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Biodiversity, Molecular Characterization, and Phylogenetic Analysis of L–Asparaginase Producing Microalgae from Thamarai Kulam Freshwater Pond, Tiruvannamalai, Tamil Nadu

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ABSTRACT

Freshwater ecosystems are important reservoirs of microalgal diversity and a valuable source of industrially relevant biomolecules. The present study explored the biodiversity of microalgae from Thamarai Kulam Freshwater Pond, Tiruvannamalai, Tamil Nadu, India, with particular emphasis on identifying L-asparaginase (L-ASNase) producing strains. This is the first report describing the physicochemical characteristics of this pond, which revealed favorable conditions for microalgal growth, including suitable pH, temperature, water turbidity, light intensity, chemical oxygen demand (COD), total dissolved solids (TDS), total nitrogen, and phosphate concentrations. A total of nine microalgal isolates (FMAS1–FMAS9) were recovered and subjected to qualitative and quantitative screening for L-ASNase production. Among them, isolated FMAS2 exhibited the highest enzyme activity (75.54 ± 0.07 IU/mL) and was selected for further investigation. Morphological characterization using light microscopy identified FMAS2 as a filamentous cyanobacterium belonging to the genus Limnospira. Genomic DNA extracted from FMAS2 showed high purity, with A260/A280 and A260/A230 ratios of 2.165 and 2.246, respectively, and a DNA concentration of 150.5 µg/mL. Molecular characterization based on 16S rRNA gene sequencing confirmed the isolate as Limnospira maxima FMAS2, and the sequence was deposited in the NCBI GenBank database under accession number PX525663.1. Phylogenetic analysis demonstrated a close relationship with authenticated L. maxima strains, supported by a bootstrap value of 100%. Evaluation of different culture media revealed SAG medium as the most effective, yielding maximum L-ASNase production (89.43 ± 0.08 IU/mL). These findings highlight the biotechnological potential of freshwater microalgal biodiversity and identify L. maxima FMAS2 as a promising source of L-ASNase.

Keywords: *Microalgal biodiversity; Limnospira maxima FMAS2; L-asparaginase; Molecular characterization; Freshwater Pond.*

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Introduction

Microalgae represent a highly diverse group of photosynthetic microorganisms inhabiting freshwater, marine, and terrestrial ecosystems, contributing significantly to global carbon fixation, oxygen evolution, and ecological nutrient cycling. Their remarkable metabolic versatility has attracted increasing attention for applications in biotechnology, pharmaceuticals, nutraceuticals, and environmental remediation. Among microalgal metabolites, bioactive enzymes have emerged as promising biomolecules with therapeutic and industrial relevance. L-asparaginase (EC 3.5.1.1), an amidohydrolase enzyme that catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia, is of particular biomedical importance due to its established use in the treatment of acute lymphoblastic leukemia and other hematological malignancies. Commercial L-ASNase is predominantly derived from bacterial sources such as *Escherichia coli* and *Erwinia chrysanthemi*; however, these microbial enzymes are often associated with immunogenicity, hypersensitivity reactions, and reduced therapeutic efficacy, prompting the search for alternative sources with improved biocompatibility and functional characteristics [1][2].

Microalgae have recently emerged as a promising alternative source of therapeutic enzymes due to their phylogenetic diversity, rapid growth, eco-friendly cultivation, and ability to synthesize structurally unique biomolecules. Few studies have demonstrated the potential of cyanobacteria and eukaryotic microalgae to produce industrial important enzymes with notable anticancer activity and comparatively lower adverse effects than conventional bacterial enzymes [3]. Nevertheless, the biodiversity of L-ASNase -producing microalgae remains largely unexplored, particularly in ecologically diverse habitats where unique strains may possess enhanced enzyme productivity and biochemical properties. Biodiversity assessment serves as a critical step in identifying novel microalgal taxa with desirable enzymatic potential, while enabling conservation and systematic cataloging of valuable microbial resources.

Morphological identification alone is often insufficient due to phenotypic plasticity and overlapping taxonomic features among microalgal genera, necessitating integrative molecular approaches for accurate species delineation [4].

Phylogenetic analysis based on molecular markers such as 18S rRNA, ITS, rbcL, and tufA gene sequences has become an indispensable tool for resolving taxonomic ambiguities and understanding evolutionary relationships among microalgae [5]. Molecular phylogeny provides insights into lineage diversification, genetic similarity, and species-level classification, thereby facilitating accurate identification of novel enzyme-producing strains. In enzyme bioprospecting, phylogenetic characterization also helps correlate taxonomic affiliation with metabolic capabilities, enabling targeted screening of phylogenetically related organisms for enhanced bioactive compound production. The integration of biodiversity exploration with phylogenetic analysis has proven effective in uncovering novel microbial resources for industrial biotechnology, yet such comprehensive investigations focusing specifically on L-ASNase producing microalgae remain scarce. Therefore, combining morphological, biochemical, and molecular approaches offers a robust strategy for identifying promising microalgal candidates for therapeutic enzyme production [6][7].

In this context, biodiversity assessment and phylogenetic characterization of L-ASNase producing microalgae constitute an important research avenue for discovering novel strains with potential biomedical applications. Exploring diverse environmental niches for enzyme-producing microalgae may reveal taxonomically unique organisms capable of producing L-ASNase with superior catalytic efficiency, reduced immunogenicity, and improved therapeutic compatibility. Furthermore, phylogenetic insights can strengthen the understanding of evolutionary distribution of L-ASNase production across microalgal taxa and support future metabolic engineering efforts. Thus, the present study aims to isolate and assess the biodiversity of microalgal strains with L-ASNase producing potential and to establish their phylogenetic relationships using molecular characterization, thereby contributing to the development of alternative and sustainable enzyme sources for pharmaceutical applications.

Materials and Methods

Chemical

All analytical-grade chemicals and reagents used for media preparation, L- ASNase activity assay, 16s rRNA sequencing were procured from reputable suppliers, including HiMedia, Merck, and SRL (Mumbai, India).

Study area

The present study was conducted at Thamarai Kulam (Fig.1), a prominent and culturally significant freshwater water body located in Thamarai Nagar, Tiruvannamalai District, Tamil Nadu, India. The pond serves as an important local ecological hotspot and supports diverse aquatic flora and microbial communities. Due to the continuous discharge of domestic wastewater from nearby residential areas, the pond has become nutrient-rich and exhibits eutrophic conditions. The elevated organic load and nutrient availability provide a favorable environment for the growth and proliferation of diverse microalgal populations. Therefore, Thamarai Kulam was selected as the study site for the isolation, screening, and characterization of L-ASNase producing microalgae.

The geographical coordinates (latitude and longitude) of the sampling site were recorded using a Global Positioning System (GPS) device during waster sample collection.

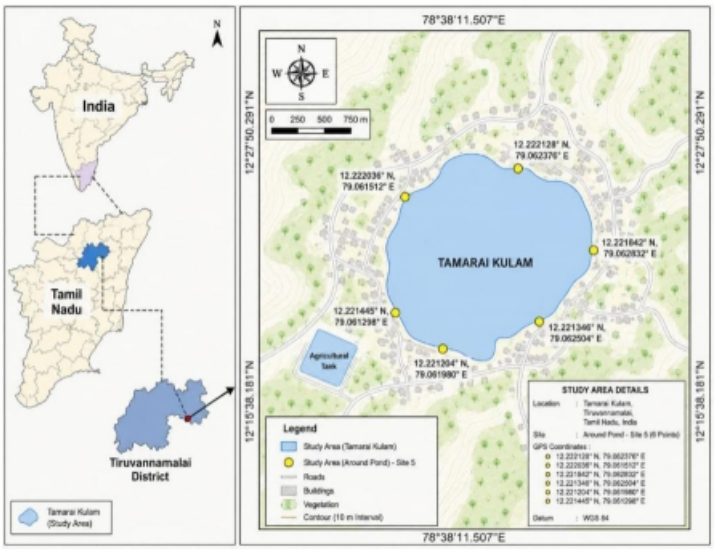

Figure 1. Location map of study area, Tamarai kulam, Tiruvannamalai district, Tamil Nadu, India

Water Sample Collection and Physicochemical Characterization

Water samples were collected from the domestic wastewater-polluted freshwater pond, Thamarai Kulam, Tiruvannamalai District, Tamil Nadu, India, for the isolation of indigenous microalgal strains. Sampling was performed using sterile polypropylene containers from different locations of the pond to obtain representative samples. The collected samples were transported to the laboratory within 2 h under aseptic conditions for immediate microalgal isolation and analysis.

On-site physicochemical parameters including pH, temperature, total dissolved solids (TDS), water transparency, and ambient light intensity, were measured at the sampling location using a portable digital pH meter, digital thermometer, TDS meter, Secchi disk, and lux meter, respectively. The pH and temperature were recorded immediately to avoid alterations due to environmental changes during transport. For laboratory-based characterization, water samples were analyzed for chemical oxygen demand (COD), total organic carbon (TOC), total nitrogen (TN), and total phosphate (TP) concentration following the Standard Methods for the Examination of Water and Wastewater [8].

Isolation and Purification of Microalgae

Microalgae (phytoplankton) were isolated from the collected freshwater samples using the serial dilution and plating technique following standard microbiological and phycological procedures for obtaining unialgal cultures [9][10]. Briefly, the collected water samples were serially diluted aseptically using sterile distilled water up to appropriate dilution levels (10^{-3}–10^{-5}) to reduce microbial density and facilitate the isolation of individual microalgal colonies. Aliquots (0.1 mL) from suitable dilutions were spread evenly onto sterile Blue Green-11 (BG-11) agar medium plates for cyanobacterial isolates and Bold's Basal Medium (BBM) agar plates for green microalgal isolates using a sterile glass spreader. The inoculated plates were incubated under controlled laboratory conditions at 25 ± 2 °C under a 12:12 h light/dark photoperiod with a light intensity of approximately 60 µmol photons m^{-2} s^{-1} until visible colonies developed.

Distinct microalgal colonies exhibiting differences in morphology, pigmentation, and growth characteristics were carefully selected and repeatedly subcultured on fresh agar plates to obtain pure unialgal cultures. Purity of the isolates was confirmed by repeated microscopic examination using a light microscope based on cellular morphology and absence of contaminating microorganisms. Each isolated microalgal strain was assigned a voucher number and preserved in the Postgraduate and Research Department of Botany, Kalaignar Karunanidhi Government Arts College, Tiruvannamalai, Tamil Nadu, India. The purified isolates were maintained in the respective microalgal culture medium under the same culture conditions for further screening and characterization. The cultures were revived after every month.

Screening for L-ASNase Production in Microalgae

L-ASNase production by microalgal isolates was evaluated using two approaches: qualitative screening and quantitative enzyme assay methods.

Qualitative method of primary screening of L-ASNase production

Purified microalgal isolates were screened for L-ASNase production using a qualitative assay based on ammonia release from L-ASNase hydrolysis, employing phenol red as a pH indicator. The isolates were cultured in appropriate growth media according to their taxonomic group, namely BG-11 medium for non-nitrogen-fixing cyanobacteria [11], nitrogen-free BG-11_0 medium for nitrogen-fixing cyanobacteria, Guillard's F/2 medium for diatoms [12], and BBM [13] for green microalgae. For screening, the respective media were supplemented with L-asparagine (10 g/L) as the substrate for enzyme production, and phenol red (0.009%, final concentration) as the pH indicator. The pH of the medium was adjusted to 6.8 before sterilization.

Actively growing purified microalgal cultures were aseptically inoculated into sterile screening tubes containing the respective supplemented media and incubated under controlled culture conditions at 25 ± 2 °C, with a 12:12 h light/dark photoperiod and a light intensity of 60 µmol photons $m^{-2}s^{-1}$ for 10 days. Phenol red remains yellow under acidic to neutral conditions but changes to pink under alkaline conditions due to ammonia liberation resulting from enzymatic hydrolysis of L-asparagine by L-ASNase producing isolates. The cultures were monitored periodically for colour change, and isolates exhibiting a distinct yellow-to-pink transition were considered positive for L-ASNase production and selected for further quantitative analysis.

Quantitative Estimation of Extracellular and Intracellular L-ASNase Activity

L-ASNase activity of the screened positive microalgal isolates was quantitatively determined by measuring the amount of ammonia released during the hydrolysis of L-asparagine using the Nesslerization method [14] [15]. Briefly, actively growing positive microalgal isolates were inoculated into their respective sterile culture medium (BG-11, BG-11_0, F/2, BBM) supplemented with L-asparagine (10 g/L) and incubated under controlled culture conditions at 25 ± 2°C, with a 12:12 h light/dark photoperiod and light intensity of 60 µmol photons $m^{-2}s^{-1}$ for 10 days.

After incubation, the cultures were centrifuged at 10,000 rpm for 10 min at 4°C to separate the culture supernatant and biomass. The clear supernatant was collected and used as the crude extracellular enzyme extract. The harvested biomass was washed twice with sterile phosphate buffer (0.05 M, pH 7.0) to remove residual medium components and resuspended in the same buffer. For the extraction of intracellular L-ASNase, the biomass was disrupted by sonication (or homogenization) under chilled conditions, followed by centrifugation at 12,000 rpm for 15 min at 4°C to remove cell debris. The resulting clear supernatant was collected as the intracellular crude enzyme extract.

Extraction of extracellular and intracellular crude L-ASNase

After 10 days of cultivation, 10 mL of the culture was centrifuged at 10,000 rpm for 10 min, and the resulting supernatant was collected for the estimation of extracellular L-ASNase activity. The biomass pellet was freeze-dried and used for intracellular enzyme extraction. Approximately 100 mg of the dried biomass was suspended in 0.8 mL of sonication buffer (50 mM Tris-HCl and 10 mM EDTA, pH 7.5) in a 1.5 mL thin-walled microcentrifuge tube. Cell lysis was performed using a probe sonicator equipped with a 2 mm microtip, operated at 50 Hz for 10 min on ice. The lysate was centrifuged at 12, 000 rpm for 20 min at 4 °C to remove cell debris, and the clarified supernatant was used to determine intracellular L-ASNase activity. Total enzyme activity was calculated by combining the extracellular and intracellular crude L-ASNase activities.

L-ASNase activity assay

L-ASNase activity was quantified by measuring the ammonia released from the hydrolysis of L-asparagine a UV-Visible spectrophotometer [16]. The reaction mixture consisted of 900 µL of 0.05 M L-asparagine prepared in 0.05 M Tris-HCl buffer (pH 7.5) and 100 µL of appropriately diluted cell-free extract. The mixture was incubated at 37 °C for 10 min, after which the reaction was terminated by adding 500 µL of 15% trichloroacetic acid (TCA). The mixture was maintained at room temperature for an additional 10 min and centrifuged at 10,000 rpm for 10 min. A 150 µL aliquot of the supernatant was transferred to a fresh tube and diluted with 1.4 mL of distilled water, followed by the addition of 200 µL of Nessler's reagent. After 10 min, the absorbance of the resulting color was measured at 480 nm using a UV-Visible spectrophotometer. Control reactions were performed under identical conditions, except that TCA was added before the enzyme sample to inhibit the reaction. One unit of L-ASNase activity was defined as the amount of enzyme required to liberate 1 µmol of ammonia per minute under the assay conditions (Equation 1). Ammonium chloride was used to generate the standard calibration curve.

$$\text{Enzyme activity (IU/mL)} = \left(\frac{\text{Absorbance}_{480} \times \text{Total reaction volume}}{(\varepsilon \times l \times t \times \text{Sample volume}}\right) \text{------(Eq. 1)}$$

where, ε is the molar extinction coefficient of ammonia (12,300 $M^{-1}cm^{-1}$), *l* is the path length (1 cm), and t is incubation time (min).

Morphological Identification of L-ASNase -Producing Microalgae

The microalgal isolate exhibiting the highest L-ASNase production was selected for taxonomic identification based on morphological and microscopic characteristics using standard algal identification keys and monographs.

Actively growing cultures were harvested during the exponential growth phase, and a small aliquot of the culture was placed on a clean glass slide, covered with a coverslip, and observed under a light microscope (Olympus CX21i-LED, Binocular Version) at different magnifications (400× and 1000×) for detailed morphological examination. The observed morphological characteristics were examined and compared with established taxonomic descriptions and standard cyanobacterial monographs for preliminary identification up to the genus and species level [17][18][19]. The morphological identification of the selected high L-ASNase producing microalgal isolate was subsequently confirmed through molecular characterization and phylogenetic analysis.

Phylogenetic Identification of the selected L-ASNase Producing Isolate

Genomic identification of the selected high L-ASNase producing cyanobacterial isolate was carried out through molecular characterization and phylogenetic analysis based on 16S rRNA gene sequencing. Actively growing isolate biomass was harvested by centrifugation at 10,000 rpm for 10 min. The collected biomass was washed thoroughly with sterile distilled water or phosphate-buffered saline to remove residual culture medium.

Genotypic characterization

Genomic DNA was extracted from the selected high L-ASNase producing isolate using a modified method involving soft mechanical lysis followed by enzymatic disruption of the cell wall [20]. Briefly, actively growing cyanobacterial biomass was harvested by centrifugation and washed to remove residual culture medium. The collected cells were resuspended in 0.5 mL of 0.15 M NaCl containing 0.1 M EDTA and transferred into sterile cryogenic vials. To facilitate disruption of the robust filamentous cyanobacterial cell wall, the cell suspension was subjected to three freeze–thaw cycles, alternating between immersion in liquid nitrogen and thawing in a 37°C water bath.

Following physical disruption, the cells were recovered by centrifugation at 8,000 rpm for 10 min, resuspended in 0.5 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and transferred to fresh sterile microcentrifuge tubes. Enzymatic cell wall lysis was performed by adding 100 µL lysozyme (50 mg/mL) and incubating the mixture at 37°C for 30 min. Subsequently, protein degradation was carried out by adding 5 µL proteinase K (50 mg/mL) along with sodium dodecyl sulfate (SDS) to a final concentration of 2%, followed by incubation at 37°C for 1 h.

To remove polysaccharides, proteins, and cell wall debris, selective precipitation was performed using the CTAB–NaCl method. Briefly, 150 µL of 5 M NaCl was added to the lysate, followed by 0.1 volume of 10% CTAB solution, and the mixture was gently inverted and incubated at 65°C for 10 min. Nucleic acids were then purified by extraction with an equal volume of chloroform: isoamyl alcohol (24:1, v/v). The samples were incubated on ice for 30 min to facilitate precipitation of CTAB complexes and centrifuged at 8,000 rpm for 10 min. The resulting aqueous supernatant was carefully transferred to a fresh tube.

Genomic DNA was precipitated by adding 0.6 volume of ice-cold isopropanol and gently mixing until visible DNA precipitation occurred. The DNA pellet was recovered by centrifugation at 15,000 rpm for 10 min at 4°C, washed with 1 mL of chilled 70% ethanol, and centrifuged again at 15,000 rpm for 5 min at 4°C to remove residual salts.

The ethanol was discarded, and the DNA pellet was air-dried and resuspended in 100 µL TE buffer. To eliminate RNA contamination, the DNA preparation was treated with 1 µL RNase (10 mg/mL) and incubated at 37°C for 1 h. The purified genomic DNA was stored at −20°C for subsequent PCR amplification and phylogenetic analysis.

DNA Quantification and Purity Analysis

The concentration and purity of the extracted genomic DNA were determined by spectrophotometric analysis [20]. An aliquot of 5µL of the extracted DNA sample was diluted 10-fold with TE buffer to ensure that the absorbance readings fell within the linear range of the instrument. The spectrophotometer was first calibrated using TE buffer as a blank. The absorbance of the diluted DNA sample was then measured at 260 nm, 280 nm, and 230 nm wavelengths against the blank. The concentration of genomic DNA was estimated based on absorbance at 260 nm, while DNA purity was assessed by calculating the A260/A280 and A260/A230 absorbance ratios. An A260/A280 ratio greater than 1.8 and an A260/A230 ratio above 2.0 were considered indicative of high-quality, contaminant-free genomic DNA suitable for downstream molecular applications [21]. Lower A260/A280 values indicated possible contamination with proteins or phenolic compounds, whereas reduced A260/A230 values suggested the presence of residual organic compounds, carbohydrates, salts, or other extraction contaminants. The DNA concentration (µg/mL) was calculated using the formula:

$$\text{DNA concentration } (\mu g/mL) = A_{260} \times 50 \times \text{dilution factor}$$

where 50 µg/mL corresponds to an absorbance of 1.0 for double-stranded DNA.

PCR amplification and sequencing

PCR amplification of the partial 16S rRNA genes was performed using the following primer set: 8F (5′- AGA GTT TGA TCC TGG CTC AG -3′) and 492R (5′- GGT TAC CTT GTT ACG ACT T-3′). The PCR reaction was performed in a 20 µL reaction containing approximately 20-50 ng of isolated DNA, 0.25 µM from each forward and reverse primers, and 4 µL of 5 x HOT FIREPol Blend Master Mix following the manufacturer's instructions (Solis BioDyne, Estonia). The thermal cycles started with an initial denaturation step at 94°C for 4 min, followed by 40 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 2 min, and a final extension step at 72 °C for 7 min. The PCR amplicon was purified using PCR purification kit- Roche according to the manufacture's protocol and subsequently sent to a commercial sequencing facility for Sanger dideoxy DNA sequencing [22]. The finalized sequence was deposited in NCBI GenBank.

Phylogenetic analysis of sequences

The phylogenetic relationship of the selected L-ASNase producing cyanobacterial isolate was determined by comparing the obtained 16S rRNA gene sequence with closely related reference sequences retrieved from the NCBI GenBank database using the BLAST algorithm. Sequences showing ≥95% similarity for genus-level identification and ≥99% similarity for species-level identification were selected for comparative analysis. Multiple sequence alignment was performed using the integrated alignment tool in MEGA version X (Molecular Evolutionary Genetics Analysis) software, and the phylogenetic tree was constructed using the Neighbour-Joining (NJ) method with appropriate bootstrap analysis to assess branch reliability [23].

Screening of Different Culture Medium for L-ASNase Production by selected microalga

To evaluate the influence of different culture medium on L-ASNase production, the selected high-producing isolate was cultivated in three different media, namely BG-11 medium [11], modified Jourdan medium (MJM), standard Zarrouk Medium [24] and SAG medium [25]. The chemical composition of the respective media is presented in Table 1. Freshly grown inoculum of isolate was prepared under standard cultivation conditions, and an inoculum volume (10% v/v) was transferred aseptically into 250 mL Erlenmeyer flasks containing 100 mL of each sterile culture medium supplemented with L-asparagine (10 g/L) as the substrate for induction of L-ASNase production.

The inoculated flasks were incubated under controlled culture conditions at 25 ± 2°C, with a 12:12 h light/dark photoperiod, light intensity of 60 µmol photons m^{-2} s^{-1}, and orbital shaking at 120 rpm for 10 days. At the end of the incubation period, cultures were harvested by centrifugation at 10,000 rpm for 10 min at 4°C to separate the biomass and culture supernatant. The supernatant was used for extracellular L-ASNase estimation, while the harvested biomass was washed with sterile phosphate buffer and disrupted by sonication for the extraction of intracellular enzyme. L-ASNase activity in both extracellular and intracellular extracts was quantified by Spectrophotometrically [16]. The medium that supported the highest enzyme production was selected as the optimal culture medium for further studies.

Table 1: Chemical composition of BG-11, MJM and SAG medium

Components	BG-11 Medium (g/L)	MJM (g/L)	SAG Medium (g/L)	SZM (g/L)
$NaHCO_3$	–	5.0	16.8	16.8
KOH	–	1.6	–	-
$NaNO_3$	1.5	5.0	2.5	2.5
K_2HPO_4	0.04	2.0	0.5	0.5
K_2SO_4	–	0.4	1.0	1.0
NaCl	–	1.0	1.0	1.0
$MgSO_4.7H_2O$	0.075	0.4	0.2	0.2
$CaCl_2.2H_2O$	0.036	0.027	0.04	0.04
Citric acid	0.006	–	–	
Ferric ammonium citrate	0.006	–	–	
Ferric citrate	–	–	0.01	
$FeSO_4.7H_2O$	–	0.01	–	0.01
EDTA	0.001	0.16	0.08	0.08
Na_2CO_3	0.02	–	–	4.03
Trace element solution (1 mL/L stock solution)	**g/L**	**mg/L**	**g/L**	**g/L**
EDTA-Na_2	–	250	–	
H_3BO_3	2.86	57	2.86	2.86
$MnCl_2.4H_2O$	1.81	25.3	1.81	1.81
$ZnSO_4.7H_2O$	0.222	110	0.22	0.222
$CoCl_2.6H_2O$	–	8.05	–	-
$CuSO_4.5H_2O$	0.079	7.85	0.08	0.079
$(NH_4)6Mo_7O_{24}.4H_2O$	–	5.5	–	-
$Na_2MoO_4.2H_2O$	0.390	–	0.02	0.0177
Distilled watse	1000 mL	1000 mL	1000 mL	1000 mL
Final pH	7.0	9.5	9.5	9.0

Statistical Analysis

All experiments were performed in triplicate, and the data are presented as mean ± standard deviation (SD). Statistical significance was assessed using one-way analysis of variance (ANOVA) at a confidence level of $p < 0.05$. All analyses were carried out using MINITAB software (version 15).

Results and Discussion

Physicochemical Characteristics

The physicochemical characteristics of water samples collected from different locations of Thamarai Kulam Pond exhibited only minor variations (Table 2), indicating a relatively homogeneous freshwater ecosystem suitable for the growth and proliferation of microalgae. The sampling sites were located at 12.22° N latitude and 79.06° E longitude, confirming that all samples originated from the same aquatic habitat. Water temperature ranged from 27 to 28°C, while pH values varied between 7.9 and 8.1. These conditions are considered favorable for freshwater microalgal growth, as most microalgae exhibit optimum growth at temperatures between 20 and 30 °C and under neutral to slightly alkaline conditions (pH 7.0–8.5). Such environmental conditions promote photosynthetic efficiency, nutrient uptake, cellular metabolism, and pigment synthesis [26][27][28]. Water transparency ranged from 0.4 to 0.5 m, indicating moderate turbidity, while the recorded light intensity was approximately 2300 lux at all sampling sites. Adequate light availability is essential for photosynthesis, chlorophyll production, and biomass accumulation, and variations in light intensity have been reported to significantly influence microalgal growth and productivity [29].

The TDS content ranged from 574 to 576 mg/L, suggesting the presence of sufficient dissolved minerals and nutrients necessary for microalgal growth. The COD values ranged from 7.88 to 7.91 mg/L, while TOC levels varied between 13.79 and 13.88 mg/L, indicating moderate organic matter availability in the pond ecosystem. These values collectively suggest a nutrient-rich environment capable of supporting diverse microalgal communities. The total nitrogen concentration ranged from 2.74 to 2.76 mg/L, whereas total phosphate concentrations were comparatively high, ranging from 9.73 to 9.77 mg/L. Nitrogen and phosphorus are essential macronutrients that regulate microalgal growth, biomass production, photosynthetic activity, and cellular metabolism. Elevated concentrations of these nutrients often stimulate phytoplankton development and contribute to increased microalgal diversity and productivity. Previous studies have demonstrated that nutrient availability, particularly nitrogen and phosphorus, strongly influences microalgal biomass yield and community composition in freshwater ecosystems [30].

Table 2: Geographical position, physicochemical parameters of water samples collection sites of Thamarai Kulam Pond for isolation of microalgae

Parameters	Water samples collection site				
	S1	S2	S3	S4	S5
Longitude	79.06˚ E	79.06˚ E	79.06˚ E	79.06˚ E	79.06˚ E
Latitude	12.22˚ N	12.22˚ N	12.22˚ N	12.22˚ N	12.22˚ N
Temperature (°C)	27±0.03	28±0.04	28±0.03	27±0.05	27±0.04
pH	7.9±0.03	8.1±0.05	8.0±0.04	8.1±0.07	7.9±0.05
Water transparency (m)	0.4±0.07	0.5±0.04	0.4±0.05	0.5±0.05	0.4±0.07
Light Intensity (Lux)	2300±0.05	2300±0.08	2300±0.08	2300±0.07	2300±0.06
TDS (mg/L)	575±0.04	574±0.05	574±0.04	576±0.06	574±0.08
COD (mg/L)	7.88±0.05	7.90±0.06	7.89±0.05	7.91±0.04	7.89±0.04
TOC (mg/L)	13.87±0.04	13.86±0.04	13.88±0.05	13.86±0.05	13.79±0.07
Total nitrogen (mg/L)	2.75±0.05	2.74±0.07	2.76±0.06	2.760.06±	2.75±0.06
Total phosphate (mg/L)	9.76±0.03	9.73±0.04	9.75±0.04	9.77±0.05	9.75±0.04

Notably, no previous scientific reports are available regarding the physicochemical characteristics of Thamarai Kulam Pond, Tiruvannamalai District. Therefore, the present investigation represents the first documented assessment of the water quality parameters of this freshwater ecosystem.

The relatively high phosphate concentration observed in the pond may be attributed to anthropogenic influences such as urban runoff, domestic sewage intrusion, and nutrient-rich surface drainage entering the water body. Such nutrient enrichment can enhance primary productivity and support the proliferation of indigenous microalgal populations. Consequently, the favorable physicochemical conditions recorded in Thamarai Kulam Pond likely contributed to the establishment of diverse microalgal communities and facilitated the successful isolation of potential L-ASNase producing strains in the present study.

Screening of Isolated Microalgae for L-ASNase Production

A total of nine microalgal strain were isolated from Thamarai Kulam Pond and designated as FMAS1 to FMAS9. All the isolated were screened for L-ASNase production using a qualitative assay. As shown in the Table 3, among the isolates, only FMAS2 exhibited a positive response, producing a distinct color change due to ammonia release during L-asparagine hydrolysis, while all other isolates (FMAS1 and FMAS3–FMAS9) were negative. Following qualitative screening, the positive isolate FMAS2 was subjected to quantitative enzyme assay, which revealed an L-ASNase activity of 75.54 ± 0.07 IU/mL. This result confirmed the strong enzyme-producing potential of the isolate FMAS2. The occurrence of enzyme activity in only one isolate indicates that L-ASNase production is highly strain-dependent among freshwater microalgae and may be influenced by genetic and physiological factors [31]. The relatively high enzyme activity observed in FMAS2 suggests efficient L-asparagine metabolism and highlights its potential as a promising source of therapeutic L-ASNase. The favorable physicochemical conditions of Thamarai Kulam Pond, including suitable nutrient availability, pH, and temperature, may have contributed to the development of metabolically active microalgal populations capable of enzyme production [30]. These findings demonstrate the potential of freshwater microalgae as alternative sources of pharmaceutically important enzymes and identify FMAS2 as a promising candidate for further molecular characterization, optimization, and biotechnological applications [32] [33].

Table 3: Screening isolated microalgae for L-ASNase production

Voucher number (Culture Code)	Qualitative screening
FMAS1	Negative
FMAS2	Positive
FMAS3	Negative
FMAS4	Negative
FMAS5	Negative
FMAS6	Negative
FMAS7	Negative
FMAS8	Negative
FMAS9	Negative

Morphological Identification of L-ASNase -Producing Isolate FMAS2

The isolate FMAS2 was initially identified based on its morphological characteristics using light microscopy. Microscopic examination revealed that the isolate consisted of long, unbranched, multicellular filaments arranged in a helical or spiral configuration.

The trichomes were cylindrical, regularly coiled, and composed of closely packed vegetative cells without true branching. The filaments exhibited a distinct blue-green coloration characteristic of cyanobacteria. Similar morphological features have been widely reported for members of the genus *Limnospira* [18][19]. The trichomes of FMAS2 appeared flexible and uniformly coiled with rounded terminal cells. No heterocysts, akinetes, or specialized reproductive structures were observed during microscopic examination. The absence of heterocysts is a distinguishing feature of the genus *Limnospira* and differentiates it from several nitrogen-fixing filamentous cyanobacteria [34]. The observed morphology was consistent with previous descriptions of *Limnospira maxima*, which is characterized by open left-handed helices, cylindrical cells, and non-heterocystous filamentous trichomes [35]. Hence, the isolate FMAS2 was morphologically identified as *Limnospira maxima.*

Morphological identification remains an important preliminary tool for cyanobacterial taxonomy because features such as trichome shape, helix diameter, cell dimensions, terminal cell morphology, and pigmentation provide valuable taxonomic information. However, environmental factors may influence morphological traits, resulting in phenotypic variability among strains. Therefore, morphological observations are generally combined with molecular analyses for accurate species identification [34][36].

Estimation of DNA Purity and Concentration of Isolate FMAS2

The quality and concentration of genomic DNA extracted from the isolate FMAS2 were evaluated using UV-Visible spectrophotometric analysis. The absorbance values recorded at 260 nm, 280 nm, and 230 nm were 0.301 ± 0.06, 0.139 ± 0.04, and 0.134 ± 0.07, respectively (Table 4). Spectrophotometric assessment is a widely accepted method for determining DNA purity and concentration because nucleic acids exhibit maximum absorbance at 260 nm, whereas proteins and phenolic compounds absorb predominantly at 280 nm and 230 nm, respectively [37]. The extracted DNA exhibited an A260/A280 ratio of 2.165, indicating minimal protein contamination. Generally, pure DNA preparations exhibit A260/A280 ratios ranging from 1.8 to 2.0, while values slightly above this range may occur in cyanobacterial and microalgal DNA preparations due to the presence of residual RNA or other nucleic acid components [38]. The observed ratio therefore suggests that the DNA extracted from FMAS2 was of sufficient quality for PCR amplification and sequencing.

The DNA concentration of FMAS2 was determined to be 150.5 µg/mL, indicating a high yield of genomic DNA. High DNA concentration is essential for molecular characterization because it ensures sufficient template availability for PCR amplification, sequencing, and phylogenetic analyses. Previous studies have reported that DNA concentrations above 50–100 µg/mL are generally adequate for successful amplification of cyanobacterial 16S rRNA genes and other molecular markers [35] [36].

Table 4: Estimation of purity and DNA concentration of isolate FMAS2

A_{260}	A_{280}	A_{230}	A_{260}/A_{280}	A_{260}/A_{230}	DNA Concentration (µg/mL)	Remarks
0.301±0.06	0.139±0.04	0.134±0.07	2.165	2.246	150.5	High purity DNA

Molecular Identification and Phylogenetic Analysis of Isolate FMAS2

The isolate FMAS2 was subjected to molecular identification through 16S rRNA gene sequencing. The amplified 16S rRNA gene sequence (1337 bp) was purified, sequenced, and submitted to the NCBI GenBank database, where it was assigned the accession number PX525663.1 (Figure 2). The obtained nucleotide sequence was analyzed using the BLAST available at the NCBI GenBank database, which revealed a high degree of similarity with members of the genus *Limnospira*. Based on sequence homology and phylogenetic analysis, the isolate was identified as *Limnospira maxima* FMAS2. Molecular characterization using 16S rRNA gene sequences is widely regarded as a reliable approach for cyanobacterial identification and taxonomic classification because it provides high phylogenetic resolution among closely related taxa [34] [36].

The phylogenetic tree (Figure 3) was constructed using the Neighbor-Joining method with representative *Limnospira* and *Arthrospira* sequences retrieved from GenBank. The resulting topology demonstrated that FMAS2 clustered within the *Limnospira maxima* clade and showed a close evolutionary relationship with *L. maxima* SISCA (OR195505), supported by a bootstrap value of 100%. High bootstrap values indicate strong statistical support for phylogenetic relationships and reflect a close genetic association among the analyzed taxa [39]. Furthermore, FMAS2 grouped with other *L. maxima* strains, including *L. maxima* BJ 2000 (GQ206141), forming a distinct lineage supported by a bootstrap value of 98%. The clustering pattern strongly confirms that FMAS2 belongs to the species *L. maxima* and shares a recent common ancestor with other members of this species. Similar phylogenetic relationships among *Limnospira* strains have been reported through both 16S rRNA gene sequence analysis and whole-genome studies [19][35].

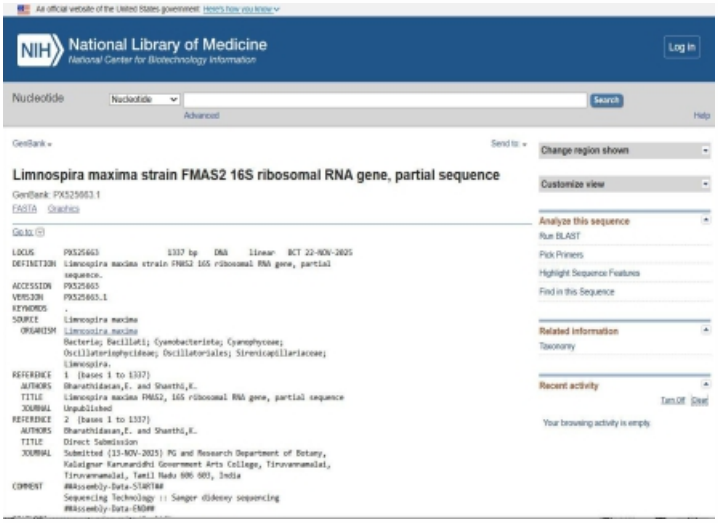

Figure. 2: Amplified 16S rRNA gene (1337 bp) sequence submitted to the NCBI GenBank database

The close phylogenetic relationship of FMAS2 with authenticated *L. maxima* strains suggests that the isolate possesses genetic characteristics typical of this species. Members of the genus *Limnospira* are well known for producing a diverse array of bioactive compounds, including proteins, phycobiliproteins, vitamins, antioxidants, polysaccharides, and several industrially important enzymes [40][41]. The molecular identification of FMAS2 as *L. maxima* therefore, provides strong evidence supporting its potential as a promising source of L-ASNase.

This molecular confirmation establishes a robust taxonomic foundation for subsequent investigations on optimization of enzyme production, purification, biochemical characterization, and evaluation of therapeutic applications.

Screening of Suitable Culture Medium for L-ASNase Production by *L. maxima* FMAS2

The effect of different culture media on L-ASNase production by *Limnospira maxima* FMAS2 was evaluated using four commonly employed cyanobacterial growth media, namely BG-11, MJM, SZM, and SAG medium (Table 5). Significant variations in enzyme production were observed among the tested media, indicating that medium composition plays a crucial role in regulating enzyme biosynthesis. Among the media evaluated, SAG medium supported the highest L-ASNase production (89.43 ± 0.08 IU/mL), followed by SZM (85.43 ± 0.06 IU/mL), MJM (83.23 ± 0.05 IU/mL), and BG-11 medium (78.54 ± 0.07 IU/mL). The results demonstrate that the nutritional composition of SAG medium was more favorable for enzyme production by *L. maxima* FMAS2 than the other tested media.

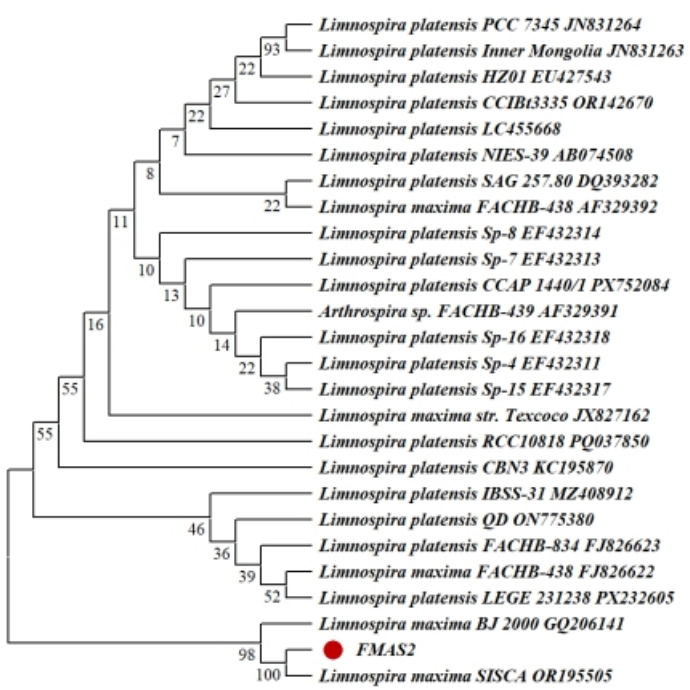

Figure 3: Phylogenetic tree for L-ASNase producing isolate L. maxima FMAS2

Table 5. Screening the medium suitability for L-ASNase producing isolate L. maxima FMAS2

Medium	L-ASNase (IU/mL)
BG-11	78.54±0.07
MJM	83.23±0.05
SZM	85.43±0.06
SAG	89.43±0.08

The comparatively lower enzyme production observed in BG-11 medium may be attributed to its relatively simple nutrient composition. Although BG-11 is widely used for the cultivation of freshwater cyanobacteria, its nutrient balance may not adequately support enhanced synthesis of extracellular enzymes. The availability of nitrogen, phosphorus, trace elements, and carbon sources strongly influences cellular metabolism and enzyme production in cyanobacteria [42]. The highest enzyme activity recorded in SAG medium (89.43 ± 0.08 IU/mL) indicates that this medium provided optimal nutritional conditions for L-ASNase biosynthesis by FMAS2.

The enhanced enzyme production may be associated with a balanced supply of macro- and micronutrients that support efficient nitrogen assimilation and amino acid metabolism. Since L-ASNase production is closely linked to nitrogen metabolism, the availability of suitable nitrogen sources and trace elements in SAG medium likely stimulated enzyme synthesis [31][43].

The increase in enzyme activity from 78.54 IU/mL in BG-11 medium to 89.43 IU/mL in SAG medium represents approximately a 13.9% enhancement in L-ASNase production, demonstrating the importance of medium selection for maximizing enzyme yield. Similar findings have been reported in cyanobacterial and microalgal studies where optimization of culture media significantly improved the production of enzymes, pigments, and other bioactive metabolites [42][44]. The results suggest that SAG medium provides the most suitable nutritional environment for the growth and L-ASNase production of *L. maxima* FMAS2.

Conclusion

The present study demonstrated that Thamarai Kulam Pond provides favorable physicochemical conditions for the growth and diversity of freshwater microalgae, leading to the isolation of nine distinct microalgal strains. Among them, FMAS2 was identified as the only L-ASNase producing isolate, exhibiting an enzyme activity of 75.54 ± 0.07 IU/mL. Morphological, molecular, and phylogenetic analyses confirmed the isolate as *L. maxima* FMAS2, while spectrophotometric analysis revealed high-quality genomic DNA suitable for molecular characterization. Evaluation of different culture medium showed that SAG medium supported the highest L-ASNase production (89.43 ± 0.08 IU/mL). These findings highlight the rich microalgal biodiversity of freshwater ecosystems and establish *L. maxima* FMAS2 as a promising source of L-ASNase with potential pharmaceutical and biotechnological applications.

Authors' contributions

Ekambaram Bharathidasan: Methodology, Validation, Formal Analysis, Investigation, Data Curation, Writing – Original Draft, Visualization.

Kanagasabai Shanthi: Data Curation, Conceptualization, Formal Analysis, Investigation, Writing – Review & Editing, Project Administration, Supervision.

Conflict of interest

The authors declare that no conflicts of interest.

Ethical approval

Not applicable

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