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Optimization of ultrasound-assisted solvent extraction of phycocyanin and phenolics from *Arthospira platensis* var. 'lonor' biomass

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ABSTRACT

This study describes the enhanced and simultaneous extraction of phycocyanin and phenolics from *Arthospira platensis* biomass. Ultrasound-assisted solvent extraction was investigated to determine its efficacy regarding phycocyanin and phenolics yield. An optimization experiment using response surface methodology revealed that the variables ethanol concentration (20%–95%, v/v), extraction temperature (15°C–65°C), sonicator amplitude (20%–100%) and extraction time (60–300 s) have a significant effect on phycocyanin and phenolics yield. The maximum yield of phycocyanin (29.9 mg/g) and total phenolics (2.4 mg/g) was predicted to occur at 40% ethanol concentration, 34.9°C extraction temperature, sonicator amplitude of 95% and extraction time of 104.7 s. The resultant extract exhibited a dose-dependent antioxidant response with an IC_{50} value of 85.75 µg/ml. This extract can be incorporated into functional foods as the extracting solvents ethanol and water have GRAS (generally recognized as safe) status.

Keywords

Arthospira platensis
Phycocyanin
Phenolics
Ultrasound-assisted extraction
Antioxidant activity

Introduction

Employing sustainable food production techniques using natural ecosystems will help increase productivity and protect the earth's natural resources. Microalgae cultivation is a new method of ecological food production. Microalgal technology for food and feed as well as for nutraceuticals and pharmaceuticals has rapidly advanced over the last two decades. Many species of algae can be induced to produce high concentrations of chosen compounds, such as proteins, carbohydrates, lipids, glycerol and pigments, that are of commercial value. *Arthospira* is a type of blue-green alga normally found in alkaline freshwater lakes with a high carbonate content. The extensive use of *Arthospira* sp. is based on its fast growth rate, zero toxicity, excellent assimilability (85%–95%), rich protein content (60%–70%), amino acid profile, high vitamin content, and variety of functionally bioactive agents. This cyanobacterium has attracted interest because of its ability to produce various chemicals and biologically active compounds such as proteins, lipids, carotenoids, pigments, phenolics and polysaccharides [1, 2]. These biochemicals have demonstrated health-enhancing functional properties [3] as well as antimicrobial, antidiabetic and antioxidant activity [4–6]. Phycocyanin, a blue-coloured pigment, is a water-soluble protein which has anti-inflammatory and anti-oxidizing effects. β-Carotene, an orange-yellow pigment found in *Arthospira* biomass, was shown to reduce the number and size of oral cancer tumours in hamsters [7]. However, the phenolic compounds found in blue-green algae have been less researched than those found in higher plants [8, 9]. Microalgal phenolic compounds were shown to scavenge free radicals [10]. In vitro studies demonstrated that the *Arthospira* and *Nostoc* species have several biological properties, which can help scavenge superoxide and hydroxyl radicals and restrict lipid peroxidation [11, 12].

Various solvent extraction techniques are employed for the isolation of bioactives from *Arthospira* biomass. Sarada *et al* [13] conducted experiments on solvent extraction of phycocyanin from biomass using three different methods: freezing and thawing, homogenization using a mortar and pestle, and simple water extraction. The authors reported that homogenization using a mortar and pestle yielded the highest

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phycocyanin content of 19.66 mg/100 mg. Duangsee *et al* [14] tested two different methods of extraction (ultrasound-assisted solvent extraction and freezing and thawing) of biochemicals from *Arthospira* biomass and stated that ultrasound-assisted solvent extraction had better extraction efficiency (22.1%) than freezing and thawing (15.6%), which findings are in accordance with the literature. The extraction efficiency of biochemicals was significantly ($p<0.05$) affected by various process parameters: temperature during extraction (°C), time of extraction (min) and sonicator amplitude (%). Ultrasound-assisted extraction (UAE) of bioactives from algal biomass is recognized as an efficient extraction technique that reduces long extraction times to a few minutes, produces higher yields than conventional extraction technologies, is less expensive and easily applied, and retains extract quality. Consequently, it was proposed that optimization of solvent extraction process parameters with ultrasound assistance would further increase biochemical yield from *Arthospira platensis* biomass.

Materials and methods

Microorganism and cultivation conditions

The microalga *A. platensis* var. lonor was purchased from Spirulina Production Research and Training Centre, Madurai, Tamil Nadu, India. The organism was cultured in 4,000 ml Haffkine flasks containing 2,000 ml of Zarrouk's medium [15]. The *A. platensis* culture was incubated for 16 days in the growth chamber which was maintained at 33±2°C and 67.5±0.5 µmol/m^2/s light intensity [16]. All chemicals were procured from Merck Specialities, India. The culture media and all glassware were sterilised using an autoclave. The biomass was harvested by centrifugation and later freeze-dried at −55±5°C and 0.001–1,000 mbar vacuum for 6–8 hours to reach a final moisture content of 5% (db) and stored in desiccators under vacuum.

Experimental design

A five-level (−2, −1, 0, +1, +2) central composite rotatable design (CCRD) was applied to four variables (Table 1) and used to predict the different combinations of the variables which would produce the maximum yield of phycocyanin and phenolics from *Arthospira* biomass. A total of 30 combinations were examined using Design Expert 7.1 statistical software (Stat-Ease, MN, USA), among which six were central points. The results were calculated using regression by developing a second order polynomial equation as given in Eq. 1:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j x_j + \sum_{j=1}^{k} \beta_{jj} x^2_j + \Sigma \sum_{i<j=2}^{k} \beta_{ij} x_i x_j \quad (1)$$

where Y is the response variable, x_i and x_j are independent process variables, β_0 is a fixed term, β_j is a linear coefficient, β_{ij} is the interaction coefficient, and β_{jj} is a quadratic factor. k denotes the number of independent parameters. In the experiment, the process variables were coded as A, B, C and D. The goodness-of-fit in the second order polynomial equation was denoted via the coefficient of determination (R^2). The fitted equation was demonstrated in 3D plots to elucidate the interaction effect between the variables and the responses. The obtained optimized levels were tested by conducting experiments and correlating the responses with the predicted values.

Coded factors	Name	Units	Low actual	High actual	Low coded	High coded
A	Ethanol concentration	%	30	70	−2	+2
B	Extraction temperature	°C	5	45	−2	+2
C	Sonicator amplitude	%	80	100	−2	+2
D	Extraction time	sec	60	180	−2	+2

Table 1 - Summary of factors used as independent variables and their levels

UAE of phycocyanin and total phenolics from *Arthospira* biomass

A Hielscher ultrasonic processor (model no. UP50H; Hielscher, Teltow, Germany) was used for sonication. The instrument operates at 30 kHz frequency and uses 50 W power. A titanium sonotrode (Model MS3) with a tip diameter of 3 mm, approximate length of 80 mm and sample capacity of 5–100 ml, was used. The amplitude of the ultrasonic processor can be varied from 20% (36 µm) to 100% (180 µm). The maximum amplitude corresponds to an acoustic intensity of 460 W/cm^2. UAE was carried out by exposing the sample containing the solvent to ultrasound waves emitted by the sonotrode at 30 kHz for 2 min. For each experiment, 0.1 g of sample dissolved in 10 ml of ethanol was used. Multi-wavelength scanning using a UV-Vis spectrophotometer (Varian Cary 50 Bio) from 400 to 800 nm was employed to identify the sample containing the most extracted phycocyanin and phenolic compounds.

Effect of ethanol concentration, temperature, ultrasound amplitude and time on phycocyanin and phenolics yield

The extraction solvent was prepared using different amounts of ethanol–water mixtures. Phyco-

cyanin and total polyphenols were extracted from *Arthospira* biomass using increasing concentrations of ethanol ranging from 20% (v/v) to 95% (v/v). The extraction temperature, ultrasound amplitude and extraction time were maintained at 25±2°C, 100% and 1 min, respectively.

Algal biomass (0.1 g) was dissolved in 50% (v/v) ethanol and ultrasonicated at 100% amplitude for 1 min at temperatures ranging from 15°C to 65°C. The extracts were cooled to 5±2°C, centrifuged and stored under refrigeration (4±1°C) until analysis.

The extraction efficiency of ultrasound-assisted solvent extraction was also examined at different ultrasound amplitudes ranging from 20% (36 µm) to 100% (180 µm). Ethanol–water mixtures, extraction temperature and time were maintained at 40%, 25±2°C and 1 min, respectively.

Arthospira biomass was extracted with 50% (v/v) ethanol and sonicated at 100% amplitude at 25±2°C for different times ranging from 1 to 5 min. The obtained extracts were cooled to 5±2°C, centrifuged and stored under refrigeration (4±1°C) until analysis.

Determination of phycocyanin content

The concentration of phycocyanin in the crude extract of *Arthospira* was measured using a spectroscopic method. The crude extract was subjected to purification steps as described by Boussiba and Richmond [17]. The optical density of the extract was measured at 615 and 652 nm using a UV-Vis spectrophotometer (Varian Cary 50 Bio). The final phycocyanin concentration (*PC*) was calculated according to Eq. 2 [18]:

$$PC = \frac{OD_{615} - 0.474(OD_{652})}{5.34} \quad (2)$$

where *PC* is phycocyanin concentration (mg/ml), and OD_{615} and OD_{652} are the absorbance of the sample at 615 nm and 652 nm, respectively. The yield (Y) of the phycocyanin (mg/g) extraction [19] was defined as:

$$Y = \frac{PC \times V}{B} \quad (3)$$

where *PC* is phycocyanin concentration (mg/ml), *V* is volume of solvent (ml) and *B* is biomass (g).

Determination of total phenolic and antioxidant content

The total polyphenolic content of *Arthospira* biomass samples was spectrophotometrically determined by the Folin-Ciocalteu method [20]. Gallic acid solution (20–200 µg/ml) was used as standard. All experiments were performed three times for each biomass sample to avoid handling errors.

The antioxidant capacity of the extracts was calculated using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) as described by Chatterjee *et al* [21] with suitable modification. Different amounts of extract (0–100 µg/ml) were added to 0.9 ml of 0.004% ethanol solution of DPPH and the final volume made up to 1 ml with 50% aqueous methanol. After 30 min of reaction time at room temperature, a decrease in colour in terms of absorbance was measured at 517 nm. The solvent used for dilution was used as blank. The OD value of the DPPH radical without the addition of antioxidant was used as control. The rate of inhibition (I%) of free radicals by DPPH was calculated using Eq. 4:

$$I\% = (A_{blank} - A_{sample} / A_{blank}) \times 100 \quad (4)$$

where A_{blank} is the OD value of control (all reagents without the extracts), and A_{sample} is the OD value of the test compound. IC_{50} was calculated from I% values. The IC_{50} value is the concentration of a metabolite needed to decrease radical formation by 50%.

Statistical analysis

Design-Expert v. 7.0.0 software (Stat-Ease, Minneapolis, USA) was used to design the experiments, build models and conduct statistical analysis. All experiments were carried out in triplicate and the data are presented as the mean of triplicate values. Analysis of variance (ANOVA) was used to predict significant difference in mean values ($p<0.05$).

Results and discussion

Yield of phycocyanin and total phenolics from *Arthospira* biomass extracted with UAE

Multi-wavelength scanning (400–800 nm) of extract samples obtained by UAE demonstrated that all samples showed maximum absorbance at 765 nm, which is the absorbance maximum for phenolic compounds [22]. Ultrasound extraction yielded 1.55 mg/g GAE total phenolics content. In UAE, breakage of cells might facilitate access of solvents to intracellular substances [23]. Comparative studies on cell rupture showed that ultrasound is more effective than repeated freezing and thawing for breaking cells [14].

Effect of ethanol concentration on phycocyanin and total phenolics yield

The effects of various ethanol concentrations on phycocyanin and phenolics yield from *Arthospira* biomass are shown

in Fig. 1A and B. As the ethanol concentration gradually increased from 20% to 40%, the phycocyanin yield increased from 12.9 to 13.1 mg/g, which was not significant. However, beyond 40% ethanol there was a significant decrease in yield. Phycocyanin can solubilize completely in a polar solvent such as water.

However, as the polarity of the extraction solvent decreases, so does extraction efficiency [24]. The maximum yield of phenolics (0.94 mg/g) was observed at 60% ethanol concentration. Yu *et al* [25] reported that adding water to ethanol improves phenolics yield. However, beyond 60% ethanol concentration there is a gradual decrease in yield, which might be due to the extraction of non-targeted compounds, such as lipids, limiting phenolics extraction. A similar trend was observed in the extraction of phenolic compounds from peanut skins [26].

Effect of extraction temperature on phycocyanin and total phenolics yield

Figure 1C and D shows associations between extraction temperature and phycocyanin and total polyphenol yield from *Arthospira* biomass. The maximum phycocyanin yield (15.6 mg/g) was seen at lower temperature (25°C), with higher temperatures of 25°C to 65°C showing a significant decrease in phycocyanin yield from 15 to 7 mg/g. Phycocyanin is heat sensitive and its stability decreases with an increase in temperature beyond 40°C [13].

Phenolics are comparatively heat stable compared to phycocyanin. A significant increase phenolic content from 0.9 to 1.2 mg/g GAE was reported as extraction temperature increased from 15°C to 25°C. This is mainly due to the efficient diffusion of solvent occurring as a result of an increase in heat [27]. However, beyond 35°C there was a gradual

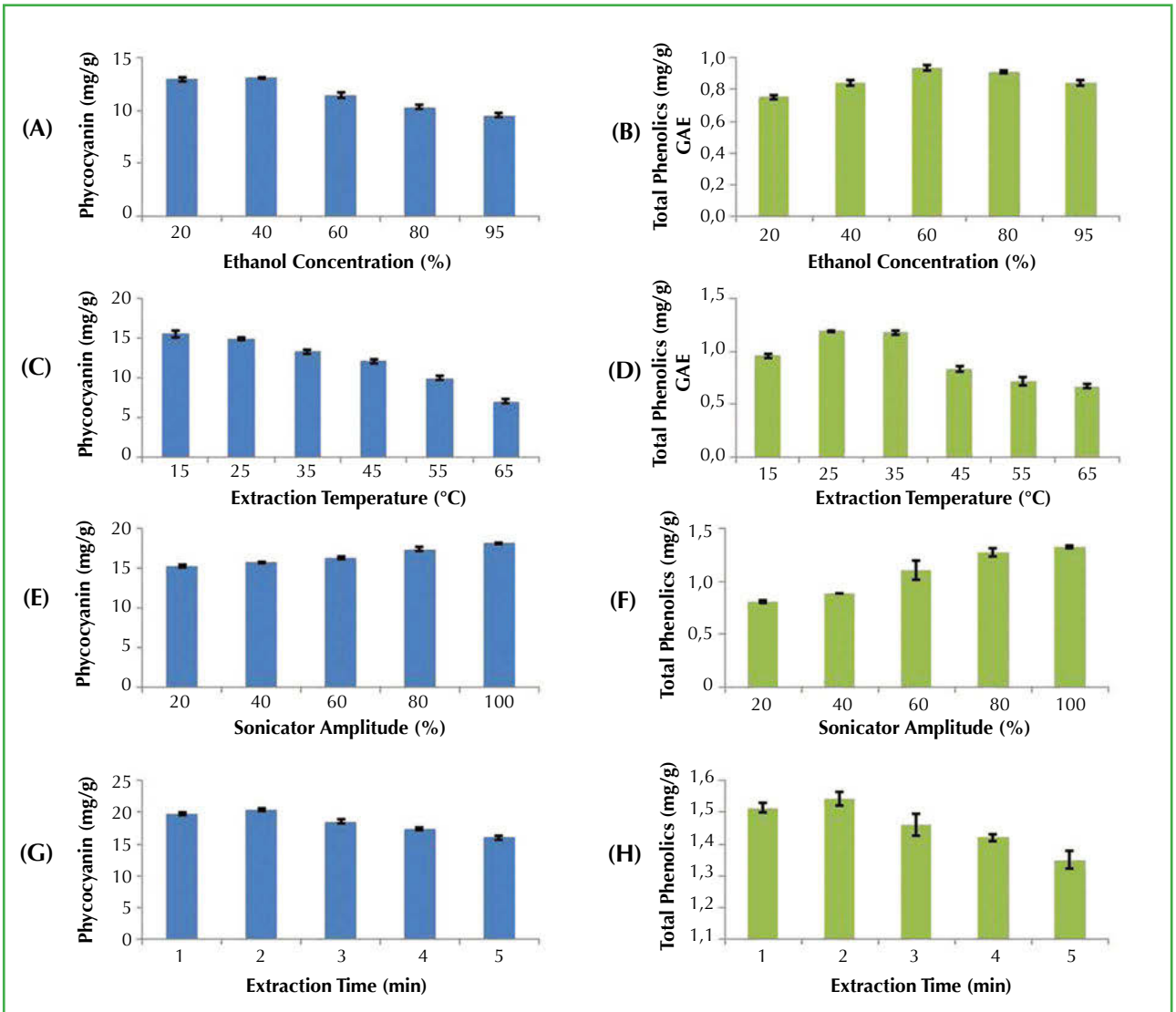

Figure 1 - Effect of (A) ethanol concentration on phycocyanin yield, (B) ethanol concentration on total phenolics yield, (C) extraction temperature on phycocyanin yield, (D) extraction temperature on total phenolics yield, (E) sonicator amplitude on phycocyanin yield, (F) sonicator amplitude on total phenolics yield, (G) extraction time on phycocyanin yield and (H) extraction time on total phenolics yield from *Arthospira platensis* biomass

decrease in phenolics content, which may have been be due to denaturation or degradation [28].

Effect of ultrasound amplitude on yield of phycocyanin and total phenolics

Figure 1E and F shows the amount of phycocyanin and total phenolics extracted at different sonicator amplitudes (20%–100%). An increase in amplitude improved the efficiency of both phycocyanin and total phenolics extraction from *Arthospira* biomass. The increase in phycocyanin yield was not significant, but total phenolic content rose significantly with an increase in amplitude from 40% to 80%. Maximum phycocyanin (18.17 mg/g) and total phenolics (1.33 mg/g GAE) yields were observed at 100% sonicator amplitude. An increase ultrasound wave amplitude causes cavitation bubbles to form, which results in effective cell disintegration and enhances the mass transfer of solvent into the cell [29].

Effect of time on the extraction yield of phycocyanin and total polyphenol content

Figure 1G and H shows the amount of phycocyanin and total phenolics extracted with different extraction times ranging from 1 to 5 min. Both components showed maximum yield at 2 min of sonication (phycocyanin 20.32 mg/g and total phenolics 1.54 mg/g GAE), with a decrease in yield seen beyond 2 min, maybe because of degradation of intracellular components by the ultrasound waves [30].

Optimization of extraction conditions

To optimize the parameters for phycocyanin and total phenolics extraction from *Arthospira* biomass, an ethanol concentration of 50% (v/v), extraction temperature of 25°C, sonicator amplitude of 75% and extraction time of 2 min were taken as the middle point for the CCRD experiment. The variable values and their responses are given in Table 2. The phycocyanin and total phenolics values are expressed in terms of mg/g of biomass.

Statistical analysis of the data revealed that all four factors had a significant effect on results. The most important factors ($p<0.001$) affecting phycocyanin content were ethanol concentration, extraction temperature and extraction time (Table 3). For total polyphenols, ethanol concentration and extraction temperature were the most important factors. Non-linear second order regression equations as a function of coded values of independent parameters were developed for phycocyanin and total phenolics, respectively:

$$\text{Phycocyanin} = 26.04 - 3.03A - 1.04B - 0.39C - 1.03D - 0.47AB + 0.53BC - 0.39BD + 0.19CD + 0.37A^2 + 0.33D^2 \quad (R^2\ 0.97) \quad (5)$$

$$\text{Total phenolics} = 2.60 + 0.40A + 0.16B + 0.018C + 0.021D - 0.22AB + 0.29BC - 0.19A^2 - 0.35B^2 - 0.22D^2 \quad (R^2\ 0.96) \quad (6)$$

here *A, B, C,* and *D* are ethanol concentration (%), extraction temperature (°C), sonicator amplitude (%) and extrac-

Process variables				Responses	
Ethanol concentration (%)	Extraction temperature (°C)	Ultrasound amplitude (%)	Extraction time (s)	Phycocyanin yield (mg/g)	Phenolics yield (mg/g)
70	25	90	120	21.11±0.16	2.66±0.04
60	35	95	150	21.44±0.04	2.48±0.02
50	25	90	120	24.92±0.88	2.86±0.04
30	25	90	120	33.74±0.43	1.06±0.04
50	25	90	120	25.98±0.60	2.06±0.04
60	15	95	150	23.17±0.97	1.92±0.05
50	25	90	180	25.84±0.29	1.78±0.09
50	45	90	120	24.03±0.70	1.52±0.05
50	25	90	60	28.63±0.24	1.66±0.06
60	35	95	90	23.02±0.35	2.62±0.07
40	35	95	150	27.21±0.47	2.22±0.07
50	25	90	120	27.08±0.16	2.76±0.08
50	25	100	120	24.83±0.11	2.78±0.07
60	15	95	90	24.5±0.22	2.13±0.03
40	15	95	90	30.42±0.27	1.02±0.06
50	25	90	120	26.34±0.13	2.58±0.08
50	5	90	120	27.87±0.04	0.92±0.05
50	25	80	120	26.12±0.18	2.89±0.07
50	25	90	120	26.2±0.19	2.67±0.03
60	35	85	90	24.41±0.05	1.82±0.05
50	25	90	120	25.73±0.03	2.65±0.08
60	15	85	150	25.97±0.31	2.56±0.07
40	35	95	90	31.16±0.23	2.31±0.10
60	15	85	90	27.44±0.21	2.77±0.09
40	35	85	150	27.63±0.18	1.75±0.04
40	15	95	150	29.22±0.10	0.86±0.05
40	35	85	90	30.17±0.13	1.33±0.02
60	35	85	150	19.78±0.13	2.07±0.06
40	15	85	90	31.92±0.16	1.1±0.09
40	15	85	150	29.54±0.10	1.5±0.07

Table 2 - Actual levels of independent variables (for extraction) and corresponding responses (phycocyanin and total phenolics yield)

Source	Sum of squares		df		p Value	
	Phyco.	Total phenolics	Phyco.	Total phenolics	Phyco.	Total phenolics
Model	295.21	11.82	14	14	<0.0001	<0.0001
A-Ethanol concentration	220.83	3.74	1	1	<0.0001*	<0.0001*
B-Extraction temperature	26.13	0.65	1	1	<0.0001*	0.0005*
C-Sonicator amplitude	3.60	0.01	1	1	0.0188*	0.0259*
D-Extraction time	25.34	0.01	1	1	<0.0001*	0.0500*
AB	3.52	0.77	1	1	0.0200*	0.0002*
AC	1.11	0.04	1	1	0.1638	0.2851
AD	0.07	0.05	1	1	0.7182	0.2416
BC	4.41	1.36	1	1	0.0107*	<0.0001*
BD	2.50	0.02	1	1	0.0445*	0.4038
CD	0.55	0.13	1	1	0.3207	0.0613
A^2	3.83	0.94	1	1	0.0160*	<0.0001*
B^2	0.00	3.26	1	1	0.9727	<0.0001*
C^2	0.36	0.09	1	1	0.4205	0.1088
D^2	2.92	1.33	1	1	0.0316*	<0.0001*
Residual	7.79	0.49	15	15		
Lack of fit	5.24	0.10	10	10	0.5221	0.9974
Pure error	2.55	0.39	5	5		
Cor total	303.00	12.31	29	29		
*Significant						

Table 3 - ANOVA data for quadratic response surface model for phycocyanin (Phyco.) and total phenolics content

tion time (s), respectively. The interaction factors with a significant effect on phycocyanin yield are presented in Fig. 2A–C. The interaction of extraction temperature and ethanol concentration had a significant effect ($p<0.02$) on phycocyanin yield. An increase in both these factors showed an adverse effect, which may have been due to the change in polarity of the extraction solvent with increased ethanol concentration [29] and degradation of phycocyanin with increased temperature [17]. The interaction effect of sonicator amplitude and extraction temperature showed that phycocyanin yield was highest at a lower temperature (15°C) and higher amplitude (100%). The quality of bubbles developed at higher amplitudes results in better extraction [31]. Phycocyanin is quite stable below 25°C, slowly starts to lose its stability and colour above 30°C, and is highly unstable above 40°C. Disappearance of the maximum absorbance peak of phycocyanin at 50°C was reported earlier [17].

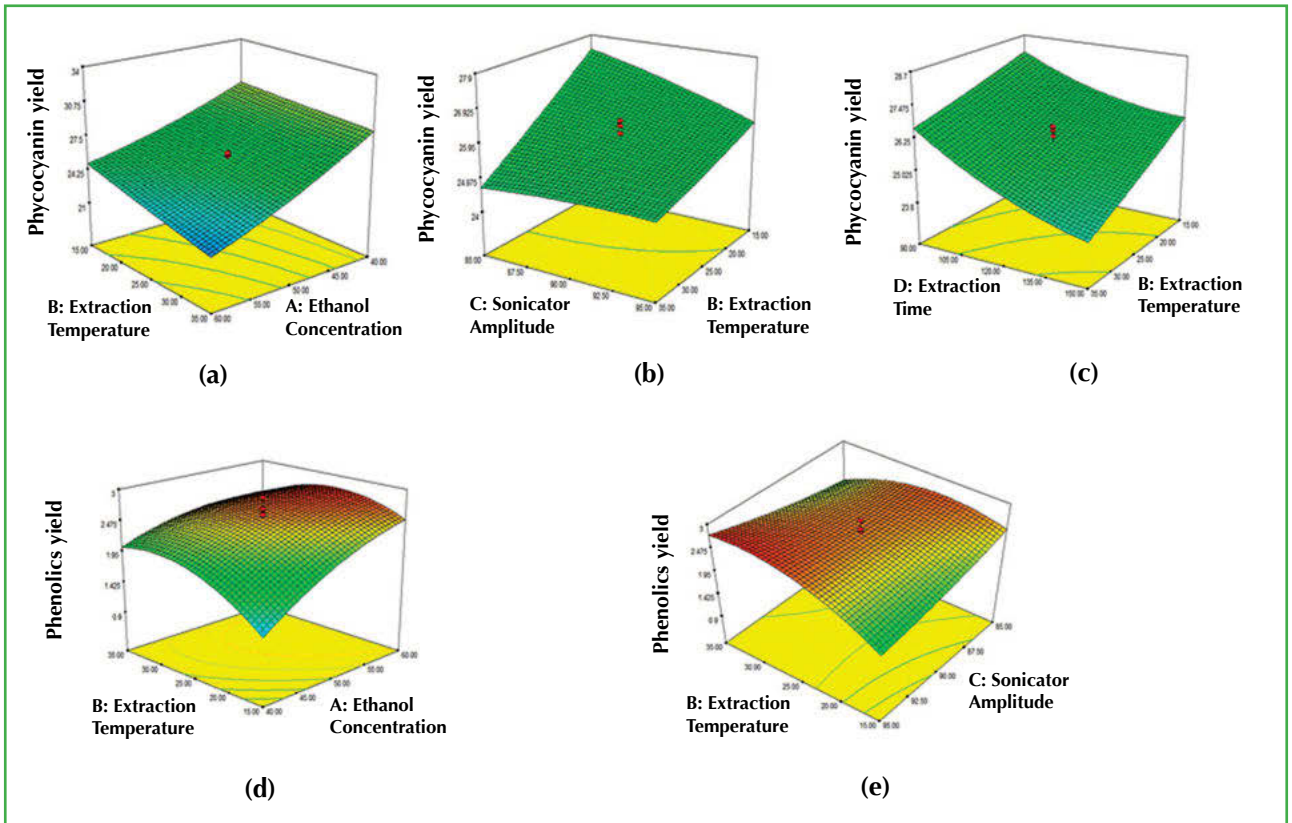

Figure 2 - Response surface graphs showing the interaction effect of independent variables on phycocyanin yield (A, B and C) and on total phenolics yield (E, F)

The positive coefficients of the first order terms of variables in Eq. 6 indicated that the increase in these variables results in an increase in total phenolics yield. The interaction factors with a significant effect on total phenolics yield are presented in Fig. 2D and E. The figure reveals that extraction temperature together with ethanol concentration and sonicator amplitude had a significant effect on phenolics yield. Generally, heat is applied to expedite the diffusion process during extraction of bioactive compounds from natural resources. An increase in temperature lowers the surface tension and viscosity of the solvent. Higher temperature disrupts and expands the cell wall and thus facilitates the solvent reaching the matrix more quickly. Moreover, high temperatures can weaken the integrity of the cell wall and membrane, helping the solvent to penetrate faster and extract bioactive compounds. However, beyond 35°C there was a gradual decrease in phenolics content, which might be due to degradation or denaturation.

The maximum yield of phycocyanin (29.9 mg/g) and total phenolics (2.4 mg/g) was predicted to occur at 40% ethanol concentration, 34.9°C extraction temperature, sonicator amplitude of 95% and extraction time of 104.7 s. To validate the second order polynomial model, optimized experiments were conducted at the predicted parameter values for phycocyanin and total phenolics which showed yields of 29.2 and 2.3 mg/g, respectively.

Anti-oxidant activity of the extract

Different concentrations of *Arthospira* extracts (0–100 µg/ml) and standard BHT (0–50 µg/ml) had varying antioxidant activity with that of *Arthospira* extract seen to be dose dependent (Fig. 3).

The scavenging ability of standard BHT was higher than that of extract samples. The IC_{50} values of *Arthospira* extracts and standard BHT were 85.75 and 33.91 µg/ml, respectively, which shows that extracts retain their activity even after exposure to ultrasound. In the case of *Arthospira*, the main components contributing to antioxidant activity include total carotenoids, α-tocopherol, chlorophyll and phycocyanin, which are all excellent radical scavengers due to their hydrogen donating ability [16]. The results indicated that *Arthospira* is a potential free radical scavenger and participates in the radical scavenging reaction in a dose-dependent manner.

The present work was carried out in a research laboratory using a very small amount (100 mg) of *A. platensis* biomass for the optimization of extraction process parameters. Although the cost of the proposed ultrasound-assisted technology is higher than conventional solvent extraction methods, the extracted compounds have high purity and can be used by food processing industries, thus justifying the extra expense. The cost of manufacturing usually decreases as extractor capacity increases, which is a major advantage of large-scale production [32].

Conclusion

This present study indicates that *A. platensis* biomass could be a primary source of phycocyanin and phenolic compounds and that the extraction yield of these bioactives can be increased using UAE. Ethanol concentration, extraction temperature, sonicator amplitude and time for extraction significantly affect phycocyanin and total polyphenol yield. The optimum process parameters, demonstrated by

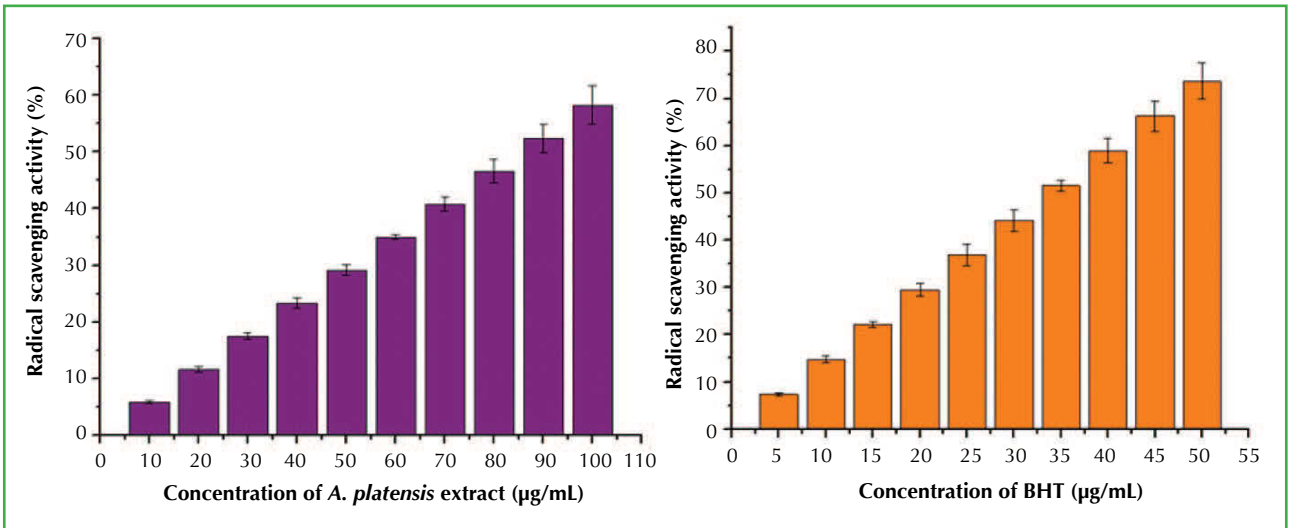

Figure 3 - Effect of different concentration of (A) *Arthospira platensis* extract and (B) standard BHT on DPPH scavenging assay

response surface methodology, for maximum phycocyanin (29.2 mg/g) and total phenolics (2.3 mg/g) yield, were 40% ethanol concentration, 34.9°C extraction temperature, sonicator amplitude of 95% and extraction time of 104.7 s. The extract obtained by this method had better antioxidant activity than extract obtained using conventional processes.

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