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Characterization of a novel thermophilic cyanobacterial strain from Taian hot springs in Taiwan for high CO_2 mitigation and C-phycocyanin extraction

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ABSTRACT

The photosynthetic thermophiles have advantage in sequestering CO_2 emitted from the energy sector due to their adaptation to high temperatures, growth at high concentrations of CO_2, and economically important metabolites. The characterization of such a microorganism, a cyanobacterium from Taian hot springs in Taiwan is described here. This thermophilic cyanobacterium is rod-shaped with a size of 1.2–2.5 μm × 6.0–9.0 μm. A comparison of the 16S RNA and cpcBA-IGS sequences revealed that it is closely related to *Thermosynechococcus elongatus* BP-1 and so named as *Thermosynechococcus elongatus* TA-1. This cyanobacterium has better growth at 10% and 20% CO_2, at 50 °C with 6000 lx light intensity, at a starting pH of 7–9 and in a medium with 20 mM NaCl. The preferred nitrogen source is $NaNO_3$ of which the minimal requirement is 10 mM. The purified phycocyanin (C-PC) from TA-1 is still kept native and active at a wide range of temperatures (4–60 °C) with a 65.65% activity even at 60 °C, as well as pH values from 4 to 9 and thus exhibiting a good thermal and acid–base stability. This thermophilic cyanobacterium could make integration of CO_2 mitigation from industrial flue gas and production of economically important product, like C-PC, more feasible.

1. Introduction

The accumulation of excessive CO_2 emitted from the energy sector into the atmosphere has seriously increased global greenhouse effects. Among various techniques that are being proposed to sequester CO_2 and mitigate the effects of greenhouse gases [1–4], the microalgal photosynthesis systems have significant advantage in CO_2 sequestration by fixing CO_2 to produce biomass that can be converted into economically important byproducts [3,5]. Since most of the photosynthetic microalgae, presently explored, have an optimal growth temperature at the range of 20–30 °C [6,7], they fail when the working temperature is above 40 °C. Therefore, the microalgae that are adapted to grow at high temperatures are desirable. A few thermo-tolerant microalgal strains that are capable of fixing CO_2 have been reported. A green microalga, identified as *Chlorella sorokiniana*, isolated from Japan, was able to grow very well at 40 °C sparging with an airstream containing 10% CO_2 [8]. Several *Chlorella* strains isolated from hot springs in Japan grew and utilized CO_2 at temperatures up to 42 °C [9]. A thermophilic cyanobacterial species of *Chlorogleopsis* from Yellowstone National Park has been reported to grow well at 50 °C and at 5% CO_2 [10]. A thermophilic cyanobacterium *Synechocystis aquatilis* SI-2 was used to evaluate a vertical flat-plate photobioreactor for CO_2 fixation and outdoor biomass production [11,12]. The tolerance of these microalgal strains to high temperature makes them potentially the appropriate photosynthesis system for CO_2 mitigation from flue gas.

A microalgal photosynthesis system not only reduces CO_2 but also provides a large amount of potentially beneficial biomass which is used in production of biofuels, soil stabilizers, fertilizers, feedstock, healthy ingredients, or fine chemicals [13,14]. Among the derived products from the microalgal biomass, C-phycocyanin (C-PC), a blue light-harvesting pigment with fluorescent property, is considered to be one of the important economic products, which is included as a healthy ingredient in cyanobacteria-based food products. There are no reliable published statistics on the C-PC market size and price; however, the market value of C-PC was estimated to be US$ 10–50 million per annum [15,16] and the price of food-grade C-PC is Austrlian $ 500 per kg [17,18]. Recently, C-PC extracted from cyanobacteria has been demonstrated to have anti-inflammatory [19–21], antioxidant [22–24], skin care [24] and hepatoprotective [21] properties. The findings of the most recent

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study by Chang et al. [25] implicated that PC from red alga *Bangia atropurpurea* promoted activation and maturation of cultured dendritic cells, and enhanced the immunological function toward Th1 activity. Phycocyanin has been considered as a potential pharmaceutical source for anti-allergic therapeutics [26]. Eriksen [58] has reviewed the production and applications of C-PC in biology, biotechnology, foods and medicine.

The thermophilic photosynthetic cyanobacteria are widely distributed over hot springs [27,28] and are primarily responsible for the CO_2 fixation of carbon cycling. These thermophilic cyanobacteria can be good candidates for CO_2 mitigation in the microalgal photosynthesis systems. A thermophilic cyanobacterium with abilities for high CO_2 mitigation and be a source of an economically important byproduct like C-PC will be highly desirable. In this paper, we report of a novel thermophilic cyanobacterial strain, belonging to genus *Thermosynechococcus*, isolated from Taian hot springs in Taiwan with the potential for high CO_2 mitigation and C-PC extraction. The identity and growth characteristics of the *Thermosynechococcus* isolate and the thermostability and acid–base stability of C-PC extracted from its biomass have been discussed.

2. Materials and methods

2.1. Isolation and maintenance

The cyanobacterial samples were collected from Taian hot springs located at the central part of Taiwan. The samples were cultured and maintained in BG11 medium [29] consisting of $NaNO_3$ 1.5 g/l, K_2HPO_4 0.04 g/l, $MgSO_4{\cdot}7H_2O$ 0.075 g/l, $CaCl_2{\cdot}2H_2O$ 0.036 g/l, citric acid 0.006 g/l, ferric ammonium citrate 0.006 g/l, EDTA 0.001 g/l, Na_2CO_3 0.02 g/l and trace metal stock solution 1 ml. The compositions of trace metal stock solution are $FeCl_3{\cdot}6H_2O$ 3.150 g/l, $MnCl_2{\cdot}4H_2O$ 0.180 g/l, $ZnSO_4{\cdot}7H_2O$ 0.022 g/l, $CoCl_2{\cdot}6H_2O$ 0.01 g/l, $CuSO_4{\cdot}5H_2O$ 0.010 g/l, and $Na_2MoO_4{\cdot}2H_2O$ 0.006 g/l. The solidified BG11 medium with 2.0% agar was used for the isolation of cyanobacterial colonies. To isolate thermophilic cyanobacteria for high CO_2 mitigation, the samples were enriched with BG11 medium at 50 °C under the light intensity of 6000 lx by bubbling airstream containing 20% CO_2. The growth cultures were transferred to fresh media under the same conditions three times in order to select for the cyanobacteria that is suitable to fix high concentration of CO_2. Then the growth cultures were spread on the BG11 solidified medium for the isolation by selecting individual single colonies repeatedly till necessary purity of microorganisms with an identical morphology by microscopic observation has been achieved. Many thermophilic cyanobacterial isolates were obtained in this study and maintained in BG11 medium at 50 °C under the light intensity of 6000 lx for further analyses. One of the isolates, tentatively named as TA-1, was further analyzed.

2.2. Atomic force microscopy (AFM)

The cells of cyanobacterium TA-1 were performed with a Nanoscope IIIa multimode scanning probe microscope (Veeco Instruments, USA) in non-contact modes. Commercial 100 μm non-contacting cantilevers (NanoProbe) were used for non-contacting mode measurements with 200–300 kHz resonant frequencies. The image was processed using NanoScope Image software.

2.3. Genomic DNA isolation, PCR amplification, and sequencing

The pellet of cyanobacterial culture was obtained by centrifuging at 13,000 rpm for 15 min and then genomic DNAs from cell pellets were purified using a MasterPure™ Gram Positive DNA Purification Kit (EPICENTRE, USA). The pellets were frozen and thawed five times with liquid nitrogen to efficiently enhance DNA extraction before genomic DNA purification.

The near full-length sequences of 16S rRNA gene were amplified from genomic DNA by PCR under the following conditions: 35 amplification cycles of 94 °C (denaturing) for 1 min, 55 °C (annealing) for 1 min, and 74 °C (elongation) for 2 min, and then 72 °C for 10 min after the last cycle. The sequences of 16S rRNA gene primers were forward HL16SF-GCAGAGTTTGAAAGCCAAGG and reverse HL16SR-GAAACGAGTCCCGAACCTAGA. A part of the phycocyanin operon (*cpc*) including intergenic spacer (IGS) between cpcB and cpcA, and the corresponding flanking regions (cpcBA-IGS) has been successfully used to determine the phylogenetic relationships between cyanobacterial strains [30,31]. The cpcBA-IGS locus was PCR amplified from genomic DNAs under the following conditions: 35 amplification cycles of 94 °C (denaturing) for 1 min, 62 °C (annealing) for 1 min, 74 °C (elongation) for 1 min, and then 72 °C for 10 min after the last cycle. The sequences of cpcBA-IGS primer set were forward *cpcBA*-IGS-FP-AGCAACCCCAACTGATCCAACC and reverse *cpcBA*-IGS-RP-GGTCGTGGTGTAGGGGAATTTTTG [31]. A total 100 μl of the PCR reaction mixture contained 10 μl of 10× PCR buffer, 10 μl dNTPs at concentrations of 10 mM, each primer at a concentration of 0.5 μM, 0.5U of *Taq* DNA polymerase (Protech Technology, Taiwan) and 76.5 μl of ddH_2O. The PCR was performed in a MyCycler™ thermal cycler (Bio-Rad, USA). The PCR products were purified by using the VIOGENE Gel-MTM Gel Extraction System purification kit before TA cloning and sequencing. The purified PCR products were directly ligated into a pCR II vector from TOPO TA Cloning® Kit of Invitrogen (San Diego, CA, USA) according to the manufacturer's protocol. The sequencing was completed by Genomics BioScience & Tech. Co., Ltd., Taiwan.

2.4. Phylogenetic analysis

To construct the phylogenetic tree, the 16S rRNA sequence of the cyanobacterial isolate was used to carry out an initial BLAST search against the NCBI GenBank database to provide candidate sequences for relatedness analyses. Multiple alignments of the 16S rRNA and the candidate sequences selected from GenBank were then created by using the Clustal X. The neighbor-joining (NJ) method in MEGA4 were used to construct the phylogenetic tree [27].

2.5. Growth conditions and nutrient requirement

The various growth conditions including concentration of sparging CO_2, temperature, light intensity, pH and nutrient requirement of NaCl and nitrogen source were tested to obtain the optimal conditions necessary for proliferation and CO_2 fixation of this cyanobacterial isolate. The growth of the cynobacterial isolate was examined with concentrations of sparging CO_2 at air (containing about 0.039% CO_2), 5%, 10%, 20%, 40%, 60%, and 80%; at temperatures 20, 25, 30, 40, 50, 60, 70, and 80 °C; light intensities at 0, 2000, 6000, 12,000, and 18000 lx; and the starting pH at 3, 5, 7, and 9. To establish the nutrient requirement of this isolate, BG11 medium with 11 different concentrations (0, 5, 10, 15, 20, 30, 40, 50, 100, 500, and 1000 mM) of NaCl and 5 different concentrations (2, 5, 10, 17.5, and 25 mM) of $NaNO_3$ were tested for optimal growth. Urea, $NaNO_3$ (17.5 mM) and NH_4Cl were tested to establish the best nitrogen source for this isolate. All experiments were carried out at 50 °C except in the tests for the growth temperature, and under the illumination of 6000 lx except when tested for the light intensity. The growth of TA-1 was monitored by evaluating the change in optical density at 600 nm (OD_{600}).

2.6. Extraction and purification of C-phycocyanin

The thermophilic cyanobacterial isolate was grown to the early stationary phase in flasks containing BG11 medium at 50 °C under light intensity of 6000 lx. The cells were harvested to carry out the extraction of C-PC by a modified method according to Moraes and Kalil [32]. The cell pellet on ice was suspended with 1× PBS at a biomass-to-1× PBS ratio of 1:10. The suspension was implemented with sonication for 30 s with a break for 10 s at each cycle within 2 h, and then centrifuged at 13,000 rpm for 20 min to remove debris. The supernatant was used for the precipitation of C-PC.

Ammonium sulfate was added to the supernatant containing C-PC until 30% saturation was obtained. The solution was maintained for 2 h and then centrifuged at 13,000 rpm for 20 min. The supernatant was further added with ammonium sulfate until 70% saturation was obtained. The solution was maintained for 2 h and then centrifuged at 13,000 rpm for 20 min. Then the precipitate, rich in C-PC, was dissolved in 1× PBS (pH 7.0) and the solution was concentrated by using Amicon Ultrafiltration Normal-Filtrate Devices with 5 kDa, 44.5 mm Ultrafiltration Disc membrane (Cat. No. PLCC04310, Millipore, USA).

The concentrated solution was loaded into a Q-Sepharose Fast Flow column (GE Healthcare, Sweden) previously equilibrated in 30 ml of 0.2 M NaCl buffer (pH 7.0) at 3 cm/h. After the solution containing non-adsorbed proteins was removed, elution was carried out using 15 ml of 0.3 M NaCl buffer (pH 7.0). In total, 50 ml of elution sample was collected from a Q-Sepharose Fast Flow column. The elution sample was removed from NaCl and concentrated by using Amicon Ultrafiltration Normal-Filtrate Devices with 5 kDa, 44.5 mm Ultrafiltration Disc membrane, by addition of with 0.25% 1× PBS (pH 7.0).

The concentrated elution sample, rich in C-PC and without NaCl, obtained from the previous step was loaded onto hydroxyapatite column (Bio-Rad Laboratories, CA, USA) and then fractions were collected in 1 ml volume each tube by eluting with 5 ml of 10 mM NaCl in 20 mM Na-phosphate buffer (pH 7.0). The fractions were removed from NaCl and concentrated by using Amicon Ultrafiltration Normal-Filtrate Devices with 5 kDa, 44.5 mm Ultrafiltration Disc membrane, by addition of 20 mM Na-phosphate buffer (pH 7.0). All the fractions were pooled together into a tube further for the analyses of optical absorbance and fluorescence emission. The absorbance of the sample was recorded at 280, 620 and 650 nm using spectrophotometer (Thermo Fisher Scientific, USA). The purity of C-PC was calculated as the ratio of optical density (OD) at 620 and 280 nm. The amount of C-PC in the sample was calculated using the following calculation according to [33]:

$$\text{C-PC}\,(\text{mg/ml}) = \frac{\text{OD}_{620} - 0.7\text{OD}_{650}}{7.38} \quad (1)$$

2.7. Thermostability and acid–base stability of C-phycocyanin

The C-PC is characteristics of the optical properties of the absorption maxima at approximate 620 nm and fluorescence emission at 642 nm wavelengths. In the study, the relative intensity of absorbance at 620 nm and fluorescence emission at