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An efficient extraction of phycocyanin by ultrasonication and separation using 'sugaring out'

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Abstract

Phycocyanin is high value, blue natural pigment with anti-aging, antioxidant and anti-inflammatory activities. Conventional methods used for extraction of phycocyanin are mortar-pestle, freezing-thawing and high pressure low temperature. Some of these methods are time consuming, whereas others do result into low yield. In the present work, phycocyanin was extracted using ultra-sonication at low temperature; further extracted phycocyanin was separated from chlorophyll using 'sugaring-out- a new phase separation method'. Effect of different parameters such as amplitude, time and duty cycle on yield of phycocyanin was assessed. Maximum phycocyanin yield (33.73 mg/g) and minimum energy (4.5×10^{-5} mg/J) required for extraction was achieved at 80% amplitude, 66% duty cycle in 4 minutes. The ultrasound assisted extraction and separation of phycocyanin using sugaring out significantly reduces time and demonstrates the ability to be cost effective solution for large scale production.

Keywords: Aqueous two phase separation, cell disruption, phycobiliproteins, Spirulina plantesis

Introduction

Phycocyanin is commercially important pigment and is used as food colorant (Furuki et al.2003; Priyadarshani and Rath 2012) nutraceutical and in immunodiagnostic applications. Various methods tried for extraction of phycocyanin are mortar-pestle ((Prabhakaran and Ravindran 2013), freezing and thawing (Doke 2005), high pressure low temperature homogenization processes (Seo et al. 2013; Moraes et al. 2011). Most of these extraction methods are time-consuming (Dey and Rathod 2012) however Ultrasound Assisted Extraction (UAE) may be an alternative to these methods.

Ultrasonic extraction is cavitation based phenomenon, where ultrasound is passed through any fluid forms millions of micro-bubbles at different locations in a medium. Cavitation results in the generation of high temperature (in the range of $1000-15000^0$K) and pressure (500−5000 bar) at thousands of locations in the reactor create higher micro-streaming and shock waves in liquid medium (Gole and Gogate, 2012). Physical effects are beneficial for enhancing the mass transfer limitation of extraction process. Various applications of ultrasound assisted extraction of protein, lipid, carotenoids and aromatic compound from the plant sources have been investigated (Cravotto et.al, 2008; Chen et. al, 2007; Ma et.al, 2009). UAE offers increased rate of extraction in less time with decreasing temperature and solvent volume which is very useful for extraction of heat labile compound.

Separation of phycocyanin should be in minimum possible time (Furuki et. al, 2003) to avoid contamination and degradation due to time and temperature. Sugaring out a new phase separation method, in which a monomeric carbohydrate or disaccharide was used to trigger phase separation in an Acetonitrile (ACN). ACN- water mixture created two phases, one solvent rich and the other aqueous phase separated by sugar which does not alter environment conditions (Dhamole et al 2010a; Dhamole et al 2010b; Dhamole et al 2016). In this work attempts have been made to extract PC in minimum possible time and separation of PC from other pigments and cell debris using sugaring out method.

Materials and Methods

Chemicals

Standard phycocyanin was provided by Hash Biotech Lab, Chandigarh, India. D-glucose (extrapure, AR), sucrose (pure), HPLC grade ACN (acetonitrile purity 99.8 %), Potassium dihydrogen phosphate (extrapure AR),

Dipottasium hydrogen phosphate (extrapure AR) were procured from sisco Research Laboratories Pvt. Ltd. (Mumbai, India).

Culture

Spirulina plantesis was cultivated in Zarrouk's medium under continuous supply of fluorescent light (3000 lux) for 10 days (Bachchhav et al 2016). After 10 days biomass was collected by centrifugation and washed with water. Freeze dried biomass found to be suitable. Washed *S. plantesis* biomass was freeze dried using Lyophilizer (Operon) at - 80^{o}C for 5-6 hrs. Sample powder was prepared by grinding in a pestle mortar for further process of extraction.

Extraction of Phycocyanin using ultrasound

Freeze dried powder (15mg) of *S. plantesis* was suspended in 50mM Phosphate buffer having pH 6.8 was kept for soaking for 10-15 minutes. To avoid over heating of reaction mixture, all experiments were performed at a below 5^{0}C temperature. Sonication was performed using sonochemical reactor VCX-130 with an ultrasonic probe operating at maximum frequency of 20 kHz with rated power of 130W. Ultrasonic probe was fitted with PZT transducer with tip diameter 2 mm. An amplitude 20µm is at 100%, experiments were conducted for different levels of amplitude such as 20, 40, 60, 80 and 100 % , time 2, 4, 6, 8, 10 and 12 minutes, operating at 33 %, 53 %, 66 % of duty cycle. After treatment of ultra-sonication sample was centrifuged at 6160 x g for 10 minutes.

Separation of Phycocyanin by sugaring out

Phycocyanin was separated from chlorophyll by sugaring out using new phase separation method (Dhamole et al 2010). Optimum phase separation condition for type of sugar, sugar concentration, solvent amount and temperature was obtained for a system containing 0.14g/L of aqueous phycocyanin solution. Phycocyanin solution (5ml) containing required amount of sugar (mass fraction- 0.1 to 0.2 in aqueous sugar solution) was mixed with equal amount of ACN. Both ACN and aqueous phase were mixed thoroughly on a vortex (Remi cyclomixer CM 101) and incubated for 3-4 hrs at 4^{0}C, 10^{0}C, 15^{0}C and 20^{0}C. Effect of temperature was studied because of heat sensitivity of phycocyanin. Samples were collected from top phase and bottom phase with a syringe. Volumes of top phase and bottom phase were recorded for calculation. Optimized separation conditions were applied for separation of phycocyanin from *Spirulina plantesis* by using following equation.

$$\text{PC Separation} = \frac{Amount\ of\ C-PC\ in\ Aqeous\ phase}{Total\ amount\ of\ PC} \times 100 \quad (1)$$

Analysis

Amount of phycocyanin was determined by a UV-visible spectrophotometer (Optima 3000 plus) optical density at 620nm. A standard graph for various concentration of phycocyanin at Optical density of 620 nm was plotted in order to evaluate a coefficient.

$$\text{C-PC Concentration} = \frac{OD\ at\ 620}{2.8536} \quad (2)$$

Purity

An absorbance maximum of C-phycocyanin is 620 nm and of total protein is 280 nm (Patil and Raghavrao 2007). Hence, the purity of phycocyanin was calculated as the ratio of OD at 620nm to 280nm.

$$\text{C-PC Purity} = \frac{\text{O.D at 620}}{\text{O.D at 280}} \quad (3)$$

Energy

Energy required for PC extraction is calculated by multiplying the power to extraction time. Phycocyanin extracted per energy is analyzed.

Results and Discussion

Effect of amplitude and time on Phycocyanin extraction

Effect of extraction time and amplitude on phycocyanin yield was evaluated. It was observed that phycocyanin yield is maximum 33.73 mg/g at 80% amplitude in 4 minutes and 32.13 mg/g at 100% amplitude in 2 minutes. However, after 6 minutes phycocyanin yield decreases after 40% amplitude. This may be mainly due to short duration between cell wall disruption and the release of pigment from *Spirulina* cells. With an increase in the extraction time, the concentration gradient may decrease, as the mass transfer was increased with continuous exposure to ultrasound as well as heating of cell components. The continuous increase in the release of phycocyanin due to extraction solvent became saturated with it and heating causes loss of phycocyanin yield (Fig 1).

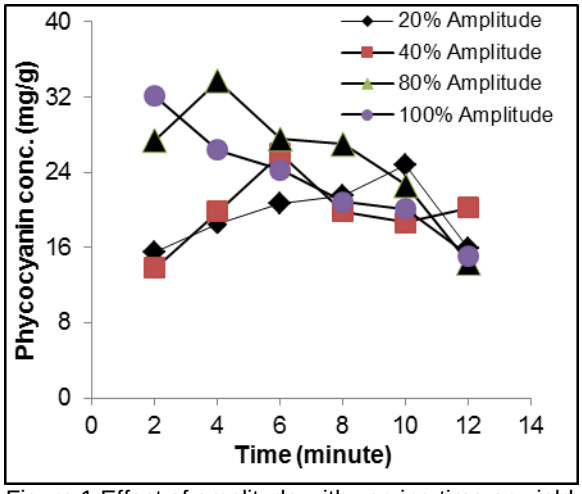

Figure 1 Effect of amplitude with varying time on yield of Phycocyanin

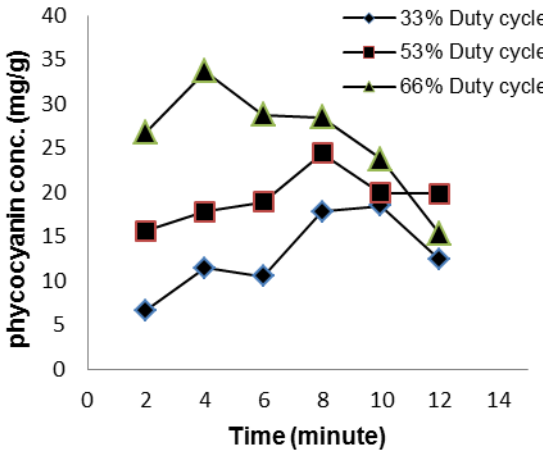

Figure 2 Effect of Duty cycle at 80% amplitude on yield of phycocyanin

Effect of Duty Cycle

The effect of duty cycle on the extraction yield of phycocyanin was studied at 33 % (i.e. 5s ON 10s OFF), 53 % (i.e. 8s ON 7s OFF) and 66 % (i.e. 10s ON and 5s OFF). Fig. 2 depicts that extraction yield of phycocyanin increases with a duty cycle (i.e. from 33% to 66 %), however, it increases with time up-to 4 minutes and then shows decreasing trend. This may be because of operating ultrasonicator in a continuous pulse mode, which caused heating of cell components leading to loss of Phycocyanin. Also, it creates the problem of erosion of the probe tip. An increase in the ON timing leads to excessive heating and unnecessary power consumption.

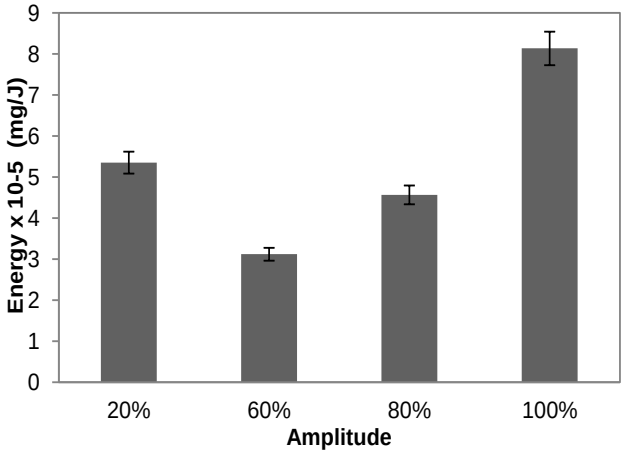

Figure 3 Maximum energy consumed at different amplitude for highest phycocyanin

Effect of amplitude on energy consumption

It was observed that extraction yield is maximum at 80 % amplitude with 4 minutes sonication time; however energy consumption at different amplitude shows decreasing trend with respect to time (Fig. 3). At 100 % amplitude energy requirement was more due to heat generation in the reaction mixture which may affect on yield of phycocyanin, as it is heat sensitive.

Evaluation of optimum condition for separation of PC using sugaring out

Separation conditions such as type of sugar and its concentration, temperature were optimized individually to achieve maximum separation efficiency with a system consisting of aqueous solution of 0.14 g/L are demonstrated as follows:-

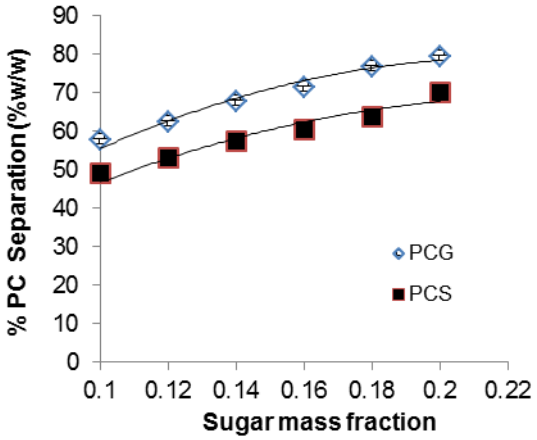

Figure 4 a Effect of type of sugar (monosaccharide-glucose and disaccharide: sucrose) on PC separation

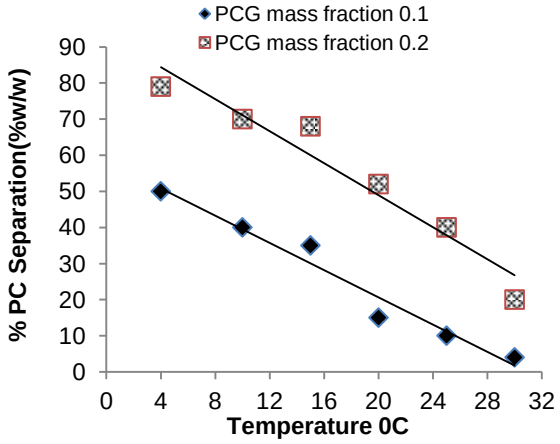

Figure 4 b Effect of temperature on separation of phycocyanin at 0.1 and 0.2 mass fraction

Effect of type of sugar and its concentration

Two types of sugar i.e. (glucose and sucrose) were used in an aqueous solution 0.14g/L of PC and its effect on separation efficiency was plotted (Fig. 4 a). It was found that separation efficiency was higher in case of glucose than sucrose by 20 %. The separation efficiency of phycocyanin increased with increasing concentration of glucose. Hence, glucose was chosen with mass fraction of 0.1 and 0.2 for further experimentation. According to the mass of sugar (200g/L), the mole of glucose (1.11mole) is more than sucrose (0.584). Sucrose can form eight hydrogen bonds, whereas glucose can form five. Thus it is anticipated that the number of hydrogen bond formed by same mass of glucose and sucrose, will be more in case of glucose, separating more amount of acetonitrile and resulting into higher separation.

Effect of temperature

The effect of temperature on phase separation at two different glucose mole fractions (0.1 and 0.2) was studied. The phase separation improved significantly by reducing the temperature from 30^0C to 1^0 C when glucose mole fraction was 0.1 whereas significant change in phase separation efficiency observed at glucose mole fraction of 0.2 (Fig.4 b). This could be credited to water structure at low temperature. The degree of hydrogen bonding depends on temperature. As the temperature is lowered, the distance between nearest neighboring water molecule is decreased. This means ACN will have a less chance to replace water molecule at lower temperature than at higher temperature. Further in an ACN-water-glucose mixture, glucose may replace the hydrogen bonds between ACN and water molecule. An effect of this may be translated to a better phase separation in an ACN-water system when sugar is added at low temperature (Dhamole et al 2010a).

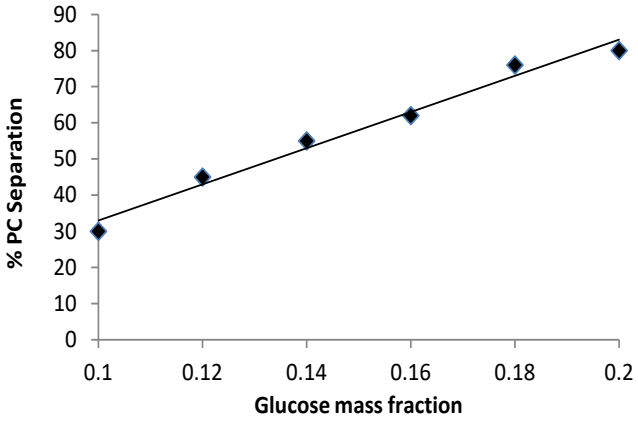

Figure 5 Separation of phycocyanin from *Spirulina plantesis* using sugaring out (Temperature 4^0C, ACN: buffer ratio 1)

Effect of Sugaring out on phycocyanin extraction

Optimized condition for sugaring out was used for separation of phycocyanin from *Spirulina plantesis*. During the extraction of phycocyanin by ultra-sonication disruption of cells released protein, chlorophyll, carotenoids. To separate all this debris from phycocyanin, sugaring out was tried as an alternative to ultracentrifugation. It formed two layers; the upper layer was of acetonitrile which contained all hydrophobic compounds like chlorophyll, carotenes and phycocyanin separated in the lower layer of buffer due to its hydrophilic nature (Fig.5). This method has minimum incubation time, high recovery of phycocyanin and removal of majority acetonitrile and no alteration in environmental conditions (Dhamole et al 2016).

Phenomena of sugaring out can be explained on the basis of interaction between glucose, acetonitrile and water molecules. It has been reported that acetonitrile molecules form three dimensional clusters and these clusters are surrounded by water molecule through hydrogen bonding and dipole-dipole interactions (Dhamole et al 2010b). It was thus anticipated that sugar molecules may have replaced the hydrogen bonds between acetonitrile and water molecules. This process may force ACN molecules out of the mixture resulting in a two phase formation. As a result, when sugar concentration was increased at low temperature it results in an increased in the upper phase (ACN-rich) volume as more ACN molecules are forced out.

Conclusion

In this paper detailed information regarding the effective extraction of phycocyanin from *S. plantesis* is provided and various parameters such as time, amplitude, duty cycle and energy consumed were optimized for improving the yield of phycocyanin by using ultra-sonication and sugaring out - a phase separation method. High yield of phycocyanin i.e. 33.73 mg/g obtained at 80 % amplitude within 4 minutes at 66 % duty cycle showing good purity. The process also has the advantage of using fewer steps and having a shorter process time, which may have contributed to the improvement in the yield and purity of the phycocyanin as compared with conventional method. Separation of phycocyanin from other pigments like chlorophyll and cell debris by using new separation method, i.e. sugaring out was tried and found to be cost effective. Also, separation of PC by sugaring out conditions were optimized and found the PCG at 0.2 mass fractions, at 4^0C is maximum. These results open up the possibility for large scale production and separation of phycocyanin with less possible time.

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