



Original Research Paper

Comprehensive Biochemical Characterization and Lipid Profiling of *Chlorella vulgaris* with Evaluation of Its Antioxidant and Antibacterial ActivitiesPasha Haritha¹, B. Kirthiga², Jagadeeswari. S^{3*}¹Research Scholar, PG & Research Department of Microbiology, Dwaraka Doss Goverdhan Doss Vaishnav College (Autonomous), Chennai, Tamil Nadu – 600106, India. Email: Harithaammisetty@gmail.com²Associate Professor, PG & Research Department of Microbiology, Dwaraka Doss Goverdhan Doss Vaishnav College (Autonomous), Chennai, Tamil Nadu – 600106, India. Email: Kirthiga@dgvaishnavcollege.edu.in^{3*}Associate Professor & Head- PG & Research Department of Microbiology, Dwaraka Doss Goverdhan Doss Vaishnav College (Autonomous), Chennai, Tamil Nadu – 600106, India. Email: jagadeeswari@dgvaishnavcollege.edu.in

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Key Words Abstract

Biochemical profiling, Extraction, Characterization, Antibacterial activity, Bioactive compounds

Chlorella vulgaris was cultivated under open-pond and closed-photobioreactor systems to evaluate its biochemical composition, lipid profile, antioxidant capacity and antibacterial efficacy. Photobioreactor cultivation significantly enhanced total pigments by 67.9% (8.16 vs. 4.86 mg/ g⁻¹ dry cell weight) and soluble protein by 58.5% (2.33 vs. 1.47 mg/ g⁻¹ dry cell weight). Conversely, open-pond biomass yielded two-fold higher carbohydrate content (0.40 vs. 0.20 mg/ g⁻¹ dry cell weight) and higher gravimetric lipid yield (18.40% vs. 11.20%). However, higher final biomass density in the photobioreactor resulted in superior volumetric lipid productivity (10.32 vs. 8.41 mg/ L⁻¹). FTIR analysis confirmed characteristic functional groups. Urea inclusion crystallization effectively enriched polyunsaturated fatty acids (PUFA recovery: 38.0 ± 2.0%), with GC–MS identifying eicosapentaenoic acid (EPA, 42.90%) and docosahexaenoic acid (DHA, 7.73%) as major omega-3 constituents. The PUFA fraction exhibited dose-dependent DPPH radical scavenging (15.20-48.60%; IC₅₀ = 105.80 µg/mL). In antibacterial assays against four major pathogens, the extract displayed potent, Gram-selective activity at 75 µg/mL, the zone of inhibition against *Staphylococcus aureus* (2.2 cm) surpassed the Streptomycin control (2.0 cm), alongside moderate inhibition of *Bacillus subtilis* (1.6 cm), *Escherichia coli* (1.5 cm) and *Salmonella enterica* (1.3 cm). These results highlight the strong influence of cultivation systems on microalgal metabolism and establish *C. vulgaris* PUFA extract as a promising Gram-selective antimicrobial and antioxidant candidate for nutraceutical, pharmaceutical and cosmeceutical applications.

INTRODUCTION

Chlorella vulgaris is a unicellular freshwater green microalga belonging to the division Chlorophyta. It is widely investigated because of its ability to produce proteins, carbohydrates, lipids, pigments, vitamins, minerals and other biologically active compounds. These characteristics have contributed to the use of *C. vulgaris* in food, nutraceutical, pharmaceutical, cosmetic and environmental biotechnology applications [1–3].

The biochemical composition of microalgal biomass is influenced by strain characteristics and cultivation conditions, including light intensity, temperature, pH, nutrient availability, aeration, mixing and cultivation system [4]. Open-pond systems are relatively inexpensive but are exposed to environmental fluctuations and contamination. Closed photobioreactors provide better control of cultivation conditions and may improve biomass consistency and

product quality [5]. Lipids are among the most important components of microalgal biomass because they may contain saturated, monounsaturated and polyunsaturated fatty acids. Some of these fatty acids have nutritional, antioxidant, antimicrobial and industrial value [6,7]. The relative distribution of fatty acids may change in response to cultivation conditions and environmental stress.

Fourier-transform infrared spectroscopy provides information about the major functional groups present in a complex lipid extract. However, FTIR does not generally identify individual fatty acids. Gas chromatography–mass spectrometry of fatty-acid methyl esters provides compound-level information about the fatty-acid composition. Therefore, the combined use of FTIR and GC–MS provides information about the chemical characteristics of microalgal lipids [8–10].

The present study involved lipid extraction, fatty acid

methyl ester (FAME) preparation, Fourier-transform infrared spectroscopy (FTIR) and gas chromatography mass spectrometry (GC–MS) characterization. The antioxidant activity of the lipid extracts was evaluated using the DPPH radical-scavenging assay, while their antibacterial potential was assessed against selected bacterial strains. These investigations provide useful information on the influence of cultivation conditions on the biochemical composition and bioactive properties of *C. vulgaris*, supporting its potential applications in health-related and industrial fields.

2. Materials and Methods

2.1 Sample Collection, Isolation and Molecular Identification

Sample collection, isolation and molecular identification were performed according to the procedure described in our previous study. Briefly, water samples were enriched in BG-11 medium and the microalgal isolate was purified by serial dilution, plating and repeated subculturing. The purified isolate was identified by microscopic examination and *rbcL* gene-based molecular analysis. The detailed PCR sequencing and phylogenetic procedures are provided in our previous publication [11].

2.2 Cultivation and Biomass Harvesting

The identified *C. vulgaris* isolate was cultivated in BG-11 medium under open-pond and closed bioreactor conditions. Culture growth was monitored by optical density and changes in culture colour. Biomass was harvested at the stationary phase by centrifugation, washed with distilled water and dried before biochemical and lipid analyses [11]. The final biomass concentrations were 0.64 g/L for the open-pond culture and 1.29 g/L for the closed-bioreactor culture.

2.3 Pigment Estimation

10mg of dried *Chlorella vulgaris* biomass were resuspended in 10 mL of 90% ethanol. The mixtures were centrifuged at 4000 rpm for 10 min and the supernatants were collected. This extraction step was repeated until the cell pellet became completely colourless. The combined ethanol extracts were brought to a final volume for spectrophotometric analysis. Absorbance was measured at 663, 645 and 470 nm using 90% ethanol as the blank. Chlorophyll a, chlorophyll b and total carotenoids were calculated using standard equations and expressed as mg/g dry cell weight [12].

$$\begin{aligned} \text{Chlorophyll a (mg/g DCW)} &= \frac{(12.70 \cdot A_{663} - 2.69 \cdot A_{645}) \times V_{\text{ethanol}}}{W_{\text{dry biomass}}} \\ \text{Chlorophyll b (mg/g DCW)} &= \frac{(22.90 \cdot A_{645} - 4.68 \cdot A_{663}) \times V_{\text{ethanol}}}{W_{\text{dry biomass}}} \\ \text{Total Carotenoids (mg/g DCW)} &= \frac{\left[\frac{1000 \cdot A_{470} - 1.91 \cdot C_a - 95.2 \cdot C_b}{225} \right] \times V_{\text{ethanol}}}{W_{\text{dry biomass}}} \end{aligned}$$

2.4 Total Carbohydrate Estimation

Total carbohydrate content was evaluated using the phenol-sulfuric acid colorimetric method following acid hydrolysis. 15 mg of dry microalgal biomass was

hydrolyzed with concentrated hydrochloric acid (HCl) at a 1:2.5 biomass-to-acid ratio. The resulting hydrolysate was neutralized with 1 M sodium carbonate (Na_2CO_3) until the acidic odor dissolved and subsequently centrifuged at 10,000 rpm for 5 min to yield a clear supernatant.

1 mL aliquot of the supernatant was transferred to a glass reaction tube, mixed with 1 mL of 5% aqueous phenol solution and rapidly injected with 3 mL of 96% concentrated sulfuric acid (H_2SO_4). The mixture was incubated in the dark at room temperature for 20 min to enable full complex colour development. Optical density was recorded at 490 nm (A_{490}) using a UV-Vis spectrophotometer against a distilled water reagent blank [13]. Carbohydrate concentrations were derived using a D-glucose standard calibration curve and calculated as follows:

$$\begin{aligned} \text{Carbohydrate Content (mg/g DCW)} &= \frac{C_{\text{glucose}} \times V_{\text{extract}}}{W_{\text{dry biomass}}} \end{aligned}$$

2.5. Total Soluble Proteins Estimation

Total soluble proteins were estimated using the Lowry's method. 15 mg of dried biomass was suspended in lysis buffer and incubated at 45°C for 30 min. A small quantity of sodium dodecyl sulfate (SDS) was added to facilitate complete cell wall disruption and membrane solubilization. The mixtures were centrifuged at 12,000 rpm for 5 min and the clear protein-containing supernatant was collected.

1 mL aliquot of the supernatant was combined with an equal volume of Reagent 1 (prepared by mixing 2% sodium carbonate in 0.1 N NaOH, 1% sodium potassium tartrate and 0.5% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in a 48:1:1 volume ratio). After thorough mixing, an equal volume of Reagent 2 (Folin-Ciocalteu reagent) was added and the reaction mixtures were incubated in the dark at room temperature for 20 min for colour development. Absorbance was measured at 660 nm. Bovine serum albumin was used as the standard and protein concentration was expressed as mg g^{-1} dry cell weight [14].

$$\text{Protein Content (mg/g DCW)} = \frac{C_{\text{protein}} \times V_{\text{extract}}}{W_{\text{dry biomass}}}$$

2.6 Total Lipid Extraction (Folch Method)

Total lipids were extracted according to Folch method. 2g of dried biomass were homogenized with 40 mL of chloroform: methanol mixture at a ratio of 2:1 (v/v) and stirred for 30 min at room temperature. Phase separation was induced by adding 0.73% sodium chloride solution. Following centrifugation at 3000 rpm for 10 min, the lower organic phase was collected and evaporated under reduced pressure using a rotary evaporator at 35°C. The dried lipid residue was weighed [15]. Lipid yield was calculated as:

$$\text{Lipid Yield \%} = \left(\frac{\text{Oil weight}}{\text{Dry cell weight}} \right) \times 100$$

2.7 FTIR analysis of lipid extracts

The crude lipid extracts were analysed using a Bruker FTIR spectrometer. Spectra were recorded over the range of 4000–400 cm^{-1} . The spectra were used to

identify major functional groups associated with the extracted lipids, including aliphatic C–H bonds, ester carbonyl groups and unsaturated carbon–carbon bonds [16]. FTIR analysis was used to characterize the chemical classes and functional groups present in the extracts, individual fatty acids were identified separately by GC–MS.

2.8 Urea inclusion crystallization

Polyunsaturated fatty acids (PUFAs) were enriched using urea inclusion crystallization based on Hayes method. One gram of crude lipid extract was saponified with ethanolic potassium hydroxide at 60°C for 1 h under nitrogen purging, acidified to pH ~2 with hydrochloric acid and extracted into n-hexane to yield free fatty acids (FFAs). The FFAs were mixed with methanol-dissolved urea (1:3–1:4 lipid-to-urea ratio, w/w), heated at 60°C until clear, cooled to room temperature and incubated at 20°C for 12 h. The solid urea complexes containing saturated and monounsaturated fatty acids were removed by vacuum filtration. The filtrate containing uncomplexed PUFAs (α -linolenic and linoleic acids) was washed with acidified water, re-extracted into n-hexane and evaporated under nitrogen gas at 35°C to recover the purified PUFA fraction [17].

2.9 FAME preparation and GC–MS analysis

Fatty acid methyl esters (FAMES) were prepared according to standard transesterification procedures. An aliquot of the lipid extract was treated with methanolic NaOH at 80°C for 15 mins to liberate free fatty acids, followed by refluxing with boron trifluoride–methanol (BF₃–MeOH) reagent at 80°C for 10 min to yield FAMES. After cooling, 1-bromotetradecane was added as an internal standard, followed by n-heptane and a saturated sodium chloride solution. The mixture was vortexed thoroughly and the upper n-heptane layer containing the purified FAMES was collected and injected into a gas chromatography–mass spectrometry (GC–MS) system equipped with an HP-5MS capillary column (30 m × 0.25 mm × 0.25 μ m) using helium as the carrier gas (1.0 mL/min). Fatty acids were identified by matching their mass spectra against the NIST spectral database and their relative abundances were calculated from the integrated peak areas relative to the internal standard [18].

2.10 Evaluation of Antioxidant Activity (DPPH Assay)

The free radical-scavenging capacity of the enriched *Chlorella vulgaris* polyunsaturated fatty acid (PUFA) extract was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Serial concentrations of the PUFA concentrate (20,40,60,80, and 100 μ g/mL) were mixed with a 0.1 mM methanolic DPPH solution.

The reaction mixtures were vortexed thoroughly and incubated in the dark at room temperature (25 $\pm$ 2 °C) for 30 mins. Absorbance was recorded at 517 nm using a UV–Visible spectrophotometer against a methanol blank. Ascorbic acid served as the positive reference standard and a sample-free DPPH–methanol solution was used as the negative control. All measurements were conducted in triplicate (n = 3). The DPPH radical-scavenging percentage was calculated using Eq. (1):

$$\text{DPPH Scavenging Activity (\%)} = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100$$

The half-maximal inhibitory concentration (IC₅₀) was determined via linear regression analysis of the concentration response curve.

2.11 Evaluation of Antibacterial Activity (Agar Well Diffusion)

The antibacterial activity of the *Chlorella vulgaris* PUFA enriched extract was evaluated using the agar well diffusion method against four representative bacterial pathogens two Gram-positive strains (*Staphylococcus aureus* and *Bacillus subtilis*) and two Gram-negative strains (*Escherichia coli* and *Salmonella enterica*). Mueller Hinton agar (MHA) plates were inoculated with standardized bacterial suspensions to form uniform lawns. Wells of 6 mm diameter were punched into the agar using a sterile cork borer and loaded with 100 μ L of the PUFA extract at varying concentrations (25, 50 and 75 μ g/mL) solubilized in dimethyl sulfoxide (DMSO). Streptomycin served as the positive antibiotic control. The plates were incubated at 37°C for 24 h and the resulting net zones of inhibition (including well diameter, measured in cm) were recorded [20].

3. Results

3.1 Pigment Estimation

Cultivation under closed photobioreactor conditions significantly enhanced microalgal pigment accumulation compared to the open-pond system (Table 1). Photobioreactor-derived biomass yielded 5.72 mg/g chlorophyll a, 1.25 mg/g chlorophyll b and 1.19 mg/g total carotenoids, raising total pigment content to 8.16 mg/g DCW. In comparison, open-pond biomass contained 3.40 mg/g chlorophyll a, 0.79 mg/g chlorophyll b and 0.67 mg/g total carotenoids (4.86 mg/g total pigments). Overall, the photobioreactor system increased chlorophyll a, chlorophyll b and total carotenoid concentrations by 68.2%, 58.2%, and 77.6%, respectively, demonstrating superior photosynthetic efficiency and light utilization under controlled cultivation conditions.

Table 1. Pigment yields of *Chlorella vulgaris* by cultivation system

Culture system	Chlorophyll a (mg/g ⁻¹ DCW)	Chlorophyll b (mg/g ⁻¹ DCW)	Total carotenoids (mg/g ⁻¹ DCW)	Total pigments (mg/g ⁻¹ DCW)
Open pond	3.40	0.79	0.67	4.86
Bioreactor	5.72	1.25	1.19	8.16

3.2 Total Carbohydrate Estimation

Open pond biomass exhibited a two-fold higher total carbohydrate content ($0.40 \text{ mg/g}^{-1} \text{ DCW}$, $A_{490} = 0.268$) compared to the closed bioreactor ($0.20 \text{ mg/g}^{-1} \text{ DCW}$, $A_{490} = 0.158$) (Table 2). This elevated accumulation in open ponds is attributed to unmanaged environmental stress, which shifts microalgal metabolism from cell division toward storage carbohydrate synthesis.

Table 2. Carbohydrate yield of *Chlorella vulgaris* by cultivation system

Culture system	Absorbance at 490 nm	Carbohydrate content ($\text{mg/g}^{-1} \text{ DCW}$)
Open pond	0.268	0.40
Bioreactor	0.158	0.20

3.3. Total Soluble Proteins Estimation

Bioreactor derived biomass exhibited a 58.5% higher soluble protein content ($2.33 \text{ mg/g}^{-1} \text{ DCW}$, $A_{660} = 0.629$) compared to open-pond biomass ($1.47 \text{ mg/g}^{-1} \text{ DCW}$, $A_{660} = 0.455$) (Table 3). Controlled bioreactor conditions such as optimal light availability and managed temperature promoted nitrogen metabolism and active protein synthesis. In contrast, environmental stress in open ponds shifted microalgal metabolism away from protein production and toward storage molecules.

Table 3. Soluble protein content of *Chlorella vulgaris* across cultivation systems

Culture system	Absorbance at 660 nm	Protein content ($\text{mg/g}^{-1} \text{ DCW}$)
Open pond	0.455	1.47
Bioreactor	0.629	2.33

3.4. Total Lipid Extraction

Total lipid content and daily volumetric productivity varied notably between the two cultivation systems (Table 4). Based on an initial dry biomass input of 2g, open pond biomass yielded $0.368 \pm 0.012 \text{ g}$ of total lipids ($18.40 \pm 0.60\%$), whereas closed-bioreactor biomass produced $0.224 \pm 0.008 \text{ g}$ ($11.20 \pm 0.40\%$). The elevated lipid percentage in the open pond is attributed to unmanaged environmental stress triggering neutral lipid storage. However, due to double the final biomass density, the closed bioreactor achieved significantly higher volumetric lipid productivity ($10.32 \pm 0.37 \text{ mg} \cdot \text{L}^{-1}$) than the open pond ($8.41 \pm 0.27 \text{ mg/L}^{-1}$).

Table 4. Comparative biomass density, gravimetric lipid yield and volumetric lipid productivity of *Chlorella vulgaris* across cultivation systems

Parameter	Open Pond System	Bioreactor System
Final Biomass Density (g/L)	0.64	1.29
Initial Sample Biomass (g)	2.00	2.00
Extracted Lipid Mass (g)	0.368 ± 0.012	0.224 ± 0.008
Total Lipid Yield (%)	18.40 ± 0.60	11.20 ± 0.40
Cultivation Duration (days)	14	14
Volumetric Lipid Productivity (mg/L)	8.41 ± 0.27	10.32 ± 0.37

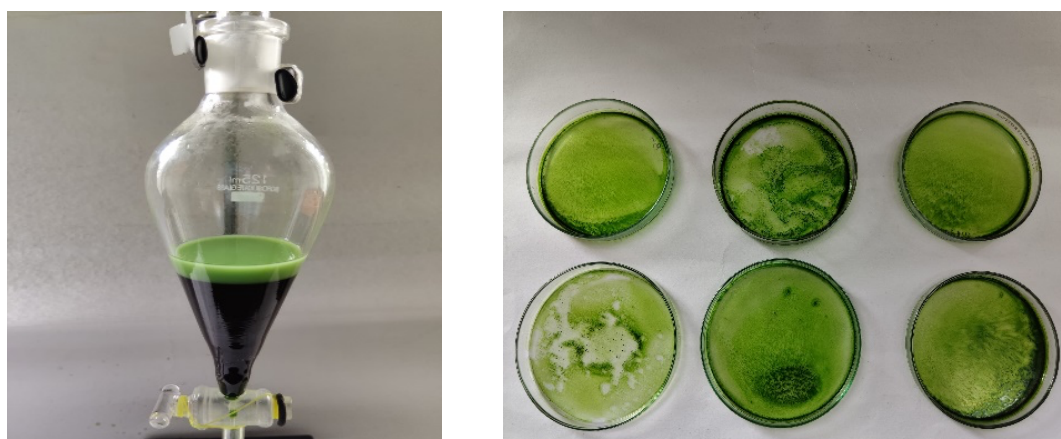


Figure 1: Lipid extraction by Folch Method

3.5. FTIR analysis of lipid extracts

The FTIR spectrum of *Chlorella vulgaris* lipid extract showed key functional groups (Table 5). A broad peak at 3276.88 cm^{-1} indicates O–H stretching of carboxylic acids. The peak at 2923.13 cm^{-1} represents aliphatic C–H stretching. Bands at 1629.21 cm^{-1} and 1538.67 cm^{-1} correspond to N–H bending of primary amines and N–O asymmetric stretching, respectively. Additionally, peaks at 1394.26 cm^{-1} (C–H bending) and 1041.83 cm^{-1} (C–N stretching) confirm the presence of alkanes and amine compounds.

Table 5. FTIR spectral peaks and functional groups of *Chlorella vulgaris* lipid extract

S. No	Frequency cm ⁻¹	Range	Bond	Functional group
1.	3276.88	3400–3250	O-H stretch	Carboxylic acids
2.	2923.13	3100–3000	C–H stretch	aromatics
3.	1629.21	1650–1580	N–H bend	primary amines
4.	1538.67	1550–1475	N–O asymmetric stretch	nitro compounds
5.	1394.26	1370–1350	C–H bend	alkanes
6.	1041.83	1335–1250	C–N stretch	aromatic amines

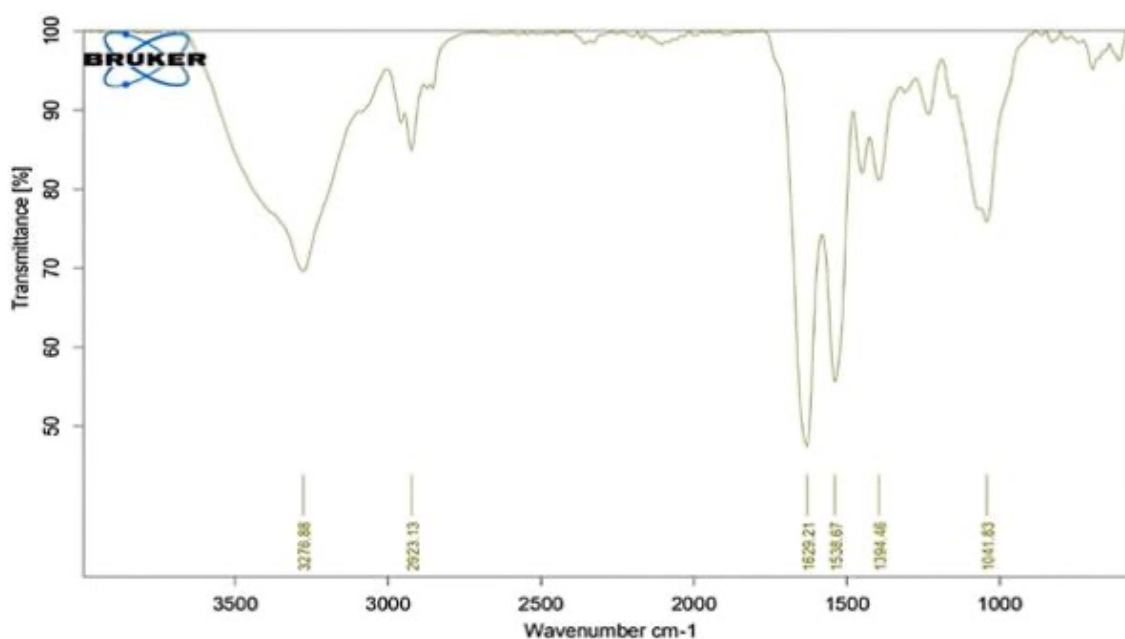


Figure 2: FTIR analysis of *Chlorella vulgaris*

3.6. Urea inclusion crystallization

Urea inclusion crystallization effectively enriched long-chain polyunsaturated fatty acids (PUFAs) from the saponified free fatty acid fraction of *Chlorella vulgaris*. From 1g of crude lipid extract, the liquid filtrate fraction yielded 0.38 ± 0.02 g of purified PUFA concentrate, corresponding to a recovery yield of $38.0 \pm 2.0\%$ (Table 6). Low-temperature crystallization at 20 °C successfully complexed straight chain saturated and monounsaturated fatty acids into solid urea adducts, leaving bulky, multi double bond polyunsaturated fatty acids concentrated in the liquid filtrate for subsequent GC–MS identification.

Table 6. Yield parameters of *Chlorella vulgaris* PUFAs post-urea inclusion crystallization

Parameter	Value
Initial Crude Lipid Input (g)	1.00
Purified PUFA Yield (g)	0.38 ± 0.02
PUFA Recovery Yield (%)	38.0 ± 2.0
Enriched Chemical Class	Long-chain polyunsaturated fatty acids (PUFAs)

3.7. FAME preparation and GC–MS analysis

Transesterification and GC–MS analysis confirmed significant enrichment of long-chain omega-3 PUFAs in *Chlorella vulgaris* lipids (Table 7). The primary component was eicosapentaenoic acid methyl ester (EPA, 42.90%, RT 17.89 min), followed by docosahexaenoic acid methyl ester (DHA, 7.73%, RT 36.39 min) and methyl 2-hydroxy-octadeca-9,12,15-trienoate (12.90%, RT 17.90 min). Together, target omega-3 PUFAs constituted over 63.5% of the total integrated peak area.

Table 7. GC–MS characterization of *Chlorella vulgaris* FAMES and lipid constituents

Peak no.	Retention time	% Area	Compound name	Molecular formula	Molecular Weight
1	3.8937	6.98	Cyclohexene, 3-(bromomethyl)-	C ₇ H ₁₁ Br	177.07
2	9.8065	3.4	6-Methoxy-3-methyl-2-benzofurancarboxylic acid	C ₁₁ H ₁₀ O ₄	214.2
3	12.7446	17.8	p-(Benzylideneamino) pheno	C ₁₃ H ₁₁ NO	197.2
4	17.8945	42.9	5,8,11,14,17-Eicosapentaenoic acid, methyl ester, (all-z)-	C ₂₁ H ₃₂ O ₂	344.52
5	17.9027	12.9	Methyl 2-hydroxy-octadeca-9,12,15-trienoate	C ₁₉ H ₃₂ O ₃	308.5
6	24.4218	27.07	4-Amino-2,6-dihydropyrimidine	C ₈ H ₁₈	138.12
7	30.1812	10.5	Pentacosane	C ₂₅ H ₅₂	352.68
8	36.3885	7.73	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-	C ₂₃ H ₃₄ O ₂	366.57

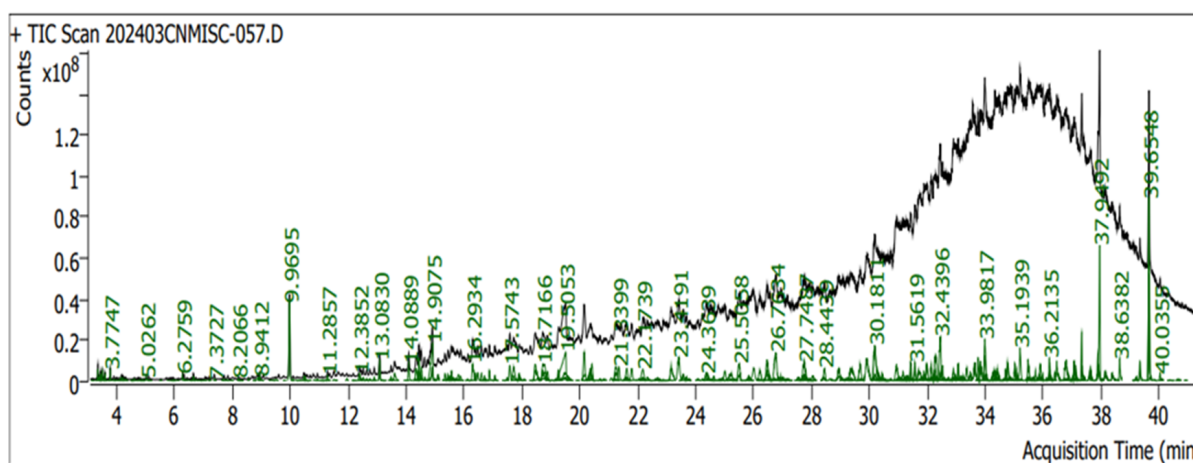


Figure 3: GC–MS analysis of *Chlorella vulgaris*

3.8. Evaluation of Antioxidant Activity

The enriched *Chlorella vulgaris* PUFA extract exhibited concentration dependent DPPH radical scavenging activity across the tested range (20–100 µg/mL) (Table 8). Radical neutralization increased progressively from $15.20 \pm 1.10\%$ at 20 µg/mL to $48.60 \pm 1.90\%$ at 100 µg/mL. Linear regression yielded an IC₅₀ value of 105.80 µg/mL for the PUFA concentrate, compared to 24.60 µg/mL for the ascorbic acid standard. This free radical-scavenging capacity is directly attributed to the high concentration of enriched omega-3 PUFAs, specifically EPA (42.90%) and DHA (7.73%), whose multiple double bonds act as hydrogen donors to stabilize DPPH radicals.

Table 8. DPPH radical-scavenging activity

Concentration (µg/mL)	PUFA Extract Scavenging (%)	Ascorbic Acid Scavenging (%)
20	15.20 ± 1.10	45.80 ± 1.50
40	26.40 ± 1.30	68.20 ± 1.80
60	34.80 ± 1.60	82.40 ± 2.00

80	41.50 ± 1.40	91.10 ± 1.20
100	48.60 ± 1.90	95.50 ± 0.80
IC ₅₀ (µg/mL)	105.80	24.60

3.9. Evaluation of Antibacterial Activity

The *Chlorella vulgaris* PUFA extract demonstrated concentration dependent antibacterial activity across all tested pathogens, displaying higher efficacy against Gram-positive strains. Among Gram-positive bacteria, the highest sensitivity was observed in *Staphylococcus aureus*, where the extract at 75 µg/mL produced an inhibition zone of 2.2 cm, as Streptomycin positive control (2.0 cm). Against *Bacillus subtilis*, the extract achieved a maximum inhibition zone of 1.6 cm at 75 µg/mL compared to 2.2 cm for Streptomycin. For Gram-negative pathogens, the extract exhibited moderate, dose-dependent growth inhibition. At the maximum concentration of 75 µg/mL, inhibition zones reached 1.5 cm for *Escherichia coli* and 1.3 cm for *Salmonella enterica*. In both Gram-negative assays, the extract's antibacterial activity remained below that of the Streptomycin control (2.2 cm and 2.0 cm, respectively).

Target Pathogen	Gram Stain	Positive Control (Streptomycin)	Extract Conc. (25 µg/mL)	Extract Conc. (50 µg/mL)	Extract Conc. (75 µg/mL)
<i>Staphylococcus aureus</i>	Gram-positive (+ve)	2.0	1.2	1.8	2.2
<i>Bacillus subtilis</i>	Gram-positive (+ve)	2.2	1.0	1.4	1.6
<i>Escherichia coli</i>	Gram-negative (-ve)	2.2	1.1	1.4	1.5
<i>Salmonella enterica</i>	Gram-negative (-ve)	2.0	0.9	1.0	1.3

Table 9: Antibacterial activity

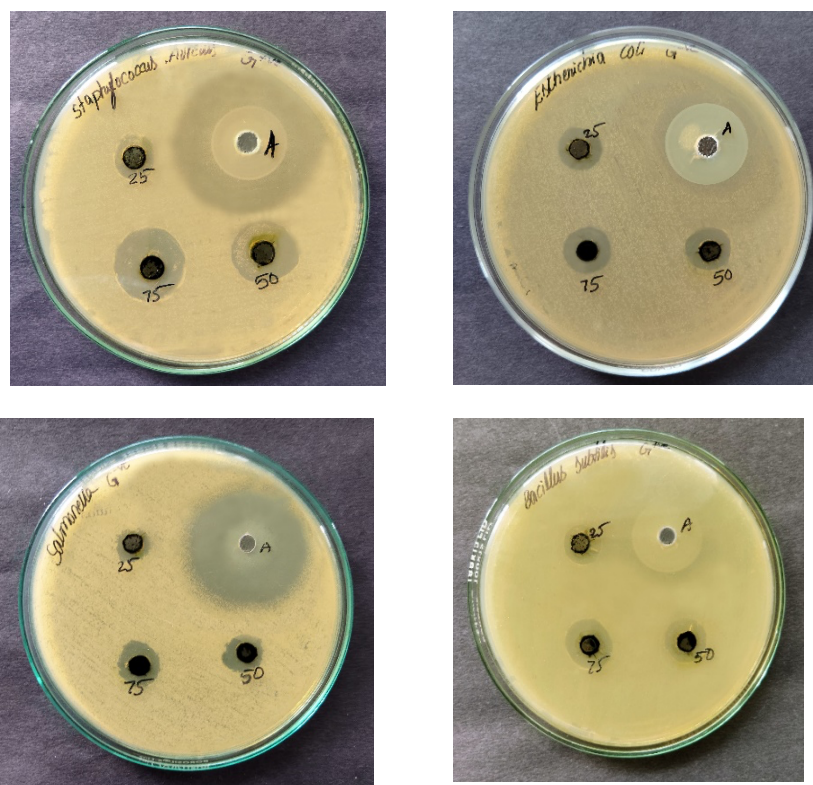


Figure 4. Agar well diffusion assay showing antibacterial activity of the PUFA-enriched *C. vulgaris*

4. Discussion

The comparative cultivation trial demonstrated that photobioreactor conditions markedly enhanced photosynthetic pigment and soluble protein accumulation in *Chlorella vulgaris* relative to the open pond (Tables 1 and 3), consistent with reports that

closed photobioreactors provide more stable light, temperature and nutrient conditions that support efficient photosynthetic and nitrogen-assimilation activity [4,5]. The increases of 68.2%, 58.2% and 77.6% in chlorophyll a, chlorophyll b and total carotenoids, respectively, together with the 58.5%

increase in soluble protein under bioreactor cultivation, indicate that the controlled environment favoured active biomass growth and macromolecule synthesis rather than stress-driven storage metabolism. Conversely, the two-fold higher carbohydrate content recorded in open-pond biomass (Table 2) aligns with the understanding that unmanaged environmental fluctuations in open systems shift microalgal metabolism from active cell division toward storage carbohydrate synthesis as an energy reserve [4,6]. A similar stress-related pattern was observed for total lipid content, with the open pond yielding a higher gravimetric lipid percentage ($18.40 \pm 0.60\%$) than the photobioreactor ($11.20 \pm 0.40\%$) (Table 4), agreeing with observations that environmental stress promotes neutral lipid accumulation in microalgae as a survival mechanism [6,7]. However, when expressed as volumetric productivity, the photobioreactor outperformed the open pond (10.32 vs. $8.41 \text{ mg L}^{-1} \text{ day}^{-1}$), because its substantially higher final biomass density (1.29 vs. 0.64 g L^{-1}) more than compensated for its lower lipid percentage. This underscores that volumetric productivity is the primary metric when evaluating microalgal cultivation systems for downstream applications [5]. FTIR spectroscopic analysis of the total lipid extract (Table 5, Figure 2) revealed key functional groups including carboxylic O–H stretching (3276.88 cm^{-1}), aliphatic C–H stretching (2923.13 cm^{-1}), and amine-associated N–H and C–N vibrations, confirming a chemically complex lipid extract containing both non-polar fatty acyl chains and polar functional groups [15,16]. Following urea inclusion crystallization, which concentrated polyunsaturated fatty acids away from saturated counterparts with a recovery yield of $38.0 \pm 2.0\%$ (Table 6) [17], GC–MS analysis identified eicosapentaenoic acid (EPA, 42.90%) and docosahexaenoic acid (DHA, 7.73%) as major constituents (Table 7). Although *C. vulgaris* is typically dominated by C16–C18 fatty acids [25], microalgal lipid profiles exhibit high metabolic plasticity under specific cultivation regimes and fractionation steps [7,24]. The enriched PUFA extract displayed dose-dependent DPPH radical-scavenging activity (15.20 – 48.60% ; $\text{IC}_{50} = 105.80 \text{ } \mu\text{g/mL}$) (Table 8). This radical-scavenging capacity is attributed to the high concentration of enriched omega-3 PUFAs, as the bis-allylic hydrogens flanking the multiple double bonds in EPA and DHA readily donate hydrogen atoms to neutralize free radicals [6,26]. In the antibacterial evaluations, the PUFA-enriched extract exhibited concentration-dependent activity with a pronounced selectivity for Gram-positive pathogens (Table 9, Figure 4). At $75 \text{ } \mu\text{g/mL}$, the extract's inhibition zone against *Staphylococcus aureus* (2.2 cm) surpassed that of the Streptomycin control (2.0 cm), while also showing substantial efficacy against *Bacillus subtilis* (1.6 cm). Efficacy against Gram-negative pathogens was moderate, reaching 1.5 cm for *Escherichia coli* and 1.3 cm for *Salmonella enterica*. This differential susceptibility is directly explained by the mechanism of

action of free fatty acids [21]. Free PUFAs readily insert into and disrupt the single peptidoglycan-phospholipid membrane architecture of Gram-positive species such as *S. aureus* and *B. subtilis*, leading to membrane leakage, electron transport uncoupling and rapid cell lysis [21,22]. In contrast, the additional lipopolysaccharide outer membrane of Gram-negative bacteria (*E. coli* and *S. enterica*) acts as a selective permeability barrier, restricting foreign fatty acid penetration [21]. The strong anti-staphylococcal activity exceeding a standard clinical antibiotic underscores the potential of *C. vulgaris* PUFA fractions as potent natural antimicrobial candidates [21–23].

5. Conclusion

This study provides a comprehensive biochemical, chemical and bioactivity characterization of *Chlorella vulgaris* cultivated under open-pond and closed-photobioreactor systems. Photobioreactor cultivation enhanced photosynthetic pigment and soluble protein accumulation while achieving superior volumetric lipid productivity. Open pond cultivation favoured carbohydrate accumulation and gravimetric lipid yield, reflecting distinct metabolic adaptations to environmental stress. Spectroscopic and Chromatographic analyses confirmed that urea inclusion crystallization successfully enriched long-chain omega-3 polyunsaturated fatty acids (EPA and DHA). The enriched PUFA extract demonstrated concentration-dependent DPPH antioxidant capacity and potent, Gram-selective antibacterial activity, notably displaying superior inhibition against *Staphylococcus aureus* compared to the standard antibiotic Streptomycin. Moderate inhibitory effects were also confirmed against *Bacillus subtilis*, *Escherichia coli* and *Salmonella enterica*. These findings highlight *C. vulgaris* PUFA extracts as promising natural candidates for nutraceutical, pharmaceutical and cosmeceutical formulations targeting Gram-positive bacterial pathogens.

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Conflict of interest:

The authors declare no conflict of interests

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