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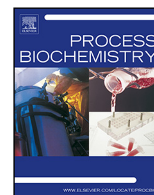

# Scaling-up of a B-phycoerythrin production and purification bioprocess involving aqueous two-phase systems: Practical experiences

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ABSTRACT

One of the most attractive segments in food and cosmetic industries is that of natural pigments. Since some synthetic pigments have been reported to be hazardous for humans, natural pigments obtained through biotechnological processes represent an attractive alternative. Our research group has previously worked on the development of an aqueous two-phase system (ATPS)-based prototype process for the recovery of B-phycoerythrin (BPE), a natural high-value pigment obtained from *Porphyridium cruentum*. Detailed studies describing the scaling up of ATPS processes from bench scale to pilot plant facilities are not common. In this paper experiences derived from the scale-up of a previously developed process for production and recovery of highly purified (purity defined as the absorbance ratio $A_{545}/A_{280} > 4$) BPE are described, where a scale-up factor of 850× was implemented. Characterization of cell disruption with a pilot-scale bead mill allowed efficient BPE release at 2900 rpm, 10% (w/v) sample load, 60% (v/v) bead load and 0.5 mm glass beads and 22 min of residence time with a yield of 1.35 mg BPE/g of wet biomass. BPE was recovered and purified using a strategy comprising isoelectric precipitation, aqueous two-phase fractionation and ultrafiltration. A 54% global BPE recovery yield, with final purity of 4.1, was achieved under optimal process conditions. Considering total costs for raw materials and energy expenditures for one batch, it was determined that the production cost of BPE was of $1.17 USD/mg, which is underneath the commercial price of a BPE standard (>$30 USD/mg).

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## 1. Introduction

The biotechnological industry has grown exponentially in the past years, and one of the most attractive segments is that of natural pigments with application on food, cosmetics and molecular biology [1,2]. On the other hand, the market industry of synthetic pigments has experienced a strict regulation regime due to the risks and detrimental effects that several of these pigments have been found to exert on human health [3,4]. The development of mental illness, allergies and diverse type of cancers associated with artificial pigments has been raised in previous reports [1,2,5]. In this context, large scale production of natural pigments has drawn the attention of several research groups in order to satisfy their demand worldwide.

Phycobiliproteins are a family of colored proteins that can be used as natural pigments. These molecules are proteins with linear prosthetic groups (bilins) that are linked to specific cysteine residues. These compounds are primarily found in biflagellate eukaryotic algae, cyanobacteria (blue-green algae) and rhodophyta (red algae). Phycobiliproteins are present in a photosynthetic complex called phycobilisome and work as accessory pigments to allow light absorption. Based on their absorption patterns, these proteins are classified in: (i) phycoerythrins (PE with $\lambda_{max}$ = 540–570 nm), (ii) phycoerythrocyanins (PEC with $\lambda_{max}$ = 570 nm), (iii) phycocyanins (PC $\lambda_{max}$ = 610–620 nm) and (iv) allophycocyanins (APC $\lambda_{max}$ = 650–655 nm) [2].

These protein-based pigments have been used in the food and cosmetic industry as natural colorants. However, their main application is found in molecular biology and biomedicine as fluorescent biomarkers [1,2,6,7]. Consequently, the design of novel and efficient bioprocesses for the recovery and purification of phycobiliproteins, particularly B-phycoerythrin (BPE), is of great interest. BPE at its highest purity level has a considerably high commercial value (>30 USD/mg depending on the nature of the final presentation of the product) [8,9]. In terms of expression systems used for BPE production, the red algae *Porphyridium cruentum* constitutes one of the best workhorses since its phycobilisome is principally constituted by BPE [10]. Structurally, the BPE molecule from *P. cruentum* presents 3 molar subunits $\alpha$, $\beta$ and $\gamma$ with a molar relation of 6:6:1 (a total of 13 subunits) with molecular weights of 18, 18 and 29 kDa respectively [7], with a total molecular mass of 245 kDa.

The design and application of bioprocesses for the recovery and purification of RPE and BPE has been previously reported [7,11,12]. Nevertheless, those protocols involved a high number of steps

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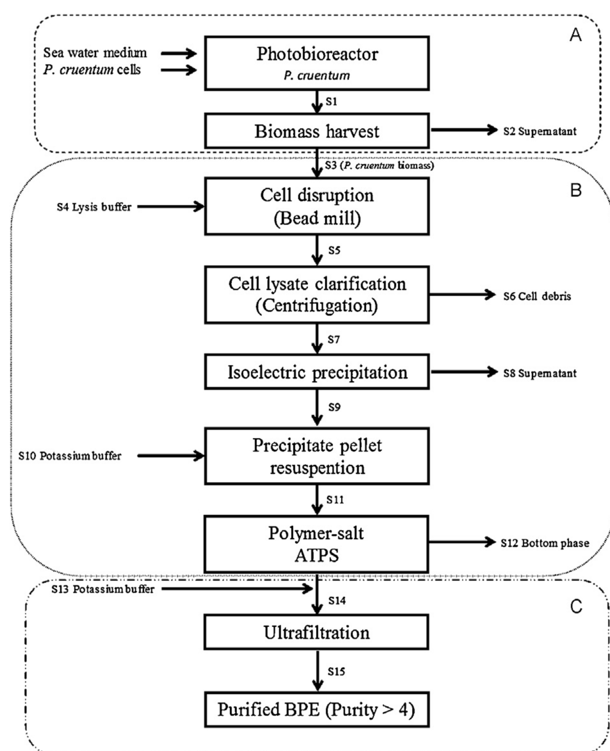


**Fig. 1.** Simplified representation of the proposed scaled process using isoelectric precipitation and aqueous two-phase systems as primary recovery and purification techniques to obtain BPE. After *P. cruentum* fermentation, a recovery stage is used to remove the supernatant. The biomass concentrate is re-suspended and then added to the bead mill for cell disruption. Isoelectric precipitation is performed by HCl addition until a pH level of 4 is attained. The precipitate pellet is re-suspended with dibasic phosphate buffer and mixed with potassium phosphate salts and PEG 1000 to form the proposed ATPS. Finally the upper phase is processed through an ultrafiltration unit to obtain highly purified BPE (purity = 4.2) and a total recovery of 54%. Streams involved in the bioprocess are numbered from S1 to S15.

which hampered the industrial scalable potential of the designed methodologies. The scale-up feasibility of a process depends on the nature of the unit operations involved [13]. The design of a novel and efficient prototype for the recovery and purification of BPE has been previously reported by our research group at lab scale [13–15]. The developed process comprised few unit operations in order to maximize overall recovery yield (~72%) without compromising BPE purity (defined as an absorbance ratio $A_{545}/A_{280} > 4$). From the bench-scale developed prototype process a pilot plant bioprocess is defined. This bioprocess is comprised by five main unit operations (Fig. 1): (1) photobioreactor, (2) cell disruption with a bead mill, (3) isoelectric precipitation, (4) aqueous two-phase systems (ATPS), and (5) cross-flow ultrafiltration.

Bead mills constitute an attractive option for cell disruption at industrial level since they have been adequately studied and characterized, thus allowing increased throughputs in bioprocess implementation [16,17]. Isoelectric precipitation techniques are readily scalable and do not require of complex instrumentation at industrial levels [15]. Liquid–liquid extraction systems such as ATPS allow rapid recuperation and purification of biological products, integration and intensification of bioprocesses, development of a biocompatible medium for biologic compounds and scale-up feasibility [13,18]. In addition, cross-flow filtration in final polishing steps provides important advantages to designed processes since membrane polarization is reduced when compared to plug-flow filtration. Transmembrane pressure is relatively constant and high throughput operations can be implemented with the proper industrial equipment [19]. Although the aforementioned techniques are well known for its scaling-up, it is crucial to characterize and optimize the process at pilot plant level before reaching commercial scale. Therefore, proper characterization of the scaling-up of the designed lab bioprocesses for the production, recuperation and purification of BPE from *P. cruentum* is necessary.

The present research work presents the practical experiences derived from the scaling up of a BPE production and purification bioprocess using *P. cruentum* as expression system at pilot plant scale. A scale-up factor of 850X was implemented when comparing our previous prototype process at lab scale and proposed pilot-plant scale strategy. The results obtained, practical experiences and main challenges are discussed in order to establish the first step for the commercial implementation of the optimized bioprocess.

## 2. Materials and methods

### 2.1. Biological material

*P. cruentum* (UTEX 161) was obtained from the Centro de Investigaciones Biológicas del Noroeste (CIBNOR), México.

### 2.2. Chemicals and reagents

Polyethylene glycol 1000 (PEG 1000 industrial grade) was purchased from Polioles S.A. de C.V. (Lerma, Mexico). Monobasic and dibasic potassium phosphate and all salts used for culture media were obtained from Desarrollo de Especialidades Químicas (Monterrey, Mexico).

### 2.3. Culture medium and cultivation conditions

*P. cruentum* was cultivated in artificial seawater culture medium as described before [7,20]. For 1 L of culture media the following components were added to bidistilled water: 24.53 g NaCl, 5.20 g $MgCl_2$, 4.09 g $Na_2SO_4$, 1.16 g $CaCl_2$, 0.70 g KCl, 0.20 g $NaHCO_3$, 0.10 g KBr, 0.03 g $H_3BO_3$, 170 mg $NaNO_3$, 13.80 mg $NaH_2PO_4$, 0.14 mg $ZnCl_2$, 0.20 mg $MnCl_2$, 0.24 mg $Na_2MoO_4$, 0.01 mg $CoCl_2$, 0.03 mg $CuSO_4$, 9.90 mg EDTA disodium salt and 4.50 mg $Fe_3(C_6H_5O_7)_2$. Briefly, cells were grown in batch mode in a 250 L (maximum capacity) acrylic photobioreactor (length 100 cm, depth 50 cm and height 50 cm) at 23–25 °C under artificial light conditions (wide spectra JBL Solar Marine Blue 35 W UV lamps) with a 24 h photoperiod. Agitation and aeration were provided with an air flow rate of 83 $cm^3/s$ enriched with 20 $cm^3/s$ of $CO_2$ (HAGEN, Optima Pumps). Biomass was allowed to grow for 30 days and later harvested by centrifugation at 3100 × *g* for 10 min. Biomass yield achieved under such conditions was 6.0 ± 0.7 g of wet biomass per L (Thermo Scientific IEC CL40R, WaltHam, USA).

### 2.4. Cell disruption

Cellular disruption of *P. cruentum* was performed using a bead mill (Dyno Mill-Multi Lab, Muttenz, Switzerland) equipped with a cooling jacket that allowed to maintain constant the temperature at 14 °C through processing. The release of BPE from *P. cruentum* using the bead mill device has not been reported in literature, and therefore a characterization of the effect of different process parameters on BPE release was necessary. Loaded cell suspension consisted on 50 mM dibasic phosphate potassium buffer with 20% (w/w) *P. cruentum* cells. Cell suspension was fed to the bead mill using a variable speed peristaltic pump (Manostat Vera Pump, Thermo Scientific) at a constant flow rate of 6 $cm^3/s$. The grinding chamber was initially filled with a 70% (v/v) bead load.

In order to characterize and optimize *P. cruentum* disruption using a bead mill, experiments were first done to determine the effect of rotational speed and bead size in BPE liberation yield. Rotational speeds of 2300, 2900 and 4000 rpm were employed. In order to determine the effect of bead size on BPE release efficiency two different bead diameters (0.5 and 1.2 mm) were tested. Once optimal rotation speed and bead size were determined, the effect of bead load (60–80%, v/v) and biomass load (10–30%, w/w) were studied. The homogenate obtained from the cell disruption stage (including cell debris) was referred to as BPE crude extract. BPE release constant ($k$) under optimal process conditions was calculated using Eq. (1) [21], where $t$ is disruption time, $R$ is the release of BPE at time $t$, and $R_m$ is the maximum achievable BPE release (1.5 mg BPE/g wet biomass, considering that after 25 min of cell disruption BPE release was constant in this value, hence being the maximum observed yield).

$$\ln \frac{R_m}{R_m - R} = kt \tag{1}$$

### 2.5. Isoelectric precipitation

The BPE crude extract was centrifuged at 3100 (or 18,000) × *g* for 15 min (Thermo Scientific IEC CL40R, WaltHam, USA) to remove cell debris and obtain a

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