Algal Research 44 (2019) 101685

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

A simple method for rapid purification of phycobiliproteins from *Arthrospira platensis* and *Porphyridium cruentum* biomass

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ARTICLE INFO

Keywords:
Phycobiliprotein purification
Membrane chromatography
Hydrophilic microfiltration polyvinylidene fluoride (PVDF) membrane
Ammonium sulphate
Arthrospira
Porphyridium

ABSTRACT

Membrane chromatography was exploited to purify allophycocyanin, phycocyanin and B-phycoerythrin from aqueous extracts of fresh or freeze-dried biomass of *Arthrospira platensis* (Cyanobacteria) and *Porphyridium cruentum* (Rhodophyta). The method can be tuned to obtain products having various degree of purity and avoids expensive and time consuming column chromatography and ultrafiltration steps. A commercial polyvinylidene fluoride (PVDF) microfiltration membrane (hydrophilic and low protein binding) was used as an ammonium sulphate responsive stationary phase to carry out the purification process. Analytical grade purity was achieved for phycocyanin (purity = 4.2–4.5, yield = 75–82 %) and B-phycoerythrin (purity = 4.5–4.8, yield = 69–71 %). Good purification, even if lower than analytical grade, was achieved for allophycocyanin (purity = 3.3–3.7, yield = 42–47%).

1. Introduction

Phycobiliproteins are brilliantly colored, highly fluorescent pigments of the photosynthetic light-harvesting antenna complexes of cyanobacteria and some algae, such as Rhodophyta and Cryptomonads. Phycobiliproteins are formed of a complex between proteins and covalently bound linear tetrapyrrole groups (i.e., the chromophores) [1]. The most common phycobiliproteins are phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE). These water soluble bright blue (phycocyanin and allophycocyanin) and fuchsia (phycoerythrin) pigments are valuable products, with several commercial applications [2–4]. Phycobiliproteins are used as natural cosmetic dyes or as fluorescent probes (allophycocyanin and phycoerythrin in particular), examples of practical applications are flow cytometry and immunoassays. Phycocyanin (and allophycocyanin) aqueous extracts obtained from *Arthrospira platensis* (Spirulina) are approved by EFSA (Regulation (EU) No. 1333/2008 and No. 231/2012) as coloring foodstuff. The US FDA classifies phycocyanin (21CFR73.1530) as a food natural color additive. Currently, natural food coloring market size is largely increasing worldwide, and is expected to reach US$1.77 billion within 2021 [3]. Moreover, phycobiliprotein therapeutic activities as antioxidant, anti-inflammatory, neuroprotective, anti-cancer and immunomodulatory has been reported [2,5–10].

The commercial value of a phycobiliprotein is strongly dependent on its purity grade, which is usually evaluated by the ratio between the value of phycobiliprotein absorbance maximum (within 540–570 nm for phycoerythrin, 615–620 nm for phycocyanin, and around 650 for allophycocyanin) and the absorbance value at 280 nm, which is related to the total amount of proteins detectable in the product. Phycobiliprotein purity greater than 0.7 is considered as food grade, greater than 1.5 as cosmetic grade, greater than 3.9 as reactive grade and greater than 4.0 as analytical grade [11,12].

Many methods have been proposed to purify phycobiliproteins and particularly phycocyanin from cyanobacterial or algal biomass sources [2,5,7–47]. Usually, high purity is attained through a number of purification steps, often involving different column chromatographic methods, which reduce product yield and increase the costs, thus preventing the exploitation at a large scale [3,5]. A few simplified chromatographic purification procedures, more suitable to be used at a large scale production, have been proposed [17,21,23–27,31,34,35]. Conversely, only a few protocols completely avoiding the expensive and time consuming column chromatography purification steps have been proposed [11,12,36–38,41–47]. Among these, only procedures applying (1) aqueous two phase extraction steps coupled to ultrafiltration (which also can be a time consuming step) [12,36,42] or (2) membrane chromatography (MC) [47,48] provide analytical grade products. Examples of phycobiliprotein purification procedures available in the literature are listed in Table 1, with the crucial purification steps

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https://doi.org/10.1016/j.algal.2019.101685
Received 16 May 2019; Received in revised form 2 September 2019; Accepted 26 September 2019
Available online 31 October 2019

Table 1
Procedures applied to purify phycobiliproteins from cyanobacterial and algal extracts.

Crucial purification treatments *	Species	Purity (A_{Phs}/A_{280}) ***	Y(%)	Ref.
Column Chromatographic methods				
PC				
1. $(NH_4)_2SO_4$ precipitation 2. Hydroxyapatite chromatography 3. Ion exchange chromatography	*A. platensis*	4.15	–	[14] Boussiba et al. (1979)
1. Hydrophobic interaction chromatography 2. Ion exchange chromatography	*Synechochoccus* sp.	4.85	76.6	[15] Abalde et al. (1998)
1. Five rivanol treatments 2. $(NH_4)_2SO_4$ precipitation 3. Gel filtration chromatography 4. $(NH_4)_2SO_4$ precipitation	*A. fusiformis*	4.30	45.7	[16] Minkova et al. (2003)
1. Two rivanol treatments 2. $(NH_4)_2SO_4$ precipitation 3. Gel filtration chromatography 4. $(NH_4)_2SO_4$ precipitation	*A. africanum*	4.52	55.0	[17] Minkova et al. (2007)
1. Three $(NH_4)_2SO_4$ precipitation steps 2. Ion exchange chromatography	*Spirulina* sp.	4.42	45.6	[18] Patel et al. (2005)
1. Three $(NH_4)_2SO_4$ precipitation steps 2. Ion exchange chromatography	*Phormidium* sp.	4.43	35.2	[18] Patel et al. (2005)
1. Three $(NH_4)_2SO_4$ precipitation steps 2. Ion exchange chromatography	*Lyngbya* sp.	4.59	36.8	[18] Patel et al. (2005)
1. $(NH_4)_2SO_4$ precipitation 2. Hydroxyapatite chromatography	*A. flos-aquae*	4.78	–	[19] Benedetti et al. (2006)
1. Two $(NH_4)_2SO_4$ precipitation steps 2. Gel filtration chromatography 3. Ion exchange chromatography	*O. quadripunctulata*	3.31	44.2	[20] Soni et al. (2006)
1. Two $(NH_4)_2SO_4$ precipitation steps 2. Hydrophobic interaction chromatography	*P. fragile*	4.52	62.0	[21] Soni et al. (2008)
1. Expanded bed adsorption chromatography 2. Ion exchange chromatography	*A. platensis*	3.64	8.7	[22] Niu et al. (2007)
1. Two $(NH_4)_2SO_4$ precipitation steps 2. Ion exchange chromatography	*A. platensis*	4.00	–	[23] Moraes et al. (2009)
1. Ion exchange chromatography in Expanded bed mode	*Spirulina* sp.	1.60	79.0	[24] Moraes et al. (2015)
1. Ion exchange chromatography in Fixed bed mode	*Spirulina* sp.	1.70	62.0	[24] Moraes et al. (2015)
1. Two $(NH_4)_2SO_4$ precipitation steps 2. Ion exchange chromatography	*A. platensis*	5.59	67.0	[25] Yan et al. (2011)
1. Chitosan affinity precipitation 2. Activated charcoal adsorption 3. Ion exchange chromatography	*A. platensis*	4.30	42.3	[26] Liao et al. (2011)
1. Expanded bed adsorption chromatography 2. Ion exchange chromatography	*A. platensis*	> 4.00	59.0	[27] Bermejo et al. (2012)
1. Two $(NH_4)_2SO_4$ precipitation steps 2. Triton X-100 precipitation 3. Gel filtration chromatography 4. Ion exchange chromatography	*Lyngbya* sp.	5.53	60.2	[8] Sonani et al. (2014)
1. Two $(NH_4)_2SO_4$ precipitation steps 2. Ion exchange chromatography	*Synechococcus* sp.	4.03	–	[28] Sonani et al. (2017)
APC				
1. Rivanol treatment 2. $(NH_4)_2SO_4$ precipitation 3. Gel filtration chromatography 4. $(NH_4)_2SO_4$ precipitation	*A. africanum*	2.41	35.0	[17] Minkova et al. (2007)
1. Two $(NH_4)_2SO_4$ precipitation steps 2. Hydroxyapatite chromatography 3. Ion exchange chromatography	*A. platensis*	5.00	43.0	[29] Su et al. (2010)
1. Two $(NH_4)_2SO_4$ precipitation steps 2. Ion exchange chromatography	*A. platensis*	5.19	80.0	[25] Yan et al. (2011)
1. Two $(NH_4)_2SO_4$ precipitation steps 2. Gel filtration chromatography 3. Ion exchange chromatography	*Lyngbya* sp.	5.43	71.9	[8] Sonani et al. (2014)
PE				
1. $(NH_4)_2SO_4$ precipitation 2. Ion exchange chromatography 3. $(NH_4)_2SO_4$ precipitation	*P. cruentum*	> 4.00	32.7	[30] Bermejo-Roman et al. (2002)
1. Expanded bed adsorption chromatography 2. Ion exchange chromatography	*P. cruentum*	> 4.00	66.0	[31] Bermejo et al. (2003)
1. Hydroxyapatite chromatography 2. Gel filtration chromatography	*C. elongata*	6.67	20.3	[32] Rossano et al. (2003)
1. Two $(NH_4)_2SO_4$ precipitation steps 2. Gel filtration chromatography 3. Ion exchange chromatography	*Lyngbya* sp.	6.75	76.2	[8] Sonani et al. (2014)

(*continued on next page*)

Table 1 (*continued*)

Crucial purification treatments *	Species	Purity (A_{Phs}/A_{280}) ***	Y(%)	Ref.
1. Expanded bed pulsing chromatography	*P. cruentum*	> 4.00	–	[33] Gonzalez-Ramirez et al. (2014)
1. Two $(NH_4)_2SO_4$ precipitation steps 2. Gel filtration chromatography	*Phormidium sp. A27DM Halomicronema sp. A32DM*	4.26 3.73	70.5 60.9	[10] Madamwar et al. (2015)
1. Expanded bed chromatography (vortex flow reactor)	*P. cruentum*	> 4.00	78.1	[34] Ibañez-Gonzalez et al. (2016)
1. Ultrafiltration 2. Ion exchange chromatography	*P. cruentum*	5.10	68.5	[35] Tang et al. (2016)
Aqueous two-phase extraction				
PC				
1. Three aqueous two-phase extraction steps	*A. platensis*	4.02 **	78.6	[36] Patil et al. (2008)
APC				
1. Two aqueous two-phase extraction steps	*A. platensis*	0.75 **	51.7	[36] Patil et al. (2008)
PE				
1. Aqueous two-phase extraction	*P. cruentum*	3.20 **	90.0	[37] Benavides et al. (2006)
1. Isoelectric precipitation 2. Aqueous two-phase extraction	*P. cruentum*	4.10 **	72.0	[38] Hernandez-Mireles et al. (2006)
Aqueous two-phase extraction + Column Chromatographic methods				
PC				
1. Chitosan affinity precipitation 2. Activated charcoal adsorption 3. Aqueous two-phase extraction	*A. platensis*	5.10 **	66.0	[39] Patil et al. (2006)
1. Chitosan affinity precipitation 2. Activated charcoal adsorption 3. Aqueous two-phase extraction 4. Ion exchange chromatography	*A. platensis*	6.69	–	[39] Patil et al. (2006)
1. Ion exchange chromatography 2. Aqueous two-phase extraction	*Nostoc* sp.	3.55 **	–	[40] Johnson et al. (2014)
Aqueous two-phase extraction + Membrane process				
PC				
1. Two aqueous two-phase extraction steps 2. Ultrafiltration 3. $(NH4)_2SO_4$ precipitation	*A. maxima*	3.80	29.5	[41] Rito-Palomares et al. (2001)
1. Three aqueous two-phase extraction steps 2. Ultrafiltration	*A. platensis*	4.05	85.0	[12] Patil et al. (2007)
1. Three aqueous two-phase extraction steps 2. Ultrafiltration	*A. platensis*	4.02	–	[36] Patil et al. (2008)
APC				
1. Two aqueous two-phase extraction steps 2. Ultrafiltration	*A. platensis*	1.5	–	[36] Patil et al. (2008)
PE				
1. Isoelectric precipitation 2. Aqueous two-phase extraction 3. Ultrafiltration	*P. cruentum*	4.10	54.0	[42] Ruiz-Ruiz et al. (2013)
1. Isoelectric precipitation 2. Aqueous two-phase extraction 3. Ultrafiltration	*P. cruentum*	–	54.6	[43] Torres-Acosta et al. (2016)
Membrane process: Micro- and Ultrafiltration				
PC				
1. Two microfiltration steps 2. Ultrafiltration	*Spirulina* sp.	1.22	100.0	[44] Chaiklahan et al. (2018)
1. Microfiltration 2. Ultrafiltration	*A. platensis*	1.60	93.4	[11] Sala et al. (2018)
PE				
1. Ultrafiltration	*G. turuturu*	1.07	100.0	[45] Denis et al. (2009)
1. Microfiltration 2. Two ultrafiltration steps	*P. cruentum*	2.30	48.0	[46] Marcati et al. (2014)
Membrane process: Membrane chromatography				
PC				
1. Two membrane chromatography steps	*A. platensis*	4.20	67.0	[47] Lauceri et al. (2018)

* "Subsidiary" treatments, such as centrifugation or dialysis, are not reported.

*** A_{280} = absorbance at 280 nm; A_{Phs} = absorbance at Phs maximum (Phs = phycobiliproteins, i.e., PC or APC or PE).

** If aqueous two-phase extraction is the unique or the last purification step, polyethylene glycol (PEG) has still to be removed before product utilization.

adopted and the final yield and purity achieved for phycobiliproteins. Table 1, although incomplete, evidences traditional column chromatography as the most exploited purification approach, while, to the best of our knowledge, there is only an example dealing with the application of membrane chromatography to the purification of phycobiliproteins, though MC has emerged as a cost-effective alternative to column chromatography [49] and has gained growing importance, particularly for purifying large biomolecules (e.g. nucleic acids, proteins, antibodies) including viruses. Applications, advantages and drawbacks of MC versus packed-bed chromatography have been discussed in references [50–52].

Lauceri et al. [47] proposed for the first time MC as a simple and rapid method to purify phycocyanin from *Arthrospira platensis* (Spirulina), using a commercial hydrophilic, low protein binding, microfiltration membrane (hydrophilic PVDF membrane, Durapore) as an ammonium sulphate responsive chromatographic device to perform MC, and a relatively simple and cheap laboratory equipment.

Here we report evidences that MC can be also used to purify other phycobiliproteins of commercial interest, such as B-phycoerythrin (B-PE) and allophycocyanin. Freeze-thawing, and freeze-thawing coupled to sonication were adopted as extraction methods. They were tested with both fresh and freeze-dried biomass of *A. platensis* (one of the main sources of PC and APC [3]) and *P. cruentum* (one of the main sources of B-PE [42]) for a preliminary evaluation of the efficiency of the MC purification method in the presence of different contaminants. It is shown that the method can be opportunely tuned to obtain products of various degree of purity, that is, from cosmetic to analytical grade, turning out to be advantageous for various applications.

2. Materials and methods

2.1. Organism and culture conditions

Arthrospira platensis, strain GL and strain M2M, and the marine red microalga *Porphyridium cruentum* of the culture collection of the CNR - Istituto per la Bioeconomia at Sesto Fiorentino (Italy) were used. Cyanobacteria strains were grown in Zarrouk medium [53], while the marine strain in F medium [54]. All the strains were cultivated in glass columns (i.d. = 50 mm, 400 mL working volume) immersed in a thermostatic water bath to maintain optimal culture temperatures (30 °C and 25 °C for *A. platensis* and *P. cruentum*, respectively) and operated in a batch mode. Cultures were exposed to continuous photon flux density (PFD) of 150 μmol m^{-2} s^{-1} supplied from one side.

2.2. Chemicals and laboratory equipment for extraction and purification

Sodium phosphate monobasic dihydrate, analytical grade, and sodium chloride, ACS reagent, were purchased from VWR Chemicals; ammonium sulphate, RPE analytical grade, was purchased from Carlo Erba Reagents. Phosphate buffer, NaCl and $(NH4)_2SO_4$ solutions were prepared with 18 MΩ Milli-Q water.

In order to reduce the risk of PVDF membrane clogging during the MC process, disposable Corning bottle-top vacuum filter systems (cellulose acetate membrane, average pore size 0.22 μm, catalogue code CLS430767) were used to filter all the solutions as well as the deionized water used to clean the PVDF membranes.

MC procedure was performed with hydrophilic PVDF microfiltration membranes (Durapore, average pore size 0.45 μm, diam. 47 mm, thickness 125 μm, catalogue code P1938, filter code HVLP 04700) purchased from Sigma-Aldrich, using a 47 mm glass (or stainless steel) vacuum microfiltration assembly and a water vacuum aspirator pump (Fig. 1). A stainless steel Millipore filter holder (model MIL-XX2004720) was used for B-PE because of the pigment large adsorption on the sintered glass support.

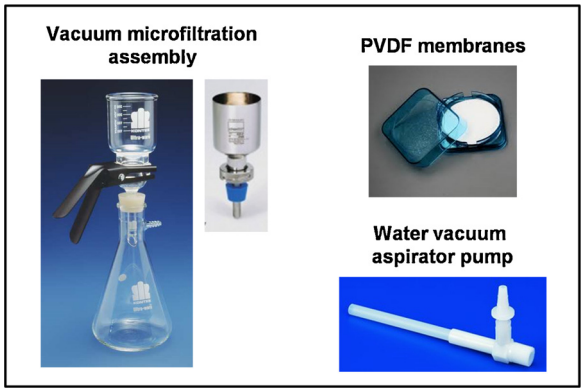

Fig. 1. Purification equipment.

2.3. Phycobiliprotein extraction procedure

2.3.1. Extraction of phycobiliproteins from freeze-dried biomass

Phycobiliprotein crude extract was obtained suspending freeze-dried cyanobacterial or algal biomass powder (typically about 100 mg) in 10–11 mL of extracting solution (NaCl 0.1 M for *A. platensis* biomass, PBS (phosphate buffered saline: 10 mM phosphate, 100 mM NaCl, pH 7) for *P. cruentum* biomass). The suspension was or (1) freeze/thawing only treatment: freeze-thawed once (frozen at −20 °C for 2 h and thawed at room temperature (20–25 °C)), maintained at 4 °C for 24 h, and then centrifuged for 45 min (12,000x*g*, T = 10 °C) to obtain the phycobiliprotein crude extract (the supernatant) or (2) freeze/thawing + sonication treatment: sonicated four times for 60 s (power 75%, pulse 60%, sonotrode S2, Hielscher Ultrasonic Processor UP200S, 200 W, 24 kHz) in water/ice bath, with 60 s pause in-between (to avoid phycobiliprotein degradation), freeze-thawed once and after maintained at 4 °C for 24 h. The suspension was finally centrifuged for 45 min (12,000x*g*, T = 10 °C) and the supernatant containing the phycobiliproteins (crude extract) was collected and stored at 4 °C.

2.3.2. Extraction of phycobiliproteins from fresh biomass

An aliquot of cyanobacterial or algal culture was centrifuged for 10 min (12,000x*g*, T = 10 °C) and the supernatant removed. The cyanobacterial or algal pellet was suspended and washed with pure 18 MΩ Milli-Q water, then centrifuged for 10 min (12,000x*g*, T = 10 °C) and the supernatant removed. The pellet was suspended in 10 mL of extracting solution (NaCl 0.1 M for *A. platensis* biomass, PBS (phosphate buffered saline: 10 mM phosphate, 100 mM NaCl, pH 7) for *P. cruentum* biomass) and was or (1) freeze/thawing only treatment: freeze-thawed three times between −20 °C and room temperature (20–25 °C), or (2) freeze/thawing + sonication treatment: sonicated four times for 60 s (power 75%, pulse 60%, sonotrode S2, Hielscher Ultrasonic Processor UP200S, 200 W, 24 kHz) in water/ice bath, with 60 s pause in-between, and then freeze-thawed three times. The freezing cycles lasted 2 h, except the last cycle that lasted all night. After the freeze-thawing treatment the suspension was maintained at 4 °C for 24 h and then centrifuged for 45 min (12,000x*g*, T = 10 °C). The supernatant containing the phycobiliproteins (crude extract) was collected and stored at 4 °C.

2.4. Phycobiliprotein purification procedure

The general extraction/purification processes of phycobiliproteins are schematized in Fig. 2.

Each purification step is characterized by a particular ammonium sulphate (AS) concentration ($[AS]_n$). The purification process includes:

1 **Cleaning MC step**: it is an **optional** MC step, but it is useful to increase the product purity. The cleaning step aims at removing

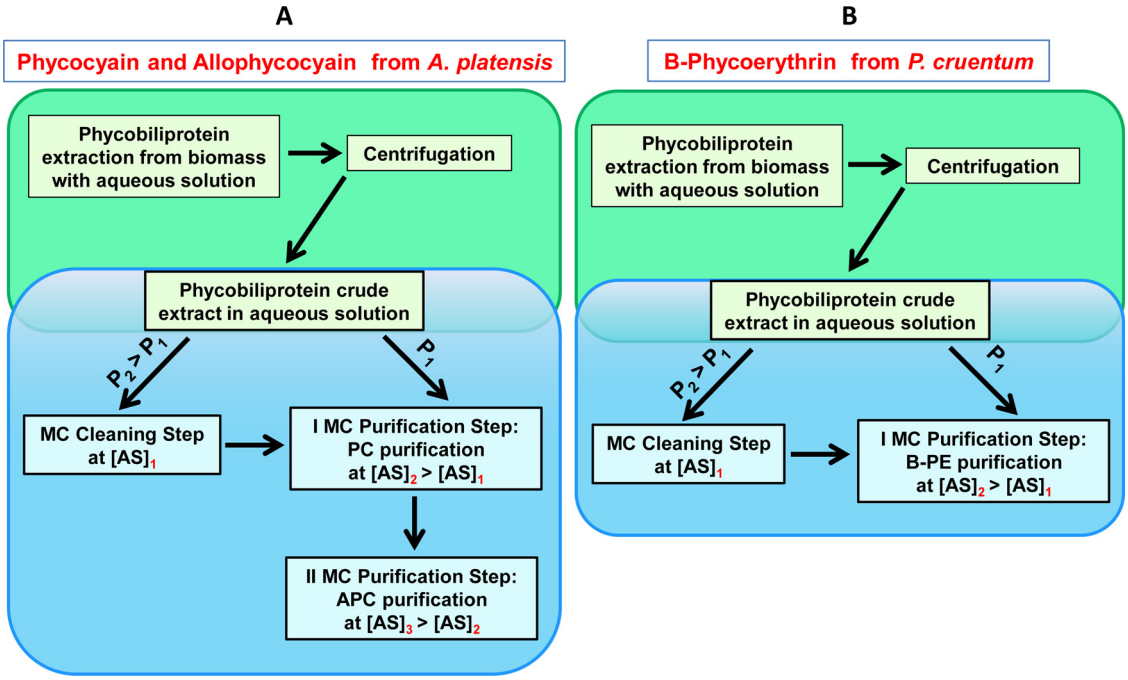

Fig. 2. General schemes of the extraction/purification processes for PC and APC (A) and B-PE (B). The extraction of the phycobiliproteins is outlined in the green box, while the purification procedure in the blue one (see the text for exhaustive explanations). P = purity; MC = membrane chromatography; [AS] = ammonium sulphate concentration; PC = phycocyanin; APC = allophycocyanin; B-PE = B-phycoerythrin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

from the phycobiliprotein extract those contaminant biomolecules requiring a lower ammonium sulphate concentration than the phycobiliproteins to bind to the membrane: ammonium sulphate (concentrated stock solution) was added to the aqueous phycobiliprotein extract to have a final AS concentration $[AS]_1$, sufficiently low to avoid phycobiliprotein binding to the membrane, but still suitable to promote the binding of some contaminant biomolecules. The phycobiliproteins filter through the membrane and the filtrate (called permeate) is recovered for the subsequent MC purification step.

2 **PC and B-PE purification:**

I MC purification step: ammonium sulphate (concentrated stock solution) is added to the aqueous phycobiliprotein extract or to the permeate obtained from the cleaning step to a final AS concentration $[AS]_2 > [AS]_1$, to induce the selective binding of the target phycobiliprotein (PC or B-PE) to the membrane (called retentate). The target phycobiliprotein is then desorbed and recovered as filtrate with an aqueous solution (e.g. deionized water, salt solutions, buffers).

3 **APC purification** (from *A. platensis* extracts):

I MC purification step – second cycle: before applying the second MC (II MC) purification step, the permeate containing APC is loaded again on the membrane and filtered to eliminate residues of PC still possibly present in solution (which binds to the membrane and it is after desorbed with an aqueous solution).

II MC purification step: ammonium sulphate (concentrated stock solution) is added to the permeate obtained from the second cycle of the I MC purification step to have a final AS concentration $[AS]_3 > [AS]_2$, in order to induce the selective binding of APC to the membrane (retentate). APC is then desorbed and recovered as filtrate with an aqueous solution (e.g. deionized water, salt solutions, buffers).

Summarizing, to achieve highly purified products, phycocyanin and allophycocyanin were separated and purified sequentially from the same extract (the total phycobiliprotein amount (PC + APC) loaded on the membrane was about 1 mg) following the general process described above and schematized in Fig. 3A, while B-phycoerythrin purification was executed following the procedure schematized in Fig. 3B (about 0.5 mg B-PE were loaded on the membrane). The details of the purification procedures are described in Appendix A, paragraphs "*1.1. PC and APC purification*" and "*1.3. B-PE Purification*".

In addition, simplified procedures (no cleaning MC step) at various ammonium sulphate concentrations were applied to purify PC and B-PE (Appendix A, paragraphs "*1.2. PC one step MC purification process*" and "*1.4. B-PE one step MC purification process*").

Ammonium sulphate 3 M was used as stock solution. NaCl 100 mM and PBS (phosphate buffered saline: 10 mM phosphate, 100 mM NaCl, pH 7) were used as extracting as well as desorbing (i.e., eluent) solutions. NaCl 100 mM was used in the purification process of *A. platensis* extracts, while PBS pH 7 was used in the purification process of *P. cruentum* extracts.

2.5. Spectrophotometric determination of the phycobiliproteins

PC and APC were determined using Eqs. (1) and (2) [55], B-PE was determined using the molar absorptivity coefficient at B-PE absorbance maximum, 547 nm, ($\varepsilon_M = 2.41 \times 10^6\ M^{-1}\,cm^{-1}$, i.e., $\varepsilon_{mg} = 10.04\ mL\ mg^{-1}\,cm^{-1}$) [56]. The purity (P) of phycobiliproteins (Phs) was evaluated using Eq. (3) and the separation factor (SF) between PC and APC (from *A. platensis* extracts), and between PC and B-PE (from *P. cruentum* extracts; the tiny amount of APC was not taken into account) using Eq. (4a) and (4b), respectively. Separation factors are defined as found for some commercial products (see for example, Sigma-Aldrich, Phyco-Biotech or ProZime product specifications), while in literature the reciprocal values are usually reported [18,36]. Finally, phycobiliprotein recovery yield (Y%) was determined by applying Eq. (5) [23], using the ratio between the amount of recovered phycobiliprotein and the amount of phycobiliprotein loaded on the membrane (i.e., the crude extract phycobiliprotein content was considered 100%).

$$[PC] = (A_{615} - 0.474\ A_{652})/5.34 \quad (1)$$

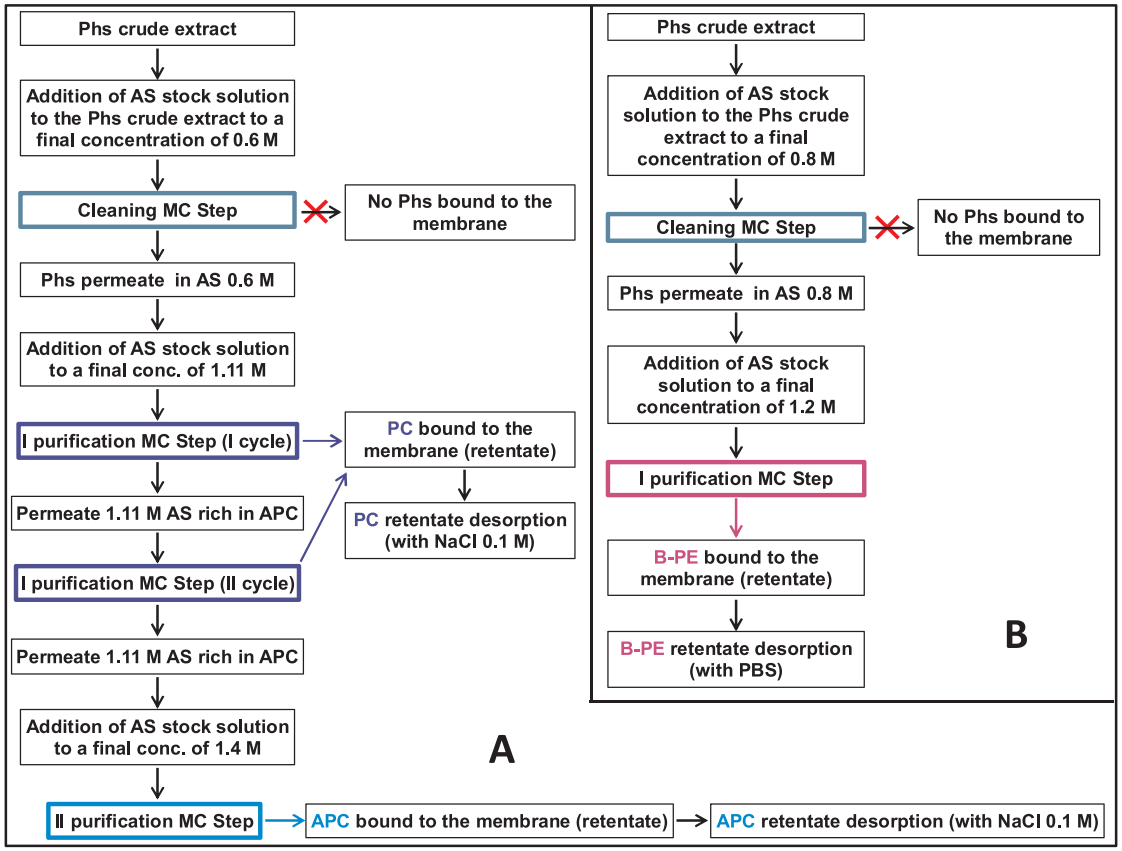

Fig. 3. Detailed purification procedures of (A) PC, APC and (B) B-PE. Phs = phycobiliproteins; AS = ammonium sulphate; MC = membrane chromatography; Phs = phycobiliproteins; PC = phycocyanin; APC = allophycocyanin; B-PE = B-phycoerythrin; PBS = phosphate buffered saline, pH 7.

$$[APC] = (A_{652} - 0.208\ A_{615})/5.09 \quad (2)$$

$$P = A_{Phs}/A_{280} \quad (3)$$

$$SF_{PC\ or\ APC} = A_{652}/A_{615} \quad (4a)$$

$$SF_{B\text{-}PE} = A_{615}/A_{547} \quad (4b)$$

$$Y\ (\%) = \frac{Phs\left(\frac{mg}{mL}\right) x\ collected\ volume\ (mL)}{Phs\ initial\ extract\left(\frac{mg}{mL}\right) x\ initial\ volume\ (mL)} x\ 100 \quad (5)$$

A_{615}, A_{652} and A_{547} are the absorbances at 615 nm (PC absorbance maximum), 652 nm (APC absorbance maximum), 547 nm (B-PE absorbance maximum), respectively; A_{Phs} is the absorbance maximum of PC, APC or B-PE and A_{280} is the absorbance at 280 nm (absorbance related to the whole protein content).

Absorbance measurements were carried out with an UVmc2 spectrophotometer (SAFAS). Suprasil quartz 1 cm light path cuvettes were used.

3. Results

The different content of contaminants due to the particular extraction procedure used (paragraphs 2.3.1 and 2.3.2) is easily evidenced by the spectrophotometric analyses of the crude extracts, as depicted in Fig. 4.

Highly purified products were obtained applying the procedure reported in paragraph 2.4 and schematized in Fig. 3. PC, APC and B-PE purity, separation factor and recovery yield are reported in Table 2.

The yield and the purity of phycobiliproteins, as well as the separation of the phycobiliproteins present in the extracts, depend not only on the number of MC steps, but also on the ammonium sulphate concentration. Purity, yield and separation factor of PC (extracted from *A. platensis)* and B-PE (extracted from *P. cruentum*) at various ammonium sulphate concentrations, purified by applying only one MC step, are reported in Fig. 5. The different separation of the phycobiliproteins contained in the extracts can be also easily deduced from the absorbance spectra. Some of them are reported in Fig. 6 as an example. The figure also shows, for comparison, the absorbance spectra of the crude extracts and of phycobiliproteins purified by applying two MC steps.

4. Discussion

In this work we have exploited MC to purify not only phycocyanin but also allophycocyanin and B-phycoerythrin, which are valuable phycobiliproteins having many commercial applications [3].

The results achieved confirm that MC is a valid method to obtain analytical grade PC with high yield (Table 2). Moreover, the method can be applied to purify APC with good purity, although lower than analytical grade, and with a yield roughly half of that attained for PC (Table 2). To the best of our knowledge, only column chromatography methods provide better results (see Table 1). Considering APC, a higher purity can be achieved by applying various cycles of the II MC purification step at ammonium sulphate 1.4 M (see Fig. 3), or carrying out the II MC purification step at a lower ammonium sulphate concentration. However, inescapably, a higher purity is attained only at the expense of the yield. For example, carrying out the II MC purification step at ammonium sulphate 1.3 M, APC purity increased from 3.3 (see Table 2: strain M2M, freeze dried biomass, freeze-thawing treatment) to 3.7 (± 0.2, no. = 2), while the yield decreased from 45% (see Table 2) to 34% (± 2, no. = 2). An even higher APC purity was achieved by applying two purification cycles at ammonium sulphate 1.4 M (P = 4.0 (± 0.1, no. = 2)), but always at the expense of the yield, which decreased to 29% (± 1, no. = 2). APC yield is also affected by the low binding capacity (about 3.7 mg/mL) of PVDF membrane for this

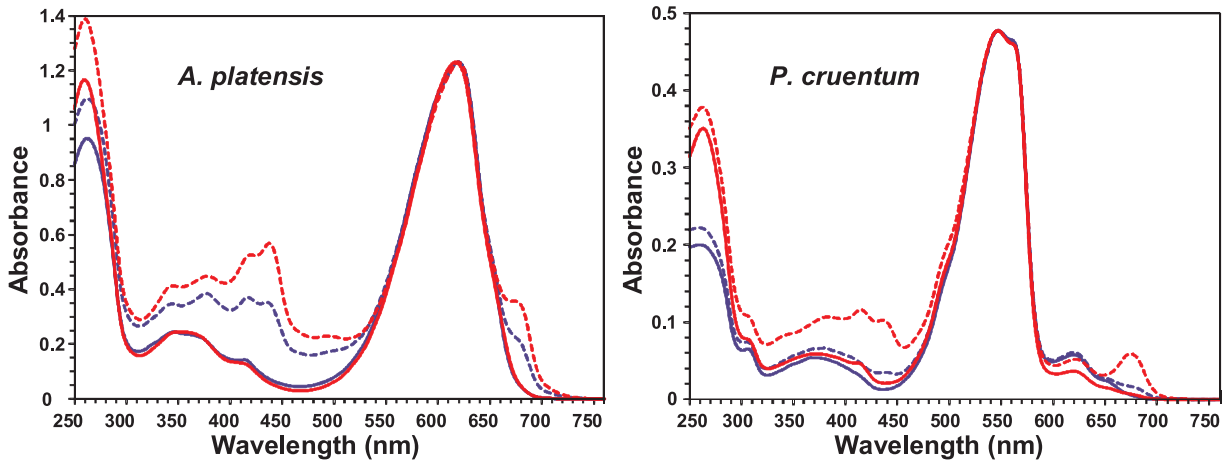

Fig. 4. Normalized (with respect to phycobiliprotein maximum) absorbance spectra of the extracts of the freeze-dried (red curves) and the fresh (blue curves) biomass of *A. platensis* (strain GL) and *P. cruentum*, prepared by freeze-thawing (continuous lines) and freeze-thawing + sonication (dashed lines). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

phycobiliprotein (see Appendix A, paragraph "*2. Evaluation of APC maximum binding capacity of PVDF membrane*"), that is only about 1/3 of that determined for PC (10.5 mg/mL) at AS 1.113 M (AS optimal concentration to purify PC and separate it from APC is 1.11–1.12 M) [47].

Good results were also obtained in the purification process of B-PE extracted from *P. cruentum*. The procedure permitted to obtain analytical grade B-PE with high yield (Table 2), however the method resulted to be not sufficiently selective to efficiently separate B-PE from PC and APC also present in *P. cruentum* cells (see Fig. 6 as an example; PC and APC bands are clearly visible around 620 and 650 nm, respectively).

Indeed, generally, selective separation is one of the main drawbacks of MC respect to column chromatography. Inadequate selective separation is one of the main reasons that has limited MC applications essentially to polishing or intermediate purification processes [29].

The moderate selectivity of MC induced us to consider its effect on the efficiency of the method (i.e., the purity of the product obtained). In particular, a preliminary investigation concerning the efficiency of the MC purification procedures (described in paragraph 2.4 and schematized in Fig. 3) was conducted using phycobiliprotein crude extracts (prepared from fresh and freeze-dried biomasses) containing various amounts of contaminants as a consequence of the extraction procedure adopted. Freeze-thawing and freeze-thawing plus sonication were adopted as extraction methods. In fact, aqueous extracts from fresh biomass contain different contaminants than the extracts from freeze-dried biomass, and cell breaking procedures less "gentle" than simple freeze-thawing can cause a greater extraction of contaminants as well (Fig. 4). Nevertheless, Table 2 shows that analytical grade PC was always achieved, with the exception of PC purified from the sonicated (both fresh and freeze dried biomass) extracts of *A. platensis* strain M2M. Analytical grade purity was also achieved for B-PE, even if significant amounts of PC and APC were still present in the purified products, due to the insufficient selectivity of the method when it was applied to *P. cruentum* extracts. On the contrary, analytical grade purity was never achieved for APC (with the exception of the application of multiple purification cycles at ammonium sulphate 1.4 M (II MC step)). APC of similar purity was obtained from all the extracts, while the yield was usually higher when freeze-thawing extraction was applied respect to freeze-thawing coupled to sonication (Table 2).

However, some applications of phycobiliproteins (for example, as food or cosmetic dye) do not require high purity products or the separation of one phycobiliprotein from the other, while it is important to maintain high yield of the product to reduce production costs.

The results obtained show that the MC method could benefit both applications requiring analytical grade purity and applications requiring less pure phycobiliproteins. As shown in Fig. 5 (one step MC procedure), the yield of PC increases with ammonium sulphate concentration until reaching a plateau, while purity initially increases and then decreases, and the separation factor (SF) initially decreases and then increases (a lower SF factor, as defined for PC and B-PE in paragraph 2.4 and in Fig. 5, corresponds to a more selective separation. Commercial analytical grade PC and B-PE usually has a specification SF < 0.3 and SF < 0.01, respectively; see, for example, Phyco-biotech or Sigma-Aldrich products). Concerning B-PE purification, it was not possible to apply MC procedure to *P. cruentum* extracts at ammonium sulphate concentration higher than 1.2 M, because of severe membrane clogging. Membrane clogging occurs also increasing B-PE concentration at AS 1.2 M, so that it was not possible to evaluate B-PE maximum binding capacity of the membrane. However, the data collected on B-PE purity and yield suggest a trend similar to that observed for PC, while B-PE separation from the other phycobiliproteins remains poor, which potentially hinders those applications that require high separation of B-PE from other phycobiliproteins.

In general, whatever the target phycobiliprotein, at low ammonium sulphate concentration the interaction of the phycobiliprotein with the membrane, which is crucial for its purification, is reduced. The membrane binding capacity increases with ammonium sulphate concentration, determining the increase of the product yield, but at the expense of phycobiliprotein purity and selective separation.

Lastly, comparing the one step (Fig. 5) and the two step (Table 2) MC procedures, the results evidenced that the one step MC procedure enables a higher yield but a lower purity and selectivity than the two step procedure. For example, the worst SF obtained for PC in the two step MC procedure (SF = 0.2) is however better (i.e., lower) than the best value obtained applying the one step MC procedure (see Fig. 5, $SF_{PC} > 0.25$ at any ammonium sulphate concentration). The same is true for B-PE.

5. Conclusions

The results obtained in this study show that MC can be exploited to purify various phycobiliproteins of commercial interest, such as phycocyanin, B-phycoerythrin and allophycocyanin. The method, applied to the crude extracts of fresh and freeze-dried biomasses of *A. platensis* and *P. cruentum* (obtained by applying two different extraction procedures), allowed us to achieve high yield and, usually, analytical grade purity for PC and B-PE. A lower yield and purity were achieved for APC.

In conclusion, the limited binding capacity of the membrane (particularly for APC) as well as poor selective separation (particularly for B-PE) are the main drawbacks of the method. Membrane clogging can be also a problem, especially at high AS concentration. However the

Table 2
PC, APC and B-PE purity and recovery yield.

Microalga	Biomass: Treatment	PC Purity (± SD, no. = a**)	PC SF* (± SD, no. = a**)	PC Yield % (± SD, no. = a**)	APC Purity (± SD, no. = a*)	APC SF* (± SD, no. = a**)	APC Yield % (± SD, no. = a**)	B-PE Purity (± SD, no. = a**)	B-PE SF* (± SD, no. = a**)	B-PE Yield % (± SD, no. = a**)
A. platensis Strain GL	Fresh: 3 Freeze/thawing	4.5 (± 0.2, no. = 8)	0.17 (± 0.01, no. = 8)	77 (± 6, no. = 8)	3.7 (± 0.3, no. = 8)	1.6 (± 0.2, no. = 8)	47 (± 3, no. = 8)	- - -		- - -
A. platensis Strain GL	Fresh: 3 Freeze/thawing + Sonication	4.2 (± 0.2, no. = 8)	0.166 (± 0.005, no. = 8)	76 (± 6, no. = 8)	3.6 (± 0.2, no. = 8)	1.6 (± 0.2, no. = 8)	38 (± 2, no. = 8)	- - -		- - -
A. platensis Strain GL	Freeze dried: 1 Freeze/thawing	4.3 (± 0.1, no. = 8)	0.19 (± 0.01, no. = 8)	81 (± 2, no. = 8)	3.4 (± 0.1, no. = 8)	1.6 (± 0.1, no. = 8)	46 (± 4, no. = 8)	- - -		- - -
A. platensis Strain GL	Freeze dried: 1 Freeze/thawing + Sonication	4.2 (± 0.1, no. = 8)	0.182 (± 0.004, no. = 8)	79 (± 1, no. = 8)	3.3 (± 0.1, no. = 8)	1.59 (± 0.03, no. = 8)	36 (± 2, no. = 8)	- - -		- - -
A. platensis Strain M2M	Fresh: 3 Freeze/thawing	4.4 (± 0.1, no. = 8)	0.20 (± 0.02, no. = 8)	82 (± 2, no. = 8)	3.5 (± 0.1, no. = 8)	1.77 (± 0.04, no. = 8)	42 (± 6, no. = 8)	- - -		- - -
A. platensis Strain M2M	Fresh: 3 Freeze/thawing + Sonication	3.9 (± 0.1, no. = 8)	0.17 (± 0.01, no. = 8)	81 (± 2, no. = 8)	3.3 (± 0.2, no. = 8)	1.69 (± 0.03, no. = 8)	50 (± 4, no. = 8)	- - -		- - -
A. platensis Strain M2M	Freeze dried: .1 Freeze/thawing	4.22 (± 0.04, no. = 8)	0.184 (± 0.004, no. = 8)	75 (± 5, no. = 8)	3.3 (± 0.1, no. = 8)	1.55 (± 0.01, no. = 8)	45 (± 1, no. = 8)	- - -		- - -
A. platensis Strain M2M	Freeze dried: 1 Freeze/thawing + Sonication	3.8 (± 0.2, no. = 8)	0.185 (± 0.003, no. = 8)	76 (± 3, no. = 8)	3.3 (± 0.2, no. = 8)	1.53 (± 0.07, no. = 8)	35 (± 1, no. = 8)	- - -		- - -
P. cruentum	Fresh: 3 Freeze/thawing	- - -		- - -	- - -		- - -	4.8 (± 0.3, no. = 12)	0.05 (± 0.02, no. = 12)	71 (± 4, no. = 12)
P. cruentum	Fresh: 3 Freeze/thawing + Sonication	- - -		- - -	- - -		- - -	4.8 (± 0.4, no. = 12)	0.05 (± 0.02, no. = 12)	69 (± 3, no. = 12)
P. cruentum	Freeze dried: 1 Freeze/thawing	- - -		- - -	- - -		- - -	4.5 (± 0.3, no. = 12)	0.03 (± 0.01, no. = 12)	69 (± 5, no. = 12)
P. cruentum	Freeze dried: 1 Freeze/thawing + Sonication	- - -		- - -	- - -		- - -	4.5 (± 0.3, no. = 12)	0.03 (± 0.01, no. = 12)	62 (± 6, no. = 12)

* SF = Separation Factor. For PC and APC SF = A_{652}/A_{615}; for B-PE SF = A_{615}/A_{547}.

** Data are the average of 2 or 3 independent (i.e. extraction + purification) experiments. In each experiment (i.e., using the same crude extract) 4 purification replicates were carried out.

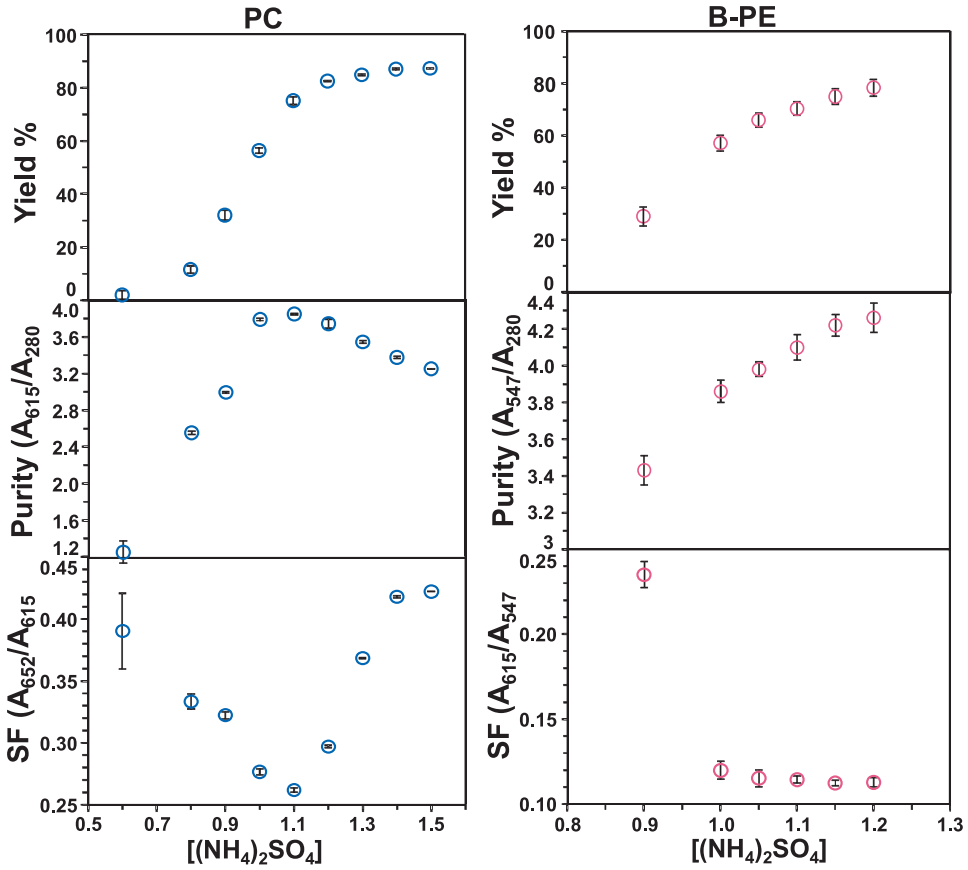

Fig. 5. One step membrane chromatography purification process. Phycocyanin (PC) and B-phycoerythrin (B-PE) purity and yield (%) as a function of ammonium sulphate concentration. Data are the average of 2 independent (i.e. extraction + purification) experiments.

simplicity of the procedure, which combines the power of chromatography with the speed of microfiltration (MC purification procedure requires about 10 min for PC or B-PE and about 15 min for APC, including membrane washing and extract solution manipulation), avoiding column chromatography and ultrafiltration steps, and the possibility to tune the procedure itself to obtain products of various purity grade, maximizing the yield, make the method attractive for obtaining products for various applications.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

Author contributions

All Authors declare that they have contributed to the manuscript in the following three sections: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data; (2)

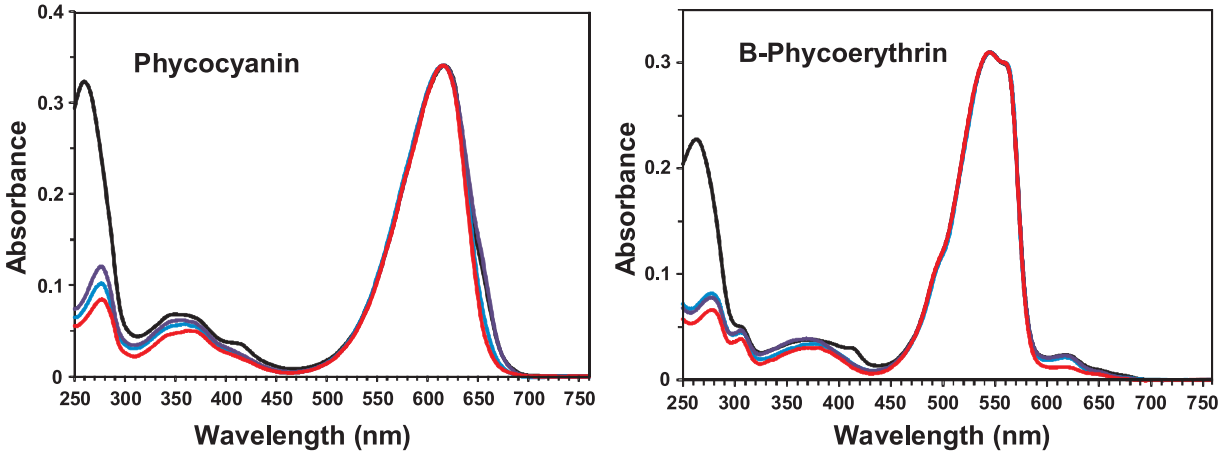

Fig. 6. Normalized (with respect to phycobiliprotein maximum) absorbance spectra of purified phycocyanin and B-phycoerythrin. Phycocyanin: one step MC at ammonium sulphate 1.1 M (light blue curve) and 1.5 M (blue curve), two step MC (red curve). The absorbance spectrum of *A. platensis* crude extract is also reported (black curve). B-phycoerythrin: one step MC at ammonium sulphate 1.1 M (light blue curve) and 1.2 M (blue curve), two step MC (red curve). The absorbance spectrum of *P. cruentum* crude extract is also reported (black curve). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be submitted.

Declaration of Competing Interest

The authors declare that they have submitted a patent application.

Acknowledgements

The authors wish to thank Mr Bernardo Cicchi for the preparation of the microalgal biomass and lyophilization.

Funding

The work has been partially supported by the H2020 project "SABANA" funded from the European Union's Horizon 2020 research and innovation program under grant agreement No 727874.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.algal.2019.101685.

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