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Ultrasound-assisted extraction of phycobiliproteins from *Spirulina* (*Arthrospira*) *platensis* using protic ionic liquids as solvent

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ABSTRACT

A new method that combines ultrasonic assisted extraction (UAE) with ionic liquids (ILs) was proposed to extract phycobiliproteins from the microalgae *Spirulina* (*Arthrospira*) *platensis*. Extraction of the pigments was carried out in an ultrasonic bath at 25 °C and at a frequency of 25 kHz. The effects of pH and solvent:biomass ratio were evaluated through a central rotational composite design and response surface methodology were used to determine the best extraction conditions. Solvents used were protic ionic liquids (PILs) 2-hydroxy ethylammonium acetate (2-HEAA), 2-hydroxy ethylammonium formate (2-HEAF), their equimolar mixture (2-HEAA + 2-HEAF) (1:1 *v*/v) and the commercial ionic liquid, 1-butyl-3-methylimidazolium chloride [Bmim][Cl] and sodium phosphate buffer (0.1 M) as a control. Results showed that the PILs were able to extract the phycobiliproteins from microalgae. The pH was the most significant variable. Solvent:biomass ratio was also significant in the extraction process. The highest concentrations of phycobiliproteins were observed using 2-HEAA + 2-HEAF as solvent at pH 6.50 and solvent:biomass ratio 7.93 $mL \cdot g^{-1}$ within 30 min of extraction. Allophycocyanin was the pigment extracted in greater quantity (6.34 $mg \cdot g^{-1}$), followed by phycocyanin (5.95 $mg \cdot g^{-1}$) and phycoerythrin (2.62 $mg \cdot g^{-1}$). Scanning electron microscopy (SEM) revealed that the ultrasound affect the cellular structure of the microalgae.

1. Introduction

Microalgae are organisms capable of synthesizing and accumulating valuable chemical compounds, such as polysaccharides, unsaturated fatty acids and a range of pigments, among them phycobiliproteins [1]. Phycobiliproteins are proteins that capture light and act as accessory photosynthetic pigments in cyanobacteria and red algae. They are divided into three subgroups: phycocyanin, allophycocyanin and phycoerythrin, each with its own characteristic color, absorbing light in a specific region of the spectrum, 615 nm, 652 nm and 562 nm, respectively [2]. These pigments are commonly used in cosmetic [3], pharmaceutical [4,5] and food industries [6–8] by their color and interesting spectral and physiological properties, such as antioxidant [9,10], anti-inflammatory [11,12] and anticancer activity [13]. Have also been used as fluorescent probes in histochemistry, flow cytometry, microscopy and fluorescence immunoassays [14]. More recently, phycobiliproteins have called attention by their potential application in the energy field, namely in the production of “dye-sensitized solar cells” (DSSCs). DSSCs are non-tracking concentrators that redirect solar radiation into simple slab waveguides to be collected by a photovoltaic cell mounted at the edge of the slab [15].

Some microalgae, such as *Spirulina* (*Arthrospira*) *platensis*, have a rigid cell wall that makes it difficult to extract specific components. To overcome this barrier, a cellular disruption operation is required in order to allow access to the internal components, facilitating the extraction process. This step is particularly important, because the contents of the extracted biomolecules are determined according to the disruption method and device used, and using an appropriate method and device is a key factor in increasing the biomolecule extraction efficiency. Therefore, many breaking techniques have been proposed: grinding, sonication, microwave, enzymatic treatment and high pressure cellular disruption to recover different components [16]. Ultrasound assisted extraction (UAE) has been used successfully in the extraction of various compounds and is easily implemented for large-scale industrial application [17,18]. The main phenomenon that occurs in exposure to ultrasound is cavitation. The cavitation is characterized by the violent collapse of bubbles in an alternating pressure field. Cavitation bubbles in the aqueous suspension of algae produce severe and localized short-term pressure increases as well as microstreaming effects (movement of liquid around gas bubbles formed by cavitation)

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and shock waves that promote the rupture of algae cells [19]. Some authors have reported the use of UAE in the extraction of phycobiliproteins from different species of microalgae [20–23].

In order to maximize the biotechnological and commercial potential of phycobiliproteins, new more efficient, economical and environmentally safe extraction methods must be developed. In this context, ionic liquids appear as an attractive and innovative alternative for the extraction of these biomolecules. Taking into account its wide versatility and capacity for reuse. ILs are salts, composed only of ions, with melting points below 100 °C, have low vapor pressure, high solvability and better chemical and thermal stability when compared to organic solvents. IL shave adjustable properties since their physical properties (polarity, hydrophobicity and viscosity) can be controlled by the exchange or combination of cations and anions. Due to their wide window of polarity, they are used in the most varied processes: from organic synthesis [24], extraction of metals [25,26], biocatalysis [27,28] to pretreatment of biomass [29,30]. Despite the many interesting features, most ILs have high cost disadvantages and low biodegradability. However, they have the advantage that they can be regenerated and reused [31]. ILs are classified into two groups: aprotic (AILs) and protic (PILs). AILs are mainly based on imidazolium and pyridine cations that are easier to use when compared to PILs. To get an idea, 250 g of the AIL used in this work, [Bmim][Cl], cost US$ 2823 [32] while 1 L of PIL 2-HEAF, considering only the value of the reagents, costs approximately US$ 9.28. PILs are synthesized by the transfer of protons from a Brønsted acid to a Brønsted base [33,34]. Among the advantages in the use of PILs we can mention: simplified synthesis, possibility of recycling and reuse and greater biodegradability in comparison to AILs [35].

In this context, a new method that combines UAE with protic ionic liquids has been proposed to obtain phycobiliproteins from *Spirulina* (*Arthrospira*)*platensis*. The performance of extraction using different protic ionic liquids was compared with sodium phosphate buffer and the aprotic ionic liquid ([Bmim][Cl]). The variables pH and solvent:-biomass ratio were optimized in order to maximize the concentration of the pigments. The recovery and reuse of the ionic liquids were evaluated. As the extraction of phycobiliproteins using protic ionic liquid is a new methodology, the data on the reuse of ILs in the extraction of phycobiliproteins are scarce. In this study, initial recovery trials using ammonium sulfate and dialysis were performed. Fractional precipitation of two steps ammonium sulfate promoted the salting out of unwanted proteins while concentrating the proteins of interest [36]. Ammonium sulfate was chosen as the precipitating agent because it maintains protein integrity, precipitates readily the phycobiliproteins, helps to reduce the amount of sample to be handled, and it is highly soluble at low temperatures [37].

2. Material and methods

2.1. Microalgae and cultivation conditions

The Cyanophyte *Spirulina* (*Arthrospira*) *platensis* was obtained from an external monoculture carried out in a recirculation system in polyethylene boxes with a volume of 500 L at 30 ± 2 °C for 20 days. The culture was enriched with urea [CH_4N_2O] (0.1 g·L^{-1}) and triple superphosphate [$Ca(H_2PO_4)2H_2O$] (0.01 g·L^{-1}) (SYNTH) with aquaculture effluents to replace losses by evaporation. Alkalinity of the selective medium was controlled by addition of 10 g·L^{-1} sodium bicarbonate [$NaHCO_3$]. The optical density of the culture medium was monitored at 680 nm using a spectrophotometer (HACH, DR2700, PORTUGAL) and the biomass of *S. platensis* was filtered daily through a blanket of synthetic glass fiber with 60 μm of pore size. The biomass was then dried in oven with air recirculation at 60 °C for 24 h and ground until a fine powder was obtained and particles of 0.25 mm to 0.84 mm diameter were selected. The biomass powder was stored in capped flasks at 25 °C until studies were carried out.

2.2. Synthesis and characterization of protic ionic liquids

Protic ionic liquids 2-hydroxy ethylammonium acetate (2-HEAA) and 2-hydroxy ethylammonium formate (2-HEAF) were synthesized through an acid-base neutralization reaction as described by [30,38]. Acetic acid (≥99.85%), formic (88–91%), monoethanolamine (99%) and aprotic ionic liquid [Bmim][Cl] (≥98%) acids were purchased by Sigma-Aldrich and were not subjected to further purification processes. After the synthesis, the PILs were stored at 25 ± 2 °C in dark bottles to avoid degradation by light. The product was characterized by nuclear magnetic resonance analysis and physico-chemical methods: pH, density and viscosity.

Density and viscosity were measured using a digital densimeter (ANTON PAAR, DSA 5000, AUSTRIA) and a digital U-tube oscillation viscometer (ANTON PAAR, SVM 3000, AUSTRIA) at 25 °C, respectively. The pH of the PILs was obtained by a pHmeter (Tecnal TEC-5, São Paulo, Brazil). ^{1}H NMR spectra were obtained on a 600 MHz Agilent DD2 (USA) spectrometer equipped with a reverse-detection 5 mm internal diameter (H-F/15N-31P) one-probe and z-axis field gradient. One-dimensional ^{1}H spectra were acquired with a time of acquisition of 1 s, gain of 26, acquisition of 16 transients in a spectral window of 16 ppm and 32 k number of points. Data was processed using the TopSpin 3.0™ program.

2.3. Extraction and determination phycobiliproteins

S. platensis was immersed in different solvent (protic ionic liquids (PILs): 2-hydroxy ethylammonium acetate (2-HEAA) and 2-hydroxy ethylammonium formate (2-HEAF); their equimolar mixture (2-HEAA + 2-HEAF) (1:1 *v*/v); the commercial ionic liquid, 1-butyl-3-methylimidazolium chloride [Bmim][Cl] and sodium phosphate buffer (0.1 M) as a control) and the extraction of phycobiliproteins was assessed using an ultrasonic device (UNIQUE, USC-1450,BRAZIL) with a frequency of 25 kHz and 25 ± 2 °C, for 30 min. A DCCR 2^2 experimental design was used to evaluate of the effect of dependent variables pH and solvent-to-biomass ratio in the obtained concentrations of phycobiliproteins. The levels and ranges adopted in the planning are summarized in Table 1. The design used 4 factorial points, 4 axial points and 3 repetitions at the central point, resulting in 11 assays by solvent. The pH was chosen as a variable in the study because it is a critical factor in the extraction processes and it directly affects the solubility of biomolecules [1]. In addition to interfering with solubility, pH affects the kinetic constants of proteins. High pH values alter the net charge of proteins, causing electrostatic repulsion and disruption of some hydrogen bonds, destabilizing them [1]. Silveira et al. [39], on the other hand, optimizing the process of extraction of the phycocyanin of *S. platensis* by the conventional method with different solvents, showed that the solvent:biomass ratio strongly influenced the pigment extraction process and that the maximum value was obtained using the lowest solvent:biomass ratio.

After extraction, 0.5 mL aliquots were withdrawn from the reaction medium and centrifuged at 6000 rpm for 10 min (GMCLAB GILSON, CAPSULEFUGE PMC-880, JAPAN). The optical density of the supernatants was determined by spectrophotometry (BIOCHROM, LIBRA S11, UK) at wavelengths 562 nm, 615 nm and 652 nm. The concentrations of phycocyanin, allophycocyanin and phycoerythrin were

Table 1
Variables and levels for central rotational compound design 2^2 (DCCR) using ultrasound in the extraction of phycobiliproteins from *Spirulina* (*Arthrospira*) *platensis*.

Variables	Unit	Levels				
		–α	–1	0	+1	+α
pH	–	4.38	5.00	6.50	8.00	8.62
Solvent:biomass ratio	(mL·g^{-1})	7.93	10.00	15.00	20.00	22.07

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