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Evaluation of growth and phycobiliprotein composition of cyanobacteria isolates cultivated in different nitrogen sources

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Abstract

Phycobiliproteins, light-harvesting pigments found in cyanobacteria and in some eukaryotic algae, have numerous commercial applications in food, cosmetic, and pharmaceutical industries. Colorant production from cyanobacteria offers advantages over their production from higher plants, as cyanobacteria have fast growth rate and high photosynthetic efficiency and require less space. In this study, three cyanobacteria strains were studied for phycobiliprotein production and the influence of sodium nitrate, potassium nitrate and ammonium chloride on the growth and phycobiliprotein composition of the strains were evaluated. In the batch culture period of 12 days, *Phormidium* sp. and *Pseudoscillatoria* sp. were able to utilize all tested nitrogen sources; however, ammonium chloride was the best nitrogen source for both strains to achieve maximum growth rate $\mu = 0.284 \pm 0.03$ and $\mu = 0.274 \pm 0.13$ day^{-1}, chlorophyll *a* 16.2 ± 0.5 and 12.2 ± 0.2 mg L^{-1}, and phycobiliprotein contents 19.38 ± 0.09 and 19.99 ± 0.14% of dry weight, whereas, for *Arthrospira platensis*, the highest growth rate of $\mu = 0.304 \pm 0.0$ day^{-1}, chlorophyll *a* 19.1 ± 0.5 mg L^{-1}, and phycobiliprotein content of 22.27 ± 0.21% of dry weight were achieved with sodium nitrate. The phycocyanin from the lyophilized cyanobacterial biomass was extracted using calcium chloride and food grade purity (A_{620}/A_{280} ratio > 0.7) was achieved. Furthermore, phycocyanin was purified using two-step chromatographic method and the analytical grade purity (A_{620}/A_{280} ratio > 4) was attained. SDS-PAGE demonstrated the purity and presence of two bands corresponding to α- and β-subunits of the C-phycocyanin. The results showed that *Phormidium* sp. and *Pseudoscillatoria* sp. could be good candidates for phycocyanin production.

Keywords Chromatography · Cyanobacteria · Extraction · Phycocyanin · Purification

Introduction

Cyanobacteria (blue-green algae) have emerged as an important source of high-value products, counting important bioactive and biotechnologically significant chemicals like phycobiliprotein (PBP), carotenoids, and polyunsaturated fatty acids with potential biotechnological application in food, cosmetic, pharmaceutical, and nutraceutical industries (Begum et al. 2016). PBP are water-soluble light-harvesting antenna complexes found in cyanobacteria, rhodophytes (red algae), and cryptophytes (Glazer and Apell 1977), which absorb energy in portions of visible spectrum that poorly utilized by chlorophyll and transfer electrons to the photosynthetic reaction centers to drive photosynthetic electron transport (Kirilovsky 2015). Based on their spectral properties, PBP are categorized into three main types, namely phycoerythrin (PE) (bright pink: λ_{max} 490–570 nm), phycocyanin (PC) (dark cobalt blue: λ_{max} 610–625 nm), and allophycocyanin (APC) (brighter aqua blue: λ_{max} 650–660 nm) (Kuddus et al. 2013; Singh et al. 2015; Manirafasha et al. 2016). Cyanobacteria species are an intense and cost-effective source of the PBP, especially PC. Phycobilin pigments have gained importance as natural colorants in food and biotechnological industries by virtue of toxicity and carcinogenicity of synthetic colorants (Amchova et al. 2015; Dasgupta 2015). In addition, PC also exhibits several biological activities like antioxidant, anti-carcinogenic, anti-inflammatory, anti-angiogenic, and neuroprotective effects (Romay et al. 2003).

Cyanobacteria are considered as a potential candidate for PC production because of their rapid growth rate, CO_2 fixation ability, high production capacity, growth on non-arable

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lands, and a wide variety of water sources, and at the same time, some also can survive under extreme environmental conditions (Encarnação et al. 2015). There are reports on many cyanobacterial species that are known to produce PC (Fatma 2009; Kuddus et al. 2013). Recently, cyanobacteria of the genus *Scytonema* isolated from various terrestrial habitats have also been studied for the composition and distribution of PBP (Asencio and Hoffmann 2013). Despite the huge variety of cyanobacteria containing PC, *Arthrospira* (*Spirulina*) *platensis* (Lee et al. 2016), *Synechococcus* (Gupta and Sainis 2010), *Anabaena* (Chakdar et al. 2014), *Nostoc* (Lee et al. 2017), and *Oscillatoria* (Soni et al. 2006) are currently used for commercial production of PC. The rhodophyte *Galdieria sulphuraria* has also been proposed for commercial production of PC; however, this species contains the cellulose-rich cell wall, causing difficulties in cell disruption and pigment extraction. Therefore, red algal strain has not yet been used for commercial production of PC (Buchweitz 2016).

The growth, biochemical composition, and pigment content of cyanobacteria vary from species to species and are affected by various physicochemical parameters (Manirafasha et al. 2016). The effect of physicochemical parameters, i.e., temperature, pH, light quality and intensity, nutrient limitation, and their synergistic interaction effect on PBP synthesis was extensively studied and reported by many researchers for cyanobacteria and rhodophyta (Fatma 2009; Chen et al. 2010; Johnson et al. 2014; Rizzo et al. 2015; Baer et al. 2016). Nitrogen is considered as one of the most important macronutrients for cyanobacterial growth and PBP synthesis since it is a fundamental element for the proteins, chlorophyll, and nucleic acid formation. Studies have shown that cyanobacteria are able to assimilate a variety of nitrogen sources including ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), urea, and amino acids with several species also capable of fixing N_2 (Herrero et al. 2001). However, comparatively few studies have investigated the effect of different nitrogen sources on growth and PBP content of cyanobacteria (Choi et al. 2003; Rückert and Giani 2004; Flores et al. 2005; Simeunović et al. 2013; Deschoenmaeker et al. 2017). To the best of our knowledge, the influence of different nitrogen sources on marine *Phormidium* and *Pseudoscillatoria* spp. growth and PBP composition (PC, APC, and PE) has not been studied. Therefore, this study was undertaken to test marine *Phormidium* sp., *Pseudoscillatoria* sp., and a brackish, alkaliphilic *A. platensis* strain for their abilities to produce PBP and to evaluate the influence of three different nitrogen sources ($NaNO_3$, KNO_3, NH_4Cl) on growth and PBP content of these strains.

The specific objectives of this study were (i) to characterize marine cyanobacteria from Aegean Sea, Urla, Izmir, Turkey; (ii) to investigate the influence of different nitrogen sources on growth, and phycobiliprotein (PBP: PC, APC, and PE) composition of three cyanobacterial strains; and (iii) to develop a simple and efficient method for the extraction and purification of PC.

Material and methods

Strain, media, cultivation, and identification

The cyanobacterial strains were obtained from Ege University Microalgae Culture Collection (http://www.egemacc.com/cultures.php), Turkey (*Arthrospira platensis* EGEMACC 30, *Phormidium* sp. EGEMACC 72, and *Pseudoscillatoria* sp. EGEMACC 74). *Phormidium* sp. and *Pseudoscillatoria* sp. originated from Aegean Sea (Izmir/Turkey). Collection, isolation, and purification of strains were described in our previous study (Montalvão et al. 2016). The marine isolates were maintained and cultivated in BG-11 plus 25 g L^{-1} sea salt (Marinium reef sea salt) (indicated as sea salt BG-11) (Costa et al. 2016; Rippka et al. 1979) and *A. platensis* in Zarrouk's medium (Zarrouk 1966) at 22 ± 1 °C under continuous illumination of 80 μmol photons m^{-2} s^{-1}. Morphological observations were made using bright field (Olympus BX53, Japan) with × 60 and scanning electron microscopy (SEM) (Philips XL-30S FEG). The species were classified morphologically according to Desikachary (1959), Bourrelly (1970), and Rasoulouniriana et al. (2009).

DNA extraction and amplification

Isolated samples were harvested by centrifugation at 3500×*g* for 10 min. The concentrated cells were transferred into 1.5-mL microtubes and stored at − 20 °C. Genomic DNA was extracted using Quick-DNA Fungal/Bacterial MiniPrep kit (Zymo Research) according to the manufacturer's instruction. Amplification was performed in 25 μL reactions using primers 27F (5′-AGAG TTTGATCCTGGCTCAG-3′) and 809R (5′-GCTT CGGCACGGCTCGGGTCGATA-3′). The PCRs were performed using HelixAmp HyperSense DNA polymerase kit (NanoHelix), and conditions included preheating at 95 °C for 2 min, 35 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min, followed by another 5 min extension at 72 °C. The resulting PCR products were verified by gel electrophoresis (1.0% agarose gel). The nucleotide sequences of PCR products were determined by a genetic analyzer (Applied Biosystems 3130XL with16-capillary array). The obtained sequences were compared and aligned with GenBank nucleotide sequences of some known

cyanobacteria using NCBI-BLAST program to find the closest related organisms (http://www.ncbi.nlm.nih.gov/BLAST).

Inoculum preparation

Cultures were grown in 500-mL Erlenmeyer flasks containing 200 mL of standard (Zarrouk's medium for *A. platensis* and sea salt BG-11 for *Phormidium* and *Pseudoscillatoria* spp.) medium on a rotary shaker at 120 rpm, 25 ± 2 °C, and 80 μmol photons m^{-2} s^{-1}. Cells were harvested during the exponential growth phase, centrifuged, and pellet washed with physiological solution (0.9% NaCl) to remove nitrate. The recovered cells were then resuspended in nitrogen-free Zarrouk's and sea salt BG-11 medium and used as inoculum at 10% volume for all experiments.

Characterization of growth and biochemical composition of the strains under different nitrogen sources

The strains were characterized in presence of different nitrogen sources to study their impact on growth and biochemical composition (chlorophyll *a* (Chl-*a*), total protein content, PBP). In the present study, Zarrouk's and BG-11 media were selected as cultivation media since they were reported to be very convenient and effective for *A. platensis* and other cyanobacteria (Sassano et al. 2007; Manirafasha et al. 2016). The influence of different nitrogen sources was studied by replacing the original nitrogen source ($NaNO_3$) in the Zarrouk's (29.4 mM) and BG-11 (17.6 mM) media with equimolar concentration of KNO_3 and NH_4Cl in the experimental flasks, respectively. A set of three 500-mL conical flasks which contain 250 mL respective medium and 10% *v*/*v* inoculum for each nitrogen source was prepared and cultivated under previously optimized culture condition temperature of 33 ± 2 °C, irradiance of 44 μmol photons m^{-2} s^{-1} (LED CATA, Cool white), and agitation speed of 120 rpm (Khazi et al. 2018) over the period of 12 days. The growth, Chl-*a*, and phycobiliprotein content were monitored at 2-day intervals and the cultures were harvested after 12 days. High phycocyanin yielded culture biomass was taken for extraction and purification of phycocyanin.

Biomass and specific growth rate determination

Five milliliter of culture suspension was filtered through pre-dried and pre-weighed Whatman GF/C glass microfiber filters (Whatman UK) and washed with distilled water. Dry cell weights (DCWs) were calculated in g L^{-1} after the membrane was dried at 65 °C to a constant weight.

The specific growth rate (μ) and doubling time (t_d) were determined at the exponential growth phase of cultures through Chl-*a* concentration and time (Li et al. 2014) using Eqs. 1 and 2 respectively.

$$Specific\ growth\ rate\ \mu = \frac{(lnX_2 - lnX_1)}{t_2 - t_1} \quad (1)$$

where X_2 represents the Chl-*a* concentration (mg L^{-1}) at time t_2 (days) and X_1 represents the initial concentration (mg L^{-1}) at time t_1 (days).

$$t_d = \ln 2/\mu \quad (2)$$

Chl-*a* extraction and quantification

Five milliliter of culture was centrifuged for 5 min at 10,000×*g*. The pellet was suspended in 5 mL methanol (100%) for 20 min in a water bath at 65 °C, followed by centrifugation at 4500×*g* for 5 min. The optical density of the supernatant was determined at 665 and 750 nm. The Chl-*a* concentration was calculated with the following equation (Eq. 3) (Demirel et al. 2015).

$$\text{Chl-}a\left(\text{mg L}^{-1}\right) = 13.9\ (A_{665} - A_{750}) \quad (3)$$

Total protein quantification

Protein concentration was measured by the Lowry method (Lowry et al. 1951) using bovine serum albumin as a standard.

Total protein content (%) was determined as indicated in Eq. 4:

$$P\ (\%of\ \text{dry}\ weight) = \frac{X}{Y} \times 100 \quad (4)$$

where P (%) is the percent protein, X is total protein content, and Y is total biomass.

Spectroscopic determination of phycobiliprotein

Phycobiliproteins (PBP) (PC, APC, and PE) were extracted from cultures by the following method. Five milliliter of culture was centrifuged for 5 min at 10,000×*g*. The pellet was suspended in 5 mL of 100 mM Na phosphate buffer (pH 7). The suspension was sonicated (Bandelin, Sonopuls HD2070) at a frequency of 20 kHz for 2 min then centrifuged at 4500×*g* for 5 min. The PC, APC, and PE concentration in mg mL^{-1} was calculated from the optical densities at 562 nm (A_{562}), 652 nm (A_{652}), and 615 nm (A_{615}), using Eqs. 5, 6, and 7 (Bennett and

Bogorad 1973). The total phycobiliprotein content was calculated as shown in Eq. 8.

$$\mathrm{PC}\left(\mathrm{mg\ mL^{-1}}\right) = \frac{\{A_{615} - (0.474\,A_{652})\}}{5.34} \quad (5)$$

$$\mathrm{APC}\left(\mathrm{mg\ mL^{-1}}\right) = \frac{\{A_{652} - (0.208\,A_{615})\}}{5.09} \quad (6)$$

$$\mathrm{PE}\left(\mathrm{mg\ mL^{-1}}\right) = \frac{\{A_{562} - (2.41\,C\text{–}PC) - (0.849\,C\text{–}APC)\}}{9.62} \quad (7)$$

$$\textit{Total}\ \mathrm{PBP} = \mathrm{PC} + \mathrm{APC} + \mathrm{PE} \quad (8)$$

$CaCl_2$ extraction, purification, and SDS-PAGE analysis of C-PC

PC extraction using $CaCl_2$ solution was performed by a modified method (Herrera et al. 1989), in which, 50 mg of lyophilized cell mass was suspended in 5 mL of 1.5% $CaCl_2$ solution and kept overnight (12 h) for extraction and centrifuged at 6000×*g* for 15 min. Then, the crude extract of PC was fractionated by precipitation with ammonium sulfate first at 30% and then at 70% saturation with continuous stirring for 2 h followed by centrifugation at 6000×*g* for 15 min and the residue was dissolved in 50 mM Na phosphate buffer (pH 7.0). Afterwards, ammonium sulfate precipitates were subjected to dialysis for overnight against 1 L Na phosphate buffer using dialysis tubing cellulose membrane (Sigma-Aldrich). Dialyzed sample was further purified by passing through Sephadex G-25 media packed in column (3 × 30 cm) pre-equilibrated and eluted with 50 mM Na phosphate buffer (pH 7.0). Collected fractions of size exclusion chromatography were further subjected to DEAE-Sephadex column (3 × 30 cm) and absorbed PC was eluted with a linearly increasing ionic concentration gradient of NaCl from 0.1 to 0.25 M. The purity of the fractions collected after each step was calculated according to the purity ratio (A_{620}/A_{280}) and the fraction showing maximum purity was selected for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The purity and estimation of molecular weight of PC subunits were determined by SDS-PAGE in a vertical slab gel apparatus (Kuru Gel verti 1010YK, VWR) with 12.5% polyacrylamide slab gel (5% stacking gel) 1 mm thickness, according to the method described by Laemmli (1970). Electrophoresis was performed at 120 V and 20 mA for about 2–3 h at room temperature. After electrophoresis, bands were stained with Coomassie brilliant blue R-250. The sizes of the C-PC subunits bands were determined by comparison with standard PC (Sigma-Aldrich) and protein markers with a range of molecular weight from 7 to 175 kDa (New England BioLabs, USA).

Statistical analysis

All the experiments were conducted in triplicate and the data calculated were expressed as mean ± standard deviation. Statistical significance ($p < 0.05$) for the data obtained on the 12th day was analyzed using one-way analysis of variance (ANOVA) (biomass, Chl-*a*, total protein, and phycobiliprotein content as the response variables for the factors $NaNO_3$, KNO_3, and NH_4Cl). When significant differences among groups were identified, multiple comparisons among means were made using the Tukey post hoc test with IBM SPSS Statistics version 23.0 software.

Results

Identification of cyanobacterial strains

Preliminary morphological observation of cyanobacterial cultures by bright field and scanning electron microscopy (SEM) analysis showed that the isolates (EGEMACC 72 and EGEMACC 74) belonged to the genus *Phormidium* and *Pseudoscillatoria*. The cells of isolate EGEMACC 72 were filamentous, filaments unbranched, more or less straight or curved, blue-green, 1.4–2 μm broad, thin layer of sheath, non-lamellated; trichomes 3–3.5 μm broad, trichomes not constricted at cross-walls, not granulate, end cells not attenuated, end cell rounded, and cross-wall not visible (Fig. 1a, c). The cells of isolate EGEMACC 74 were cylindrical, unbranched, blue-green, blunt cells at the ends, and trichomes 2.5–3.5 μm broad and were found to leave a thin mucilaginous trail as they glide, mucilaginous sheath absent (Fig. 1b, d). Furthermore, the isolates were identified based on partial 16 rRNA gene sequences, reviewed with BLASTn. The partial 16S rDNA gene sequence coding for the ribosomal RNA of the strains was sequenced and submitted to GenBank (Accession Numbers: HQ916865, HQ916861). The BLAST analysis of the corresponding sequences showed that the isolate EGEMACC 72 was closely related to *Phormidium* sp. (HQ916865) with sequence similarity of 92% as well as it was the representative of the genus *Phormidium*. The comparison of rDNA gene sequence (781 bp) revealed that the isolate EGEMACC 74 was closely related to *Pseudoscillatoria* sp. (HQ916861) with maximum sequence similarity of 97%.

Variation in growth rate, biomass, and Chl-*a* concentration with different nitrogen sources

The impact of different nitrogen sources on growth, biomass production, and Chl-*a* content of cyanobacteria strains, i.e., *A. platensis*, *Phormidium* sp., and *Pseudoscillatoria* sp., were investigated. The time course profiles of the Chl-*a* concentration obtained in different nitrogen sources are shown in Fig. 2.

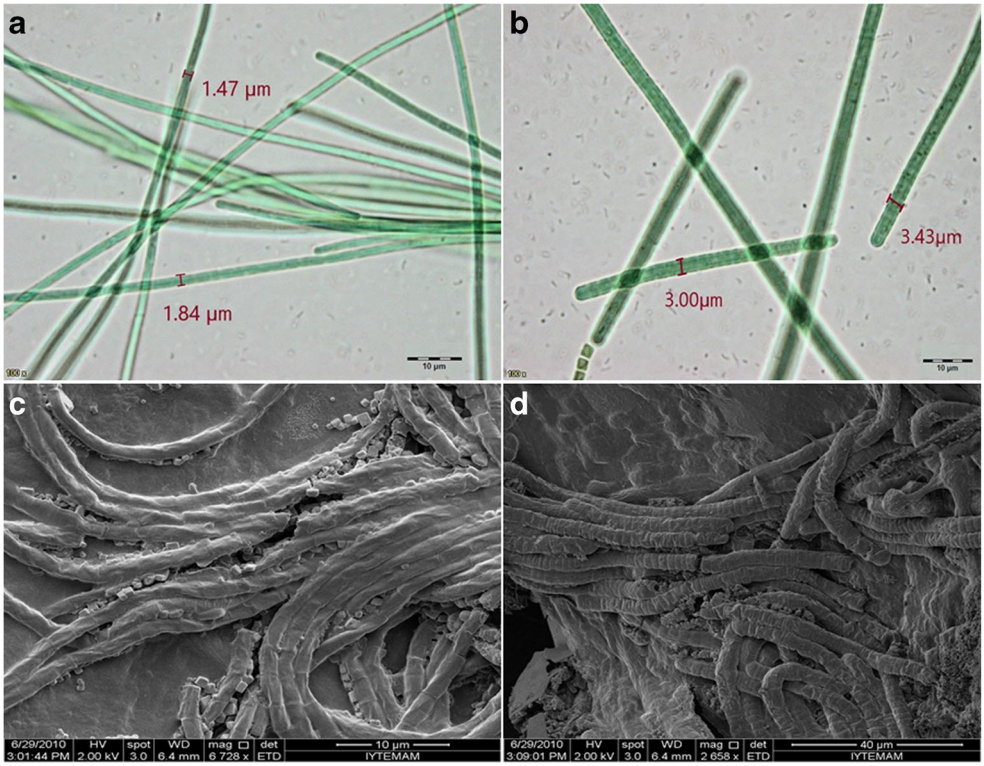

Fig. 1 Microphotographs of **a** *Phormidium* sp. and **b** *Pseudoscillatoria* sp. Scanning electron microscopic images of **c** *Phormidium* sp. and **d** *Pseudoscillatoria* sp.

The brackish and alkaliphilic *A. platensis* exhibited the fastest growth rate of 0.304 ± 0.0 day^{-1} in Zarrouk medium containing $NaNO_3$ and reached the highest biomass of 1.56 ± 0.1 g L^{-1} and Chl-*a* content of 19.1 ± 0.5 mg L^{-1}. This was followed by biomass of 1.08 ± 0.06 g L^{-1} and Chl-*a* of 16.27 ± 0.7 mg L^{-1} with the growth rate of 0.261 ± 0.0 day^{-1} in the medium with KNO_3. The differences between the growth rate (ANOVA: $d.f. = 1$, $F = 44.431$, $p = 0.003$) and Chl-*a* (ANOVA: $d.f. = 1$, $F = 31.756$, $p = 0.005$) using $NaNO_3$ and KNO_3 were statistically significant. Conversely, the given NH_4Cl concentration of 29.42 mM exhibited toxic effect on the growth of *A. platensis*. The marine isolates *Phormidium* sp. *and Pseudoscillatoria* sp. cultured in sea salt BG-11 medium with NH_4Cl (17.6 mM) grew better and provided significantly higher biomass (1.225 ± 0.0 and 1.12 ± 0.01 g L^{-1}), Chl-*a* (16.2 ± 0.5 and 12.2 ± 0.1 mg L^{-1}), and the growth rates (0.284 ± 0.03 and 0.274 ± 0.13 day^{-1}) than that with $NaNO_3$ and KNO_3 (Tukey's post hoc: $p = 0.000$) (Table 1).

Variation in total protein and PBP content

Total protein profiles of examined cyanobacteria strains cultivated with three nitrogen sources are shown in Table 1. Protein content % (based on DCW) showed a gradual enhancement with the period of incubation for all investigated nitrogen source. The three examined strains had protein contents in the range of 35–45% under investigated culture conditions and nitrogen sources. The alkaliphilic *A. platensis* exhibited the highest protein content (45.5 ± 0.2% of dry weight) in Zarrouk's medium with $NaNO_3$ as a nitrogen source, followed by KNO_3 (43.4 ± 0.1% of dry weight). Since *A. platensis* growth inhibition occurred with NH_4Cl, the total protein and PBP content measurements were not conducted for these flasks, whereas marine isolates *Pseudoscillatoria* sp. and *Phormidium* sp. showed the maximum protein contents of 43.2 ± 0.2 and 38.3 ± 0.1% of dry weight in sea salt BG-11 medium with NH_4Cl as nitrogen source. The total protein content of *Pseudoscillatoria* sp. (38.4 ± 0.1% of dry weight) with $NaNO_3$ as nitrogen source was similar with NH_4Cl (38.3 ± 0.1% of dry weight) as nitrogen source for *Phormidium* sp.

According to Table 1 and Fig. 3, *A. platensis* cultured in Zarrouk medium with $NaNO_3$ as nitrogen source provided a significantly higher total PBP (22.27 ± 0.2% of dry weight) with the high PC content of 14.53 ± 0.16%, APC of 5.89 ± 0.05%, and PE of 1.84 ± 0.0% of dry weight under provided culture conditions (temperature of 33 ± 2 °C, irradiance of 44 µmol photons m^{-2} s^{-1}, and agitation speed of 120 rpm) (ANOVA: $d.f. = 1$, $F = 10.515$, $p = 0.032$). This was followed by KNO_3 (PBP 21.73 ± 0.19% of dry weight). The total PBP content of two marine strains grown with three different nitrogen sources was significantly different. *Pseudoscillatoria* sp. cultured in sea salt BG-11 medium with NH_4Cl exhibited higher PBP content of 19.99 ± 0.14% with the PC (14.22 ± 0.15), APC (4.4 ± 0.01), and PE (1.36 ± 0.02) % of dry weight than those with other nitrogen sources (Tukey's post hoc: $p =$

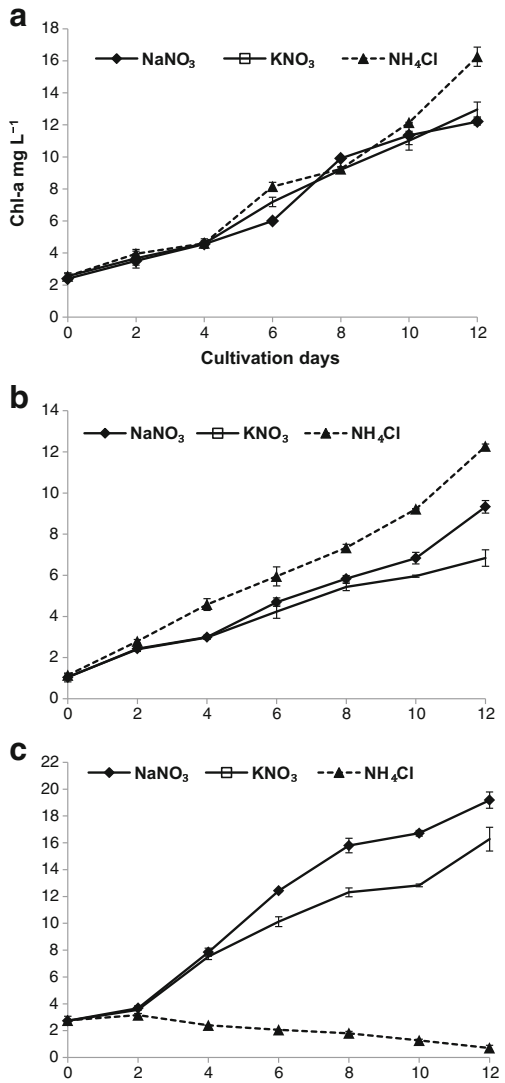

Fig. 2 Growth profile of **a** *Phormidium* sp., **b** *Pseudoscillatoria* sp., and **c** *A. platensis* cultivated under different nitrogen sources

0.000), whereas *Phormidium* sp. cultured in sea salt BG-11 with NH_4Cl produced 19.38 ± 0.09% PBP with PC (13.36 ± 0.10), APC (4.34 ± 0.06), and PE (1.67 ± 0.06) % of dry weight.

Extraction and purification of phycocyanin

After 12 h incubation of biomass with $CaCl_2$ solution on rotary shaker, the supernatant was collected by centrifugation the purity ratio (A_{620}/A_{280}) of PC in crude extracts was observed as greater than 1 for all the tested samples (Table 2). In order to achieve reactive grade purity, the C-PC from crude extract was further purified by a three-step sequential method consisting of fractional ammonium sulfate precipitation, Sephadex G-25 gel filtration, and DEAE-Sephadex ion-exchange column chromatography. The results of the purification profile of C-PC are summarized in Table 2. The crude extract of PC was fractionated by precipitation with ammonium sulfate (first at 30% and then at 70% saturation). The resulting ammonium sulfate precipitates were dialyzed and the purity (A_{620}/A_{280}) after this step was calculated as greater than 2. The dialyzed samples were further purified by passing through Sephadex G-25 media, which resulted in increased purity ratio greater than 3, and after purification by DEAE-Sephadex, the attained purities of C-PC for *A. platensis*, *Phormidium* sp., and *Pseudoscillatoria* sp. were 4.33, 4.14, and 4.4, respectively.

Purity and molecular weight confirmation

The purity of fractions obtained after DEAE-Sephadex ion-exchange chromatography was confirmed using SDS-PAGE (Fig. 4). The α-PC subunits of *A. platensis* and *Phormidium* sp. have the same molecular weight of 18 kDa, whereas the

Table 1 Summary of specific growth rate, biomass concentration, Chl-*a*, total protein, and PBP content (values obtained on the 12th day) of examined cyanobacteria strains cultivated with various nitrogen sources. Values represent the mean ± SD of three replicates

Strain	Nitrogen source	Specific growth rate based on Chl-*a* (μ_{max} day^{-1})	$t_{d(day)}$	Biomass concentration DW (g L^{-1})	Chl-*a* (mg L^{-1})	Total protein (%DW)	PBP (%DW)
A. platensis	$NaNO_3$	0.304 ± 0.00	2.2	1.56 ± 0.1	19.1 ± 0.5	45.5 ± 0.2	22.27 ± 0.21
	KNO_3	0.261 ± 0.00	2.6	1.08 ± 0.06	16.27 ± 0.7	43.4 ± 0.1	21.73 ± 0.19
	NH_4Cl	*	*	*	*.	*	*
Phormidium sp.	$NaNO_3$	0.250 ± 0.00	2.7	1.18 ± 0.01	12.2 ± 0.2	35.5 ± 0.1	18.53 ± 0.31
	KNO_3	0.221 ± 0.03	3.1	1.04 ± 0.03	12.8 ± 0.4	36.2 ± 0.1	18.74 ± 0.17
	NH_4Cl	0.284 ± 0.03	2.4	1.225 ± 0.00	16.2 ± 0.5	38.3 ± 0.1	19.38 ± 0.09
Pseudoscillatoria sp.	$NaNO_3$	0.264 ± 0.03	2.6	0.87 ± 0.04	9.3 ± 0.3	38.4 ± 0.1	18.9 ± 0.27
	KNO_3	0.204 ± 0.00	3.3	0.69 ± 0.03	6.8 ± 0.4	34.7 ± 0.2	16.92 ± 0.21
	NH_4Cl	0.274 ± 0.13	2.5	1.12 ± 0.01	12.2 ± 0.1	43.2 ± 0.2	19.99 ± 0.14

*Not detected because of growth inhibition

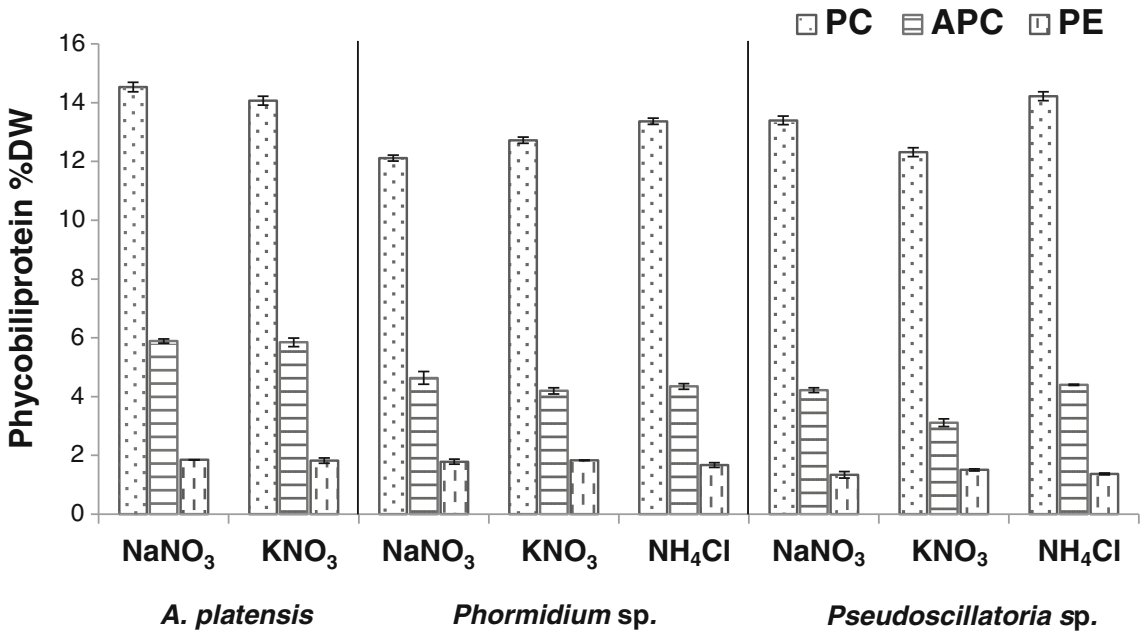

Fig. 3 Phycobiliprotein content of examined cyanobacteria strains grown under various nitrogen sources

molecular weights of β-subunits were 22 and 21.5 kDa (lanes B, C, and D), respectively. The molecular weights of α- and β-PC subunits of *Pseudoscillatoria* sp. were slightly different and calculated to be 17.5 and 21 kDa, respectively (lane E).

Discussion

Among the nutrients required for the growth of cyanobacteria, nitrogen is considered as an important element which is required for the growth and synthesis of proteins and other cellular components. Changes in nitrogen source and amount of cultivated media limit the growth and alter their pigment and biochemical composition. Growth on different nitrogen sources had different effects on the growth rate of studied cyanobacteria strains, and there was a significant difference among cultures containing $NaNO_3$ and the other nitrogen sources (KNO_3 and NH_4Cl) in terms of the final cell density and Chl-*a* concentration. In the present study, it was found that nitrate salts ($NaNO_3$ and KNO_3) were more favorable for the growth of *A. platensis* than that of ammonium salts. The ammonium chloride (29.42 mM) in the medium changed the *A. platensis* biomass color from green to yellow and the cells died. Ferreira et al. (2010) also reported the similar problem during *A. platensis* growth with ammonium (15–16 mM) as nitrogen source. Regarding the mechanism of ammonia toxicity, it has been reported that ammonia affects the oxygen evolving complex (OEC) by displacing a water ligand to the outer manganese cluster of the OEC, leading to loss of PSII function (Drath et al. 2008; Gutierrez et al. 2016; Markou and Muylaert 2016). The growth inhibition/cell lysis of *A. platensis* with NH_4Cl can be attributed to the fact that the ammonium concentration above 10 mM in the medium results in the decrease of pH by releasing H^+ ions during ammonium utilization and uncontrolled ammonium influx (Converti et al. 2006; Kim et al. 2016). Therefore, when using ammonium salts as a nitrogen source for *A. platensis* and for other cyanobacteria, the concentration must be kept low (Ferreira et al. 2010), and the acidification of the medium during growth on NH_4Cl should be resolved by increasing the buffering capacity of the growth medium by addition of HEPES-NaOH to the medium (Liotenberg et al. 1996; Sakamoto et al.

Table 2 Summary of C-PC purification from examined cyanobacteria strains

Strain	Nitrogen source	$CaCl_2$ crude extract (A_{620}/A_{280})	70% NH_4SO_4 fractionation (A_{620}/A_{280})	Dialysis (A_{620}/A_{280})	Sephadex-25 (A_{620}/A_{280})	DEAE-Sephadex (A_{620}/A_{280})
A. platensis	$NaNO_3$	1.18	1.77	2.31	3.28	4.33
	KNO_3	1.07	2.03	2.19	3.0	4.11
Phormidium sp.	NH_4Cl	1.15	2.34	2.44	2.94	4.14
Pseudoscillatoria sp.	NH_4Cl	1.12	1.92	2.18	3.31	4.4

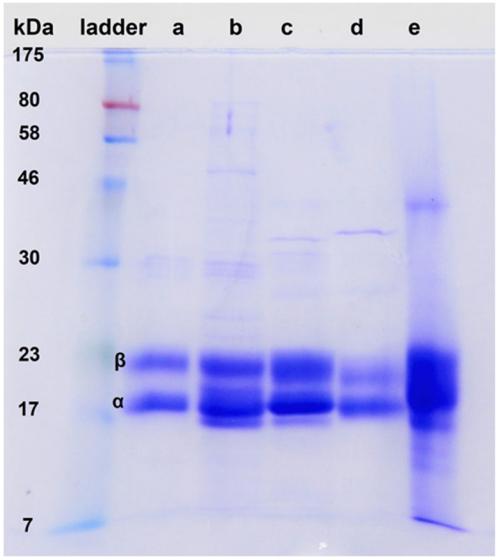

Fig. 4 SDS-PAGE profile of purified C-PC: ladder; lane a—*A. platensis* C-PC obtained from $NaNO_3$ culture; lane b—*A. platensis* C-PC obtained from KNO_3 culture; lane c—*Phormidium* sp. C-PC obtained from NH_4Cl culture; lane d—*Pseudoscillatoria* sp. C-PC obtained from NH_4Cl culture; lane e—C-PC standard

1998). In a review article, Collos and Harrison (2014) reported that the optimal ammonium concentration for cyanophyceae was 3000 μM, whereas 13,000 μM concentration was toxic.

On the other hand, the ammonium concentration of 17.6 mM in sea salt BG-11 medium did not show growth inhibitory effect and it was the most favorable nitrogen source for the cell growth and biomass production of *Phormidium* sp. and *Pseudoscillatoria* sp. than $NaNO_3$ and KNO_3. The higher growth rates and Chl-*a* contents of these strains with NH_4Cl may be due to higher uptake and assimilation rates on ammonia than nitrate. Since ammonium is the reduced form of nitrogen, cell incorporates ammonia directly into the nitrogen metabolism whereas nitrate or nitrite must first be reduced to ammonium prior to its utilization.

According to Madkour et al. (2012), the protein biosynthesis in *A. platensis* increases with an increase of nitrogen concentration in the cultivation medium, whereas low nitrogen concentrations lead to a decrease in the level of these compounds. In the present study, 29.42 mM concentration of nitrogen source in Zarrouk's and 17.6 mM in sea salt BG-11 medium yielded the total protein contents of 45.5 ± 0.2 and 43.5 ± 0.2% of dry weight in *A. platensis* and *Pseudoscillatoria* sp., respectively. The total percentage of protein was less when compared to other reported cyanobacterial strains. Rizzo et al. (2015) found a protein content value of 59% in *A. platensis* using Zarrouk's medium after 18 days of growth under green light at an irradiance of 50 μmol photons m^{-2} s^{-1}, whereas the present experiments were carried out at 44 μmol photons m^{-2} s^{-1} over the period of 12 days, so the obtained protein contents were lower than the results previously reported. The PBP contents of three cyanobacterial strains obtained in the present study was ranging from 16.92 ± 0.2–22.27 ± 0.2% of dry weight, which are remarkable in comparison with previously reported PBP contents of several cyanobacterial strains (8.4 ± 0.4–38.3 ± 1.9% of dry weight) (Khattar et al. 2015). The effect of using different nitrogen sources and their concentrations on PBP of *A. platensis* and other cyanobacterial strains has also been evaluated by other researchers (Vieira Costa et al. 2001; Madkour et al. 2012; Ajayan et al. 2012). With an increase in nitrogen concentration in the culture medium, PBP content in cyanobacteria increases to a certain extent and further increase does not affect the PBP production. It has been reported that the maximum C-PC content of *A. platensis* increased from 10% to nearly 12% as the initial nitrogen source concentration rose from 0.03 to 0.045 M, while further increasing the $NaNO_3$ concentration did not enhance C-PC content or productivity (Chen et al. 2013). In contrary, nitrogen limitation in the medium triggers the degradation of PBP, since PBP serves as a nitrogen storage compound (Boussiba and Richmond 1980). Therefore, the availability of sufficient nitrogen is known to be an important factor for PBP production in cyanobacteria. In the present study, 29.42 mM concentration of $NaNO_3$ in Zarrouk's medium and 17.6 mM concentration of NH_4Cl in sea salt BG-11 medium exhibited better results on PBP production of *A. platensis* (22.27 ± 0.21% of dry weight) and marine isolates *Phormidium* and *Pseudoscillatoria* (19.38 ± 0.09 and 19.99 ± 0.14% of dry weight).

PC is the main PBP in *A. platensis* and comprises up to 20% of its dry weight (Cuellar-Bermudez et al. 2015). The PC values found in the present study ranged from 12.1 ± 0.08 to 14.5 ± 0.16% of dry weight under different nitrogen sources evaluated. Similar to our findings, Ajayan et al. (2012) reported the PC values of *A. platensis* in the range of 9.92 ± 0.13–14.81 ± 0.2% which were obtained with KNO_3 and urea as a nitrogen source, respectively. The three strains of *Arthrospira* were analyzed for their PC content by Bhattacharya and Shivaprakash (2005) and values obtained were within the range of 9.4–14.8%. The results of the present study demonstrated that PC content changed markedly depending on the nitrogen source. The PC content of *Phormidium* and *Pseudoscillatoria* sp. cells grown with ammonium was 5–15% higher than those cultured with nitrate salts. Similar to our results, phycobilisomes isolated from ammonium-grown *Synechococcus* sp. PCC 7002 cultures contained 10–20% more PC than those grown with nitrate (de Lorimier et al. 1992). The nitrogen source in culture medium markedly influenced the PE and PC content of the *Calothrix* sp. and observed that PE was 35% lower in ammonium compared to nitrate-grown cells, whereas PC was 46% higher (Liotenberg et al. 1996). In the present study, although the PC content was significantly higher in *A. platensis* compared with two marine strains, it is also important to consider the cost of nitrogen and other nutrients for developing cost-effective algal pigment

production. To overcome high production costs, cheap resources should be found. Our study evaluated the possibility of using ammonium salts by marine strains, which were cheaper than nitrate salt. Therefore, *Phormidium* sp. and *Pseudoscillatoria* sp. could be a good candidate for large-scale PC production.

Since the maximum PBP yield was found in the cultures of *Phormidium* sp. and *Pseudoscillatoria* sp. with NH_4Cl and *A. platensis* grown with $NaNO_3$ and KNO_3, these culture biomasses were selected to carry out extraction and purification steps. Many methods for PC extraction and purification have been described in the literature (de Jesús et al. 2016; Eriksen 2008). The efficiency of PC extraction may vary depending on the properties of the buffer, the duration of the extraction, biomass concentration, mode of cell disruption (physical/chemical cell disruption), and species of cyanobacteria. Therefore, no standard procedure was available for maximal extraction of PC from cyanobacterial cells (Sobiechowska-Sasim et al. 2014; Horváth et al. 2013). Among the various extraction methods, freezing and thawing was reported to be the most efficient method for PC extraction (Soni et al. 2006), whereas using ultrasonic bath in the presence of glass pearls in the *A. platensis* biomass has been reported to have 57% higher extraction efficiency than freezing and thawing method (Moraes et al. 2011). However, all these methods involved cell disruption which needs to supply some extra energy to promote cell breakage. In addition, mechanical methods of disintegration release a large number of contaminants from the cells which resulted in high viscosity and low purity in the crude extract. To circumvent these problems, we tested the extraction method using calcium chloride 1.5% salt solution without applying any cell disruption methods. The commercial use of PC in the food does not require high purity. Therefore, $CaCl_2$ utilized in this study was found to be an effective salt solution to attain food grade purity (A_{620}/A_{280} ratio greater than 0.7) in crude extracts. Various methods have been reported for the purification of PC from cyanobacteria but all these methods involve the combination of chromatographic and non-chromatographic methods such as aqueous two-phase extraction (Patil et al. 2008), ultrafiltration (Singh et al. 2009), expanded bed chromatography (Moraes et al. 2015; Jian-Feng et al. 2007), hydroxyapatite chromatography, ammonium sulfate precipitation, ion-exchange, and gel permeation chromatography (Zhang and Chen 1999; Kumar et al. 2014). Patil et al. (2008) employed a non-chromatographic (aqueous two-phase extraction) method to purify PC from *A. platensis* and achieved the final purity of 3.23, whereas with the combination of aqueous two-phase extraction and ion-exchange chromatography, the purity of 6.69 was achieved from *A. platensis* (Patil et al. 2006). Similarly, Singh et al. (2009) combined ultrafiltration and ion-exchange chromatography to obtain C-PC with A_{620}/A_{280} of 4.1 from *Phormidium ceylanicum*. Chakdar et al. (2014) reported the purity of 2.75 from *Anabaena variabilis* CCC421 after precipitation with ammonium sulfate and DEAE-cellulose ion-exchange chromatography; however, this method does not satisfy the standard of reactive or analytical grade purity (A_{620}/A_{280} ratio greater than 3.9 and 4). Depending on the application of PC, the method should be selected based on purification need. In the present study, our procedures having shown efficient separation and purification of C-PC from *A. platensis*, *Phormidium* sp., and *Pseudoscillatoria* sp. with purity ratio greater than 4 satisfy the standard for analytical grade. Therefore, this purification procedure was found to be suitable for small scale and an alternative for industrial scale.

In conclusion, our results showed that three different nitrogen sources had different effects on the growth and PBP accumulation in *A. platensis*, *Phormidium* sp., and *Pseudoscillatoria* sp. Among the three nitrogen sources, NH_4Cl was found as a favorable source for growth and PC production of *Phormidium* and *Pseudoscillatoria* sp., whereas $NaNO_3$ was found as an optimal nitrogen source for *A. platensis*. Calcium chloride utilized in the present study was found to be an effective salt solution to extract C-PC and achieve food grade purity (A_{620}/A_{280} ratio greater than 0.7) in crude extracts, while reactive/analytic grade purity (A_{620}/A_{280} ratio greater than 3.9) was achieved after Sephadex and DEAE-Sephadex ion-exchange chromatography. The strains *Phormidium* sp. and *Pseudoscillatoria* sp. isolated in the present study may prove to be promising cyanobacteria for C-PC production.

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