

# Recovery of pure C-phycoerythrin from a limestone drought tolerant cyanobacterium *Nostoc* sp. and evaluation of its biological activity

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## Resumen

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*Recuperación de C-ficoeritrina de la cianobacteria tolerante a la sequía Nostoc sp. en caliza y evaluación de su actividad biológica*

Se realizó la caracterización de ficoeritrina de la cepa A5 de *Nostoc* sp., seguida de investigación de su actividad biológica para aplicaciones biotecnológicas. Para la extracción de ficoeritrina, el uso de tampón acetato (pH 5.1) produjo  $65.04 \mu\text{g mL}^{-1}$ , y se identificó como C-ficoeritrina. Los resultados de su actividad antioxidante sugirieron su acción como un potente eliminador de radicales libres. Además, la C-ficoeritrina de *Nostoc* mostró una capacidad notable como agente antibacteriano y antifúngico, con estabilidad significativa de hasta 10 días. La glucosa ( $4 \text{ mg mL}^{-1}$ ) fue un buen conservante para la C-ficoeritrina a 25 y 4 °C. Se obtuvo por primera vez una C-ficoeritrina estable de *Nostoc* sp. en condiciones de sequía en piedra caliza, lo que demuestra la necesidad de estudiar microorganismos de ambientes extremos.

**Palabras clave:** C-ficoeritrina (C-PE); Cianobacterias tolerantes; *Nostoc*; Actividad biológica.

## Abstract

The phycoerythrin characterization from *Nostoc* sp. strain A5 was done, followed by investigation of its biological activity for biotechnological applications. For phycoerythrin extraction, the use of acetate buffer (pH 5.1) resulted in  $65.04 \mu\text{g mL}^{-1}$ , and C-phycoerythrin was identified. Results of its antioxidant activity suggested action as a potent free radical scavenger. In addition, *Nostoc*'s C-phycoerythrin showed noteworthy ability for antibacterial and anti-fungal agents with significant stability up to 10 days. Glucose ( $4 \text{ mg mL}^{-1}$ ) was a good preservative for C-phycoerythrin at 25 and 4 °C. A stable C-phycoerythrin from *Nostoc* sp. was obtained for the first time from limestone drought conditions, showing the need of studying microorganisms from extreme environments.

**Key words:** C-phycoerythrin (C-PE); Tolerant cyanobacteria, *Nostoc*; Biological activity.

## Introduction

Cyanobacteria are prokaryotes with oxygenic photosynthetic ability that possess a wide range of coloured components, including carotenoids, chlorophyll and phycobiliproteins. Phycobiliproteins are water-soluble supramolecular protein aggregates involved in light harvesting during photosynthesis and may comprise from 40 to 60 % of the total soluble proteins found in cyanobacteria (Kumar *et al.* 2014). They are highly diverse in structure and pigment composition, acting as cell protection against photo-oxidative damage under stress conditions (Pandey *et al.* 2013). Moreover, these colored proteins have considerable importance as natural dyes and possess health-promoting properties in addition to pharmaceutical applications as antimicrobial and antioxidants agents (Liu *et al.* 2014, Nowruzi *et al.* 2018a). Antioxidant molecules are important to prevent or delay hepatotoxicity, heart diseases and cancer (Kim *et al.* 2018). The use of artificial oxidants has reduced due to their potentially carcinogenic nature (Sen *et al.* 2017). Thus, natural antioxidants are desirable and have been intensively examined (Maurya *et al.* 2014).

Phycobiliproteins can be divided into three classes based on their spectral properties: phycoerythrin ( $\lambda_{\text{max}} \sim 565$  nm), phycocyanin ( $\lambda_{\text{max}} \sim 620$  nm), and allophycocyanin ( $\lambda_{\text{max}} \sim 650$  nm). A fourth phycobiliprotein known as allophycocyanin B ( $\lambda_{\text{max}} \sim 670$  nm) has also been shown to be present in cyanobacteria in low amounts (Kumar *et al.* 2014). Light absorbed by phycoerythrin is transferred to phycocyanin, then to allophycocyanin and ultimately to chlorophyll during the photosynthetic process (Seo *et al.* 2013). Phycoerythrins (PEs) are highly sensitive to variations in pH, salt concentration, temperature change, desiccation and light stress (Kesheri & Sinha 2011).

*In vitro* processes, such as PEs extraction and purification, difficult its commercial applications. The amount of phycoerythrin can change due to a chromatic adaptation mechanism. Under low light intensity, synthesis of phycoerythrin is promoted to elongate rod structures (Wada *et al.* 2013). There are typical protocols for PE extraction and purification. Chakdar & Pabbi (2012) reported that precipitation with 65 % ammonium sulfate resulted in 85.81 % recovery of PE with a purity of 2.81 (OD562/OD280). Ranjitha & Kaushik

(2005) obtained 85 % recovery of PE from *Nostoc muscorum* C.Agardh ex Bornet & Flahault with a purity of 2.89 (A562/A280) using 55 % ammonium sulfate precipitation. Around 80 % recovery of PE content with a purity ratio of around 1.5 was shown for young and old cultures of *Phormidium* sp. A27DM, *Lyngbya* sp. A09DM and *Halomicronema* sp. A32DM after treatment of crude extract with 70 % ammonium sulfate (Parmar *et al.* 2011). Similarly or higher than reported by Tchernov *et al.* (1999), Reis *et al.* (1998) and Parmar *et al.* (2011), PE purity of 4.95 was achieved by Chakdar & Pabbi (2012) during chromatographic separation. Moreover, chromatography on DEAE cellulose-52 column gave pure PE (A 562 /A 280=8.12) from *N. muscorum* (Ranjitha & Kaushik, 2005). Tripathi *et al.* (2007) shown a PE purity of 5.25 from *Lyngbya arboricola* Brühl & Biswas using a procedure involving acetone precipitation and gel filtration in addition to ammonium sulfate precipitation and DEAE-Cellulose column chromatography. However, the recovery (62.5 %) was not as higher as published by Ranjitha & Kaushik (2005) and Parmar *et al.* (2011).

Using different process, phycoerythrin may be used as natural colorants by industries engaged in food and feed production. The potential from soil and fresh water cyanobacteria to produce bioactive compounds has been widely explored (Cadel *et al.* 2008, Nowruzi *et al.* 2018b). However, extraction and purification of phycoerythrins from calcareous environments especially that associated with limestone surfaces are underexplored. In the present studied, the *Nostoc* isolate was obtained from Cretaceous nodular chalk limestone rocks, where the light intensity is extremely high and water is scarce. We hypothesize that the drought tolerant *Nostoc* sp. A5 produces a stable phycoerythrin (red pigment) to absorb the green light. Hence, this study was conducted in order to understand the properties and degree of stability of PE after extraction and purification from a limestone cyanobacterium and evaluate its biological activities (e.g., antioxidant, antimicrobial activity).

## Material and methods

### Cyanobacterial isolation and growth conditions

*Nostoc* sp. strain A5 was isolated from a cliff face

in the North-West Mountains of Khozestan province ( $34^{\circ}25'04''N$ ,  $47^{\circ}00'59''W$ ), Iran, which consists of Cretaceous nodular chalk limestone. In order to obtain a cyanobacterial monoculture, samples were spread into sterile Petri dishes containing solid BG-11 medium (Allen 1968), without a nitrogen source, pH 7.1, and incubated in a growth chamber for two weeks at  $28\pm1$  °C under constant artificial illumination of 1500–2000 lux. After 14 days of growth, selected colonies were washed three times with deionized water, and transferred to a fresh solid medium. For bacteria-free cultures, colonies were tested for bacterial contamination in dextrose-peptone broth and caseinate-glucose agar media. The selected bacteria-free colony was maintained on different agar slants. After 20 days, the isolate was washed with sterile deionized water, and transferred to 1 L of freshly prepared liquid BG11<sub>0</sub> medium.

#### Morphological characterization of the studied strain

The 10-days-old cyanobacterial strain A5 was examined under a Leica DM750 microscope and morphological descriptions were made according to the classification system devised by Komárek 2013.

#### Molecular analysis

Genomic DNA was extracted from living cells using the EZNA SP Plant DNA mini kit (Omega Bio-Tek) according to the manufacturer's instructions. PCR reactions were performed on genomic DNA using the oligonucleotide primers pA (5'-AGAGTTGATCCTGGCTCAG-3') and B23S (5'-CTTCGCCTCTGTGTGCCTAGGT-3') (Stoyanov *et al.* 2014) that target the 16S rRNA gene sequence. Reactions were cycled with an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 55 °C and 30 s extension at 72 °C, and final extension step at 72 °C for 5 min. Amplicons were verified by gel electrophoresis on a 1 % agarose gel stained with ethidium bromide. PCR products were purified using the Geneclean® Turbo kit (Qbiogene, Inc.) prior to sequencing. Sequencing reactions were carried out using the refined PCRs products in a ABI Prism 310 Genetic Analyzer (Applied Biosystems, Life Technologies). A total volume of 10 µL of the PCR master mix included 1 µL of forward or reverse primers (10 µM), 1 x sequencing buffer, 1 µL of

Big dye and 100 ng (1 µL) of DNA. The cycle sequencing reaction was carried out using 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 minutes, followed by storage overnight at 4 °C. After the completion of the sequencing reactions, the sequenced products were precipitated by adding 40 µL of 0.125 M NaCl and 2.5 x volume of cold 100 % ethanol, followed by vortexing and centrifugation at 13000×g for 10 min at 4 °C. Once the supernatant was removed, a 5x volume of 70 % ethanol was added, and the sample centrifuged at 13000×g for 5 minutes (4 °C). The supernatant was removed, and the pellet was dried at 37 °C. The purified reaction was resuspended in 12 µL of HiDi-formamide, the mixture was spun down, denatured for 2 min at 94 °C and subjected to sequencing. The runtime for each reaction was 45 min with a running voltage of 15 kV at a temperature of 50 °C and the polymer used was POP-6™ (Applied Biosystems, Life Technologies). The 16S rRNA gene sequences obtained in this study was used to construct a consensus sequence in BioEdit version 7.0. Positions with gaps, as well as undetermined and ambiguous sequences were removed. BLAST searches (<http://www.ncbi.nlm.nih.gov/BLAST>) of the partial 16S rRNA gene sequence were used to identify similar sequences available in the GenBank database of NCBI (National Center for Biotechnology Information).

#### Chemicals

All chemicals and protein molecular weight marker used in this study were of analytical grade, purchased from the Hi-Media, Merck and Sigma manufacturers. All buffers and reagents used were prepared in double distilled water.

#### Extraction and estimation of phycobiliproteins

Phycoerythrin (PE) were extracted from 500 mL of homogenized log phase (14 days old) culture after centrifuged at 4,000 rpm to obtain a pellet. The pellet was suspended in 100 mL of 20 mM acetate buffer (pH 5.1). Extraction was carried out by repeated freezing (-20 °C) and thawing (room temperature) method for 4 days until cell biomass became dark purple. Cell debris was removed by centrifugation at 5,000 rpm for 10 min, and a crude extract was obtained. In addition to acetate buffer (pH 5.1; 0.1 M), optimization of PE extraction was tested using different buffers [citrate buffer (pH 5.0; 0.1 M); carbonate buffer (pH 9.6; 0.1

M); tris buffer (pH 7.2; 0.05 M), and sodium phosphate buffer (pH 7.0; 0.1 M)] as published previously by Afreen & Fatma (2018).

## Purification

Purification was carried out according to Afreen & Fatma (2018). Solid ammonium sulphate was added to the crude extract slowly for achieving 65 % saturation by continuous stirring. The resulting solution was allowed to stand for 12 h under cold room, and centrifuged at 4,500×g for 10 min. The pellets were re-suspended in a small volume of 50 mM acetic acid–sodium acetate buffer (pH 7.1), and dialyzed overnight. The extract was recovered from the dialysis membrane and filtered through 0.45 µm filter (Chakdar & Pabbi 2012, Tiwari *et al.* 2015).

DEAE-Cellulose 11 from Sisco Research Laboratory (SRL) was used for anion exchange chromatography. A column of 30 cm × 2 cm dimension was prepared for purifying the PE protein. The column was equilibrated with 150 mL of acetate buffer (pH 5.1) and 10 mL of dialysed, and filtered sample was placed on the top of the DEAE-cellulose column with the help of a syringe. Acetate buffer (50 mL, pH 5.1) was applied to the column to wash out any unbound protein. A linear gradient of acetate buffer with pH ranging from 3.76 to 5.1 was used to develop the column and eluates were collected in 5 mL fractions. Flow rate was kept at 20 mLh<sup>-1</sup> (Román *et al.* 2002, García-Falcón & Simal-Gándara 2005).

SDS-PAGE was carried out in a vertical slab gel apparatus (Miniprotean III, Bio-Rad) according to Zhao *et al.* (2013) using a 16.5 % polyacrylamide slab gel containing 0.1 % (w/v) SDS with a stacking gel of 4 % polyacrylamide. Samples were pre-incubated with 4 % (w/v) SDS, 12 % (v/v) glycerol, 2 % (v/v) mercaptoethanol, 0.025 % (w/v) bromophenol blue, and 50 mM Tris, pH 6.8, for about 5 min at 95 °C. Gels were run at room temperature, visualized by staining for 30 min with 0.1 % (w/v) Coomassie Brilliant Blue R-250, 40 % methanol (v/v) with 7 % (v/v) acetic acid, and de-stained in dilute acetic acid. The molecular weight of the purified protein was determined by running a Novex Sharp pre-stained protein marker along with the sample.

The absorption spectrum was determined by scanning the sample in a range of 300-750 nm wavelengths by Specord 200 spectrophotometer

(Analytik Jena, Germany).

The amounts of PE (phycoerythrin), PC (phycocyanin) and APC (allophycocyanin) in the different extracts and biliprotein (PBPs) containing solutions were calculated from measurements of the absorbance at 565, 620 and 650 nm using the following equations Román (2002):

$$PC(\mu\text{gmL}^{-1}) = \frac{OD_{620\text{nm}} - 0.7 OD_{650\text{nm}}}{7.38}$$

$$APC(\mu\text{gmL}^{-1}) = \frac{OD_{650\text{nm}} - 0.19 OD_{620\text{nm}}}{5.65}$$

$$PE(\mu\text{gmL}^{-1}) = \frac{OD_{565\text{nm}} - 2.8[R - PC] - 1.34[APC]}{12.7}$$

Purity of PE was calculated at each step as purity ratio (A565/A280). Absorbance at 565 nm and 280 nm indicated the concentration of PE and proteins, respectively.

These equations were established by using the simultaneous equations of Bennett & Bogorad (1973) and the extinction coefficients from Bryant *et al.* (1979).

## Effect of temperature and preservatives on the optical properties of purified PE

Stability of purified PE (710 µg mL<sup>-1</sup>) was obtained by measuring loss (%) of PE without adding additives and also with sucrose and glucose as preservatives (4 mg mL<sup>-1</sup>) at different temperatures (25, 30, 35, 40, 45, 4 and -20 °C) by recording its absorption spectrum for 10 hours (Setyoningrum & Nur 2015, Gonzalez-Ramirez *et al.* 2014, Rastogi *et al.* 2015).

## Biological activity of purified PE

### Evaluation of antifungal and antibacterial activity

The purified PE (710 µgmL<sup>-1</sup>) was tested for its antifungal and antibacterial activities using the paper disk diffusion method. Muller Hinton Agar (MHA) (25 mL) and Saubouraud's dextrose agar were used for antibacterial and antifungal activities, respectively. Agar plates were inoculated with a standardized quantity of suspension containing 1.5×10<sup>8</sup> CFUmL<sup>-1</sup> bacteria corresponding to 0.5 MacFarland standard according to Wikler *et al.* (2006). Final inoculum density from 0.5×10<sup>5</sup> to 2.5×10<sup>5</sup> CFUmL<sup>-1</sup> *Candida albicans* (C.P. Robin) Berkout and 0.4×10<sup>4</sup>–5×10<sup>4</sup> CFUmL<sup>-1</sup> *Aspergillus niger* P.E.L. van Tieghem were calibrated by using a hemocytometer cell counting chamber. Plates were incubated overnight at 37 °C for 18-24 h (bacteria) and at 35 °C for 24-48 h (fungi). The microorganisms used in assays were

obtained from the Iranian Research Organization for Science and Technology (IROST). Three gram-positive bacteria *Staphylococcus aureus* Rosenbach (PTCC 1112), *Bacillus subtilis* (Ehrenberg) Cohn (PTCC 1023), *Bacillus cereus* Frankland & Frankland (PTCC 1015), three Gram-negative bacteria *Escherichia coli* (Escherich) (PTCC 1047), *Pseudomonas aeruginosa* (Schroeter) Migula (PTCC 1310), *Salmonella typhimurium* (ex Kauffmann & Edwards) Le Minor & Popoff (PTCC 1609), a yeast *Candida albicans* (ATCC 10231) and a phytopathogenic fungus *Aspergillus niger* (ATCC 16404) were employed in the study. The diameter of the zones with complete inhibition of growth was measured to the nearest millimeters using a ruler and expressed in mm. All the tests were performed under sterile conditions and repeated three times. The antibacterial agents tetracycline (30 µg) and gentamicin (10 µg), and antifungal agent nystatin (10 µg) were included in the assays as positive standard antibiotic control (Espinel-Ingroff 2007).

For determination of stability of purified PE, filter discs were stored in Petri dishes at room temperature for 10 days and the antibiogram bioassays were performed again (Nowruzi *et al.* 2012).

Bioassays for additional evaluation of results taking into consideration the quantity of compound with antimicrobial activity by minimum inhibitory concentration (MIC) of µg mL<sup>-1</sup> were carried out according to the standard reference method (Wayne, 1999).

Minimum inhibitory concentrations of purified PE were determined by the broth dilution method. The MIC for fungi was performed in 10 tubes (16×160 mm) containing 1 mL Saubouraud's dextrose broth with 1 mL of dilutions of purified PE and 100 µL of suspension containing 10<sup>4</sup> spore mL<sup>-1</sup> of fungi cultures. The required concentration of the purified PE was dissolved in 1 mL of Saubouraud's dextrose broth and diluted to serial two-fold dilution ranging from 250 to 0.97 µg mL<sup>-1</sup>. The tubes of 11 and 12 were considered as controls containing 1 mL Saubouraud's dextrose broth with 1 mL of purified PE (500 mg mL<sup>-1</sup>) and 1 mL Saubouraud's dextrose broth with 100 µL of fungi cultures, respectively. Tubes were incubated at 27 °C for 2 days. Fungi growth in tubes was assessed visually. One (1) µL of supposed tested broth was placed on the sterile Saubouraud's dextrose agar as the lowest concentration of the com-

pound inhibiting the visual growth of the test cultures on the agar plate. If there was no visible growth, MIC was considered as minimum bactericidal concentration (MBC). For bacteria, MIC was determined according to the standard CLSI guidelines. Mueller-Hinton agar plates with active principle (250 - 0.97 µg mL<sup>-1</sup>) were spotted with 2 µL bacterial inocula (10<sup>7</sup> CFU mL<sup>-1</sup>) and incubated at 37 °C for 20 h. MIC was determined as the lowest concentration of purified PE resulting in complete inhibition of fungi and bacteria growth after the incubation time. There were three replicates per assay and each test was repeated at least twice.

The data of all the parameters were statistically analyzed using the one-way ANOVA with 95% confidence limits ( $p<0.05$ ) and results expressed as mean± SE.

#### **Antioxidant activity of purified PE**

##### **2, 2 Diphenyl-1-Dipicrylhydrazyl (DPPH) assay**

This test was conducted following the method described by Shanab *et al.* (2012) with modifications. An amount of 710 µg mL<sup>-1</sup> purified PE was mixed with 1 mL of DPPH reagent. After incubating for 30 min in the dark at room temperature, the absorbance was measured at 517 nm. Ascorbic acid (100 µg mL<sup>-1</sup>) was used as positive control.

$$\text{Activity (\%)} = \frac{\text{Ac} - \text{At}}{\text{Ac}} \times 100$$

Where At was the absorbance of sample, and Ac the absorbance of DPPH.

##### **ABTS assay**

ABTS+ radicals (7 mM) were produced by adding potassium persulphate (2.45 mM) in the dark for 12–16 h. The resulting solution was diluted with ethanol up to an absorbance of 0.5 at 734 nm. An aliquot of 3 mL of ABTS + solution was added to 50 µL of the PE sample (710 µg mL<sup>-1</sup>) and standards, and the absorbance was recorded at 734 nm against ethanol as blank (Re, 1999). ABTS+ solution was taken as positive control and BHT as standard.

$$\text{Activity (\%)} = \frac{\text{Ac} - \text{At}}{\text{Ac}} \times 100$$

Where At was the absorbance of sample, and Ac the absorbance of ABTS.

##### **Superoxide anion radical scavenging activity assay**

Superoxide anion radical (SOR) assay followed as described by Afreen & Fatma (2018). Reaction mixture was prepared by adding 0.2 mL methio-

nine (200 mM), 1.5 mL Na<sub>2</sub>CO<sub>3</sub> (1 M), 0.1 mL nitro blue tetrazolium, 0.1 mL riboflavin (60 µM); 0.1 mL EDTA (3 mM) and 0.2 mL distilled water. The mixture was kept in the presence of light for 1 h during which changed its color to blue. A volume of 0.88 mL Tris-HCl (50 mM) was added to 0.2 mL of the PE sample (710 µg mL<sup>-1</sup>) including standard, and incubated in a water bath at 25 °C for 10 min. A volume of 2.2 mL of the reaction mixture was added to test tubes and the absorbance was measured at 560 nm against distilled water as blank, reaction mixture as positive control and vitamin C as standard (1 mM stock). The percentage of the SOR scavenging was calculated using the following equation:

$$\text{Superoxide anion radical scavenging effect (\%)} = (A_0 - A_1/A_0) \times 100$$

Where A<sub>0</sub> was the absorbance of the control, and A<sub>1</sub> was the absorbance of the test sample. The actual decrease in absorption induced by the test was compared with the positive control. IC<sub>50</sub> values were determined by plotting the graph of PE concentration versus the percentage of scavenging free radicals.

#### Nitric oxide (NO) scavenging activity

The nitric oxide radical scavenging activity was measured by using Griess reagent according to Kamble *et al.* (2013). Volumes of 50, 100, 150, 200 and 250 µL of purified PE (710 µg mL<sup>-1</sup>) and 500 µL of standard (vitamin C 1 mg mL<sup>-1</sup>) were taken and diluted up to 1.5 mL with distilled water in test tubes. A volume of 1.5 mL of 10 mM sodium nitroprusside was added to all tubes and incubated for 150 min at 25 °C. After incubation, 1.5 mL of the reaction mixture was transferred to

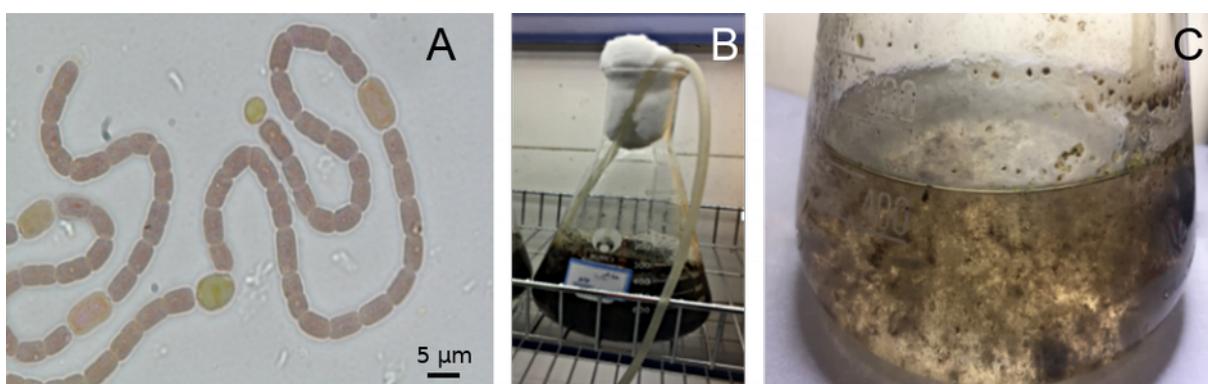
the new tubes and 1.5 mL Griess reagent (1% sulphanilamide, 2% orthophosphoric acid, 0.1% NEDD) was added to all tubes. O.D. was taken at 545 nm. Vitamin C was used as positive control and decrease in absorbance indicates high scavenging activity. The experiments were performed in triplicates and percent scavenging activity was calculated as follows:

$$\text{Scavenging (\%)} = \frac{\text{Abs of control} - \text{Abs of the test}}{\text{Abs of control}} \times 100$$

## Results

### Morphological and molecular characterization

*Nostoc* sp. strain A5 was able to grow rapidly on liquid media culture, and changed the color from light brown to dark brown under laboratory conditions (Figs. 1A-C). Microscopic observation showed brownish or dark-colored trichomes. Vegetative cells similar in form, cylindrical, 3.5–4.0 µm wide, 7.0–11.0 µm in length, brownish. Heterocysts somewhat spherical or oblong, 5.0–6.5 µm wide, 6.0–12.5 µm long. Akinetes were ellipsoidal to oblong, 5.0–5.5 µm wide, 10.0–12.0 µm long. The size of the spherical colonies ranged from 0.5 to 5.0 mm. The unicellular culture and dry type material were deposited into AL-BORZ herbarium and Cyanobacteria Culture Collection (CCC) of Science and Research Branch, Islamic Azad University, Tehran, respectively. The studied strain was further characterized based on the 16S rRNA gene sequence, and registered under the DDBJ accession number MG385057.



**Figura 1.** Microscopía de campo brillante con células vegetativas y heterocitos intercalares y terminales. **A:** colonias macroscópicas de color marrón oscuro en cultivos de medios líquidos (**B, C**). Barra: 5 µm

**Figure 1.** Bright field microscopy with vegetative cells and intercalary and terminal heterocysts. **A:** macroscopic dark brown colonies in liquid media cultures (**B, C**). Bar: 5 µm.

### Extraction, purification and characterization of PE

PE extraction from *Nostoc* sp. A5 was optimized for its maximum recovery and acetate buffer (pH 5.1) was found to generate better result, extracting  $65.04 \text{ } \mu\text{g mL}^{-1}$  by freezing and thawing method (Table 1). Purity ratio is an indicator of the purification degree in which 0.7 is considered as food grade, 3.9 as reactive grade and greater than 4.0 as analytical grade (Delgado-Vargas & Paredes-López 2002). As revealed from the high final purity ( $\text{OD565}/\text{OD280}=1.5$ ), PE extraction using acetate buffer (pH 5.1) was efficient to result in high purity of PE in comparison to other buffers (Table 1). For this reason, further PE extraction was done in acetate buffer only.

Steps	Vol. (mL)	PE ( $\mu\text{g mL}^{-1}$ )	Purity of PE ( $\text{OD565}/\text{OD280}$ )
Crude extract	Acetate (pH 5.1)	65.04	1.5
	Carbonate (pH 9.6)	55.10	0.9
	Citrate (pH 5.0)	100	0.5
	Sodium phosphate (pH 7)	20.04	0.15
	Tris (pH 7.2)	8.3	0.03
Ammonium Sulfate precipitation	10	532	4.82
Dialysis	10	550	4.97
DEAE-Cellulose column chromatography	5	710	6.22

Tabla 1. Purificación gradual de PE de *Nostoc* sp. A5.

Table 1. Stepwise purification of PE from *Nostoc* sp. A5.

At each purification step, concentration and purity of PE were checked as shown in table 1. During successive steps of purification, involving ammonium sulfate precipitation followed by a single step anion exchange chromatography using DEAE cellulose-11 column and acetate buffer, the purity ratio increased from 1.5 up to 6.22. It was found to be enhanced after each purification step (Fig. 2, step I to step IV).

From crude extract to purified PE, purity was increased by almost four times, which showed the efficiency of the method to obtain high purity phycoerythrin. According to the absorption spectra, PE have categorized into three types: (i) B-PE,  $\lambda_{\text{max}} \sim 540-560 \text{ nm}$ , shoulder at  $\sim 495 \text{ nm}$ ; (ii) R-PE,  $\lambda_{\text{max}} \sim 565, 545$  and  $495 \text{ nm}$ , and (iii) C-PE,  $\lambda_{\text{max}} \sim 563/543$  and  $492 \text{ nm}$  (Sun *et al.* 2009). Absorption spectra of purified PE showed maximum absorption at 565 nm, which clearly indicates that the purified phycoerythrin is of C-PE nature (Fig. 2, step I to step IV). Precipitation of phycobiliproteins with 65% saturation of

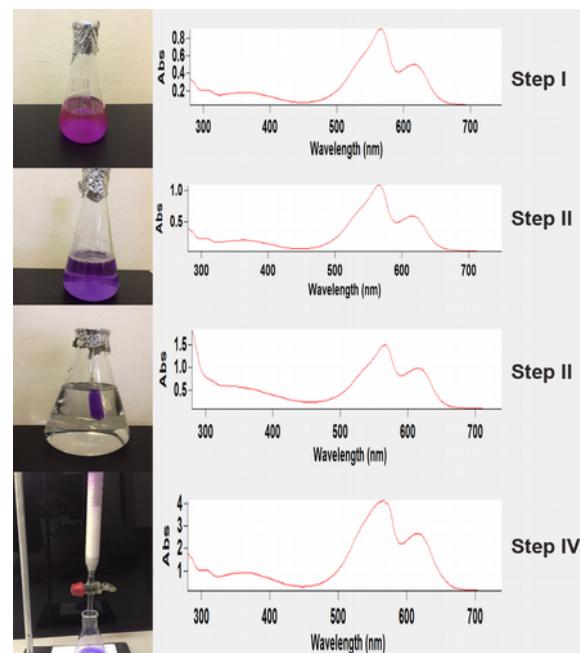


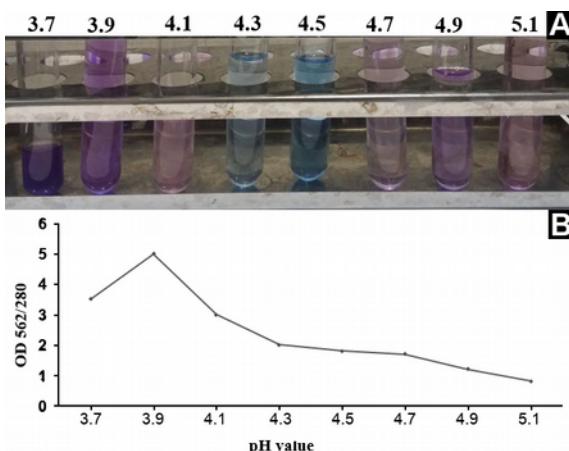
Figura 2. Preparación y espectros de absorbancia de PE purificado en varias etapas de purificación. Paso I, preparación del extracto crudo; Paso II, precipitación con sulfato de amonio, Paso III, diálsis; PASO IV, cromatografía de intercambio aniónico.

Figure 2. Preparation and absorbance spectra of purified PE at various stages of purification. Step I, preparation of the crude extract; Step II, ammonium sulfate precipitation, Step III, dialysis; STEP IV, anion exchange chromatography.

ammonium sulfate resulted in a purity of 4.82, although no significant increase in purity was observed after dialysis (4.97) (Table 1). During the chromatographic separation, it was possible to obtain a different fraction of phycobiliproteins blue, pink and violet. C-PE with maximum purity ( $\text{OD565}/\text{OD280}=6.22$ ) and concentration of  $710 \text{ } \mu\text{g/mL}$  was eluted as a pinkish-violet-color solution at pH 3.9 (Figs. 3A, B; Table 1). The SDS-PAGE analysis of the purified PE (pH 3.9) revealed two bands of  $\sim 15 \text{ KDa}$  and  $17 \text{ KDa}$  corresponding to  $\alpha$  and  $\beta$  subunits, respectively (Fig. 4).

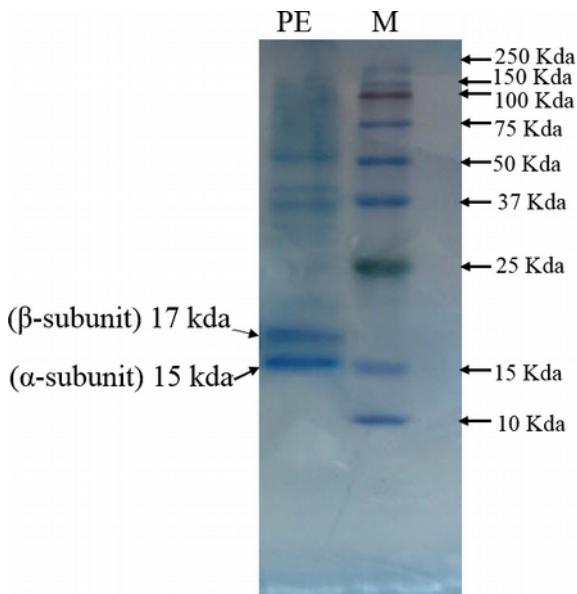
### Effects of different temperatures and preservatives on PE obtained from *Nostoc* sp. A5

The absorption spectrum was recorded with or without preservative at 25, 30, 35, 40, 45, 4 and -20 °C. The visible absorption spectra of C-PE showed that the loss of color was slightly lower at 45 °C and very high at -20 °C in ten hours (Fig. 5A). The maximum rate of decrease was found with the presence of sucrose (Fig. 5B) at 35, 40 and 45 °C, while there was drastic change at 45 °C



**Figura 3.** A: Eluados coloreados obtenidos durante el desarrollo de la columna a diferentes pH; B: Propiedades ópticas de PE en el rango de pH (b).

**Figure 3.** A: Coloured eluates obtained during developing the column at different pH; B: Optical properties of PE in the pH range.



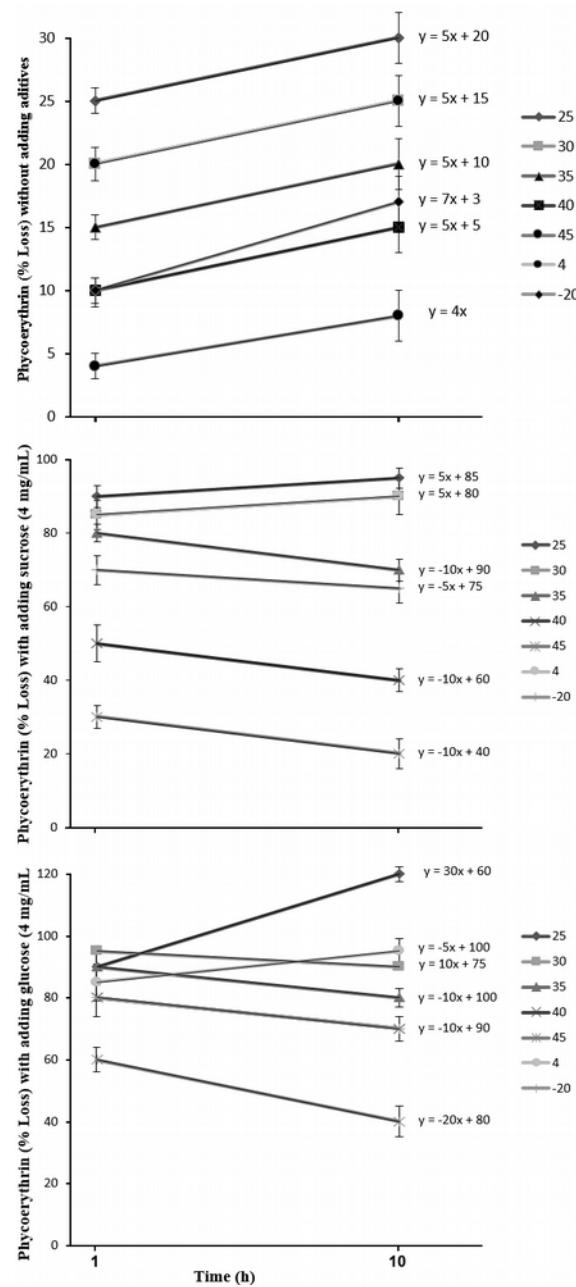
**Figura 4.** Análisis de SDS-PAGE de ficoeritrina purificada. M: marcador de peso molecular de la proteína, PE - ficoeritrina purificada.

**Figure 4.** The SDS-PAGE analysis of purified phycoerythrin. M - Protein molecular weight marker, PE- purified phycoerythrin.

with glucose (Fig. 5C). In some cases, the use of additives increased the absorbance of C-PE. For instance, with sucrose at 25 and 4 °C, the absorbance increased very slowly, while the highest increase of slope was found with glucose.

#### Biological activity of purified PE

Antibacterial and antifungal activities were demonstrated in all of the tested Gram positive and Gram negative bacteria and fungi. It was found that PE had the strongest inhibitory effect



**Figura 5.** A: Pérdida (%) en la cantidad de PE puro obtenido de *Nostoc* sp. A5 sin agregar aditivos. Presencia de sacarosa (B) y glucosa (C) para evaluar sus efectos conservantes sobre las propiedades ópticas de C-PE a diferentes temperaturas (25, 30, 35, 40, 45, 4 y -20 °C) en diez horas.

**Figure 5.** A: Loss (%) in the amount of pure PE obtained from *Nostoc* sp. A5 without adding additives. Presence of sucrose (B) and glucose (D) to evaluate their preservative effects on the optical properties of C-PE at different temperatures (25, 30, 35, 40, 45, 4 and -20 °C) in ten hours.

against *Staphylococcus aureus* and *Aspergillus niger* (Tables 2 and 3, respectively).

The antimicrobial activity produced by purified PE measured by minimum inhibitory concentration has shown in tables 4 and 5 for bacteria and fungi, respectively. The results indicated that

the MICs of purified PE was  $3.9 \text{ } \mu\text{g mL}^{-1}$  against *Bacillus subtilis* and *Pseudomonas aeruginosa*, whereas it was more than  $7.81 \text{ } \mu\text{g mL}^{-1}$  against *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhimurium* (Table 4). Furthermore, results demonstrated that the MICs of the purified PE was  $3.9 \text{ } \mu\text{g mL}^{-1}$  against *Candida albicans*, whereas it was  $7.81 \text{ } \mu\text{g mL}^{-1}$  against *Aspergillus niger* (Table 5).

Statistical analysis revealed there was no significant decrease in the inhibitory effect of the biomass after 10 days (Tables 2 and 3). As it is

illustrated in the results section, some variations were observed from experiment to experiment and it became clear that a given bioactive isolate did not necessarily show clearing at all the times.

### Antioxidant activity of purified PE

Free radical scavenging potential of *Nostoc* sp. A5 PE in all of the four methods was found to be concentration dependent.  $\text{IC}_{50}$  value in DPPH method was  $0.038 \text{ mg mL}^{-1}$  for PE and  $0.032 \text{ mg mL}^{-1}$  for ascorbic acid (vitamin-C) that was used as standard (Fig. 6A). For ABTS method,  $\text{IC}_{50}$  values were

Microorganism	PE=710 ( $\mu\text{g mL}^{-1}$ )	After 10 days
<i>Bacillus subtilis</i> (PTCC 1023)	$10.9 \pm 0.16$	$9.9 \pm 0.46$
<i>Bacillus cereus</i> (PTCC 1015)	$12.16 \pm 0.44$	$11.5 \pm 0.18$
<i>Escherichia coli</i> (PTCC 1047)	$10.3 \pm 0.88$	$9.56 \pm 0.37$
<i>Staphylococcus aureus</i> (PTCC 1112)	$12.33 \pm 0.33$	$12 \pm 0.15$
<i>Pseudomonas aeruginosa</i> (PTCC 1310)	$9.83 \pm 0.44$	$9 \pm 0.2$
<i>Salmonella typhimurium</i> (PTCC 1609)	$12.5 \pm 0.76$	$12 \pm 0.49$

**Tabla 2.** Actividad antibacteriana del PE purificado presentado por diámetro de zona de inhibición (mm). Resultados son medias ± error estándar.

**Table 2.** Antibacterial activity of the purified PE presented by inhibition zone diameter (mm). Results are means ± SE.

Microorganism	PE= 710 ( $\mu\text{g mL}^{-1}$ )	After 10 days
<i>Aspergillus niger</i> (ATCC 16404)	$13.55 \pm 0.18$	$10.9 \pm 0.1$
<i>Candida albicans</i> (ATCC 10231)	$12.33 \pm 0.37$	$10.5 \pm 0.18$

**Tabla 3.** Actividad antifúngica del PE purificado presentado por el diámetro de la zona de inhibición (mm). Resultados son medias ± error estándar.

**Table 3.** Antifungal activity of the purified PE presented by inhibition zone diameter (mm). Results are means ± SE.

Microorganism	250	125	62.5	31.25	15.62	7.81	3.9	1.95	0.97	MIC
<i>Bacillus subtilis</i> (PTCC 1023)	-	-	-	-	-	-	-	+	+	3.9
<i>Bacillus cereus</i> (PTCC 1015)	-	-	-	-	-	-	+	+	+	7.81
<i>Escherichia coli</i> (PTCC 1047)	-	-	-	-	-	-	+	+	+	7.81
<i>Staphylococcus aureus</i> (PTCC 1112)	-	-	-	-	-	-	+	+	+	7.81
<i>Pseudomonas aeruginosa</i> (PTCC 1310)	-	-	-	-	-	-	-	+	+	3.9
<i>Salmonella typhimurium</i> (PTCC 1609)	-	-	-	-	-	-	+	+	+	7.81

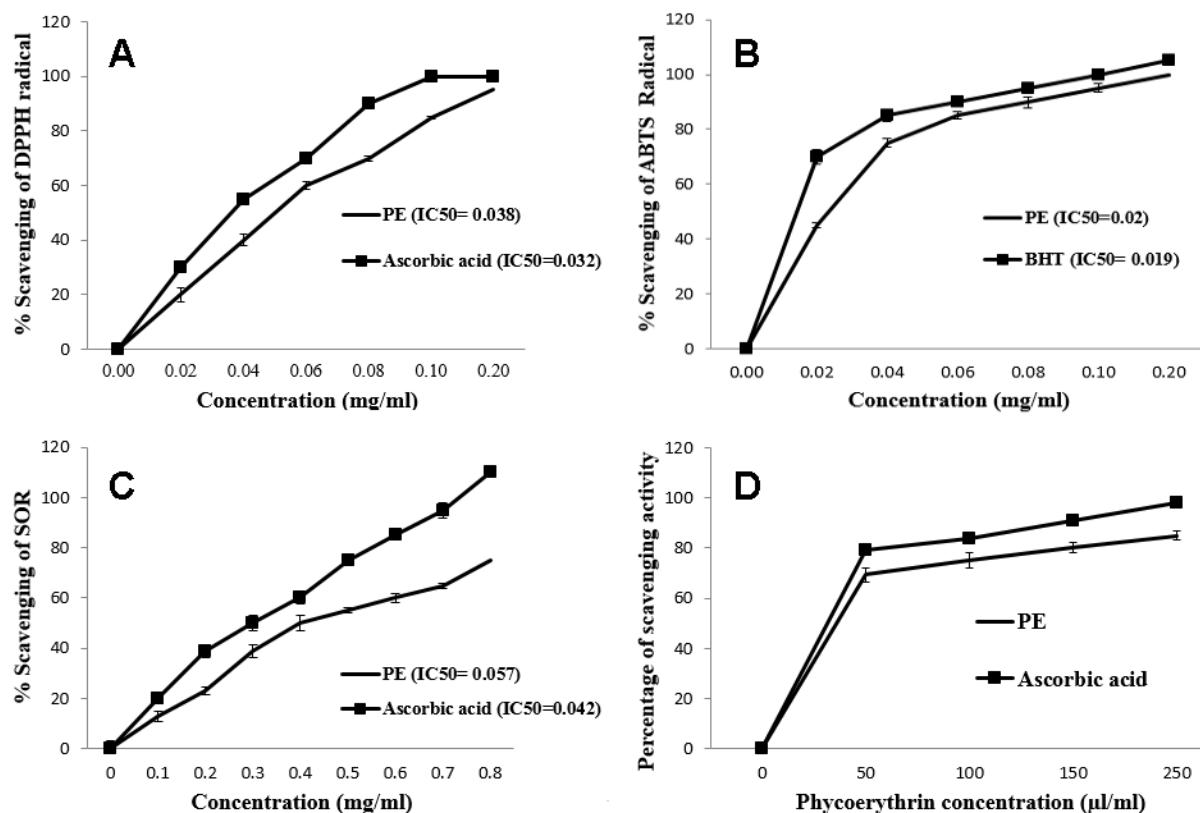
**Tabla 4.** Concentración inhibitoria mínima (CIM) del PE purificado. (-) Sin crecimiento; (+) Crecimiento. Concentración en  $\mu\text{g/mL}$ .

**Table 4.** Minimum Inhibitory Concentration (MIC) of the purified PE. (-) No growth observed; (+) Growth observed. Concentration of extracts in  $\mu\text{g/mL}$ .

Microorganism	250	125	62.5	31.25	15.62	7.81	3.9	1.95	0.97	MIC
<i>Aspergillus niger</i> ATCC 16404	-	-	-	-	-	-	+	+	+	7.81
<i>Candida albicans</i> TCC 10231	-	-	-	-	-	-	-	+	+	3.9

**Tabla 5.** Concentración inhibitoria mínima (CIM) del PE purificado. (-) Sin crecimiento; (+) Crecimiento. Concentración en  $\mu\text{g/mL}$ .

**Table 5.** Minimum Inhibitory Concentration (MIC) of the purified PE. (-) No growth observed; (+) Growth observed. Concentration of extracts in  $\mu\text{g/mL}$ .



**Figura 6.** Potencial de eliminación de radicales libres de PE de *Nostoc* sp. A5. A: Método DPPH; B: Método ABTS; C: Método SOR; D: método del óxido nítrico.

**Figure 6.** Free radical scavenging potential of *Nostoc* sp. A5 PE. A: DPPH method; B: ABTS method; C: SOR method; D: nitric oxide method.

0.02 mg mL<sup>-1</sup> and 0.019 mg mL<sup>-1</sup> for PE and standard BHT (butylated hydroxyl toluene), respectively (Fig. 6B). SOR anions were weak oxidants but generated powerful and harmful radicals. The IC<sub>50</sub> for scavenging rate of PE and ascorbic acid were 0.057 and 0.042 mg mL<sup>-1</sup>, respectively (Fig. 6C). In addition, the figure 6D illustrates the percentage inhibition of nitric oxide generation by PE in volumes of 10, 25, 50, 100, 150 and 250 µL, which significantly scavenged 79 %, 84 %, 91 % and 98 % the nitric oxide radicals, respectively.

## Discussion

Although a number of reports are available for PE purification and characterization from different cyanobacterial and red algal strains, exploitation and optimization of PE production from the genus *Nostoc* Vaucher ex Bornet & Flahault are limited. PE has a great potential for pharmaceutical and food industry applications (Mishra *et al.* 2010, Tan *et al.* 2016). However, due to limited stability, its application has become a challenging endeavor. In order to find out a PE with high resis-

tance and stability, the present investigation was carried out using *Nostoc* sp. A5 as a drought tolerant cyanobacterium. The strain was isolated from Cretaceous nodular chalk limestone and the region characterized by high light intensity as well as less than 0.5 % of water. These unfavorable environmental conditions may enhance the stability and resistance of PE.

Many studies have reported that cyanobacteria were able to tolerate prolonged periods of drought (Simeunović *et al.* 2013). Furthermore, studies of pigment contents in cyanobacterial strains from different habitats may contribute to the understanding of cyanobacteria-specific acclimation processes and responses to environmental change conditions. Bioactive compounds isolated from cyanobacteria growing in unfavorable conditions maybe important for the production of biotechnologically valuable molecules, such as pigments (Johnson *et al.* 2014, Nowruzi *et al.* 2020a, 2020b, Nowruzi & Blanco 2019). In this sense, two phycoerythrin-rich *Synechococcus* isolates shown different responses against light intensity (Postius 1998). In the present work, the absorption

spectra indicated that the purified PE is of C-PE nature (Fig. 2 (step I to step IV)). Despite the occurrence of PE in *Nostoc* (Ranjitha & Kaushik 2005) this is the first report of C-PE extracted from the genus *Nostoc* isolated from a limestone drought environment. The PE purity was investigated in a range of pHs with different buffers. Only acetate buffer (pH 5.1) resulted in high PE purity, indicating the complex chemical nature of biliproteins (PBPs). In fact, changes in pH values differentially affect the PBPs from different sources. R-PE from *Polysiphonia urceolata* (Lightfoot ex Dillwyn) Greville (Rhodophyta) showed purity at pH 3.5 (Liu *et al.* 2009). At low pH, denaturation or dissociation of PBPs into individual subunits may occur, and severe changes in pH values may disturb the electrostatic properties and hydrogen bonding involved in protein association leading to changes in chromophore structure (Liu *et al.* 2009). Variation in pH affects different processes such as solubility and bioavailability of nutrients, transport of substances across the cytoplasmic membranes, the activity of both intra and extracellular enzymes, photosynthetic electron transport and the osmotic potential of the cytoplasm.

Molecular weights of PE subunits may vary from organism to organism (Vásquez-Suárez *et al.* 2018). The SDS-PAGE analysis of the purified PE revealed two bands for *Nostoc* sp. A5 PE of ~15 KDa and 17 KDa corresponding to  $\alpha$  and  $\beta$  subunits, respectively (Fig. 4). A 19.4 KDa band were reported for PE in *Nostoc muscorum* by Ranjitha & Kaushik (2005). Using preservative for PE grade is indispensable because it is highly sensitivity towards temperature, and the process should be commercially viable (Liu *et al.* 2009). Results from the present study showed that without adding additives, PE absorbance decreased as the temperature changed. At 25 °C and 4 °C with the presence of glucose, PE stability was found to be better reached. Moreover, we have demonstrated that almost complete loss of C-PE content occurred at 40 °C. However, Galland-Irmouli *et al.* (2000) have demonstrated the thermostability of R-PE from *Palmaria palmata* (L.) F. Weber & D. Mohr (Rhodophyta) up to 60 °C.

Many studies have recorded the PE antimicrobial effects with moderate inhibition to *C. albicans*, *A. niger*, *P. aeruginosa*, *E. coli*, and *Staphylococcus* (Afreen & Fatma 2018, Najdenski *et al.* 2013, Medina-Jartiz *et al.* 2011) similarly to

observed in the present study.

The purified PE from *Nostoc* sp. A5 exhibited stability after 10 days, and this evidence may also be used for determining the resistance of pigment.

Phycoerythrin can be largely useful as food colorant (Sun *et al.* 2009). Although its security has been questioned, it is a strong antioxidant (Dufossé *et al.* 2005, Macedo *et al.* 2017, Jerez-Martel *et al.* 2017), which highlights its advantage. Solani *et al.* (2015) have recently reported the anti-oxidant based anti-ageing activity and anti-Alzheimer potential of PE isolated from *Lynghbya* sp. A09DM in wild type and transgenic *Caenorhabditis elegans* Maupas, 1900 (free-living nematode). However, little investigation about the effect of PE antioxidant activity is available in the literature.

Our results of antioxidant surveys suggest that PE has efficient scavenging effects ( $IC_{50}=0.038$ , 0.02 and 0.057 mg mL<sup>-1</sup> for DPPH, ABTS and SOR anions, respectively) compared to ascorbic acid ( $IC_{50}=0.032$  and 0.042), and is a potent free radical scavenger acting as antioxidant molecule. Similarly, Afreen & Fatma (2018), reported  $IC_{50}=0.043$  mg mL<sup>-1</sup> using the extract of *Michrochaete Thuret* ex Bornet & Flahault for DPPH test. Furthermore, Valuta *et al.* (2015) demonstrated that extracts of *Nostoc linckia* Bornet ex Bornet & Flahault (70 % inhibition of ABTS radical) at 5 mg L<sup>-1</sup> and ABTS radical scavenging capacity increased with the increasing phycobiliproteins concentration. Extracts of *Nostoc sphaeroides* Kützing ex Bornet & Flahault showed considerable superoxide radical inhibiting activity according to Kuriakose (2007). The superoxide anion scavenging activity of the extract may be due to the direct scavenging of superoxide anion generated from photo illumination of riboflavin. Nitric oxide (NO) is an important chemical mediator generated by *Staphylococcus* Rosenbach y endothelial cells, macrophages, neurons, among others and involved in the regulation of various physiological processes. Excess concentration of NO is associated with several diseases, such as septic shock, atherosclerosis, ischemia-reperfusion, neurodegenerative disorders like Alzheimer's and Parkinson's diseases, cancer and diabetes (Wojcik *et al.* 2010). Oxygen reacts with the excess nitric oxide to generate nitrite and peroxynitrite anions, which act as free radicals. In the present study, the purified C-PE competed with oxygen to react with nitric oxide and inhib-

bited the generation of the anions.

## Conclusions

*Nostoc* sp. A5 is a limestone drought tolerant cyanobacterium with potential for bioprospecting uses. A stable C-PE produced by the strain A5 was successfully extract, purified, characterized and evaluated for in vitro stability under different temperatures, additives and pH conditions. Significant antioxidant activities of C-PE shown its feasibility for future applications as colorant in food industries and for pharmaceutical purposes. However, toxicological studies must be carried out for commercial production, since *Nostoc* is known as a toxing-producing genus. Furthermore, studies on its conformational behavior under high stress conditions will be extremely necessary to explore and enhance its molecular stability for industry applications. In addition, *Nostoc*'s PE also shown ability for natural effective antibacterial and antifungal agents with significant stability up to 10 days. This highlights the need to explore microorganisms from unfavorable environmental conditions due to their potential for a wide variety of biological activities.

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