Evaluation of Different Extraction Methods for Phycocyanin Extraction from *Spirulina platensis*

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Abstract

C-Phycocyanin is a natural pigment that has several applications in food and cosmetic industry. Recent studies have also revealed the medicinal effects of this natural blue dye. In this study, Comparison effect of mechanical (freezing and thawing, sonication and bead milling) along with chemical (inorganic acid, buffers and sea salt) extraction methods were carried out on C-Phycocyanin concentrations to achieve the optimum conditions of phycocyanin extraction from *Spirulina platensis*. Our result authenticated that repeating temperature cycles obtained higher phycocyanin concentration which could observed clearly by unaided eye. The optimum condition for extraction is freezing and thawing method under -20°C condition in five repeated cycles in comparison with liquid nitrogen at the same condition. The best solvent was determined sea salt solution then distilled water, PBS and TE buffer, respectively. Under optimize condition phycocyanin was extracted with a concentration of 0.29 mg/ml and purity ratio (A_{620}/A_{280}) of 3.42.

Key words: *Spirulina,* C-phycocyanin, Solvent, Freeze-thaw extraction, Sonication

Introduction

Currently, the demand of natural colorants is growing rapidly due to their beneficial applications in food and pharmaceutical industry (Chethana et al., 2015; Patil et al., 2006). Among of different colorant, the natural blue dye is more important than the others in confectionary and drinks industry due to rarity in nature (Martelli et al., 2014).

Phycobiliproteins are an excellent colorant water-soluble proteins bearing covalently attached with linear tetrapyrrole prosthetic groups known as phycobilins. Based on their visible absorption properties, the phycobiliproteins have been classified to four spectroscopic classes: phycoerythrocyanin, phycocyanins, allophycocyanins and phycoerythrins (Antelo et al., 2008). phycobilins are found not only in cyanobacteria but also in red algae and crytomonads (Moraes et al., 2011). They are mainly different in their protein structure and pigment color: red (phycoerythrins), purple

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(phycoerythrocyanin, R-Phycocyanin), dark blue (C-Phycocyanin) and blue-green (allophycocyanins) (Silva et al., 2009)

Among of all microalgae, the *Spirulina* genus is applied as a rich, inexpensive and natural source of phycocyanin pigment for food and cosmetics (Bhaskar et al., 2005; Furuki et al., 2003) which approved legally by Europe, Japan, USA and Brazil (Colla et al., 2016). It was estimated up to 20% of protein fraction of *Spirulina platensis* is phycocyanin (Silveira et al., 2007). Phycocyanin has been demonstrated some therapeutic properties including anti-inflammatory and anti-cancer effects (Dupuis et al., 2017; Mitra et al., 2015). The purity of C-PC which is figured out by the absorbance ratio of A620/A280, is essential factor to determine final application, so as purity of 0.7, 3.9 and above 4 consider as food, reactive and analytical grade, respectively (Patil et al., 2006; Rito- Palomares et al., 2001).

a wide range of C-PC extraction methods through physical or enzymatic treatments, have been reported which an industrial process involve physically cell disruption (Furuki et al., 2003). However, it is essential to present an appropriate technique for phycocyanin extraction from *Spirulina platensis* in large scale which is able to produce C-PC with high purity and concentration without any further purification requirement.

Due to many applications of phycocyanin in various fields, the aim of this study was to examine an extraction method with maximum yield of pigment and improvement of efficiency and purity.

Materials and Methods:

Culture conditions of Spirulina platensis

The algae was donated from algae collection of research institute of applied science of ACECR, Tehran, Iran. Algal cell cultivated in Zarrouk (Vonshaket al., 1982) medium at pH 9 using white lamp, shaked 100 rpm and aerated with atmospheric air.

Extraction methods

C-PC was extracted from the wet biomass of *Spirulina platensis* by using following methods:

Inorganic acid extraction

The wet biomass was treated with hydrochloric acid (5 M) in the proportion 5:1 (g biomass: ml hydrochloric acid) and thenwas subjected three alternative different methods: Without any treatment was placed in room temperature for 24 hours (Moraes et al., 2011). Repeated freeze- thaw in -20°C (3 cycles) Ultrasonic treatment for 30 min, temperature control to be under 30°C by exchanging with freshwater.

Mechanical extraction

The *Spirulina* cells were subjected 2 methods to disrupt cell wall mechanically: either using glass beads with mortar for homogenizationor freeze-thaw at first in -20°C (3 cycles) and followed by liquid nitrogen (3 cycles). TE buffer was used as a solvent in both methods.

Freeze-thaw optimization

In the present of Tris-HCl buffer (pH=8), *Spirulina* biomass was frozen in liquid nitrogen (3 cycles) or -20°C (3 or 5 cycles) and thawing at room temperature in order to compared together.

Comparing different solvents

To find out the best solvent for phycocyanin

extraction, *Spirulina* biomass was suspended in distilled water, different buffer (50mM sodium phosphate buffer pH 7 and Tris-HCl buffer in pH 8) and 1% sea salt separately. It was followed by Freezing (-20°C) and thawing (4°C) three repeated cycles.

In all above experiments the extracts were centrifuged at 10000 rpm for 5 min to remove cell debris and phycocyanin concentration ($mgml^{-1}$) was evaluated in their supernatants using the following equation 1 (Moraes et al., 2011; Sarada et al., 2011; Siegelman et al., 1978):

(1) $$CPC : \frac{(OD620 - 0.474OD652)}{5.34}$$

The purity and yield were calculated by following equation 2 and 3, respectively:

(2) $$\text{Purity: } \frac{OD620}{OD280}$$

(3) $$\text{Yield: } \frac{(CPC)V}{DB}$$

Where in OD620 is the maximum absorbance of C-Phycocyanin and OD280 is the total protein absorbance. V is the total solvent volume (ml) and DB is the dry biomass (g).

Results

This study compared three different types of extractions using solvent, ultrasonic and freeze-drying extraction.

Using HCL (5M) as an inorganic acid solvent in different PC extraction method (without any treatment, Sonication and freeze-thaw) showed no phycocyanin absorption and all of supernatants were colorless. The result exhibited that proper extraction method was freezing and thawing method with applied of TE buffer. However, applying homogenization in mortar and pestle with glass bead in TE buffer showed low amount of PC which we could not consider as a valuable data.

In comparison of freezing and thawing in-different temperature, the highest amount of C-PC was obtained by applying freeze-thaw in -20°C (5 cycles). Furthermore, The purity in this method was the highest in compared to other methods were used. According to Table 1, liquid nitrogen method yield was significantly higher than others, almost two times of -20°C (3 cycles). Our research experiments showed 3–5 cycles freeze–thaw were quite enough to achieve the high extraction efficiency. Although liquid nitrogen frozen in the presence of extraction medium for 3 cycles obtained the highest yield but the purity is lower than one. To choose suitable buffer for maximum phy-

Table1. Comparison of different freeze-thaw methods for C-Phycocyanin extraction

Amounts	Method		
	-20 (3 Cycles)	-70 (3 Cycles)	-20 (5Cycles)
CPC* (mg/ml)	0.20	0.31	0.33
yield (mg/g)	2.89	4.46	3.39
purity	0.80	0.97	1.31

*CPC means phycocyanin

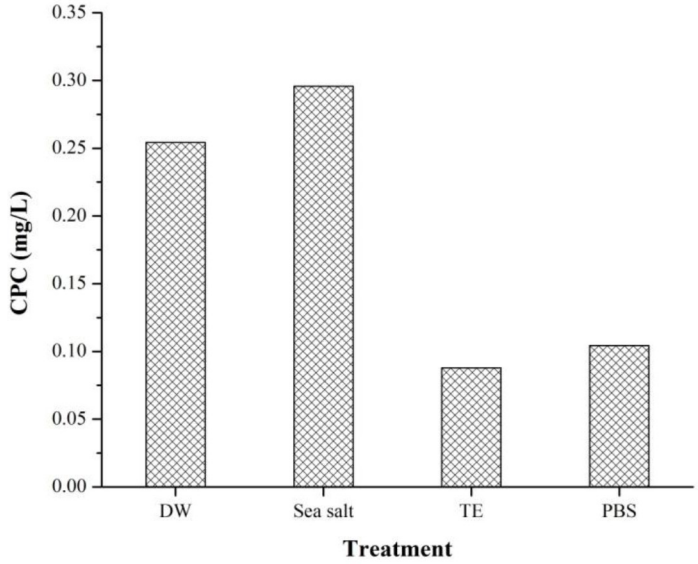

Fig. 1. C-Phycocyanin extraction by using different solvent

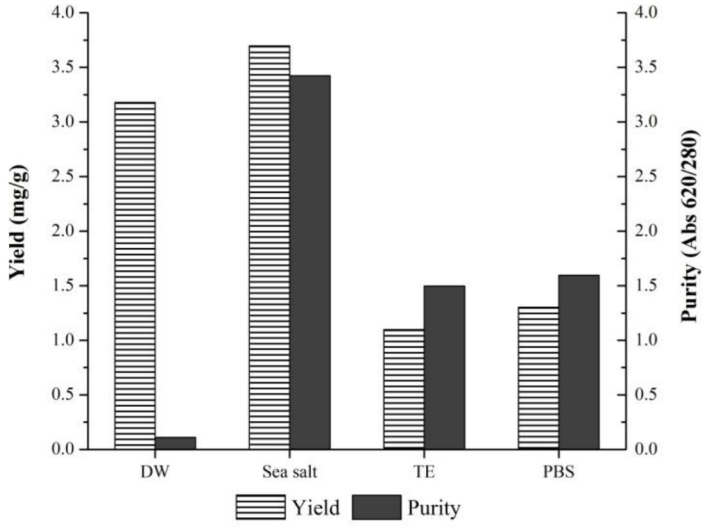

Fig. 2. Comparison of extraction purity and yield of C-phycocyanin by applying different solvent.

cobiliproteins extraction, Freezing (-20°C) and thawing at 4°C was done in present of PBS, TE, distilled water and sea salt. The highest amount of C-Phycocyanin was extracted when sea salt and distilled water were used as a solution, respectively (Fig. 1).

Similarly, the highest yield and purity showed in sea salt extraction. The comparison result of different C-phycocyanin extraction methods demonstrated in Figures 1 and 2, as shown the highest C-Phycocyanin extraction concentration (Figure 1), yield and purity (Figure 2) were estimated approximately 0.29 $mgml^{-1}$, 3.69 mgg^{-1} and of 3.42, respectively. The highest phycocyanin purity obtained from sea salt solution. PBS buffer at pH 7.0 exhibited phycocyanin yield slightly.

Discussion

One of the most important requirements to obtain phycobiliproteins from algae is optimizing the extraction method. A perfect method should be included rapid and sufficient disruption for a quantitative extraction and recovery of the released pigment. Our study showed that among different physically cell disruption methods, the best method for PC extraction are freeze-thaw which is similar with Moraes (2011) and Sarada et al. (1999) research reports. They indicated that HCl concentration lower than 8M resulted in the least amount of PC and also proved that the best method for disrupting cell wall was freezing and thawing. In addition, another study showed that high concentration of HCl was not suggested for separation of pigment from phycobiliprotein (O'hEocha, 1963; Sarada et al., 1999) and also proved that phycocyanin had low stability in pH below 5 (Sarada et al., 1999). Siavasankari and Ravindran (2014) reported that HCl (12M) showed significantly poor yield of phycocyanin in comparison with freezing and thawing method. Moreover, some papers reported that the freeze-thaw was the best method for C-phycocyanin extraction from wet biomass (Hemlata and Fareha, 2011; Kissoudi et al., 2017; Soni et al., 2008). The freeze–thaw method exhibited all mention advantages in addition to cost-effective without significant deprivations of the protein biological capacity (Moraes et al., 2011). In comparison withliquid nitrogen or -20°C, Freeze-thaw in -20°C showed the highest purity. As PC concentration depends straightly on the cell envelope disruption (Avila Jerley and Prabu, 2015), whenever the cell is frozen, the ice crystals formed during freezing and resulting in damage to the cell wall, promoting a better extraction of the phycobiliprotein (Soniet al., 2008). The more cell disruption, the higher protein extraction which effect on phycocyanin purity, however maximum 5 cycles freeze–thaw were enough to achieve the highest purity. It was sameas Zhu et al. (2007) and Horváth et al. (2013) reports.

Silveria et al. (2007) obtained the highest amount of phycocyanin using water as a proper solvent among applying different solvents which is similar to our result. It was concluded that the highest yield and purity was obtained by applying sea salt solution whereas the main content of sea salt is sodium nitrate and sodium hydrogen carbonate. Herrera et al. (1989) findings have already confirmed that sodium

nitrate solution is the best solution for phycocyanin extraction with high level of purity. On the contrary, our result about PBS extraction, Kissoudi et al. (2017) reported, sodium phosphate buffer show the best function in extraction.

In the present investigation, a new method has been examined for the C-PC extraction, which is not only simple but also is rapid and more efficient in compare with existing methods.

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