

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

エチオピアのソーダ湖に生息するリムノスピラ属菌株の分子同定と培養、模擬屋外光条件下での成長評価

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SYNOPSIS

Malnutrition, especially among children with limited dietary diversity, is a significant problem in regions like Ethiopia. *Arthrospira*, a protein-rich cyanobacterium, could enhance nutrition through biomass production, but its use as food in Ethiopian soda lakes is underexplored. This study involved morphological isolation, growth rate screening, 16S rRNA identification, and biochemical analysis of *Arthrospira*-like isolates (Chapter II, Study 1). It was followed by an evaluation of the effects of varying simulated light conditions (full sunlight, greenhouse, mid-day shade in a greenhouse, and full-time shade in a greenhouse) on the growth and biochemical composition of *Limnospira fusiformis* strain LC-30, selected from Study 1 (Chapter III, Study 2). A total of 100 *Arthrospira*-like strains were isolated, with four strains exhibiting the highest specific growth rates selected for molecular identification, all confirmed as *Limnospira fusiformis*. These strains underwent biomass screening and biochemical analysis, showing results consistent with previous studies. Notably, strains LC-30 and LA-08 exhibited high protein and phycocyanin contents (Study1). Whole-time shade resulted in the highest protein (64.80% ash-free dry weight) and phycocyanin content (97.20 mg g⁻¹). The two-stage cultivation method for phycocyanin demonstrated a 32.7% increase in content compared to the simulated study (study 2). Identifying fast-growing *Limnospira fusiformis* strains and optimizing light conditions for maximizing biomass, phycocyanin and nutritional value highlights the potential of indigenous strains as nutritional supplements.

Keywords: Biochemical composition, *Limnospira fusiformis*, molecular identification, nutritional supplementation, simulated outdoor light, Shading strategies.

Introduction

Malnutrition, a widespread global health issue, significantly impacts immunity, well-being, and cognitive development. In Ethiopia, 38% of children experience stunting and 9% suffer from wasting¹. To address the persistent issue of insufficient dietary diversity, exploring innovative solutions is necessary. Microalgal cultivation emerges as a promising approach to addressing malnutrition². This might offer a sustainable, nutrient-dense solution that can be scaled to meet the dietary needs of vulnerable populations.

Commercial cultivation of microalgae such as *Arthrospira* and *Chlorella* has progressed, providing vital nutrients like proteins, carbohydrates, lipids vitamins, minerals, and pigments³. *Arthrospira*, with its high protein content (50-77%) and rapid growth, highlights the need for screening fast-growing strains to maximize biomass production efficiency. Soda lakes in Ethiopia, rich in indigenous *Arthrospira* species, offer a unique opportunity to enhance food security⁴. To achieve sustainable nutritional solutions, identifying and utilizing local strains with rapid growth and high biomass yields is crucial. *Arthrospira* excels in producing biomass in alkaline-saline environments with minimal contamination risk due to its adaptability to pH levels between 8 and 11

and temperatures between 25–35 °C⁵. Therefore, isolating and identifying strains with rapid growth and high biomass yields is vital for maximizing the potential benefits of microalgal cultivation.

Traditionally, *Arthrospira* species have been identified using morphological characteristics. However, due to the similarities among strains and the influence of environmental factors on morphology, this method presents challenges. For instance, advances in molecular and ultrastructure analyses have led to the reclassification of *Arthrospira* as the genus *Limnospira*, which includes *L. fusiformis*, *L. maxima*, and *L. indica*⁶. As a result, accurate identification now requires combining traditional morphological methods with molecular techniques like 16S rRNA gene sequencing. Accurate identification of strains is crucial, but optimizing growth conditions is essential for maximizing biomass production and nutrient content in large-scale cultivation.

Arthrospira spp. thrives under specific culture conditions, requiring optimized light intensity, dissolved inorganic carbon, pH, and temperature to enhance biomass production. Optimizing laboratory cultures identifies ideal growth parameters for *Arthrospira* spp., while indoor simulations facilitate their application to outdoor cultivation⁷. Intense sunlight can stress *Arthrospira* spp.,

reducing productivity and altering biomass composition. Assessing light intensity indoors is crucial for optimizing *Arthrospira* spp. growth and productivity outdoors. Previous studies largely focused on controlled labs, with limited exploration of outdoor light simulation⁸. While studies have replicated outdoor conditions for species like *Chlorella sorokiniana* and *Nannochloropsis salina*^{9,10}, optimizing fluctuating light for outdoor cultivation of *Limnospira fusiformis* from Ethiopian soda lakes remains underexplored¹¹.

The main goal of this PhD thesis is to identify and evaluate *Limnospira* strains for their biomass yield, nutritional value, and growth potential under varying environmental conditions. The specific objectives are Chapter 2: Screen and identify *Limnospira* strains from Ethiopian soda lakes, assessing their biomass and nutritional values. Chapter 3: Assess the growth and biochemical composition of *L. fusiformis*, including phycocyanin, under simulated outdoor light intensities, and evaluate two-stage cultivation for phycocyanin enhancement.

Materials and Methods

Sample collection and strain maintenance: Water samples were collected from Lake Chitu and Lake Arenguede in Ethiopia. Cyanobacterial strains were cultured in a modified Spirulina Ogawa Terui (SOT) medium, calibrated for optimal growth, and adjusted to pH 9.8. The strains were grown in 500 mL Erlenmeyer flasks with an effective volume of 250 mL, incubated at 35 °C under cool-white, fluorescent lamps at 160 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, with light cycles of 12-h L/12-h D and continuous mixing at 0.25 vvm.

Study 1. Isolation, screening, and molecular identification of *Arthrospira*

Morphological isolation: Serial dilutions were prepared and dispensed into 96- and 12-well microtiter plates. Individual trichomes were isolated using the capillary pipette method, transferred to 12-well plates with sterile SOT medium, and incubated at 35 °C under 160 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ with 12-h L/12-h D. Cultures were examined under an inverted microscope (Axioskop-2 Plus, Carl Zeiss). Morphological parameters such as coil count and length were evaluated using an optical microscope with a digital camera and analyzed with ImageJ software.

Screening and selection of potential strains: Cultures of 100 morphologically distinct *Arthrospira*-like isolates were grown in 40 mL of SOT medium in test tubes. Specific growth rates were measured at 750 nm, and high-performing *Arthrospira*-like isolates were selected for morphological and molecular analysis.

DNA extraction and sequencing of 16S rRNA gene: Cells from cultures of four isolates (LC-30, LC-23, LA-08, and LA-32) were collected by centrifugation for genomic DNA extraction using the lysis buffer method¹². The 16S rRNA gene was amplified with primers 27F and 1492R using a PCR mixture of 10 μL 2 \times PCR premix reagent

(EmeraldAmp® MAX PCR Master Mix, TAKARA BIO), 1 μL of each primer, 7 μL of sterile water, and 1 μL of DNA. PCR products were analyzed via 1% agarose gel electrophoresis and sequenced by Eurofins Genomics (Japan), with sequences compared to the NCBI database using BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Biomass dry weight (DW): Molecularly identified strains were screened for biomass concentration in 500 mL Erlenmeyer flasks with 250 mL of medium, maintained at 35 °C under white, fluorescent light (160 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, 12-h L/12-h D) with continuous mixing at 0.25 vvm. DW was determined by filtering cultures through pre-combusted, pre-weighed glass microfiber filters (0.7 μm , GF/F; Whatman). Filters were washed with NH_4HCO_3 solution, dried at 60 °C for 24 h, and weighed.

Protein and phycocyanin content analysis: The protein content of LC-30 and LA-08 strains was measured using the Lowry method with bovine serum albumin as a standard. Phycocyanin content was assessed by centrifuging the culture, washing cells, and freeze-thawing, followed by spectrophotometric measurement at 620 nm.

Study 2. Evaluation of growth and biochemical composition of *L. fusiformis* under simulated outdoor light conditions

Microalgal strain and pre-culture conditions:

Potential strain *L. fusiformis* LC-30, selected from study 1, was maintained in a modified SOT medium and grown in 250 mL Erlenmeyer flasks under 160 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ light (12-h L/12-h D), with a pH of 10. Pre-cultures were incubated at 30 °C in 300 mL Erlenmeyer flasks under 400 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ light with continuous mixing.

Design and simulated light experiments: Outdoor light intensity data from Bahir Dar, Ethiopia, was used to simulate varying conditions (Table 1).

Time (24 h)	Average light intensity ($\mu\text{mol m}^{-2} \text{ s}^{-1}$)			
	T1	T2	T3	T4
0601–0700	200 \pm 103	170 \pm 88	170	150
0701–0800	400 \pm 201	300 \pm 173	300	250
0801–0900	1000 \pm 72	900 \pm 62	900	750
0901–1000	1500 \pm 138	1300 \pm 118	1300	1000
1001–1100	1900 \pm 46	1600 \pm 39	1300	1000
1101–1200	2000 \pm 83	1700 \pm 71	1400	1400
1201–1300	2000 \pm 165	1700 \pm 142	1400	1400
1301–1400	1800 \pm 86	1600 \pm 74	1300	1000
1401–1500	1400 \pm 154	1200 \pm 133	1200	1000
1501–1600	900 \pm 155	800 \pm 133	800	700
1601–1700	400 \pm 171	300 \pm 157	300	250
1701–1800	50 \pm 29	40 \pm 26	40	30
1801–0600	0	0	0	0

*Maximum average mid-day light intensity measurements, T1: Full sunlight (2000 $\mu\text{mol m}^{-2} \text{ s}^{-1}$), T2: Greenhouse (1700 $\mu\text{mol m}^{-2} \text{ s}^{-1}$), T3: Mid-day shade in greenhouse (1400 $\mu\text{mol m}^{-2} \text{ s}^{-1}$), T4: Whole-time shade in greenhouse (1400 $\mu\text{mol m}^{-2} \text{ s}^{-1}$).

Four glass-column photobioreactors were used for semi-continuous cultivation at 30 ± 2 °C, starting at 0.06 OD₇₅₀.

Daily samples measured growth, biomass, and biochemical composition (protein, carbohydrates, lipids, and phycocyanin).

Result and Discussion

Study 1. Isolation, screening and molecular identification of *Arthrospira*

Morphological characteristics of selected strains: From 100 *Arthrospira*-like strains, four with the highest growth rates (0.94 ± 0.22 to 1.04 ± 0.20 d⁻¹) were selected for morphological and molecular analysis. They displayed varied coil tightness and traits such as trichome length (133–244 µm), helix diameter (17–25 µm), pitch (25–57 µm), trichome diameter (4–6 µm), and coil count (8–12) ($p < 0.05$; Fig. 1). Our findings align with established *Arthrospira* traits^{4,13}, confirming their resemblance to strains from Ethiopian soda lakes. However, the coiling degree, influenced by environmental factors and physiological conditions, varies within species over time, complicating morphological classification. This variability highlights the critical importance of integrating molecular markers, such as 16S rRNA sequencing, with morphological analysis to ensure accurate and reliable taxonomic identification.

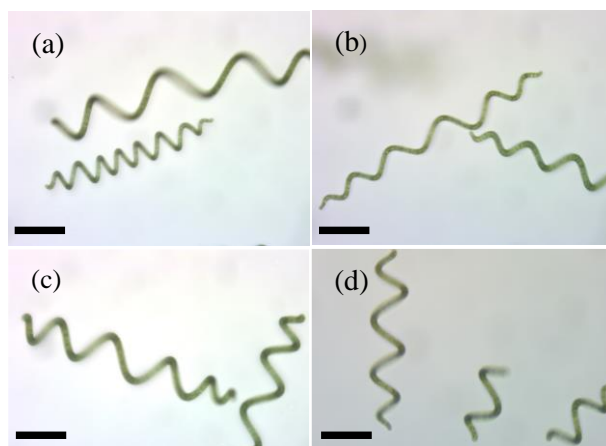


Fig. 1. Microscopic view of *Arthrospira*-like isolates: LC-30 (a), LC-23 (b), LA-08 (c), and LA-32 (d)

Molecular identification of selected strains: DNA sequencing of the 16S rRNA gene confirmed that all *Arthrospira*-like strains (LC-30, LA-08, LC-23, and LA-32) belong to the genus *Limnospira* with 99.62–100% similarity. The phylogenetic position of the strains is shown in Fig. 2. Our findings align with the reclassification of *Limnospira* from *Arthrospira*¹⁰. *Limnospira* thrives in alkaline habitats, which is advantageous for mass production and offers resilience against contamination¹⁶. Identifying strains adapted to Ethiopian soda lakes is crucial for sustainable cultivation, offering advantages in mass production, contamination resistance, and improving local food security.

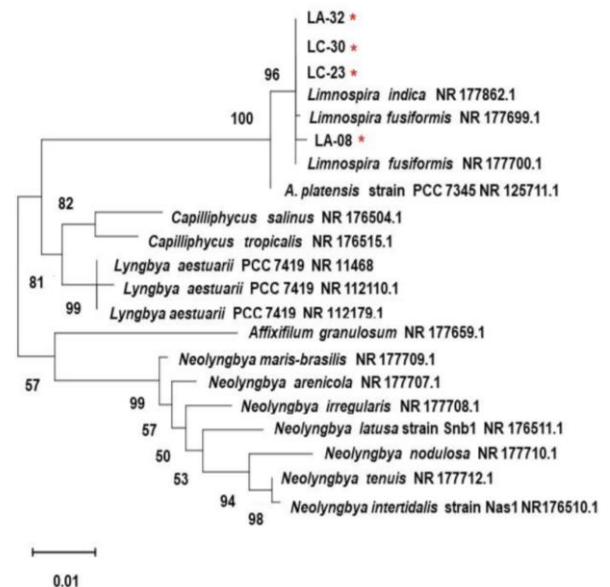


Fig.2. Phylogenetic position of the four *Limnospira* strains

Biomass dry weight: Among the screened *Limnospira* strains, LC-30 exhibited the highest biomass (3.27 ± 0.51 g L⁻¹) and growth rate (1.63 ± 0.05 d⁻¹), followed by LA-08 (2.31 ± 0.12 g L⁻¹; 1.12 ± 0.07 d⁻¹) respectively (Fig. 3). LC-30 outperformed previous reports for *Spirulina platensis*, indicating significant potential for enhanced yields under optimized conditions.

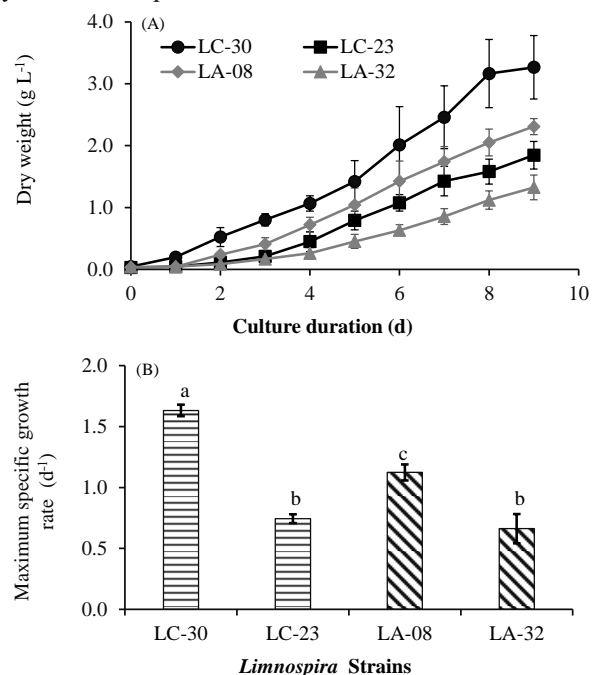


Fig. 3. Biomass dry weights and specific growth rates

Protein and phycocyanin content: Strains LC-30 and LA-08 exhibited high protein (582.80 ± 0.07 and 557.90 ± 0.09 mg g⁻¹) and phycocyanin (78.49 ± 0.00 and 70.36 ± 0.00 mg g⁻¹) contents, surpassing typical *Arthrospira platensis* values and traditional protein sources. These results highlight their potential to address nutrient

deficiencies¹⁴, with room for further phycocyanin optimization.

Study 2. Growth and biochemical composition of *L. fusiformis* under simulated outdoor light conditions

Growth: The highest dry weight for *L. fusiformis* was 3.22 g L⁻¹ under full sunlight (T1), but photoinhibition reduced biomass over time¹⁵. Shaded conditions (T3: 2.97 g L⁻¹; T4: 2.82 g L⁻¹) mitigated photoinhibition, ensuring more stable biomass production, highlighting shading's role in optimizing yield (Fig. 4).

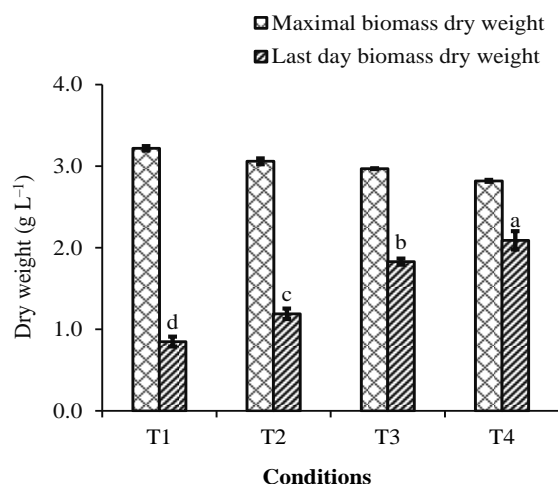


Fig.4. Biomass dry weight of *L. fusiformis*

Biochemical composition: Under varied light conditions (T1–T4), whole-time shade (T4) yielded the highest protein (64.80%) and phycocyanin (97.20 mg g⁻¹) levels, while full sunlight (T1) and greenhouse light (T2) resulted in higher carbohydrates but lower protein. Lipid content peaked in T2 (10.87%) and was lowest in T4 (7.43%) (Table 2). Shading (T3, T4) preserved protein and phycocyanin by mitigating oxidative stress, whereas high light (T1, T2) induced reactive oxygen species¹⁶, degrading protein and increasing carbohydrate storage. Conversely, under optimized light conditions, energy from photosynthesis is efficiently used for higher protein synthesis¹⁷. Optimizing light conditions is critical to maximizing *L. fusiformis* LC-30's biochemical composition and biomass production for outdoor cultivation.

Table 2. Maximal biochemical composition (% AFDW) and phycocyanin content (mg g⁻¹) of *L. fusiformis* LC-30.

Condition	Protein	Carbohydrate	Lipid	Maximum PC	Last day PC
T1	56.29 ^d	19.18 ^a	9.22 ^b	75.96 ^d	26.11 ^d
T2	54.81 ^c	20.57 ^a	10.87 ^a	86.78 ^c	59.01 ^c
T3	60.50 ^b	17.80 ^a	9.72 ^{ab}	94.77 ^b	71.87 ^b
T4	64.80 ^a	13.22 ^b	7.43 ^c	97.20 ^a	84.38 ^a

* AFDW; ash free dry weight, PC; phycocyanin

Two stage culture strategy to enhance phycocyanin

The two-stage cultivation of *Limnospira fusiformis* significantly enhanced growth and phycocyanin (C-PC) production. Stage 1 (400 μmol m⁻² s⁻¹) supported uniform biomass growth (~3.1 g L⁻¹), while Stage 2 (100 μmol m⁻² s⁻¹) optimized C-PC synthesis, achieving a peak productivity of 50.83 mg L⁻¹ d⁻¹ and a maximum C-PC content of 129.02 mg g⁻¹. This aligns with Yu et al. who noted that lower light intensities enhance light-harvesting efficiency, optimizing pigment synthesis¹⁸. Biomass peaked at 4.5 g L⁻¹ under 600 μmol m⁻² s⁻¹, highlighting *L. fusiformis* resilience to light stress, with low-light conditions proving more efficient for pigment production.

Conclusions

- 1) *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.
- 2) The observed differences in growth rates and biomass production among strains highlight the need for rigorous screening to select the most productive strains.
- 3) 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.
- 4) High light intensity led to carbohydrate accumulation.
- 5) Optimized shading and light conditions are essential for improving biochemical composition, productivity, and the transition of *Limnospira* strains from indoor to outdoor cultivation
- 6) The two-stage cultivation approach increased C-PC production by 32.7% compared to simulated light conditions. This scalable strategy effectively balances high-value pigment yields with robust biomass production, offering significant potential for industrial applications.

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