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RESEARCH ARTICLE

Molecular Identification and Biochemical Characterization of *Graesiella emersonii* LDC1 as a Potential Source of Omega -3 Fatty Acids

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Abstract

Objectives: Omega-3 fatty acids are poly-unsaturated and essential with significant health benefits. The inability of conventional sources like plants and fish oil to meet the growing demand for omega-3 fatty acids geared up the search for alternative sources. The current study has focused on assessing the potential of a freshwater microalga, *Graesiella emersonii* LDC1, to produce Polyunsaturated Fatty Acids (PUFAs), particularly concerning omega-3 fatty acids. **Methods**: The taxonomy of the isolate was assessed using a combination of molecular techniques and in silico tools, such as BLAST, CLUSTAL OMEGA, and MEGA 11. The isolate was characterized in terms of its growth pattern and lipid productivity. Additionally, its fatty acid methyl ester profile was analyzed using Gas chromatography-mass spectrometry. **Findings**: The isolated microalga was identified as *Graesiella emersonii* due to a 99.72% sequence similarity and phylogenetic tree analysis. The fatty acid methyl esters profile demonstrated the dominance of poly-unsaturated fatty acids (61.7%), followed by saturated fatty acids (22.4%). Mono-unsaturated fatty acids contribute a notable 5.6%, with other compounds enriching the mix at 10.3%. These results also underscored the potential of the isolate to produce essential omega-3 fatty acids, including α-linolenic acid (36.0%), 8,11,14-Docosatrienoic acid (28.3%), stearidonic acid (1.9%) and docosahexaenoic acid (6.1%). **Novelty**: *G. emersonii* LDC1 has a promising path for docosahexaenoic acid production through the Omega-3 fatty acid biosynthesis route. This study has produced the second scientific report on a natural source of 8,11,14-docosatrienoic acid (C22:3), a rare omega-8 fatty acid with potential health benefits, such as cardioprotective functions.

Keywords: α-linolenic acid; Docosahexaenoic Acid; Graesiella emersonii; Omega-3 Fatty Acids and Polyunsaturated Fatty Acids

1 Introduction

Fatty acids are biological compounds exhibiting varied nutritional benefits as food and feed supplements due to their health-promoting biochemical functions as energy sources, metabolites of pathways and building blocks of biological structures[1]. Especially Omega-3 fatty acids, including α-linolenic acids (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), were found to show a long list of health benefits including their ability to support the maintenance of cardiovascular health, mental health and strong immune system[2]. Omega-3 fatty acids are crucial structural and functional components of various organs and systems, including the nervous system, eyes, bones, and muscles. They offer a wide range of health benefits as both food and feed ingredients. However, since the human body cannot synthesize these fatty acids on its own, there is a need for dietary supplementation to ensure adequate intake[3].

Fish oil is the primary source of omega-3 fatty acids, but its high cost has raised concerns. This high price, along with issues such as overfishing, variability in omega-3 content, and unexpected environmental factors, has made the sustainable supply of fish oil-based omega-3 fatty acids questionable[4]. To overcome this gap in the demand and supply chain of omega-3 fatty acids, wild-type and engineered microalgae are proposed as viable alternatives. The characteristics of microalgae like faster growth rate, high lipid content per unit weight of dry cells and ability to thrive in varied culture conditions, make them ideal candidates for ensuring a continuous supply of omega-3 fatty acids[5].

Freshwater microalgae strains are considered ideal sources of bio-products important in various industries, including antioxidants (such as carotenoids), omega-3 and omega-6 fatty acids as nutraceuticals, and lipids for biodiesel feedstock due to their high productivity, low land use, and adaptability. Application of indigenous strains was especially recommended as they ensure dominance and high adaptability to the environmental and climatic conditions of their native habitat. While efforts are underway to explore new sources of omega-3 long-chain PUFAs (n-3 LC-PUFAs) and to reduce the burden of production cost through genetic engineering and transgenic technology, the complexity of metabolic pathways and dietary habits continues to challenge the adequacy of PUFA supply. Addressing these gaps requires a multifaceted approach, including improved dietary strategies, an intensive search for new potential sources of PUFAs and innovative biotechnological solutions[6,7].

Graesiella emersonii is a freshwater green microalga that belongs to the class Chlorophyceae. It exists in unicellular forms and is reported to have high lipid productivity. This microalga is being explored for its potential to produce biodiesel and other bio-products that offer health benefits and have environmental applications[8]. Thus, in the current study, a locally isolated, freshwater microalgal strain *Graesiella emersonii* LDC1 was tested for its ability to produce lipids and Polyunsaturated fatty acids (PUFAs) in batch culture mode by assessing its Fatty Acid Methyl Esters (FAME) profile with the GC-MS analysis.

2 Methodology

2.1 Isolation and Morphology

Graesiella emersonii LDC1 was isolated during a preliminary study conducted in the Department of Zoology at Lady Doak College in Madurai, Tamil Nadu. A pure culture was obtained from the initial culture sample using the streak plate method on solid BG11 medium plates[9]. Single colonies were chosen for establishing axenic cultures of

the isolated strain in liquid BG11 medium with gradual scaling up from 5ml to 400ml culture volume. The morphology of the isolated strain was examined microscopically using an Olympus CH20i Model Binocular light microscope with 100x objective and 10x eyepiece with immersion oil.

2.2 Molecular identification

Genomic DNA (gDNA) was extracted for molecular analysis using QIAGEN DNeasy Blood & Tissue Kit (Cat. No. / ID: 69504) and its quality was evaluated on 1.0 % agarose gel, a single band of high-molecular weight DNA has been observed. A fragment of 18S rRNA gene was amplified using universal eukaryotic primers, NS1 (GTA GTC ATA TGC TTG TCT C) and NS4 (CTT CCG TCA ATT CCT TTA AG). The reaction mixtures for PCR amplification comprise 2 μL of template (~ 50ng of DNA), 1 μL of primers (~ 2.5 pmol) and 7 μl of master mix. PCR amplification was performed under the following thermal cycling conditions: denaturation at 95 °C for 3 minutes and 30 seconds followed by 40 cycles of 95 °C for 30 s, annealing at 50°C for 30 seconds and extension at 72 °C for 3 minutes for 35 cycles. A single discrete PCR amplicon band of 1100 bp was observed when resolved on agarose gel (Figure 2). PCR product was purified using the QIAGEN QIAquick PCR Purification Kit (Cat. No. / ID: 28104) and subjected to Sanger sequencing using BDT v3.1 Cycle sequencing kit on Applied Biosystems^{TM} MiniAmp^{TM} Plus Thermal cycler and analyzed on ABI 3730xl Genetic Analyzer. A consensus sequence of the 18S rRNA gene was created by combining forward and reverse sequences, trimming noise, and verifying mismatches. Only 100% aligned sequences with no insertions or deletions were validated and compared using NCBI's BLASTn algorithm. After manual accuracy checks, the sequence was submitted to NCBI GenBank and assigned an accession number (PQ801144). The phylogenetic analysis employed both Neighbor Joining and Maximum Likelihood methods, with one hundred bootstrap simulations conducted. Aligned sequences were exported to MEGA11 for further analysis. Members of the genus *Coelastrella* belonging to the order Sphaeropleales and family Scenedesmaceae of class Chlorophyceae were used as outgroups for constructing the phylogenetic tree. The smaller subunit ribosomal RNA partial gene sequence of *G. emersonii* LDC-1 was submitted to GenBank with the accession number (PQ801144)[10,11].

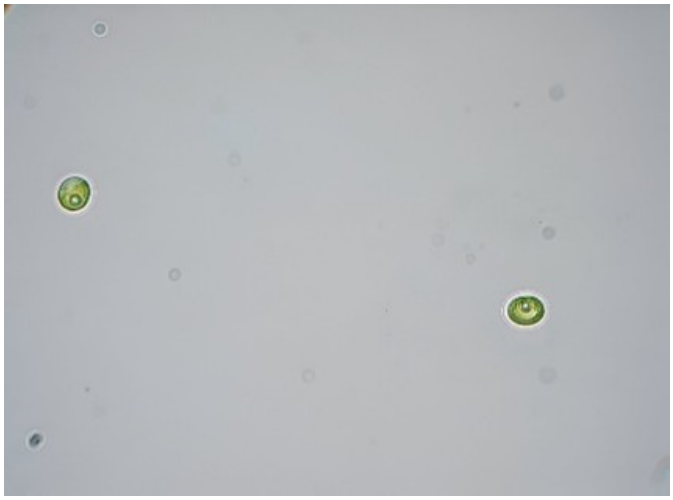

Fig 1. Morphology of *Graesiella emersonii* LDC-1. Microscopic image of the isolated strain was captured with an Olympus CH20i Model Binocular light microscope (1000x). The cells were found to be spherical, solitary, with single nucleus and no prominent vacuoles

2.3 Culture conditions

The cultures were maintained in batch culture mode in 1L Erlenmeyer flasks with BG11 medium. For biomass production, exponentially growing microalgal culture was transferred to flasks with fresh sterile medium with 20% v/v inoculum[12]. Cultures were illuminated by tubular fluorescent lamps and the light intensity at the surface of the cultures was maintained at 2000lux with a photoperiod of 12:12 light: dark at 25$\pm$1°C of temperature and a pH of 7.1.

2.4 Assessment of growth pattern and pigment production

The growth pattern of *Graesiella emersonii* in batch culture mode was monitored by determining the cell density using a Neubauer counting chamber, where cell numbers were estimated at 48 h intervals and the growth curve was developed by plotting the cell density against the incubation time. The algal biomass was harvested on alternate days, centrifuged at 5000rpm for 15 minutes, and dried in a hot air oven at 50°C for cellular dry weight estimation (CDW g L^{-1}) using an analytical balance with a precision of 0.1 mg. Chlorophyll and carotenoid content was estimated by the method of Lichtenthaler and Wellburn (1983) with culture samples collected every 48 hours from day 1 to day 15 of the incubation period[13].

2.5 Lipid productivity

G. emersonii, was cultured in the BG11 medium in batch culture mode in sterile Erlenmeyer flasks and the biomass was harvested every 48 hours from day 1 to day 13. Lipid production and accumulation were confirmed through Nile red staining.Total lipid content was extracted and estimated using the Bligh and Dyer method.[14].

2.6 FAME Preparation and GC-MS

Fatty acid methyl ester (FAME) analysis was carried out with 40ml of culture samples collected every other day from day 1 to day 13 of the culture duration. After centrifugation at 5000 rpm for 10 mins, the harvested biomass was dried in a hot air oven at 50°C until constant weight, and 50mg of biomass from each sample was used for lipid extraction using chloroform: methanol (2:1). Dry total lipids were dissolved in chloroform. FAME preparation was done by adding methanol: acetyl chloride (9.5:0.5) at 80°C for 3 hours. Subsequently, cooled to room temperature and added 1- 2ml of hexane. After the esterification step, 15ml of 5% NaCl and 5ml of hexane were added and allowed to separate into two layers. The upper aqueous layer was collected and washed with 15 ml of a 2% potassium bicarbonate solution. Next, 5 ml of hexane was added and thoroughly mixed, allowing the sample to separate into two distinct phases. The upper aqueous layer was collected in a fresh tube and the samples were dried using the rotary vacuum evaporator. Finally, the samples were stored at -20°C. Before injecting the samples in gas chromatography-mass spectrometry (GC-MS), they were dissolved in HPLC-grade hexane and centrifuged at 5000 rpm for 5 minutes to remove the debris. The samples were analyzed using GC-MS with a non-polar capillary column (RX-5SIL MS) from Shimadzu, Japan. The column measured 30 meters in length, with an inner diameter of 0.25 mm and a thickness of 0.25 μm. The program was set to 120 °C for an initial 5 minutes, followed by a temperature increase of 5 °C per minute, ultimately reaching a peak of 280 °C for 10 minutes.[15].

3 Results and Discussion

3.1 Phylogeny and molecular characterization

Morphological features of the new isolate were examined microscopically and as found to be spherical to ellipsoidal with an 8.8 to 14.4 μm diameter. The cells of the isolate had a cup-shaped chloroplast and a single visible pyrenoid (Figure 1). The BLAST results from the homology search showed a high percentage of identity (99.72%) with *Graesiella emersonii* strain MSP2 (OR056297.1). Molecular phylogeny was inferred from sequence analyses using CLUSTAL OMEGA for multiple sequence alignment and MEGA11 for phylogenetic tree construction (**Supplementary Figure**) and evolutionary analysis. The phylogenetic tree constructed to analyze the evolutionary history of *G. emersonii* LDC-1 revealed that *G. emersonii* LDC-1(PQ801144) and *Graesiella emersonii* strain MSP2 (OR056297.1) share a most recent common ancestor within the same clade and distantly related to *Coelastrella* sp. Therefore, the isolate is classified as a member of the *Graesiella emersonii* group and deposited at the National Facility for Marine *Cyanobacteria* (NFMC), Bharathidasan University, Tamil Nadu and obtained the culture ID (BDUGD005).

A homology search for the 18s rRNA sequence of *G. emersonii* LDC-1 was carried out using the nucleotide BLAST tool of NCBI. Sequences showed the highest score were chosen, and phylogenetic analysis was performed using CLUSTAL OMEGA and MEGA11. The phylogenetic tree constructed using maximum likelihood method. showed that the isolated strain was found to have a close association with the *Graesiella emersonii* strain MSP2 (OR056297.1). The numbers on the nodes indicate bootstrapping value done for 100 replicates.

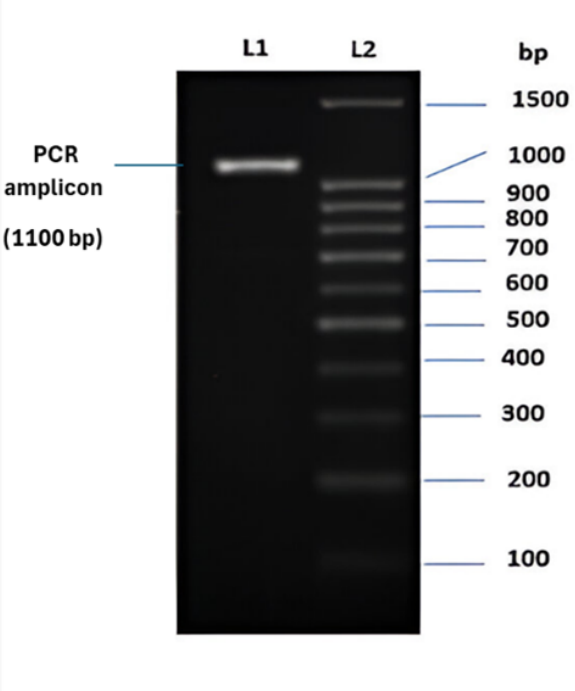

Fig 2. Agarose gel electrophoresis of 18S rRNA PCR product. A Fragment of 18S rRNA gene was amplified with NS1 and NS4 primers. A single discrete PCR amplicon band of 1104 bp was observed when resolved on agarose gel. L1 indicates the PCR amplicon and L2 indicates the DNA ladder

3.2 Growth pattern and pigment production

Graesiella emersonii LDC-1, a unicellular (Figure 1) freshwater green microalga was cultured in BG11 medium in batch mode and its growth pattern was recorded through cell density assessment. The values were plotted and the resulting growth curve showed a log phase duration of 8 days (Day 3 to Day 11), a stationary phase for two days (Day 12, Day 13) and entered into the decline phase on Day 15 (Figure 3).

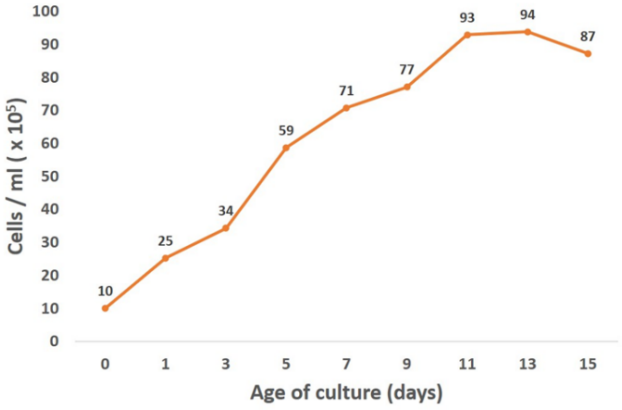

Fig 3. Growth pattern of *G. emersonii* LDC-1 derived with cell count using Neubauer Chamber

The growth pattern of *G. emersonii* LDC-1 was recorded with cell count on 48 hours interval from day 1 to day 13 of culture incubation. The observed growth pattern indicated that the culture was in lag phase on day 1 (25 x 10^5 cells/ml) and day 2, showed exponential growth through log phase that extended from day 3 to day 11 (34 x 10^5 cells/ml to 93 x 10^5 cells/ml)and in

stationary phase on day 12 and 13 (94 x 10^5 cells/ml) and entered into decline phase on day 15 (87 x 10^5 cells/ml)

Preliminary pigment analysis of *G. emersonii* LDC-1 biomass showed that the chlorophyll a and b content were estimated to be 1.0mg/L and 0.45mg/L on day 1 and 6.32mg/L and 2.44mg/L on day 15 respectively, with an increasing trend from day 1 to 15 (Figure 4). The estimated amount of carotenoids was also found to show an increasing trend from day1 (0.16mg/L) to day 15 (1.55mg/L) of incubation (Figure 4).

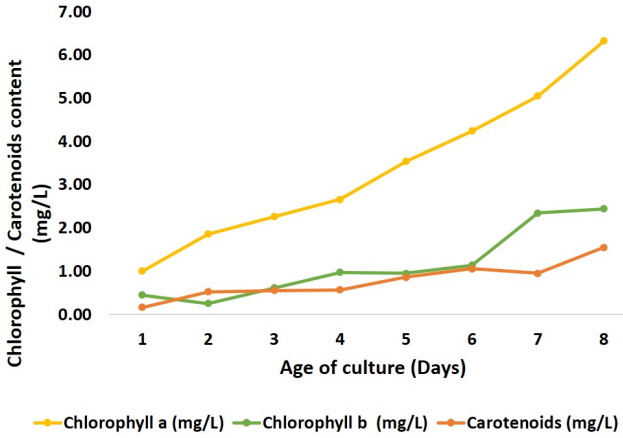

Fig 4. Chlorophyll and Carotenoids content in *G. emersonii* LDC-1. Chlorophyll content was estimated by Lichtenthaler and Wellburn method. Chlorophyll a and b content were estimated to be on an increasing trend from day 1 (1.0 mg/L and 0.45 mg/L) to day 15 (6.3 mg/L and 2.3 mg/L) in a trend aligning the observed growth pattern of *G. emersonii* LDC-1. Carotenoid content was also estimated by Lichtenthaler and Wellburn method and found to show an increasing trend from day1 to day 15 (0.16 mg/L to 1.55mg/L) along the growth pattern of *G. emersonii* LDC-1

Lipid production in *G. emersonii* LDC-1 was estimated with culture maintained in batch mode in triplicates, culture samples were collected from each flask on alternate days for the analysis. Lipid yield was found to increase from day 1 to day 11 of the culture period with the maximum yield on day 11 (46.6 $\pm$ 1.0 mg/gm Dry weight of biomass).

3.3 Lipid Productivity

The estimated lipid content of *G. emersonii* LDC-1 biomass harvested every 48 hours from day 1 to day 13 (Figure 6), showed an increase from 1.4% Dry Weight (DW) to 4.7% DW. Additionally, the Nile Red staining results confirmed lipid droplet accumulation in *G. emersonii* LDC-1, which appeared as bright yellow spots under a fluorescent microscope (Figure 7).

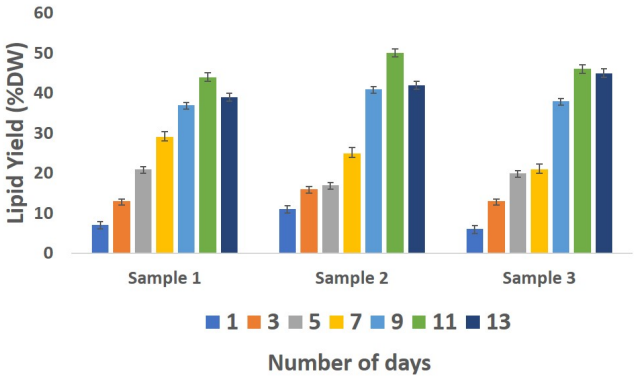

Fig 5. Lipid production in *G. emersonii* LDC-1

Nile red staining was carried out with the day 11 culture sample with highest lipid accumulation. Intracellular lipid droplets of microalgae were observed as bright yellow-colored droplets after staining in a fluorescence microscope.

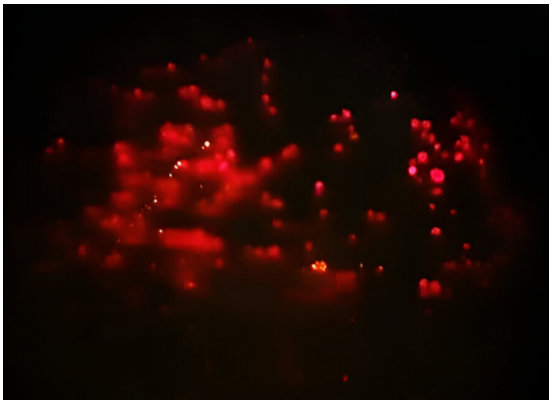

Fig 6. Nile red staining of *G. emersonii* LDC-1

FAME profile of *G. emersonii* LDC-1 was analyzed to assess the proportion of different types of fatty acids and other compounds. Saturated fatty acids (SFAs) were found to dominate during day 1 (40.70%) to day 3(44.90%) (lag phase and initial log phase). Polyunsaturated fatty acids (PUFA) content was found to be the highest in proportion among the entire FAME compounds from day 5 (47.5%) to day 13(61.1%). Monounsaturated fatty acids (MUFA) content was found to be highest on Day1(17.8%)) and then followed a decreasing trend till day 13.

3.4 FAME Profile of *G. emersonii* LDC-1

The fatty acid methyl ester (FAME) profile of *G. emersonii* LDC-1, as determined through Gas Chromatography/Mass Spectroscopy (GC/MS) analysis, showed a significant increase in fatty acid production towards the end of the exponential phase on day 11. The composition was predominantly made up of Polyunsaturated Fatty Acids (PUFAs), accounting for 61.7% of the total. This was followed by Saturated Fatty Acids (SFAs) at 22.4%, then Monounsaturated Fatty Acids (MUFAs) at 5.6%, with other compounds making up the remaining 10.3% in the FAME profile.

Do et al. isolated *Graesiella emersonii* from freshwater samples collected on Ulleungdo Island, South Korea, as part of their study on its biodiesel properties. They characterized FAME profile of this isolated strain and reported that it produced only 40.25% polyunsaturated fatty acids (PUFAs), which is significantly lower than the PUFA content of the current isolate, *G. emersonii* LDC-1 (61.7%)[16].

Palmitic acid (C16:0) was the predominant saturated fatty acid, making up 26.15% during the lag and early log phases (Figure 7). In the monounsaturated fatty acids, cis-10-nonadecenoic acid dominated at 9.02% in the lag phase, while elaidic acid represented 3.96% during the log phase.

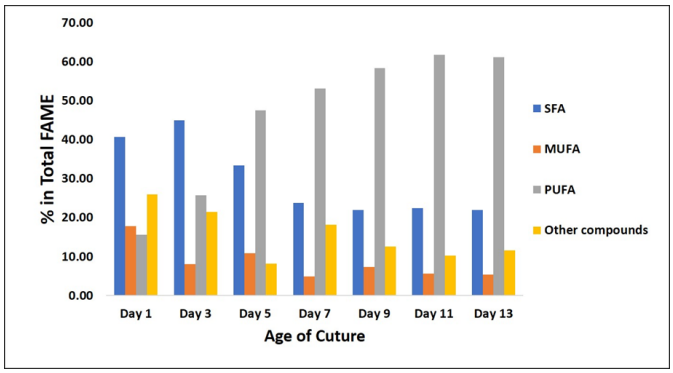

Fig 7. Saturated fatty acids, unsaturated fatty acids and other compounds in FAME profile of *G. emersonii* LDC-1

The major fatty acids of *G. emersonii* LDC-1, defined as those contributing over 50% of total SFAs, MUFAs, and PUFAs, include palmitic and stearic acids (SFAs), oleic acid (MUFA), and linoleic acid (C18:2), α-linolenic acid (C18:3), 8,11,14-docosatrienoic acid (C22:3), and docosahexaenoic acid (C22:6) from the FAME profile (Table 1).

Table 1. Major fatty acids in the FAME profile of *G. emersonii* LDC1

Name	Day 1	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13	Note
Palmitic Acid(C16:0)	24.4	26.2	24.2	18.6	18	18.6	18.9	SFA
Stearic Acid(C18:0)	5.3	6.4	2.9	2.6	2.3	2.6	1.8	SFA
Oleic acid(C18:1)	6.4	3.3	5.6	4	4.4	3.9	3.7	MUFA
Linoleic Acid(C18:2)	4.4	6.4	10.3	15.2	13.5	16.9	16.5	PUFA (n-6)
Alpha Linolenic acid(C18:3)	0	0	0	3.3	34	36	35.7	PUFA (n-3)
8,11,14-Docosatrienoic acid(C22:3)	11.2	17.5	28.3	27.3	0	0	0	PUFA
Docosahexaenoic acid(C22:6)	0	0	5.32	2.8	6	6.1	6	PUFA (n-3)

Fatty acids that contributed more than 50% of the total SFAs, MUFAs and PUFAs in the FAME profile of *G. emersonii* LDC1 were chosen as major fatty acids and the data on the amount of individual fatty acids produced on different days of culture incubation were subjected to one way ANOVA and p value of 0.02 showed the statistical significance.
SFA- Saturated Fatty Acid, MUFA- Mono Unsaturated Fatty Acid, PUFA- Poly Unsaturated Fatty Acid, n-3 – Omega-3 Fatty acid, n-6 –Omega- 6 Fatty acid

Table 2. Fatty acid methyl ester profile of *Graesiella emersonii* LDC1 screened through different days of growth cycle

Fatty Acid (FA)	Day 1 %FA	Day 3 %FA	Day 5 %FA	Day 7 %FA	Day 9 %FA	Day 11 %FA	Day 13 %FA
Caproic acid(C6:0)		1.5 ± 0.0	2.4 ± 0.0				
Pelargonic acid(C9:0)	2.0 ± 0.0						
Lauric Acid(C12:0)	3.4 ± 0.0	2.0 ± 0.0	0.8 ± 0.0	1.4 ± 0.0		0.5 ± 0.0	0.5 ± 0.0
Tridecanoic acid(C13:0)		3.0 ± 0.0	1.2 ± 0.0				
Myristic Acid(C14:0)	3.0 ± 0.0	2.5 ± 0.0	1.9 ± 0.3	1.2 ± 0.1	0.6 ± 0.0	0.7 ± 0.2	0.7 ± 0.2
Myristoleic acid(C14:1)			3.6 ± 0.0				
Palmitic Acid(C16:0)	24.4 ± 2.5	26.2 ± 3.2	24.2 ± 2.0	18.6 ± 3.4	18.0 ± 0.6	18.6 ± 0.9	18.9 ± 0.4
Palmitoleic acid(C16:1)	2.2 ± 0.0	0.8 ± 0.0	1.7 ± 0.0	0.9 ± 0.0	0.4 ± 0.0		
7,10-Hexadecadienoic acid(C16:1)			1.5 ± 0.5	3.3 ± 0.3	2.3 ± 0.0		
cis-10-Heptadecenoic acid(C17:1)		3.9 ± 1.8					
Stearic Acid(C18:0)	5.3 ± 0.0	6.4 ± 0.5	2.9 ± 0.2	2.6 ± 0.5	2.3 ± 0.5	2.6 ± 0.4	1.8 ± 0.5
Oleic acid	6.4 ± 0.0	3.3 ± 0.4	5.6 ± 0.8	4.0 ± 0.9	4.4 ± 0.2	3.9 ± 0.3	3.7 ± 0.3
Vaccenic acid(C18:1)					2.5 ± 0.0		
Linoleic Acid(C18:2)	4.4 ± 0.4	6.4 ± 0.9	10.3 ± 0.4	15.2 ± 0.9	13.5± 0.9	16.9 ± 0.9	16.5 ± 0.8
Alpha Linolenic acid(C18:3)				3.3 ± 0.1	34.0 ± 4.3	36.0 ± 1.1	35.7 ± 1.7
Gamma linolenic acid(C18:3)						0.5 ± 0.1	0.5 ± 0.1
Stearidonic acid(C18:4)				1.2 ± 0.0	1.3 ± 0.2	1.7 ± 0.3	1.9 ± 0.1
cis-10-Nonadecenoic acid(C19:1)	9.2 ± 0.0					1.7 ± 0.0	1.7 ± 0.0
Arachidic acid(C20:0)		1.1 ± 0.0					
Dihomo-alpha-linolenic acid(C20:3)		1.8 ± 0.0	2.1 ± 0.4				
Arachidonic acid(C20:4)					1.2 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
Heneicosanoic acid(C21:0)	2.6 ± 0.0						
8,11,14-Docosatrienoic acid(C22:3)	11.2 ± 2.3	17.5 ± 3.7	28.3 ± 1.4	27.3 ± 1.7			
Docosahexaenoic acid(C22:6)			5.32 ± 0.2	2.8 ± 0.4	6.0 ± 1.2	6.1 ± 1.1	6.0 ± 0.1
Montanic acid(C28:0)					1.0 ± 0.0		
Melissic acid(C30:0)		2.2 ± 0.0					
Percentage of SFA	**40.7**	**44.9**	**33.4**	**23.8**	**21.9**	**22.4**	**21.9**

Continued on next page

Table 2 continued

Percentage of MUFA	**17.8**	**8**	**10.9**	**4.9**	**7.3**	**5.6**	**5.4**
Percentage of PUFA	**15.6**	**25.7**	**47.52**	**53.1**	**58.3**	**61.7**	**61.1**
Percentage of FA in Total FAME	**74.1**	**78.6**	**91.82**	**81.8**	**87.5**	**89.7**	**88.4**
Percentage of Others in Total FAME	**25.9**	**21.4**	**8.18**	**18.2**	**12.5**	**10.3**	**11.6**

***FA** – Fatty Acids **, %FA-** Fatty acids composition in percentage of total FAME components (Values are indicated as Mean ± Standard Deviation)
FAME profile of the isolate indicated that the production of SFAs was higher till day 3 (44.9%) and then the production of PUFAs was found to be higher than SFAs and MUFAs from day 5 (47.52%) to day 13 (61.7%).

Notably, 8,11,14- Docosatrienoic acid, a much unexplored omega-8 fatty acid with potential health benefits like cardioprotective function was found to dominate (28.3%) the FAME profile of *G. emersonii* LDC-1 till the early log phase (till Day 7) [17]. 8,11,14-Docosatrienoic acid(C22:3), is a physiologically functional, but, a rare ω-8 fatty acid, was reported as a major fatty acid accounting for 20% of total lipid content in only a green microalga that was isolated from a Salt Lake in Tibet Plateau, China, classified as genus Desmodesmus. So, the current study is the second scientific report on fresh water green microalgae with this rare ω-8 fatty acid as a major fatty acid contributing to 28.3% of total fatty acids [18].

A one-way ANOVA was conducted to assess the differences in fatty acid production over the culture period, yielding a p-value of 0.002. Since this value is below 0.01, the differences are statistically significant. The highest levels of polyunsaturated fatty acids (PUFAs) were observed on day 11, so the biomass harvested on this day was used for further studies.

During the late exponential phase and till the stationary phase of the culture growth, the production of α-linolenic acid, an omega-3 PUFA, dominated the FAME profile (36.03%), which was comparatively greater than other *G. emersonii* isolates that were previously reported in the literature including *Graesiella emersonii* GEGS21 (22.1 %) and *Graesiella emersonii* MM0036 (27.2%), but lesser than an already reported strain *Graesiella emersonii* NFW2 (45.04%).

Pharmacological research showed that α-Linolenic acid has anticancer, anti-inflammatory, anti-oxidant, anti-obesity, and neuroprotection functions. Also was reported to regulate the properties of intestinal microflora [19]. The novelty of the FAME profile of *G. emersonii* was the confirmation of its ability to produce Docosahexaenoic acid (DHA) (up to 6.10%) during the exponential phase (Days 5 -13) of the culture growth, since no other *Graesiella emersonii* strains isolated and characterized so far including *Graesiella emersonii* NFW2, were reported to produce DHA [20].

Compared to *G. emersonii* GEGS21 which was isolated and characterized from Geumgang Estuary, Korea, the isolate of the current study *G. emersonii* LDC-1 was found to accumulate less amount of linoleic acid (14%) but more amount of linolenic acid (36.03%). Docosahexaenoic acid (DHA, $C_{22:6}$ n-3) was not detected in the strain *G. emersonii* GEGS21, but in the current study, *G. emersonii* LDC-1 was found to produce DHA (6.1%), an omega-3 fatty acid with potential health benefits. Omega-3 fatty acids are long-chain polyunsaturated fatty acids (LC-PUFAs) that were found to have their first double bond at the position of the third carbon from the omega end of the fatty acid chain. Omega-3 fatty acids that are much explored for their biological function and biomedical applications are α-linolenic acid (ALA, C18:3), eicosapentaenoic acid (EPA C20:5), and docosahexaenoic acid (DHA C22:6) [21].

The unique structural property of the omega-3 LC-PUFAs including long carbon chains and double bonds at multiple positions provides special properties for omega-3 fatty acids, especially for EPA and DHA, that may further strengthen their role towards health benefits. Research reports indicate that a daily dietary intake of 2–4 g of combined EPA and DHA decreases the risk of cardiovascular health issues in individuals with cardiovascular disease by their ability to influence the rate of oxidation and signal transduction pathway by altering lipid rafts of the plasma membrane by interacting with the surrounding phospholipids and thus lowering cholesterol accumulation in the cell membrane [22,23]. In an embryological study involving bovine models, it was reported that embryo elongation in the omega-3-treated group was enhanced compared to the omega-6-treated group [24].

Humans are unable to synthesize linoleic acid (LA) and α-linolenic acid (ALA) from palmitic acid (PA) and oleic acid (OLA) because they lack delta-12 desaturase (D12Des), an essential enzyme. The conversion of LA and ALA into EPA and DHA is also inefficient in the human body, leading to recommendations for daily dietary supplementation of these fatty acids for better health. This has resulted in a growing market demand for omega-3 fatty acids. Although fish oil is currently the main source, it cannot meet this demand due to supply fluctuations and cost issues. Thus, exploring alternative sources, such as microalgae, is necessary for a consistent supply of omega-3 fatty acids [25,26].

Elongases and desaturases are the enzymes that play a crucial role in the biosynthesis of long-chain PUFA from precursor PUFA. Among the elongases and desaturases involved in the PUFA biosynthesis Delta-5 desaturase (D5D) and delta-6 desaturase (D6D) were reported as two key enzymes playing a vital role in the synthesis of long-chain PUFA. These two enzymes

D5D and D6D are encoded by fatty acid desaturase 1 (FADS1) and FADS2 genes, respectively [27]. D5D especially was reported to play a significant role in the long chain PUFA synthesis by the conversion of linoleic acid (LA, 18:2 n-6) and α-linolenic acid (ALA, 18:3 n-3) to arachidonic acid (AA, 20:4 n-6) and eicosapentaenoic acid (EPA, 20:5 n-3), respectively, thus projects its role as the sole enzymatic source of endogenous arachidonic acid and eicosapentaenoic acid [28].

G. emersonii LDC-1 FAME profile revealed that it is a good source of PUFAs especially Omega-3 FAs, since its FAME profile highlighted its ability to produce three among the four Omega-3 PUFAs including linoleic acid, α-linolenic acid, and DHA. It has also provided a scope for the production of DHA, a long-chain Omega-3 PUFA as 6.1% of DHA was reported in the FAME profile of *G. emersonii* LDC-1. The isolate could have utilized the n-3 fatty acid biosynthesis pathway to synthesize DHA, given that the precursor (ALA) and intermediate compounds (Eicosatrienoic acid, ERA, and Eicosatetraenoic acid, ETA) of this pathway were identified in the FAME profile. This has given a scope for increasing the production of DHA in *G. emersonii* by upregulating the expression of the delta 5 desaturase and delta 5 elongase to increase the rate of conversion of ERA and ETA to Eicosapentaenoic acid (EPA) and Docosapentaenoic acid (DPA). As immediate precursors of docosahexaenoic acid, upregulation of EPA and DPA production can increase the DHA production in *G. emersonii* LDC-1.

Modifying the expression of key enzymes in metabolic pathways at the genetic level can drive carbon flow toward the product direction, thereby increasing product yield [29]. In the future, *G. emersonii* LDC-1 can be metabolically engineered to serve as a promising source of omega-3 fatty acids with potential health benefits.

4 Conclusion

The FAME profile analysis of *G. emersonii* LDC-1 indicates that it is a valuable source of polyunsaturated fatty acids (PUFAs), particularly Omega-3 fatty acids, because it has demonstrated the capability to synthesize three out of the four omega-3 PUFAs, which are linoleic acid, α-linolenic acid, and DHA. Especially the noteworthy, 6.1% content of DHA that was reported in the FAME profile of *G. emersonii* LDC-1, indicating promising prospects for DHA production. It is notable that, *G. emersonii* LDC-1 employs the n-3 Omega-3 FA biosynthesis pathway for DHA production, with the precursors (ALA) and intermediates (Eicosatrienoic acid, ERA, and Eicosatetraenoic acid, ETA) in its FAME profile. Consequently, upregulation of delta 5 desaturase and delta 5 elongase expression in *G. emersonii* LDC-1 can open avenues in the future, to enhance DHA production, thereby augmenting the conversion rate of ERA and ETA to eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA). In the future, metabolic engineering and standardization of the mass cultivation protocol of *G. emersonii* LDC-1, can support its ability to serve as a promising source of omega-3 fatty acids. 8,11,14-Docosatrienoic acid(C22:3), a very long chain omega-8 PUFA, though reported to be physiologically functional, as a rare fatty with low yield from natural sources, further research for exploration of its applications or manipulation of production is limited. Thus, this study has provided an opportunity to explore the possible applications of this rare fatty acid with *G. emersonii* LDC-1 as a supportive natural source through bioprocess engineering and large-scale cultivation.

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