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Simple and efficient method for extraction of C-Phycocyanin from dry biomass of *Arthospira platensis*

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ABSTRACT

Conventional methods for primary extraction can extract only 50–60% of the total C-phycocyanin (C-PC) present in a given biomass because of the resistance offered by the cell membrane for its disruption. Current practice for extraction of C-PC is from wet biomass of *Arthospira platensis,* which is highly perishable. Drying of biomass increases the shelf life and helps the small scale industries as the need for considerable space and expertise for cultivation of biomass is avoided. However, dry biomass was reported to be unsuitable for C-PC extraction due to its higher resistance to cell disruption. So, the main objective of the current study is to develop a method for primary extraction of C-PC from dry biomass of *A. platensis*. Practically such reports are scarce. Conventional methods such as homogenization, maceration, freezing and thawing were attempted besides ultrasonication. A presoaking step (0–150 min) of biomass introduced prior to extraction has significantly increased the efficiency of all the extraction methods. Standardization of process parameters such as solid-liquid ratio (1:6, 1:8 and 1:10) and processing time was carried out besides amplitude (10–70%) and time (0–3.0 min) of ultrasonication. When ultrasonication was employed in combination with the conventional primary extraction methods, significant synergy was observed. Ultrasonication in combination with 'Freezing and thawing' resulted in 30% increase (maximum) in extraction efficiency over 'freezing and thawing' alone. Among all the methods employed, 'ultrasonication + freezing and thawing' resulted in the highest extraction efficiency of 92% followed by 'ultrasonication + maceration' (83.45%). Studies on extraction kinetics and energy requirement are also carried out. In any given primary extraction method, colour measurements showed a qualitative correlation of the extraction efficiency of C-PC with the extent of discoloration of spent biomass. These synergistic methods can be applied for extraction of biomolecules from other microalgae.

1. Introduction

Phycobiliproteins are accessory photosynthetic pigments that participate in an extremely efficient energy transfer chain in photosynthesis and responsible for about 50% of light capitation from blue-green algae and red algae. Chloro-phycocyanins (C-PC) constitute major portion of the phycobiliproteins in *Arthospira platensis* when compared to Allo-phycocyanins (A-PC) and phycoerythrins (PE) put together [1]. C-PC has many applications in pharmaceutical (fluorescent marker), cosmetic and also in food industry (as natural blue colorant). A few studies have demonstrated the neuroprotective [2], hepatoprotective [3], anti-inflammatory [2] and antioxidant [2,4] properties of C-PC. A few reports on upstream processing in terms of improving the concentration of C-Phycocyanin in biomass [5,6] and also on downstream processing with respect to separation and purification of C-PC are available [7–10]. The common practice for evaluating the extraction efficiency of downstream processing is based on the C-PC content in the crude extract. Most of the primary extraction methods were observed to extract only 50–60% of the total C-PC present in the biomass. The possible reason could be the resistance offered by the cell membrane for its disruption [11]. Improving the C-PC content in biomass will be useful only if one can extract most of this available C-PC in stable form. Hence, it is very important to achieve higher yields in primary extraction.

Growing biomass requires a lot of space which is a major constraint (availability and cost) for the industries located in metropolitan and urban places. As an economic alternative, *A. platensis* can be cultivated at remote/rural country side and can be transported to the industry in dried form for further processing in view of the highly perishable nature of wet biomass. It will immensely benefit the bioindustry, including small and medium scale industries, as the need for both expenditure as well as expertise for cultivation of biomass is avoided, since they can do

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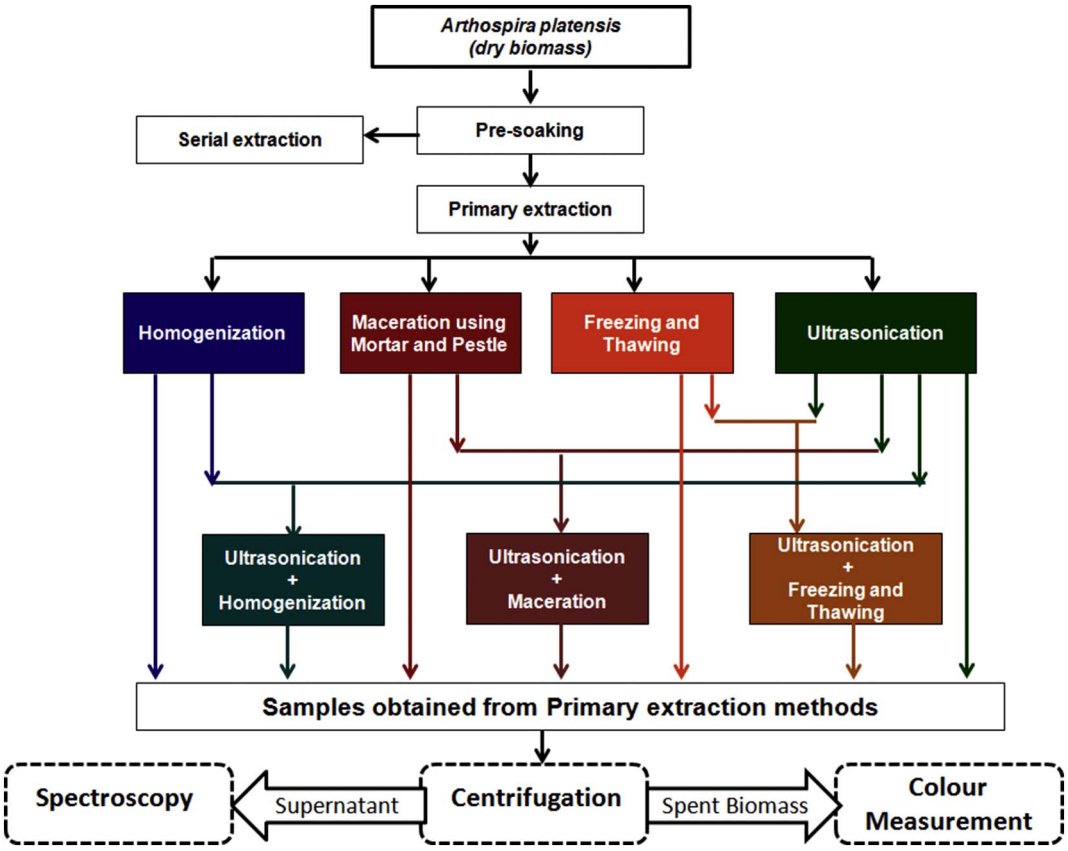

Fig. 1. Overall work plan for ultrasound assisted extraction of C-PC from dry *Arthospira platensis*.

the downstream processing with dry biomass as starting material. However, the cell membrane of wet biomass is easily disrupted during extraction, while that of dry biomass will be rigid and resistant, resulting in lower degree of extraction of C-PC. Inadequate disruption of cells results in hindered release of C-PC. Although, higher degree of cell disruption can result in higher release of C-Phycocyanin, it has a drawback of lower C-PC purity (due to the release of undesired biomolecules located in cytoplasm and other cell organelles). C-PC being a shear sensitive biomolecule, tends to denature when subjected to harsher processing conditions or to processing for longer durations [12,13]. Further, higher degree of disruption leads to fine cell debris which poses problems in further downstream processing. Thus, it is required to disrupt cell membrane sufficiently enough to release maximum possible C-PC from that is present in cell biomass without compromising on its purity and integrity.

Standardization of primary extraction for extraction of Phycobiliproteins from wet biomass of *Spirulina* sp. employing for methods such as homogenization [14], lysozyme digestion [15], *Klebsiella pneumonia* bacteria [16], ultrasonication [17], ultrasonication in presence of glass pearls [18] and pulsed electric field [19] has been reported. There are only a few studies on the standardization of C-PC primary extraction by different methods from dry biomass of *A. platensis* such as extraction using acids [20], water [21] and microwave [22].

Ultrasound can be used as an efficient cell disruption method. The research works carried out on ultrasound based extraction methods can be classified into two categories 1) ultrasound assisted methods where ultrasound alone is used for the disruption of cells and enhanced extraction and 2) ultrasound synergized methods where ultrasound is carried out along with other cell disruption methods to achieve synergy thereby increasing yield. Good number of reports are available where ultrasound assisted extraction [23,24] was explored in extraction of R-Phycoerythrin from *Grateloupia Turuturu* [25] and *Gelidium pusillum* [26], phycobiliproteins [14,27,28] and phenolic compounds from *A. platensis* [29]. On the other hand, reports on ultrasound synergized extraction methods for C-PC extraction are scarce.

Accordingly, the aim of this study was to evaluate different physical/mechanical methods in order to find the best protocol for primary extraction of C-Phycocyanin from dry biomass of *Arthospira platensis*. Besides ultrasonication, various conventional extraction methods such as homogenization, maceration, freezing and thawing alone and in combination were investigated. Process parameters such as pre-soaking time, solid-liquid (S/L) ratio and processing time for all the methods were standardized besides amplitude and time of ultrasonication.

2. Materials and methods

2.1. Materials

2.1.1. Algal biomass

Dry biomass of *Arthospira platensis*, reportedly grown in Zarrouk medium [30], was procured from M/s Parry Nutraceuticals, Chennai, India and stored in aluminum phthalate pouches at cold conditions (4 ± 2 °C).

2.1.2. Chemicals

K_2HPO_4 and KH_2PO_4 (phosphate salts) of analytical grade were procured from Merck, Bangalore. All other chemicals (analytical grade) used were procured from Merck, Bangalore.

2.2. Methods

2.2.1. Preparation of phosphate buffer

Phosphate buffer of 0.1 M and pH 6.8 [31], used for primary extraction of C-PC was prepared using double distilled water. Even in our earlier studies, pH of 6.8 was found to be the most suitable [8].

2.2.2. Primary extraction methods

Different conventional methods for primary extraction such as homogenization, maceration using mortar and pestle, freezing and thawing were attempted besides ultrasonication to arrive at the best protocol for the primary extraction of C-PC from dry biomass of *A. platensis*. Further, ultrasonication was also performed in combination with these conventional methods in order to explore the synergy. The schematic diagram of overall work plan is shown in Fig. 1 and the methods are explained in detail in the following sections.

2.2.2.1. Serial extraction. For estimating the maximum extractable C-PC content present in the dry biomass, repeated extraction (serial extraction) was carried out in the following manner. Dry biomass (5 g) was added to 0.1 M phosphate buffer (35 mL) of pH 6.8 at 1:8 S/L ratio (the middle value of S/L ratio range studied).

The suspension was soaked at room temperature (27 ± 2 °C) with intermittent stirring for 4 h. The suspension was then subjected to repeated cycles of freezing and thawing (4 h of freezing and 1 h of thawing) followed by centrifugation (REMI, PR 24) at 11,200 × *g* for 30 min at 4 °C. The supernatant was removed and the pellet was re-suspended in fresh batch of extraction buffer for Freezing and Thawing. After each cycle, the supernatant was analyzed for C-PC content. This extraction procedure was repeated until no further increase in C-PC content in the supernatant of a given cycle. The supernatant obtained from these repeated cycles was pooled for the estimation of C-PC content by spectrophotometric analysis. The C-PC extraction efficiency (η) of all the primary extraction methods was estimated considering this as the reference (100%) as per the Eq. (1) given below.

$$\text{C-PC extraction efficiency } (\eta) = \left[\frac{\text{C-PC from a given primary extraction method}}{\text{C-PC content from serial extraction}}\right] \times 100 \quad (1)$$

2.2.2.2. Pre-soaking of dry biomass. The dry biomass before subjecting to any primary extraction method was rehydrated by soaking in buffer in order to soften it for effective disruption of cells. Extraction of C-PC was carried out for samples of 1:8 solid-liquid (S/L) ratio (the middle value of S/L ratio range studied) at different soaking times (0, 15, 30, 60, 90, 120 and 150 min). This standardization of pre-soaking was done for two typical primary extraction methods namely, homogenization and ultrasonication. In case of homogenization, the samples were homogenized (Ultra-Turrax IKA 25T disperser with 18G tool, Germany) at 25,200 × *g* for 6 min (the middle value of extraction time employed for conventional methods). For ultrasonication, the samples were processed at ultrasonication time of 2 min, ultrasonication amplitude of 50% (the middle value of amplitude feasible with ultrasonication unit employed in the present study) at an ultrasonication pulse "on and off" cycle of 1/1 s.

2.2.2.3. Homogenization. Homogenization is a widely used industrial method for wet cell disruption. Predetermined weights of dry biomass of *A. platensis* were mixed with different required volumes of phosphate buffer (0.1 M, 6.8 pH) to result in slurries of three different S/L ratios, namely, 1:6, 1:8 and 1:10. The same S/L ratios are maintained in all the primary extraction methods employed in the present study. The samples were pre-soaked (for standardized duration) before subjecting to homogenization (Ultra-Turrax IKA 25T disperser with 18G tool, Germany) at 25,200 × *g* over time durations of 0, 2, 4, 6, 8, 10 and 12 min. Homogenization was carried out while cooling the extraction buffer in order to avoid its temperature rising above room temperature (27 ± 2 °C). The suspensions thus obtained were centrifuged in a refrigerated centrifuge at 4 °C and 11,200 × *g* for 30 min. Supernatant was taken for spectrophotometric analyses and spent biomass for colour analysis.

2.2.2.4. Maceration using mortar and pestle. Predetermined weights of dry biomass of *A. platensis* was mixed with different required volumes of phosphate buffer (0.1 M, 6.8 pH) resulting in slurries of three S/L ratios. The samples were pre-soaked (for standardized duration) before subjecting to maceration using mortar and pestle over time durations of 0, 2, 4, 6, 8, 10 and 12 min. The suspensions thus obtained were centrifuged at 11,200 × *g* for 30 min and 4 °C in a refrigerated centrifuge. Supernatant was taken for spectrophotometric analyses and the spent biomass for colour analysis.

2.2.2.5. Freezing and thawing. Freezing and thawing is a widely used laboratory method for extraction of phycobiliproteins [18]. In freezing and thawing, pressure is exerted on cell wall by freezing intracellular fluids as well as extracting buffer, leading to development of pores in the cell wall, facilitating leaching of the intracellular contents into the extraction buffer. Predetermined weights of dry biomass was mixed with different required volumes of phosphate buffer (0.1 M, 6.8 pH) resulting in slurries of three S/L ratios, namely, 1:6, 1:8 and 1:10. The samples were pre-soaked (standardized duration) and suspension was subjected to 6 cycles (extraction of C-PC significantly reduced after 6 cycles during serial extraction) of freezing and thawing. Each cycle involves freezing at −40 ± 2 °C for 4 h and thawing to room temperature (27 ± 2 °C) for 1 h. On centrifugation of the suspension, the supernatant was taken for spectroscopic analysis while spent biomass to colour analysis.

2.2.2.6. Ultrasonication. Different process parameters that affect the extraction of C-PC such as ultrasonication amplitude (10, 20, 30, 40, 50, 60 and 70%) and ultrasonication time (0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3 min) were standardized. Predetermined quantity of dry biomass was added to phosphate buffer (0.1 M, pH 6.8) to form slurries of three S/L ratios. The suspension was then pre-soaked (for standardized duration) before subjecting to ultrasonication (IKA Labortechnik, U200S control) at an ultrasonication pulse "on and off" cycle of 1/1 s. These ultrasonicated samples were centrifuged at 11,200 × *g* for 30 min at 4 °C and supernatant was taken for spectroscopy while spent biomass to colour analysis.

i. Standardization of process parameters

a. Ultrasonication amplitude

In order to study the effect of ultrasonication amplitude, extraction of C-PC from dry biomass of *A. platensis* (1:8 S/L ratio) was carried out by subjecting to ultrasonication for 2 min at different amplitudes (10, 20, 30, 40, 50, 60 and 70%) for pre-soaked (for standardized pre-soaking time) samples.

b. Ultrasonication time

In order to study the effect of ultrasonication time, extraction of C-PC was carried out by subjecting the samples of dry biomass of *A. platensis* at three S/L ratios pre-soaked (for standardized pre-soaking time) at standardized amplitude (standardized in previous section) for different ultrasonication times (0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 min).

c. Extraction kinetics

The following equation describes the extraction of C-PC from the dry biomass of *A. platensis* [32].

$$\frac{dC_L}{dt} = k(C_S - C_L)^2 \quad (2)$$

and the concentration terms involved are "C_L" and "C_s", the concentration of C-PC in the extraction (mg/mL) at any given time "t" (min) and the equilibrium concentration of C-PC in the extract

(mg/mL). "k" is the second order extraction rate constant (mL/mg·min).

separating the variables of Eq. (2), will result in the following.

$$\frac{dC_L}{(C_S - C_L)^2} = k \cdot dt \tag{3}$$

Integration of the above equation employing the limits of "t" (0 to t) and "C_L" (0 to C_L) and linearization of results in the following form.

$$\frac{t}{C_L} = \frac{t}{C_S} + \frac{l}{kC_S^2} \tag{4}$$

It may be noted that Eq. (4) is in the form of a straight line y = mx + c. The kinetic parameters namely, 'k' and 'C_s' the second order rate constant and equilibrium/saturation concentration, respectively, can be inferred from the Intercept and the slope of the plot of 't/C_L' versus "t". As 't' tends to "0", that is, at low values of 'C_L', 'R_i', the initial extraction rate (mg/mL. min.) can also can be estimated as $R_i = kC_s^2$.

d. Energy calculations

Sound waves that propagate through liquid medium during ultrasonication leads to rise in the temperature of the medium (extraction buffer in the present case). This happens because during ultrasonication, a portion of power gets converted into heat. The heat dissipated in the medium was estimated by calorimetric measurements [33]. The mean temperature rise with respect to time was recorded at adiabatic condition at standardized condition of ultrasonication using thermocouple [34]. The energy imparted by ultrasonication to the medium was estimated from this data.

Ultrasonic power (P) was calculated using the following equation.

$$P = mC_p\left(\frac{dT}{dt}\right) \tag{5}$$

where 'm', 'C_p' and 'dT/dt' are mass of the solvent (g), the specific heat of the solvent, (J/g/°C) and change in temperature with time (°C/min), respectively.

Ultrasonic intensity (UI, W/cm^2) and Acoustic energy density (AED, W/cm^3) [34] were calculated using following equations.

$$UI = \left(\frac{4P}{\pi D^2}\right) \tag{6}$$

$$AED = \left(\frac{P}{V}\right) \tag{7}$$

where, 'D' and 'V' are the probe diameter (cm) and the volume of the solvent in (cm^3), respectively.

2.2.3. Ultrasound synergized extraction methods

It was thought prudent to employ ultrasonication in combination with the conventional methods (homogenization, maceration, and freezing and thawing) in order to explore the synergy. Accordingly, experiments were carried out and the procedures are explained in the following sections.

2.2.3.1. Ultrasonication with homogenization. Predetermined weights of dry biomass of *A. platensis* were pre-soaked (for standardized pre-soaking time) with desired volumes of phosphate buffer (0.1 M, 6.8 pH) resulting in slurries of three S/L ratios. The slurries were subjected to ultrasonication (for standardized ultrasonication amplitude and time). The suspensions obtained after ultrasonication were subjected to homogenization for different time durations of 0, 2, 4, 6, 8, 10 and 12 min followed by centrifugation in a refrigerated centrifuge at 4 °C and 11,200 ×*g* for 30 min. Supernatant was taken for spectrophotometric analysis and spent biomass for colour analysis.

2.2.3.2. Ultrasonication with maceration. Predetermined weights of dry biomass of *A. platensis* were soaked (for standardized rehydration time) with desired volumes of phosphate buffer (0.1 M, 6.8 pH) resulting in slurries of three S/L ratios. The slurries were subjected to ultrasonication (for standardized ultrasonication time and amplitude). The suspensions obtained after ultrasonication were subjected to maceration using mortar and pestle for different time durations of 0, 2, 4, 6, 8, 10 and 12 min. The samples thus obtained were centrifuged in a refrigerated centrifuge at 4 °C and 11,200 ×*g* for 30 min. Supernatant was taken for spectrophotometric analysis and spent biomass for colour analysis.

2.2.3.3. Ultrasonication with freezing and thawing. Predetermined weights of dry biomass of *A. platensis* were pre-soaked (for standardized pre-soaking time) with required volumes of phosphate buffer (0.1 M, 6.8 pH) resulting in slurries of three S/L ratios. The slurries were subjected to ultrasonication (for standardized duration). The suspensions thus obtained were subjected to 6 cycles of freezing and thawing. Each cycle involved 4 h of freezing at −40 ± 2 °C and 1 h of thawing to room temperature (27 ± 2 °C). The suspension was subjected to centrifugation and supernatant to spectroscopic analysis while spent biomass to colour analysis.

2.2.4. Analysis

2.2.4.1. Spectroscopy. The concentration of C-Phycocyanin (C-PC) was determined using UV-spectrophotometer (Shimadzu, model UV1601, Japan) by measuring the optical density at 620 nm (λ_{Max} for C-PC) and 650 nm (λ_{Max} for A-PC). The concentration and purity of C-PC were calculated by the following equations [35].

$$\text{Concentration (mg/ml)} = \frac{[A_{620} - 0.7(A_{650})]}{7.38} \tag{8}$$

$$\text{Purity} = \frac{A_{620}}{A_{280}} \tag{9}$$

wherein A_{620} is the absorption maxima of C-PC and A_{280} is the absorbance of total proteins.

2.2.4.2. Colour measurement. Commission Internationale de L'Eclairage (CIE) L*, a* and b* values of spent biomass for C-PC, obtained from different primary extraction methods, were measured using a colorimeter (Konica Minolta CM-5, Japan). The values were measured using illuminant D65 and 10° observer angle. The instrument was calibrated using a standard white reflector plate. L* indicates the lightness of the colour where value '100' indicates white and value '0' indicates black. +a* value indicates redness and –a* indicates greenness of the sample. +b* value indicates yellowness and –b* indicates blueness of the sample [36].

2.2.4.3. Statistical analysis. All the experiments were carried out in triplicates (n = 3). Means and standard deviations (SD) are given for three independent experiments. Multiple comparison tests were carried out by ANOVA at 95% confidence level (p b 0.05) using Microsoft excel-2007.

3. Results and discussion

Ultrasonication, besides different conventional methods for primary extraction (homogenization, maceration using mortar and pestle and freezing and thawing) were attempted for enhancing the degree of primary extraction of C-PC from the dry biomass of *A. platensis*. Ultrasonication in combination with these conventional methods was also employed. The results are discussed in terms of yield (mg/g), purity and extraction efficiency (%) in the following sections.

3.1. Serial extraction

C-PC content in microalgal biomass varies from batch to batch depending on season and geographical location [20]. In order to account for this variation, expressing the results as extraction efficiency was thought to be prudent rather than alone the yield and purity of C-PC. The maximum extractable C-PC content of the biomass needs to be estimated for this purpose and hence serial (repeated) extraction was carried out. Repeated freezing and thawing was carried out (as described in Section 2.2.2.1) until no detectable C-PC content in the supernatant was observed. This formed the reference (100%) for the estimation of extraction efficiency of a given method.

No noticeable C-PC was observed in the buffer after the 9thcycle of serial extraction. The pooled extract of all the cycles was analyzed and total C-PC content was estimated to be 119 mg/g of dry biomass and the same was used for the estimation of the efficiency of any given primary extraction method (as per Eq. (1)).

3.2. Pre-soaking of dry biomass

Lower yield of C-PC in dry *A. platensis* was observed by Sarada et al. [20]. Dry biomass when directly used for extraction without pre-soaking results in very low yield because of the resistance offered by the hardened dry cell membrane. So, the biomass was soaked (as described in Section 2.2.2.2) for different times (0, 15, 30, 60, 90, 120 and 150 min). This standardization was carried out for two typical primary extraction methods, namely, homogenization and ultrasonication. For both these primary extraction methods attempted, 120 min of rehydration time was found to be the most suitable. Further increase in soaking time had no effect on extraction of C-PC (for both homogenization and ultrasonication). Hence the same pre-soaking time was maintained for all the primary extraction methods.

3.3. Homogenization

It is desirable to soften the dry biomass before extracting C-PC which was achieved by rehydrating the dry biomass by soaking. Pre-soaking time was standardized. The results of the same are presented in Fig. 2a. It can be seen from the figure that the samples without soaking resulted in the lowest yield of 29.01 mg/g of dry biomass after homogenizing for 6 min at 25,200 ×*g*. Yield of C-PC increased with an increase in soaking time till 120 min (2 h). Further increase in soaking time was observed not to have any effect on extraction of C-PC. At the conditions studied (soaking time of 120 min, homogenization time of 6 min, S/L ratio of 1:8), a maximum C-PC content of 52.26 mg/g dry biomass could be extracted which is ~23 mg/g more when compared to the results obtained from unsoaked biomass. Hence 120 min of pre-soaking was considered for extraction of C-PC in all the conventional primary extraction methods, including homogenization. The effect of pre-soaking time on extraction yield was found to be statistically significant during Homogenization ($p < 0.05$). The extract at pre-soaking time ($T = 0$) was considered as control.

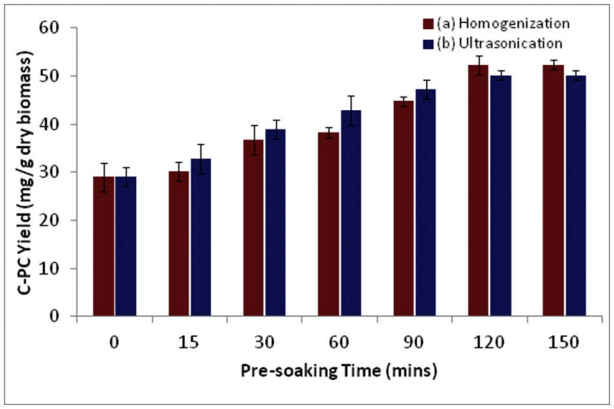

Fig. 2. Effect of pre-soaking on extraction of C-PC by (a) homogenization, (b) ultrasonication.

In order to standardize S/L ratio and homogenization time, the dry biomass (pre-soaked for 120 min) at three S/L ratios was subjected to homogenization at 25,200 x g for different time durations. The results of these experiments are presented in Fig. 3a. It can be seen that for all the three S/L ratios attempted, increase in the degree of extraction (mg/g dry biomass) was observed with an increase in homogenization time. A maximum yield of 53.84 mg/g dry biomass with a purity of 0.38 was observed at 1:10 S/L ratio at homogenization time of 6 min. Practically the same yield (52.11 mg/g dry biomass) could be achieved at 1:6 S/L ratio at homogenization time of 8 min, however, with higher purity of 0.6. Based on the value of serial extraction, the extraction efficiency of homogenization at standardized conditions was estimated to be 45.24 and 43.79% for 1:10 and 1:6 S/L ratios, respectively. The effect of extraction time on extraction yield and purity were found to be statistically significant ($p < 0.05$) for all the three S/L ratios attempted during Homogenization. Homogenization was observed to be the best among the conventional methods when applied individually for primary extraction of R-phycoerythrin (R-PE) from macroalgae *Gelidium pusillum*" with an extraction efficiency of 63% [26].

3.4. Maceration using mortar and pestle

The dry biomass (pre-soaked for 120 min) at three S/L ratios was subjected to maceration using mortar and pestle and the results are shown in Fig. 3b. It can be seen from the figure that the yield (mg/g dry biomass), at all the three S/L ratios employed, increased with an increase in maceration time. A maximum yield of 57.14 mg/g dry biomass could be observed at 1:10 S/L ratio at maceration time of 4 min. Practically the same yield (55.91 mg/g dry biomass) could be achieved at 1:6 S/L ratio at maceration time of 8 min. However, higher purity (0.63) could be achieved at 1:6 over 1:10 S/L ratio (0.54). The extraction efficiency of maceration at standardized conditions estimated based on the value of serial extraction was 48.01 and 46.98% for 1:10 and 1:6 S/L ratios, respectively. The effect extraction time on extraction yield and purity were found to be statistically significant for all the S/L ratios attempted during Maceration ($p < 0.05$).

3.5. Freezing and thawing

The dry biomass (pre-soaked for 120 min) at three different S/L ratios was subjected to freezing and thawing (for 6 cycles, each cycle involving 4 h of freezing at −40 ± 2 °C and 1 h of thawing to room temperature) and the results are shown in Fig. 4. It can be seen from the figure that the yield increased with number of cycles up to 5th cycle above which it became constant.

Steady state could be achieved quickly in case of higher S/L ratios. A maximum yield of 74.51 mg/g dry biomass with a purity of 0.56 could be observed in freezing and thawing at 1:10 S/L ratio after 4 cycles of (higher compared to that of homogenization and maceration, 53.84 and 57.32, respectively). Practically the same yield (73.73 mg/g dry biomass) could be achieved at 1:6 S/L ratio after 4 cycles, however, with higher purity (0.66). Based on the value of serial extraction, the extraction efficiency of freezing and thawing at standardized conditions was estimated to be 62.61 and 61.9% for 1:10 and 1:6 S/L ratios, respectively. The effect of extraction time on extraction yield and purity were found to be statistically significant ($p < 0.05$) for all the S/L ratios attempted also during freezing and thawing.

In case of homogenization and maceration, higher purity without compromising much on yield was observed in 1:6 S/L ratio. In all these methods 1:8 S/L ratio was observed to result in intermediate values of yield and purity of 1:10 and 1:6 S/L ratios. It appears that, in freezing

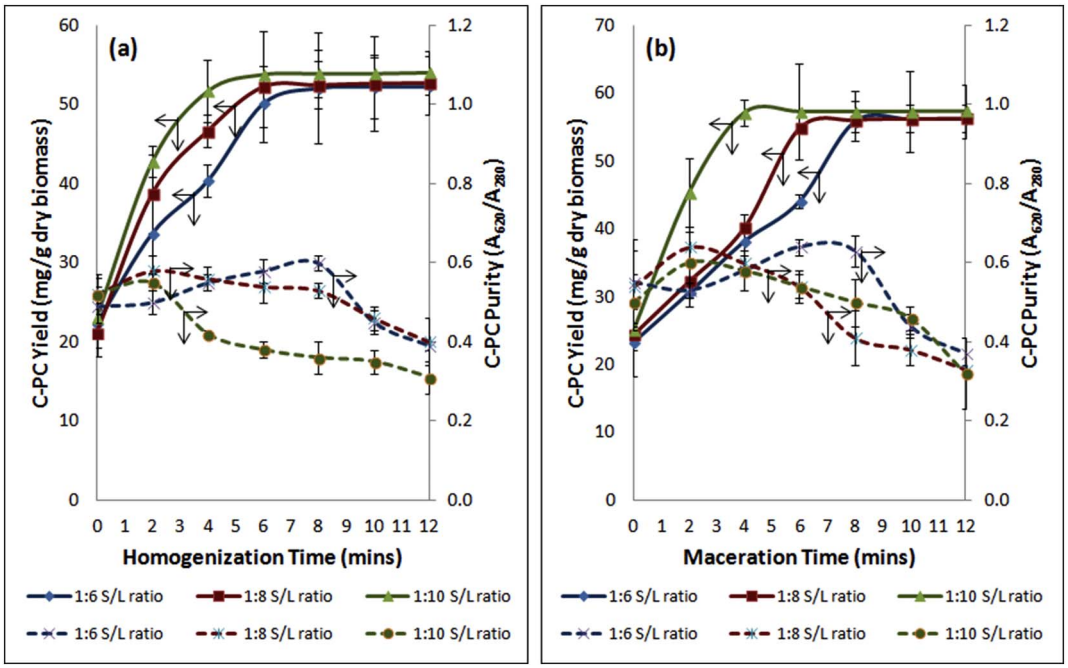

Fig. 3. Extraction of C-PC by (a) homogenization, (b) maceration.

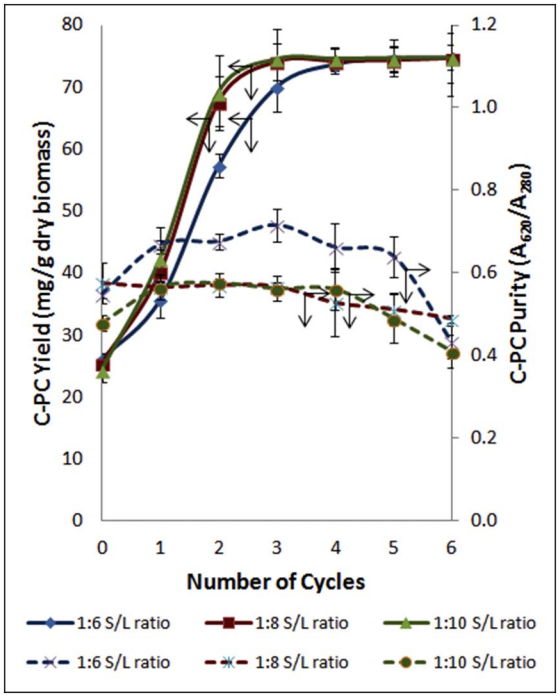

Fig. 4. Extraction of C-PC by freezing and thawing.

and thawing, during 4th cycle, the cell membrane of the biomass was broken sufficiently to release C-PC but not much of the other contaminant proteins from cytoplasm and other organelles resulting in maximum purity and yield. At freezing and thawing cycles higher than 4 cycles, even though slight increase in C-PC yield was observed, purity reduced indicating the release of contaminant proteins.

3.6. Ultrasonication

3.6.1. Standardization of different parameters

For standardization of pre-soaking time, ultrasonication time of 2 min, ultrasonication amplitude of 50% (the middle value of amplitude feasible with ultrasonication unit employed in the present study) was used. Ultrasonication pulse 'on and off' cycle (1/1 s) was used in the present work.

The results of pre-soaking experiments during ultrasonication are presented in Fig. 2b. It can be seen from the figure that the samples without soaking resulted in the lowest yield of 29.12 mg/g of dry biomass after sonicating for 2 min. It was observed that the yield of C-PC increased with an increase in soaking time till 120 min (2 h). Further increase in soaking time was observed not to have any effect on extraction of C-PC. At the conditions studied (rehydration time of 120 min, ultrasonication time of 2 min, ultrasonication amplitude of 50%, S/L ratio of 1:8), a maximum C-PC yield of 50.1 mg/g dry biomass could be obtained which nearly twice the amount obtained than that from unsoaked dry biomass. Hence, 120 min of soaking was considered for further ultrasonication experiments. The effect of pre-soaking time on extraction yield was found to be statistically significant during ultrasonication time of 2 min at 50% amplitude and 1:8 S/L ratio ($p < 0.05$). The extract at pre-soaking time ($T = 0$) was considered as control.

3.6.1.1. Ultrasonication amplitude. Ultrasonication amplitude was standardized by carrying out the extraction of C-PC at different amplitudes (10, 20, 30, 40, 50, 60 and 70%) for ultrasonication time of 2 min at 1:8 S/L ratio, pre-soaked for 120 min. The results are shown in Fig. 5. It can be inferred that a maximum yield of C-PC of 48.88 mg/g dry biomass was observed at 50% ultrasonication amplitude. Increase in degree of extraction was not observed with further increase in amplitude. Accordingly, further extraction experiments were carried out at ultrasonication amplitude of 50% (incidentally the middle value of the range). The effect of ultrasonication amplitude on extraction yield was found to be statistically significant ($p < 0.05$) during presoaking time of 120 min, ultrasonication time of 2 min at 50% amplitude and 1:8 S/L ratio [26].

3.6.1.2. Ultrasonication time. In order to study the effect of ultrasonication time, extraction was performed from dry biomass (pre-soaked for 120 min) of three different S/L ratios for different ultrasonication times of 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 min at ultrasonication amplitude of 50%. The results are shown in Fig. 6. It

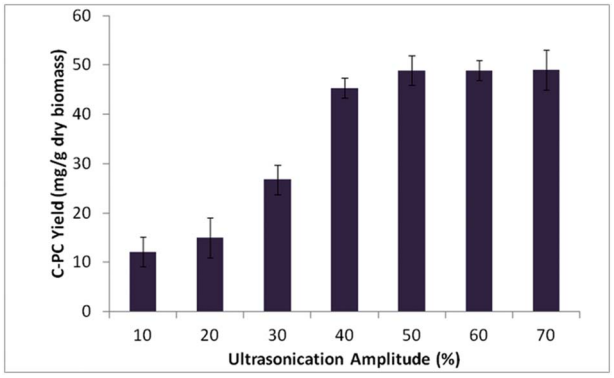

Fig. 5. Effect of ultrasonication amplitude on extraction of C-PC by ultrasonication.

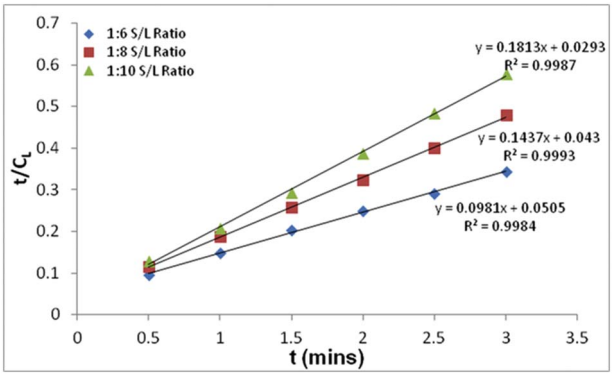

Fig. 7. Kinetics of C-PC extraction by ultrasonication.

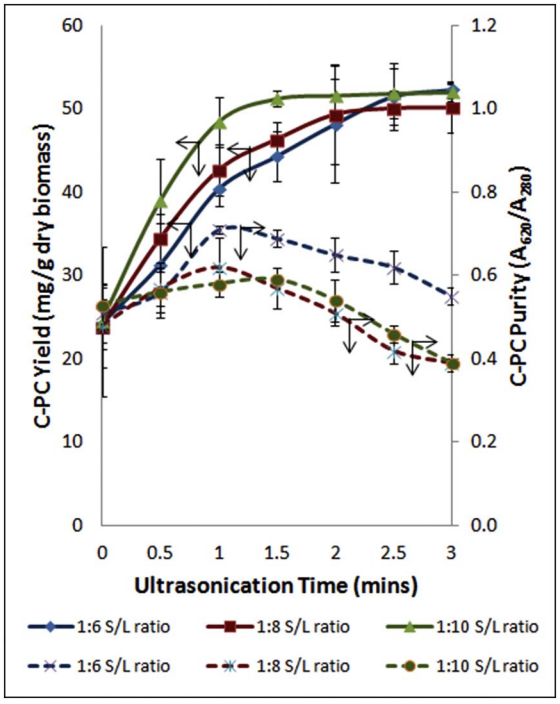

Fig. 6. Extraction of C-PC by ultrasonication.

can be seen from the figure that the yield (mg/g dry biomass), for all the three S/L ratios attempted, increased with an increase in ultrasonication time. A maximum yield (51.23 mg/g dry biomass) with a purity of 0.59 was observed at 1:10 S/L ratio at ultrasonication time of 1.5 min. Practically the same yield (51.51 mg/g dry biomass) but with a higher purity of 0.62 could be achieved at 1:6 S/L ratio after 2.5 min of ultrasonication. Based on serial extraction, the extraction efficiency of ultrasonication at standardized conditions was estimated to be 43.05 and 43.26% for 1:10 and 1:6 S/L ratios, respectively. Even in ultrasonication, 1:8 S/L ratio is observed to result in intermediate yield and purity values of 1:10 and 1:6 S/L ratios. A similar extraction efficiency of 42.65% was observed by Zhu et al. [16] using ultrasonication for primary extraction of C-PC from wet biomass of *A. platensis*. Reports on positive impact of ultrasonication are available on extraction of C-PC from *A. platensis* [17].

Higher yields perhaps could have been achieved if ultrasonication was carried out for higher times. However, ultrasonication was never allowed to exceed 3 min because further increase in ultrasonication time resulted in an increase in temperature (despite cooling with the help of a jacket) which is detrimental to the stability of C-PC. Hence, ultrasonication was carried out in combination with other conventional methods Hence, for ultrasonication, S/L ratio of 1:6, soaking time of 120 min, ultrasonication amplitude of 50% and ultrasonication time of 2.5 min can be inferred as the most suitable set of conditions. The effect of ultrasonication time on extraction yield and purity were found to be statistically significant ($p < 0.05$) for all the three S/L ratios attempted during ultrasonication. Ultrasonication has been successfully applied for enhanced extraction of different biomolecules such as Anthocyanins [37], R-Phycoerythrin [25] and phenolic compounds [29].

3.6.1.3. Extraction kinetics. C-PC concentrations obtained at different extraction times have been plotted according to the Eq. (4) as shown in Fig. 7. The kinetic parameters namely, C_S equilibrium or saturation concentration (which gives extraction capacity), k, the extraction rate constant and R_i, initial extraction rate at different S/L ratios, are given in Table 1. A strong influence of S/L ratio on the kinetic parameters can be seen from the table. The highest Ri and k values of 34.13 and 1.12, respectively were obtained at S/L ratio of 1:10. An increase in the kinetic parameters was observed with increase in in S/L ratio. This observation can be explained by the fact that the system has higher availability of solvent for solute (C-PC in the present work) at higher S/L ratios. Kinetics at different S/L ratios was reported for extraction of phenolic compounds from pomegranate peels [38]. Kinetics of extraction (second-order) of several biomolecules from natural sources such as antioxidants from *Fumaria officinalis* L. [39] and *Picea abies* bark [32] have been reported in literature.

3.6.1.4. Energy calculations. During ultrasonication, a portion of power gets converted to heat, leading into rise in the temperature of the medium. The heat dissipated in the medium during ultrasonication was estimated by calorimetric measurements. Ultrasonication was carried out at standardized conditions. The temperature was observed to rise from 27 to 37.4 °C after 2.5 min of ultrasonication. The power, ultrasound intensity and acoustic power density were estimated, based on these measurements, to be 29.02 W, 102.7 W/cm^2 and 0.29 W/cm^3, respectively.

3.7. Ultrasound synergized extraction methods

When individual primary extraction methods are not sufficient to achieve desired yield, they are carried out in combination [40–42]. In

Table 1
Kinetic parameters of C-PC extraction from dry *Arthospira platensis*. Values used for kinetic study are means of three independent experiments ($n = 3$).

S/L ratio	Slope	$C_S = \frac{1}{\text{Slope}}$	Intercept	$R_i = \frac{1}{\text{Intercept}}$	$k = \left(\frac{Ri}{C_S^2}\right)$	R^2
1/6	0.0981	10.1937	0.0505	19.802	0.1906	0.9984
1/8	0.1437	6.95894	0.043	23.256	0.4802	0.9993
1/10	0.1813	5.51572	0.0293	34.13	1.1218	0.9987

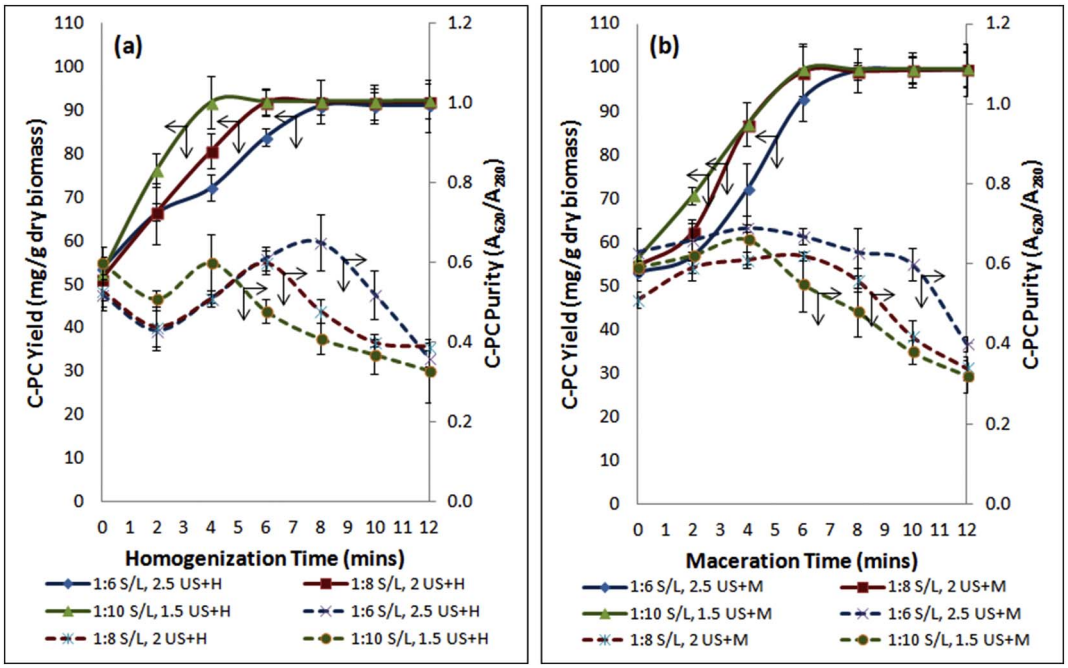

Fig. 8. Extraction of C-PC by (a) ultrasonication in combination with Homogenization, (b) ultrasonication in combination with Maceration.

the present work, primary extraction methods resulted in low yield of C-PC when compared to serial extraction (119 mg/g of dry biomass). Ultrasonication could not be employed for longer duration as it leads to an increase in temperature. Hence, ultrasonication was carried out in combination with other conventional methods to exploit synergy and the results are discussed in the following sections.

3.7.1. Ultrasonication with homogenization

The dry biomass (pre-soaked for 120 min) at three S/L ratios was subjected to ultrasonication (for 2.5 min, standardized during ultrasonication alone) followed by homogenization (ultrasonication + homogenization) for different time periods (0, 2, 4, 6, 8, 10 and 12 min). The results are shown in Fig. 8a. An increase in extraction yield from 52.44 to 91.88 mg/g dry biomass can be observed at ultrasonication (2.5 min) + homogenization (4 min) at 1:10 S/L ratio (translating into an increase in extraction efficiency from 44.06 to 77.21%). Practically the same yield (91 mg/g dry biomass) with a higher purity of 0.65 could be achieved at 1:6 S/L ratio after ultrasonication for 2.5 min followed by homogenization for 8 min. Based on the value of serial extraction, the extraction efficiency of 'ultrasonication + homogenization' at standardized conditions was estimated to be 77.21 and 76.47% for 1:10 and 1:6 S/L ratio, respectively. The ANOVA was applied for the results obtained during homogenization (after 2.5 min of ultrasonication) and the effect of extraction time on extraction yield and purity were found to be statistically significant ($p < 0.05$) for all the three S/L ratios attempted.

'Ultrasonication + homogenization' resulted in higher extraction efficiency compared to ultrasonication or homogenization when employed alone (43.05 and 43.28%, respectively). This could be due to, more of cutting rather than shearing of biomass taking place during homogenization (unit used in the present work). As a consequence, the overall surface area perhaps did not increase to the desired extent. Similarly, ultrasonication, when employed alone, appeared to be not enough to produce sufficient cracks on the cell membrane. On the other hand, cavitation comes into play in 'ultrasonication + homogenization' (comprising compression and decompression resulting in sonoporation) during ultrasonication followed by cutting of biomass during homogenization which facilitates release of C-PC.

3.7.2. Ultrasonication with maceration

The dry biomass (pre-soaked for 120 min) at three S/L ratios was subjected to ultrasonication (for 2.5 min, standardized during ultrasonication alone) followed by maceration (ultrasonication + maceration) for different time durations (0, 2, 4, 6, 8, 10 and 12 min). The results are shown in Fig. 8b. An increase in extraction yield from 56.27 to 99.41 mg/g dry biomass can be observed at ultrasonication (2.5 min) + maceration (6 min) at 1:10 S/L ratio (translating into an increase in extraction efficiency from 47.28 to 83.53%). Practically the same yield (99.31 mg/g dry biomass) could be achieved at 1:6 S/L ratio after ultrasonication for 2.5 min followed by maceration for 8 min. However, higher purity (0.63) could be achieved at 1:6 over 1:10 S/L ratio (0.55). Based on the value of serial extraction, the extraction efficiency of 'ultrasonication + maceration' at standardized conditions were estimated to be 83.53 and 83.45% for 1:10 and 1:6 S/L ratios, respectively. In case of 'ultrasonication + homogenization' also C-PC of higher purity without compromising much on yield was observed at 1:6 S/L ratio. The ANOVA was applied for the results obtained during maceration (after 2.5 min of ultrasonication) and the effect of extraction time on extraction yield and purity were found to be statistically significant ($p < 0.05$) for all the S/L ratios attempted.

'Ultrasonication + maceration' resulted in higher extraction efficiency (83.53%) when compared to ultrasonication or maceration when employed alone (43.05 and 46.01%, respectively). This could be due to attrition, the only action involved in maceration which perhaps did not reduce the size of biomass to the desired extent. Ultrasonication, when employed alone, appeared to be not enough to produce sufficient pores on the cell membrane. In contrast, cavitation (compression and decompression resulting in sonoporation) comes into play in 'ultrasonication + maceration' followed by attrition (during maceration) of the biomass which facilitates release of C-PC. Ultrasound synergized with maceration is reported to result in higher yield during extraction of oil from oleaginous seeds [43]. Meullemiestre et al. [41] in their work observed ultrasound assisted maceration to yield 40% more when compared to maceration alone during extraction of phenolic compounds from maritime pine saw wood dust waste.

3.7.3. Ultrasonication with freezing and thawing

The dry biomass (pre-soaked for 120 min) at three S/L ratios was

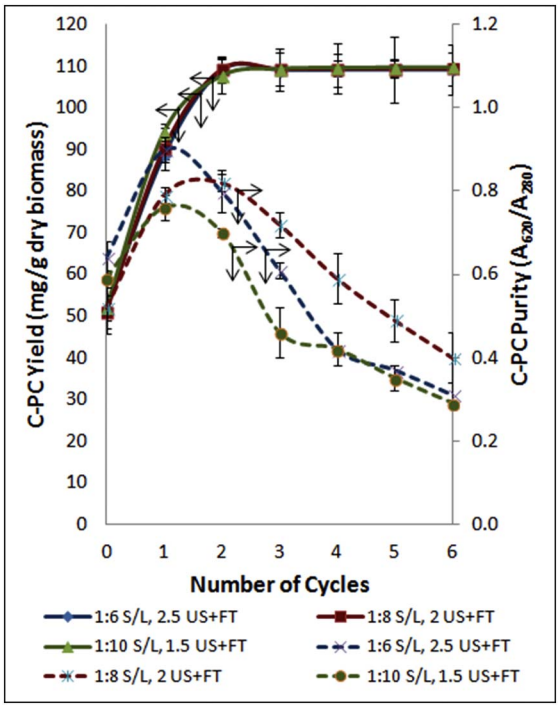

Fig. 9. Extraction of C-PC by ultrasonication in combination with freezing and thawing.

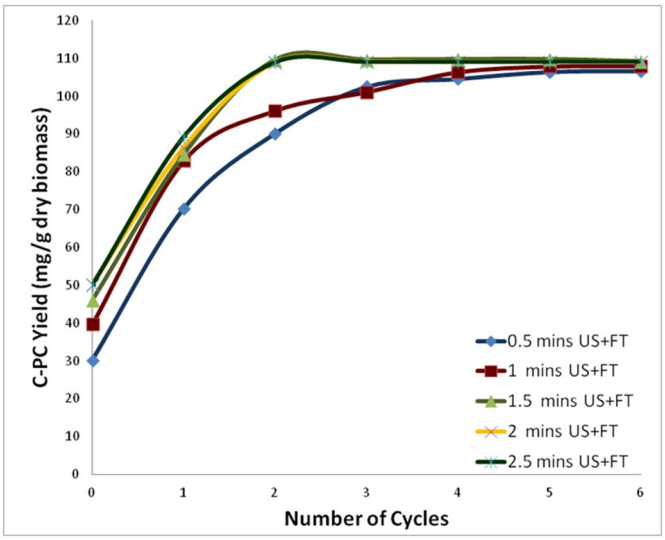

Fig. 10. Standardization of ultrasonication time in extraction of C-PC by ultrasonication and freezing and thawing.

subjected to ultrasonication (for 2.5 min) followed by freezing and thawing (6 cycles, 4 h of freezing and 1 h thawing). The results are shown in Fig. 9. An increase in extraction yield from 51.91 to 109.3 mg/g dry biomass (with a corresponding increase in purity from 0.59 to 0.7) can be observed at ultrasonication (2.5 min) + freezing and thawing (3 cycles) at 1:10 S/L ratio (translating into an increase in extraction efficiency from 43.62 to 91.84%). Practically the same yield (109.03 mg/g dry biomass) with higher purity of 0.8 could be achieved at 1:6 S/L ratio after ultrasonication for 2.5 min followed by freezing and thawing (2 cycles). Based on the value of serial extraction, the extraction efficiency of 'ultrasonication + freezing and thawing' at standardized conditions were estimated to be 90.41 and 91.62% for 1:10 and 1:6 S/L ratios, respectively. In case of other primary extraction methods (homogenization, maceration, freezing and thawing, and ultrasonication) when employed alone and ultrasonication when employed in combination with these methods (ultrasonication + homogenization and ultrasonication + maceration) also higher purity without compromising much on yield at 1:6 S/L ratio was observed. In all these methods 1:8 S/L ratio was observed to result in intermediate values of yield and purity of 1:10 and 1:6 S/L ratios. The ANOVA was applied for the results obtained during freezing and thawing cycles (after 2.5 min of ultrasonication) and the effect of extraction time on extraction yield and purity were found to be statistically significant ($p < 0.05$) for all the three S/L ratios attempted.

"Ultrasonication + freezing and thawing" resulted in higher extraction efficiency (91.62%) when compared to ultrasonication or freezing and thawing alone (43.05 and 62.56%, respectively). In fact it was observed to be the best of all the methods studied. Power for 2.5 min of ultrasonication based on calorimetric measurements was estimated to be 29.02 W. Higher extraction efficiency (92%) achieved by this method could be due to, cavitation of the cell membrane during ultrasonication [44] followed by development of pores (permeabilization) in the cell membrane because of pressure exerted by freezing intracellular fluids as well as extracting buffer on membrane during freezing and thawing [45]. This is expected to result in more number of pores and cavities on membrane resulting in higher extraction yield as well as achieving higher purity. Higher purity is because of the fact that C-PC being membrane bound protein needs only disruption of membrane to leach out into the buffer. These methods like ultrasonication (at low ultrasonication times) and freezing and thawing involves permeabilization of membrane. On the other hand, methods like homogenization and maceration results in cutting or shearing of the cells leading to release of also the other contaminant proteins from cytoplasm and other organelles of cells, in turn resulting in lower purity of C-PC.

"Ultrasonication + freezing and thawing" (at 1:6 S/L ratio) was observed to be the best method in terms of yield and purity. In order to explore the possibility of reducing the ultrasonication time below 2.5 min, further standardization experiments were carried out. The dry biomass (pre-soaked for 120 min) at 1:6 S/L ratios was subjected to different time periods of ultrasonication (0–2.5 min) followed by 6 cycles of freezing and thawing (4 h freezing and 1 h thawing). The results are shown in Fig. 10.

The yield of C-PC gradually increased with ultrasonication time (0 to 1.5 min) followed by freezing and thawing. Significant increase in C-PC content could not be observed with further increase in ultrasonication time (2.0 and 2.5 min). The highest yield of 109.57 mg/g of dry biomass (translating into extraction efficiency of 92.07%) could be achieved at 1.5 min of ultrasonication followed by 2 cycles of freezing and thawing (1:6 S/L ratio). The ANOVA was applied for the results obtained during standardization of "Ultrasonication + freezing and thawing" and the ultrasonication time on extraction yield was found to be statistically significant ($p < 0.05$). It can be inferred from Fig. 6 that when ultrasonication was employed alone, the highest yield could be achieved at 2.5 min of ultrasonication time. Freezing and thawing when employed alone, the highest yield could be achieved after 4 cycles (Fig. 4). By further standardization of ultrasonication time, 1 min of ultrasonication time (1.5 instead of 2.5 min) and 2 cycles (2 instead of 4 cycles, that is, 10 h) of freezing and thawing could be reduced while achieving similar yield without compromising on the purity of C-PC. Reduction in extraction time during ultrasound assisted extraction is reported for extraction of taurine from *Porphyra yezoensis* [46]. Power required for 1.5 min of ultrasonication based on calorimetric measurements was estimated to be 14.88 W (half of the power required for 2.5 min of ultrasonication). Silveira et al. [21] in their work achieved a maximum concentration and purity of 3.68 mg/mL and 0.46, respectively at solvent ratio of 1:12 and extraction time of 4 h at 25 °C. Recently, Aftari et al. [22] achieved 2.2 mg/mL concentration of C-PC by ultrasound assisted extraction of *A. platensis*. However, relatively higher concentration of 4.54 mg/mL and a purity of 1.27 could be achieved by microwave assisted extraction. Coward et al. [47] subjected the freeze

dried samples to a minimum of five freeze and thaw cycles at −20 °C until frozen (2 h) followed by sonication on ice for 10 min. The samples were then vortexed and frozen at −20 °C. These steps were repeated. The methodology followed was observed to be reasonably effective. The main focus of the work was on increasing the phycobiliprotein in the biomass and the methodology mentioned above was used only for analytical purpose. Patel et al. [48] followed a similar procedure for primary extraction of C-PC. They used freeze-dried biomass suspended in sodium-phosphate buffer. The suspension was subjected to sonication for 60 s followed by repeated freezing at −20 °C and thawing to room temperature in the dark. This procedure was repeated to result in good yield with purity similar to the present work. However, the focus of the work was on purification of C-PC rather than on primary extraction. Abalde et al. [49] obtaining a C-PC concentration 27 μg/mL from wet biomass of *Synechococcus* sp. *IO201* by freezing and thawing method. Minkova et al. [50] achieved a C-PC concentration of 1.28 mg/mL from fresh biomass of *Scaphyglottis fusiformis*.

3.8. Effect of solid liquid ratio

In all the primary extraction methods employed (when employed alone and when employed in combination with ultrasonication), a general trend can be seen that yield of the C-PC to be the highest at 1:10 S/L ratio (in the order of 1:10 > 1:8 > 1:6). It can also be observed that the rate of extraction to be higher at 1:10 S/L ratio enabling to reach maximum yield in shorter time. This can be due to higher availability of solvent for solute (C-PC in the present work) at higher S/L ratios. Xu and Pan, [51] in their study on extraction of all-trans-lycopene observed that yield and extraction rate increased with an increase in S/L ratio. On the other hand, in all the primary extraction methods attempted 1:6 S/L ratio has resulted in the highest purity (purity in the order of 1:6 > 1:8 > 1:10). Lower purity at higher S/L ratio could be attributed to the availability of higher quantity of solvent resulting in extraction of other contaminant proteins also from cytoplasm and other organelles of biomass. So, S/L ratio must be judiciously selected as higher S/L ratio is preferred for higher yield and lower S/L ratio for higher purity. In view of higher C-PC concentration in the extract and purity with similar yield, S/L ratio of 1:6 was considered to be most suitable S/L ratio for extraction of C-PC by all the primary extraction methods. The best results of all the primary extraction methods are summarized in Table 2.

3.9. Colour measurement

The colour analysis were carried out for spent biomass (after each primary extraction method), in an attempt to correlate with the extent of extraction. The results are shown in Table 3. A clear relation showing a decrease in blue colour in spent biomass could be observed with an increase in C-PC extraction efficacy from Table 3 for a given primary extraction method.

Considerable difference in blue colour (−b*) between algal biomass (before C-PC extraction) and spent biomass obtained after primary extraction of C-PC can be observed. The biomass that was not subjected to any primary extraction method (control) exhibited highest b* value (−b* = −4.42). On the other hand, colour measurement of the spent biomass obtained after serial extraction indicated the lowest b* value (−b* = −0.22) indicating maximum C-PC extraction.

The colour measurement of spent biomass corresponding to ultrasonication (2.5 min) + freezing and thawing (2 cycles) reached close (−b* = −0.91) to that of serial extraction (−b* = −0.22), which conforms this to be the best primary extraction method. From the Table 3, −b* value of the spent biomass can be seen to be in the increasing order of 'ultrasonication + freezing and thawing' (−b* = −0.91), 'ultrasonication + maceration' (−b* = −1.74), 'ultrasonication + homogenization' (−b* = −2.18), 'freezing and thawing alone' (-b* = −2.49), 'maceration alone' (−b* = −3.4), 'homogenization alone' (−b* = −3.9) and 'ultrasonication alone' (−b* = −3.9). It can be seen from Table 2 that this order tallies with the order of decreasing extraction efficiency of all primary extraction methods.

Meanwhile, release of C-PC made the spent biomass lighter (L* value) compared to the dry biomass (control) used for extraction. L* value was observed to be lowest for dry *A. platensis* biomass (control) indicating the darkest among all the samples (because of presence of C-PC).

3.10. Synergistic effect of ultrasonication

It can be seen from Table 2 and Fig. 11 that homogenization, maceration, freezing and thawing and, ultrasonication when employed alone did not result in high efficacy. However, when employed in combination with conventional methods homogenization, maceration and freezing and thawing resulted in extraction efficiency of 76.47, 83.45 and 91.62%, respectively which are significantly higher than when these conventional methods are employed alone indicating synergistic effect.

The synergy of "ultrasonication + freezing and thawing" can be described based on the inefficiency of the individual methods to achieve high yields. C-PC, being a membrane bound pigment, will not leach into extraction buffer on getting detached from membrane until and unless cell membrane is ruptured. Primary extraction methods when employed alone appear not sufficient enough to achieve the adequate disruption of membrane. Colour analysis discussed in previous section supports this inference. The profound and synergistic effect of ultrasound when employed in combination with other

Table 2
Comparison of C-PC primary extraction methods from dry *Arthospira platensis*. Yield values are means ± SD of the three independent experiments (n = 3). Extraction efficiency is calculated for the mean values of yield.[a]

Extraction method	Yield (mg/g of dry biomass)	Efficiency (%)	Purity
Serial extraction	119.00 ± 1	100.00	–
1.5 min US + 2 cycles FT, 1:6 S/L	109.57 ± 1	92.08	0.8
2.5 min US + 2 cycles FT, 1:6 S/L	109.03 ± 3	91.62	0.8
2.5 min US + 8 min M, 1:6 S/L	99.31 ± 2	83.45	0.63
2.5 min US + 8 min H, 1:6 S/L	91.00 ± 2	76.47	0.65
FT for 4 cycles, 1:6 S/L	73.73 ± 1	61.96	0.66
M for 8 min,1:6 S/L	55.91 ± 3	46.98	0.63
H for 10 min, 1:6 S/L	52.11 ± 4	43.78	0.6
US for 2.5 min, 1:6 S/L	51.51 ± 2	43.29	0.62

[a] US—ultrasonication; H—homogenization; M—maceration; FT—freezing and thawing.

Table 3
Colour Analysis of spent biomass obtained after primary extraction. Values are the results obtained from single experiment ($n = 1$).[a]

Primary extraction method	L*(D65)	a*(D65)	b*(D65)
Serial extraction	15.48	−5.25	−0.22
US + FT	14.98	−4.7	−0.91
US + M	14.73	−4.56	−1.74
US + H	14.8	−4.49	−2.18
FT	14.51	−4.36	−2.49
Method	13.57	−4.29	−3.4
H	13.46	−3.36	−3.9
US	13.08	−2.01	−3.9
Untreated	9.73	−0.64	−4.42

[a] US—ultrasonication; H—homogenization; M—maceration; FT—freezing and thawing.

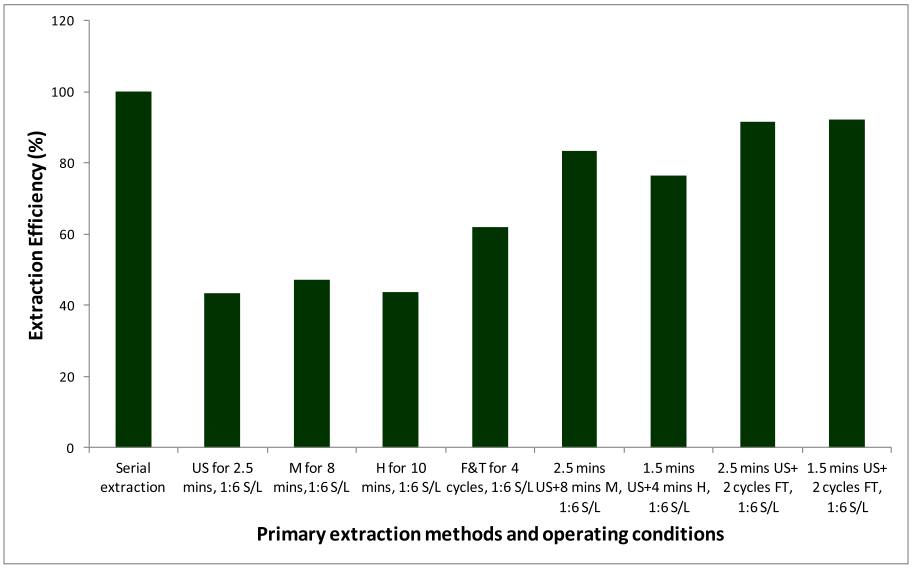

Fig. 11. Comparison of different primary extraction methods for C-PC extraction from dry biomass of *Arthospira platensis*.

conventional methods can be designated to increased cell disruption and penetration of solvent into the matrix of solid which are essentially the mechanical effect [52] thereby improving the mass transfer, reduced solvent consumption and lower extraction time [53–55]. The ultrasound waves which propagate into the liquid media during sonication of liquids especially at high intensities, cause alterations in the low and high pressure cycles with rates that depend on the amplitude and frequency. Ultrasonic waves of high intensity, during low-pressure cycle, generate small voids or vacuum bubbles in the liquid. These bubbles on attaining a certain volume cannot absorb any further energy, and collapse in the high pressure cycle [56]. Such implosion of cavitation bubble [43], leads to the formation of strong currents of very high velocity (> 400 km/h) hit the cells in water [57] generating the shear stress which in turn break the cell envelops [58].

In the present study, higher extraction efficiency observed may be by the combination of two mechanisms, namely, sonoporation and sonocapillary effect which occur due to permeability or disruption of cell membranes. Sonoporation would result in release of cellular contents in the extraction medium. Meanwhile, sonocappillary effect comes into action, which leads to increase in depth and velocity of penetration of solvent into the canals through pores. In the subsequent operation of freezing, intracellular ice formation occurs promoting further (as membrane is already disrupted during ultrasonication) disruption of cell membrane during thawing. This results in a higher extraction of intracellular substances [45]. Ultrasound synergized methods for extraction of C-PC are reported. The synergistic disruptive effect of ultrasonic vibration and ethanol solvent on *A. platensis* improved the extraction performance of C-PC as compared to the conventional soxhlet extraction. UAE method achieved a yield of 15.66% within 20 min extraction time, while the soxhlet extraction obtained a yield of 11.13% in 4 h [59]. Meullemiestre et al. [41] in their work observed ultrasound assisted maceration to yield 40% more when compared to maceration alone during extraction of phenolic compounds from maritime pine saw wood dust waste. Meullemiestre et al. [60] in their work on extraction of oil from wet yeast (*Yarrowia lipolityca*) explained the mechanism of enhanced extraction during ultrasound assisted methods is attributed to the pore formation on its membrane (observed in SEM) due to cavitation. The efficacy of ultrasonication for enhanced extraction of C-PC [17] and other different natural colours such as anthocyanin [37] and all-trans-lycopene [51] has been reported in the literature.

Ultrasound synergized methods employed for extraction of C-PC in present work would easily qualify to be a 'green technology'. Ultrasonication is a mechanical method. As and does not require any additional chemical solvent, it does not result in generation of any additional waste. In fact, it employed water/buffer, an eco-friendly solvent, for the extraction of C-PC. The additional power of 14.88 W (for 1.5 min of ultrasonication), spent during 'ultrasonication + freezing and thawing' is worth expending, considering the increase in yield (when compared to freezing and thawing alone) of this high value low volume compound (from 61% to 92%) in lower extraction time.

Ultrasonic devices are applicable to laboratory scale use or can be scaled-up and operated continuously [61]. For probe type ultrasonication systems, probes of different feed handling capacity with wide range power from 50 to 400 W for lab scales and from 500 to 16,000 W for industrial scales are available. The other alternative is the bath type ultrasonication systems with a larger radiating surface and an agitation system. Bath type ultrasonication systems of feed handling capacity (pilot scale of 30 to 50 L and industrial scale of 500 to 1000 L) are also available. A few of the advantages of ultrasound assisted extraction are Low operational costs [62,63] and facile equipment, versatile, flexible [62] and the operation of equipment does not need extensive technical training [63]. However, ultrasound assisted extraction requires precise standardization of process parameters as most of the biomolecules are thermolabile and heat generation during ultrasonication (either because of higher frequency or amplitude or exposure time employed) will lead to thermal degradation of biomolecules [12,13]. For the scaleup of the present work, these systems may be suitably selected based to the scale of operation [44].

3.11. Stability of C-PC

The extent of degradation of C-PC during ultrasonication is expected to be very low in the present study as the operating conditions employed for extraction are very mild, that is, 1.5 min ultrasonication time, 50% amplitude and 20 kHz frequency. Absorption maxima of C-PC remaining unchanged after ultrasound synergized extraction is the confirmation for the structural stability of C-PC. Degradation was not observed during ultrasound assisted extraction of polyphenols from orange peels at lower frequency of 20 kHz [64]. In addition, it has no negative impact on other cell component like chlorophyll [65]. However, degradation of bioactive compounds was observed when higher frequencies (80–100 kHz) and extraction times are used [12,13,64].

4. Conclusions

Primary extraction of C-PC from dry biomass of *A. platensis* by conventional methods like homogenization, maceration and, freezing and thawing, alone and in combination with ultrasonication are studied. A presoaking step (120 min after standardization) of biomass introduced prior to extraction has significantly increased the efficiency of all the extraction methods. Ultrasonication alone, in spite of optimization did not show good extraction efficiency. However, when employed along with other pre-treatment methods, synergistic effect was observed resulting in increased extraction yield/efficiency. 'Ultrasonication + freezing and thawing' resulted in the highest extraction efficiency (92.08%). 'Ultrasonication + maceration' was observed to be the second best method (83.45%). Kinetic parameters were estimated for extraction of C-PC by ultrasonication considering second order kinetics of mass transfer. Colour measurement studies indicated a clear correlation between the extraction efficiency of C-PC and degree of discoloration of spent biomass in a given primary extraction method. Based on calorimetric measurements, power, ultrasound intensity and acoustic power density were estimated to be 14.88 W, 52.67 W/cm^2 and 0.148 W/cm^3, respectively. Further standardization of ultrasonication time during 'ultrasonication + freezing and thawing' resulted in reduction of ultrasonication time by 1 min and reduction in freezing and thawing by 2 cycles (2 instead of 4 cycles during freezing and thawing alone) with overall reduction in processing time by ~10 h. Ultrasonication for 1.5 min (pre soaked for 120 min, 1:6 S/L ratio and ultrasonicating at 50% amplitude and 20 kHz frequency) followed by freezing and thawing for 2 cycles (freezing for 4 h at −40 °C and thawing to room temperature for 1 h) was found to be the best methodology for primary extraction of C-PC from dry biomass of *A. platensis*.

Contribution of authors

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Design of experimental plan. Execution of experiments. Analysis and interpretation of the results. Drafting of the manuscript.

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Design of experiment. Interpretation of the results. Critical revision of the article for important intellectual content.

Authors agreement for authorship and submission of manuscript for peer review.

All the four authors agree to be an author of this paper. We also agree to the submission of the manuscript for peer review.

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