Appl Biol Chem (2017) 60(6):631–636
DOI 10.1007/s13765-017-0320-x

Online ISSN 2468-0842
Print ISSN 2468-0834

ARTICLE

Isolation and purification of phycocyanin from cyanobacteria of a mangrove forest

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Received: 1 August 2017 / Accepted: 16 September 2017 / Published online: 25 September 2017

Abstract A cyanobacterium is an ancient group of photosynthetic prokaryotes that are thought to be the first organisms to carry out oxygenic photosynthesis. Phycocyanin (PC) is used to capture light energy for photosynthesis, unique to cyanobacteria. Sundarbans, a mangrove forest, is situated in the delta of Ganges, Meghna and Brahmaputra rivers, India. It has been declared as world heritage centre for its biodiversity. We would be the first to report on the photosynthetic pigment, phycocyanin of any strain of cyanobacteria isolated from a mangrove forest, Sundarbans. We have isolated the photosynthetic pigment from a cyanobacterial strain, AP24. Sequential precipitation of crude extract was done by the use of ammonium sulphate. Dialysis of the protein sample, anion exchange chromatography on a DEAE-cellulose DE 52 column and gel filtration chromatography with Sephadex G-100 column chromatography increased the purity ratio of phycocyanin. The purification was done both by DEAE-cellulose [purity ratio of the eluted cyanobacterial phycocyanin (C-PC) (A_{620}/A_{280}) increased to 4.31] and by Sephadex column. The molecular weight of purified C-PC was found to be 72 kDa, which include two subunits α (17 kDa) and β (19 kDa) in a dimer association $(\alpha\beta)_2$. The purified C-PC shows antioxidant activity. It was observed that the rate of haemolysis is decreased with increasing concentration of C-PC.

Keywords Antioxidant activity · Cyanobacteria · Phycocyanin · Sundarbans

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Introduction

Cyanobacteria are a varied group of prokaryotic microorganisms and exist in different forms including unicellular, filamentous, planktonic or benthic and colonial (coccoid) ones. They can grow fast due to their uncomplicated structure, frequently called blue-green algae. They are characterized by a great morphological diversity, unicellular as well as filamentous species, being included with a cell volume ranging over more than five orders of magnitude [1]. Cyanobacteria are an ancient group of photosynthetic prokaryotes that are thought to be the first organisms to carry out oxygenic photosynthesis. All cyanobacteria are capable of oxygenic photosynthesis, but a number of cyanobacterial species can switch to sulphide-dependent anoxygenic photosynthesis [2]. They are considered to be the ancestors of chloroplasts. These organisms can inhabit a range of habitats including freshwater, marine and soil environments, as well as extreme habitats such as hot spring water, and Arctic and Antarctic environments [1, 3].

Cyanobacteria are rich source of structurally novel and biologically active metabolites, which are shown to exhibit antibacterial, antifungal, anticancer or cytotoxic, antimalarial and other pharmacological activities [4].

Cyanobacteria are named for their high concentration of phycocyanin (PC), which is the major component of phycobiliprotein. Due to their capacity for chromatic adaptation, ratios between phycocyanin and phycoerythrin within the phycobilisomes can be altered, allowing the cyanobacteria to efficiently capture light energy despite the restrictions of their habitat [5]. PC is used to capture light energy for photosynthesis, is unique to cyanobacteria and is responsible for the distinctive deep blue colour. PC is a

water-soluble phycobiliprotein capable of exerting potent antioxidant protection and anti-inflammatory activities [6]. PC represents a significant component of blue-green algae, as it can constitute up to 15% of its dry weight when harvested. It is well documented that cyanobacterial phycocyanin (C-PC) selectively inhibits cyclooxygenase-2 activity [6]. In terms of its antioxidant activity, in vitro studies have reported PC scavenging alkoxyl, hydroxyl and peroxyl radicals, as well as reacting with peroxynitrite and hypochlorous acid [7], thus reducing cellular oxidative damage. Other phycobiliproteins are phycoerythrin (deep red) and allophycocyanin (bluish green). These three phycobiliproteins are classified according to their different absorption maxima, phycocyanin (λ_{max} at 620 nm), phycoerythrin (λ_{max} at 562 nm), phycoerythrocyanin (λ_{max} at 568 nm) and allophycocyanin (λ_{max} at 652 nm) [8–10]. α (low mw polypeptide) and β (high mw polypeptide) subunits are associated in trimers $(\alpha\beta)_3$ and hexamers $(\alpha\beta)_6$. The estimated molecular weight of C-PC is 140–210 kDa. The methods usually tried for purification of C-PC involve density gradient centrifugation, ammonium sulphate precipitation, ion exchange chromatography and gel filtration chromatography.

Sundarbans has been declared as world heritage centre for its biodiversity. It is situated in the delta of Ganges, Meghna and Brahmaputra rivers, India. It is the largest halophilic mangrove in the world. It is a rich source of large number of marine plants, animals and micro-organisms including cyanobacteria. Our strain (cyanobacterium AP24; EU908683.1, GI: 196050488) was isolated from Sundarbans. The 16S rRNA gene sequence of the isolate was submitted to GenBank, and accession number was found to be EU908683. The dendrogram of the isolate indicated species richness based on the morphological, biochemical and physiological characteristics. The strain is taxonomically related to LPP group B [11]. Very few studies have been conducted related to cyanobacteria from Sundarbans [12, 13]. But no studies have been conducted on phycocyanin or any other photosynthetic pigments of cyanobacteria from Sundarbans. This study is very important and unique in this respect. We would be the first to report on the photosynthetic pigment, phycocyanin of any cyanobacterial strain from Sundarbans.

Materials and methods

Components of ASNIII media was purchased from HiMedia, Mumbai, India, and all are of AR grade. DEAE-cellulose DE 52 was purchased from HiMedia, Mumbai, India. Sephadex G-100 was obtained from Sigma, Bangalore, India. Standard protein marker was obtained from HiMedia, Mumbai, India, and AAPH (2, 2′-Azobis(2-amidinopropane) dihydrochloride) was obtained from Merck, Mumbai, India.

The marine cyanobacterial culture was maintained in artificial seawater nutrient media (ASNIII) media at 25 °C in 12-h light and dark cycle.

AP 24 cells were collected from culture media after 30 days of incubation. The cell mass was washed with distilled water repeatedly, and then, 5 g freeze-dried cell mass was homogenized by a hand-held Homogenizer (Remi, Kolkata, India). This homogenized cell mass was suspended in 100 mL of 20 mM Tris–HCl buffer (containing 10 mM EDTA, pH 6.5, lysozyme of 1 mg/mL). Total mixture was sonicated for 60 s by using sonicator (Piezo-U-Sonic, Kolkata, India). This sonicated cell mass was incubated at 4 °C for 10 h. It was then subjected to repeat freeze-thawing at −20 °C and 4 °C. Release of phycobiliprotein was observed by the appearance of reddish blue colour. The mixture was centrifuged at 10,000 g for 30 min at 4 °C. Clear supernatant was collected.

The supernatant was taken, and absorbance was measured by UV–VIS spectrophotometer (LI-295UV visible, Intech, Chandigarh, India). Phycocyanin gave a characteristic peak at 620 nm. The purity ratio of phycocyanin was determined by measuring the ratio of absorbance at 620 and 280 nm (A_{620}/A_{280}). 620, 652 and 562 nm show maximum absorbance for C-PC, APC and PE, respectively. Using the following equation, we can calculate the concentration of C-PC, APC and PE [14]:

$$\text{C-PC}\,(\text{mg/mL}) = [A_{620} - 0.0474\,(A_{652})]/5.34,$$

$$\text{APC}\,(\text{mg/mL}) = [A_{652} - 0.208\,(A_{620})]/5.09,$$

$$\text{PE}\,(\text{mg/mL}) = [A_{562} - 2.41\,(\text{PC}) - 0.849\,(\text{APC})]/9.62$$

The entire procedure was carried out at 10 °C. Solid ammonium sulphate was gradually added in 100 mL of crude extract to achieve 25% ammonium sulphate saturated and allowed to stand at 4 °C for 9 h. The resulting solution was centrifuged at 10,000 g for 30 min at 4 °C (Centrifuge 5430R Eppendorf, Hamburg, Germany). The supernatant was further treated with solid ammonium sulphate until it reached 50% saturation and kept overnight at 4 °C. The resulting solution was centrifuged at 10,000 g for 30 min. The pellet mainly containing C-PC was mostly dissolved in acetate buffer (0.1 M, pH 4.5). The resulting mixture was again centrifuged at 10,000 g for 30 min. The small pellet (mainly basic proteins) was discarded, and supernatant was further brought to ammonium sulphate precipitation to achieve 50% saturation and allowed to kept overnight at 4 °C. The precipitated C-PC was dissolved in 10 mL of 20 mM Tris–HCl buffer (pH 8.1) and dialysed for 10 h at 4 °C against same buffer. The buffer was changed twice for complete removal of ammonium sulphate.

DEAE-cellulose-52 was used for anion exchange chromatography. 20 cm × 2.5 cm column was prepared for C-PC purification. The column was pre-equilibrated with 20 mM Tris–HCl buffer (pH 8.1). The dialysed sample of C-PC was loaded on the column. Then, the column was washed with 10-bed volume of the same buffer. The column was first eluted with 10-bed volume of 0.05 M NaCl in 20 mM Tris–HCl buffer (pH 8.1). The elution was performed by subsequent addition of different concentrations of NaCl (0.1, 0.15, 0.2 and 0.25 M) in 20 mM Tris–HCl buffer (pH 8.1). The C-PC was then eluted between 0.15 and 0.25 M NaCl concentration, and sample was collected in 2-mL microcentrifuge tube. The flow rate of sample was 0.5 mL/min. Scanning of all fraction was determined in the range of 250–800 nm. Purity ratio of $A_{620}/A_{280} > 3.5$ was pooled together. The resulting solution was brought to 50% ammonium sulphate saturation. The pellet was mixed with 10 mL 20 mM Tris–HCl buffer (pH 8.1) and dialysed against water at 4 °C. The sample was freeze-dried.

Gel filtration chromatography was prepared for the determination of molecular weight of native protein. The column was prepared by Sephadex G-100. The column was pre-equilibrated with 10 mM potassium phosphate buffer and calibrated using egg albumin (45 kDa), bovine serum albumin (67 kDa) and bovine γ-globulin (158 kDa).

SDS-PAGE (1.5 mm thick) carried out according to Laemmli [15] using a 15 and 5% polyacrylamide as resolving and stacking gels, respectively. Samples were mixed with equal volume of sample buffer containing 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.002% (w/v) bromophenol blue and 60 mM Tris–HCl (pH 6.8), and boiled for 8 min. Electrophoresis was carried out at room temperature. Proteins were visualized with Coomassie brilliant blue (G 250) staining. Prestained protein ladder (MBT092 from HiMedia, Mumbai, India) was used as reference.

Native PAGE under non-denaturing condition separates proteins based on the combination of molecular properties including size, shape and charge as they move through a polyacrylamide gel matrix. In native PAGE (1.5 mm thick), 5 and 10% acrylamide were used as stacking and resolving gels, respectively. Tris–HCl (pH 8.8) was used as running buffer. Proteins were visualized with Coomassie brilliant blue (G250) staining.

Zinc acetate staining was used to observe fluorescent property of the protein. The native gel without Coomassie blue staining was incubated with 20 mM zinc acetate solution for 5 min at room temperature [16]. The fluorescence was observed under UV light.

Blood samples were obtained from healthy volunteers by venipuncture. Blood was collected in heparinized tube. RBC was isolated by centrifugation at 1000 g for 10 min. Sample was washed with three times with phosphate buffer saline. RBC was suspended in same buffer to achieve 5% haematocrit level.

In this experiment, RBC suspension with phosphate buffer saline acts as a control. RBC suspension was pre-incubated for 15 min at 37 °C with different concentrations (10–150 μM) of C-PC. Then, the samples were incubated with 50 mM AAPH and then subjected to haemolysis for 3 h.

An aliquot of reaction mixture (1 mL) was taken and centrifuged at 3000 g for 2 min. The absorbance of supernatant was determined at 540 nm to obtain the extent of haemolysis [17]. RBC was incubated with distilled water (100% haemolysis) and taken act as the reference value.

The aqueous extract containing 50 μM C-PC was incubated with AAPH for 40 min. The spectral changes were recorded between 300 and 800 nm every 10-min interval [6].

Results

The quantitative evaluation of the phycocyanin content is summarized in Table 1. UV–visible absorption spectra of each step of purification are shown in Fig. 1 and SDS-PAGE in Fig. 2A and native PAGE in Fig. 2B. Fluorescent property of C-PC is shown in Fig. 3. The effect of AAPH in C-PC solution is shown in Fig. 4 and antioxidant activity of C-PC in Fig. 5.

Harvested AP 24 cells were washed with 20 mM Tris–HCl buffer (containing 10 mM EDTA, pH 6.5) repeatedly, and 5 gm of freeze-dried cell mass was homogenized. These homogenized cell masses were suspended in 100 mL of 20 mM Tris–HCl buffer [containing 10 mM EDTA, pH 6.5 and lysozyme (1 mg/mL)]. Cell debris was removed by cold centrifugation, and dark cobalt blue supernatant was collected.

Sequential precipitation of crude extract was done by the use of ammonium sulphate. Dialysis of the protein sample, anion exchange chromatography on a DEAE-cellulose column increased the purity ratio of phycocyanin. C-PC was precipitated by the three-step ammonium sulphate precipitation reactions. Spectroscopic measurements showed that pellets (25% ammonium sulphate saturation) contain no C-PC but contain other unwanted proteins. After 50% ammonium sulphate saturation, precipitated C-PC was collected and dissolved in acetate buffer to precipitate basic proteins. After complete dialysis, C-PC was loaded on DEAE-cellulose-52 column. The column was first washed with 10-bed volume of 20 mM Tris–HCl buffer (pH 8.1). C-PC was eluted between 0.15 and 0.25 M NaCl concentrations. The recovery of the eluted C-PC was 48% with purity ratio (A_{620}/A_{280}) of 4.31. The quantitative

Table 1 Data of the purification and separation of C-PC from AP 24

Purification step	Purity ratio A_{620}/A_{280}	Separation factor A_{620}/A_{652}	C-PC (mg ml^{-1})	Recovery of C-PC (%)
(1) Crude extract	0.8	2.4	0.2	100
(2) Ammonium sulphate precipitation with 25% saturation	0.9	2.4	0.3	93
(3) Ammonium sulphate precipitation with 50% saturation	2.4	2.9	0.3	83
(4) DEAE-cellulose-52	4.3	4.6	0.4	48

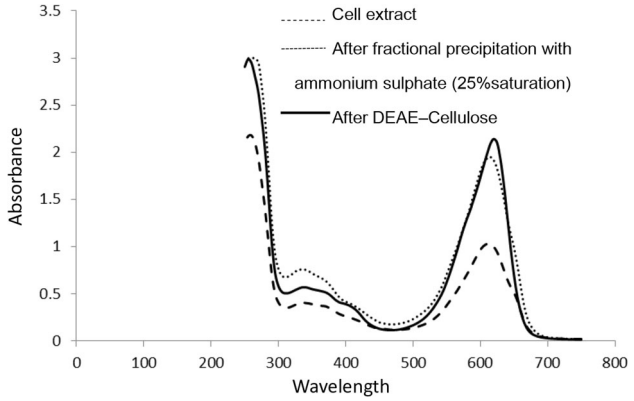

Fig. 1 Overlay absorption spectra of C-PC at each stage of purification

evaluation of the phycocyanin content is summarized in Table 1.

Purity ratio (A_{620}/A_{280}) increased in every step of purification, and absorption spectrum of C-PC increased with each step of purification (Fig. 1) and as well as separation factor (A_{620}/A_{652}). High separation factor is due to low contamination of APC.

The SDS-PAGE picture shows that C-PC is composed of two subunits α and β corresponding to molecular weight 17 and 19 kDa. The molecular weight of native purified C-PC was determined by gel filtration on Sephadex G-100 column chromatography. Purified C-PC elutes out after bovine serum albumin or BSA (molecular weight 67 kDa) and before bovine gamma globulin (molecular weight 158 kDa). The protein subunits are in the form $(\alpha\beta)_2$. This confirms the molecular weight to be 72 kDa (2α 17 kDa each and 2β 19 kDa each). The presence of a single band on native PAGE (Fig. 2B) indicates purity and subunit integrity of C-PC.

The presence of fluorescence indicates that C-PC contains bilin-linked polypeptide (Fig. 3).

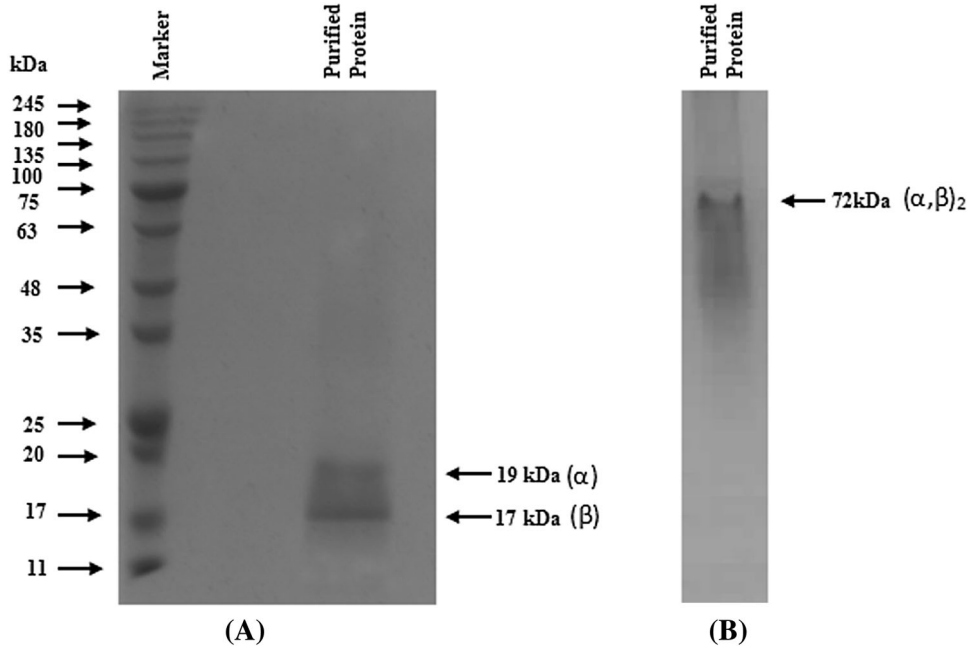

Fig. 2 (**A**) SDS-PAGE of C-phycocyanin: lane 1: molecular marker, lane 2: purified C-PC, (**B**) native PAGE of C-PC

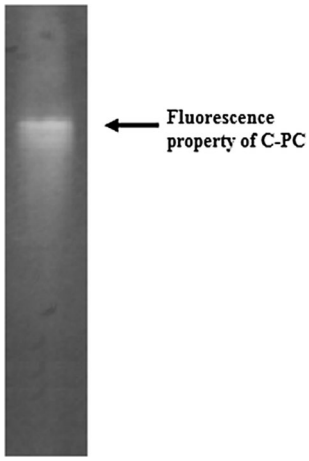

Fig. 3 Fluorescence emission by phycobiliprotein containing C-PC

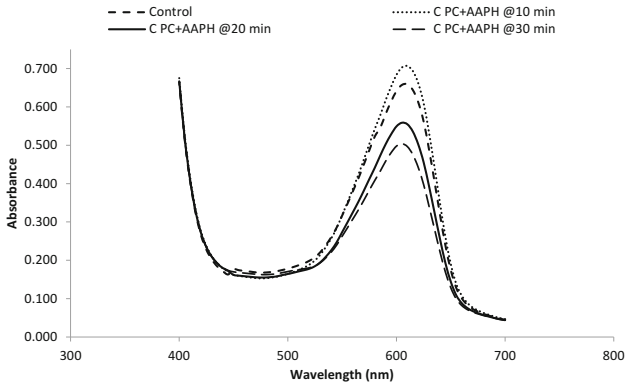

Fig. 4 Spectroscopic measurement of C-PC incubated with AAPH in a time-dependent manner

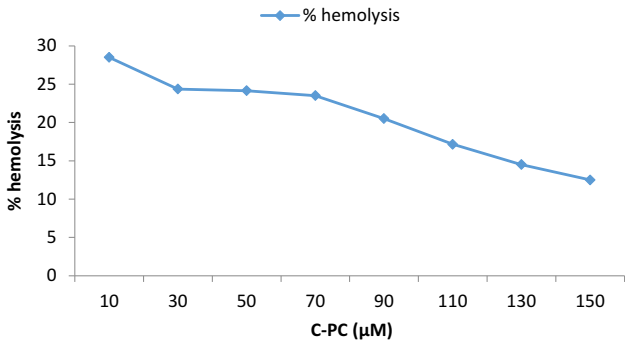

Fig. 5 Percentage of haemolysis in human RBCs pre-incubated with different concentrations of C-PC and then subjected to haemolysis by 50 mM AAPH for 3 h

C-PC shows maximum absorbance at 620 nm, but when AAPH was added in C-PC solution, there was a progressive decrease in absorbance at 620 nm (Fig. 4).

When RBC was incubated with PBS buffer at 37 °C for 3 h, negligible haemolysis was observed as PBS is isotonic to human RBC. AAPH, a water-soluble free radical generator, induces oxidative haemolysis of RBC suspension, but when C-PC was added to this solution, the rate of haemolysis is reduced. Rate of haemolysis is decreased with increasing concentration of C-PC. This result is shown in Fig. 5.

Discussion

Cyanobacteria are aquatic and photosynthetic, and they are one of the most genetically varied organisms considered. A new strain of cyanobacterium (16S rRNA gene sequence accession number: EU908683 taxonomically related to LPP group B) was isolated from marine sea water from Sundarbans. Our present study focussed on separation, efficient purification of C-PC and antioxidant activity of the purified phycocyanin from a not well-documented marine cyanobacterium. During the purification process, cyanobacterial cells are homogenized and freeze-thawed at −20 °C. The cell lysate was precipitated with 25% saturated ammonium sulphate, which removed some unwanted protein, and as a result purity ratio (A_{620}/A_{280}) also increased. Next, 50% saturated ammonium sulphate mainly precipitated C-PC. This C-PC containing pellet was dissolved in acetate buffer which removed some basic protein which increased purity ratio (A_{620}/A_{280}) of C-PC. Finally, 50% saturated ammonium sulphate in acetate buffer precipitated C-PC. This precipitated C-PC was dialysed overnight, which removed salts and low molecular weight proteins. Ultimate purification was achieved when this dialysed sample was loaded on DEAE-cellulose column. Between 0.15 and 0.25 M NaCl concentrations, C-PC was eluted. Purity ratio of the eluted C-PC (A_{620}/A_{280}) increased to 4.31. Purity was also confirmed by the SDS-PAGE. The molecular weight of purified C-PC was 72 kDa which include two subunits α (17 kDa) and β (19 kDa) in a dimer association $(\alpha\beta)_2$.

The purified C-PC was dialysed for removal of salts and freeze-dried for further use. For the measurement of antioxidant activity AAPH, a strong free radicals generator was used as oxidant. When AAPH was added on C-PC containing solution, it reduced absorbance of C-PC at 620 nm in a time-dependent manner. Further, it caused disappearance of characteristic blue colour of C-PC. Human RBC suspension was incubated with varying concentration C-PC. It was found that the rate of haemolysis decreased when C-PC concentration was increased.

We are the first group to purify a photosynthetic dye from a cyanobacteria isolated from Sundarbans. Our strain (cyanobacterium AP24; EU908683.1, GI: 196050488) is taxonomically related to PP group B. Cyanobacteria from Sundarbans [12, 13] are not so well studied. It should be noted that no studies have been done on phycocyanin or any other photosynthetic pigments of cyanobacteria from

Sundarbans. As Sundarbans has been declared as world heritage centre for its biodiversity, this study is novel and unique in this respect. As the result suggests, this dye molecule is quite similar to other phycocyanin found in different cyanobacteria, when the molecular weight and subunit composition are concerned.

Different reports about antioxidant activity of phycocyanin have been cited. We also found that the phycocyanin from AP24 shows antioxidant activity.

As the strain as well as the phycocyanin is till now not well studied, exhaustive studies should be carried out about different aspects of this dye and other bioactive compounds present in AP24.

Acknowledgments This study was supported by University Grants Commission, Government of India (F.No. 42-227/2013 (SR) dated 22.03.2013). The authors thank Professor Joydeep Mukherjee, School of Environmental Studies, Jadavpur University, Kolkata, India, for providing the cyanobacteria strain AP24.

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