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Hyperproduction of phycobiliproteins by the cyanobacterium *Anabaena fertilissima* PUPCCC 410.5 under optimized culture conditions

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ABSTRACT

This study was targeted to find new sources of phycobiliproteins (PBP) as these are important as natural colours, antioxidants and fluorescent markers. Here we report *Anabaena fertilissima* PUPCCC 410.5 as a good producer of PBP. Control cultures of the cyanobacterium produced 383 μg PBP mg^{-1} dry biomass on day 8. The pH of the medium, supplementation of nitrogen source, sugar and illumination of cultures with different colours of light were optimized for PBP production. Optimized individual parameters were: pH of the medium 7.5; supplementation of 2 mmol nitrite L^{-1} or 0.5% sucrose; and illumination of cultures with blue light. Total PBP production under these conditions ranged between 627 and 696 μg mg^{-1} dry biomass, resulting in nearly 1.6 fold increase, with a significant increase in phycocyanin content. Incubation of 2 mmol nitrite L^{-1} supplemented cultures in blue light resulted in nearly 4.5 fold increase in phycoerythrin compared to the control cultures. The PBP/phycocyanin/phycoerythrin production under the optimized conditions by *Anabaena fertilissima* PUPCCC 410.5 is significantly higher than the PBP production by the other cyanobacterial strains reported in literature. Thus this cyanobacterial strain is a promising candidate for PBP production at commercial level.

1. Introduction

Cyanobacteria are the oxygenic photosynthetic prokaryotes that appeared approximately 2.4 billion years ago [1]. These organisms possess a wide variability of physiological strategies which allow them to occupy the diverse ecological habitats in the terrestrial, fresh water and marine surroundings with the widely fluctuating environmental factors such as light intensity and quality, temperature, nutrient availability and water activity [2–6]. Although cyanobacteria are prokaryotic, they are similar to the plants in having chlorophyll *a* and the ability to perform oxygenic photosynthesis. Cyanobacteria have phycobiliproteins (PBP) as accessory pigments responsible for the absorption of different wavelengths of light for photosynthesis [3]. These chromoproteins may account for up to 60% of the cellular proteins and also serve as an additional source of nitrogen reserves in the cyanobacteria. The PBP are water soluble, highly fluorescent and are classified based on spectroscopic properties into three groups: Phycoerythrin (PE) (λ_{max} 540–570 nm), Phycocyanin (PC) (λ_{max} 590–630 nm) and Allophycocyanin (APC) (λ_{max} 620–655 nm) [7–10].

Cyanobacteria have gained increasing interest as an attractive source of bioactive substances, like polyunsaturated fatty acids, β-carotene and other pigments with antioxidant activity [11–16], antivirals [17], antimicrobials [18], and microsporine like amino acids and scytonemin as photoprotectants [19]. Phycobiliproteins being non-toxic and non-carcinogenic, have wide applications in food and cosmetic industries (as natural colourants), in pharmaceutical (as antioxidants and anticancerous agents) and in the field of immunology (as fluorescent markers) [20–22].

The commercial exploitation for the PBP is limited to *Spirulina* and *Porphyridium* only [21]. Since PBP have an array of applications, there is a need to explore more cyanobacteria for large scale PBP production. Here we report the isolation and characterization of *Anabaena fertilissima* as a hyperproducer of phycobiliproteins.

2. Material and methods

2.1. Organism and culture conditions

The cyanobacterial strain PUPCCC 410.5 isolated from the rice fields of Kum Kalan village (30° 54′ 48″ N; 76° 3′ 56″ E) of Ludhiana district in Punjab, India was raised into axenic cultures through plating technique [23]. The pure cultures were grown in Chu-10 medium [24], slightly modified by replacing calcium nitrate with an equimolar amount of calcium chloride, defined as the basal medium ($CaCl_2 \cdot 2H_2O$, 0.232; K_2HPO_4, 0.01; $MgSO_4 \cdot 7H_2O$, 0.025; Na_2CO_3, 0.02; $Na_2SiO_3 \cdot 5H_2O$, 0.044; ferric citrate, 0.0035; citric acid, 0.0035 g L^{-1}, pH 7.5). The stock and experimental cultures were incubated in a culture room at 28 °C ± 2 °C and illuminated for 14 h daily with a light intensity 44.5 μmol photon flux density m^{-2} s^{-1} at the surface of culture vessels

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Table 1
Amount of phycobiliproteins produced by cyanobacterial strains on day 8.

S. No.	Organism	Total PBP (% of dry biomass)	S. No.	Organism	Total PBP (% of dry biomass)
1	*Anabaena fertilissima*	38.3 ± 1.9	11	*Lyngbya* sp.	14.5 ± 0.7
2	*Anabaena* sp. 1	27.0 ± 1.3	12	*Nostoc microscopicum*	13.2 ± 0.6
3	*Anabaena naviculoides*	26.4 ± 1.3	13	*Nostoc linckia*	12.3 ± 0.6
4	*Nostoc calcicola*	25.3 ± 1.2	14	*Nostoc* sp.	11.9 ± 0.5
5	*Nostoc muscorum*	23.2 ± 1.1	15	*Synechocystis pevalekii*	11.8 ± 0.5
6	*Anabaena variabilis*	22.5 ± 1.1	16	*Westiellopsis prolifica*	11.4 ± 0.5
7	*Nostoc palludosum*	21.3 ± 1.0	17	*Calothrix* sp.	10.4 ± 0.5
8	*Anabaena* sp. 2	19.9 ± 0.9	18	*Nostoc punctiforme*	10.0 ± 0.5
9	*Phormidium molle*	16.2 ± 0.8	19	*Leptolyngbya foveolarum*	8.5 ± 0.4
10	*Oscillatoria formosa*	15.2 ± 0.7	20	*Nostoc spongiaeforme*	8.4 ± 0.4

The strains were grown in basal medium for 8 d and amount of PBP determined. Data are means ± SD of three replicates.

unless otherwise stated. Exponentially growing 8–10 days old stock cultures were used in the study.

2.2. Identification of cyanobacterium isolate

The cyanobacterium was identified based on morphological characters following Desikachary [25]; Komárek [26], combined with the partial 16S rRNA gene and phycocyanin *cpcB*-intergenic spacer (IGS)-*cpcA* gene sequencing.

Genomic DNA was extracted by using HiPurA™ Plant Genomic DNA Miniprep Purification Spin Kit (HiMedia, Mumbai, India). Cyanobacterial 16S rRNA gene fragment of 667 nucleotides was amplified using 5 pmol of the cyanobacteria specific primers CYA106F and CYA781R [27]. A total 50 μL PCR reaction mixture was comprised of 200 μmol L^{-1} dNTPs, 50 μmol L^{-1} each primer, 1X PCR buffer, 3 U Taq polymerase, and 100 ng genomic DNA. The thermo-cycling conditions involved an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 52 °C, and 2 min at 72 °C and final extension at 72 °C for 8 min.

The phycocyanin IGS and flanking coding regions were amplified using the primers PCβF (5′-GGCTGCTTGTTTACGCGACA-3′) and PCαR (5′ CCAGTACCACCAGCAACTAA-3′) [28]. A PCR reaction mixture of 25 μL contained 50 ng DNA template, 1X GoTaq® Green Master Mix (Promega Corporation, Madison, USA), and 25 μmol L^{-1} of each primer. Amplification was done by initial denaturation at 94 °C for 5 min, followed by 40 cycles of 10 s at 94 °C, 20 s at 55 °C and, 40 s at 72 °C and final extension at 72 °C for 10 min. The gel-purified product was obtained using GeneJET Gel Extraction Kit (Fermentas, Lithuania). The sequencing was done using BigDye® Terminator v3.1 Cycle Sequencing Kit and an ABI Prism 310 Genetic Analyser (Applied Biosystems, USA). The sequences were analysed using the gapped BLASTn (http://www.ncbi.nlm.nih.gov/Blast) search algorithm and aligned to the near neighbours. The phylogenetic trees were constructed using MEGA6.06 software package [29].

2.3. Optimization of conditions for growth and PBP production

The exponentially growing stock cultures after two washings with sterilized double distilled water were inoculated in 100 mL basal medium in 250 mL Erlenmeyer flasks to 0.1 initial absorbance at 750 nm. Immediately 10 mL of cultures were withdrawn and centrifuged at 5000 *g* for 10 min. Each cell pellet was washed thrice with distilled water, oven dried at 70 °C for 24 h and weight of biomass was recorded. The growth was monitored at regular intervals as increase in the dry weight of biomass with time. The effect of pH on growth and PBP production was studied by setting pH of the medium at 6.5, 7.5, 8.5 and 9.5. The effect of red, blue, green and yellow light on growth and PBP production was studied by illuminating the culture vessels wrapped with the cellophane papers of respective colours. The concentrations 2 and 5 mmol L^{-1} each of KNO_3 and KNO_2 were achieved in the basal medium to study their effect on growth and PBP production. Glucose, sucrose and fructose were added to the basal medium separately to a final concentration of 0.5% to study the effect of sugars on growth and PBP production by the cyanobacterium.

2.4. Extraction and quantification of phycobiliproteins

Phycocyanin and phycoerythrin from the cultures grown under varied conditions were extracted in water by the freeze-thaw method. The biomass pellet obtained by centrifugation at 5000 *g*, was washed thrice with double distilled water and resuspended in distilled water. The contents were subjected to 10–12 freeze-thaw cycles till the water soluble pigments were completely released from the biomass. Absorbance of the supernatant, after centrifugation at 5000 *g*, was recorded at 562 nm, 615 nm and 652 nm. The PBP were quantified following the equations given by Bennett and Bogorad [30].

2.5. Chemicals

All chemicals used in the present study, unless otherwise stated, were obtained from S D FINE-CHEM Limited, Mumbai, India.

2.6. Statistical analysis

Data were statistically analysed by applying ANOVA and Tukey's post-hoc test at 95% confidence level ($P < 0.05$) using the Graph Pad 5.0 version 5.4 and data presented in results are the average ± SD of three independent experiments each with three replicates.

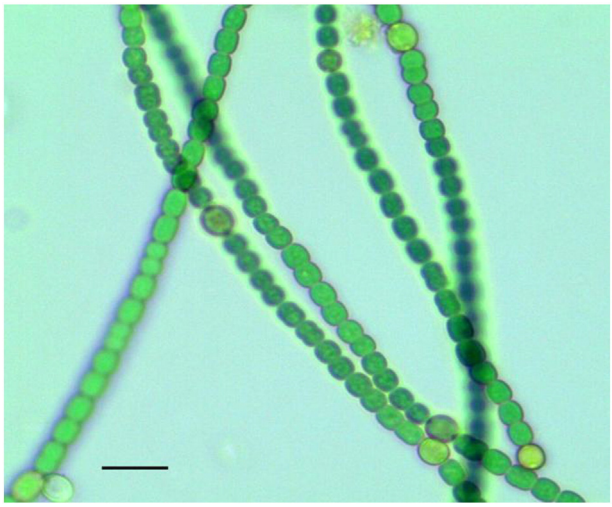

Fig. 1. Photomicrograph of *A. fertilissima* PUPCCC 410.5 (Scale bar = 10 μm).