

**Molecular identification and cultivation of indigenous
Limnospira strains from Ethiopian soda lakes: Growth
evaluation under simulated outdoor light conditions**

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ABSTRACT

Malnutrition, particularly among children, remains a critical issue in Ethiopia due to limited dietary diversity and widespread nutritional deficiencies. *Arthrospira*, a protein-rich cyanobacterium known for its rapid growth in extreme conditions, has significant potential for addressing these challenges through sustainable biomass production. However, the precise identification and optimization of indigenous strains for large-scale cultivation in Ethiopia have not been fully explored. A single criterion is insufficient for selecting potential *Arthrospira* strains; a comprehensive approach is necessary, including morphological screening, growth rate evaluation, and biochemical analyses. Such a multifaceted strategy is essential for identifying strains that are both fast-growing and nutritionally superior, ensuring their suitability for sustainable cultivation and their role in mitigating malnutrition in regions like Ethiopia. Outdoor cultivation using natural sunlight is an efficient method for producing valuable microalgal products such as proteins, lipids, carbohydrates, and antioxidants. However, a major challenge in large-scale outdoor cultivation is managing photoinhibition caused by intense sunlight, which can significantly reduce productivity. To address this, shading strategies and light management are crucial. Simulating varying light intensities refines outdoor cultivation, providing a more sustainable approach. The main objective of this PhD study is to identify and optimize indigenous *Arthrospira* strains from Ethiopian soda lakes using a holistic screening approach and to develop effective shading strategies for large-scale outdoor cultivation, aiming to improve biomass productivity and nutritional quality to address malnutrition.

Firstly, in Chapter II, the isolation and screening of 100 *Arthrospira*-like strains were conducted, focusing on the morphological characterization of four promising isolates from Ethiopian soda lakes using established criteria. These strains were further evaluated for their

morphological traits, biomass accumulation, and specific growth rates, with LC-30 emerging as the top performer, exhibiting the highest dry weight ($3.27 \pm 0.51 \text{ g L}^{-1}$) and specific growth rate ($1.63 \pm 0.05 \text{ d}^{-1}$). DNA analysis (16S rRNA gene sequencing) confirmed all four strains as *Limnospira fusiformis*, not *Arthrospira*. Additionally, biochemical composition analysis of two potential strains, LC-30 and LA-08, revealed impressive protein and phycocyanin levels, with LC-30 showing 58.28% ash-free dry weight and LA-08 reaching 55.79%. This study highlights the potential of these indigenous strains for large-scale biomass production and nutritional supplementation.

Secondly, in Chapter III, the effects of varying outdoor light intensities on the growth and biochemical composition of *Limnospira fusiformis* were investigated to optimize outdoor cultivation strategies. Four light scenarios were tested: full sunlight ($2000 \mu\text{mol m}^{-2}\text{s}^{-1}$), greenhouse light ($1700 \mu\text{mol m}^{-2}\text{s}^{-1}$), mid-day shade ($1400 \mu\text{mol m}^{-2}\text{s}^{-1}$), and whole-time shade ($1400 \mu\text{mol m}^{-2}\text{s}^{-1}$). Whole-time shade yielded the highest last day dry weight (2.10 g L^{-1}), protein content (63.10% ash-free), and phycocyanin productivity ($0.11 \text{ g L}^{-1}\text{d}^{-1}$), while high light intensities led to increased carbohydrate accumulation but reduced protein synthesis and cell growth, indicating photoinhibition. The findings highlight the need to optimize light intensity, particularly through shading, to enhance biomass yield and improve nutritional quality, making large-scale production more feasible for food industry applications. The two-stage cultivation method for phycocyanin demonstrated a 32.7% increase in content compared to the simulated study.

Chapter IV discussed the molecular identification and reclassification of Ethiopian *Limnospira fusiformis*, its growth potential and nutritional value, shading strategies for enhancing protein and phycocyanin production, two-stage cultivation for phycocyanin enhancement, the feasibility of raceway ponds for large-scale cultivation, and future research directions addressing outdoor challenges, strain diversity, and energy efficiency.

1 CHAPTER I General Introduction

1.1 Overview of malnutrition and the role of microalgae

Malnutrition, driven by insufficient dietary diversity, climate change, conflict, and rapid population growth, is a critical global issue, particularly in developing countries (Lindgren et al., 2018). It leads to severe micronutrient deficiencies, stunting, and acute malnutrition, causing millions of child deaths each year. In Ethiopia, where 38% of children are stunted and 9% suffer from wasting (EDHS, 2019; Deyessa and Ayele, 2019; Mengesha et al., 2020), the crisis is especially acute (Fig. I-1). Malnutrition, often due to inadequate dietary intake, is a major contributor to childhood mortality, responsible for nearly half of all deaths in children under five (UNICEF/WHO/World Bank Group –, 2023). This situation leads to long-term consequences, including reduced educational attainment and cognitive development, ultimately diminishing human capital. The imbalance between population growth and agricultural production has further exacerbated reliance on unbalanced diets, worsening the crisis (Demisu and Desisa Benti, 2018). Tackling this challenge necessitates robust measures to enhance dietary diversity and improve nutritional intake (Woldegebriel et al., 2020). One promising solution is the cultivation of microalgae and, which are rich in essential nutrients and can be sustainably produced (Hug and von der Weid, 2012). Microalgal biomass has a long history of use as human food, particularly as dietary supplements in industrialized countries to address micronutrient deficiencies (Koyande et al., 2019). However, in developing countries like Ethiopia, such resources remain significantly underutilized. Expanding the use of microalgae and cyanobacteria in these regions could be crucial for combating malnutrition and improving public health.

Microalgae have been consumed for over 2,000 years, with early use by Chinese people and indigenous cultures during times of famine. The first recorded use dates to when the Chinese

collected *Nostoc* from lakes to survive famine conditions (Spolaore et al., 2006). Despite this long history, only a few species, such as *Arthrospira platensis* (*Spirulina*), *Chlorella vulgaris*, and *Aphanizomenon*, have been domesticated for human consumption. Large-scale cultivation of microalgae is a more recent development. In the 1940s, concerns over rapid population growth and the potential for severe food shortages prompted researchers to explore microalgae as a sustainable source of human nutrition (Ferreira et al., 2012). This led to intensive studies on the outdoor cultivation of *Chlorella* species as a human protein source (Oren, 2010; Borowitzka, 2016). By the 1950s, large-scale outdoor cultivation technologies emerged, beginning in Japan during the post-World War II recovery period and later expanding to the U.S. and Europe (Lafarga et al., 2020). These technologies focused on mass cultivation for protein production, with certain microalgae strains capable of accumulating up to 70% protein during rapid growth. Today, microalgae are extensively utilized in both biological and industrial applications, including human foods, functional ingredients, cosmeceuticals, pharmaceuticals, fatty acids, and carotenoids. The process, as outlined in Fig. I-2, highlights the current trends in large-scale production and application, showing the key steps from sampling in water bodies to final product development (Kaur et al., 2023). This approach is critical for ensuring that selected microalgal strains are optimized for large-scale cultivation, focusing on characteristics like high biomass yield, nutrient composition, and resistance to environmental factors. Optimized strains are then applied across various industries, supporting the sustainable and efficient production of nutritional supplements, bioactive compounds, and other industrial products, ultimately meeting the growing global demand (Mehariya et al., 2021). Their high biochemical composition and significant nutritional and health benefits have heightened public awareness and intensified scientific focus on the cultivation, biomass production, and enhancement of microalgal metabolites. This growing interest is driven by their potential as sustainable, nutrient-

rich food sources (Table I-1), meeting global demand while simultaneously promoting health and environmental benefits.

Over the past 60 years, the commercial cultivation of cyanobacteria species like *Arthrospira*, *Chlorella* (Chlorophyta), *Dunaliella salina*, *Isochrysis galbana*, and *Scenedesmus obliquus* has advanced significantly, leading to the production of essential molecules such as proteins, carbohydrates, lipids (Table I-1), pigments, vitamins, and minerals (Lee, 1997). Among the potential species, *Arthrospira* and *Chlorella vulgaris* stand out as particularly notable, being marketed as protein-rich food ingredients and supplements, as well as functional foods, due to their high nutrient content. Their nutrient-rich compositions and favorable essential amino acid profiles have propelled them to become dominant players in the global microalgae market, with protein content ranging from 46-71% in *Arthrospira* and 51-58% in *Chlorella* (Table I-1), surpassing traditional protein sources like eggs, beef, and soybeans (Hug and von der Weid, 2012; Abate and Belachew, 2019). *Arthrospira* is highly valued for its rapid growth rate and swift doubling time, making it an ideal candidate for biomass cultivation (Ogbona et al., 2007; Gani et al., 2019), highlighting its potential for large-scale production and sustainable applications.

1.2 Nutritional significance of *Arthrospira* cultivation

Arthrospira, commonly known as *Spirulina*, is a versatile cyanobacterium thriving in diverse aquatic environments, including freshwater, brackish, and alkaline saline lakes (Torzillo and Vonshak, 2013). Globally, *Arthrospira* is extensively cultivated for human nutrition with major production centers in Japan, the USA, India, China, and Myanmar (Olabi et al., 2023). Its cultivation has shown an increasing trend year after year, driven by growing demand for sustainable, nutrient-rich food sources and its expanding applications in health and wellness industries (Fig. I-3). It has

garnered significant attention for its exceptional nutritional and health benefits, making it a key ingredient in food supplements, pharmaceuticals, and cosmetics. With a rich nutritional profile that includes 60-70% protein, 15-25% polysaccharides, 3-9% lipids (Fig. I-4, Table I-1), essential pigments like phycocyanin and carotenoids, vitamins (B12, pro-vitamin A), minerals (notably iron), gamma-linolenic acid (GLA), and antioxidants such as phycocyanin and β -carotene, *Arthrospira* is highly valued for its contributions to human health (Bhattacharya and Shivaprakash, 2005). The balanced amino acids in *Arthrospira* proteins can be hydrolyzed into bioactive peptides, enhancing their use in functional foods, pharmaceuticals, and nutraceuticals (Lupatini et al., 2017). Despite its proven safety, good taste, and potent nutritional properties, the focus on *Arthrospira* research and production is unevenly distributed, with developed nations rapidly advancing in large-scale cultivation and application, while many developing countries, particularly in regions where nutritional interventions are most needed, have not yet fully embraced its potential (Amadou and Lawali, 2022). This disparity highlights the need for increased awareness and investment in *Arthrospira* research and production to ensure its benefits are accessible worldwide including developing nations.

Arthrospira has been particularly effective in treating malnutrition in children due to its high nutritional composition. It is utilized in various forms, including powders, tablets, extracts, and supplements, and is often incorporated into traditional food products such as yogurt, green tea, and bakery goods, enhancing their nutritional value without compromising taste (Matondo et al., 2016; Wu et al., 2023). Several studies have demonstrated its efficacy in improving protein, iron, and retinol levels, thereby enhancing hematological health and intellectual development in school-aged children (Sachdeva et al., 2004; Lucas et al., 2023). Its abundant β -carotene content also significantly boosts vitamin A stores, a critical factor in combating vitamin A deficiency (Bhattacharya and Shivaprakash, 2005). Regular supplementation with *Arthrospira* has been shown

to promote weight gain in young preschool children and improve overall nutritional status in undernourished children (Matondo et al., 2016; Barennes et al., 2022). Moreover, prolonged consumption of *Arthrospira* granules has led to substantial nutritional improvements, highlighting its potential as a long-term solution for malnutrition (Kashyap et al., 2022). Therefore, *Arthrospira* is widely recognized for its significant nutritional benefits due to its rich chemical composition, making it an important resource in the fight against malnutrition. However, its potential remains largely untapped in Ethiopia, where developing and integrating Spirulina-fortified foods into national nutrition programs could effectively address problem of dietary diversity existed in the country. Thus, expanding the utilization of *Arthrospira* in traditional diets holds great potential to enhance human nutrition and meet the growing demand for functional foods in Ethiopia. This can be achieved by exploring indigenous *Arthrospira* strains from Ethiopian soda lakes, offering a sustainable solution to enhance dietary diversity and improve food security.

1.3 Ethiopian soda lakes as optimal habitats for *Arthrospira*

Soda lakes are highly alkaline water bodies with pH levels often exceeding 9, characterized by high concentrations of sodium carbonate and bicarbonate. These lakes, typically located in arid or semi-arid regions, experience significant evaporation with limited freshwater inflows, contributing to their alkalinity and salinity (Kebede, 1997; Lanzén et al., 2013). Despite their extreme conditions, soda lakes provide unique habitats for organisms like *Arthrospira*, a cyanobacterium that thrives in extreme environments. Key limnological features of soda lakes include high salinity, electrical conductivity, elevated temperatures, and nutrient variability, particularly nitrogen and phosphorus, all of which influence the productivity and ecology of the lakes (Girma et al., 2012; Mebrat and Etisa, 2023). Soda lakes in Ethiopia, including lake Chitu,

lake Arenguade, lake Beseka and lake Shalla are part of the East African Rift Valley system and are known for their high salinity, alkalinity, pH and different nutrient levels (Table I-2) (Misztak et al., 2021; Melese and Debella, 2023). These conditions favor species like *Arthrospira fusiformis*, which dominates microbial communities in lakes such as Chitu (Melese and Debella, 2024). However, Lake Arenguade, being less extreme in terms of alkalinity and salinity, has seen changes in its physicochemical characteristics, including increased nutrient loading and fluctuating salinity (Table I-2). These shifts have led to more frequent algal blooms, with *A. fusiformis* increasingly overshadowed by other cyanobacteria, altering the lake's phytoplankton structure (Girma et al., 2012; Getachew et al., 2019; Melese and Debella, 2023; 2024). This highlights the need for comprehensive assessment studies in these lakes to evaluate the status of *A. fusiformis* and other species.

Phytoplankton communities in soda lakes are typically dominated by cyanobacteria like *Arthrospira*, which thrives in alkaline, saline conditions (Fig. I-5). Cyanobacteria play a major role in primary production, while other phytoplankton groups, such as diatoms (*Cyclotella*, *Navicula*) and green algae (*Monoraphidium minutum*, *Coelastrum astroidem*), are less dominant but can increase in abundance during periods of high nutrient availability, especially after rainy seasons (Melese and Debella, 2023; 2024). Bacillariophyceae (diatoms) predominate in lakes Shala and Beseka due to lower salinity, stable nutrient levels in lake Shala, and higher silica concentrations in lake Beseka, creating favorable conditions for their growth (Fig. I-5). In contrast, the more extreme salinity and alkalinity in lakes Chitu and Arenguade favor the dominance of cyanobacteria like *Arthrospira fusiformis* (Melese and Debella, 2024). Although much is known about the limnology of soda lakes and their ecological roles, there are significant gaps in understanding the biochemical potential of indigenous *Arthrospira* strains sourced from these lakes. Few studies have examined

the biochemical profiles of *Arthrospira* strains across seasons, their adaptability to environmental stress, or their genetic diversity (Li et al., 2001; Getachew et al., 2019). Furthermore, research on optimizing large-scale cultivation methods tailored to these species is limited, despite their clear commercial and nutritional potential.

Indigenous *Arthrospira* strains from Ethiopian soda lakes offer significant potential for addressing food security and malnutrition, given their high adaptability to extreme environments, rich nutrient content, and rapid growth rates (Assaye et al., 2018; Karssa et al., 2018; Woldie et al., 2024). Their ability to thrive in highly alkaline and saline conditions minimizes the risk of contamination, making them ideal for large-scale cultivation. Therefore, there is a need to explore the locally adapted *Arthrospira* strains from Ethiopian soda lakes, updating molecular data to assess their potential as a sustainable and nutritionally rich food source, which could play a critical role in enhancing dietary diversity and combating malnutrition in Ethiopia.

1.4 Taxonomic challenges and advancements in *Arthrospira* classification

The classification of microalgae and cyanobacteria is primarily based on morphological observations through microscopy. However, environmental variations can induce phenotypic plasticity, particularly in cyanobacteria, making morphology-based identification potentially misleading and prone to misidentification (Hicks et al., 2021). Studies suggest that a significant number of cyanobacterial strains in culture collections may be misidentified (Anagnostidis and Komárek, 1988). Therefore, alongside morphological assessments, molecular methods are increasingly employed to improve the taxonomic accuracy of cyanobacterial species (Komárek and Mareš, 2012). Commonly, *Arthrospira* species have been identified based on their morphological characteristics, as outlined by Ciferri (Ciferri, 1983). However, this process presents significant

challenges due to the close similarities among strains and the influence of environmental conditions on their morphology, leading to various morphological forms (Cheng et al., 2018). These limitations indicate that relying solely on microscopy for accurate identification is insufficient.

Recent advancements in molecular and ultrastructural analyses have significantly reshaped the taxonomy of *Arthrospira*. Initially described as *Arthrospira jenneri* by Stizenberger and Gomont in 1892 (Gomont, 1892), the genus underwent various reclassifications, transitioning to *Spirulina* in 1925 (Geitler, 1925), reverting to *Arthrospira* in 1989 (Castenholz, 1989), and finally being split into two lineages in 2019. *Arthrospira jenneri* retained its original classification, while the newly defined genus *Limnospira* was established for species such as *L. fusiformis*, *L. maxima*, and *L. indica*, previously known as *A. fusiformis*, *A. maxima*, and *A. indica* (Nowicka-Krawczyk et al., 2019). However, *A. platensis* was not reassigned to *Limnospira* due to insufficient information on its type strain, highlighting the critical need for updated research on the taxonomy of *Arthrospira*.

In a study by Roussel et al. (2023), a total of 92 *Limnospira* strains were analyzed for taxonomic review, including 72 new isolates from the Camargue region in the Rhône delta, France (Cellamare et al., 2018), 13 from Lake Dziani Dzaha (Leboulanger et al., 2017; Cellamare et al., 2018), four from Lake Natron, two from a historical collection, and one from *Spirulina* Solutions ([https:// spirulinasolutions.fr/](https://spirulinasolutions.fr/)). Additional reference strains, such as *L. indica* PCC 8005, *A. platensis* PCC 7345 (Pasteur Culture Collection of Cyanobacteria, France), and *L. fusiformis* SAG 85.79 (Culture Collection of Algae at Göttingen University, Germany), were used to identify new isolates. The current lack of information on the taxonomy of Ethiopian *Arthrospira* species highlights the need for further research in this area. None of the previous findings have provided detailed reports specifically addressing the molecular taxonomy of *Arthrospira* strains from

Ethiopia, underscoring the importance of conducting comprehensive genetic studies to better understand these indigenous strains.

Key features that reliably distinguish *Arthrospira* from *Limnospira* include the arrangement of thylakoids within cells and the presence of a calyptra or thickened cell wall in apical cells. *Arthrospira* contains radially arranged thylakoids, while *Limnospira* exhibits irregular, whirl-like thylakoid sections in the central part of the cell (Nowicka-Krawczyk et al., 2019). Although environmental factors may influence the number or density of thylakoids, their arrangement remains a stable characteristic within genetic clusters identified by the 16S rRNA gene (Misztak et al., 2021). Additionally, the presence of a thickened cell wall or calyptra is unique to *Limnospira* and has not been observed in *Arthrospira* trichomes, nor was it mentioned in the original description of *A. jenniferi* (Nowicka-Krawczyk et al., 2019). Most strains and species within the genus *Limnospira*, including *L. fusiformis*, prefer alkaline habitats, which facilitates large-scale cultivation by reducing the risk of contamination from other organisms (Nowicka-Krawczyk et al., 2019; Misztak et al., 2021; Roussel et al., 2023). These unique traits make *Limnospira* an ideal candidate for large-scale food production. Therefore, accurate taxonomic identification is crucial for selecting high-performing strains, ensuring optimal productivity and quality, and enhancing the overall efficiency and reliability of mass cultivation in the food industry.

1.5 Optimization of light intensity for sustainable outdoor *Arthrospira* cultivation

Optimizing the cultivation of *Arthrospira* is necessary for maximizing biomass yield, improving product quality, and ensuring efficiency from laboratory to commercial scales. *Arthrospira* species thrive under specific conditions, making the optimization of factors such as light intensity, dissolved inorganic carbon, pH, and temperature essential for biomass production

(Kishi and Toda, 2018). Significant progress has been made in understanding *Arthrospira* growth in laboratories, but translating these conditions to outdoor cultivation is challenging due to fluctuating light and temperature, necessitating strain-specific optimization.

Optimized sunlight plays a crucial role in enhancing the growth and biochemical accumulation of *Arthrospira* species (Jian-Fei et al., 2023). Tailoring light conditions in outdoor cultivation, particularly in tropical countries with abundant sunlight, can significantly boost *Arthrospira* biomass production (Jesus et al., 2018; Karssa et al., 2018; Sukumaran et al., 2018; Sung et al., 2021). Studies show that while growth rates increase with rising light intensity, this effect plateaus once light saturation is reached (Xue et al., 2011). Exposure to excessive light, particularly during intense midday sunlight, can lead to photoinhibition, where Photosystem II (PSII) becomes overburdened, reducing photosynthetic efficiency and overall growth (Vonshak, 1987; Carneiro et al., 2018; Hidasi and Belay, 2018). The stress is often linked to the production of reactive oxygen species (ROS), which damage cellular structures and exacerbate physiological stress (Carneiro et al., 2018). Excessive photosynthetically active radiation (PAR) can lead to reduced growth rates and altered biochemical composition, highlighting the importance of optimizing light conditions for effective *Arthrospira* cultivation (Hidasi and Belay, 2018).

Phycocyanin, a valuable light-harvesting pigment in *Arthrospira*, is particularly sensitive to excessive light exposure (Jesus et al., 2018). While light optimization for biomass production may not always be ideal for phycocyanin induction, further refinement is required to balance light intensity for both growth and pigment (Jiang et al., 2023; Yu et al., 2023). Thus, enhancing phycocyanin content requires further refinement of light conditions to achieve high pigment yield without compromising overall biomass production. Therefore, optimizing light exposure is necessary for maximizing both biomass yield and phycocyanin content, emphasizing the need for cultivation strategies that carefully balance light conditions to achieve optimal results. The species

used in this study, *Limnospira fusiformis*, was found in our prior findings from Ethiopian soda lakes (Woldie et al., 2024), has yet to be fully optimized for outdoor light intensity. By refining light conditions, sustainable and large-scale cultivation of *Limnospira fusiformis* can be achieved, ensuring both high productivity and quality.

1.6 Objectives of the study

This PhD thesis aims to address the significant challenges and unexplored opportunities in the cultivation and utilization of *Limnospira* strains from Ethiopian soda lakes, with a particular focus on their potential for large-scale biomass production and their nutritional contributions, especially in combating malnutrition. This will be accomplished by achieving the following specific objectives:

- 1) Isolate, screen, and characterize *Limnospira* strains from Ethiopian soda lakes, with a focus on evaluating growth and biomass production, protein content, and overall nutritional value (Chapter II),
- 2) Evaluate the growth potential and biochemical composition, including phycocyanin content, of indigenous *Limnospira fusiformis* under simulated outdoor light intensities (Chapter III),
- 3) Develop and refine a two-stage cultivation strategy to enhance phycocyanin content in *Limnospira fusiformis* strains and evaluate its impact and optimization for large-scale production (Chapter III). By achieving the above objectives, this PhD research lays a solid foundation for scalable and sustainable microalgae cultivation, addressing malnutrition, enhancing biotechnological innovations, and optimizing large-scale production systems tailored to Ethiopia's unique environmental conditions.

Tables

Table I-1. Biochemical composition of dry biomass of different microalgae species.

Microalgae species	Composition (% dry matter)			References
	Proteins	Lipids	Carbohydrates	
<i>Anabena cylindrica</i>	43–56	4–7	25–30	Becker, 2004
<i>Aphanizomenon flos-aquae</i>	62	3	23	Christaki et al., 2011
<i>Botryococcus braunii</i>	39–40	25–34	19–31	Tibbetts et al., 2015
<i>Chaetoceros gracilis</i>	12	7.2	4.7	Brown, 1991
<i>Chlamydomonas reinhardtii</i>	48	21	17	Spolaore et al., 2006
<i>Chlorella pyrenoidosa</i>	57	2	26	Chisti, 2007
<i>Chlorella vulgaris</i>	51–58	14–22	12–17	Mata et al., 2010
<i>Dunaliella salina</i>	57	6	32	Sousa et al., 2008
<i>Isochrysis galbana</i>	50–56	12–14	10–17	Silva and Aranda, 2013
<i>Nannochloropsis sp.</i>	30	22	10	Kent et al., 2015
<i>Phaeodactylum tricornutum</i>	34.8	16.1	16.8	Tibbetts et al., 2015
<i>Scenedesmus dimorphus</i>	8–18	16–40	21–52	Kent et al., 2015
<i>Scenedesmus obliquus</i>	50–56	12–14	10–17	Cai et al., 2013
<i>Spirogyra sp.</i>	6–20	11–21	33–64	Bruton, 2009
<i>Spirulina maxima</i>	60–71	6–7	13–16	Becker, 2007
<i>Spirulina platensis</i>	46–63	4–9	8–14	Sousa et al., 2008
<i>Tetraselmis chuii</i>	31–46	25	12	Tibbetts et al., 2015
<i>Tetraselmis maculata</i>	52	3	15	Schwenzfeier et al., 2011

Table I-2. Limnological characteristics of four Ethiopian soda lakes (Melese and Debella, 2023).

Parameters	Lakes			
	Arenguade	Beseka	Chitu	Shala
Temperature (°C)	22.20	25.90	22.80	22.90
pH	10.04	8.55	10.46	10.12
DO (mg L ⁻¹)	3.33	6.61	5.44	7.36
Alkalinity (meq L ⁻¹)	50.20	36.20	426.70	305.70
Salinity (g L ⁻¹)	2.91	1.88	38.40	16.78
Turbidity (NTU)	37.70	35.90	30.30	15.05
NO ₃ -N (µg L ⁻¹)	40.60	98.40	69.50	27.12
NH ₃ -N (µg L ⁻¹)	161.90	56.40	113.00	89.20
SiO ₂ (mg L ⁻¹)	39.40	58.40	2.59	2.37
Soluble reactive phosphorus (mg L ⁻¹)	1.04	0.44	1.75	1.05
Total phosphorus (mg L ⁻¹)	2.08	1.15	2.41	1.59
HCO ₃ ⁻ (meq L ⁻¹)	51.34	27.10	313.10	140.73
CO ₃ ²⁻ (meq L ⁻¹)	67.60	29.53	279.90	141.82

Notes: NTU– Nephelometric Turbidity Unit, meq L⁻¹– milliequivalents per liter

Figures

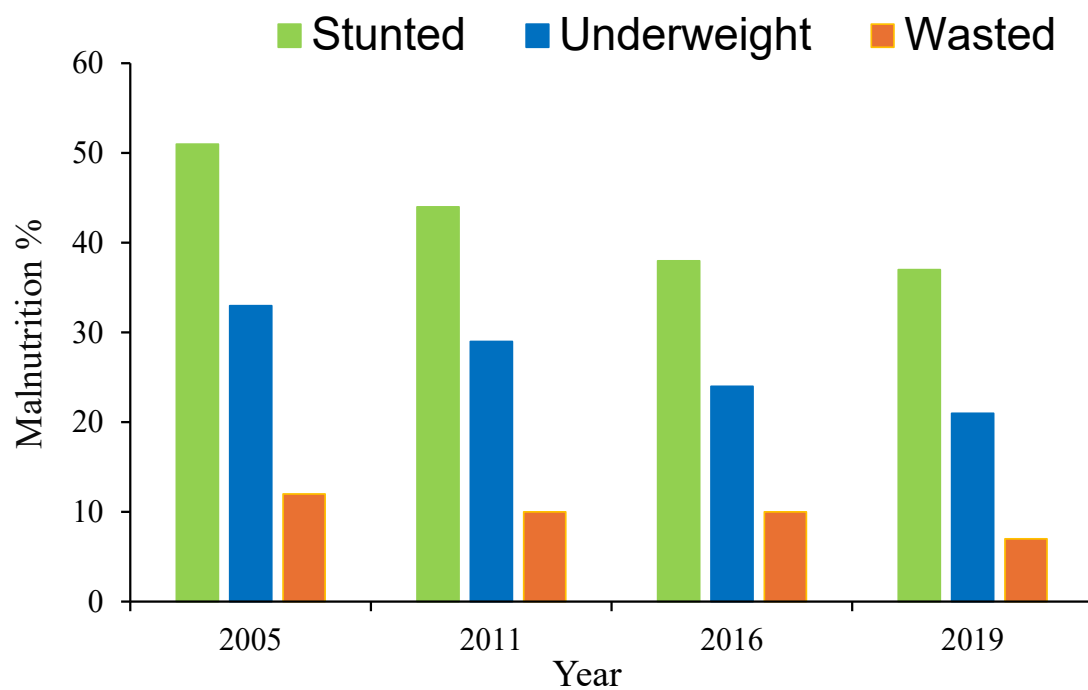


Fig. I-1. The percentage of malnourished children under 5, showing trends in stunting, wasting, and underweight prevalence (EDHS, 2019).

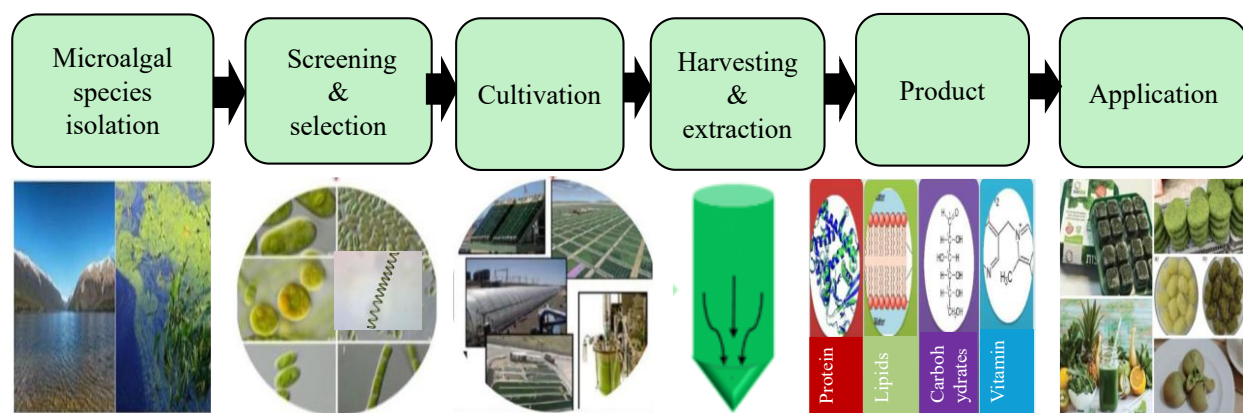


Fig. I-2. Microalgal cultivation process (modified from Kaur et al., 2023).

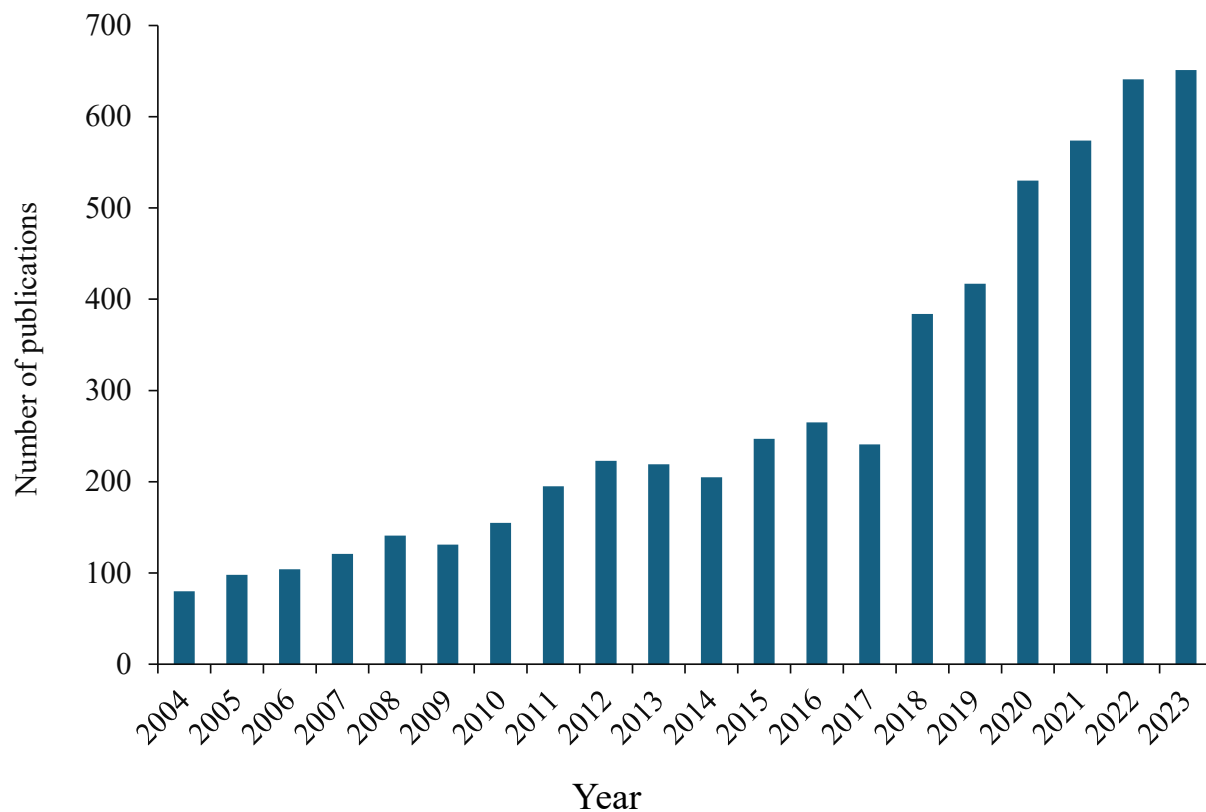


Fig. I-3. Number of publications in the last twenty years identified by searching specific keywords: "*Spirulina*" and "*Arthrospira*" combined with terms related to food and functionality (Scopus.com)

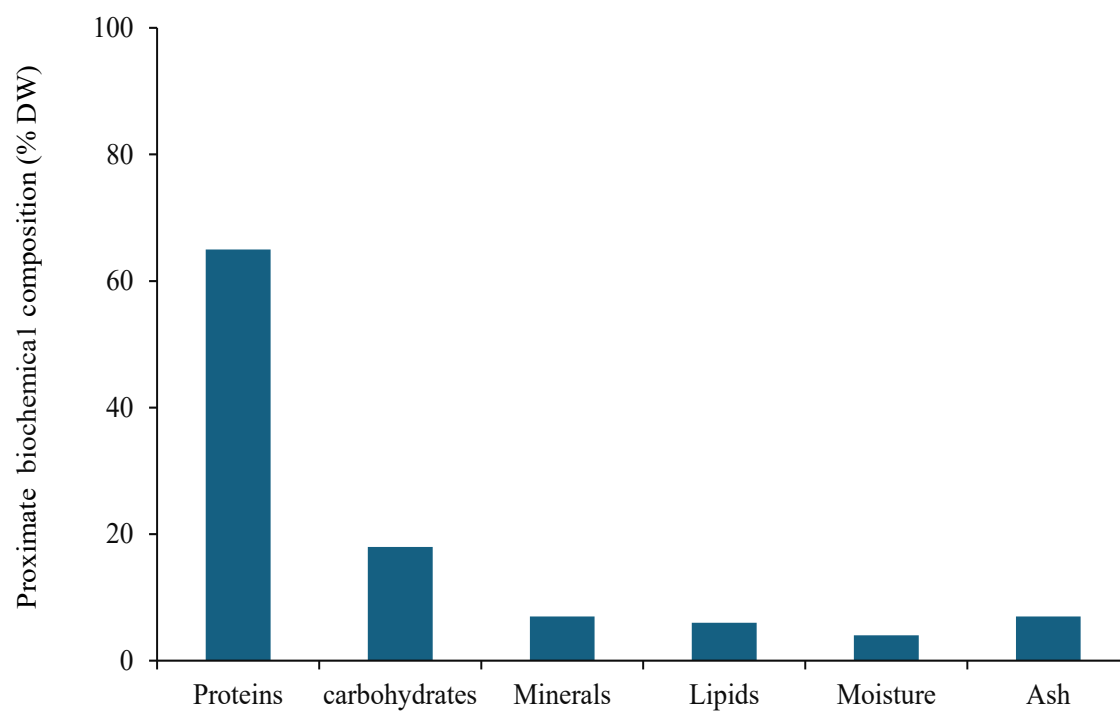


Fig. I-4. Proximate biochemical composition of *Arthrospira* species (Aouir et al., 2017).

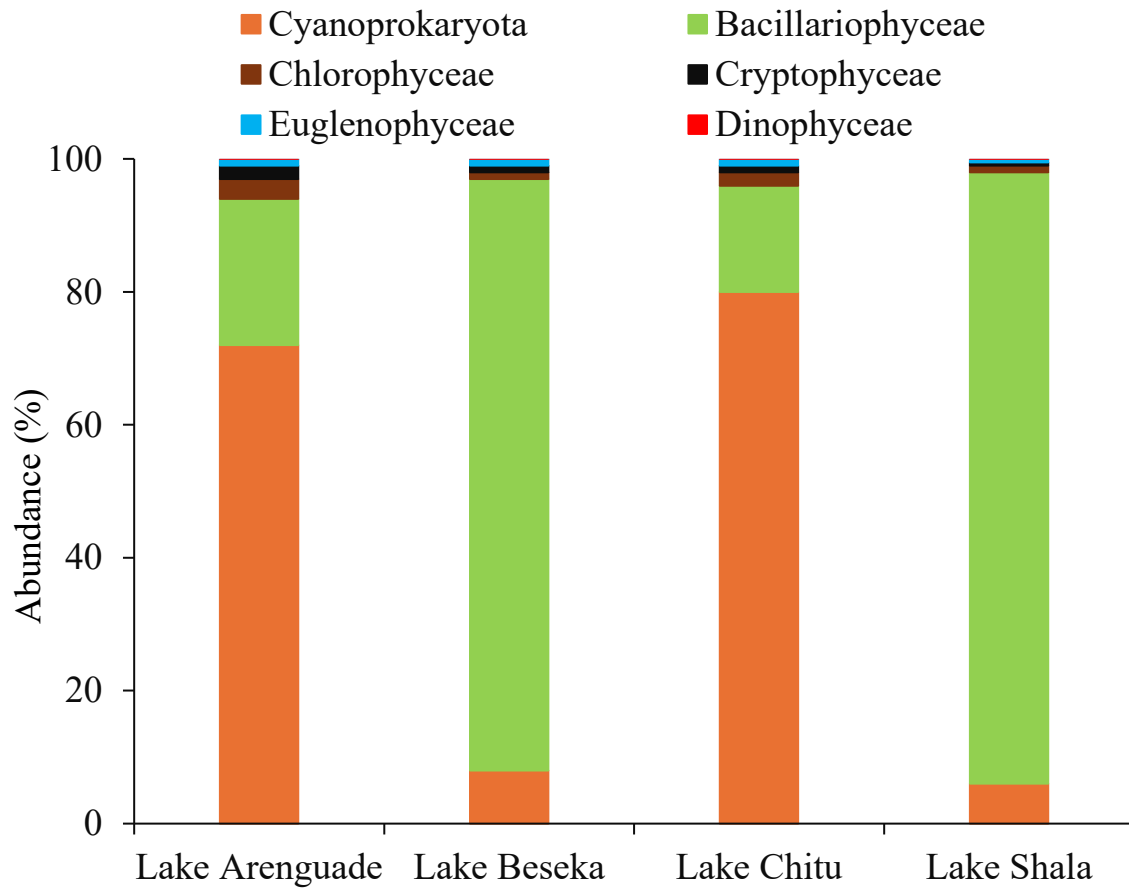


Fig. I-5. Abundance of phytoplankton groups in four Ethiopian soda lakes (modified from Melese and Debella, 2024).

2 CHAPTER II Exploration of indigenous *Arthrospira* strains with high biomass and nutritional value

2.1 Introduction

Despite the potential of indigenous *Arthrospira* strains, their full utilization for human nutrition in Ethiopia remains limited, highlighting the need for further research and exploration. The success of *Arthrospira* cultivation for sustainable nutrition depends on identifying strains with fast growth rates and high biomass production (Zhang et al., 2022). Given the diverse characteristics of different strains, including growth rates, nutritional content, and adaptability to specific culture conditions, it is essential to select the most suitable strains for large-scale production (Vonshak, 1987). Isolation and screening of microalgae strains have long been recognized as critical steps in maximizing the potential of local species for various applications. Previous studies demonstrated the importance of isolating and screening microalgae from natural habitats to identify those with the highest biomass productivity (Lee et al., 2014; Najeeb et al., 2024). Screening enables the selection of *Arthrospira* strains that demonstrate high growth rates, maximum biomass production, and resilience to environmental fluctuations in light, temperature, and nutrient availability (Zaki et al., 2021; Huesemann et al., 2023a). By selecting strains suited for outdoor cultivation, stable production can be achieved while minimizing the need for controlled environments. Bhattacharya and Shivaprakash (2005) evaluated three *Spirulina* species—*S. platensis*, *S. laxissima*, and *S. lonar*—under identical conditions, finding *S. platensis* to be the top performer due to its faster growth, higher biomass production, and elevated levels of essential pigments like chlorophyll-*a* and C-phycocyanin, as well as high biomass concentration, making it ideal for large-scale cultivation.

In addition to growth rates and biomass production, morphological characteristics such as trichome length and helix pitch are critical screening criteria. These traits directly impact biomass yield and harvesting efficiency. Cheng et al.(2018) demonstrated that increasing trichome length and helix pitch improves biomass harvesting efficiency and enhances carbon dioxide fixation rates, both crucial for large-scale production. Longer trichomes correlated with higher biomass growth, while larger helix pitches facilitated easier and more efficient harvesting by preventing trichomes from passing through filter systems. Optimizing these morphological traits can significantly enhance both productivity and harvesting efficiency, making them key factors in selecting strains for sustainable large-scale *Arthrospira* cultivation. This highlights the importance of screening to identify strains with superior growth and biomass, ensuring optimal productivity for food and biotechnology applications. Strains that are well-suited to local environmental conditions and food applications can greatly enhance cultivation techniques, contributing to efforts aimed at addressing nutritional deficiencies in Ethiopia.

Morphological isolation of *Arthrospira* strains, traditionally used for classification, has significant drawbacks due to the close resemblance between strains and the influence of environmental factors on their morphology (Li et al., 2001). This often leads to misidentifications, resulting in variations in growth rates, nutrient compositions, and product quality, thereby compromising the reliability of large-scale cultivation (Kaggwa et al., 2013). Recent advancements in molecular and ultrastructural analyses have transformed *Arthrospira* taxonomy, highlighting the need for up-to-date molecular data to accurately represent strain diversity (Nowicka-Krawczyk et al., 2019). The lack of comprehensive ultrastructural data and ongoing taxonomic revisions emphasize the necessity of advanced genetic analyses, such as 16S rRNA sequencing, to accurately categorize and identify strains (Ludwig and Klenk, 2001). Accurate taxonomic identification is

essential for optimizing the nutritional and commercial potential of *Arthrospira*, ensuring consistent product quality and reliability in large-scale cultivation. Misidentification can lead to inconsistencies in product quality, yield, and unpredictable outcomes, undermining the benefits of using specific strains. Given the taxonomic uncertainties, particularly in ecological and biotechnological contexts, molecular identification and potential reclassification of *Arthrospira* species from Ethiopian soda lakes is necessary for reliable and efficient production in the food industry.

Although molecular identification of fast-growing *Arthrospira* strains is a critical foundational step, it alone does not suffice for ensuring successful large-scale outdoor cultivation. A novel, comprehensive screening approach is necessary, focusing on the quantification of key nutritional components such as protein and phycocyanin, a pigment crucial for human nutrition. This advanced method goes beyond traditional identification techniques by integrating molecular, biochemical, and physiological assessments, enabling the selection of strains that not only grow rapidly but also possess superior nutritional profiles (Leca et al., 2023). This innovative and thorough screening process is vital for enhancing *Arthrospira* cultivation, establishing it as a reliable and sustainable solution for food and health applications (Dinpazhooh et al., 2022). This PhD study aims to isolate and screen locally adapted *Arthrospira* strains from Ethiopian soda lakes. By identifying strains with superior growth rates, resilience, and enhanced nutritional profiles, the goal is to optimize *Arthrospira* cultivation for sustainable large-scale production. Therefore, to identify and improve representative strains specifically suited for outdoor cultivation and to ensure consistent productivity and high-quality biomass, comprehensive biochemical analysis is essential.

This chapter determined the potential of indigenous *Arthrospira* strains from Ethiopian soda lakes through a series of focused investigations. The study began by isolating and characterizing the

strains based on their morphological traits. The growth rates and biomass yields of the isolated strains were then evaluated under controlled laboratory conditions. Molecular identification techniques, specifically 16S rRNA sequencing, were employed to accurately classify the strains. Finally, the nutritional composition of the selected strains was assessed, with particular emphasis on protein and phycocyanin contents. These steps were helpful in identifying and optimizing strains with high growth performance and nutritional value for large scale biomass production trials.

2.2 Materials and Methods

2.2.1 Sample collection and strain maintenance

Water samples were collected from two soda lakes situated in Ethiopia, Lake Chitu (LC) and Lake Arenguade (LA), on March 12, 2020. In the field, we measured the pH using a portable pH meter (SO10, HORIBA) and assessed the light intensity using a digital lux meter (HTC LX-101, HTC). Additionally, both the water and atmospheric temperatures were recorded *in situ* using a thermorecorder (TR-52, T&D Corporation). Lake Chitu is a volcanic explosion crater lake at a latitude of 7°24'30"N, a longitude of 38°25'30"E, and an altitude of 1540 m above sea level (Fig. II-1). Lake Arenguade is also a member of the crater lakes, located at a latitude of 8°42.10"N and longitude of 38°58.30"E, and an altitude of approximately 1900 m above sea level (Fig. II-1). Water samples were collected using a bucket and then filtered with a 100 µm size mesh to remove the zooplankton. The water samples were transferred into 100 mL plastic bottles and taken to the laboratory at Bahir Dar University, Ethiopia.

The cyanobacterial strains were cultivated in a modified *Spirulina* Ogawa Terui (SOT) medium containing (in mg L⁻¹): Na₂CO₃ (12,189), NaHCO₃ (9,660), K₂HPO₄ (500), NaNO₃ (2,500), Na₂SO₄ (815), NaCl (1,000), MgSO₄·7H₂O (200), CaCl₂ (30), FeSO₄·7H₂O (10), Na₂EDTA·2H₂O (80),

H₃BO₃ (2.86), MnSO₄·5H₂O (2.17), ZnSO₄·7H₂O (0.222), CuSO₄·5H₂O (0.079), and Na₂MoO₄·2H₂O (0.021). The initial pH of the medium was meticulously adjusted to 9.8 and optimized for *Arthrospira* strain cultivation (Kishi and Toda, 2018).

To maintain sterility, all culture apparatus underwent autoclaving at 121 °C for 20 min using an autoclave (SX-500, TOMY). The modified SOT medium was further sterilized by filtration through a 0.22-μm membrane filter (GPWP, Millipore). Cyanobacterial strains were then cultured in 500-mL Erlenmeyer flasks, each containing approximately 250 mL of the modified SOT medium and incubated at 35 °C for 7 days. Illumination was provided by cool-white, fluorescent lamps (FL20SEX-N-HG, NEC) at an irradiance of 160 μmol photons m⁻² s⁻¹ photosynthetic photon flux density. The culture was subjected to alternate light cycles of 12-h L/12-h D with continuous mixing at a flow rate of 0.25 vvm. The optimal culture conditions (medium, temperature, and pH) were determined based on previous studies specifically tailored for *Arthrospira* strain cultivation (Kishi and Toda, 2018).

2.2.2 Morphological isolation and selection of potential strains

After maintaining the isolates in the SOT medium, morphological characterization was performed by placing an inoculum drop on a glass slide and examining it under an inverted microscope (Axioskop-2 Plus, Carl Zeiss). Serial dilutions were then meticulously prepared and dispensed into both 96- and 12-well microtiter plates with 200 μL and 4 mL SOT medium, respectively.

Individual trichomes were isolated and treated as distinct strains by using the capillary pipette method. *Arthrospira*-like trichomes were subsequently transferred to 12-well microtiter plates

containing sterile SOT medium. The cultures were incubated under light conditions of $160 \mu\text{mol m}^{-2} \text{s}^{-1}$ (12-h L/12-h D cycle) at 35°C .

Morphological parameters including coil count, helix diameter, pitch, overall diameter, length, and trichome abundance were meticulously evaluated. Quantitative measurements of cell morphology (in μm) were based on measuring a minimum of 10 trichomes of varying lengths using an optical microscope (Axioskop-2 Plus; Carl Zeiss) equipped with a digital camera (EOS Kiss X7i, Canon). The ImageJ 1.53 software (National Institute of Health, Bethesda, MD, USA) was used for subsequent analyses. The screening process focused on assessing purity and optical density values to ensure the selection of pure strains.

Cultures of 100 morphologically distinct isolates were performed in test tubes containing 40 mL of SOT medium. These were maintained at a constant temperature of 35°C with a light intensity of $160 \mu\text{mol m}^{-2} \text{s}^{-1}$ (12-h L/12-h D), and continuous mixing was facilitated by an air compressor (TC120246, Total Tools) throughout a 7-d cultivation period. To identify the most promising strains, we meticulously assessed maximum specific growth rates by measuring the optical density at 750 nm (OD_{750}) using a UV-Vis spectrophotometer (UV2450, Shimadzu). The maximum specific growth rate (μ_{max} ; day^{-1}) was calculated from the growth curve during the log-growth phase using Eq. (1) (Baily and Ollis, 1986).

$$\mu_{\text{max}} (\text{d}^{-1}) = \frac{\ln(X2) - \ln(X1)}{t2 - t1}$$

Where X1 and X2 are the biomass concentrations (OD_{750}) at times $t1$ and $t2$ respectively.

Out of the 100 cultured isolates, only 81 were grown and compared. It is noteworthy that the remaining 19 strains did not exhibit growth during the comparison test experiments. Among the 81 (37 from lake Arenguade and 44 from lake Chitu) morphologically identified *Arthrospira*-like strains, maximum specific growth rates exhibited variation, ranging from 0.23 ± 0.12 to 1.04 ± 0.20

d^{-1} (Table II- I). The overall average maximum specific growth rate was $0.64 \pm 0.23 \text{ d}^{-1}$ (Fig. II-1), highlighting considerable variability among the isolates in terms of growth potential. Following this, cells from the four selected strains demonstrating robust maximum specific growth rates during the exponential growth phase were transferred into 500 mL Erlenmeyer flasks, each with an effective volume of 250 mL. The flask cultures were maintained at 35°C under white, fluorescent light with a light intensity of $160 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (12-h L/12-h D), and continuous mixing at a flow rate of 0.25 vvm. The experiments were performed in triplicate to ensure the robustness of the results. Regular measurements of the biomass dry weight (DW) were recorded after sampling every 24 hours. As a methodological procedure, systematic screening helps assess microorganisms and identify promising candidates for further study by scrutinizing specific characteristics, such as growth rate and biomass accumulation (Uma et al., 2020).

2.2.3 DNA extraction and sequencing of the 16S rRNA gene

Cells from four strains (LC-30, LC-23, LA-08, and LA-32) were used for further analysis. The cells were first collected by centrifuging 1 mL of *Arthrospira*-like culture at $20,627 \times g$ at 4°C for 5 min (Janssen et al., 2010) and then used for genomic DNA extraction using the lysis buffer method (Lee and Frost, 2002). The 16S rRNA gene was amplified using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The lysis buffer, essential for DNA extraction, was meticulously prepared by combining 200 $\mu\text{g/mL}$ Proteinase K, 0.5% Tween 20, 10 mM Tris at pH 8.3, and 50 mM KCl (Lee and Frost, 2002; Kok et al., 2012). This carefully formulated lysis buffer facilitated effective dispersion and ensured comprehensive extraction of genetic material for downstream analyses. Subsequently, a polymerase chain reaction (PCR) mixture (20 μL) was prepared to contain 10 μL of $2\times$ PCR premix reagent

(EmeraldAmp® MAX PCR Master Mix, TAKARA BIO), 1 µL of each primer, sterile pure water (7 µL), and 1 µL of extracted DNA. The PCR mixture was processed under the following conditions: 94 °C for 3 min, 30 cycles of 94 °C for 30 sec, 58 °C for 30 sec, and 72 °C for 1 min 40 sec; and a final extension at 72 °C for 5 min.

The resulting PCR products were subjected to 1% agarose gel electrophoresis, and base pair length and concentration were assessed. DNA ladders of (1kb DNA Ladder PLUS NE-MWD1P, Nippon Genetics) were used as indicators of amplicon size. Sanger sequencing of PCR products was conducted by Eurofins Genomics (Eurofins, Japan) to verify the identification of cyanobacteria. To confirm taxonomic identity, the amplified DNA samples were specifically targeted for 16S rRNA molecular markers. The resulting sequences were subjected to comprehensive analysis, alignment, and comparison with the NCBI gene database using the Basic Local Alignment Search Tool (BLAST) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Genetic information retrieved from the database of the four strains was used to construct a phylogenetic tree, which was visualized using MEGA11 (Tamura et al., 2021).

2.2.4 Analytical parameters

2.2.4.1 Cell growth

The optical density of the cells was measured at 750 nm using a portable spectrophotometer (DR3900, HATCH) and a UV-VIS spectrophotometer (UV2450, Shimadzu). The pH of the culture was determined using a tabletop pH meter (D-74, HORIBA), and a thermal logger (TR-52, T&D Corporation) was used to record the temperature of the culture during growth.

To determine the biomass dry weight, the culture was filtered through pre-weighed glass fiber filters (pore size 0.7 µm, GF/F; Whatman) that were pre-combusted in a muffle furnace (KDF S-70,

KDF) at 550 °C for 6 h (Liu, 2019; Tanaka et al., 2020). Subsequently, the filters were washed with 0.28 M NH₄HCO₃ solution to remove excess salt (Zhu and Lee, 1997) and dried for over 24 h at 60 °C, then weighed. The maximum specific growth rate (μ_{\max} ; day⁻¹) for the biomass dry weight of four potential strains was calculated from the biomass curve during the log-growth phase using Eq. (2) (Baily and Ollis, 1986).

$$\mu_{\max} (\text{d}^{-1}) = \frac{\ln(X_2) - \ln(X_1)}{t_2 - t_1}$$

Where X1 and X2 are the biomass concentrations (gDW L⁻¹) at times t1 and t2 respectively.

2.2.4.2 Protein and phycocyanin content analysis

The Lowry method was used to measure the protein content of the pretreated biomass of the two selected strains (LC-30 and LA-08) using bovine serum albumin as a standard (Lowry et al., 1951). *Limnospira* culture suspension (0.9 mL) extracted on Day 8 of the cultivation period was mixed well with Milli Q water (MQW) (8.1 mL) and added to a centrifuge tube. The mixture was later centrifuged and then tested for alkaline hydrolysis in 1 mL of NaOH (1N) and dried at 60 °C for 3 h (Rausch, 1981). After centrifuging at 2,580 × g for 10 min, the supernatant was carefully discarded, and the remaining biomass underwent two drying phases, first at 60 °C and second, following the addition of 1 N NaOH, in a thorough drying process. The dried sample was rehydrated with 3.5 mL of MQW via persistent stirring. For the analysis, the rehydrated sample (1.0 mL) was placed in a HACH glass vial, mixed with 5.0 mL of sodium carbonate alkaline copper solution, and left for 10 min. Subsequently, 0.5 mL of 1/2 diluted phenol reagent was added and mixed quickly. After a minimum of 30 min at room temperature, the absorbance was measured at 750 nm (Homandberg, 1997).

Phycocyanin content was assessed following an established protocol (Tan et al., 2020). After

centrifuging the *Limnospira* culture at $10,000 \times g$ for 15 min, cells were washed with double-distilled water to eliminate adherent salts and then dried at 50 °C. Next, the cells (0.50% w/v) were subjected to a freeze-thaw process: they were initially frozen at -80 °C for 2 h and then thawed at 25 °C for 24 h in double-distilled water at pH 7. The resulting solution was centrifuged at $10,000 \times g$ at 4 °C for 30 min to isolate the clear supernatant containing phycocyanin. The phycocyanin content was quantified with high precision as previously described (Bennett and Bogobad, 1973) and the absorbance of the supernatants at 620 nm was measured using a UV-VIS spectrophotometer (UV2450, Shimadzu). The purity of phycocyanin was assessed by calculating the ratio of absorbance at 620 nm to that at 280 nm (Antelo et al., 2010).

2.2.5 Statistical analysis

Measurements were conducted in triplicate, and the data are presented as mean \pm standard deviation (SD). Significance was determined by one-way analysis of variance, followed by Tukey–Kramer multiple comparison tests using Sigma Plot (v.13) (<https://systatsoftware.com/download-sigmaplot-v13/>). Statistical significance was set at $p < 0.05$.

2.3 Results

2.3.1 Morphological identification and screening of *Arthrospira*-like strains

Using a bioprospecting approach, we morphologically identified 100 isolates initially presumed to be *Arthrospira*-like strains, providing a comprehensive analysis of Ethiopian soda lakes. Morphological characterization followed the guidelines outlined by Gomont (Gomont, 1982; Komárek and Anagnostidis, 1989). In both Chitu and Arenguade lakes, the numerous *Arthrospira*-like strains showed different levels of coil tightness, including highly and loosely coiled

morphologies (Fig. II- 3).

The morphological characteristics of *Arthrospira*-like strains, including trichome length (133–244 µm), helix diameter (17–25 µm), helix pitch (25–57 µm), trichome diameter (4–6 µm), and coil count (8–12 per trichome), highlight the need for prioritizing the understanding of morphological diversity, particularly in large-scale cultivation ($p < 0.05$; Fig. II- 4). Molecular confirmation of strains identified as potential candidates for biomass production is essential (Mishra et al., 2022).

2.3.2 Molecular identification and phylogenetic analysis of selected strains

The integration of DNA sequencing and phylogenetic analysis serves as a cornerstone in unraveling the genetic intricacies among *Arthrospira* strains, which is crucial for precise taxonomic delineation and subsequent applications (Ballot et al., 2004; Mishra et al., 2022). The evolutionary journey of taxonomic classification, transitioning from *Arthrospira* to *Spirulina*, reverting to *Arthrospira* (Castenholz, 1989), and culminating in its reclassification as *Limnospira* in 2019 (Nowicka-Krawczyk et al., 2019), highlights the dynamic nature of cyanobacterial taxonomy, setting the stage for our investigation.

Our study generated a phylogenetic tree (Fig. II- 5) highlighting the distinct taxonomic positions of four potential strains: LC-30, LA-08, LA-23, and LA-32. This visual representation provides a comprehensive overview of the genetic relationships, revealing significant genetic congruence among these strains. Strains LC-30, LC-23, and LA-32 exhibited remarkable genetic resemblance with similarities of 100% and 99.88% to *L. fusiformis* and *L. indica*, respectively, confirming their distinct taxonomic affiliations within *Limnospira*. Notably, strain LA-08 showed marked similarities to all *L. fusiformis*, *L. indica*, and *L. fusiformis* with percentages reaching

99.62%, 99.50%, and 99.50%, thereby enhancing the robustness of our taxonomic insights (Table II-2).

After a thorough screening and molecular identification, four strains were selected: LC-30 and LC-23 from Lake Chitu, and LA-08 and LA-32 from Lake Arenguede which showed notable specific growth rates were subjected to additional screening tests for biomass concentration and growth. Notably, LC-30 exhibited the highest cell dry weight of $3.27 \pm 0.51 \text{ g L}^{-1}$, followed closely by LA-08 with $2.31 \pm 0.12 \text{ g L}^{-1}$. Strains LC-23 and LA-32 demonstrated slightly low dry weights of 1.85 ± 0.22 and $1.33 \pm 0.20 \text{ g L}^{-1}$, respectively (Fig. II-6A). Specific growth rate, a fundamental metric for assessing cellular biomass accumulation, showed similar trends. Strain LC-30 boasted the highest specific growth rate of biomass dry weight at $1.63 \pm 0.05 \text{ d}^{-1}$, followed by LA-08 with $1.12 \pm 0.07 \text{ d}^{-1}$. Strains LC-23 and LA-32 exhibited marginally lower specific growth rates, registering 0.74 ± 0.04 and $0.66 \pm 0.12 \text{ d}^{-1}$, respectively ($p < 0.05$; Fig. II- 6B).

2.3.3 Protein and phycocyanin content analysis

Owing to their potential for rapid growth, increased biomass concentration, elongated trichomes, and higher helix pitch, only two strains, LC-30 and LA-08, were selected for protein and phycocyanin content analysis. The protein analysis of these strains revealed significant differences in protein levels between the two strains at 58.28 ± 0.07 and $55.79 \pm 0.09 \text{ % AFDW}$, respectively ($n=3$, $p < 0.05$; Fig. II- 7A). These findings have significant implications for the potential use of these strains to address protein deficiencies and improve nutritional outcomes.

As a valuable pigment-protein combination, phycocyanin has many uses, including as a nutritional supplement, antioxidant, and natural food color (Furmaniak et al., 2017; Renugadevi et al., 2018). Following biomass extraction from strains LC-30 and LA-08, the phycocyanin levels

were determined to be $78.49 \pm 0.00 \text{ mg g}^{-1}$ and $70.36 \pm 0.00 \text{ mg g}^{-1}$, respectively. The observed variations in phycocyanin concentrations between these strains ($n=3$, $p < 0.05$; Fig. II- 7B) suggest strain-specific diversity in pigment production. Findings of previous studies and our current study showed that relatively fast-growing strains of *Arthrospira* and *Limnospira* exhibit substantial protein accumulation, which was directly related to the specific growth rate of the strain ($p < 0.05$; Fig. II-7A). Maximum specific growth rate and phycocyanin accumulation in *Arthrospira* and *Limnospira* strains revealed a direct relationship, however, the correlation was not significant ($p > 0.05$; Fig. II-7B). Notably, the concentrations, coupled with the purity ratings of 0.75 for LA-08 and 0.79 for LC-30, fell within the food-grade range for phycocyanin (Herrera et al., 1989; Rito-Palomares et al., 2001).

2.4 Discussions

First, in the present study, cyanobacterial isolates were traditionally identified by performing a thorough assessment of their morphological features following accepted criteria. The morphology of these strains closely aligned with well-established and previously reported morphological characteristics of *Arthrospira* (Ogato and Kifle, 2014; Karssa et al., 2018). The diameter has been reported to vary from 15 to 60 μm , trichome width is approximately between 2.5 and 16 μm , and helix pitch is typically between 0 and 80 μm (Vonshak et al., 1996a). Our findings were consistent with these previous reports, confirming the similarity of our strains to *Arthrospira* from Ethiopian soda lakes (Karssa et al., 2018). Notably, strains with long trichomes and high helix pitches may exhibit different growth rates, light capture, and nutrient uptake efficiencies, potentially influencing their suitability for biomass production (Cheng et al., 2018). Previous research has highlighted the significance of optimizing helix pitch and elongated trichome length in *Arthrospira* strains, leading

to substantial improvements in biomass harvesting efficiency and CO₂ fixation rates, resulting in a remarkable increase of 75.6% in harvested biomass yield (Cheng et al., 2018). Thus, we prioritized the screening of strains with extended trichomes and optimized helix pitches, aligning to identify strains with high specific growth rates and biomass concentration potential.

The reclassification of *Limnospira* from *Arthrospira* in 2019 (Nowicka-Krawczyk et al., 2019) marks a paradigm shift in cyanobacterial taxonomy, and our study aligns with this taxonomic evolution. Accurate taxonomic classification hinges on discerning features that distinguish *Arthrospira* from *Limnospira*. *Arthrospira* exhibits radially arranged thylakoids, whereas *Limnospira* showcases irregular whirl-like patterns in the central part of the cell (Gomont, 1893). The presence of a thickened cell wall or calyptra appears exclusive to *Limnospira* and is absent in *Arthrospira* trichomes and was not mentioned by the author in the original description of *A. jenneri*. Although environmental factors may influence the number or density of thylakoids, their arrangement remains remarkably stable within the genetic clusters defined by the 16S rRNA gene (Komarek, 2006). Strains/species within *Limnospira* are renowned for their affinity for alkaline habitats. This characteristic simplifies the mass production of varieties widely used in the food industry, offering increased resilience against contamination by other organisms (Carvalho et al., 2004; Koru, 2012; Roussel et al., 2023). Our findings challenge previous assumptions regarding the prevalence of *A. fusiformis* in lake Chitu and its reported decline in lake Arenguade (Girma et al., 2012). Contrary to expectations, our study identified *L. fusiformis* in both lakes, suggesting potential shifts in environmental conditions over time. Advanced molecular techniques, notably 16SrRNA, are indispensable for identifying cyanobacterial species, emphasizing the importance of accurate identification, especially for *Arthrospira*-like species that have not been definitively characterized through morphological studies including in Ethiopia.

The biomass concentration of our isolated LC-30 (3.27 g L⁻¹) is higher than previously reported

results for *Spirulina platensis* ranging from 0.42 to 2.27 g L⁻¹ (Travieso et al., 2001; Azgin et al., 2014; Zhu et al., 2018; Khannapho et al., 2021). Since the remarkable 4.95 and 5.82 g L⁻¹ dry mass reported in the *S. platensis* strain using optimized tubular photobioreactors (Travieso et al., 2001; Azgin et al., 2014), higher yields would be obtained in four strains in this study by optimizing the design and parameters of the culture system. The maximum specific growth rate in terms of dry weight in the LC-30 strain, 1.63 ± 0.05 d⁻¹, also exceeded the reported rates in other studies, where maximum specific growth rates for various *Arthrospira* strains ranged from 0.4 to 1.14 d⁻¹ (Vonshak et al., 1996b; Gordillo et al., 1998; Bhattacharya and Shivaprakash, 2005; Pumas and Pumas, 2016; Pan-utai et al., 2020) (Table II- 2). Additionally, LA-08 exhibited a specific growth rate that surpassed previous reports ranging from 0.42 to 1.10 d⁻¹. Note that the introduction of a flat-type photobioreactor led to a substantial increase in the specific growth rate of *S. platensis* (1.7 d⁻¹) (Chen et al., 2013), as seen in studies beyond the scope of our research using traditional flasks. The results of yield and growth rate in our study underscore the importance of specific growth rates in determining biomass yield, aligning with prior research that emphasizes the superior biomass accumulation of fast-growing strains. For example, the study by Azgin et al.(2014) demonstrated that strains with higher specific growth rates (1.02 d⁻¹) result in greater biomass accumulation (0.79 g L⁻¹) than strains with lower specific growth rates (0.69 d⁻¹), which led to a lower biomass concentration of 0.53 g L⁻¹. Through rigorous morphological screening, LC-30 and LA-08, distinguished by their fast growth rates and superior biomass accumulation, emerged as exceptionally promising candidates, surpassing documented rates for *Arthrospira* strains, and highlighting the significance of specific growth rates in achieving higher biomass production.

The protein levels of *Limnospira* strains in our study exceeded the typical range reported for *Arthrospira platensis* in various studies, which generally falls between 285 to 551 mg g⁻¹ (Gordillo et al., 1998; Pumas and Pumas, 2016; Pan-utai et al., 2020) (Table II- 3). Furthermore, the strains

in our study demonstrated protein levels closely aligned with previously published values, including 561, 591, and 610 mg g⁻¹ (Vonshak et al., 1996b; Pumas and Pumas, 2016). When magnetic fields are applied, startling protein content (73.2 0%) is reported (Deamici et al., 2016). Future research should explore magnetic field treatment on *Limnospira* strains, investigating field strength, exposure duration, and growth stages to optimize protein yield. A striking observation was the parity in protein content between commercial *A. platensis* and *Limnospira* strains, with both exhibiting higher protein levels than in traditional protein sources. In the current study, the protein content surpassed those of conventional protein sources like egg, beef, fish, chicken, and soybean (ranging from 130–430 mg g⁻¹) (Soletto et al., 2005; Hug and von der Weid, 2012; Koru, 2012; Caporgno and Mathys, 2018; Abate and Belachew, 2019). This high protein content, as demonstrated by the *Limnospira* strains in our study, has substantial potential for addressing nutrient deficiencies related to a lack of dietary diversity, particularly in developing countries. By integrating protein-rich strains into local dietary practices (Kilimtzidi et al., 2019), communities can access abundant and sustainable reservoirs of essential nutrients.

The phycocyanin concentrations obtained in our study were like those reported in other studies on *A. platensis* strains. Previous studies documented values as low as 41 and 66 mg g⁻¹ (Gordillo et al., 1998; Pumas and Pumas, 2016), making the concentrations from strains LC-30 and LA-08 notably higher. The fact that the phycocyanin concentration in our study was lower than values previously published in the literature (96, 114, 120, and 148 mg g⁻¹) (Bhattacharya and Shivaprakash, 2005; Pumas and Pumas, 2016) must be addressed (Table II- 3).

Previous reports demonstrated higher concentrations, up to approximately 148 mg g⁻¹, utilizing various enhancement techniques such as adjusting salt concentrations, applying magnetic fields, and using specific photobioreactor designs as well as intrinsic factors of the species (Bhattacharya

and Shivaprakash, 2005; Chen et al., 2013b; Deamici et al., 2016; Pumas and Pumas, 2016). In this study, we obtained food-grade concentrations of phycocyanin using traditional flask-based methods. However, the results suggest the potential for further improvement. Future studies on LC-30 and LA-08 could leverage successful strategies from previous research, enhancing strain growth rates for improved phycocyanin production (Setyoningrum and Nur, 2015).

2.5 Conclusions

Arthrospira-like isolates from Ethiopian soda lakes were identified as *Limnospira* strains adapted to alkaline environments. Strain LC-30 showed the highest biomass, growth rate, and superior protein and phycocyanin content, indicating strong potential for large-scale cultivation. The observed differences in growth rates and biomass production among strains highlight the need for rigorous screening to select the most productive strains.

Tables

Table II-1. Maximum specific growth rate (μ_{\max} ; day⁻¹) of 81 isolates at OD_{750nm}

Isolate name	(μ_{\max} ; day ⁻¹)	Isolate name	(μ_{\max} ; day ⁻¹)	Isolate name	(μ_{\max} ; day ⁻¹)
LC-30	1.041 ± 0.20	LA-38	0.665 ± 0.16	LC-37	0.525 ± 0.60
LA-08	0.954 ± 0.12	LA-30	0.647 ± 0.21	LC-57	0.525 ± 0.05
LA-32	0.945 ± 0.02	LA-21	0.646 ± 0.23	LA-03	0.518 ± 0.21
LC-23	0.914 ± 0.22	LC-19	0.644 ± 0.04	LA-20	0.508 ± 0.20
LA-31	0.904 ± 0.23	LC-18	0.642 ± 0.06	LC-25	0.506 ± 0.52
LA-18	0.896 ± 0.28	LC-17	0.629 ± 0.13	LA-06	0.495 ± 0.47
LA-10	0.895 ± 0.32	LA-19	0.628 ± 0.18	LA-17	0.493 ± 0.35
LC-11	0.837 ± 0.31	LC-55	0.624 ± 0.15	LA-29	0.480 ± 0.11
LA-09	0.804 ± 0.02	LA-40	0.620 ± 0.32	LC-27	0.479 ± 0.29
LC-21	0.788 ± 0.26	LC-44	0.607 ± 0.43	LC-36	0.479 ± 0.06
LA-22	0.786 ± 0.16	LA-34	0.602 ± 0.52	LC-05	0.478 ± 0.13
LC-12	0.782 ± 0.19	LC-24	0.599 ± 0.27	LA-39	0.461 ± 0.02
LC-22	0.775 ± 0.32	LA-16	0.599 ± 0.20	LC-50	0.453 ± 0.63
LA-05	0.770 ± 0.13	LC-13	0.595 ± 0.29	LA-01	0.450 ± 0.05
LC-34	0.765 ± 0.16	LC-53	0.594 ± 0.26	LA-36	0.447 ± 0.11
LC-35	0.765 ± 0.53	LC-39	0.593 ± 0.37	LA-27	0.436 ± 0.74
LC-08	0.754 ± 0.51	LA-42	0.585 ± 0.24	LA-25	0.423 ± 0.19
LC-31	0.751 ± 0.07	LA-41	0.582 ± 0.28	LA-33	0.416 ± 0.67
LC-45	0.741 ± 0.31	LC-38	0.581 ± 0.32	LC-49	0.407 ± 0.01
LC-29	0.739 ± 0.36	LC-54	0.568 ± 0.42	LA-37	0.404 ± 0.31
LA-28	0.726 ± 0.11	LC-48	0.566 ± 0.07	LC-41	0.401 ± 0.06
LC-47	0.711 ± 0.09	LC-26	0.564 ± 0.11	LA-23	0.369 ± 0.04
LC-28	0.706 ± 0.07	LA-26	0.563 ± 0.19	LA-24	0.343 ± 0.25
LA-07	0.698 ± 0.18	LC-56	0.558 ± 0.53	LC-52	0.334 ± 0.49
LC-07	0.682 ± 0.28	LA-13	0.558 ± 0.61	LC-43	0.283 ± 0.42
LC-46	0.675 ± 0.17	LC-42	0.549 ± 0.42	LC-51	0.265 ± 0.30
LA-14	0.665 ± 0.53	LA-35	0.544 ± 0.38	LC-40	0.234 ± 0.23

Notes: LA: Lake Arenguade; LC: Lake Chitu

Table II-2. BLAST search results of *Limnospira* strains used in this study based on 16S rRNA gene sequence analysis.

Strain name	Closest in the gene bank	Similarity %	Accession No
LA-08	<i>Limnospira fusiformis</i>	99.62	NR_177700.1
	<i>Limnospira indica</i>	99.50	NR_177862.1
	<i>Limnospira fusiformis</i>	99.50	NR_177699.1
L-30, LC-23 and	<i>Limnospira fusiformis</i>	100.00	NR_177700.1
LA-32	<i>Limnospira indica</i>	99.88	NR_177862.1
	<i>Limnospira fusiformis</i>	99.88	NR_177699.1

Table II- 3. Comparison of maximum specific growth rate, protein, and phycocyanin content of *Arthrospira* strains from available literature.

Species	Strain	Cultivation conditions		Specific growth rate (μ max; d ⁻¹)	Normalized μ max	Protein (mg g ⁻¹)	Normalized protein	Phycocyanin (mg g ⁻¹)	Normalized phycocyanin	References
		Culture medium	Light (μ mol m ⁻² s ⁻¹)							
<i>S. platensis</i>	M-2	Zarrouk	200	1.44	0.80	561	0.62	NA		Vonshak et al., 1996b
<i>A. platensis</i>	UTEX-1926	Zarrouk	100	0.50	0.06	285	0.00	41	0.28	Gordillo et al., 1998
<i>S. platensis</i>		Zarrouk	120	1.39	0.76	NA		148	1.00	Bhattacharya and Shivapraakash, 2005
<i>S. laxissima</i>		Zarrouk	120	0.63	0.16	NA		96	0.65	
<i>S. lonar</i>		Zarrouk	120	0.96	0.42	NA		120	0.81	
<i>Spirulina</i>	LEB 18	Zarrouk	500	1.70	1.00	NA		140	0.95	Chen et al., 2013
	LEB 18	Zarrouk	60	NA		732	1.00	140	0.95	Deamici et al., 2016
		Zarrouk	27	1.02	0.47	610	0.73	114	0.77	Pumas and Pumas, 2016
	AARLC005	CMU02+L	27	1.18	0.60	551	0.60	66	0.45	
<i>A. maxima</i>	IFRPD	Zarrouk	162	0.48	0.05	395	0.25	NA		Pan-utai et al., 2020
	1183	Zarrouk	162	0.42	0.00	398	0.25	NA		
		Zarrouk	162	0.59	0.13	434	0.33	NA		
<i>L. fusiformis</i>	LC-30	SOT	160	1.63	0.95	583	0.67	78	0.53	This study
	LA-08	SOT	160	1.12	0.55	558	0.61	70	0.47	

NA: Not Available; CMU02: Low-cost medium; CMU02+L: Water after water treatment (L pond).

Figures

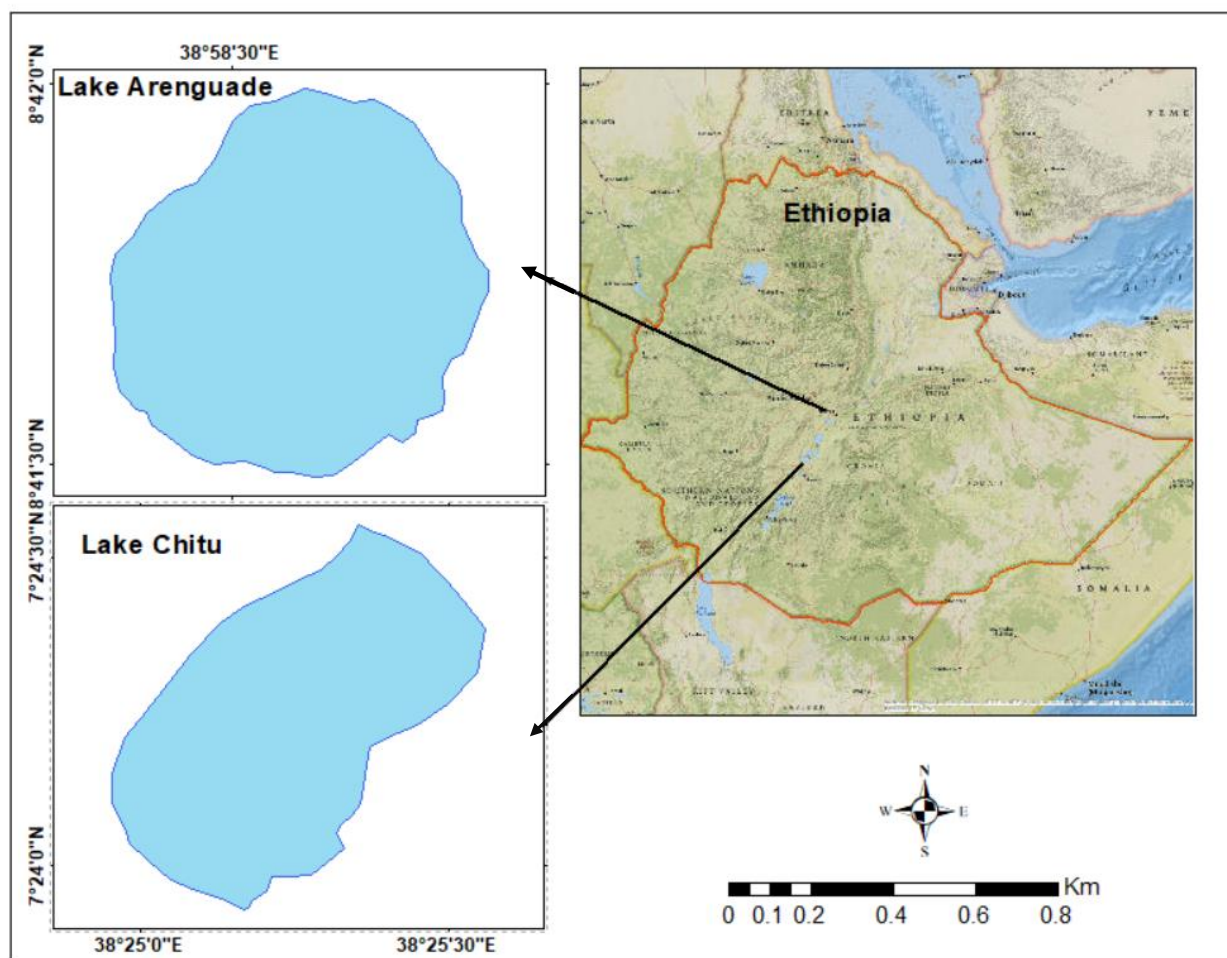


Fig. II-1. Location of Ethiopian soda lakes (Lake Arenguade and Lake Chitu): sampling sites for microalgae collected in this study.

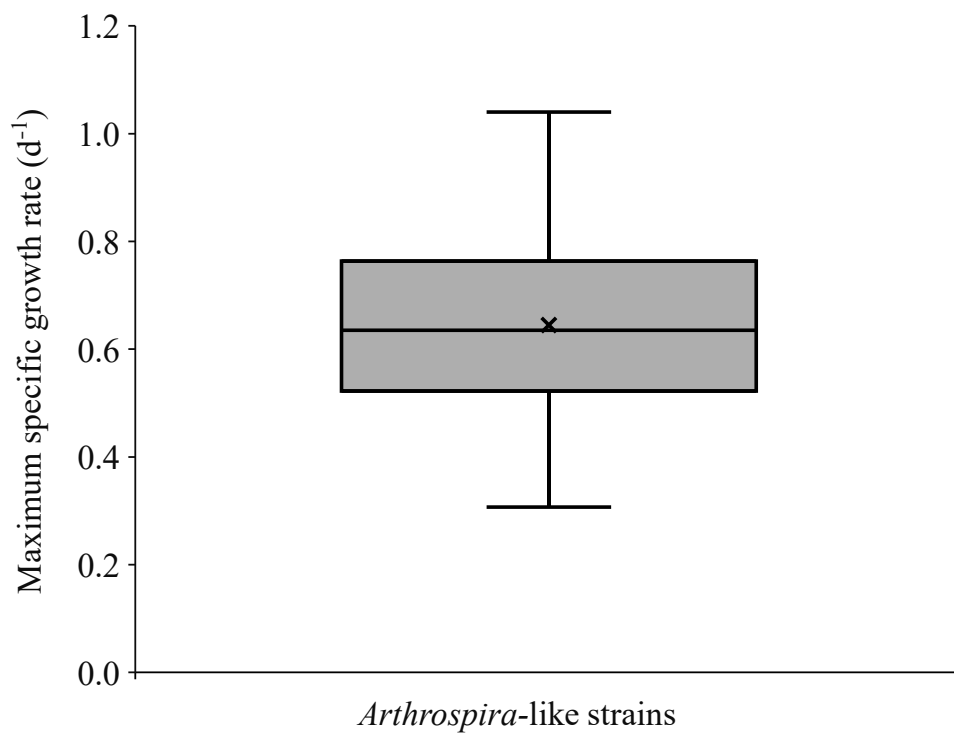


Fig. II-2. Box plot depicting the distribution of maximum specific growth rates among *Arthrospira*-like strains ($n=81$), isolated from Ethiopian soda lakes. x: the median value of the dataset.

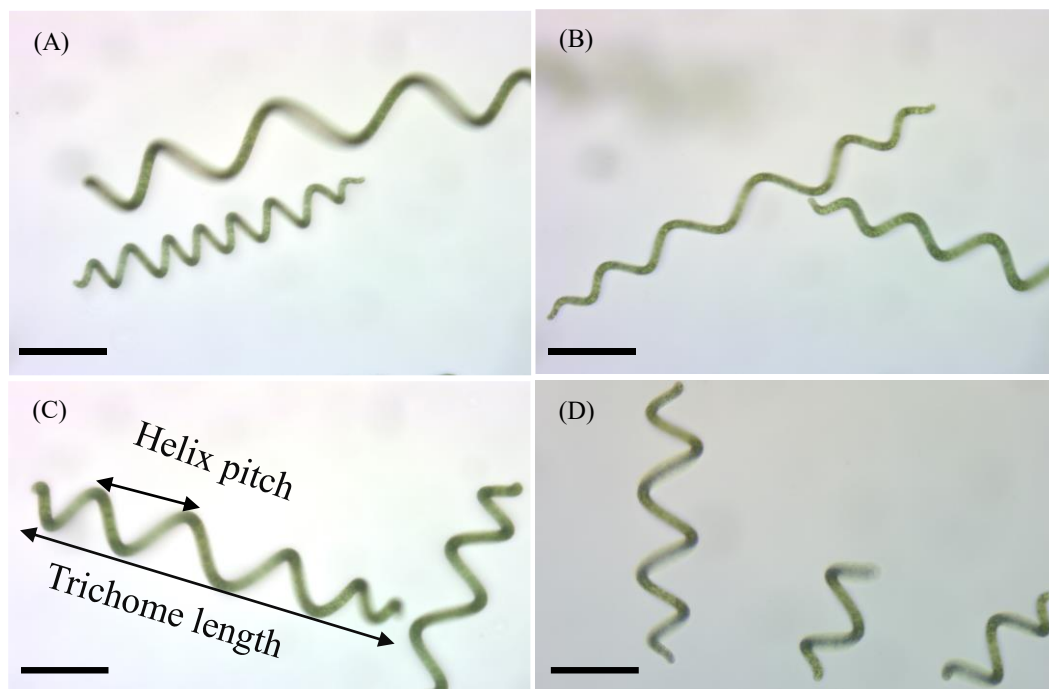


Fig. II- 3. Microscopic view of *Arthrospira* like isolates: LC-30 (A), LC-23 (B), LA-08 (C), and LA-32 (D). The scale bar represents 50 μm. LC: Lake Chitu; LA: Lake Arenguade.

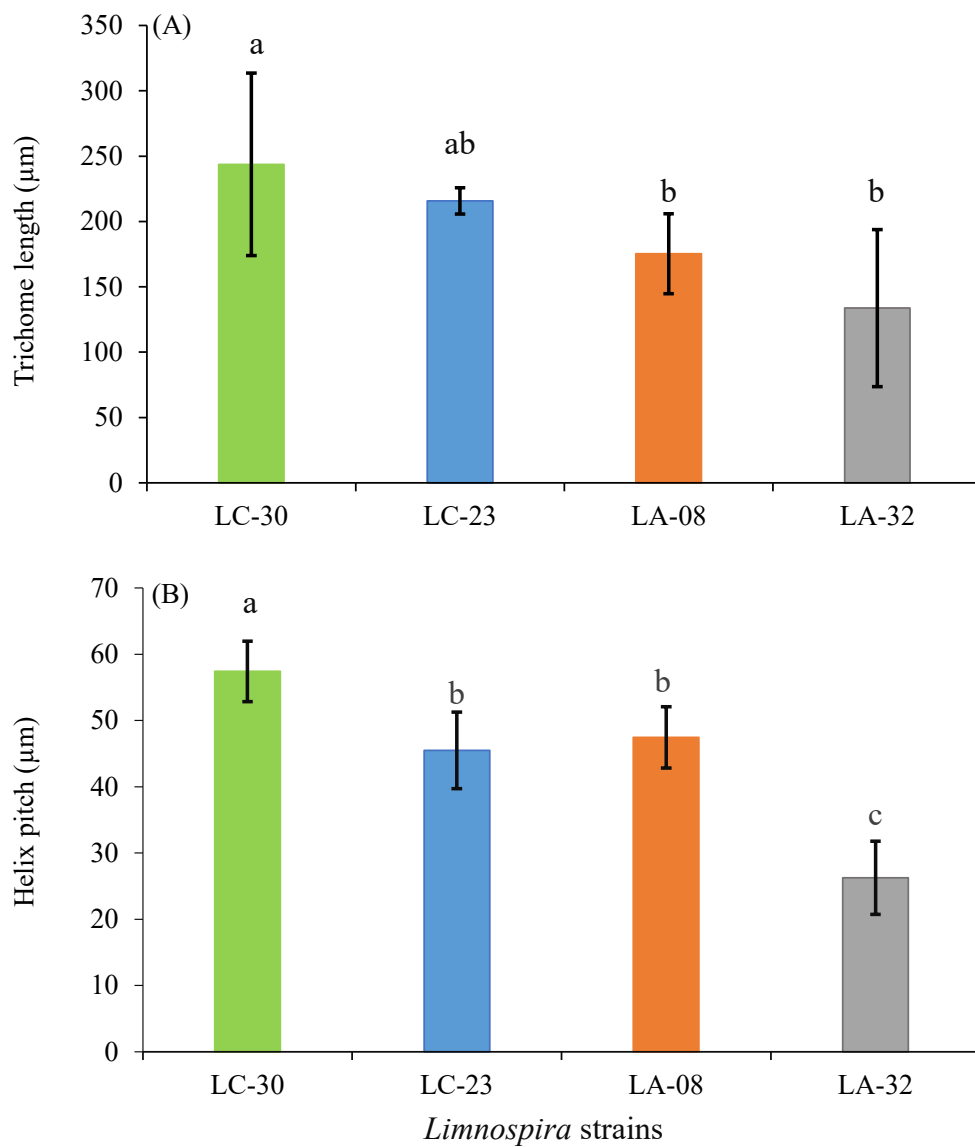


Fig. II-4. Morphological characteristics of four selected strains. The presented data are expressed as the mean \pm standard deviation. Different lowercase letters denote significant differences among strains in trichome length and helix pitch ($p < 0.05$).

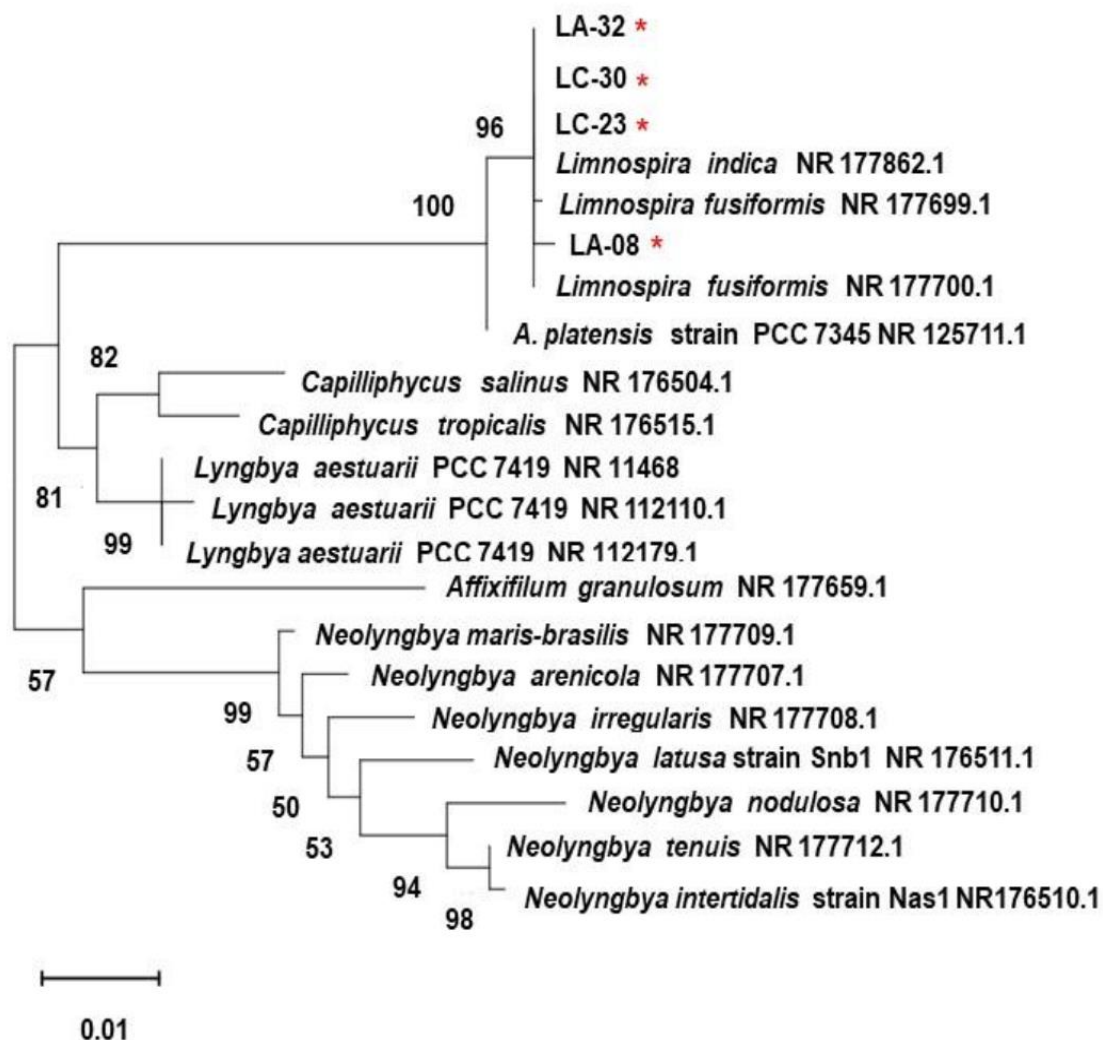


Fig. II- 5. Phylogenetic position of the four *Limnospira* strains isolated from lake Chitu and lake Arenguade in Ethiopia based on 16S rRNA gene sequences data by maximum likelihood method, 1000 bootstrap replications. *Limnospira* strains of the present study are marked by a red star (*)

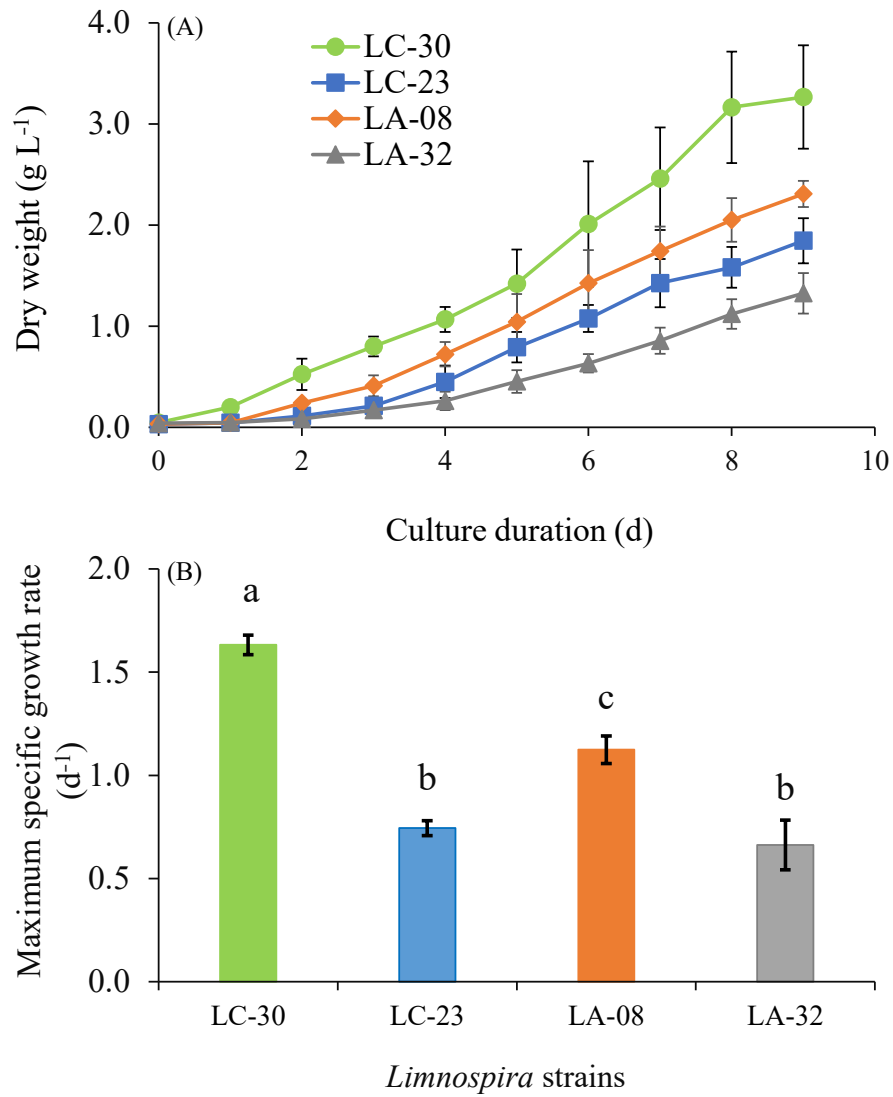


Fig. II-6. Biomass concentration and maximum specific growth rate. The presented data are expressed as the mean \pm standard deviation. Different lowercase letters denote significant differences among strains in specific growth rates in dry weight ($p < 0.05$).

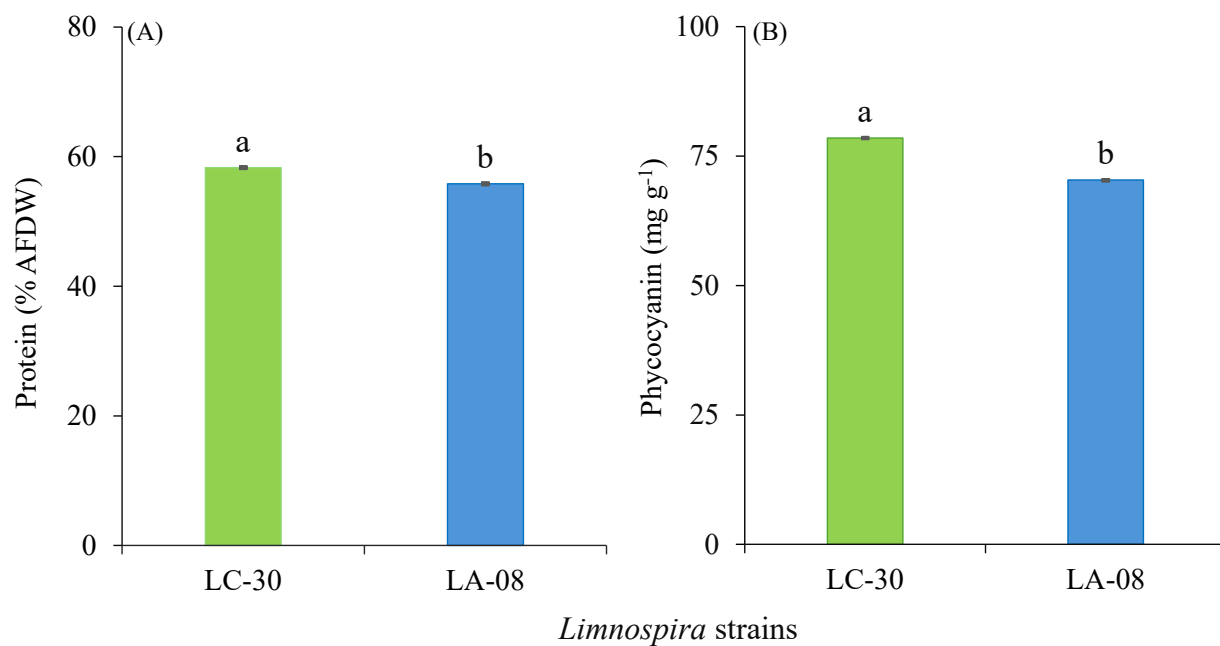


Fig. II-7. Protein and phycocyanin contents of *Limnospira fusiformis* in batch culture.

3 CHAPTER III Evaluation of *Limnospira fusiformis* growth and biochemical composition under simulated outdoor conditions

3.1 Introduction

The *Limnospira fusiformis* strain used in this study was selected through rigorous screening for its high biochemical composition and growth potential (Woldie et al., 2024). However, successful indoor cultivation does not guarantee effective performance in outdoor environments, where fluctuating light intensities and extreme mid-day sunlight can significantly impact growth rates and the production of essential metabolites like proteins and phycocyanin (Apel and Weuster-Botz, 2015). Previous studies in *Arthrospira/Limnospira* cultivation have shown that photosynthesis typically saturates at about one-third of full solar radiation (60-70% of full sunlight) (Vonshak, 1987; Adugna et al., 2024). Therefore, excessive light during peak hours can induce stress responses, leading to morphological changes, such as altered trichome length and helix pitch, which in turn affect productivity and biomass composition (Wu et al., 2005; Gao et al., 2008). Despite these findings, the correlation between the morphological changes and the biochemical composition of the strains has not been fully explored, leaving a critical gap in understanding how outdoor light conditions affect *Limnospira* cultivation. Therefore, it is essential to investigate how fluctuating outdoor light intensities and extreme sunlight exposure impact both the growth and biochemical production of the strain, particularly in tropical regions like Ethiopia. The impact of light on photosynthetic performance is commonly assessed through the maximum quantum yield of Photosystem II (Fv/Fm ratio), which typically ranges from 0.75 to 0.85 under optimal conditions (Lu and Vonshak, 1999). However, under excessive light exposure, the Fv/Fm ratio declines, indicating stress or damage to PSII, which impairs the organism's ability to effectively convert light energy into chemical energy. Moreover, high light intensity can induce additional stress, leading to morphological changes in

Arthrospira, such as spiral breakage, reduced productivity, and altered biomass composition (Wu et al., 2005; Ma and Gao, 2010).

To mitigate the adverse effects of photoinhibition, shading strategies, such as the use of shading nets, have proven effective. By deploying shading nets during the most intense sunlight periods, solar irradiance can be precisely reduced by specific percentages (e.g., 25%), creating a more balanced light environment and enhancing growth and productivity (Vonshak and Guy, 1992). This targeted approach protects cells from photoinhibition during peak sunlight hours and optimizes light exposure for sustainable outdoor cultivation. Implementing shading strategies through simulation studies is critical for ensuring a smooth transition from controlled environments to outdoor cultivation of *Limnospira fusiformis* (Lucker et al., 2014; Marsullo et al., 2015). These studies assess light management techniques to protect cells from photoinhibition, thereby supporting sustainable large-scale production (Apel and Weuster-Botz, 2015). By replicating natural outdoor light conditions, simulations allow for the evaluation of how strains respond to fluctuating light intensities and extreme sunlight, which is vital for maintaining consistent productivity, stable biochemical composition, and long-term viability in outdoor cultivation.

Previous research has focused heavily on controlled laboratory settings, often overlooking the dynamic light conditions of outdoor environments (Hidasi and Belay, 2018; Pelagatti et al., 2023). This has created a research gap in understanding how outdoor-simulated light intensities affect microalgal productivity and biochemical composition. While indoor systems like LED-lit raceway ponds and temperature-controlled photobioreactors have been used to simulate outdoor conditions for optimizing biofuel production in species such as *Chlorella sorokiniana* and *Nannochloropsis salina* (Huesemann et al., 2017a; 2023b; Beirne et al., 2023), they have not specifically addressed light optimization for *Limnospira fusiformis* in tropical regions like Ethiopia. It is essential to conduct indoor experiments that simulate outdoor light dynamics to regulate light intensity and mitigate

photoinhibition. This approach will enhance the performance of *Limnospira fusiformis* in outdoor settings, improving productivity and sustainability. Despite extensive laboratory research, there is a notable gap in studies that simulate outdoor light dynamics for large-scale, cost-effective *Arthrospira* cultivation. Developing simulation techniques that replicate natural conditions is crucial to ensure strains perform well not only in the lab but also in real-world environments. Simulated systems can mimic outdoor light intensities, helping optimize strain assessment while reducing the time and costs associated with outdoor cultivation (Apel and Weuster-Botz, 2015; Huesemann et al., 2017a,c). However, optimizing the high midday light intensities for *Limnospira fusiformis* cultivation remains underexplored, highlighting a significant area of study. Addressing this challenge is essential for improving productivity in outdoor environments, as excessive light during peak sunlight hours can lead to photoinhibition, reducing overall growth and biomass yield.

This chapter assessed the impact of varying simulated outdoor light intensities on *Limnospira* growth, biochemical composition, and morphology to identify optimal cultivation conditions. Controlled shading and light management strategies were developed to mitigate photoinhibition, particularly during mid-day hours, enhancing biomass yield and nutritional quality. These strategies provide practical guidance for optimizing *Limnospira* cultivation under outdoor conditions typical of tropical regions such as Ethiopia.

3.2 Materials and Methods

3.2.1 Microalgal strain and pre-culture conditions

A previously identified cyanobacterial strain, *L. fusiformis* LC-30, was used in this simulation study (Woldie et al., 2024). The algal stock was maintained in a modified Spirulina Ogawa Terui (SOT) medium containing (in mg L⁻¹): Na₂CO₃ (12,189), NaHCO₃ (9,660), K₂HPO₄ (500), NaNO₃

(2,500), Na₂SO₄ (815), NaCl (1,000), MgSO₄·7H₂O (200), CaCl₂ (30), FeSO₄·7H₂O (10), Na₂EDTA·2H₂O (80), H₃BO₃ (2.86), MnSO₄·5H₂O (2.17), ZnSO₄·7H₂O (0.222), CuSO₄·5H₂O (0.079), and Na₂MoO₄·2H₂O (0.021). The culture in 250 mL Erlenmeyer flasks was illuminated with a light intensity of 160 $\mu\text{mol m}^{-2}\text{s}^{-1}$ with a 12-h L/12-h D photoperiod at 25 °C and an initial pH of 10 to provide optimal growth conditions (Kishi and Toda, 2018). The culture apparatus was autoclaved at 121 °C for 20 minutes using an autoclave (SX-500; TOMY, Japan), and the medium was sterile-filtered through a 0.22- μm membrane (GPWP; Millipore, USA).

Aliquots from the stock culture were added to sterilized 500 mL Erlenmeyer flasks (effective volume: 300 mL) and diluted with a modified SOT medium until they reached the exponential growth phase for the pre-culture experiment. The culture was incubated at a constant temperature of 30 °C with illumination using cool-white, fluorescent lamps (FL20SEX-N-HG; NEC, Japan) at an intensity of 400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a 12-h L/12-h D photoperiod and continuous mixing at a flow rate of 0.2 vvm.

3.2.2 Design and simulated light experiments

The lower annual temperatures in Bahir Dar, Ethiopia ranging from 7°C to 13°C, and daily highs of 26°C to 30°C might pose cultivation challenges for *Limnospira fusiformis*, as these conditions fall below its optimal growth range of $30 \pm 2^\circ\text{C}$. To mitigate this, greenhouse plastic stabilizes water temperatures and prevents contamination. This setup maintains conditions between 17°C to 41°C, with an average of $30 \pm 2^\circ\text{C}$ applied in this study for optimal growth. The simulation experiments used outdoor light intensity data collected using data loggers (TR-74Ui Illuminance UV Recorder; T&D Corporation, Japan) inside and outside a greenhouse in Bahir Dar, Ethiopia, from February to April 2022. A Raspberry Pi-controlled LED light system with four glass column

photobioreactors (6.0 cm in diameter and height of 52 cm) was used. The four reactors with fluctuating light intensities were as follows: full sunlight intensity (T1) with a maximum of 2000 $\mu\text{mol m}^{-2}\text{s}^{-1}$; inside greenhouse conditions (T2) with a daily maximum of 1700 $\mu\text{mol m}^{-2}\text{s}^{-1}$; mid-day shade in greenhouse conditions (T3) with a maximum of 1400 $\mu\text{mol m}^{-2}\text{s}^{-1}$, corresponding to a reduction in light intensity ($\sim 20\%$ shade compared to T2); and simulated whole-time shade in greenhouse conditions (T4) with a maximum daily intensity of 1400 $\mu\text{mol m}^{-2}\text{s}^{-1}$ (Table III- 1). The levels (T1–T2) were determined using the recorded outdoor light intensity to ensure an accurate simulation of varying light conditions throughout the day. The average light intensity simulations matched hourly fluctuations from 06:00 to 18:00, accurately representing the daily outdoor conditions in Bahir Dar, Ethiopia (Apel and Weuster-Botz, 2015; Huesemann et al., 2017b; Sukumaran et al., 2018). The working volume and light-receiving area were set at 1.2 L and 0.021 m^2 , respectively. A quantum sensor (QSPL-2101, Biospherical Instruments, San Diego, CA, USA) was used to accurately measure light intensity.

Cells in the exponential growth phase from the pre-culture were inoculated into all four culture conditions (T1–T4) with an initial cell density of 0.06 (OD_{750}). After a 5-day batch culture phase, a 30-day semi-continuous cultivation was conducted. Light intensity was calibrated with hourly fluctuations between 30 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ (minimum average light during the light period) and 2000 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$, with 0 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ at night, and continuous mixing at a flow rate of 0.2 vvm. The temperature control was maintained at 30 ± 2 °C using a thermo-regulator with a water bath. After the batch culture phase, cultivation was changed to a semi-continuous mode while maintaining a 20% dilution rate through medium recycling (Bezerra et al., 2011). Samples (0.24 L) were withdrawn daily from day 6 onward and replaced with an equal volume of medium. The nutrient-supplemented filtrate, which is the liquid portion of the medium after filtration, was reintroduced directly into the reactor for recycling. Sampling was conducted every 24 h.

3.2.3 Two-stage culture strategy to enhance phycocyanin

3.2.3.1 Microalgal strain and pre-culture

The *Limnospira fusiformis* LC-30 strain used in this study was maintained in SOT medium. Seed cultures were grown in 500 mL flasks containing 300 mL of medium under a light intensity of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ with a 12-hour light/12-hour dark cycle at a controlled temperature of $30 \pm 1 \text{ }^{\circ}\text{C}$. Cells in the logarithmic growth phase were harvested and used as inoculum for experimental setups.

3.2.3.2 Effects of light intensity on growth and C-phycocyanin accumulation

of *Limnospira fusiformis* LC-30

The experiment was conducted in 500 mL Erlenmeyer flasks with effective volume of 300 mL illuminated by cool-white, fluorescent lamps (FL20SEX-N-HG, NEC). Seed cells harvested from the pre-culture were re-suspended in fresh SOT medium, and the optical density of the liquid culture at 680 nm (OD_{680}) was measured using a spectrophotometer (DR3900; Hach, USA). The initial inoculation density was standardized to $\text{OD}_{680} = 0.3$. Given that nitrogen sources in the medium are typically depleted after 4–5 days due to the rapid growth of *L. fusiformis*, the experimental duration was set to 4 days. Cultures were subjected to light intensities of 100, 200, 400, and $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ under a 12-hour light/12-hour dark cycle at a constant temperature of $30 \pm 1 \text{ }^{\circ}\text{C}$. After 4 days of cultivation, the C-phycocyanin (C-PC) content and productivity were measured to evaluate the effects of varying light intensities.

3.2.3.3 Establishment of two-stage culture strategy for *Limnospira fusiformis* LC-30

The two-stage culture experiment was conducted in 500 mL Erlenmeyer flasks with an initial inoculation density of $OD_{680} = 0.3$. Cultures were grown under a light intensity of $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ with a 12-hour light/12-hour dark cycle at a controlled temperature of $30 \pm 1 \text{ }^{\circ}\text{C}$. After 4 days of cultivation, the light intensity was reduced to 100 and $200 \mu\text{mol m}^{-2} \text{s}^{-1}$, marking the transition to the second stage of culture. Biomass concentration was measured daily throughout the culture period, and the experiment ended when growth entered the decline phase. At the end of the culture, the C-phycocyanin (C-PC) content and productivity were assessed for each treatment to evaluate the effectiveness of the two-stage strategy.

3.2.4 Analytical methods

Cell growth was assessed by measuring the optical density at 750 nm using a portable spectrophotometer (DR3900; Hach, USA). The culture was filtered through pre-combusted, pre-weighed glass fiber filters ($0.7 \mu\text{m}$, Whatman GF/F). The filters were then washed with $0.28 \text{ M NH}_4\text{HCO}_3$ solution to remove excess salt (Zhu and Lee, 1997), dried at 60°C for over 24 h, and reweighed to determine biomass dry weight. The oven-dried filters were then burnt to ashes in a furnace, cooled in a vacuum desiccator, and weighed to determine the ash-free dry weight (AFDW). The ash content was precisely determined using gravimetric methods from the AFDW and expressed as a percentage. In semi-continuous cultures, biomass productivity (P_{xs} ; $\text{g L}^{-1} \text{d}^{-1}$) was calculated by multiplying the dilution rate (D ; day^{-1}) with the average of three dry weight measurements taken during the stationary phase (X_{s} ; g L^{-1}): $P_{\text{xs}} = D \times X_{\text{s}}$ (Bezerra et al., 2011; Xia et al., 2023).

The maximum quantum yield (F_v/F_m) of photosystem II (PSII) was measured in 3 mL dark-acclimated cultures using a portable pulse-modulated fluorometer (AquaPen-C 100; PSI, Czech

Republic) over 10 min (Zaman et al., 2018) to detect photoinhibition. The accuracy of the measurements of maximum Fm in cyanobacteria was confirmed by adding 20 $\mu\text{mol L}^{-1}$ of 3-(3,4-chlorophenyl)-1,1-dimethylurea (DCMU) to the samples (Misumi et al., 2016).

The protein content in the pretreated biomass of strain LC-30 was quantified using the Lowry method (Lowry et al., 1951). The *Limnospira* culture (1 mL) was centrifuged and treated with 1 mL of 1 N NaOH for alkaline hydrolysis, followed by drying at 60°C for 3 h (Rausch, 1981). After centrifugation and removal of the supernatant, the biomass was dried in two phases: initially at 60°C, then in a final drying step after adding 1 N NaOH. The dried sample was rehydrated with 3.5 mL of Milli-Q Water (MQW). For the analysis, 1.0 mL of the rehydrated sample was mixed with 5.0 mL of a sodium carbonate alkaline copper solution in a HACH glass vial. Then, 0.5 mL of 1/2 diluted phenol reagent was added. The absorbance was measured at 750 nm after incubation at room temperature (25 ± 2 °C) for at least 30 min (Homandberg, 1997).

Carbohydrate content was determined using the phenol-sulfuric acid method (Nielsen, 2003). Firstly, 1.0 mL biomass suspension was mixed with 9.0 mL of MQW and centrifuged at $2,580 \times g$ for 10 min. The supernatant was then discarded, and the remaining biomass was dried at 105 °C for 1 d. Next, 1.0 mL of 1 N NaOH was added to the dried samples, followed by another round of drying. The samples were then rehydrated with 3.0 mL of MQW. A mixture of the rehydrated sample (1.0 mL) and 5% phenol solution (1.0 mL) was then treated with concentrated sulfuric acid (5.0 mL) to induce a color change. After cooling, the absorbance was measured at 490 nm, and calibration was performed using a glucose solution as the standard.

The sulfo-phosphor-vanillin (SPV) assay method described by Mishra et al. (Mishra et al., 2014) was used for lipid analysis. Standard lipid stocks were prepared using commercial canola oil at 20 mg in 10 mL of chloroform (final concentration: 2 mg mL⁻¹) and stored at -20°C until use. Different volumes of the lipid standard solution were added to empty tubes. The tubes were then

heated at 60°C for 10 minutes to evaporate the solvent, and 100 µL of water was added to each tube. The SPV reagent was prepared by dissolving 0.6 g of vanillin in 10 mL of ethanol, followed by the addition of 90 mL of deionized water and 400 µL of concentrated phosphoric acid. The reagent was stored in the dark until use. Fresh phospho-vanillin reagent was prepared before each experiment to ensure high activity.

For lipid quantification using the SPV reaction, GF/F *Limnospira* cells were suspended in 2 mL of water, centrifuged at 2,580 x g for 5 minutes (Chowdhary et al., 2022), and the pellet was treated with 2 mL of sulfuric acid. The mixture was then heated at 100°C for 10 minutes and cooled in an ice bath. After centrifugation, 1 mL of the supernatant was transferred to a glass vial, 2.5 mL of the phospho-vanillin reagent was added, and the mixture was incubated at 37°C for 15 minutes with intermittent shaking at 6.45 x g, every five minutes. Lipid content was quantified by measuring absorbance at 530 nm.

The effects of the simulated outdoor light intensity were confirmed by quantitatively examining the trichome length (µm) and helix pitch of the cells under each experimental condition. The trichomes were observed under an optical microscope (Axioskop-2 Plus; Carl Zeiss) equipped with a digital camera (EOS Kiss X7i; Canon). Morphological changes were analyzed for 30 trichomes from each condition using ImageJ 1.53 software (Kim et al., 2007; Wu et al., 2012).

Phycocyanin content was determined using a standardized protocol (Tan et al., 2020). *Limnospira* cells were centrifuged at 10,000 x g for 15 minutes, washed with double-distilled water, and dried at 50 °C. Biomass (0.50% w/v) underwent freeze-thaw cycles: freezing at –80 °C for 2 h, followed by thawing at 25 °C for 24 h in double-distilled water with a pH of 7. After centrifugation at 10,000 x g at 4 °C for 30 minutes, the resulting solution containing crude phycocyanin (C-PC) was obtained.

For the two-stage culture strategy to enhance phycocyanin, harvested *Limnospira fusiformis* LC-30 biomass was centrifuged, and the collected cells were dried. C-PC extraction followed the protocol by Huo et al., (2022). Briefly, 60 mg of algal biomass was added to 3 mL of 0.1 M phosphate buffer (pH 7), thoroughly mixed, and subjected to a freezing process for 4 hours, followed by thawing for 24 hours at 30 °C, repeated for two cycles to lyse the cells and extract C-PC. The mixture was then centrifuged at $5000 \times g$ for 8 minutes, and the supernatant was used to determine the C-PC content in the biomass using an equation derived from Bennett and Bogobad (Bennett and Bogobad, 1973; Antelo et al., 2010):

$$(A) \text{ Phycocyanin (mg mL}^{-1}\text{)} = (A_{620} - (0.474 \times A_{652}))/5.340$$

$$(B) \text{ Yield (mg g}^{-1}\text{)} = (\text{Phycocyanin} \times \text{Solvent volume})/ (\text{Dried biomass})$$

where, A_{620} and A_{652} are the absorbance of C-PC extract at 620 and 652 nm

The following equation was used to calculate C-PC productivity:

$$\text{PC- PC (mg L}^{-1}\text{d}^{-1}\text{)} = \text{Biomass productivity} \times \text{C-PC content}$$

3.2.5 Statistical analysis

All measurements were conducted in triplicates, and the data were presented as mean \pm standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA), followed by Tukey–Kramer multiple comparison tests, with significance set at $p < 0.05$, using Sigma Plot 15.0 Systat Software.

3.3 Results

3.3.1 Effects of simulated light intensities on *Limnospira fusiformis* growth and productivity

The results revealed that the highest dry weight of 3.22 g L⁻¹ was achieved under full sunlight conditions (T1, 2000 $\mu\text{mol m}^{-2}\text{s}^{-1}$), followed closely by the greenhouse condition (T2, 1700 $\mu\text{mol m}^{-2}\text{s}^{-1}$) with 3.06 g L⁻¹. Mid-day shade in a greenhouse (T3, 1400 $\mu\text{mol m}^{-2}\text{s}^{-1}$) resulted in 2.97 g L⁻¹, while whole-time shade in a greenhouse (T4, 1400 $\mu\text{mol m}^{-2}\text{s}^{-1}$) produced a biomass dry weight of 2.82 g L⁻¹ on day 8 (Fig. III- 1). Condition T4 exhibited the highest last day dry weight at 2.10 g L⁻¹, followed by T3, T2, and T1 with values of 1.83 g L⁻¹, 1.20 g L⁻¹, and 0.85 g L⁻¹, respectively ($p < 0.05$) (Fig. III- 1). Although the maximum biomass productivity ($\sim 0.73 \text{ g L}^{-1}\text{d}^{-1}$) was similar across all conditions with no significant difference ($p > 0.05$), T4 exhibited consistent productivity throughout the cultivation period, with significantly higher biomass productivity ($0.50 \text{ g L}^{-1}\text{d}^{-1}$) on the last day compared to that in T1–T3. Additionally, T3 demonstrated higher productivity than T1 and T2, with T2 surpassing T1 ($p < 0.05$) (Fig. III-2).

3.3.2 Chlorophyll fluorescence analysis

A decrease in photosynthetic activity (Fv/Fm) was observed under all culture conditions, indicating reduced photosynthetic efficiency under high light. Fv/Fm values below 0.70 indicate limited light energy utilization and negative impacts on PSII reaction centers (Lu and Vonshak, 1999). The significant decreases in Fv/Fm values were more pronounced in T1 (0.43) and T2 (0.52) compared to T3 (0.61) and T4 (0.66) (Fig. III- 3).

3.3.3 Morphological changes

The morphology of *L. fusiformis* (trichome length and helical pitch) underwent significant changes during the light-simulated experiments (T1–T4) (Fig. III-4B-E). Under condition T1, both trichome length and helical pitch showed a marked reduction compared to their initial measurements observed at the start of the experiment (Day 0: Fig. III-4A). Trichome length decreased from 289.5 μm to 51.3 μm , and helical pitch from 25.4 μm to 1.8 μm by Day 18 ($p < 0.05$) (Fig. III-4F, G). However, significantly higher trichome length (261.51 μm) and helical pitch (6.8 μm) were observed under T4 compared to that under T1–T3, where trichome length ranged from 51.3 to 138.5 μm and helix pitch from 1.8 to 5.3 μm respectively ($p < 0.05$), indicating a reduced impact of light intensity on morphological changes.

In this study the influence of simulated light intensity on spiral morphological changes and their correlation with phycocyanin and protein levels in *L. fusiformis* was examined (Fig. III-5). Linear regression analysis revealed strong correlations between trichome length and protein content ($R^2 = 0.90$), trichome length and phycocyanin content ($R^2 = 0.93$), helical pitch and protein content ($R^2 = 0.92$), and helical pitch and phycocyanin content ($R^2 = 0.91$).

3.3.4 Effects of simulated light intensities on *Limnospira fusiformis* biochemical composition

The average maximal biochemical composition of *L. fusiformis* was observed during the cultivation periods: T1 (days 9-15), T2 (days 12-18), T3 (days 15-21), and T4 (days 18-24) (Fig. III-6A). The protein content based on AFDW was higher in T4 (64.80%) than in T1, T2, and T3 (56.29%, 54.81%, and 60.50%, respectively; $p < 0.05$). Higher carbohydrate levels of 19.18%, 20.57%, and 17.80% AFDW were observed under conditions T1, T2, and T3, respectively, than under T4 (13.22% AFDW) ($p < 0.05$; Fig. III-6A). Variations in lipid content were also evident across different light

conditions, with T2 exhibiting the highest lipid content at 10.87% AFDW and T4 the lowest at 7.43% AFDW ($p < 0.05$) (Fig. III- 6A). The biomass ash content was quantified on the last day of cultivation which was 11.00% in T4 and 12.60% in T3 ($p < 0.05$; Table III-2).

Fig. III-6B shows the effect of light intensity on phycocyanin production in *L. fusiformis*. The highest C-PC productivity was observed in T4 ($0.11 \text{ g L}^{-1}\text{d}^{-1}$), which was significantly higher than in T1 ($0.09 \text{ g L}^{-1}\text{d}^{-1}$) and T2 ($0.10 \text{ g L}^{-1}\text{d}^{-1}$) ($p < 0.05$; Fig. III-6B).

3.3.5 Growth and phycocyanin production of *Limnospira fusiformis* under two-stage cultivation with varying light intensities

During Stage 1 (day 0 to 4), all treatments (L1, L2, L3, L4) cultivated under $400 \mu\text{mol m}^{-2} \text{ s}^{-1}$ light intensity showed uniform growth, with biomass dry weight reaching $\sim 3.1 \text{ g L}^{-1}$. In stage 2 (day 4 to 11), varying light intensities led to distinct growth patterns. L4 ($600 \mu\text{mol m}^{-2} \text{ s}^{-1}$) exhibited the highest biomass ($\sim 4.5 \text{ g L}^{-1}$), followed by L3 ($400 \mu\text{mol m}^{-2} \text{ s}^{-1}$) and L2 ($200 \mu\text{mol m}^{-2} \text{ s}^{-1}$) with intermediate values ($\sim 4.33\text{--}4.40 \text{ g L}^{-1}$). L1 ($100 \mu\text{mol m}^{-2} \text{ s}^{-1}$) showed comparable biomass ($\sim 4.01 \text{ g L}^{-1}$) on final day, indicating slower growth under lower light conditions Fig. III-7.

In this study, the effects of varying light intensities ($100\text{--}600 \mu\text{mol m}^{-2} \text{ s}^{-1}$) during the second stage of cultivation, specifically on the second day of the phycocyanin induction phase, were evaluated in terms of their impact on the growth, phycocyanin content, and productivity of *Limnospira fusiformis*, as shown in Fig. III-8. Biomass dry weight increased steadily with light intensity, ranging from 3.66 g L^{-1} at $100 \mu\text{mol m}^{-2} \text{ s}^{-1}$ to 4.10 g L^{-1} at $600 \mu\text{mol m}^{-2} \text{ s}^{-1}$. Phycocyanin productivity peaked at $50.83 \text{ mg L}^{-1} \text{ d}^{-1}$ under $100 \mu\text{mol m}^{-2} \text{ s}^{-1}$ but declined to $35.31 \text{ mg L}^{-1} \text{ d}^{-1}$ as light intensity increased to $600 \mu\text{mol m}^{-2} \text{ s}^{-1}$. C-PC content was highest at $100 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (129.02 mg g^{-1}) and decreased significantly with increasing light intensities.

3.4 Discussions

3.4.1 Effects of simulated light intensities on *Limnospira fusiformis* growth and productivity

The initial surge in maximum biomass dry weight observed under conditions T1 and T2 can be attributed to the unrestricted light availability, which provides more energy for fuel photosynthesis. Previous studies have reported that cells can still produce maximal biomass despite initially high light levels (Farahdiba et al., 2020). However, photoinhibition gradually affected the dry weight under both full sunlight and greenhouse conditions. The current finding aligns with previous studies conducted on *Limnospira indica*, which have shown that the highest level of photoinhibition occurred at irradiances exceeding $1500 \mu\text{mol m}^{-2}\text{s}^{-1}$ (Garcia-Gragera et al., 2022), with a notable decline in biomass dry weight (from 3 g L^{-1} to 0.50 g L^{-1}) during the initial cultivation period. However, in the current study, shaded greenhouse conditions (T3 and T4) positively influenced biomass dry weight throughout the cultivation period, as indicated in Fig. III- 1. The current study highlighted the role of carefully managing light conditions, particularly through shading, to mitigate the effect of photoinhibition on biomass dry weight in *L. fusiformis* cultures, which is in line with previously reported findings (Vonshak and Guy, 1992; Kilimtzidi et al., 2019). These findings highlighted those shaded cultures consistently exhibited higher biomass dry weights, indicating protection from photoinhibition. However, prolonged exposure to high light stress resulted in decreased photosynthetic efficiency. Therefore, the culture was examined on the last day of the study to elucidate the precise stress responses triggered by prolonged exposure to high light intensity on the biomass dry weight of the strain.

This observation is consistent with previous reports on *Spirulina* sp., suggesting that subsequent photoinhibition likely played a role in the decline in dry weight on the last day (Soletto

et al., 2008; Farahdiba et al., 2020). Another study, supporting the findings of the current report, observed that a photon flux density of $1800 \mu\text{mol m}^{-2}\text{s}^{-1}$ reduced the photosynthetic rate of *Spirulina platensis* by 50% (Jensen and Knutsen, 1993; Kilimtzidi et al., 2019). Indeed, the observed variations in the last-day dry weight among the different conditions in our study highlight the significant impact of subsequent photoinhibition on *Limnospira* biomass dry weight.

Despite the initially higher biomass productivity under all culture conditions (T1–T4), the sustained productivity observed in shaded conditions (T3–T4) suggested that the strain is relatively performing efficient photosynthetic activity in conditions with reduced light intensity ($1400 \mu\text{mol m}^{-2}\text{s}^{-1}$) due to shading. In line with the findings of the current study, shading, which reduced incident sunlight by 25%–30%, effectively prevented the decline in photosynthetic activity, thus averting photoinhibition. This resulted in higher biomass productivity compared to that under non-shaded conditions (Vonshak and Guy, 1992). Shading was found to significantly improve microalgal productivity by reducing exposure to PAR, which led to increased biomass dry weight. However, the observed decrease in biomass concentration and productivity over time under all conditions suggests that, while shading mitigated some photoinhibition, it was not entirely sufficient to prevent productivity declines, indicating a complex relationship between shading and biomass productivity that warrants further investigation. Although extracellular polymeric substance (EPS) accumulation was not directly measured in this study, it is anticipated due to culture medium recycling, which may influence biomass productivity. Prolonged cultivation and medium reuse can lead to increased EPS, affecting viscosity, nutrient diffusion, and cell aggregation, all of which can reduce biomass yield (De Philippis & Vincenzini, 1998; Ozturk et al., 2014). Despite lower photoinhibition in T3 and T4, the decline may be attributed to nutrient depletion and EPS buildup, with the 20% dilution rate and 5-day hydraulic retention time (HRT) marking the transition from batch to semi-continuous cultivation, possibly requiring shortening for stable biomass production. Optimizing these

parameters, as suggested by previous research (Morais et al., 2009; Bezerra et al., 2011), could enhance nutrient availability, reduce EPS accumulation, and improve productivity in future cycles.

3.4.2 Chlorophyll fluorescence analysis

Notably, non-shaded, high-light conditions (T1 and T2) resulted in a significant decrease in the Fv/Fm ratio after the batch cultivation phase. The current finding aligned with previous research on *Limnospira indica*, showing that photosynthetic efficiency declines when light intensities exceed $1700 \mu\text{mol m}^{-2}\text{s}^{-1}$ during semi-continuous cultivation (Garcia-Gragera et al., 2022). However, even under shaded conditions (T3 and T4), the Fv/Fm values remained below the optimal range in the current study, suggesting varying levels of high light stress in all conditions. This highlighted the need to reduce the light intensity or change batch cultivation to alleviate photoinhibition. Consistent with our study, previous research on *Spirulina* showed that PSII efficiency (Fv/Fm) decreased with increasing irradiance, with the most significant inhibition (Fv/Fm, 0.51) observed at noon, coinciding with peak irradiance of $2000 \mu\text{mol m}^{-2}\text{s}^{-1}$ (Lu and Vonshak, 1999). Thus, the cells were susceptible to photoinhibition in outdoor *Spirulina platensis* or *Limnospira* cultures, with a noticeable reduction in Fv/Fm towards mid-day, correlating with the daily irradiance pattern. Therefore, it is imperative to consider the potential impact of light on PSII reaction center inactivation and biomolecule and pigment synthesis by implementing suitable shading techniques to ensure the optimal growth and spiral characteristics of *L. fusiformis* cultures.

3.4.3 Morphological changes

The changes observed in spiral morphology in T1 and T2 were likely responses to high light stress, resulting in enhanced trichome rigidity and photoprotection. This phenomenon mirrors similar morphological transformations observed in *A. platensis* under high light intensity, where a transition

from a loosely coiled helix to a tightly coiled helix occurs in response to increased visible light (Ma and Gao, 2010). The observed morphological changes in response to exposure to high light intensity, such as reduced trichome length and helical pitch, are protective mechanisms against the adverse effects of high PAR by enhancing self-shading (Wu et al., 2005). However, it is necessary to note that excessive accumulation of ROS, induced by high PAR levels in outdoor cultures, can lead to filament breakage (Wu et al., 2005; Ma and Gao, 2010), significantly impacting biomass productivity and composition (Vonshak and Guy, 1992). Conversely, shade conditions with reduced light intensities maintained the original morphology, potentially optimizing light capture and CO₂ fixation (Cheng et al., 2018). These studies highlight the delicate interplay between light intensity, morphology, and cellular responses, underscoring the need for tailored cultivation strategies to enhance productivity and minimize stress-induced damage. Understanding and managing morphological changes in response to light intensity are essential for optimizing microalgal cultivation practices and improving product quality.

The high R^2 values indicated strong connections between morphological characteristics and the production of proteins and phycocyanin in *L. fusiformis*. To the best of our knowledge, this is the first report demonstrating the correlation between morphological changes induced by strong light and the biochemical composition of the strain. Under shaded conditions (T3, T4) with reduced spiral changes, protein and phycocyanin contents increased by 56.60% and 83.50%, respectively, on the last day of cultivation compared to that under non-shaded conditions (T1 and T2), which exhibited morphological changes including reduced trichome length and helix pitch. Consistent with our findings, previous reports have indicated that longer trichomes in *A. platensis* may enhance photosynthesis by enlarging the surface area and increasing the rate of CO₂ fixation (Cheng et al., 2018), consequently promoting higher metabolic activity and protein synthesis in the strain (Wu et

al., 2005). Another study reported that an optimal helix pitch in *Spirulina* cultures enhanced light penetration and nutrient diffusion, ultimately leading to increased protein levels and improved yields (Gao et al., 2008). Therefore, optimizing morphological characteristics is advantageous for outdoor cultivation practices, as it enhances biomass yield and, ultimately, product quality, ensuring a more efficient and sustainable microalgal production process.

3.4.4 Effects of simulated light intensities on *Limnospira fusiformis* biochemical composition

The highest light intensities under conditions T1 and T2 resulted in protein degradation under high light stress. According to previous studies, it is known that higher PAR levels lead to the accumulation of ROS and oxidization of lipids in the sheath or cell membrane, ultimately leading to spiral breakage (Jensen and Knutsen, 1993; Lu and Vonshak, 1999) and a decrease in the content of photosynthetic pigments with damaged photosystem II (PSII) (Ma and Gao, 2010). However, shading under conditions T3 and T4 in the current study reduced light stress and preserved last-day protein content by 54.06% and 63.10%, respectively, compared to that under the non-shaded conditions (Table III- 2). In alignment with the recent study on *A. platensis*, the shaded culture exposed to fluctuating light intensities ranging from 437 to 1406 $\mu\text{mol m}^{-2}\text{s}^{-1}$ exhibited a 65.20% protein content, surpassing that under the non-shaded condition, which had a protein content of 53.20% (Kilimtzidi et al., 2019). Therefore, shading greatly increases protein accumulation by reducing the amount of incoming light, effectively preventing photoinhibition and promoting protein synthesis. In addition to observing variations in protein content among the conditions, we also examined the carbohydrate content of the biomass.

Limnospira fusiformis tended to accumulate more carbohydrates in T1 and T2, indicating its capacity for carbohydrate synthesis under high light stress conditions. This result was similar to

previous studies where a shift from 50 to 400 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ resulted in a 34% increase in carbohydrate accumulation of its biomass dry weight in *Arthrospira maxima* (De Philippis et al., 1992). In another study, the carbohydrate content of *A. platensis* exposed to 700 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ reached 18%–45% of cell dry weight after 12 h high light exposure, which was higher than that obtained at 50 or 270 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ (Aikawa et al., 2012). Therefore, the *Limnospira* strain can store carbohydrates when exposed to environmental stress, including high light intensities, as a coping mechanism, as supported by prior reports (Chen et al., 2013c). The high carbohydrate production by *Arthrospira*, particularly its beta-glucan content, offers significant dietary advantages (Anny et al., 2024). Similarly, the carbohydrates produced by *Limnospira* cells may offer comparable nutritional advantages, consistent with findings from previous studies. However, *L. fusiformis* exhibited a consistent carbohydrate content (13.22%–15.6%) under shaded conditions, suggesting that carbohydrate metabolism is regulated to support essential cellular processes, including protein synthesis (Aikawa et al., 2012). This adaptive response highlights the versatility of *L. fusiformis* in optimizing its metabolic pathways to sustain its biochemical composition under various light conditions.

The results of the current study align with those of Raslavičius et al. (2014), where an increase in lipid content from 36.10% to 47.10% was observed with a change in illumination intensity from 55 to 400 $\mu\text{mol m}^{-2}\text{s}^{-1}$. In another study, high light intensity (962 $\mu\text{mol m}^{-2}\text{s}^{-1}$) promoted lipid accumulation as a mechanism to convert excess light into chemical energy, thereby mitigating the risk of photooxidative damage. Conversely, lower light intensity (592 $\mu\text{mol m}^{-2}\text{s}^{-1}$) tended to prioritize protein synthesis over lipid accumulation (Palanisamy et al., 2021). This highlighted the intricate interplay between light intensity and cellular metabolism in microalgae and emphasized the importance of optimizing light conditions for protein and lipid production in cultivation settings. The ash content was lower in T4 and in T3 respectively, which was lower than that in Moroccan *Spirulina*

(14.56%) (Seghiri et al., 2019). However, T1 and T2 resulted in the highest ash contents of 18.60% and 21.00%, respectively, which are comparable to previous findings of 20% in *Spirulina* sp. (Jesus et al., 2018). Therefore, shading can be considered a viable strategy for optimizing microalgal biomass quality by minimizing ash content, ultimately enhancing its suitability for diverse industrial and nutritional purposes.

Although T4 and T3 ($0.11 \text{ g L}^{-1}\text{d}^{-1}$) had approximately similar productivity, T4, which was whole time shaded during the cultivation period, showed a notable improvement in C-PC productivity compared to T1 and T2. Aligned with recent research on *A. platensis*, shaded cultures subjected to fluctuating light intensities ranging from 437 to $1406 \mu\text{mol m}^{-2}\text{s}^{-1}$ demonstrated enhanced phycocyanin productivity, achieving $0.006 \text{ g L}^{-1}\text{d}^{-1}$. This surpassed the productivity under non-shaded conditions, which yielded $0.002 \text{ g L}^{-1}\text{d}^{-1}$ (Kilimtzi et al., 2019). On the last day of cultivation, T4 exhibited the highest C-PC productivity of $0.11 \text{ g L}^{-1}\text{d}^{-1}$ (Fig. III-6B) with a content of 84.38 mg g^{-1} , followed by T3, T2, and T1, which had C-PC contents of 71.87, 59.01, and 26.11 mg g^{-1} respectively. Notably, the condition labeled T1 had the lowest last-day C-PC content among all the conditions tested (Table III- 2). This result indicated that the phycocyanin content of the strain was inversely related to light intensity, with higher light intensities leading to decreased phycocyanin accumulation (Chen et al., 2010). The current study also aligns with previous research findings, showing that shaded cultures achieve significantly higher last-day C-PC content (58.00 mg g^{-1}) than non-shaded cultures (20.00 mg g^{-1}) (Kilimtzi et al., 2019). The correlation between C-PC productivity and light intensity underscores the importance of optimizing light conditions to shape microalgal pigment composition, including phycocyanin content. Shading, when applied at T3 and T4, enhanced the accumulation of phycocyanin in *L. fusiformis*, indicating its potential for improving the production of valuable compounds. Shading likely prevents photoinhibition, safeguards cells from light-induced damage, enhances phycocyanin content, and maintains cell morphology.

3.4.5 Growth and phycocyanin production of *Limnospira fusiformis* under two-stage cultivation with varying light intensities

In this study, we investigated the growth dynamics and phycocyanin (C-PC) production of *Limnospira fusiformis* under two-stage cultivation with varying light intensities. During Stage 1 (day 0 to 4), biomass growth was uniform across all treatments under a moderate light intensity of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$, indicating that the initial growth phase was not significantly affected by light intensity. However, in stage 2 (day 4 to 11), light intensity had a pronounced impact on growth, with higher intensities (400–600 $\mu\text{mol m}^{-2} \text{s}^{-1}$) promoting increased biomass production. This finding aligns with those of Hsieh-Lo et al., (2019) and Pagels et al., (2019), who observed enhanced growth at optimal light intensities for *Arthrospira* species. Interestingly, unlike previous studies where light intensities above 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ resulted in growth inhibition due to photoinhibition, biomass growth remained high under 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in this study. This suggests that *Limnospira fusiformis* may possess a higher tolerance to light stress or be better equipped to handle elevated light conditions compared to other microalgae. While excessive light can overwhelm the photosynthetic machinery and generate reactive oxygen species (Soletto et al., 2008), the high biomass observed under 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ indicates that this strain is resilient to light stress, possibly due to adaptive mechanisms in its photosynthetic apparatus.

Phycocyanin productivity exhibited an inverse relationship with light intensity, with the highest productivity and C-PC content observed at 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. This supports the findings of García-López et al., (2020) and Yu et al., (2023), who reported that lower light intensities optimize light-harvesting efficiency, enhancing pigment synthesis. Higher light intensities beyond this threshold led to a decline in C-PC synthesis, consistent with previous studies by Rizzo et al., (2015)

and Lee et al., (2016) , which highlighted the negative impact of excessive light on pigment production. Overall, these results underscore the importance of optimizing light conditions at different stages of cultivation. While higher light intensities promote biomass growth, lower light intensities are more favorable for phycocyanin production. The two-stage cultivation method, where stage 1 supported growth and stage 2 focused on optimizing pigment yield with lower light intensities, is recommended for enhancing both biomass and C-PC production. This approach can be adapted for industrial-scale applications, where a balance between high-value pigment production and robust biomass accumulation is crucial.

3.5 Conclusions

Actual outdoor light conditions were simulated indoors, where shaded environments effectively reduced photoinhibition, resulting in higher and more stable biomass yields. Notably, whole-time shade produced the highest protein and phycocyanin contents. High light intensity led to carbohydrate accumulation. Optimized shading and light conditions are essential for improving biochemical composition, productivity, and the transition of *Limnospira* strains from indoor to outdoor cultivation. Two-stage cultivation enhances phycocyanin production in *Limnospira fusiformis* by combining moderate light for growth and low light for efficient pigment synthesis.

Tables

Table III- 1. Average daytime outdoor light intensity at various time intervals for the weather conditions in Bahir Dar, Ethiopia.

Time (24 h)	Light intensity ($\mu\text{mol m}^{-2}\text{s}^{-1}$)					
	Full sunlight intensity (T1)		Inside a greenhouse (T2)		Mid-day shade in a greenhouse (T3)	Whole-time shade in a greenhouse (T4)
	Average	Range	Average	Range	Average	Average
0601–0700	200 \pm 103	24–299	170 \pm 88	20–237	170	150
0701–0800	400 \pm 201	194–778	300 \pm 173	166–668	300	250
0801–0900	1000 \pm 72	797–1094	900 \pm 62	685–940	900	750
0901–1000	1500 \pm 138	1094–1723	1300 \pm 118	9040–1482	1300	1000
1001–1100	1900 \pm 46	1729–1882	1600 \pm 39	1486–1618	1300	1000
1101–1200	2000 \pm 83	1862–2172	1700 \pm 71	1601–1867	1400	1400
1201–1300	2000 \pm 165	1762–2404	1700 \pm 142	1515–2067	1400	1400
1301–1400	1800 \pm 86	1593–1880	1600 \pm 74	1370–1500	1300	1000
1401–1500	1400 \pm 154	1044–1585	1200 \pm 133	898–1363	1200	1000
1501–1600	900 \pm 155	628–1181	800 \pm 133	540–1015	800	700
1601–1700	400 \pm 171	102–703	300 \pm 157	87–604	300	250
1701–1800	50 \pm 29	4–91	40 \pm 26	3–78	40	30
1801–0600	0	0	0	0	0	0

* T1–T4; Outdoor light conditions

Table III- 2. Biochemical composition (protein, carbohydrate, total lipid, and ash) and crude phycocyanin (C-PC) content of *Limnospira fusiformis* (LC-30) biomass on the last day of the cultivation period under different conditions based on the ash-free dry weight percent. The data are presented as the mean \pm standard deviation, $n=3$.

Condition	Protein (%)	Carbohydrate (%)	Lipid (%)	Ash (%)	C-PC (mg g ⁻¹)
T1	34.77 \pm 7.61 ^b	25.70 \pm 0.84 ^a	8.90 \pm 4.22 ^a	18.60 \pm 4.43 ^b	26.11 \pm 1.81 ^d
T2	37.03 \pm 1.62 ^b	23.51 \pm 1.32 ^a	8.91 \pm 1.10 ^a	21.02 \pm 4.42 ^a	59.01 \pm 2.63 ^c
T3	54.06 \pm 0.91 ^a	19.60 \pm 0.11 ^b	9.71 \pm 4.41 ^a	12.62 \pm 3.91 ^c	71.87 \pm 1.31 ^b
T4	63.10 \pm 3.10 ^a	15.60 \pm 1.60 ^c	7.60 \pm 1.72 ^b	11.00 \pm 2.70 ^c	84.38 \pm 0.92 ^a

* Light intensity conditions, T1: Full sunlight; T2: Inside greenhouse; T3: Mid-day shade in a greenhouse; T4: Whole-time shade in a greenhouse (Refer table III-1).

Figures

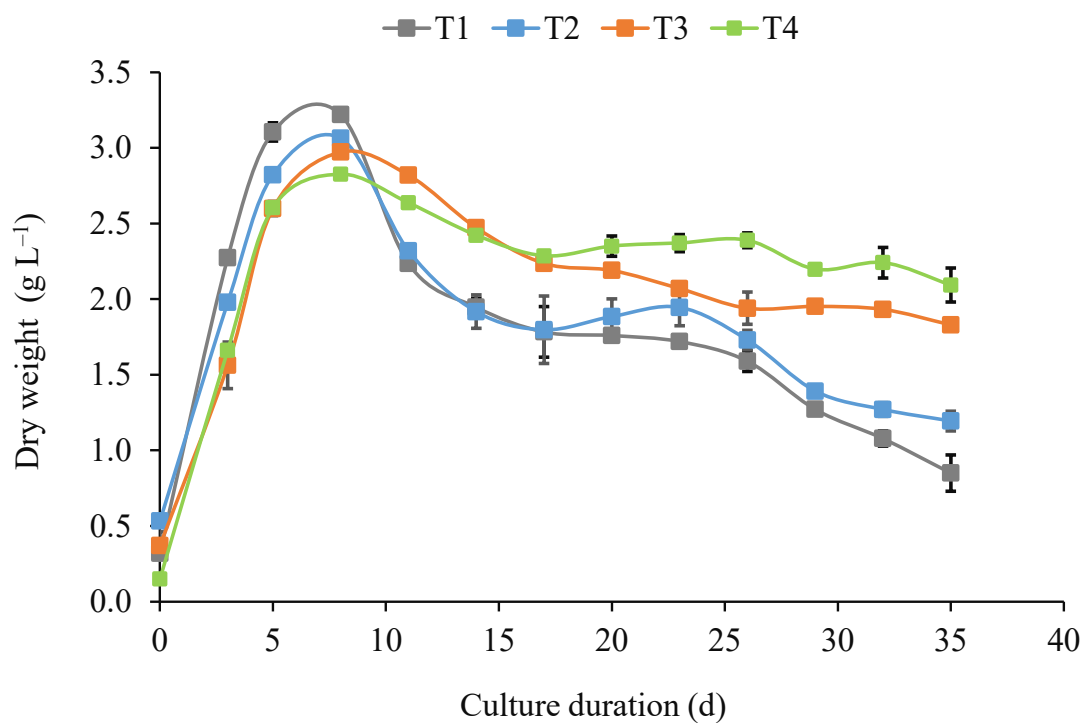


Fig. III-1. Biomass dry weight (g L^{-1}) of *Limnospira fusiformis* (LC-30) in batch and semicontinuous cultures under various simulated outdoor light intensities: in full sunlight (T1), inside a greenhouse (T2), mid-day shade in a greenhouse (T3), and whole-time shade in a greenhouse (T4). The data are presented as the mean \pm standard deviation, $n=3$.

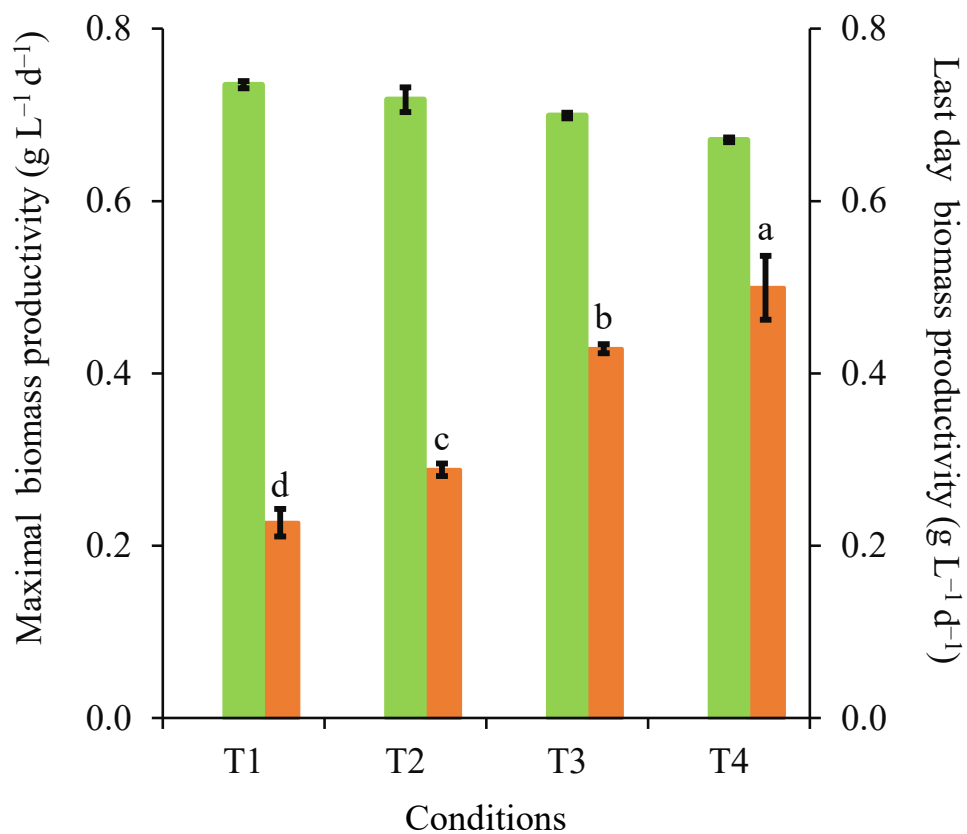


Fig. III- 2. Impact of simulated light intensity on biomass productivity of *Limnospira fusiformis* (LC-30) in full sunlight (T1), inside a greenhouse (T2), mid-day shad in a greenhouse (T3), and whole-time shad in a greenhouse (T4). The data are presented as the mean \pm standard deviation, $n=3$. Different lowercase letters denote significant differences among conditions in biomass productivity ($p < 0.05$).

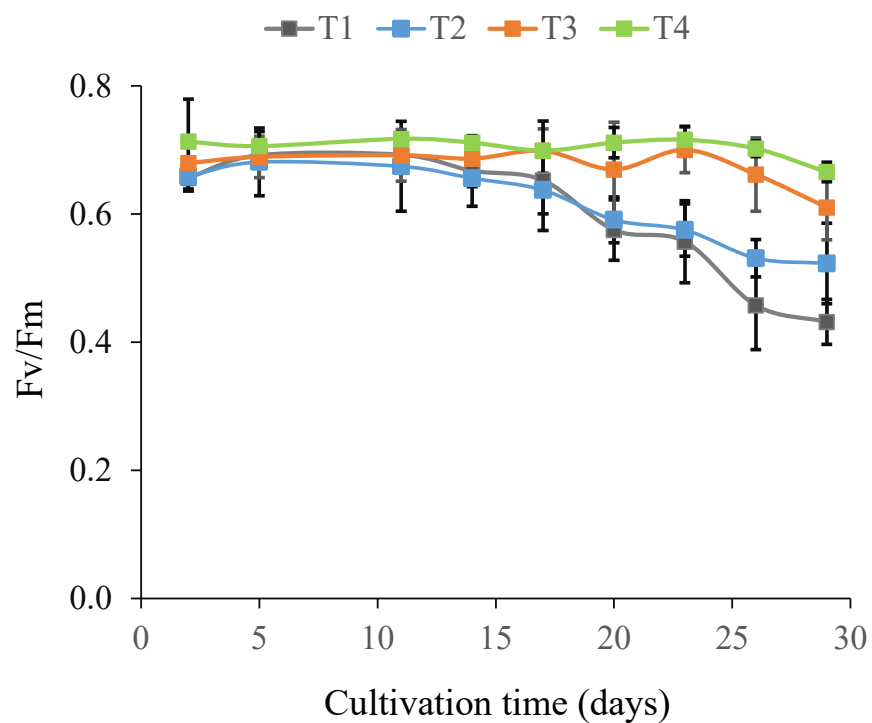


Fig. III-3: Fv/Fm ratio of *Limnospira fusiformis* (LC-30) cultured under various simulated outdoor light intensities: in full sunlight (T1), inside a greenhouse (T2), mid-day shade in a greenhouse (T3), and whole-time shade in a greenhouse (T4). The data are presented as the mean \pm standard deviation, $n=3$.

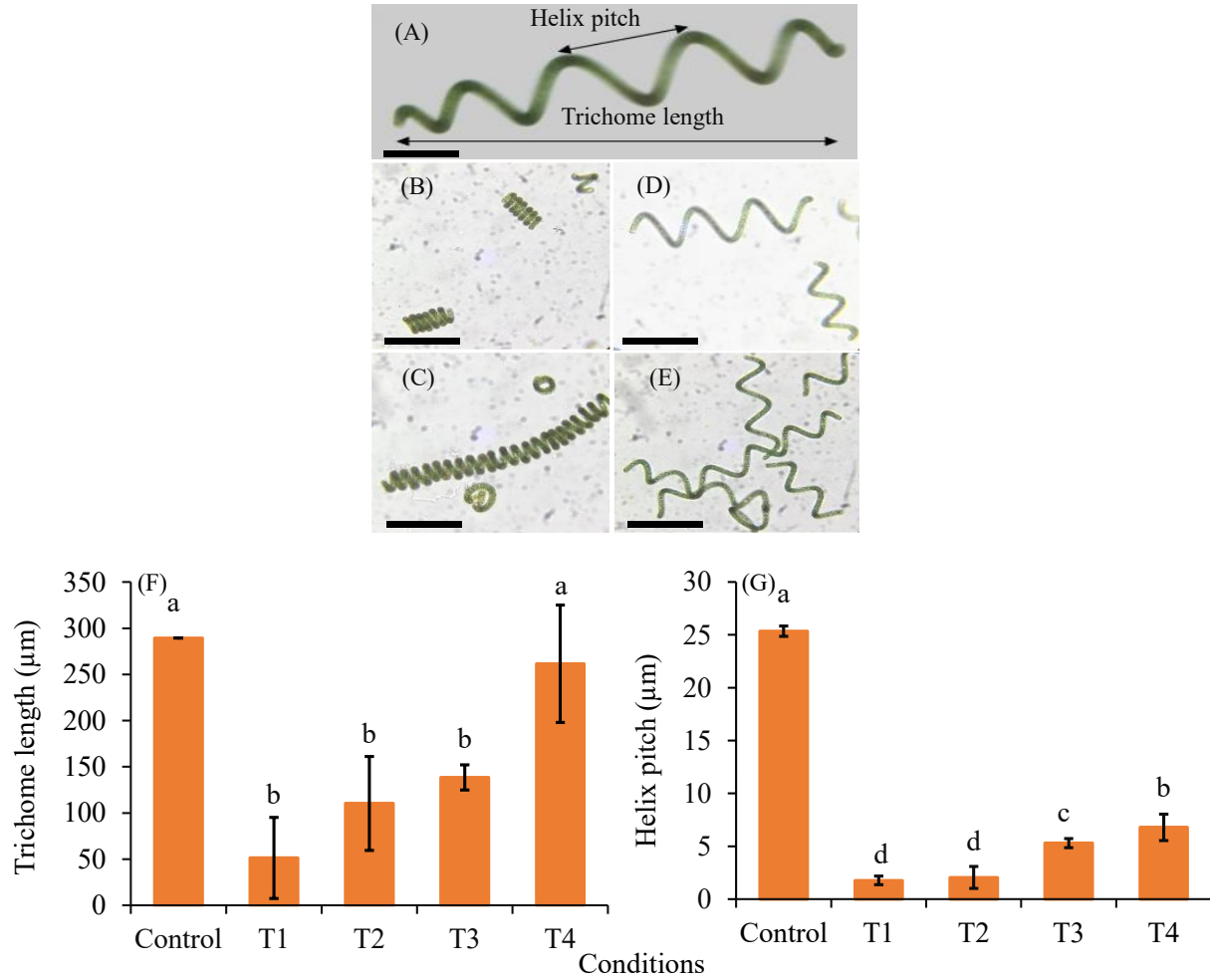


Fig. III- 4. Morphological characteristics of *Limnospira fusiformis* (LC-30) at the beginning of the experiment as control (A), on day 18 under different light intensities: in full sunlight (T1) (B), inside a greenhouse (T2) (C), mid-day shade in a greenhouse (T3) (D), and whole-time shade in a greenhouse (T4) (E). The scale bar represents 50 μm. The data are presented as the mean \pm standard deviation, $n=3$. Different lowercase letters denote significant differences among conditions in trichome length and helix pitch ($p < 0.05$).

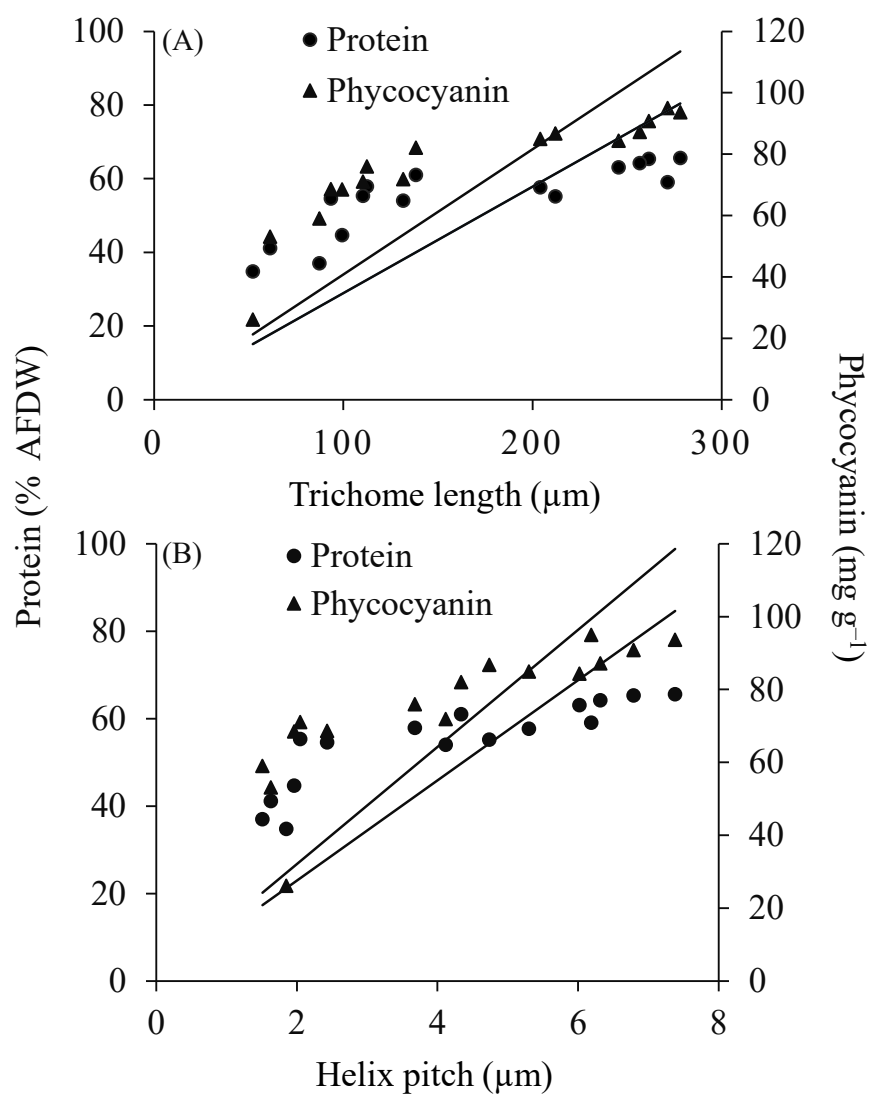


Fig. III- 5. Correlation of protein and phycocyanin with trichome length ($R^2 = 0.90, 0.93$) (A) and with helix pitch ($R^2 = 0.92, 0.91$) (B) in *Limnospira fusiformis* (LC-30). AFDW; Ash-free dry weight.

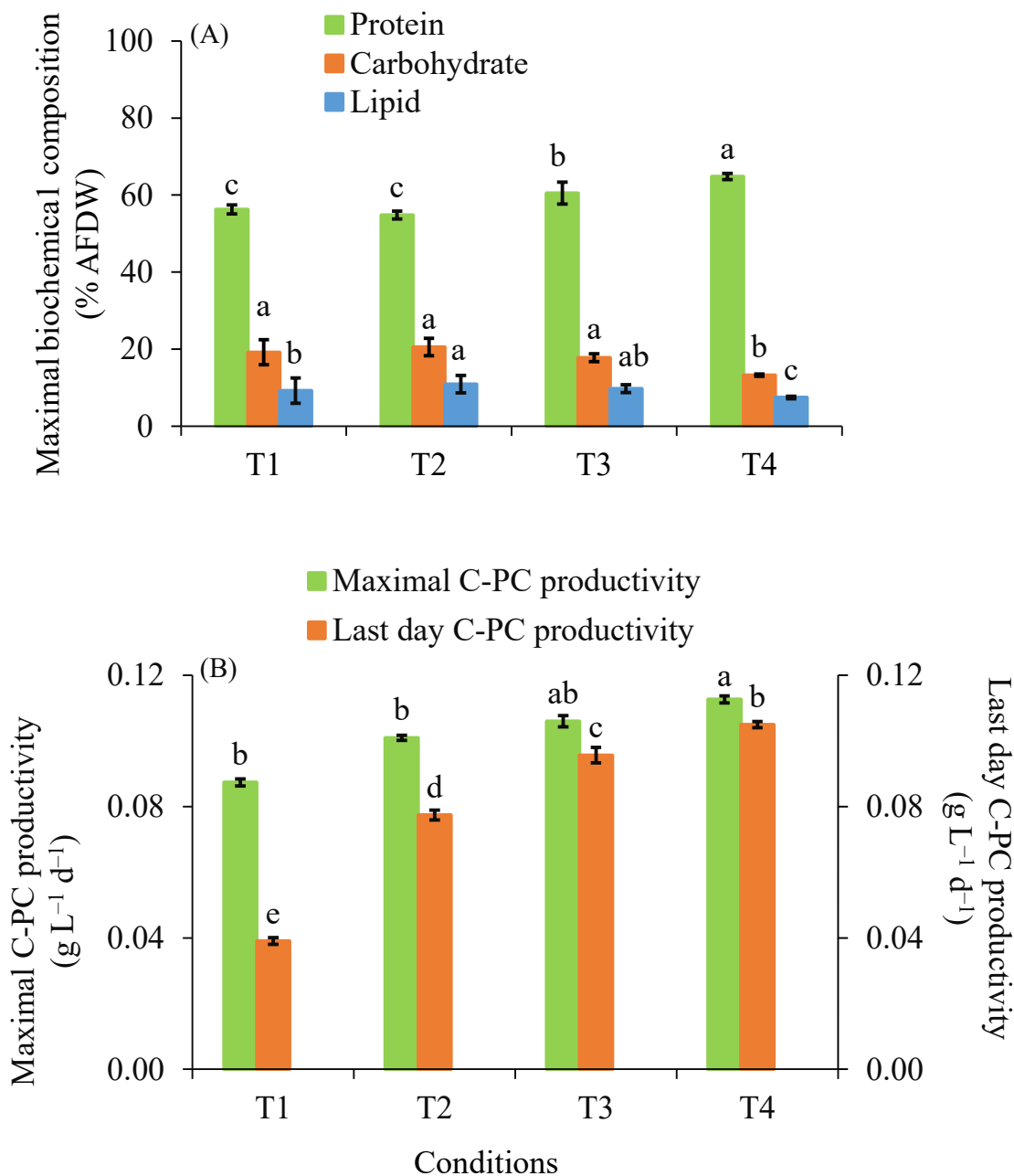


Fig. III-6. Average maximal biochemical composition (A), and crude phycocyanin (C-PC) productivity (maximal and last day) of *Limnospira fusiformis* (LC-30) under various light conditions (B): in full sunlight (T1), inside greenhouse (T2), mid-day shade in a greenhouse (T3), and whole-time shade in a greenhouse (T4). The data are presented as the mean \pm standard deviation. Statistical differences ($p < 0.05$) between conditions are denoted by different lower-case letters. AFDW; Ash-free dry weight.

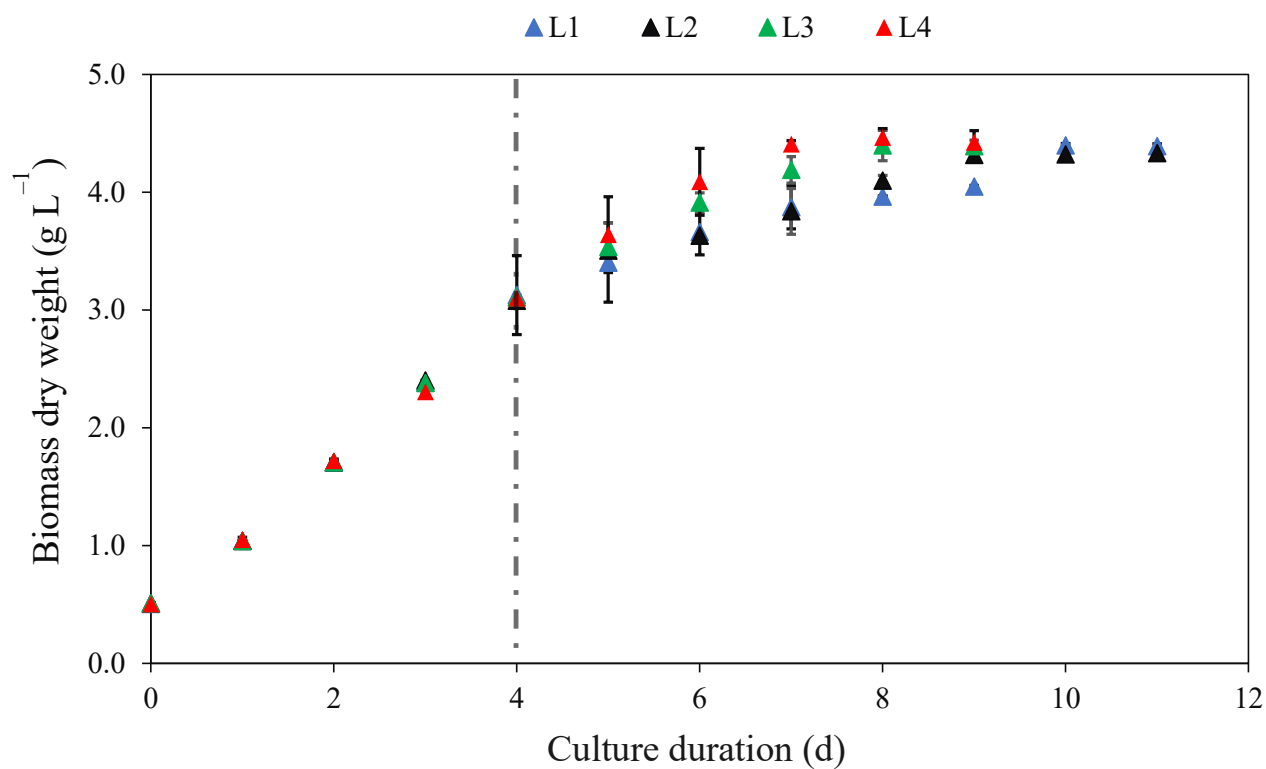


Fig. III-7. Growth curve of *Limnospira fusiformis* under the proposed two-stage cultivation with varying light intensities (L1: 100, L2: 200, L3: 400, and L4: 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$), highlighting biomass growth trends.

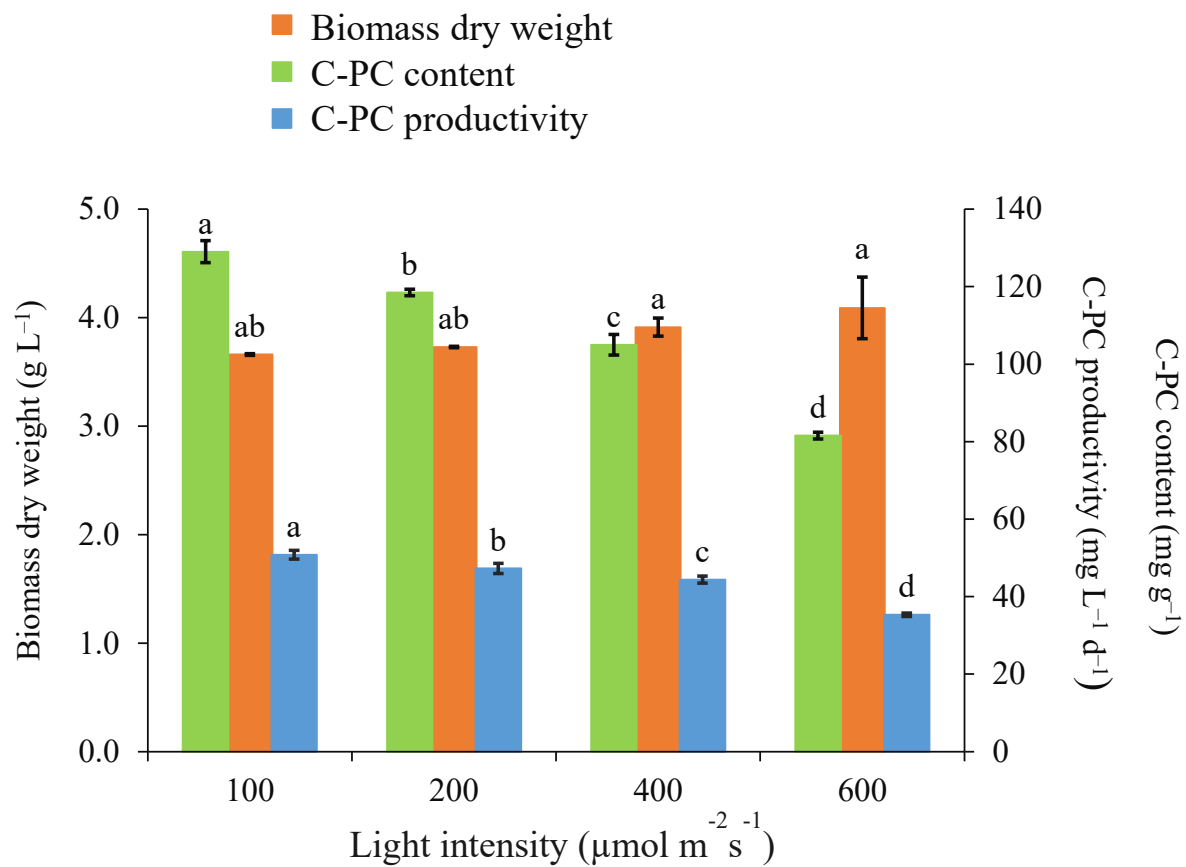


Fig. III-8. The biomass, crude phycocyanin (C-PC) content, and productivity under different light intensity. Different lower-case letters indicate significant differences ($p < 0.05$) within groups.

4 CHAPTER IV General Discussion

This PhD study focused on the isolation and identification of *Arthrospira*-like isolates from Ethiopian soda lakes, confirming them as *Limnospira fusiformis* later reclassified as *Limnospira maxima* (Clade I), a safe, non-microcystin-producing species with exceptional growth potential and high phycocyanin content (Chapter II). Investigations into light conditions revealed that shading techniques significantly enhanced protein synthesis, while two-stage cultivation strategies optimized pigment productivity and biomass yield by effectively balancing light intensities to support both growth and phycocyanin accumulation (Chapter III). Chapter IV emphasized the reclassification of *Limnospira fusiformis* into *Limnospira maxima* (Clade I) and highlighted the potential of raceway ponds as sustainable and cost-effective systems for large-scale outdoor cultivation, leveraging favorable conditions for mass production. Future considerations include seasonal variability, strain adaptability, and renewable energy integration, positioning *Limnospira fusiformis* as a key resource for combating malnutrition and supporting sustainable industrial applications.

4.1 Molecular identification and taxonomic reclassification of Ethiopian *Limnospira* strains

The molecular identification and reclassification of Ethiopian *Limnospira* strains represent a critical advancement in cyanobacterial taxonomy, paving the way for their application in human nutrition and biotechnology. The taxonomic distinction between *Limnospira* and *Arthrospira*, established by Nowicka-Krawczyk et al. (2019), utilized an integrative approach combining morphological, ecological, and molecular data to resolve longstanding ambiguities. Building on

this foundation, Ethiopian *Arthrospira*-like isolates were identified as *Limnospira fusiformis* through 16S rRNA gene sequencing, detailed analysis of their helical trichome morphology, and their adaptation to alkaline-saline environments (Woldie et al., 2024). The reclassification of Ethiopian isolates as *Limnospira fusiformis* highlights their suitability for safe, large-scale cultivation as a sustainable, nutrient-rich food resource.

4.1.1 Classification into clades and implications

Recent refinements, highlighted by Roussel et al. (2023) and Pinchart et al. (2024), divided genus *Limnospira* into two clades: clade I (*Limnospira maxima*) and clade II (*Limnospira platensis*), differentiated by specific nucleotide markers in the V3-V4 regions of the 16S rRNA gene. Phylogenetic analysis in this PhD study utilized nucleotide sequences from both Clade I and Clade II of the V3-V4 regions of the 16S rRNA gene to elucidate the relationship of Ethiopian *Limnospira* strains. This approach confirmed their placement within Clade I, aligning them closely with *L. maxima* (Fig. IV-1). These findings align with studies by Papapanagiotou and Gkelis, (2019), which demonstrated the importance of molecular markers for precise taxonomy. Classification into Clades I and II highlights the genetic, morphological, and ecological diversity within *Limnospira*, enabling more precise strain selection for specific applications, such as food production and biotechnology, while ensuring safety and scalability.

4.1.2 Safety and suitability for food applications

The food safety of *Arthrospira* has long been a critical concern due to the potential presence of microcystins, toxins produced by some cyanobacteria. Only *Arthrospira platensis* products have received approval for human consumption in countries like Canada, Australia, and the United States (FAO, 2008). Studies have confirmed the absence of detectable microcystins in pure

Arthrospira products (Manali et al., 2017), while low levels detected in supplements (Gilroy et al., 2000) were within safe regulatory limits. Ethiopian *Limnospira fusiformis* strains, classified under Clade I (*L. maxima*), are grouped in a clade that lacks the microcystin-producing genes, suggesting a lower likelihood of microcystin production (Pinchart et al., 2024; Woldie et al., 2024). However, the absence of microcystins has not yet been definitively confirmed, and future studies should focus on direct toxicity assessments to validate the safety of these strains for human consumption. Ensuring rigorous quality assurance protocols, including toxin testing, is essential for confirming the absence of harmful substances and ensuring the safety of *Limnospira* for human applications. With their nutrient-rich profile, ecological resilience, and potential safety, Ethiopian *Limnospira fusiformis* strains offer a promising and sustainable solution for addressing global food security, contingent on confirmation of their safety through thorough testing. Their adaptability to extreme environments and large-scale cultivation potential make them ideal for functional food applications, combating malnutrition, and meeting regulatory standards. Additionally, these strains hold promise for advancing environmentally friendly biotechnologies, positioning them as a transformative resource for nutritional and technological innovation.

4.2 Growth potential and optimization strategies for *Limnospira fusiformis* cultivation

4.2.1 Growth metrics under controlled conditions

Under controlled conditions, *Limnospira fusiformis* LC-30 demonstrated exceptional growth potential and nutritional value, achieving a specific growth rate of 1.63 d^{-1} at $160 \mu\text{mol m}^{-2} \text{ s}^{-1}$ light intensity (Woldie et al., 2024). This growth rate surpasses most strains reported in previous studies, such as *Arthrospira maxima* IFRPD 1183 (0.48 d^{-1}) and *Spirulina platensis* strains ($0.42\text{--}1.02 \text{ d}^{-1}$), highlight LC-30 superior adaptability to cultivation conditions (Table II-2). The strain's

protein content reached 58.3% of ash-free dry weight (AFDW), outperforming *A. maxima* IFRPD (39.8%) and approaching the highest levels reported for *A. platensis* C1 (65%) (Gordillo et al., 1998; Chaiklahan et al., 2007). In conclusion, *L. fusiformis* LC-30 robust growth, high protein content, and adaptations to extreme environments make it a promising candidate for large-scale cultivation. By implementing optimization strategies, its growth and biochemical profile can be further enhanced, positioning LC-30 as a versatile resource for sustainable biomass production and high-value pigment applications, particularly in addressing nutritional deficiencies and expanding industrial applications.

4.2.2 Light intensity and shading optimization

This PhD study research findings highlight the remarkable capacity of *Limnospira fusiformis* LC-30 for generating protein and phycocyanin (C-PC) under simulated outdoor light intensity. In simulated settings, LC-30 yielded protein levels between 35% and 65%, with peak production occurring under whole time shaded conditions ($1400 \mu\text{mol m}^{-2} \text{s}^{-1}$). This output exceeds that of comparable strains like *A. platensis* C1 (47%) and *A. platensis* SAG 21.99 (43–59%) grown in similar environments (Chaiklahan et al., 2007; Kilimtzidi et al., 2019). Nevertheless, it aligns closely with optimized *A. platensis* strains, which have reported protein concentrations up to 70% in raceway ponds (Hidasi and Belay, 2018). The outcomes highlight *Limnospira fusiformis* as an ideal candidate for scalable production, given its high protein content and environmental adaptability.

Regarding phycocyanin production, LC-30 demonstrated yields of $76\text{--}97 \text{ mg g}^{-1}$, with maximum performance under shaded conditions, comparable to *A. platensis* F&M-C256 (75 mg g^{-1}) and *A. platensis* SAG 21.99 ($58\text{--}122 \text{ mg g}^{-1}$) (Kilimtzidi et al., 2019; Zanolli et al., 2022).

However, LC-30's output falls short of *A. platensis* LEB-18, which achieved 311 mg g⁻¹ under nutrient-optimized outdoor conditions (Jesus et al., 2018), emphasizing the critical influence of geographic and climatic factors in maximizing phycocyanin production. LC-30 also outperformed *A. platensis* UTEX-1926 (41 mg g⁻¹) (Chen et al., 2013), showcasing its competitive potential for high-value nutraceutical applications. To further exploit this strain's capabilities, strategies such as two-stage cultivation approaches offer promising avenues. Yu et al., (2023) reported substantial improvements in *A. platensis* HN5 phycocyanin yields, achieving 160 mg g⁻¹ and a productivity of 59.72 mg L⁻¹ day⁻¹ under two-stage cultivation with nitrogen supplementation. Similarly, advanced cultivation systems incorporating high-light intensities for biomass growth followed by low-light, nutrient-enriched conditions for pigment synthesis could significantly enhance LC-30 productivity. For example, *A. platensis* LEB-18 achieved 140 mg g⁻¹ under optimized conditions (Deamici et al., 2016), demonstrating the potential impact of light and nutrient optimization. Although competitive, LC-30 phycocyanin content remains below some reported values, highlighting opportunities for further enhancement. Targeted optimization strategies, including light intensity modulation, nutrient supplementation, and advanced cultivation techniques, can unlock the full potential of LC-30 for industrial-scale production of high-value compounds.

4.2.3 Two-stage cultivation approaches for phycocyanin enhancement

The simulation studies in this PhD research highlighted the adaptability of *Limnospira fusiformis* LC-30 to diverse light conditions, demonstrating varying phycocyanin (PC) yields (section 4.2.2). The findings highlight the critical role of precise light intensity management in optimizing PC synthesis and provide valuable insights for developing cultivation strategies tailored to varying environmental conditions. Building on these insights, the experimental application of

the two-stage cultivation system validated its effectiveness in optimizing both biomass growth and PC production. During the first stage, conducted under high light intensities ($\sim 400 \mu\text{mol m}^{-2} \text{s}^{-1}$), rapid cell division and robust biomass accumulation were observed. This phase resulted in a biomass concentration of approximately 3.1 g L^{-1} for *Limnospira fusiformis* LC-30, establishing a strong foundation for subsequent pigment enhancement. This phase establishes a strong biomass foundation, preparing the culture for the subsequent pigment-enhancement stage (Fig. III-7). In the second stage, transitioning to lower light intensities ($\sim 100\text{--}600 \mu\text{mol m}^{-2} \text{s}^{-1}$) significantly enhanced PC synthesis. *L. fusiformis* LC-30 achieved a PC yield of 129.02 mg g^{-1} and a productivity of $50.83 \text{ mg L}^{-1} \text{ day}^{-1}$ (Fig. III-8). Similarly, *Arthrospira maxima* LJGR1, under blue LED light at $475 \mu\text{mol m}^{-2} \text{s}^{-1}$, produced a PC content of 135 mg g^{-1} (García-López et al., 2020). The two-stage strategy balances biomass growth and pigment synthesis by leveraging high light intensities for photosynthesis in the first stage and lower intensities to boost pigment accumulation in the second stage, overcoming the limitations of single-stage cultivation.

Moreover, the simulation studies emphasized the potential for dynamic light modulation techniques, such as adjustable shading or reflective materials, to optimize cultivation outcomes. Tailoring light conditions allows for maximizing biomass growth during high sunlight periods in the first stage and enhancing pigment production under lower light intensities in the second stage. The scalability and adaptability of this approach make the two-stage system particularly suitable for outdoor applications in high-light regions such as Ethiopia, where natural conditions can support industrial-scale biomass and pigment production. This method, when integrated with raceway pond systems, represents a sustainable and cost-effective framework for producing high-value pigments, aligning with the goals of enhancing biomass and PC yields for industrial and nutritional applications.

4.3 Scalability and sustainability of *Limnospira* cultivation in Ethiopia

Raceway ponds, established through the SATREPS EARTH project, are positioned as a sustainable and cost-effective solution for large-scale cultivation of *Limnospira fusiformis* in Ethiopia (Fig. IV-2). These systems leverage Ethiopia's favorable climatic conditions, including high solar irradiance and moderate temperatures, with the added benefit of greenhouse coverings to mitigate environmental fluctuations. The unique adaptations of indigenous *L. fusiformis* strains to the high salinity and alkalinity of Ethiopian soda lakes create a naturally contamination-resistant environment. This resilience not only minimizes operational costs but also enhances scalability and productivity, making these settings ideal for sustainable cultivation systems (Torzillo et al., 1991). Therefore, the established raceway ponds offer an excellent opportunity to harness Ethiopia's ideal climatic and ecological conditions for efficient, large-scale microalgae production.

In this PhD research, simulation studies demonstrated biomass yields of 2.82–3.22 g L⁻¹ under outdoor light conditions, aligning with or exceeding global benchmarks for raceway ponds (0.5–2.0 g L⁻¹ d⁻¹). In the current study, shading strategies effectively mitigated photoinhibition during midday high light intensities (>2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$), increasing protein content to 65% under optimized shaded light conditions (1400 $\mu\text{mol m}^{-2} \text{s}^{-1}$), providing valuable insights for outdoor raceway pond applications. Leveraging these insights in outdoor raceway systems will optimize light management, maximizing productivity and biochemical quality in scalable and practical cultivation settings (Vonshak & Guy, 1992). Raceway ponds provide several advantages over alternative cultivation systems. Compared to photobioreactors, which are costly and energy-intensive, and biofilm systems, which face scalability limitations, raceway ponds strike a balance between economic feasibility and productivity (Radmann et al., 2007). They are simpler in design,

cheaper to construct and operate, and highly scalable, making them ideal for large-scale cultivation in regions with favorable climates like Ethiopia. Seasonal factors, such as photoinhibition during summer or reduced growth in winter due to low temperatures, can be mitigated through effective system optimization, such as shading and temperature control (Carneiro et al., 2018). Leveraging Ethiopia's natural advantages and contamination-resistant *L. fusiformis* strains, raceway ponds, paired with advanced light management, enable sustainable, scalable microalgae production. This system supports high-quality biomass cultivation, addressing food security, malnutrition, and economic development, while advancing sustainable biotechnology.

4.4. Future directions for large scale applications

This PhD study demonstrated the importance of light management in optimizing *Limnospira fusiformis* productivity and biochemical composition. Shading significantly enhanced phycocyanin and protein content, surpassing yields reported in outdoor raceway ponds and photobioreactor studies. Transitioning from outdoor light-simulated laboratory studies to real-world cultivation presents challenges such as diurnal temperature fluctuations, wind, humidity, and nutrients. In Bahir Dar, Ethiopia, the greenhouse water temperature in the raceway pond fluctuates between 17°C and 41°C, posing a challenge to maintaining optimal *Limnospira fusiformis* growth, which thrives at $30 \pm 2^\circ\text{C}$. While the simulation experiment in the greenhouse maintained an average temperature of $30 \pm 2^\circ\text{C}$, real-world outdoor conditions are more variable, and fluctuations beyond this range can cause significant stress to the algae, leading to reduced biomass productivity. These temperature extremes can increase cellular stress, potentially causing thermal damage to the *Limnospira fusiformis* cells. High temperatures, in particular, may lead to the melting or weakening of the cell wall, a critical structural component, which impairs cellular integrity and functionality.

The loss of cell wall integrity may result in the leakage of cellular contents, which can inhibit photosynthesis and growth (Torzillo and Vonshak, 2013; Singh and Singh, 2015). Furthermore, inadequate ventilation exacerbates this stress by limiting air exchange and cooling, further impeding cellular function and growth. To mitigate these effects, future studies should focus on mechanisms of temperature regulation, such as optimizing natural ventilation (e.g., roof vents and sidewalls), using mechanical ventilation systems (e.g., fans or exhaust systems), and incorporating passive cooling strategies (e.g., shading or evaporative cooling) (Lu et al., 2011; Apel et al., 2017). These strategies will help maintain a stable environment within the raceway pond, ensuring that *Limnospira fusiformis* growth remains efficient and sustainable. Additionally, the findings in this study, which focused on February to April, do not account for seasonal variations in Ethiopia's climate, such as changes in light intensity and temperature, which significantly influence year-round productivity (Khan et al., 2018; Dinpazhooh et al., 2022). Future studies need to address these complexities to scale and adapt findings, developing robust and sustainable *L. fusiformis* production systems for outdoor settings.

This PhD study also focused on a single indigenous strain limits insights into strain-specific adaptability to diverse environmental conditions. Dinpazhooh et al., (2022) highlighted the importance of strain-specific responses to light and nutrient availability in outdoor systems, emphasizing the need for broader exploration. Expanding future research to include multiple indigenous strains will enhance the applicability of these results, contributing to the development of generalized algal cultivation systems adaptable to the diverse climatic conditions of Ethiopia.

Lastly, the energy-intensive methods employed in this PhD study, though effective, limit feasibility for resource-constrained regions. Integrating renewable energy sources, such as solar-powered systems, and employing passive shading techniques could significantly reduce

operational costs and environmental impact (Khan et al., 2018). Future research should prioritize energy-efficient, cost-effective cultivation strategies to ensure economic viability while supporting sustainable food production and addressing malnutrition challenges in Ethiopia.

In conclusion, this PhD study successfully isolated over 100 *Arthrospira*-like isolates from Ethiopian soda lakes, with molecular analysis of four fast-growing isolates confirming their classification as *Limnospira fusiformis*. Rigorous screening identified representative strains with superior growth potential and biochemical profiles, positioning them as highly suitable candidates for large-scale production to address food security challenges. Simulation experiments under outdoor-like conditions optimized light intensities, demonstrating effective strategies to enhance both biomass productivity and phycocyanin content. The successful implementation of a two-stage cultivation approach further validated the potential of *L. fusiformis* strains for scalable and sustainable phycocyanin production. By integrating systematic isolation, screening, and cultivation optimization, this research underscores the promise of *L. fusiformis* as a safe, nutrient-dense, and scalable resource. These findings pave the way for addressing malnutrition, advancing sustainable biotechnology, and developing outdoor cultivation systems tailored to Ethiopia's unique environmental conditions.

Table

Table IV-1. Comparison of biochemical composition, and crude phycocyanin (C-PC) content of *Arthrospira* and *Limnospira fusiformis* from available literature.

Species/ strain	Cultivation conditions			Composition (% dry weight)			C-PC (mg g ⁻¹)	References
	Bioreactor	Initial cell density (g L ⁻¹)	Max light (μmol m ⁻² s ⁻¹)	Protein	Carbohydrate	Lipid		
<i>A. platensis</i> / C1	Flasks	-	100	47	36	4	200	Chaiklahan <i>et al.</i> , 2007
			500	42	42	4	48	
<i>Spirulina</i> / LEB-18	RP	0.50	1841	63	6	12	311	Jesus <i>et al.</i> 2018
			559	54	10	12	74	
<i>A. platensis</i>	RP	0.60	1879	65	18	14	110	Hidasi& Belay, 2018
		0.80	1483	70	-	-	114	
<i>A. platensis</i> SAG 21.99	RP	0.06	1406	43	20	-	58	Kilimtzi <i>et al.</i> , 2019
			516	59	10	-	122	
<i>L. indica</i>	ALPBR	1.00	387	59	13	-	92	Garcia <i>et al.</i> , 2021
			932	48	16	-	84	
			1473	47	20	-	61	
<i>A. platensis</i>	RP	0.60	296	40	44	4	48	
<i>A. platensis</i> F&M-C256	AR	0.70	700	55	6	-	75	Zanolla <i>et al.</i> , 2022
<i>L. fusiformis</i> / LC-30	CBR	0.06	2000	35	26	9	76	
			1700	37	24	9	87	This study
			1400	54	20	10	95	
			1400	64	16	8	97	

Note: RP: Raceway Pond; CBR: Column photobioreactor; ALPBR: Airlift photobioreactor. Light conditions in this study-

Full sunlight, inside a greenhouse, mid-day shade in a greenhouse and whole-time shade in a greenhouse (Refer Table III-1).

Figures

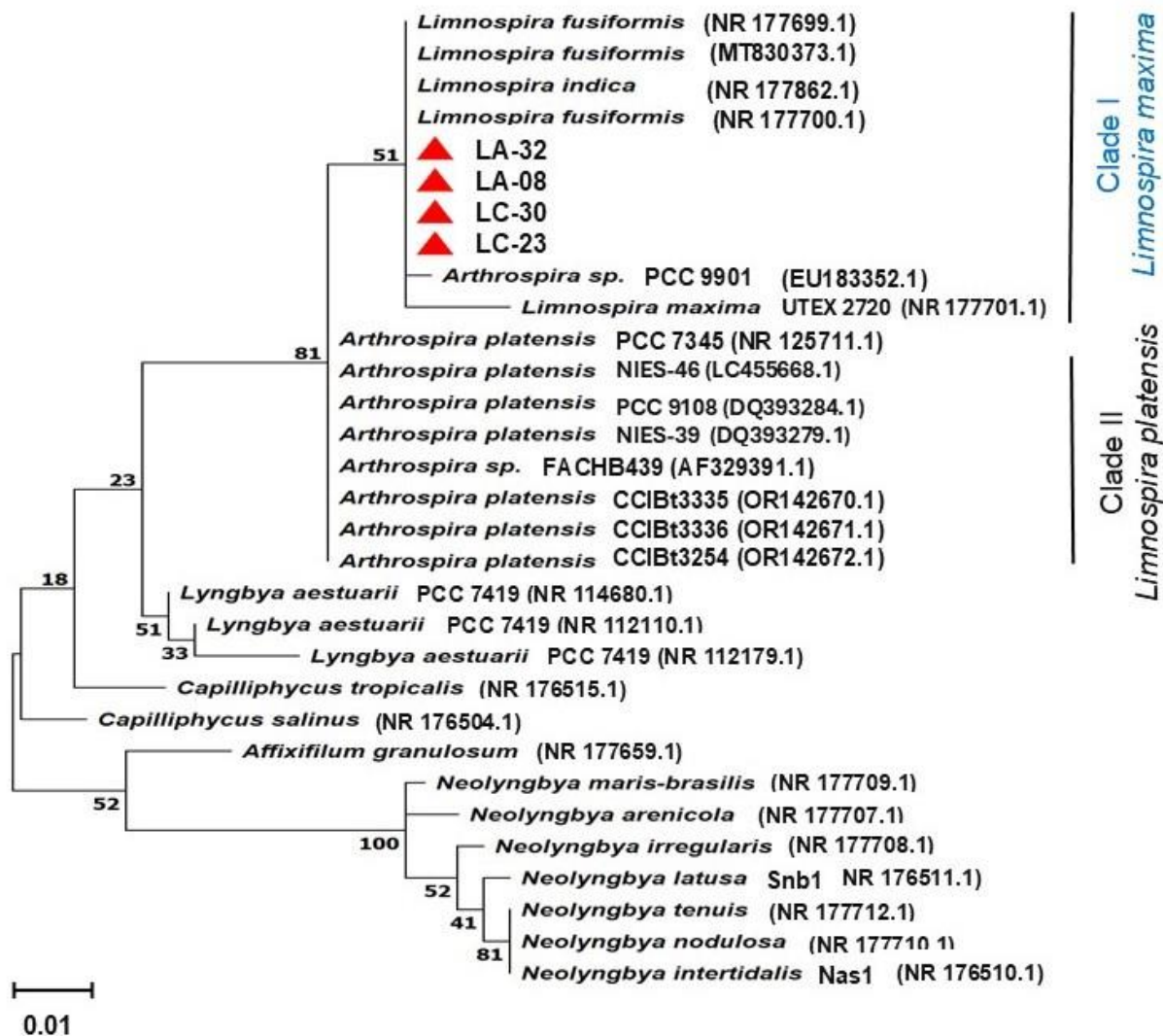


Fig. IV-1. Phylogenetic position of the four *Limnospira* strains isolated from Lakes Chitu and Arenguade in Ethiopia, based on 16S rRNA gene sequence data. The tree was constructed using the maximum likelihood method with 1000 bootstrap replications. The analysis incorporates the latest reclassification of *Limnospira fusiformis* into *Limnospira maxima* (Clade I) and includes comparisons with related species in the gene bank. The *Limnospira* strains from this study are marked with a red triangle (▲).



Fig. IV-2. Raceway ponds constructed by the SATREPS EARTH project at Bahir Dar University, Ethiopia, for the large-scale cultivation of microalgae.

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