Evaluation of biofuel potential and pigment profiling of cyanobacterial strains isolated from Freshwater regions

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***Abstract*—The increasing global demand for sustainable energy has directed attention toward third-generation biofuels derived from cyanobacteria. This study evaluated the biofuel potential and pigment profiling of three cyanobacterial strains-Nostoc punctiforme, Nostoc sp., and Anabaena sp.-isolated from freshwater sources in Tamil Nadu, India. Morphological identification confirmed strain-level differences, and pigment analyses revealed Nostoc sp. exhibited the highest chlorophyll-a (18.34 µg/mL) and phycobiliprotein content, indicating superior light-harvesting capacity. Conversely, Nostoc punctiforme showed the highest carotenoid content (0.0032 µg/mL), suggesting strong photoprotective traits. Lipid extraction using the Bligh and Dyer method identified Anabaena sp. as the top lipid producer (4.0% dry weight). Gas chromatography of fatty acid methyl esters (FAMEs) revealed a rich profile of medium- to long-chain fatty acids in Anabaena sp., including C16:0, C18:2, and C18:3, with significant concentrations of polyunsaturated fatty acids. These findings highlight the dual potential of freshwater cyanobacteria in sustainable biofuel production and high-value pigment applications, emphasizing their role as promising feedstocks for renewable energy and biotechnology.**

***IndexTerms*—Cyanobacteria, Biofuel, Lipid Extraction, Phycobiliproteins, Chlorophyll-a, Carotenoids, Gas Chromatography, Sustainable Energy, Third-generation Biofuels**

I. INTRODUCTION

The global energy demand remains heavily reliant on fossil fuels, leading to resource depletion, unpredictable climate variations, and severe environmental consequences, including oil spills and greenhouse gas (GHG) emissions [1]. The urgent need for sustainable and renewable energy alternatives has driven extensive research into biofuel production. Among these, liquid biofuels such as fatty acid esters (biodiesel) present an environmentally friendly substitute for conventional fossil fuels [2]. Developing economically viable and renewable energy sources is essential to mitigating the risks associated with fossil fuel dependence, including economic instability and environmental [3]. A primary concern with fossil fuel combustion is its significant contribution to GHG emissions, which exacerbate global climate change [4]. Projections indicate that these emissions may reach approximately 43 billion metric tons by 2040 if alternative energy solutions are not widely adopted [5]. Initially, first-generation biofuels derived from agricultural crops were considered a promising renewable energy source. However, their production raised concerns over food security due to competition for arable land [6]. To address these limitations, second-generation biofuels emerged, utilizing non-food biomass sources such as plant and animal feedstocks. These biofuels are primarily produced through transesterification, a process that converts fatty acids into biodiesel using an alcohol-based catalyst [7].

Expanding beyond these approaches, third-generation biofuels have gained prominence, particularly those derived from microalgal biomass [8],[9]. Although research on phototrophic microorganisms for biofuel production is relatively recent, significant advancements have been made over the past three decades, particularly in cyanobacterial studies. Cyanobacteria, a group of oxygenic photosynthetic prokaryotes, hold great potential for biofuel generation due to their ability to rapidly synthesize lipids and accumulate biomass through photosynthesis [10]. Unlike algae and higher plants, cyanobacteria possess a distinct lipid profile, with diacylglycerols embedded in thylakoid and photosynthetic membranes, making them an abundant and valuable source of biofuel precursors. Given their high photosynthetic efficiency, cyanobacteria have garnered considerable research interest for biofuel production. These microorganisms enable large-scale cultivation with the added advantage of mitigating atmospheric carbon dioxide levels, contributing to environmental sustainability. Additionally, cyanobacteria can be cultivated using wastewater from industrial and agricultural sources, promoting bioremediation while reducing the strain on freshwater resources. The integration of wastewater treatment with biomass cultivation presents an eco-friendly approach to biofuel production, highlighting the multifaceted applications of cyanobacteria in renewable energy and environmental management [11]. Cyanobacteria are among the earliest known oxygen-producing photosynthetic organisms, playing a crucial role in atmospheric oxygenation for over three billion years [12].

As third-generation biofuel feedstocks, cyanobacteria offer significant advantages due to their diverse biochemical transformations, leading to the production of biofuels such as biogas, cellulosic ethanol, biohydrogen, and biodiesel [13]. Their high efficiency in capturing solar energy and converting biomass into biofuels makes them a compelling alternative to traditional energy crops. Furthermore, cyanobacterial lipids can be directly converted into fatty acid methyl esters (FAMEs), the primary components of biodiesel [14]. Beyond biofuel production, cyanobacteria are valued for their high-value biochemical co-products, including pigments, secondary metabolites, and enzymes. Carotenoids such as canthaxanthin, β-carotene, and zeaxanthin serve as natural colorants, antioxidants, and dietary supplements with potential health benefits, including protection against cardiovascular diseases and cancer [15]. These value-added products enhance the economic feasibility of cyanobacterial biofuel production by creating additional revenue streams. Cyanobacteria serve as an efficient platform for producing various renewable biofuels and bio-based chemicals, utilizing sunlight, CO_2, and water as primary resources [16]. Some of the key biofuels derived from cyanobacteria include ethanol, butanol, biodiesel, and hydrogen. Cyanobacteria can naturally ferment glucose and sucrose under anaerobic conditions, resulting in ethanol production. Unlike conventional bioethanol production, which relies on yeast fermentation, cyanobacteria offer a more sustainable approach by eliminating the need for external fermentation initiators [17]. Genetically modified cyanobacterial strains have shown enhanced ethanol yields, making them a viable option for large-scale bioethanol production [18].

Cyanobacteria synthesize fatty acids that can be transesterified into biodiesel. Their ability to directly convert solar energy into lipid feedstocks makes them a promising alternative to traditional oilseed crops [19]. Hydrogen gas, considered a clean and sustainable energy carrier, can be produced by certain cyanobacterial species via nitrogenase and hydrogenase enzyme activity [20]. Genetic modifications have been explored to enhance hydrogen yields and increase the efficiency of photobiological hydrogen production [21]. Additionally, cyanobacteria produce, a variety of pigments with industrial applications that are Phycocyanin a blue phycobiliprotein, phycocyanin functions as a light-harvesting pigment in photosynthesis and possesses antioxidant and anti-inflammatory properties. It is widely used as a natural dye in the food and pharmaceutical industries. Phycoerythrin a red phycobiliprotein, phycoerythrin exhibits bright fluorescence, making it valuable in biotechnological applications such as fluorescent labelling in research and medical diagnostics. Chlorophyll a is essential for photosynthesis, chlorophylls enable cyanobacteria to convert light energy into chemical energy. They have applications as natural colorants in the food industry and as antioxidants in health care products. Carotenoids in this pigment, ranging from yellow to red, play a protective role against oxidative damage. Industrially, carotenoids are used as natural colorants and have pharmaceutical and cosmetic applications due to their antioxidant properties [22]. The present research aimed to evaluate the pigment profiling and biofuel potential of three cyanobacterial strains isolated from a freshwater region. This study focused on assessing their pigment composition, lipid accumulation, and biofuel production capacity, highlighting their potential as sustainable feedstock for third-generation biofuels.

II. MATERIALS AND METHODS

A. Sample collection, Isolation & Cultivation

The three Cyanobacterial samples were collected from two water bodies in Mulayampoondi, Tiruppur, Tamil Nadu, India, selected based on visible algal growth and suitable environmental conditions [23]. The first site, a stagnant well on Puduppai Road (GPS: 10.853852° N, 77.69167° E), it exhibited a thick green algal layer, indicating high cyanobacterial biomass. Surrounding vegetation and an irrigation system with inlet and outlet pipes suggested potential agricultural runoff [24] influencing nutrient levels. The second site, a flowing river in Karaivalasu (GPS: 10.860284° N, 77.687291° E), the river contained submerged aquatic vegetation and organic debris, with cyanobacterial presence observed along the riverbanks, interspersed with other algae and plant matter. Water movement at this site could influence the formation and distribution of cyanobacterial blooms [25]. Samples were collected from natural habitat in clean aseptic plastic containers with Protective gears such as gloves, mask and glasses. Approximately 10 mL of liquid sample were Collected from the sampling area [26].

The cyanobacterial strains were purified using serial dilution followed by plating onto sterile BG-11+ agar medium [27]. The plates were incubated at 25°C under a constant light intensity of 50 μE m^{-2} s^{-1} for up to 10 days. Once colonies developed, they were further isolated and purified using the method described by [28]. The plates were regularly examined to distinguish cyanobacterial colonies from bacterial contaminants. Axenic cyanobacterial colonies were carefully selected and subcultured into 50 mL of BG-11N_0 liquid medium, incubated under the same conditions. Culture purity was confirmed through repeated plating and regular microscopic observation.All purified cyanobacterial strains were cultivated in 500 mL Erlenmeyer flasks containing 200 mL of BG-11+ medium. The cultures were maintained at 28 ± 2°C under a 14/10-hour light/dark cycle with a light intensity of 50 μE m^{-2} s^{-1} under static conditions. To ensure proper mixing, the cultures were gently agitated by hand on alternate days.

B. Morphological Characterization of the Isolates

Pure culture was ensured by checking under Automated Inverted Leica Microscope (DMI 3000B). Pictures of isolated strains were captured at 100X magnification using Brightfield Microscope for morphological analysis and identification. It might be noted that genomic study was not performed and identifications of the strains were performed based on morphological analysis. Primary identification of the morphology was performed based on the available literature and standard book [29].

C. Pigment Analysis

For chlorophyll analysis,take 1 mL of the culture and centrifuge at 14,000 rpm for 5 minutes. Collect the pellet and resuspend it in 0.9 mL of 90% ice-cold methanol. Incubate the sample overnight at 4°C in the dark. After incubation, centrifuge again at 14,000 rpm for 5 minutes, collect the supernatant, and measure its absorbance at 663 nm [30]. For carotenoid analysis, take 1 mL of the culture and centrifuge at 14,000 rpm for 5 minutes. Collect the pellet and resuspend it in 0.9 mL of 85% acetone. Incubate the sample overnight at 4°C in the dark. After incubation, centrifuge again at 14,000 rpm for 5 minutes, collect the supernatant, and measure its absorbance at 450 nm [31]. For phycobiliprotein analysis, take 1 mL of the culture and centrifuge at 14,000 rpm for 5 minutes. Collect the pellet and resuspend it in 0.9 mL of phosphate buffer. Incubate the sample overnight at -20°C in the dark. After incubation, perform three freeze-thaw cycles at 30-minute intervals. Centrifuge again at 14,000 rpm for 5 minutes, collect the supernatant, and measure its absorbance at 562nm for Phycocyanin,615nm for Allophycocyanin and 652nm for Phycoerythrin [32].

D. Lipid Extraction

Lipid extraction was performed following the protocol outlined by [33]. A measured quantity of freeze-dried biomass was homogenized with a chloroform-methanol mixture (2:1) using a pestle and mortar. The extraction process was repeated until no visible pigments remained in the biomass. The resulting extract was filtered using Whatman No. 1 filter paper, and a third volume of distilled water was added to eliminate water-soluble contaminants. The filtrate was then vortexed and allowed to settle, forming two distinct layers. The lower lipid-rich phase was carefully separated and pooled. To ensure purity, the collected lipid extract was passed through anhydrous sodium sulfate and stored in a pre-weighed glass vial. The solvent was subsequently evaporated using a rotary evaporator (Buchi

Rotovapor R-205, Buchi, India). Finally, the total lipid content was determined gravimetrically and expressed as a percentage of the dry biomass weight.

E. Preparation of FAME (Fatty Acid Methy Ester)

The purified lipid was transferred to a round-bottom flask and preheated in a water bath to remove any residual moisture. A 3% sulfuric acid catalyst was dissolved in methanol, and the resulting solution was added to the preheated lipid at room temperature. The reaction setup included a reflux condenser, magnetic stirrer, and thermometer, with the reaction conducted at 65 ± 1°C.Upon completion, the reaction mixture was transferred to a separating funnel and left undisturbed for 30 minutes. The lower layer, containing glycerol, unreacted methanol, and the catalyst, was carefully separated, while the upper layer, consisting of methyl esters, was collected. The methyl ester was then purified through repeated washing with water until the lower aqueous layer reached a neutral pH (pH 7.0). Finally, the purified methyl ester was dried at 70°C using a rotary evaporator (Evator II) and subsequently analyzed via gas chromatography [34].

F. Gas Chromatography Analysis

The fatty acid profile was determined using capillary column gas chromatography, specifically applied to oil methyl esters [35]. The fatty acid methyl ester (FAME) samples were analyzed using a gas chromatograph (Shimadzu QP2010, Japan) equipped with a flame ionization detector (FID). A 2 µL aliquot of each sample was injected into an SP-2560 column (Supelco, USA) with dimensions of 100 m × 0.25 mm I.D. and a film thickness of 0.20 µm. The temperature program was set as follows: the oven temperature was initially held at 140°C for 5 minutes, then increased to 240°C at a rate of 4°C per minute, followed by a 15-minute hold at 240°C. Helium was used as the carrier gas at a flow rate of 20 cm/s, with the detector temperature maintained at 260°C and a split ratio of 100:1. The total run time for each sample was 55 minutes. Fatty acids were identified and quantified by comparing their retention times and peak areas with those of authentic standards from the Supelco FAME mix (C4–C24) (Bellefonte, PA, USA).

III. RESULT

A.Morphological Identification

The morphological analysis of the isolated cyanobacterial strains was conducted by observing their cell shape, size, arrangement, color, and presence of sheath and heterocysts. There are totally three cyanobacterial strains were identified (Fig.1, 2, 3) that are *Nostoc punctiforme*, *Nostoc sp.*, *Anabaena sp.*, *Nostoc punctiforme* exhibited filamentous, bead-like chains of nearly spherical cells with visible heterocysts. Akinetes were also present, aiding survival in extreme conditions. *Nostoc sp.* displayed uniseriate filaments with spherical cells (2–10 µm), green in color due to chlorophyll-a and phycobiliproteins. Heterocysts were not clearly observed. *Anabaena sp.* showed chain-like, sometimes branched trichomes with heterocysts and elongated cells. The presence of pigments like phycocyanin and chlorophyll-a confirmed its cyanobacterial nature.

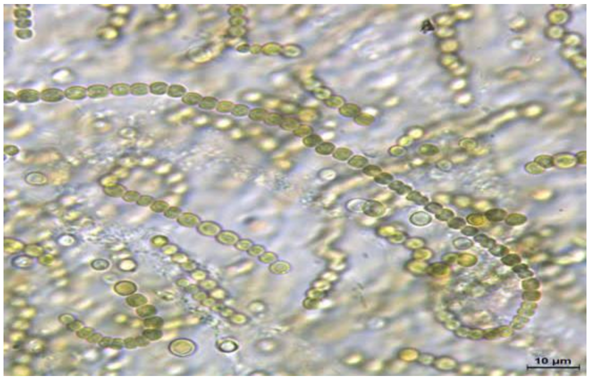


Figure.1. *Nostoc punctiforme*

Figure.2. *Nostoc sp.*,

Figure.3. *Anabaena sp.,*

B. Photosynthetic Pigments (Chlorophyll-a Analysis)

Chlorophyll-a content was measured at 663 nm using spectrophotometry. *Nostoc sp.* (Fig.4) showed the highest concentration (18.34 µg/mL), followed by *Nostoc punctiforme* (13.94 µg/mL), while *Anabaena sp.* had the lowest (3.79 µg/mL).

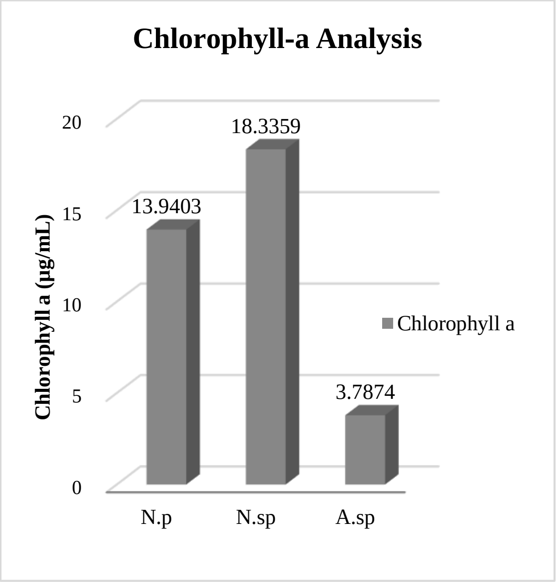


Figure.4. Chlorophyll a content in each strain

C. *Carotenoids Estimation*

Carotenoid content was estimated at 450 nm. *Nostoc punctiforme* (Fig.5) recorded the highest value (0.0032 µg/mL), significantly differing from both *Nostoc sp.* (0.0012 µg/mL) and *Anabaena sp.* (0.0010 µg/mL), which showed no significant difference between them.

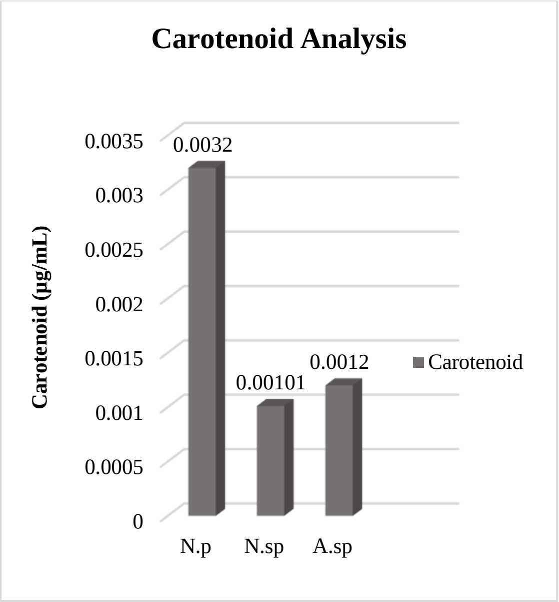


Figure.5. Carotenoid content in each strain

D. Phycobiliprotein Estimation

Phycobiliproteins, including phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE), were quantified spectrophotometrically at 562 nm, 615 nm, and 652 nm, respectively. Phycocyanin (PC): *Nostoc sp.* Showed (Fig.6) the highest PC content (1.2471 µg/mL), followed by Anabaena sp. (0.9001 µg/mL) and *Nostoc punctiforme* (0.1164 µg/mL).

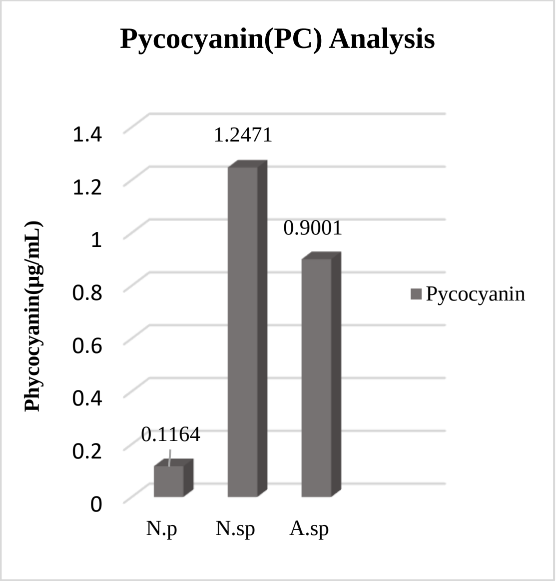


Figure.6. Phycocyanin (PC) content in each strain

Allophycocyanin (APC): *Nostoc sp.* again exhibited (Fig.7) the highest APC concentration (1.1684 µg/mL), with *Anabaena sp.* at 0.8533 µg/mL and *Nostoc punctiforme* at 0.0273 µg/mL.

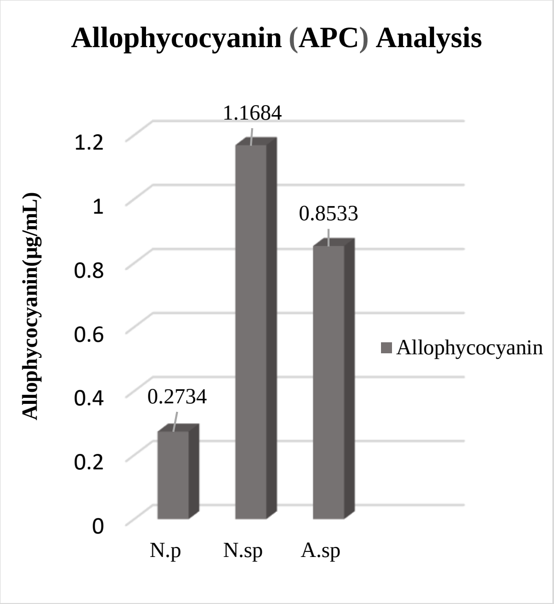


Figure.7. Allophycocyanin (APC) content in each strain

Phycoerythrin (PE): The highest PE content was recorded in *Nostoc sp.* (1.1301 µg/mL), followed by *Anabaena sp.* (0.9642 µg/mL) and *Nostoc punctiforme* (0.2029 µg/mL). All pigment concentrations showed statistically (Fig.8) significant variation among the strains.

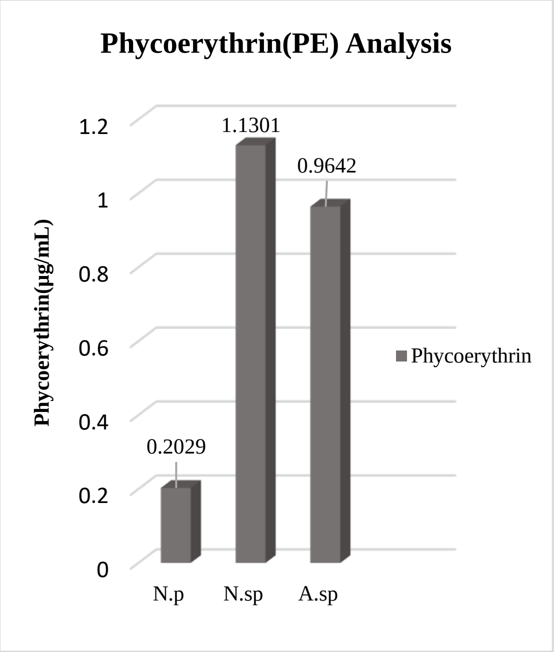


Figure.8. Phycoerythrin (PE) content in each strain

E. Comparative Analysis of Phycobiliproteins (PC, APC, PE)

Phycocyanin (PC), Allophycocyanin (APC), and Phycoerythrin (PE) play a vital role in enhancing light absorption across different wavelengths. Among the three strains, *Nostoc sp.* showed the highest concentrations (Fig.9) of all phycobiliproteins, indicating a superior pigment synthesis capacity and efficient light-harvesting potential. *Anabaena sp.* exhibited moderate levels of phycobiliproteins, while *Nostoc punctiforme* recorded the lowest, suggesting a limited ability for pigment production and adaptation to light variability.

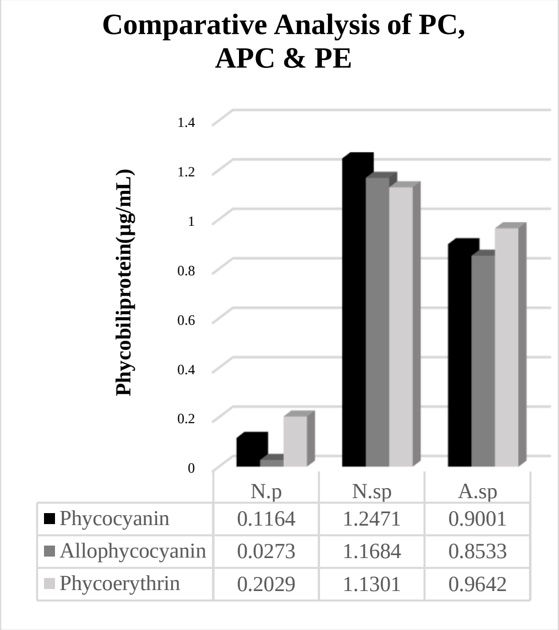


Figure.9. Pigment PC, APC & PE content in each strain

F. Comparative Analysis of Chlorophyll-a, Carotenoids, and Phycobiliproteins

Nostoc sp. emerged as the most efficient strain for light harvesting (Fig.10) due to its high levels of chlorophyll-a and phycobiliproteins. *Anabaena sp.* demonstrated moderate phycobiliprotein levels but lower chlorophyll-a, suggesting an adaptation to varied light conditions. In contrast, *Nostoc punctiforme* exhibited the highest carotenoid content, indicating strong photoprotective capabilities, though its lower chlorophyll-a and phycobiliprotein content may limit its photosynthetic efficiency.

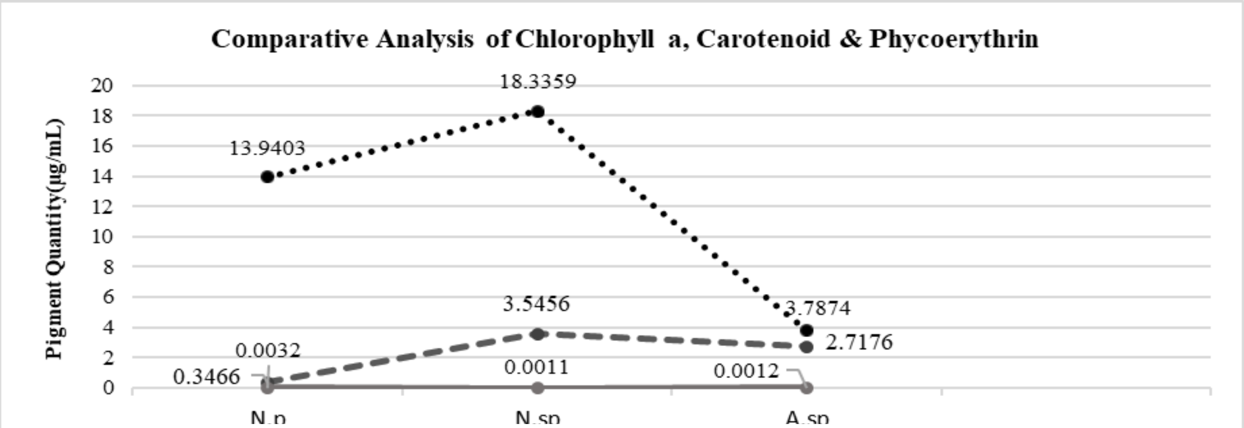
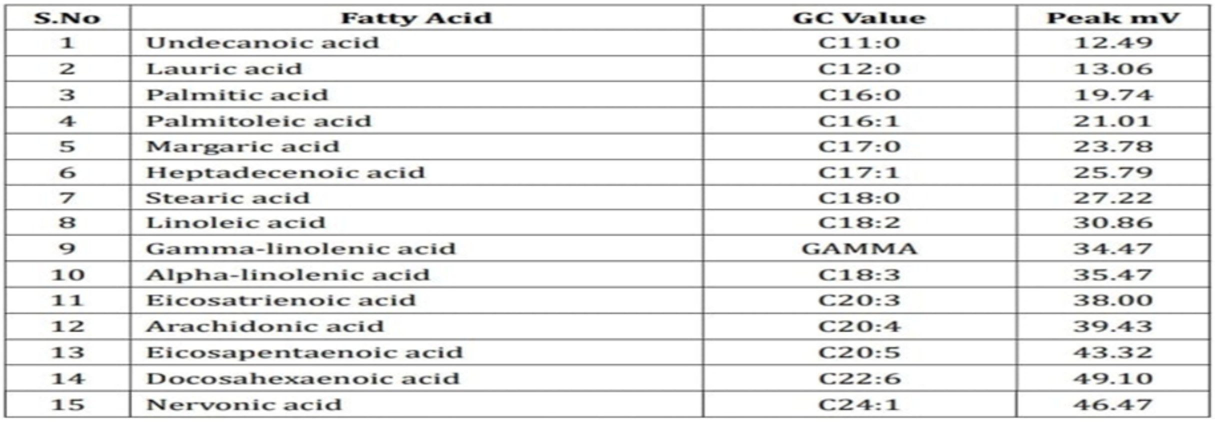


Table.1. GC Reading peak value of FAME in *Anabaena sp.*, 12

Figure.10. Comparative Analysis of Chlorophyll-a, Carotenoids, and Phycobiliproteins



S.No	Fatty Acid	GC Value	Peak mV
1	Undecanoic acid	C11:0	12.49
2	Lauric acid	C12:0	13.06
3	Palmitic acid	C16:0	19.74
4	Palmitoleic acid	C16:1	21.01
5	Margaric acid	C17:0	23.78
6	Heptadecenoic acid	C17:1	25.79
7	Stearic acid	C18:0	27.22
8	Linoleic acid	C18:2	30.86
9	Gamma-linolenic acid	GAMMA	34.47
10	Alpha-linolenic acid	C18:3	35.47
11	Eicosatrienoic acid	C20:3	38.00
12	Arachidonic acid	C20:4	39.43
13	Eicosapentaenoic acid	C20:5	43.32
14	Docosahexaenoic acid	C22:6	49.10
15	Nervonic acid	C24:1	46.47

Table.1. GC Reading peak value of FAME in Anabaena sp.,

G. Lipid Content Estimation

Lipid content was estimated using the Bligh and Dyer method. Among the strains, *Anabaena sp.* exhibited the highest lipid content (4.0%), followed by *Nostoc punctiforme* (3.0%) and *Nostoc sp.* (1.25%). These values represent lipid yield per 100g of dry biomass. The results (Fig.11) highlight *Anabaena sp.* as a promising candidate for biofuel production due to its superior lipid accumulation.

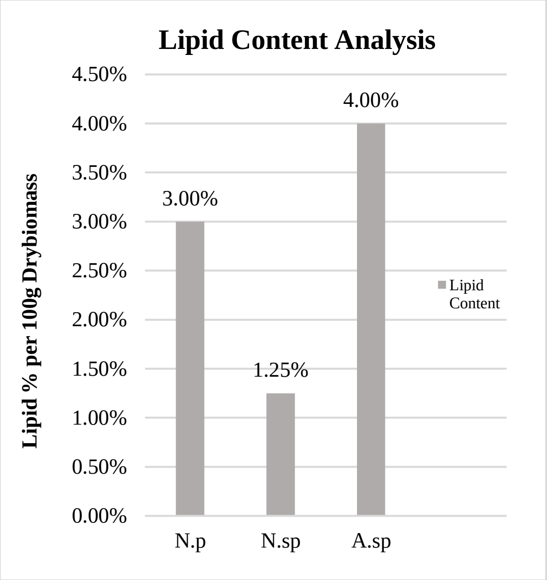
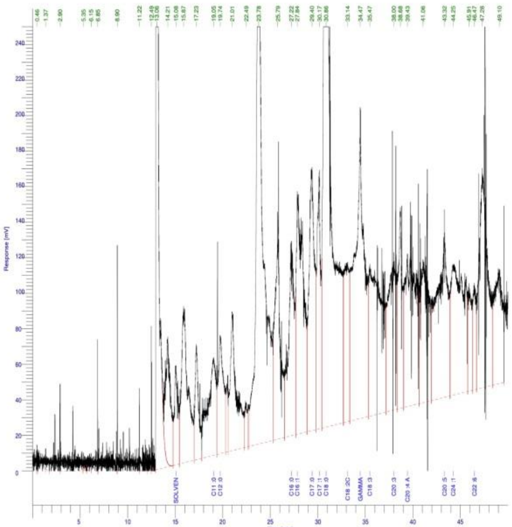


Figure.11. Lipid Content in each cyanobacterial strains

H. FAME Analysis by Gas Chromatography

GC analysis of *Anabaena sp.* revealed 15 fatty acids, (Table.1) including both saturated and unsaturated types. Dominant components included palmitic acid (C16:0), linoleic acid (C18:2), and gamma-linolenic acid (C18:3), with C16:0 showing the highest peak. The presence of polyunsaturated fatty acids (PUFAs), such as C18:3, C18:2, and C20:5, underscores the strain's potential for both nutraceutical and biofuel applications. The detection (Graph.1) of hydrocarbons further supports its viability in sustainable bioenergy production.



Graph.1. Readings of FAME in Anabaena sp.,

IV. CONCLUSION

The study demonstrates the potential of freshwater cyanobacteria as sustainable resources for biofuel and bioproduct development. *Nostoc sp.* showed high pigment productivity, while *Anabaena sp.* exhibited superior lipid accumulation and favorable fatty acid profiles, indicating its suitability for biodiesel production. Nostoc punctiforme was notable for its carotenoid content, highlighting its role in antioxidant applications. These findings support the use of cyanobacterial strains in renewable energy and industrial biotechnology.

ACKNOWLEDGMENT

I would like to thank Sengunthar Arts and Science College (Autonomous), Tiruchengode, for providing the Algal Research Laboratory Facility to carry out this project and helping with the cultivation and analysis of cyanobacterial strains. I am very grateful to my guide, Dr.P. Venkatachalam, for his support, guidance, and encouragement throughout the project.

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