CHEMICAL ENGINEERING TRANSACTIONS

VOL. 110, 2024

A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.cetjournal.it

Guest Editors: Marco Bravi, Antonio Marzocchella, Giuseppe Caputo

ISBN 979-12-81206-10-6; **ISSN** 2283-9216

DOI: 10.3303/CET24110057

Innovative Solvent-based Phycocyanin Extraction from *Arthrospira Platensis*

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Arthrospira platensis is a cyanobacterium interesting for its high phycocyanin (C-PC; 10-20% w/w) and protein content (60-70% w/w). These components can be used as food/feed ingredients and additionally C-PC can be used for pharmaceutical applications due to its antioxidative, hepatoprotective and anti-carcinogenic properties. Current methods for C-PC extraction from *A. platensis* involve cell membrane lysis to release pigments in an aqueous extraction medium. Conventional cell lysis techniques, such as freeze-thawing and bead milling require long processing times and high energy inputs and result in release of other cell components, jeopardising the C-PC extract purity. Natural Deep Eutectic Solvents (NADES) represent an emerging metabolite extraction technology which offers bio-safety, high extraction efficiency, improved stability and specificity towards the desired target compound.
The present work focused on C-PC extraction by emerging cell lysis (ultrasonication) and metabolite extraction with hydrophilic NaDES.

1. Introduction

Cyanobacteria, especially *Spirulina platensis*, have been extensively investigated for their potential applications across diverse domains, including agriculture, cosmetics, fuels, functional foods, health, environmental remediation, and waste management. Blue-green algae, renowned for their bioactive constituents and nutritional elements such as carbohydrates, minerals, vitamins, and proteins, feature prominently in this research landscape, with phycocyanin, a noteworthy phycobiliprotein, demonstrating exceptional beneficial attributes. Phycocyanin, present not only in cyanobacteria but also in red algae and cryptomonads, is localized within phycobilisomes—supramolecular complexes within the thylakoid membrane—playing a crucial role in photosynthesis through their antenna configuration.
Distinguished by absorption peaks at 650 nm and 620 nm, allo-phycocyanin (A-PC) and chloro-phycocyanin (C-PC) can be identified based on their respective wavelengths. Numerous studies have underscored the antioxidant properties of phycocyanin, encompassing anti-inflammatory, antitumor, neuroprotective, hepatoprotective, antidiabetic, and immunomodulating effects (Ashaolu et al., 2021).
The extraction of phycocyanin from fresh biomass involves various methods such as freeze-thawing, enzyme-assisted extraction, solvent-assisted extraction (maceration, soaking, percolation), and innovative techniques like Ultrasound Assisted Extraction (UAE), Microwave Assisted Extraction (MAE), High Pressure Process (HPP), Pulsed Electric Fields (PEF), and Extraction with Supercritical Fluids (SFE) (Chittapun et al., 2020). In this study, ultrasound-assisted extraction was coupled with novel hydrophilic solvents.
Specifically, innovative hydrophilic solvents, namely Natural Deep Eutectic Solvents (NaDES) were employed. Over the past decades, the scientific community has embraced neoteric solvents, comprising sustainable, economical, and biodegradable options derived from natural components.

Paper Received: 20 February 2024; Revised: 08 April 2024; Accepted: 19 May 2024
Please cite this article as: Mrotek E., Nervar A., Ferrer-Ledo N., Canziani S., Bravi M., 2024, Innovative solvent-based phycocyanin extraction from Arthrospira platensis , Chemical Engineering Transactions, 110, 337-342 DOI:10.3303/CET24110057

Deep Eutectic Solvents (DES) have emerged as a distinctive class of solvents characterized by unique properties that make them increasingly attractive in various scientific and industrial applications. Unlike conventional solvents, DES are typically formed through the combination of a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) at specific molar ratios, resulting in a eutectic mixture with a considerably lower melting point than the individual components. This low melting point, known as the eutectic point, defines the temperature at which the mixture becomes a homogeneous liquid (Gonzalez-Diaz et al., 2023)

Natural Deep Eutectic Solvents (NaDES), a subset of DES, represent a fascinating category due to their composition derived from naturally occurring components. Combinations of organic acids, such as lactic acid or malic acid, and various hydrogen bond donors like amines or alcohols, mimic the synergistic interactions found in biological systems. This inherent resemblance to substances in nature makes NaDES an environmentally friendly and sustainable alternative to traditional solvents (Dai et al., 2013).

The notable extracting power of NaDES is attributed to the unique arrangement of its constituents, allowing for a tailored design that enhances solubility and selectivity in extraction processes. This characteristic has sparked interest in utilizing NaDES as efficient and eco-friendly solvents for the extraction of bioactive compounds, such as pigments, essential oils, and other valuable components from natural sources. Understanding the composition and properties of NaDES is crucial for unlocking their full potential and advancing sustainable practices in various scientific disciplines (Liu et al., 2018).

This study aims to evaluate innovative solvents in their hydrophilic form for their potential application in a sustainable extraction process, using phycocyanin as a benchmark bioactive compound. Results are quantified in terms of extracted protein concentration, purity index, and extraction efficiency.

2. Materials and methods

2.1 Chemical and biological materials

Lactic acid (purity ≥ 85%), maleic acid (purity ≥ 99%), betaine (purity ≥ 98%), choline chloride (purity ≥ 98%), glucose (purity ≥ 99.5%) and glycerol (purity ≥ 99.5%) were all procured from Sigma-Aldrich and used without further purification. Demineralised water was used for the experiments. Biomass utilized in the study was freeze-dried *Arthrospira platensis* was obtained from Algreen B.V. (Wageningen, The Netherlands). The biomass had been stored in the dark at a temperature of 4 °C to preserve its integrity.

2.2 Solvents preparation and characterization

NaDES were prepared by heating method by mixing the components at 80 °C; LGH was composed of lactic acid (L), glucose (G) and water (H) in a molar ratio of 5:1:3 (Dai et al., 2013); CMH and BMH were made of, respectively, choline chloride (C) and betaine (B) in molar ratio 1:1:8 and 1:1:7 with maleic acid (M) and water (H) (Mitar et al., 2019). Lastly, GGH was made of glycerol (G), glucose (G) and water (H), initially in a molar ratio of 1:2:4 (Wils et al., 2021). Afterwards, the prepared GGH solvent was marked as GGH-20 (solvent containing 20% v/v of water) and additional samples of GGH-30 (30% v/v of water) and GGH-45 (45% v/v) were prepared, with molar rations of HBA:HBD:H_2O equal to 1:2:6 and 1:2:9, respectively.

The rheology of solvents was investigated using a rotoviscosimeter (RV12, Haake Rotovisco), density was calculated based on gravimetric analysis using an analytical scale and pH was measured with the standard electrode and pH meter (pH 8+ DHS Basic, XS)

2.3 Design of extraction experiments

C-phycocyanin solvents extractions from freeze-dried *Arthrospira platensis* were conducted using a solid-liquid ratio 0.02 g/mL in 50 mL falcon tubes. Before the extraction step, suspension of biomass in the solvent was subjected to ultrasound pretreatment for 1 min using a 100 W ultrasound processor (UP100H, Hielscher Ultrasonics) in a cryostated jacket reactor at 4 °C. Afterwards, samples were kept under stirring conditions in an orbital shaker (Certomat R, Braun Biotech) at 50 rpm for 24 h at room temperature in the dark. After the extraction, solid-liquid separation was carried out by centrifugation at 18,459 g for 10 minutes (Avanti® J-20XP, Beckman Coulter) at 10 °C. Prior to the analysis, aliquots of extracts were filtered using a 0.22 μm syringe filter. Extracts were analyzed spectrophotometrically in the UV-Vis range (UV1800, Mapada Instruments). Performance of innovative solvents was compared with demineralized water-based extraction under the same testing conditions.

2.4 Phycocyanin quantification

The content of allo-phycocyanin (A-PC) and chloro-phycocyanin (C-PC) was determined based on the spectrophotometric analysis according to the correlations from the literature (Kursar & Alberte, 1983).

$$A - PC\left(\frac{mg}{L}\right) = 200 \cdot Abs_{650} - 52{,}3 \cdot Abs_{620} \quad (1)$$

$$C - PC\left(\frac{mg}{L}\right) = 166 \cdot Abs_{620} - 108 \cdot Abs_{650} \quad (2)$$

The degree of purity (DP) was determined as the ratio of the absorbance measured at the wavelength of the C-PC Abs_{620}, compared to the absorbance measured at the characteristic wavelength of the proteins Abs_{280} (Tavanandi et al., 2018).

$$DP = \frac{Abs_{620}}{Abs_{280}} \quad (3)$$

Lastly, the extraction efficiency (Y) is expressed as the ratio of the mass of C-PC that was expressed to the mass used initially.

$$Y(\%) = \frac{mg\ C - PC}{mg\ biomass} \cdot 100 \quad (4)$$

3. Results and discussion

Table 1 Properties of solvents used in this work: composition, density, viscosity and pH

Solvent HBA:HBD:H_2O (mol)				Density, ϱ (g/mL)		Viscosity, μ (mPa·s)		pH	
				Experimental	Literature	Experimental	Literature	Experimental	Literature
H_2O	-	-	1	1.0	1.0	0.89	0.89	7.0	7.0
LGH	5	1	3	1.28	1.25	35.47	n.d.	0.82	n.d.
CMH	1	1	8	1.17	1.18	34.96	1.5-10.8	0.45	1.06
BMH	1	1	7	1.20	1.17	34.96	1.5-10.8	2.77	2.88
GGH-20	1	2	4	1.31	n.d.	143.94	n.d.	4.82	n.d.
GGH-30	1	2	6	1.29	n.d.	36.50	n.d.	4.65	n.d.
GGH-45	1	2	9	1.25	n.d.	34.96	n.d.	4.52	n.d.

The investigation into Natural Deep Eutectic Solvents (NaDES) for C-phycocyanin (C-PC) extraction involved a detailed analysis of solvent properties. Table 1 outlines the key characteristics, such as composition, density, viscosity, and pH, of the prepared NaDES compared to demineralized water. NaDES, being composed of organic acids and hydrogen bond donors, exhibited unique properties influenced by their composition. The eutectic nature of NaDES contributed to their distinct characteristics, including solubility and selectivity for targeted compound extraction. All of the composed NaDES present slightly higher density than water in the range from 1.17 to 1.31 g/mL. The variation in the density is attributed to the different composition of NaDES. The mixtures of components with higher molecular weight present higher density, and among the composed NaDES, GGH-20 exhibits the highest density equal to 1.31 g/mL. Prepared NaDES are characterized by much higher viscosity than pure water. This high viscosity is attributed to the presence of extensive hydrogen bonding interactions between HBA and HBD. Especially in the case of sugar-based NaDES, glucose tends to form stronger hydrogen bonds, hence causing higher viscosity of the DES. (Fuad et al., 2021). Taking into consideration eutectic properties of the HBA:HBD bonds, viscosity of GGH solvent was reduced by increasing the water content up to 45%, resulting in dynamic viscosity drop for the samples GGH-30 and GGH-45 to 36.50 mPa·s and 34.96 mPa·s, respectively, making it comparable with the rest of the tested NaDES (Dai et al., 2015). NaDES prepared with acidic compounds (lactic acid and malic acid) exhibited low pH values, as low as 0.45 for CMH, to 2.77 for BMH. It's known that for organic acid-based NaDES, addition of water increases the pH (Mitar et at., 2019). For the sugar-based NaDES, initial pH of the solvent containing 20% of water was equal to 4.82 and decreased slightly with the rising water content in the GGH solvent.

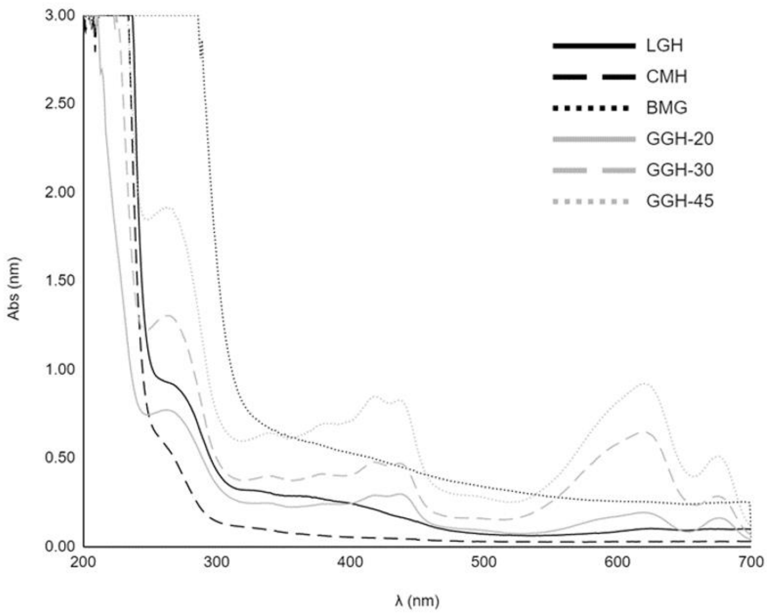

Figure 1 Absorbance spectra of NaDES-based extracts

Spectrophotometric analysis of NaDES-based extracts (Figure 1) revealed their affinity to specific groups of compounds present in *Arthrospira platensis* biomass. The organic acids-based extracts present absorbance around 280 nm, which is characteristic wavelength indicating presence of proteins (Chang et al., 2017). As for sugar-based extracts, their absorbance spectra revealed presence of pigments. GGH extracts present strong light absorbing properties for the wavelength around 620-650 nm, indicating presence of phycobiliproteins (Sobiechowska-Sasim et al., 2014) Additionally, absorbance around 420 nm and 680 nm suggests presence of chlorophylls in the extract, implying relatively broad affinity of tested solvents to wide group of pigments (Palta, 1990).

Table 2 Phycobiliproteins content, purity index, extraction yield and pH of the NaDES-based extracts

	A-PC (mg/L)	C-PC (mg/L)	C-PC (mg/g)	DP	Y (%)	pH
H_2O	658.44	2058.65	102.93	0.63	10.29	6.23
LGH	330.56	162.45	8.12	0.13	0.81	1.85
CMH	108.39	37.9	1.9	0.09	0.19	0.76
BMH	901.59	391.35	19.57	0.09	1.96	2.75
GGH-20	181.35	570.2	28.51	0.30	2.85	6.27
GGH-30	487.74	1968.3	98.42	0.60	9.84	6.33
GGH-45	959.49	2657.2	132.86	0.63	13.29	6.49

Spectrophotometric analysis of extracts revealed variance of their extractive power towards phycobiliproteins. As presented in the Table 2, for water extract, A-PC concentration equaled 658.44 mg/L and was lower than C-PC content that reached 2058.65 mg/L, this trend was different for organic acids-based and sugar-based NaDES. For LGH, CMH and BMH concentration of A-PC in the extract was over 2x higher than C-PC concentration. As presented in the Figure 2, organic acids-based extracts exhibited low degree of purity and C-PC extraction yield from 0.19% for CMH extract to 1.96% for BMH. First of the glucose-based solvents exhibited different phycobiliproteins extraction behavior, with higher extractive power towards C-PC, reaching extraction yield equal to 2.85% with extract purity of 0.3. Further modification of water content in the GGH solvent to 30% and 45% resulted in improvement of C-PC oriented extraction, with extraction efficiency equal to 9.84% and 13.29%, respectively. Solvent GGH-45 showed higher affinity to phycobiliproteins than water, as the C-PC extraction yield equal to 13.29% exceeded the efficiency of 10.29% of water extraction. At the same time, purity of both extracts was equal to 0.63.

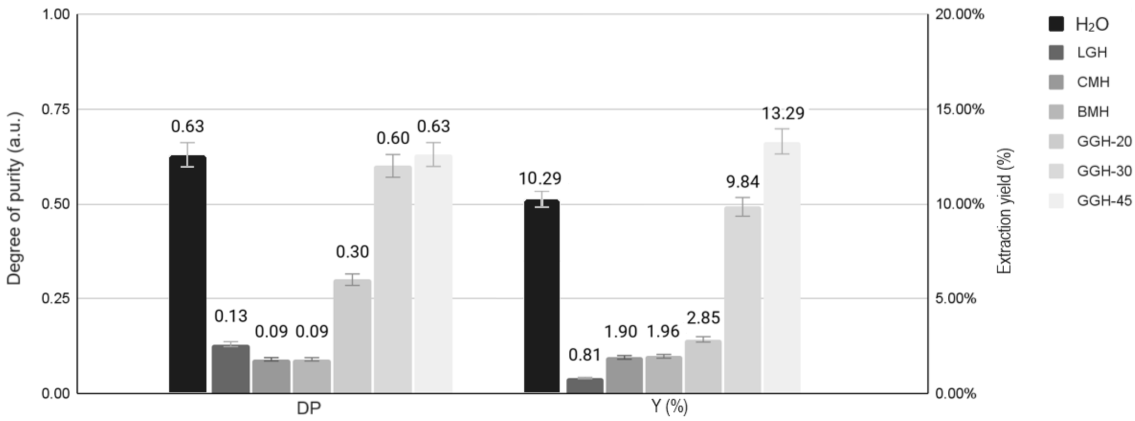

Figure 2. Degree of purity and C-PC extraction yield of water- and NaDES based extracts

Obtained results are in good correlation with the pH of the prepared solvents. The pH of NaDES played a crucial role in influencing the stability of phycocyanin during the extraction process. Organic acids-based NaDES exhibited low pH values, impacting the stability of phycocyanin. Phycocyanin stability is limited by the pH value, being the most stable under pH conditions from 5.5 to 6.0. Strong absorbance in the range below 300 nm of acidic extracts indicates precipitation of proteins and change in the conformation of proteins (Adjali et al., 2022). In contrast, glucose-based NaDES maintained suitable pH conditions in the range from 6.27 to 6.49, contributing to the enhanced stability of phycocyanin. The results indicate the promising role of NaDES, especially glucose-based formulations, in enhancing the extraction efficiency and purity of C-PC. The study aligns with the global push for greener extraction technologies, as NaDES are derived from natural components, presenting an eco-friendly and sustainable alternative to conventional solvents.

4. Conclusions

This study explored the extraction of C-phycocyanin from *Arthrospira platensis* using innovative hydrophilic solvents, NaDES, in comparison to traditional water-based extraction methods. NaDES, composed of organic acids and hydrogen bond donors, demonstrated varying properties influenced by their composition. The eutectic nature of NaDES contributed to their unique characteristics, offering potential advantages in solubility and selectivity for targeted compound extraction.

The findings highlight the potential of NaDES, specifically glucose-based formulations, in enhancing the extraction efficiency and purity of C-PC. Organic acids-based NaDES extracts showed higher concentration of allo-phycocyanin (A-PC) compared to C-PC, while glucose-based NaDES (GGH) exhibited a higher extractive power towards C-PC. Organic acids-based NaDES extracts showed lower purity index and C-PC extraction yield, indicating challenges in achieving high specificity and efficiency for C-PC. Glucose-based NaDES, especially GGH-30 and GGH-45, demonstrated improved extractive power for C-PC with higher extraction yields compared to water-based extraction. The pH of NaDES played a crucial role in influencing the stability of phycocyanin during the extraction process. Organic acids-based NaDES exhibited low pH values, potentially impacting the stability of phycocyanin, while glucose-based NaDES maintained suitable pH condition. Further optimization of NaDES compositions, including adjusting water content, opens avenues for tailoring solvents to specific extraction requirements. This research provides valuable insights for sustainable and efficient extraction methods, with implications for pharmaceuticals, food, and other industries. Future work could focus on optimizing NaDES formulations for enhanced extraction efficiency and specificity, contributing to the advancement of green and sustainable extraction practices.

Acknowledgments

This work was supported by the project PON “Ricerca e Innovazione” 2014-2020 – Action IV.5 “Dottorati su tematiche green”

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