Isolation and Characterization of Microalgae with potential as human nutrition

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Abstract

Microalgae present significant potential as feedstock for biodiesel production, industrial food applications and pharmacology, along with the production of various high-value products such as pigments and polyunsaturated fatty acids. The selection of strains is essential for maximizing economic feasibility. This study focused on the characterization of two microalgal strains isolated from the Thi Nghe River (Ho Chi Minh City, Vietnam). Phylogenetic analysis of the 18S rDNA-ITS region identified the strains as Desmodesmus armatus and Monoraphidium subclavatum. The primary objectives were to extract and to analyze chlorophyll a, chlorophyll b, carotenoids and phycocyanin from these microalgae. Ultrasound-assisted extraction methods were employed to disrupt the microalgal cells followed by pigment analysis.

The results revealed that Monoraphidium subclavatum contained significantly higher levels of pigments compared to Desmodesmus armatus. Specifically, Monoraphidium subclavatum exhibited a higher chlorophyll a content (632.817 ± 12.510 µg/g) than Desmodesmus armatus (268.840 ± 16.024 µg/g). A similar trend was observed for chlorophyll b (364.933 ± 11.801 µg/g in M. subclavatum vs. 162.147 ± 17.562 µg/g in D. armatus) and carotenoids (1899.333 ± 24.007 µg/g in M. subclavatum vs. 180.0 ± 34.044 µg/g in D. armatus). Interestingly, phycocyanin levels were higher in Desmodesmus armatus (0.360 ± 0.010 µg/g) than in Monoraphidium subclavatum (0.247 ± 0.015 µg/g). These findings underscore the potential of these strains for pigment production and biodiesel applications, with M. subclavatum showing particularly promising results in food industry.

Keywords: Microalgae, human nutrition, pigments, phycocyanin.

Introduction

Green algae are ubiquitous in terrestrial and aquatic environments, from marine shorelines to freshwater bodies. They display a remarkable diversity in their interactions with other organisms, cellular structures, reproductive strategies and physical forms compared to other algae[1]. Furthermore, green algae possess chlorophyll-based tissues akin to terrestrial plants including pyrenoids for storing photosynthetic products and a variety of photosynthetic pigments[2]. Green algae can thrive in various regions with diverse temperatures, making them highly promising for the production of biologically active compounds and offering significant economic benefits[3].

Pigments are complex molecules or macromolecules that alter the color of light they transmit or reflect by selectively absorbing specific wavelengths. In microalgae, natural pigments play a vital role in various pigmentation-dependent activities such as photosynthesis. These pigments have significant potential as biologically active compounds and are used as food colorings and nutraceutical ingredients with properties such as antioxidant, anticancer, immunomodulatory, antiangiogenic, antidiabetic and anti-inflammatory effects. Microalgae contain three main categories of pigments: (i) chlorophylls, (ii) carotenoids and (iii) phycobiliproteins (PBPs). Chlorophylls, the primary pigments responsible for photosynthesis, are divided into four types (a, b, c and d) with distinct molecular structures. These fat-soluble chlorophylls give microalgae a greenish color.

Carotenoids and PBPs act as accessory pigments. Chlorophyll b is found in green algae (Chlorophyceae), while different types of chlorophyll c occur in brown algae (Phaeophyceae). Chlorophyll d, along with accessory pigments such as R-phycocyanin (R-PC), allophycocyanin (APC) and carotenes (α/β), is found in red algae (Rhodophyta). Due to their unique molecular structures and diverse beneficial properties, microalgal pigments are considered promising eco-friendly colorants, nutraceuticals and antioxidants with significant commercial value. They hold potential in industries such as food and dairy, alternative foods, dietary supplements, pharmaceuticals, cosmetics, aquaculture, textiles and other light manufacturing sectors[4,5]. To varying extents, these pigments have been developed and applied, with some achieving efficient industrial production and significant economic value.

The goal is to identify strains that are well-suited for growth in tropical climates and are capable of adapting to constantly changing environmental conditions such as those in rivers

and seas. We have successfully isolated two microalgae strains from the Thi Nghe River in Ho Chi Minh City, Vietnam. The identification of the two strains was carried out using the 18S rDNA-ITS region. One strain was classified inside the Chlorophyceae class as *Monoraphidium subclavatum* and the other was categorized within the Scenedesmaceae family as *Desmodesmus armatus*.

Both the two strains are members of Chlorophyta, characterized by chloroplasts that originated from an initial endosymbiosis with a cyanobacterium, whose closest surviving relatives inhabit freshwater environments. They are considered as source of biomass products and have applications in valuable food pigments.

Material and Methods

Microalgae isolation: The microalgae (phytoplankton) samples will first be collected from Thi Nghe River, Ho Chi Minh City. Twenty litres of water were collected from the river's surface to pass a plankton net of mesh sizes 25 µm to collect different useful species from the sample. The collected samples will be sent to the laboratory for the isolation step. A micropipette was use to pick up individual microalgae cells and each cell was washed several times with sterile BBM (Bold-Basal Medium) until there were one individual microalgae per medium drop. This drop was transferred to sterile BBM for continous culturing.

DNA extraction and sequencing for Identification of microalgae: For genomic DNA extraction, samples were incubated in lysis buffer, supplied with final 1 mg/ml proteinase K (NEB, MA, USA) at 56°C for one hour followed by chloroform extraction and DNA precipitation with sodium acetate (pH 5) and isopropanol. The DNA pellet was dissolved in Tris-EDTA buffer.

The target DNA was amplified using primers CYA-16S-106F-CGGACGGGTGAGTAACGCGTGA and CYA-16S-781R-GACTACTGGGGTATCTAATCCCATT. The PCR amplifications were performed in 20 µL reaction mixture containing 50 ng DNA template, 250 µM of each primer and PCR Supermix (Salagene, HCM, VN). The amplification profile consisted of an initial denaturation at 95 °C for 5 min followed by 35 cycles of 20 sec at 95 °C, 20 sec at 55°C, 1 min at 72 °C and a 5 min final extension at 72 °C by using DNA engine cycler (Biorad, CA, USA).

PCR products were checked by electrophoresis on 1% agarose gel in 0.5× Tris-Acetate-EDTA (TAE) buffer. The gel was stained with 6X Gelred solution (Salagene, HCM, Vietnam) and was visualized under ultraviolet light of a transilluminator (UVP, CA, USA).

For the direct sequencing of PCR amplicon, 5 µl of amplified products were incubated for 30 min at 37^0C together with 1U of exonuclease I (NEB, MA, USA) and 5U of shrimp alkaline phosphatase (NEB). Stop at 85^0C for 15 min. to degrade primers and dephosphorylate dNTPs which remained in final product. Sequencing reactions were performed using ABI PRISM BigDye™ Terminator Cycle Sequencing Kits (Applied Biosystem, CA, USA) with the PCR primers for both directions respectively. The product was cleaned up using magnetic beads (PerkinElmer, MA, USA) and then analyzed on an automated DNA analyzer (3130XL, Applied Biosystem).

The sequencing results from both directions of each sample were assembled using CLC Main Workbench version 7.8.1 (CLC Bio, Arhus N, Denmark). The confident DNA sequence was obtained by manually verifying with the high quality electropherogram shown by CLC Main Workbench. Final assembled sequences were subjected to BLASTn analysis against the core nucleotide database (core_nt) to identify species based on the top BLAST hit[6].

Biomass collection and extraction of pigments: The phytoplankton were grown until they reached the middle of the exponential phase, with their cell count being approximately $4x10^6$ for *M. subclavatum* and *D. armatus* by day 11. The microalgae were collected at mid-exponential phase using a using a Hettich centrifuge model EBA 20 (Germany). Ethanol extracts were prepared with absolute ethanol (HPLC grade, ACS certified) (Fisher, CAS 64-17-5) following methods by Hemalatha et al[3].

Determination of chlorophylls and carotenoids: To evaluate the content of carotenoids, chlorophylls a and chlorophylls b, 0.1 g of fresh microalgae was extracted in 10 mL of 96% ethanol using an ultrasonic bath functioning at 10 minutes at 75°C with two cycles. Each collection was performed in triplicate. Spectrophotometric analysis was performed on the filtered extracts at various wavelengths at 662 nm, 645 nm and 470 nm. Chlorophyll and carotenoid levels were measured using the Lichtenthaler-Buschmann equations.

C_a, µg/mL=13.95× A_{665}- 6.88 × A_{649}
C_b, µg/mL=24.9× A_{649}- 7.32 × A_{665}
Cx+c, g/L=(1.0 ×A_{470} – 2.05 × C_a- 114.8 xC_b)/245

where C*a* is chlorophyll *a*, C*b* is chlorophyll *b* and C*(x+c)* is total carotene.

Determination of Phycocyanin: For phycocyanin extraction, 0.1 g of fresh microalgal sample was placed in 15 mL polypropylene centrifuge tube and mixed with 10 mL of saline extraction buffer. The buffer consisted of 8.77 g NaCl, 2.01 g KCl, 11.36 g Na_2HPO_4 and 3.72 g Na_2EDTA per liter of distilled water. The mixture was incubated at 4°C for 16 hours followed by sonication for 10 minutes. Afterwards, the samples were centrifuged at 4°C at 4,000 rpm for 20 minutes. Phycocyanin extracts were analyzed using an SP-UV 1100 UV-VIS spectrophotometer, measuring absorbance at 615 nm and 682 nm. Pigment concentration, yield and purity index were calculated using designated equations.

$$\text{Phycocyanin concentration, mg/ml} = \frac{(A615 - 0.475 \times A652)}{5.34}$$

Data evaluation: The chlorophyll a, chlorophyll b, carotenoid, phycocyanin and antioxidant activity assays were conducted with three replicates. The results were expressed as the mean value ± standard error of the mean. Tukey's Honestly Significant Difference test was employed to determine statistically significant differences between the samples.

Results and Discussion

Morphology of microalgae: Morphology determination of microalgae is a critical aspect of studying and identifying different species, as it provides valuable insights into their structure, function and adaptability. In this study, *Desmodesmus armatus* (*D. armatus*) cells were flat, with two or four cells arranged horizontally in a row. The cells were oval or ellipsoidal, measuring 8-9 µm in length and 3-4 µm in width. Most cells were light green with smooth surfaces and no spines were present on either the internal or external cells. *Monoraphidium subclavatum* (*M. subclavatum*) cells were typically solitary, sharp and crescent-shaped, though they were rarely sigmoid. The surface of the cells was smooth with no protuberances.

Chloroplasts occupied nearly the entire cell. The cell length ranged from 10-12 µm and the width from 4.5-5.2 µm. The cells were also light green. Microscopic observation revealed that Nile red dye successfully interacted with intracellular lipid globules in microalgae cells. As shown in the images (Figure 1), the lipid globules emitted an orange/yellow fluorescence upon interaction with the dye while chlorophyll exhibited a red autofluorescence.

The results showed that yellow fluorescence appeared in both strains, indicating that *D. armatus* and *M. subclavatum* possess the ability to synthesize lipids. This is a significant finding, as the lipid production potential of microalgae plays a crucial role in exploring new sources for food and biofuel applications. To minimize the potential discrepancies caused by morphological variations, a phylogenetic analysis of partial 18S rRNA gene sequences was conducted for both microalgal isolates. Through 18S rRNA sequencing, we were able to distinguish two separate species *Monoraphidium subclavatum* and *Desmodesmus armatus* which exhibited a remarkably high sequence similarity of 99% (Figure 2).

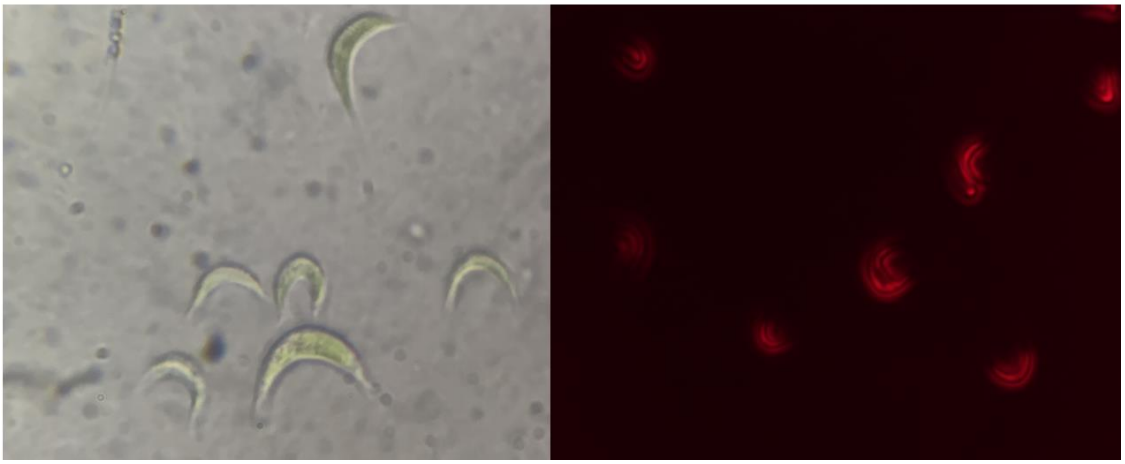

Fig. 1a: Microscopic images of a *Monoraphidium subclavatum* cell: (a) Unstained and (b) stained with Nile red dye

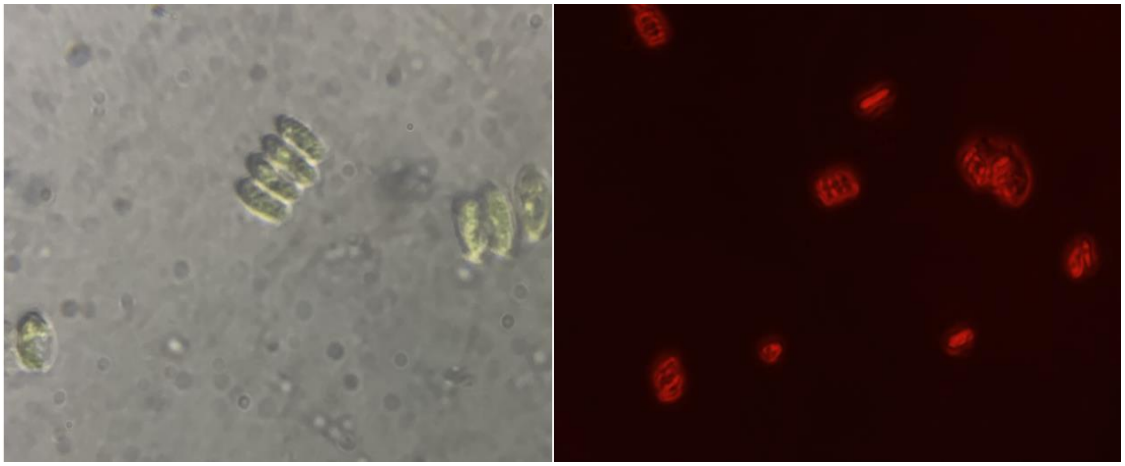

Fig. 1b: Microscopic images of a *Desmodesmus armatus* cell: (a) Unstained and (b) stained with Nile red dye.

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>Monoraphidium subclavatum strain FBCC-A409 small subunit ribosomal RNA gene, parti
spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence
Sequence ID: MT169963.1 Length: 2557
Range 1: 624 to 890

Score:483 bits(261), Expect:2e-131,
Identities:265/267(99%),  Gaps:0/267(0%), Strand: Plus/Plus

Query  3    AAGCTCGTAGTTGGATTTCGGGTGGGTTCCAGCGGTCCGCCTATGGTGAGTACTGCTGTG  62
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  624  AAGCTCGTAGTTGGATTTCGGGTGGGTTCCAGCGGTCCGCCTATGGTGAGTACTGCTGTG  683

Query  63   GCCCTCCTATCTGTCGGGAACGGGCTCCTGGGCTTCACTGTCCGGGACCTGGGTTCGACG  122
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  684  GCCCTCCTTTCTGTCGGGAACGGGCTCCTGGGCTTCACTGTCCGGGACCTGGGTTCGACG  743

Query  123  ATGATACTTTGAGTAAATTAGAGTGTTCAAAGCAAGCCTACGCTCTGAATACTTTAGCAT  182
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  744  ATGATACTTTGAGTAAATTAGAGTGTTCAAAGCAAGCCTACGCTCTGAATACTTTAGCAT  803

Query  183  GGAATATCGCGATAGGACTCTGGCCTATCTCGTTGGTCTGTAGGACCGGAGTAATGATTA  242
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  804  GGAATATCGCGATAGGACTCTGGCCTATCTCGTTGGTCTGTAGGACCGGAGTAATGATTA  863

Query  243  AGAGGGACAGTCGGGGGGTATTCGTATT  269
            |||||||||||||||| |||||||||
Sbjct  864  AGAGGGACAGTCGGGGGCATTCGTATT  890
```

Fig. 2a: The BLAST analysis was conducted to confirm the identification of *Monoraphidium subclavatum*.

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>Desmodesmus armatus isolate UMT-B20 small subunit ribosomal RNA gene, partial sequence
Sequence ID: MN879270.2 Length: 1743
Range 1: 595 to 868

Score:496 bits(268), Expect:2e-135,
Identities:272/274(99%),  Gaps:0/274(0%), Strand: Plus/Plus

Query  4    AAGCTCGTAGTTGGATTTCGGGTGGGTTTCAGCGGTCCGCCTATGGTGAGTACTGCTGTG  63
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  595  AAGCTCGTAGTTGGATTTCGGGTGGGTTTCAGCGGTCCGCCTATGGTGAGTACTGCTGTG  654

Query  64   GCCTTCCTTACTGTCGGGGACCTGCTTCTGGGCTTCATTGTCCGGGACAGGGATTCGGCA  123
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  655  GCCTTCCTTACTGTCGGGGACCTGCTTCTGGGCTTCATTGTCCGGGACAGGGATTCGGCA  714

Query  124  TGGTTACTTTGAGTAAATTGGAGTGTTCAAAGCAGGCTTACGCCGTGAACATTTTAGCAT  183
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  715  TGGTTACTTTGAGTAAATTGGAGTGTTCAAAGCAGGCTTACGCCGTGAACATTTTAGCAT  774

Query  184  GGAATAACATGATAGGACTCTGCCCTATTCTGTTGGCCTGTAGGAGTGGAGTAATGATTA  243
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  775  GGAATAACATGATAGGACTCTGCCCTATTCTGTTGGCCTGTAGGAGTGGAGTAATGATTA  834

Query  244  AGAGGAACAGTCGGGGGGTATTCGTATTCCATTGT  277
            |||||||||||||||| ||||||||| ||||||
Sbjct  835  AGAGGAACAGTCGGGGGCATTCGTATTTCATTGT  868
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Fig. 2b: The BLAST analysis was conducted to confirm the identification of *Desmodesmus armatus*

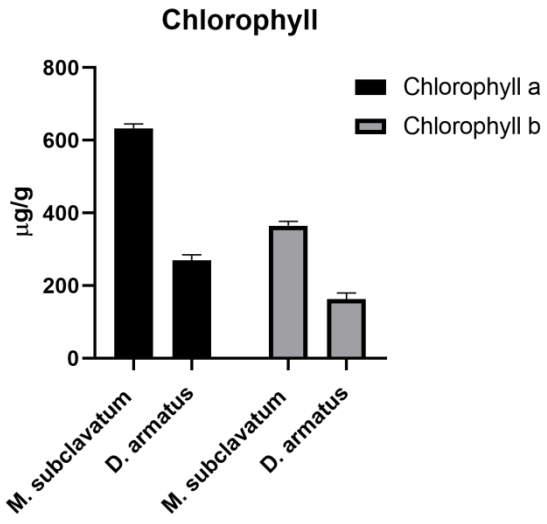

Fig. 3: Chlorophyll content in *Monoraphidium subclavatum* and *Desmodesmus armatus* wet biomass

Our analysis revealed two novel variants within each species. This observation suggests a potential reservoir of genetic diversity within their local ecology.

Chlorophyll and carotenoid determination: To determine the amount of chlorophyll extractable from *Monoraphidium subclavatum* and *Desmodesmus armatus* biomass, the chlorophyll content was measured by performing successive extractions using pure solvent ethanol. The results are shown in figure 3. Chlorophyll is the primary pigment responsible for photosynthesis in microalgae, playing a critical role in capturing light energy for the process. The chlorophyll

content in microalgae varies between species and is significantly influenced by environmental conditions such as light intensity, nutrient availability and temperature.

In this study, we cultivated microalgae in a basic medium without optimization. The results showed that the chlorophyll a and b contents in *M. subclavatum* were higher than in *D. armatus*. Specifically, the chlorophyll a content in *M. subclavatum* was 2.36 times higher than that in *D. armatus*, while the chlorophyll b content was 2.25 times higher. These findings suggest that *M. subclavatum* may have a greater photosynthetic capacity and potential for higher biomass production under the given growth conditions.

In a study conducted by Pozzobon et al[10] on the pigment composition of Desmodesmus sp., the chlorophyll a content was found to range between 76 and 1498 µg/g. The variation in chlorophyll a levels was largely influenced by environmental factors, particularly light intensity and nitrogen availability. Medium light intensity and sufficient nitrogen supply resulted in greater chlorophyll a accumulation, enhancing the photosynthetic efficiency of the microalgae.

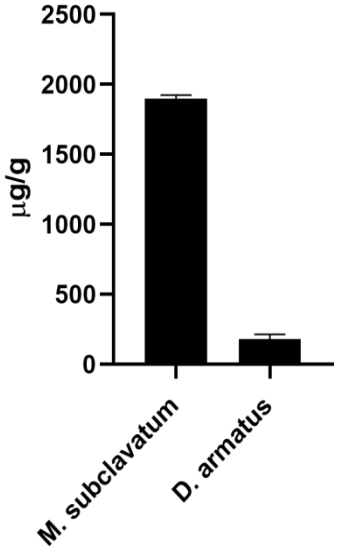

Fig. 4: Carotenoids content in *Monoraphidium subclavatum* and *Desmodesmus armatus* wet biomass

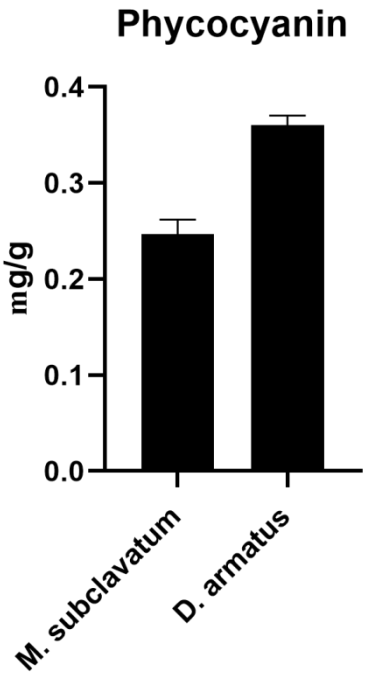

Fig. 5: Phycocyanin content in *Monoraphidium subclavatum* and *Desmodesmus armatus* wet biomass

Conversely, lower and higher light or nitrogen limitations led to a significant reduction in chlorophyll a content. The chlorophyll b content was also found to show similar trends. Like chlorophyll a, its concentration varied depending on environmental factors such as light intensity and nitrogen availability[9].

Carotenoids are also essential pigments in microalgae, playing a critical role in the photosynthetic process and cellular protection. These pigments not only assist in capturing light energy by complementing the absorption spectrum of chlorophyll but also protect the cells from damage caused by excessive light and oxidative stress[10]. The carotenoids content in *M. subclavatum* and *D. armatus* is depicted in figure 4. The results indicated a significant difference in carotenoid concentrations between the two species ($p = 0.99$). Specifically, *M. subclavatum* exhibited a higher carotenoid content compared to D. armatus, with values of 1899 ± 24.007 µg/g and 180.00 ± 34.044 µg/g respectively. The carotenoid level in *M. subclavatum* is 10.55 times higher than that in *D. armatus.*

Fernandes et al[2] used the HPLC method to determine the carotenoid content of *Chlorella sorokiniana* and *Scenedesmus bijuga*. The highest total carotenoid content was determined in the extract of *C. sorokiniana* (1408.46 µg g^{-1}) and *S. bijuga* exhibited the lowest content (1195.75 µg/g)[11]. Additionally, the carotenoid content in microalgae is influenced by light conditions and nutrient availability in the growth medium. For example, under white light, *M. braunii* primarily synthesizes lutein as the major carotenoid, with concentrations of 535 ± 22 µg g^{-1} CDW (cell dry weight). Moreover, other carotenoids and xanthophylls are also produced in significant amounts including neoxanthin (398 ± 14 µg g^{-1} CDW), violaxanthin (228 ± 8 µg g^{-1} CDW) and alpha-carotene (22 ± 1 µg g^{-1} CDW).

These variations in carotenoid synthesis highlight the adaptability of microalgae to different environmental conditions, making them valuable sources of natural pigments. The ability to manipulate light intensity and spectrum as well as nutrient composition, provides a powerful tool for optimizing carotenoid production in commercial applications.

Phycocyanin determination: Phycocyanin is a blue pigment-protein complex that belongs to the phycobiliprotein family, which is found in cyanobacteria (blue-green algae) and some types of red algae. It plays a key role in photosynthesis, functioning as an accessory pigment by capturing light energy and transferring it to chlorophylls for use in photosynthetic processes. Phycocyanin were determinated in *M. subclavatum* and *D. armatus*. The results are shown in figure 5. Our analysis found a significant difference in phycocyanin concentrations between *M. subclavatum* (0.2467 ± 0.02 mg/g) and *D. armatus* (0.36 ± 0.01 mg/g).

In a study focused on the isolation of microalgae from Bangladesh, the phycocyanin content was determined in several species, revealing varying levels of this valuable pigment. The results showed that *Monoraphidium sp*. contained 0.422 ± 0.008 mg/g of phycocyanin, *Selenastrum sp.* had 0.573 ± 0.004 mg/g, *Ankistrodesmus sp.* contained 0.222 ± 0.008 mg/g while *Scenedesmus sp*. exhibited the highest phycocyanin content at 1.539 ± 0.032 mg/g[12].

Conclusion

This study successfully identified two microalgal strains isolated from the Thi Nghe River in Vietnam. Through phylogenetic analysis of the 18S rRNA gene sequences, the strains were determined to belong to the species *Monoraphidium subclavatum* and *Desmodesmus armatus*. The molecular identification was supplemented by morphological observations, confirming the distinct characteristics of each strain. These findings are significant as both strains exhibit promising potential for biotechnological applications, particularly in lipid synthesis for biodiesel production and the extraction of high-value pigments such as chlorophyll, carotenoids and phycocyanin.

The isolation of these strains from a local aquatic environment highlights its adaptability to variable conditions, making them suitable candidates for further research and industrial-scale cultivation in similar tropical or subtropical climates. This identification serves as the foundation for exploring their use in biodiesel production, nutraceuticals, environmental sustainability and food industry efforts.

Acknowledgement

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