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ISOLATION AND CHARACTERISATION OF PHYCOBILIPROTEIN RICH MUTANT OF CYANOBACTERIUM *SYNECHOCYSTIS* SP.

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Chemical (N′ methyl-N′-nitro-N-nitrosoguanidine) mutagenesis and penicillin selection were utilised to isolate a phenotypically altered mutant of cyanobacterium *Synechocystis* sp. This mutant (ntm_{60A}) exhibited enhanced protein content and nitrogen fixing potential but lower amount of chlorophyll and nitrate reductase activity. A remarkable and significant increase was observed in the total phycobiliprotein content of the mutant, especially in relation to the amount of phycoerythrin. This strain can be exploited as a rich source of natural coloring agent such as phycobilins in the bioindustry.

Keywords: NTG mutagenesis – phycobiliproteins – *Synechocystis* sp.

INTRODUCTION

Cyanobacteria are a fascinating group of organisms which combine in themselves special features of both prokaryotes and eukaryotes. The photosynthetic apparatus of cyanobacteria houses unique supramolecular complexes (phycobilisomes), composed of a brilliantly coloured family of water soluble proteins – phycobiliproteins [15]. Cyanobacterial phycobilisomes serve as not only antenna complexes for light harvesting in the wavelength range of 500–670 ηm, but they can also be used as storage materials for reduced carbon and nitrogen [6]. It has been suggested that a very complicated network of antagonistic and/or cooperative effectors are involved in the photoregulation of both chromatic adaptation of the photosynthetic apparatus and formation of heterocysts and hormogonia [23]. In recent years phycobiliproteins have been widely applied as flourescent tags in a variety of analytical and diagnostic procedures [10], besides being used as natural protein dyes in food and cosmetic industry.

Mutagenesis of cyanobacteria using N′ methyl-N′-nitro-N-nitrosoguanidine (NTG) has been one of the most successful genetic manipulation techniques for unicellular [19] and filamentous forms [8]. Chapman and Meeks [7] described conditions for NTG mutagenesis that produce mutants of *Anabaena variabilis* ATCC 29413 at a frequency of about one in 10^4 viable cells. Most of the mutants isolated

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and characterised have been related to nitrogen fixation, auxotrophy or photoautotrophy [20]. In the present investigation, studies have been undertaken to isolate and characterise a mutant of *Synechocystis* sp. tilted towards phycobiliprotein synthesis, obtained through induction by NTG.

MATERIALS AND METHODS

Organism and culture conditions

Synechocystis sp. (ARM 340) was obtained from the germplasm collection of the National Centre for Conservation and Utilisation of Blue-Green Algae, Indian Agricultural Research Institute, New Delhi. The culture was plated and a single colony picked up and grown in BG-11 medium at 28 ± 20 °C using 16/8 light dark cycle and illumination of 2000–3000 lux [22]. This clonal population was used for mutagenesis experiments.

Mutagenesis with NTG followed by penicillin enrichment

Clonal log phase culture (4–5 days old, 10^5–10^6 cells ml^{-1}) of *Synechocystis* sp. was washed with sterile water and the pellet resuspended in phosphate buffer containing (50 mM Disodium hydrogen phosphate and 50 mM Sodium dihydrogen phosphate) in 5 : 1. The suspension were treated with 100 and 200 µg ml^{-1} of NTG and placed on shaker. At intervals of 15 minutes, the samples were removed, pelleted and washed with phosphate buffer and 0.1 ml aliquots plated on agar medium. The plates were incubated under routine growth conditions and the number of surviving colonies were counted. Based on these experiments, a concentration of 100 µg ml^{-1} and a time of 30 and 45 minutes was selected for NTG treatment, followed by penicillin enrichment (@500 µg ml^{-1}). The mutant colonies were isolated and grown in nitrogen free and nitrogen supplemented (@1.5 g l^{-1} Sodium nitrate) medium.

Growth attributes and enzymatic assays

The mutants isolated were grown in nitrogen depleted and supplemented media for 15 days after which the cultures were measured for the following parameters:

Chlorophyll: Spectrophotometric estimation of chlorophyll was carried out following the method of Mckinney [17].

Protein: Quantitative estimation of protein was done using the method of Herbert et al. [11].

Phycobiliproteins: The spectrophotometric method of Bennett and Bogorad [2] was employed for the quantification of PC (phycocyanin), APC (allophycocyanin) and PE (phycoerythrin) pigments and total phycobiliproteins.

Nitrate reductase activity: The diazocoupling method of Lowe and Evans [14] which quantifies the amount of nitrite was utilised.

Acetylene reducing activity (ARA): Gas chromatographic quantification of ethylene formed was utilised as an index of the nitrogenase activity following the method of Jewell and Kulasooriya [12]. One ml samples were injected into Nucon Model Gas Chromatograph fitted with Porapak N column and attached to Flame Ionisation Detector.

Gel electrophoretic analysis of proteins

0.5 ml of 15 days old cultures of mutant and wild type *Synechocystis* were analysed for their total protein profiles using SDS-PAGE technique [13] on 12% gel along with broad range protein molecular weight standard.

RESULTS AND DISCUSSION

Generation of mutant

The frequency of mutation using NTG was 2.2 and 2.3×10^{-4} after 45 and 60 minutes, respectively, when 100 μg ml^{-1} of NTG was used. One mutant (ntm_{60A}) was selected for biochemical and physiological characterization, on the basis of its distinct pale colour and mucilaginous appearance on agar plates. The phenotypic characteristics of this mutant proved to be stable and reproducible and the morphology and cell diameter varied significantly from the wild type. The mutants formed four to many celled aggregates with prominent mucilaginous sheath, which is characteristically different from the unicellular wild type cells, which rarely formed two celled aggregates. The size of the wild type ranged between 4.2–4.5 μm in diameter while the mutant cells were of 5.5–6 μm diameter.

Characterization of mutant

Physiological characterization of the mutant (ntm_{60A}) grown in nitrogen deficient and supplemented medium, revealed significant differences in the parameters under study (Table 1). The mutant showed significantly reduced chlorophyll content, indicative of the slow growth of the mutant. A similar reduction was also observed in relation to the nitrate reductase enzyme activity. The protein content of the mutant was found to be significantly higher in both the nitrogen depleted and nitrogen supplemented medium. Ammonia excreting potential of the mutant did not vary significantly from the wild type (parent) strain. *Synechocystis* sp., a non-heterocystous cyanobacterium was found to be capable of nitrogen fixation and this potential was found to be enhanced in this mutant. Similar reports on *Synechocystis* strains [4, 9] and other non-heterocystous strains exist in literature [3, 17, 18].

Table 1
Physiological attributes of wild and mutant strains of *Synechocystis* sp.

Strains and growth medium	Chlorophyll	Phycobiliproteins	Total proteins (×10)	Acetylene reducing activity †	Nitrate reductase activity ††	Phycobiliproteins/ chlorophyll	Percent of phycobiliproteins/ protein
Wild strain (+N medium)	6.9±0.26[b]	14.3±0.69[b]	12.3±0.69[a]	7.68±0.88[a]	0.075±0.012[b]	2.07	11.63
Wild strain (–N medium)	5.7±0.39[b]	8.2±0.26[a]	20.7±1.19[b]	33.10±3.23[b]	0.025±0.008[a]	1.40	3.96
Mutant strain (+N medium)	3.6±0.16[a]	121.3±1.9[d]	17.3±1.90[ab]	7.31±1.06[a]	0.012±0.004[a]	33.60	70.12
Mutant strain (–N medium)	2.5±0.14[a]	86.3±1.09[c]	33.7±1.51[c]	45.03±2.02[c]	0.006±0.001[a]	34.50	25.61
CD	1.51 **	4.84 **	8.12 **	11.79 **	0.02 **	–	–

All values expressed as μg ml^{-1}.
† Values in η moles C_2H_4 μg chl^{-1} hr^{-1}.
†† Values in mg NO_2 μg $Protein^{-1}$.
Different letters in superscript indicate significant ($P<0.01$) differences between treatments as determined by the test for 'F'. Overall significance between treatments indicated by $P<0.01$ = **, $P>0.01$ = NS.

The most remarkable change brought about by NTG mutagenesis was on the phycobiliprotein content of *Synechocystis* (Fig. 1). The mutant was found to show a 9–10 fold increase in the total phycobiliproteins and a drastic change in the phycobiliprotein : chlorophyll ratio from a value of 1.4/2.7 in the wild type to 33.6/34.5 in the mutant. Also, the percent phycobiliproteins (of total proteins) showed a 7–8 fold enhancement. This was found to be mainly in relation to the phycoerythrin production which shows an absorption maxima at 560–570 ηm. Phycoerythrins are known to be widely distributed among all taxonomic groups and form the most spectroscopically variable class of phycobiliproteins. It is well known that the unidirectional energy transfer between the heterogeneous components of PBS is a consequence of the energy difference of absorption between PE, PC and APC, wherein the primary absorbing pigment is PE (shortest wave length). Phycoerythrocyanins (PEC, λ_{max}=575 ηm) and phycoerythrins (PE, λ_{max}=565–575 ηm) are found at the core distal end of the peripheral rods, while Phycocyanins (PC, λ_{max}=615–640 ηm) and Allophycocyanins (APC, λ_{max}=650–655 ηm) are found in the distal end of the peripheral rods and core substructure, respectively [5].

SDS-PAGE profiles of total proteins of the mutant and wild type strains (Fig. 2) also provide evidence for qualitative and quantitative changes in the proteins of

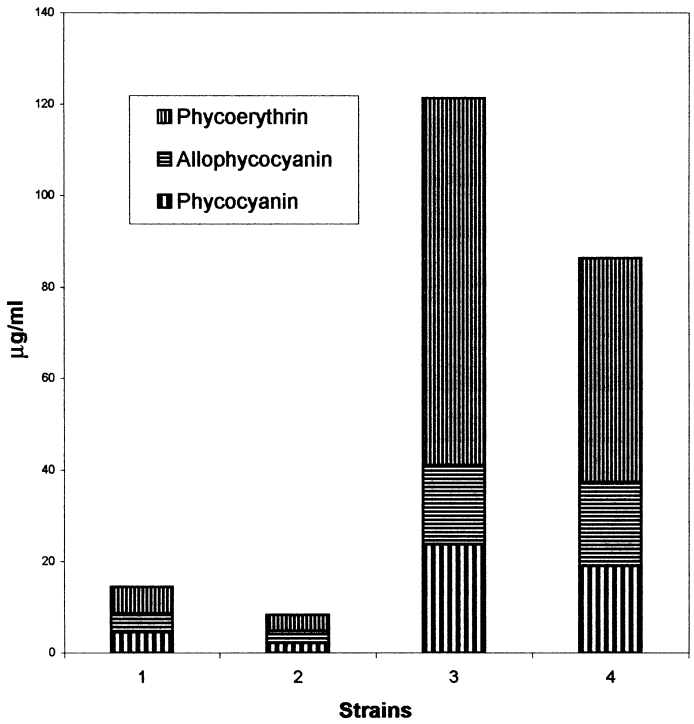

Fig. 1. Distributional pattern of phycobiliprotein components in wild and mutant strains of *Synechocystis* sp. 1. Wild (+N medium); 2. Wild (–N medium); 3. Mutant (+N Medium); 4. Mutant (–N Medium)

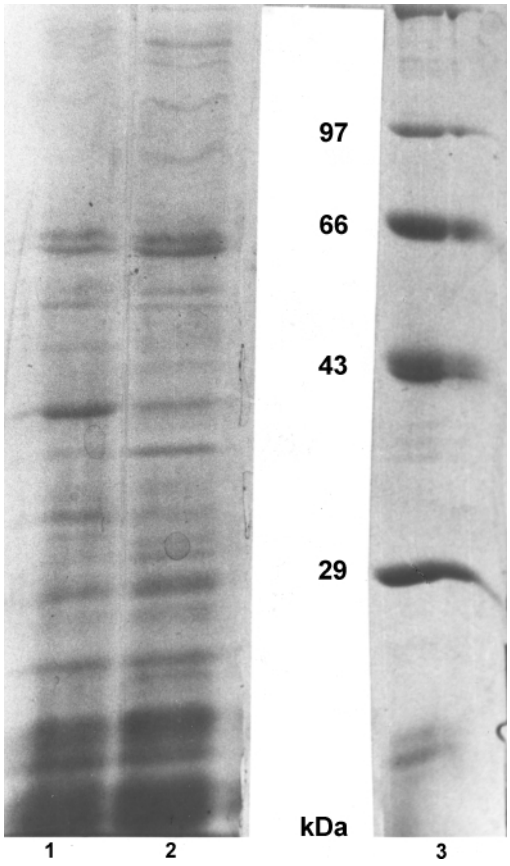

Fig. 2. Protein profiles of mutant (1) and wild type (2) strains of *Synechocystis* sp. obtained through SDS-PAGE (Lane 3, Molecular marker of broad range)

mutant. This may be indicative of alterations in the transcriptional/translational machinery of the cell [21]. Complete amino acid sequencing of the individual phycobiliproteins in the future may help in revealing the changes brought about by mutagenesis. Studies on the organization and transcription patterns for genes encoding the phycobilisome components of several cyanobacteria have revealed that these constitute 5–9 transcriptional units [6]. UV irradiation is known to bring about a large number of mutations in the light harvesting complexes in cyanobacteria and such mutants have been employed for better understanding of the structure, assembly of light harvesting proteins and light regulated expression of phycobilisome assembly [1]. The present study indicates the utility of NTG mutagenesis in obtaining pigment mutants which may contain mutations in the operons involved in phycoerythrin/phycocyanin synthesis. It is well known that molecular mechanisms exist for the operation of global nitrogen control in cyanobacteria [14] and modification of even a single gene

involved in regulation of nitrogen levels may have pleiotropic effects, such as those observed in this mutant.

The limited distribution of these phycobiliproteins in microorganisms and their wide ranging uses, underlines the need for exploitation of cyanobacteria as an attractive source of these valuable compounds. Mutagenesis is well established as a tool for the improvement of genetic and thereby industrial potential of microorganisms. This mutant strain, showing high phycobiliprotein content and aerobic nitrogen fixation has immense potential in bioindustry. The value of this mutant can be further enhanced by detailed molecular analyses, especially in relation to the regulatory mechanisms involved in the uptake, utilisation and storage of nitrogen in cyanobacteria, for the cost effective production of this mutant. This, in turn may lead to greater exploitation of cyanobacterial strains as a source of value added products, in the years to come.

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