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Original Article

STANDARDIZATION AND APPLICATION OF PCR TARGETING CHLORELLA SPECIES ISOLATED FROM ENVIRONMENTAL SAMPLES

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ABSTRACT

Objective: Identification of Chlorella species from the environment through 18s ribosomal RNA sequencing. This study was aimed to design primer targeting Chlorella and other closely related algal species targeting 18s ribosomal RNA, ITS1 region.

Methods: Sanger sequencing was carried out for the identification of algae up to the genus and species level using an in-house designed primer and optimized PCR conditions.

Results: Out of 2 algae samples identified phenotypically, one isolate identified as *Chlorella vulgaris* and other one identified as *Chlorella sorokiniana* based on the results of Basic Alignment Search Tool (BLAST).

Conclusion: To conclude, this study provided primers with PCR conditions to characterize algal samples through molecular identification with 100% accuracy than the phenotypic method.

Keywords: *Chlorella* species, Microalgae, PCR, Primer designing, Sanger sequencing, Genotyping, Environmental samples

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INTRODUCTION

Microalgae or microphytes are a ubiquitous group of fast-growing unicellular microscopic microphototrophs. Microalgae belong to phytoplankton can be isolated from freshwater and marine systems and they can survive in both the water column and sediment [1-3]. Pharmaceutically valuable products from microalgae and its industrial commercialization today is still in its infancy and can be seen as a gateway to a multibillion-dollar industry. They represent a major untapped resource of genetic potential for valuable bioactive agents and fine biochemical [4]. Compounds from microalgal extracts have been accepted as having greater biological and economic importance than dried biomass. In the sense of global population growth and availability of terrestrial food items, microalgae may provide sustainable and reliable replacements for widely used commodities of animal or plant origin. To date, however, only a limited number of strains have been utilised in nutritional and pharmaceutical purposes. Major research and development would entail the transition from a niche market to the widespread use of algal products as food commodities. In turn, this will include improving existing strains through genetic engineering, and modifications or screening new species to the growth of microalgae with increased targeted metabolite production [5]. In the environmental samples, they were found to be either as an individual or in chains or groups. Depending on the species, their sizes can range from a few micrometers (μm) to a few hundred micrometers. Environmentally, microalgae cultivation is considered a promising solution that mitigates global warming via sequestering the primary atmospheric greenhouse gas, CO_2, by photosynthesis [1-3, 6, 7]. Screening of local microalgae species with high nutritional value and potential for oil production is essential to achieve successful commercial large-scale cultures. The species of the genus Chlorella are considered cryptic species that are morphologically similar but genetically distinct [8-10]. *Chlorella vulgaris* (ChV) is a unicellular microalga which contains a wide variety of antioxidant compounds including beta-carotene, chlorophyll, alfa-tocopherol, ascorbic acid, lycopene, lutein, zeaxanthin, Vitamin C, and Vitamin E [11]. The lack of obvious morphological taxonomic characteristics in addition to an exclusively asexual reproductive cycle through autospores makes it difficult to differentiate between the species of the genus Chlorella Beijerinck depending on the traditional taxonomy [10]. Sequences for the 18S rRNA gene were used to identify several microalgal species and in particular to differentiate species of Chlorella [12, 13]. In recent years, microalgae achieve high potential as a feedstock for biofuel production due to their several advantages such as higher biomass productivity, lesser water demand, and no agricultural land requirement compared to other energy crops [14-18].

The current study aimed to isolate, identify through phenotypic method and characterization through molecular method using unique primers designed by our group and standardization of the in-house primer targeting 18s ribosomal RNA for the identification of microalgae at the genus and species level.

MATERIALS AND METHODS

Sample collection and isolation of *Chlorella*

Water sample collected from Madras Christian College, Chennai in a sterile conical flask and cultured in Bold Basal medium. The growth of algae was maintained at 28 °C with 4000 lux light intensity for 30 d. Colonies were selected based on color differences and transferred to fresh agar plates. Microscopic observations at 100X magnification with oil immersion (Olympus Microscope) and serial dilution were made until a unialgal culture was obtained. After 30 d of growth, biomass was harvested. Post-harvest, 100 mg of wet cell biomass was washed with 100 mmolTris buffer (pH 7.5) and stored at-20 °C [16-18].

Genomic DNA extraction

Frozen cells were thawed and genomic DNA extraction was performed using HiPurA™ Plant Genomic DNA Miniprep Purification kit (MB507-50PR) as per the manufacturer instruction, in brief, ground material, immediately add 400 μl of Lysis Buffer (PL) (DS0016) containing Additive-I (DS0054) (preheated to 95 °C) and mix thoroughly and transferred to Hishredder, contaminants such as cell debris, salt precipitates are removed by centrifugation through a

HiShredder and DNA isolated using HiElute Miniprep Spin Column (Capped) is based on the advanced silica binding principle presented in a microspin format and the DNA will be eluted finally using 200 µl Elution buffer.

Primer designing–In-house

To identify the Chlorella species, we downloaded all the Chlorella 18s ribosomal RNA, ITS1, 5.8S rRNA gene, ITS2, and 28S rRNA gene from NCBI databases. Multalin was performed for the downloaded set of sequences and identified the conserved region among the sequences. Further, the consensus sequence was uploaded to the Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/) to obtain the desired set of primers and to determine the amplicon size for the gene of interest with optimum annealing temperature for three targets. The most appropriate pair of primers was selected using the NCBI BLAST tool (http://blast.ncbi.nlm.nih.gov/) using megablast and primer blast. Non-primer dimer formation was analyzed with a blastn tool. Based on a sequence alignment, chosen the primer length as 20 bp optimal and annealing temperature as an average of 60oc, selected the GC content above 55% for the optimal binding efficiency targeting 18s ribosomal RNA region to amplify internal fragments with the size of 410bp (table 1) [19, 20].

Table 1: In-house designed primer used for the amplification and sequencing of blue-green algae targeting 18s ribosomal RNA and ITs 1 region

Primer name	Primer Seq 5'----3'	Annealing temp	Melting temp
Algae_NMN_SP_F	TACGTGCGTAAATCCCGACT	60	56
Algae_NMN_SP_R	ACCCGAAATCCAACTACGAG	60	56

PCR standardization

We used the Genomic DNA of known Chlorella species identified earlier microscopically. Primers were procured from Eurofins, India with 100 mmol concentration (table 2). Lyophilized primers were reconstituted using sterile 1x TE buffer to maintain the stability of the primer. The same was diluted further to obtain a 0.5 nM working concentration. 15 µl 2X Takara PCR premix (R004A) PCR Buffer containing Mgcl2, dNTPs, and Taq Polymerase were used for the PCR amplification. 5 µl of DNA added into the master mix and performed the Amplification as per the below conditions. Amplified products were analyzed by electrophoresis in a 2% agarose gel at 100 V for 1 h in 1X TAE [40 mmol Tris–HCl (pH 8.3), 2 mmol acetate and 1 mmol EDTA] containing 0.05 mg/l Ethidium bromide [19, 20].

Table 2: Thermal profile standardized for the amplification of 18s ribosomal RNA PCR

Conditions	Temperature (C)	Duration	Step	Cycle
Initial Denaturation	94	7 min	1	1
Denaturation	94	45 sec	2	40
Annealing	60	30 sec		
Extension	72	45 sec		
Final Extension	72	5 min	3	1
Hold	4	Infinity	4	1

DNA sequencing

Sanger sequencing was performed as per standard protocol [20]. Obtained results were analyzed after primary trimming of sequence with low-quality scores and subjected to blast in order to confirm the genotype of the targeted gene.

RESULTS

We isolated 15 blue-green algae colonies based on microscopical and cultural morphological studies. *Chlorella* species identified were observed macroscopically and the image is shown. Isolation of DNA was successful and found to be an average of 11ng/µl was estimated using Nanodrop (Spectrophotometer-Thermo Scientific). Primers were standardized for annealing temperature using Gradient PCR, where the optimal temperature was found to be 60 °C was fixed for further studies. Denaturation and Extension temperature was optimized as per the PCR Master mix instruction.

Two isolates of Blue-green algae were identified macroscopically, and both of them were amplified for the target 18s ribosomal RNA PCR. Confirmation of PCR standardization carried out using known Blue-green algae DNA used as Positive control, and no template control (NTC) was used to confirm the false-positive amplification and quality control of the reaction mixture. Both positive control and two test samples NMNSP1 and NMNSP2 were amplified and no amplification was found in the NTC sample (fig. 1).

DNA sequencing

Sanger sequencing was carried out using the reverse primer processed for PCR amplification of two Samples, which showed amplification, and the generated ABI file was analyzed using Bioedit software, all the chromatogram peaks were checked for the quality control (fig. 2) and the best regions were downloaded as FASTA format and the same were analyzed using NCBI Blast tool. Blast results showed the given sequence was 100% homology to *Chlorella* species (fig. 3) and above 30 targets were matching with *Chlorella vulgaris* for sample NMN_SP and *Chlorella sorokiniana* for the sample NMN_SP2, hence it was submitted to NCBI Genbank and approved by the NCBI team and released the data under NCBI Accession ID: MW847613; and MW858375 respectively.

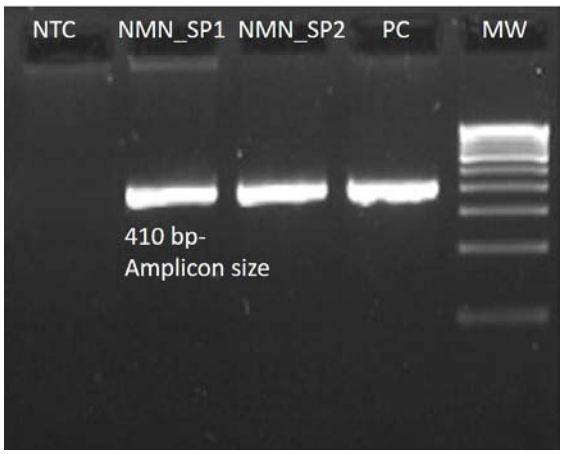

Fig. 1: Agarose gel electropherogram showing positive amplification for two isolates obtained from environmental samples labelled as NMN_SP1 and NMN_SP2. Know Algal DNA used as positive control showed amplification and NTC (Master mix+Nuclease free water as sample) showed no amplification

Fig. 2: ABI file-chromotogram of *Chlorella vulgaris*_NMN_SP

Fig. 3: NCBI blast results shows 100% homology with *Chlorella vulgaris*

DISCUSSION

The microscopic observations of this work are in agreement with those recorded for the genus *Chlorella* Beijerinck. Though Phenotypically both NMNSP1 and NMNSP2 identified as Chlorella species. But the species level identification was not accurate through the phenotypic method. Hence this study has proven the need for molecular identification of algal identification though the molecular method provides accurate identification than the phenotypic method. Basic Local Alignment Search Tool of the National Center for Biotechnology Information (NCBI BLAST) of *Chlorella* sp. NMN_SP sequence has shown close relationships (100% identity and 98% query cover) between highly dissimilar morphologies such as *Chlorella sorokiniana* NKH6 gene (LC505542.1) and *Micractinium* sp. KSF0094 (MN414469.1) demonstrating that the evolution of vegetative morphology can be rapid. Further, NMNSP2 showed 100 % homology to *Chlorella sorokiniana*, and the same has been published under NCBI database. The growth of *Chlorella vulgaris* NMN_SP1 and *Chlorella sorokiniana* NMN_SP2 reached an exponential phase at day 20, almost similar to the duration period of *Chlorella marina* reported by Muthukumar *et al.* [21]. The resulted generation time (g), the mean time required to double the population, was 2.54 d. Consequently, the growth rate constant (K), the number of generations per unit time, was at 0.393 generations/day; and the maximum biomass (M) was 135.65 × 10^6 cells/ml. This agrees to some extent with the results reported by Rosenberg *et al.* [22] for different *Chlorella* spp. The maximum biomass production is similar to that recorded for *Chlorella vulgaris* by Montoya *et al.* [23] using nitrogen-limited Bold basal medium provided with 4% CO_2-enriched air, also by El-Mohsnawy *et al.* [24]. The isolated *Chlorella vulgaris* NMN_SP has been further studied for the biosynthesis of copper oxide nanoparticles (Cuo NPs), studies reported on characterization, optimization, and antimicrobial studies of the nanoparticles by our team early [25]. The biosynthesized CuO NPs was confirmed visually by the appearance of dark brown color formation in the mixture added with copper acetate. The existence of nanoparticles was confirmed by UV visible spectroscopy at 540 nm. Biosynthesized nanoparticles were characterized by SEM, EDAX, and XRD. Further, antimicrobial potential studies of synthesized CuO NPs were carried out against selected bacteria and fungi. Literature suggests that the *Chlorella vulgaris* plays a vital role in both bioenergy production and Food with rich protein sources varies from 42 to 58% of its biomass dry weight [1-3, 6-13].

The current study aims to design the primer against universal target gene 18s ribosomal RNA and ITS1 and ITS2 region to characterize the identified algae up to the species level detection. Primer designing and synthesis were successful and the standardization of PCR was obtained with all the tested samples and the available known positive algal DNA. Further to confirm the primer has no cross binding with any other bacteria or fungal also evaluated using specificity test. Two Gram-positive bacteria–*Staphylococcus aureus* and *Streptococcus pneumonia* and two Gram-negative bacteria–*Escherichia coli* and *Pseudomonas aeruginosa*, One fungal DNA–*Aspergillus flavus* was used for specificity test and all the non-algal DNA samples found to be negative as per our expectation. Blast analysis also showed that the designed primer was highly specific to Blue-green algae, especially with *Chlorella* species. Since our target was *Chlorella*, we have chosen the region of the gene which is highly conserved among the *Chlorella* species and the same has been evaluated both phenotypically and genotypically.

CONCLUSION

The study aims to design and standardize PCR for Blue-green algae, specifically to *Chlorella* species was successfully achieved and the designed primers were optimized and amplified the target regions

without any cross-reaction, primer dimer formation, and nonspecific band formation. Further sequencing confirmed the species as Chlorella vulgaris and its unique sequence given the name NMN_SP as a strain name. Further studies on this *Chlorella vulgaris* will provide us a better understanding of the potential activities as antimicrobial, anticancer, antidiabetic, and other industrially beneficial pharmaceutical products.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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