tonnes per annum [13]. *Chlorella* strains are also used for medicinal purposes, since they have role in improved immune response, protection against renal failure, improved fertility, better weight control, healthier skin and growth promotion of intestinal lactobacillus [34,43].

2.7. Fatty Acid Profiles

Ideal microalgal candidates for biodiesel production require not only high lipid and TAG production, but also suitable fatty acid composition [44]. Fatty Acid Methyl Esters (FAME) profiles of algal strains are given in Table 4, while comparison of the lipids with respect to the saturated, monounsaturated and polyunsaturated compounds is provided in Figure 3, which indicate that these compounds varied significantly among the nine algal strains. For example, saturated fatty acids (SFAs) ranged from 17.7% to 57.9%, monounsaturated fatty acids (MUFAs) from 1.9% to 60.6%, polyunsaturated fatty acids (PUFAs) from 4.9% to 33.8%. It is suggested that quality biodiesel should contain relatively low concentrations of both long chain saturated FAME and polyunsaturated FAME for satisfactory low temperature operability and oxidative stability [22,45].

Palmitic acid (C16:0) was the predominant fatty acid present in the algal lipid extracts. The highest percentage was obtained with *C. vulgaris* LC2, *C. sorokiniana* RP1 and *Micractinium* sp. LC3, LC11. A reasonable balance for fuel could be achieved with oil containing high levels of monounsaturated fatty acids like palmitoleic acid (16:1) and oleic acid (18:1) because of their capability of giving the finest compromise between ignition quality, combustion heat, cold filter plugging point (CFPP), oxidative stability, viscosity, and lubricity, which are determined by the structure of its component fatty acids [22,45–47]. It was interesting to find out that the monounsaturated FAs composed of palmitoleic acid (C16:1) and oleic acids (C18:1) in *C. vulgaris.* LF5, LC8, LC9 and *Dictyosphaerium ehrenbergianum* IL2 presented a major percentage of about 42.4% to 60.5%, whereas other species showed small differences ranging from 0.5% to 6.6%.

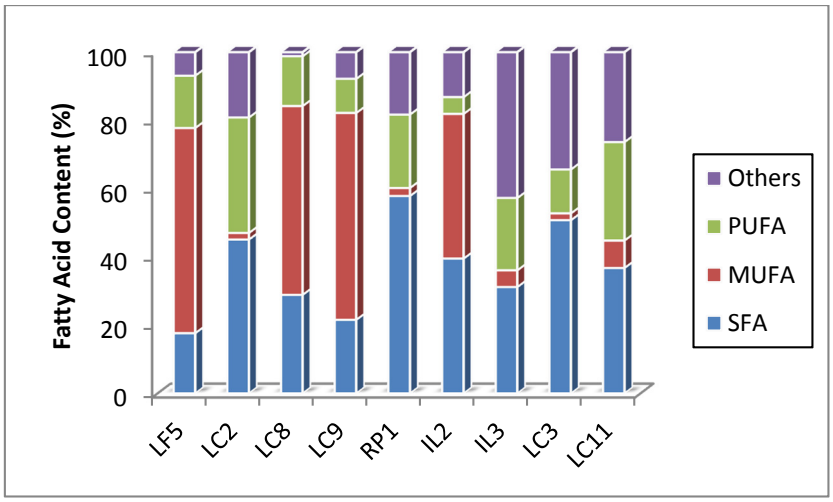


Figure 3. The fatty acid compositions of the screened microalgae: SFA—Saturated fatty acids (C9:0–C20:0); MUFA—Monounsaturated fatty acids (C14:1–C20:1); PUFA—Polyunsaturated fatty acids (C18:2, C20:2, C18:3, C18:3, C20:4).

Table 4. Fatty acid composition of the screened microalgal strains (% of total Fatty Acid Methyl Esters (FAME)).

Fatty Acids	LF5	LC2	LC8	LC9	RP1	IL2	IL3	LC3	LC11
C9:0	nd	nd	nd	nd	0.03	nd	nd	nd	0.03
C10:0	0.01	nd	0.02	0.03	0.04	0.33	nd	0.21	0.05
C12:0	0.08	nd	0.07	nd	0.1	1.73	1.53	1.07	nd
C14:0	0.15	0.61	0.15	0.16	0.82	8.46	1.34	1.6	0.6
C16:0	16.2	31.85	27.71	19.73	52.07	22.63	21.28	42.65	34.1
C17:0	0.22	0.43	0.2	0.28	0.77	nd	0.61	0.36	0.8
C18:0	0.92	4.11	0.8	1.34	3.14	3.81	4.38	3.04	0.88
C19:0	0.04	0.39	0.02	0.04	0.28	2.64	2.13	1.91	0.38
C20:0	0.09	7.78	nd	0.06	0.67	nd	nd	nd	nd
C12:1	0.05	nd	nd	nd	0.12	nd	nd	0.86	0.09
C14:1 ω5c	nd	nd	nd	nd	nd	nd	nd	nd	0.05
C15:1 ω5c	nd	nd	nd	nd	nd	nd	nd	nd	0.07
C15:1 ω6c	0.05	nd	0.04	0.05	nd	nd	nd	nd	nd
C15:1 ω8c	0.08	nd	nd	nd	nd	nd	nd	nd	nd
C16:1 ω7c	0.04	0.4	0.04	nd	0.09	36.66	0.83	nd	6.56
C17:1 ω6c	nd	nd	nd	nd	nd	nd	2.99	nd	nd
C17:1 ω8c	1.04	1.23	0.52	1.69	0.71	nd	1.08	1.13	1.26
C18:1 ω5c	9.99	nd	11.63	nd	nd	nd	nd	nd	nd
C18:1 ω9c	48.86	nd	43.04	58.8	nd	5.69	nd	nd	nd
C19:1 ω11c	nd	0.3	nd	0.05	nd	nd	nd	nd	nd
C20:1 ω9c	nd	nd	nd	nd	1.42	0	nd	nd	nd
C18:2 ω6c	15.31	33.79	14.58	9.99	18.98	3.47	21.17	11.5	26.94
C18:3 ω6c	nd	nd	nd	nd	2.51	nd	nd	1.36	1.79
C20:4 ω6c	nd	nd	nd	nd	nd	1.45	nd	nd	0.09
C16-C18	81.29	69.75	86.13	89.86	76.7	35.6	46.83	58.55	63.71

Percentages of FAME were calculated based on peak area of individual peaks in the GC spectrum. nd: Not detected.

Previous investigations [37,44] showed that the most common feedstocks suitable for biodiesel production were enriched in the five most common C16-C18 fatty acids, including C16:0 (palmitic acid), C18:0 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid), and C18:3 (linolenic acid) [45]. The data in Table 4 show that the nine algal species contained considerable amounts of C16 and C18 species, ranging from 45% to 90%, except the strain *Dictyosphaerium ehrenbergianum* IL2, which contained a substantially low value at 35.6%.

Principal Component (PC) analysis was performed using FAME composition data to understand the fatty acid distribution among the isolated strains. The FA distribution is represented as PCA biplots in Figure 4. Only four fatty acids contributed to the major variations in the fatty acid composition, based on these variations, two distinctly different groups were identified, viz., an oleic acid group and a palmitic-linoleic acid group, whereas other fatty acids showed little variations. The oleic acid group included *C. vulgaris* LF5, LC8 and LC 9, which had a FA composition more similar to that of rape seed and palm oils, with C18:1 as the most abundant fatty acid [22]. Similarly the strains *C. vulgaris* LC2, *C. sorokiniana* RP1, *Micractinum* sp. IL3 and *Micractinum* sp. LC11 presented quite a similar fatty acid profile to that of soy, characterized by a high content of C18:2 and included in the palmitic-linoleic acid group.

Microalgal fatty acid profiles vary according to individual species and environmental conditions (and also during laboratory cultivation). An important aspect of strain selection and/or improvement, although until now less considered, is the FA composition of the microalgae oleaginous biomass [25,48]. The composition and structure of fatty acid methyl esters could significantly influence the fuel properties, such as degree of unsaturation and carbon chain length, and determine the fuel properties (e.g., cetane number, viscosity, cold flow, oxidative stability, and iodine value) of biodiesel [45]. However, it is difficult to say clearly state the suitability of fatty acid profiles, due to the diversity and conflicting impacts of fatty acid profiles on biodiesel properties. Therefore a highly comprehensive analysis is urgently needed to evaluate the most important biofuel properties [22].

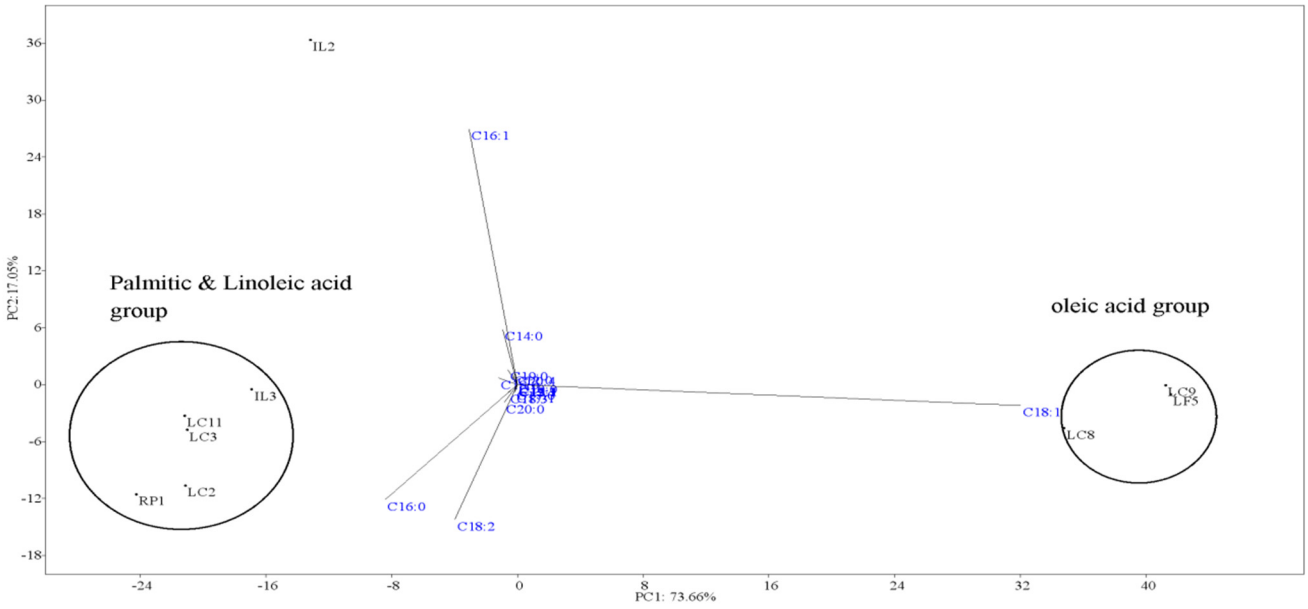


Figure 4. Principal component analysis (PCA) bi-plots for the distribution of isolated microalgal strains in relation to their FAME composition.

2.8. Predicted Fuel Properties

As shown in Table 5, eight important biodiesel properties besides average degree of unsaturation of the nine candidates were predicted**.** The most important fuel properties considered to assess the potential of biodiesel as substitute of diesel fuel are viscosity, cetane number (CN), density, cold filter plugging point (CFPP), oxidative stability, lubricity, ignition quality, combustion heat and cold flow [6,49].

The quality standards for diesel fuel require a minimum cetane number of 40; in the present study, the value of CN calculated for selected strains ranged from 56 to 61, which is in accordance with the standards reported as a minimum cetane number of 47 [45] why different values? High cetane value is one of the important fuel property indicators of better combustion, low nitrous oxide (N_2O) emissions, less occurrence of knocking and easier engine start-up [46,50].

Iodine value in the present study was found to be less than 80.39 g I_2/100 g in all strains, which satisfies the European biodiesel standards (Table 5). Higher iodine values may result in the polymerization of glycerides and deposition of lubricant in the engine [51]. The low degree of unsaturation found in these strains is crucial for the overall performance of diesel engines and an encouraging feature for biofuel production.

The melting point of saturated fatty acids is always higher than that of unsaturated fatty acid compounds. When the oil contains mostly saturated FA ester molecules, crystallization may occur at temperatures within the normal engine operation range [51], which results in poor CFPP properties. Biodiesel rich in palmitic and stearic acid methyl esters has a tendency to present a poor CFPP (equivalent to a higher plugging point temperature), because when a liquid biodiesel is cooled, these FAME precipitate first [46]. In the present study, the levels of palmitic and stearic acid (Table 4) were low (below 35%), except for *Chlorella sorokiniana* RP1, *C. vulgaris* LC2 and *Micractinum* sp. LC11 (55.2%, 36.0% and 45.7%, respectively). These low stearic and palmitic acid values may have contributed to the lower CFPP temperatures of the majority of the studied strains. The CFPP values estimated for biodiesel from the strains studied in the present work ranged from 6.91 °C (*Chlorella sorokiniana* RP1) to −9.66 °C (*Chlorella vulgaris* LF5). According to a previous report, the CFPP values obtained from microalgal oils vary from −12.3 to 20.8 °C [51] and LCSF for the microalgal isolates were in accordance with the international standards revealing the flow performance of biodiesel at low temperatures [25] without greatly affecting the cold flow properties of biodiesel [50].

3. Materials and Methods

3.1. Sampling

Microalgae were sampled from four different locations: Lacul Ciucas (LC) in Romania, Inner Lake (IL), Tihany, Lake Velencei surrounded by a reed zone (RP) and from Lake Feneketlen (LF) in Hungary (see sampling site details in Table 1). The predominant criterion for sample collection was the presence of abundant microalgae in the water [25]. Temperature, pH and conductivity of each sample were determined *in situ* with a Multi-Line P4 meter (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany) and the samples were transported to the laboratory in a thermo box in dark conditions.

Table 5. Comparison of nine properties of biodiesel from microalgal oil, biodiesel, and American Society for Testing and Materials (ASTM) biodiesel, European (EN) biodiesel standard.

Property	LF5	LC2	LC8	LC9	RP1	IL2	IL3	LC3	LC11	Biodiesel	US (ASTM D6751-08)	Europe (EN 14214)
Kinematic viscosity 40 °C ($mm^2 s^{-1}$)	4.63	4.77	4.67	4.69	4.90	4.91	5.04	5.07	4.95	4–5	1.9–6.0	3.5–5.0
Specific gravity (kg^{-1})	0.878	0.876	0.877	0.877	0.875	0.875	0.874	0.873	0.875	0.87–0.89	0.85–0.9	-
Cloud point (°C)	7.84	10.77	8.78	9.18	13.58	13.85	16.66	17.19	14.65	-	-	-
Cetane number	56.81	58.27	57.27	57.47	59.68	59.81	61.21	61.48	60.21	45–55	Minimum 47	Minimum 51–Maximum 120
Iodine value (g Iodine/100 g)	80.39	64.03	75.18	72.95	48.41	46.92	31.30	28.33	42.46	-	-	Maximum 120
HHV (MJ/kg)	40.14	39.75	40.01	39.96	39.38	39.34	38.97	38.90	39.24	-	-	-
Average Unsaturation	0.91	0.69	0.84	0.81	0.48	0.46	0.25	0.20	0.40	-	-	-
Long-chain saturated factor (LCFF)	2.17	13.02	3.171	2.703	7.447	4.188	4.318	5.785	3.85	-	-	-
Cold Filter Plugging Point (CFPP)	−9.66	2.43	−6.51	−7.98	6.91	−3.31	−2.91	1.69	−4.38	-	-	−5 to −13

3.2. Isolation and Cultivation of Microalgal Strains

Sample processing started within 3–6 h after sampling. The samples were filtered through double-layered blotting sheets to remove any solid contaminations and debris and 100 µL of filtrate were then transferred onto the BG11 (Blue Green Medium) solid culture media [52] and incubated in a light chamber at 20 ± 2 °C for two weeks. After growth, different colonies were picked, cultivated photoautotrophically in 125 mL Erlenmeyer flasks containing 60 mL of BG11 medium [53] and incubated in a light mounted shaker at 20 ± 2 °C, with shaking at 120 RPM and a light intensity of 21.2 W/m^2 using a photoperiod of 12 h light: dark, in CO_2 limited condition for 21 days. Axenicity of the isolates was confirmed by cultivation on LB plates and observation with a light microscope following the method [25].

3.3. Morphological Investigations

During the isolation process, algal strains were preliminarily identified by observation of morphological characteristics under a light microscope and using botanical approaches as described [54].

3.4. Molecular Characterization

Taxonomic determination was further confirmed by sequencing the 18S ribosomal RNA gene. Genomic DNA was extracted from the isolates with a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). PCR was performed with primer pairs Euk328f-Chlo02R [55,56] as described by Somogyi *et al.* [57]. Reactions were carried out in a final volume of 50 µL using 2 µL genomic DNA, 0.2 mM of each deoxynucleotide, 1 U LC *Taq* (*Thermus aquaticus*) DNA polymerase (Fermentas, Vilnius, Lithuania), 1 × PCR buffer (Fermentas), 2 mM $MgCl_2$, 0.325 mM of each primer and 20 µg of BSA (Bovine Serum Albumin) (Fermentas). The thermal profile consisted of a first denaturing step at 98 °C for 3 min, followed by 32 amplification cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 60 s, elongation at 72 °C for 1.30 min and a final extension step at 72 °C for 10 min. PCR product purification, sequencing reaction and capillary electrophoresis were performed by the LGC Genomics GmbH (Berlin, Germany). Chromatograms were corrected manually with Chromas 1.45 software (Technelysium Pty Ltd., South Brisbane, Australia). The generated sequences were compared to the GenBank nucleotide database using the Blast program [58]. The obtained 18S rRNA gene sequences were submitted to GenBank under the accession numbers given in Table 2. Phylogenetic analysis was performed using the Molecular Evolutionary Genetics Analysis v5 (MEGA5) software [59] using an alignment created with SINA Aligner [60].

3.5. Nile Red Staining of Neutral Lipid Droplets

To visualize TAG accumulation in microalgae, strains were stained with lipid-sensitive Nile Red fluorescent dye, observed under fluorescence microscope for detecting the presence of intracellular lipid droplets [61]. Staining was carried out on fixed (1.5% glycerol for 5 min) cells [33]. The dye was then added directly to the preparation to effect 1:50 / 1:100 dilutions and incubated for 5–10 min in the dark at room temperature [33]. The fluorescence intensity of the stained microalgal cells was measured at an excitation and emission wavelength of 450 and 510 nm, respectively, using a Nikon 80i (Nikon, Tokyo,

Japan) epifluorescence microscope equipped with a camera Q-Imaging Micropublisher 3.3 RTV (Real Time Viewing), and the software used to capture images was the Image-Pro Plus v6.0 (Media Cybernetics, Inc., Bethesda, MD, USA).

3.6. Determination of Total Lipids

The Bligh and Dyer method [62] for lipid extraction was used in this study with slight modifications. The harvested cells were disrupted using ultra sonic bath (Equitron, Mumbai, India) at frequency of 153 KHz for 1 min. All samples were extracted with 3 mL of chloroform/methanol at a ratio of 1:2 volumes for volume (V/V) by vortexing (1 min) and centrifugation at 8000 rpm for 15 min at room temperature (RT). The supernatants were collected and residues were re-extracted thrice with 2 mL of chloroform/methanol (1/1, V/V) by centrifugation as stated above. All the supernatants were pooled together, filtered with Whatman No. 1 filter paper (Whatman Inc., Clifton, NJ, USA), and washed with 2 mL of milli-Q water, followed by centrifugation using glass centrifuge tubes (model T32c Janetzki, Olympus, Japan) at 8000 rpm for 5 min. The lower organic phases were collected and evaporated to dryness. Samples were resuspended in chloroform for further analysis.

3.7. Fatty Acid Methyl Esters (FAME) Determination

Fatty acid methyl esters (FAME) present in the algal lipids were determined following the technique proposed by Freedman *et al.* [63] with some modifications. The conversion of lipids into FAME was performed by acid-catalyzed methylation. The dried lipid (50 mg) extract was added to 15–20 mL of 2% H_2SO_4 in methanol and refluxed for about 4 h 30 min. After complete conversion as monitored by thin layer chromatography (TLC), the solvent was partially removed and the remaining mixture was extracted with ethyl acetate (20 mL), and the combined ethyl acetate layers were washed with water until pH was neutral. The ethyl acetate extract was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure on a rotary evaporator (Büchi R-200, Flawil, Switzerland) to recover the FAME fraction. Methyl esters of fatty acids were analyzed using a gas chromatograph (GC-6890N, Agilent, Santa Clara, CA, USA) equipped with a flame ionization detector DB225 capillary column (30 m × 0.25 mm I.D.; 0.25 μm—Agilent Technologies). The initial oven temperature was maintained at 160 °C for 2 min with a sequential increase to 180 °C at 6 °C min^{-1} for 2 min and 230 °C at 4 °C min^{-1}. The final oven temperature was maintained at 230 °C for 10 min. Nitrogen was used as the carrier gas with a flow rate of 1.5 mL min^{-1}. The injector and FID temperatures were set at 230 and 250 °C respectively, while a split ratio of 50:1 was maintained for the analysis. Heptadecanoic acid was used as the internal standard for quantitative analysis. The components were identified by comparing their retention times and fragmentation patterns with those of the standards and expressed as a percentage of the total fatty acids identified in the oil following the description [64].

3.8. Calorimetric Determination of Total Protein

Total proteins were analysed by the Folin phenol reagent method as described by Lowry *et al.* [65]. Samples were prepared by boiling resuspended cells at 100 °C for 10 min in the presence of

1 N (Normality) NaOH. Aliquots (1 mL) were subjected to protein determination. The calibration curve was obtained using bovine serum albumin (BSA) as a reference standard.

3.9. Determination of Total Carbohydrates

Total soluble carbohydrates were analysed by the phenol-sulphuric acid method as described by Dubois *et al.* [66]. The calibration curve was obtained using D-glucose (Hi-Media Laboratories, Mumbai, India) as a reference standard.

3.10. Bioprospecting of Biodiesel Properties Based on FAME Profiles

Predictive models based on FA composition were used in this study for the calculation of critical biodiesel properties, otherwise it would require large amounts of diesel and specialized instrumentation, which are not always readily available. In recent studies, many equations based on FA composition have been built to predict the properties of biodiesel [22,51,67].

In this work, the equations of Hoekman *et al.* [22] were selected to predict the properties of biodiesel, since the calculated values using the equation are more closer to the measured values from biodiesel [37,44].

The Average Degree of Unsaturation (ADU) calculated from compositional profiles of fatty acid were calculated using Equation (1):

$$\text{ADU} = \sum \text{M} \times \text{Yi} \quad (1)$$

where M is the number of carbon–carbon double bonds in FA constituent and Yi is the mass fraction of each FA constituent respectively

The relationships between average degree of unsaturation and biodiesel properties including kinematic viscosity, specific gravity, cloud point, cetane number, iodine value and higher heating value (HHV) are as shown in Equations (2)–(7) [22]:

$$\text{Kinematic viscosity} = -0.6313\text{X} + 5.2065 \quad (2)$$

$$\text{Specific gravity} = 0.0055\text{X} + 0.8726 \quad (3)$$

$$\text{Cloud point} = -13.356\text{X} + 19.994 \quad (4)$$

$$\text{Cetane number} = -6.6684\text{X} + 62.876 \quad (5)$$

$$\text{Iodine value} = 74.373\text{X} + 12.71 \quad (6)$$

$$\text{HHV} = 1.7601\text{X} + 38.534 \quad (7)$$

where X is the ADU.

The long-chain saturated factor (LCSF) directly used to calculate Cold Filter Plugging Point (CFPP) was also estimated through Equations (8) and (9). These two factors are both related to chain saturation and length of FAME [21]:

$$\text{LCSF} = (0.1 \times \text{C}_{16}) + (0.5 \times \text{C}_{18}) + (1 \times \text{C}_{20}) + (1.5 \times \text{C}_{22}) + (2 \times \text{C}_{24}) \quad (8)$$

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

3.11. Statistical Analysis

The experiments adopted for the estimation of total protein, carbohydrate and lipids were carried out in triplicate and data are expressed as mean ± standard deviation. Principal component analysis (PCA) for the fatty acids was accomplished to explore the underlying inter-relationships between them. PCA bi-plots were generated using PAST (paleontological statistics) 2.06 statistical software [68].

4. Conclusions

Currently, screening and evaluation of potential green algal strains *viz* biomass productivity, lipid cell content, fatty acids and fuel properties are some of the key parameters that determine the economic feasibility of algal oils for biodiesel production. In this work, nine *Chlorophyceae* strains isolated from freshwater and soda lakes from Central Europe were compared according to their biomass and lipid productivities. The total lipids ratios in dry biomass varied from as low as 8.9% to ratios as high as 42.1%. The highest lipid yields were observed from the *C. vulgaris* LC8 strain with a favorable C16-C18 fatty acid profile of 77.38%, as well as desirable biodiesel properties like a high cetane number (57.3), low viscosity (4.67 mm^2/s), lower iodine number (75.2 g I_2/100 g), relative cloud point (8.8 °C) and negative cold filter plugging point (−6.5 °C). *C. vulgaris* LC8 presented one of the best combinations of desirable traits, which makes it a good candidate for further assessment for biofuel production. Results suggest that the adequate fatty acid composition of microalgal oil and the lipid productivity of strains must be priority criteria for strain selection, to make the algae-based biodiesel industry viable.

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Author Contributions

R.S. (corresponding author) conducted the experiments and analyzed the data and wrote the manuscript. E.S. supervised the study in India while T.F. supervised the study in Hungary. T.T. helped in Fatty Acid analysis. T.S. and M.T. coordinated in the writing of the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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