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Stability, bio-functionality and bio-activity of crude phycocyanin from a two-phase cultured Saharian *Arthrospira* sp. strain

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ABSTRACT

Crude phycocyanin extract (PC-E) from a two-phase cultured Saharian *Arthrospira* sp. strain with a purity grade of 1.24 (reagent grade) was characterized regarding its stability, bio-functionality and bio-activity. PC-E showed higher solubility values above pH 6.0, while the maximum stability was achieved at pH 6.0. PC-E was stable only up to 50 °C, while the addition of stabilizing agents (PEG-4000 and sorbitol) significantly enhanced thermostability. FTIR and DSC analysis revealed that thermostabilization by PEG-4000 and sorbitol addition was achieved through hydrogen bound and amorphous glass formation. Emulsifying and foaming properties of PC-E were concentration-dependent. PC-E displayed antioxidant activity in a dose dependent manner, including β-carotene bleaching inhibition, ferrous chelating effect, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of 63%, 78% and 100% at 5 mg mL^{-1}, respectively. Moreover, PC-E exhibited great antibacterial activity at 5 mg mL^{-1} against *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli* and *Pseudomonas* sp.

1. Introduction

The cyanobacterium *Arthrospira* sp. (*Oscillatoriaceae*) (commonly known as spirulina) is considered as one of the most economically important cyanobacteria, which is nowadays worldwide cultivated in large scale facilities. *Arthrospira* has gained attention due to its imposing biochemical composition and its content of various high value metabolites, which have a great potential to be used as nutraceutical or source for the production of various pharmaceuticals or fluorescent products [1,2]. Biomass of the genus *Arthrospira* typically contains about 10–20% carbohydrates, 5–10% lipids and 55–70% protein (dry weight; DW), where 15–20% of the proteins are consisted from phycobiliproteins, which are proteins complexed with covalently bound phycobilins (chromophores). In *Arthrospira*, phycocyanin (PC) (λ_{max} = 620 nm) is one of the major phycobiliproteins, which can be easily water-extracted [3].

Phycocyanin (PC) has attracted the interest as natural pigment and is already used in the food industry. Additionally, PC is of great interest because it exhibits various biological properties including antioxidant, hepato-protective, anti-inflammatory, neuro-protective and anti-proliferative activities [4–7]. Considering that crude PC extraction is a simple and of low cost technology it is of particular interest to investigate their usability in an industrial and applied context towards using it as functional food or food additives [8]. Nonetheless, the use of PC in industry is still limited because of its sensitivity towards industrial treatments, such high temperature (for example during pasteurization), pH (acidic or alkaline) or the use of oxidizing agent, all applied during their extraction, purification and preparation of products, which result in loss by precipitation or dissociation phenomena leading to the bleach of the blue color [3,9]. Despite the relative extend number of studies regarding PC thermostabilization by adding stabilizers, the structural thermostability mechanisms of PC is still not well understood and is generally approached on an empirical basis [4,10].

In a previous study, induced change in intracellular compounds, including PC content, in *Arthrospira* strain isolated form Algerian Sahara desert was performed using multifactor stress combination strategy according to a two-phase culture process [11]. Considering that the efficacy of the cyanobacterial metabolites for various

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application is affected from the environmental conditions of the site and habitat, from where the strain is isolated [5], and given that there is an increasing interest in microalgal/cyanobacterial biotechnology research for the production of different intra and extracellular high value metabolites [9], the present study aimed to investigate comprehensively and in more details the preparation of PC extract from a local strain of *Arthrospira* sp. cultivated in two-phase mode. A two-phase process leads to the production of a biomass with higher PC content, while avoiding biomass productivity restriction caused by the subjection of cells to conditions that are not favorable for cell division and multiplication (growth) [11]. Additionally, the effects of pH, temperature, initial concentration and stabilizing agents on the thermostability of the extracted PC were investigated. Functional and biological properties of the extracted PC were further assessed.

2. Material and methods

2.1. Arthrospira sp. strain cultivation

Arthrospira sp. was isolated from Tamanrasset, Algeria (1824 m altitude, 23° 06′ 11″, N5° 49′ 01″ E) and cultivated in two-step culture process according to a previous study [11]. Briefly, precultures were carried out to enhance the biomass production and conducted in externally illuminated photobioreactor consisting of 10 L sterilized glass cylindrical flask with an inner diameter of 20 cm and height of 32 cm, containing 8 L of Zarrouk medium (pH 9.0). Precultures were stirred continuously using filter-sterilized air provided by a membrane air pump at a constant flow rate of 0.1 volume air per volume of bioreactor per minute and conducted under optimal growth condition of light (80 μmol photons $m^{-2} s^{-1}$) and temperature (30 ± 1 °C). At the end of exponential phase growth (day 4), the biomass (0.69 ± 0.05 g L^{-1}) was concentrated by filtration (20 μm nylon mesh) (Phase I). The biomass from precultures was re-suspended (1 g DW L^{-1}) in 1000-mL Erlenmeyer containing 500 mL of modified Zarrouk medium in terms of NaCl (11.7 g L^{-1}), $NaNO_3$ (0.5 g L^{-1}) and K_2HPO_4 (2.6 g L^{-1}) and exposed to low light intensity (10 μmol photons $m^{-2} s^{-1}$) with a photoperiod of 16:8, to induce the PC accumulation (Phase II) [11]. After three days of induction, biomass was harvested by filtration, washed with deionized water to remove medium salts, freeze-dried, weighed and finally stored at −20 °C. Under these culture conditions, the PC yield was estimated to be 230.1 ± 18.87 mg g^{-1} of biomass (DW).

2.2. Extraction of crude phycocyanin (PC-E)

PC-E was obtained following the method of Kumar et al. [12] with slight modifications. Prior to extraction, the cultivated *Arthrospira* sp. was defatted with hexane for 2 h at 60 °C. Subsequently, the defatted residue was depigmented using acetone at ratio of 1:10 (w/v). Defatted and depigmented biomass (3.3 g) was suspended in 100 mL 20 mM of sodium acetate and 50 mM of NaCl buffer (pH 5.1) to obtain a final solid-liquid concentration of 33 mg L^{-1}. The extraction was conducted by consecutive three cycles of freezing at −20 °C and thawing at room temperature (25 ± 2 °C), during 24 h. The sample was then centrifuged at 8000 rpm for 30 min and at 4 °C, to remove residual cell debris. The supernatant was recovered and subjected to a single step precipitation process using 65% ammonium sulfate and kept overnight at 4 °C. The pellet containing PC was recovered by centrifugation at 8000 rpm, for 30 min at 4 °C, and dissolved in 50 mL of sodium phosphate buffer (pH 6.0). The extract obtained was termed as crude PC-E and dialyzed against distillated water using dialysis membrane of 3.5 kDa cut-off at 4 °C during 48 h to remove proteins and salts. The dialyzed extract was recovered and filtered through 0.45 μm filter.

The PC content of *Arthrospira* biomass was estimated spectrophotometrically according to equation of Bennett and Bogorad [13] as following:

$$PC = [OD_{620} - 0.474 \times OD_{652}]/5.34$$

The purity of phycocyanin (PC) was determined by using the following absorption ratio:

$$Purity = OD_{620}/OD_{280}$$

where OD_{620} and OD_{280} are absorbances of PC and proteins, respectively.

The yield of PC was calculated by equation described by Silveira et al. [14] as following

$$PC_{Yield} = C_{PC} \times V/DWB$$

where PC_{Yield} is expressed in mg g^{-1}, C_{PC} is phycocyanin concentration in mg mL^{-1}, V is the volume of extraction solvent (mL), and DWB is the dried weight of *Arthrospira* sp. biomass (g).

2.3. Physicochemical properties of PC-E

2.3.1. Electrophoretic analysis

A 15% polyacrylamide gel electrophoresis (SDS-PAGE) of PC-E was carried out. PC-E solution (1.5 mg mL^{-1}) was mixed with the loading buffer (2% SDS, 5% mercaptoethanol, and 0.002% bromophenol blue) in proportion of 1:3 (v/v). The mixtures were heat-denatured at 90 °C for 5 min and analyzed by polyacrylamide gel electrophoresis. Electrophoresis was run at 100 V for 1.5 h. The gel was stained with Coomassie Brilliant blue. The mobility of extracted PC-E was determined by reference to standard protein markers of known molecular weight ranging from 10 to 85 kDa.

2.3.2. Solubility of PC-E

PC-E solubility behavior was evaluated as following: PC-E (130 mg) was mixed with 10 mL of water, vortexed for 10 min at room temperature while the pH of the suspension was adjusted to range between 2.0 and 11.0 by adding 0.1 M of HCl or NaOH. The mixtures were stirred for 15 min and then centrifuged at 8000 rpm for 15 min. The resulting supernatants were recovered and used to quantify the soluble protein content by Lowry method [15]. Results were expressed in % of soluble proteins as function of pH variation. The proteins content of PC-E was determined in triplicate.

2.3.3. Effect of pH on PC-E UV–visible spectral property

To explore the pH effect, PC-E was prepared in different buffers: 50 mM phosphate-citrate buffer for pHs 3.0 and 5.0, 50 mM sodium phosphate buffer for pHs 6.0 and 7.0, and 50 mM glycine-NaOH buffer for pH 9.0. After pH exposure, aliquots of the different PC solutions were collected and analyzed by recording its absorbance over 200–800 nm at 25 ± 1 °C.

2.4. Thermostability of PC

To assess the thermal stability, PC-E solutions, at different concentrations (0.5, 2.75 and 5 mg mL^{-1}), prepared in 50 mM sodium phosphate buffer (pH 6.0) were incubated for 75 min at different temperatures from 30 to 90 °C. To further investigate the thermostability of PC as function of incubation time, PC-E solutions (pH 6.0), at different concentrations, were incubated for 120 min at 60 °C. Samples were tested at 15 min intervals. The thermal stability assessment was performed in triplicate. Thermal degradation of PC was monitored by following the decrease of the absorbance, particularly at 620 nm. The PC stability was expressed as relative concentration of PC (RC_{PC}, %), defined as the remaining concentration of PC, as shown in the following equation [10]:

$$RC_{PC}\% = (C_{PC\,(f)}/C_{PC\,(i)}) \times 100$$

where $C_{PC\,(f)}$ is the concentration after each treatment, $C_{PC\,(i)}$ is the initial concentration in the PC-E.