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Fractionation of proteins and carbohydrates from crude microalgae extracts using an ionic liquid based-aqueous two phase system

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ABSTRACT

Mild, simple and efficient recovery methods are required to obtain high-value microalgae proteins. As a promising extraction method, an Aqueous two phase system (ATPS) was used to partition proteins from crude microalgae extracts obtained from two green microalgae of industrial interest: *Neochloris oleoabundans* and *Tetraselmis suecica*. Furthermore, the Non-Random Two Liquids model (NRTL) was applied to describe both the phase diagram and the partition coefficient of total protein. It was observed that total protein preferentially concentrates in the top phase. Additionally, no significant effect on partition or extraction efficiency was noted at different tie lines. Experimental data indicate that proteins and sugars are selectively fractionated in top and bottom phases respectively. The model provided a good representation of the experimental data for the liquid-liquid equilibrium. Moreover, the model also led to a good representation of the partitioning data for two reference proteins, Rubisco and Bovine Serum Albumin (BSA), as well as for total protein from crude microalgae extracts.

1. Introduction

Due to their rich composition, microalgae are a potential source of biomolecules for food, feed, chemical and pharmaceutical products, of which proteins are of paramount industrial relevance. Microalgae can accumulate up to 60% protein under different cultivation conditions [1]. Because of their sustainability, techno-functionality and broad range of applications, algae proteins have been in the spotlight of numerous studies [2]. However, microalgae proteins are often present intracellularly or forming complexes with pigments and polysaccharides and thus, their recovery and purification still represents a challenge [3]. Several processes have been developed for the extraction and fractionation of proteins from microalgae. pH-shifting, filtration and adsorption are commonly reported [4]. However, such processes, are often characterized by low yields, poor selectivity and harsh conditions. Further research on alternative separation methods is therefore required.

Aqueous two-phase system (ATPS) is a liquid-liquid extraction method that has been presented as a mild, easily scalable, efficient and cost competitive technology for the recovery of a broad range of biomolecules [5]. Although large scale applications are reported [6], its widespread implementation has been constrained by the poor understanding of the partitioning mechanism and by the selection of the phase forming components, in terms of sustainability, recyclability and costs. Ionic liquids (ILs) have gained significant attention in the last decades as phase forming components in ATPS due to their chemical versatility and physicochemical properties. They are non-flammable and non-volatile. Moreover, their physicochemical properties (e.g. polarity, viscosity, miscibility) can be tuned by manipulating their cations and anions, allowing the tailor-made design of extraction processes [7]. Ionic liquid-based ATPS have been studied by several authors for the extraction of lipids proteins [8]. High extraction efficiencies and partition coefficients 3–4 times higher can be achieved in comparison with traditional polymer-salt systems [9].

Partitioning of proteins in IL-based ATPS is a complex phenomenon. It depends on several factors including type and concentration of phase-forming components, pH, temperature, ionic strength and chemical nature of the target molecule(s) [7]. In the case of proteins, hydrophobicity, isoelectric point, molecular weight and conformation play a critical role [10]. Significant progress has been made in the theoretical understanding of the underlying mechanisms of protein partitioning as well as phase equilibrium in ATPS. For the latter, several

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Nomenclature

Letter	Definition [Units]
a	activity[–]
CP	crude protein [–]
EE	extraction efficiency [%]
g	interaction energy [J mol^{-1}]
k	partition coefficient protei [–]
Q	penalty term [–]
R	universal gas constant [J mol^{-1} K^{-1}]
T	system temperature [K]
x	mole fraction [mol mol^{-1}]

Greek characters

α	non-randomness (NRTL model) [–]
τ	adjustable interaction parameter [–]
γ	activity coefficient [–]

Subscripts

i, k, l	component, parameters and tie lines respectively [–]
ij	refers to binary interaction between components *i* and *j*
g	free glucose [–]
T, B	top and bottom phase respectively [–]
exp, calc	experimental and calculated value respectively [–]
p	protein [–]

thermodynamic models have been successfully implemented for systems containing polymers [11], salts [12] and ILs [13]. Thermodynamic models have also been used to describe and predict the partition coefficients of model proteins in ATPS; satisfactory estimations are reported for polymer/salt systems using an extension of the Pitzer's model [12] and multicomponent Wilson model [14]. For polymer-polymer systems, modifications of the Pitzer's model [15], Flory Huggins theory [16] and UNIQUAC model [17], have been successfully implemented. A correct understanding and prediction of equilibrium and partitioning can lead to further developments in design, scale up and process optimization.

Despite the large number of publications in the field of ILs and protein extraction [8,18], the application of ATPS for the extraction of microalgae proteins and in particular for crude microalgae extracts is limited. The published research have centred mostly on extracting *C-phycocyanin* from *Spirulina* strains [19], *B*-phycoerythrin from *Porphyridium cruentum* [20] and proteins from *Chlorella pyrenoidosa* [21] and *Chlorella sorokiniana* [22]. Combination of several disintegration-extraction methods have also been described. Lee and co-workers [23] extracted proteins from *Chlorella vulgaris* using ultrasound and IL-based buffers, proving that the IL aids in the disintegration process. This was also demonstrated by Orr et al. [24] for the extraction of lipids from wet microalgae.

In the present investigation, we study the equilibrium of an IL-based ATPS and the partitioning of crude protein extracts obtained from two green microalgae: *Neochloris oleoabundans* and *Tetraselmis suecica*. *N. oleoabundans* have been extensively investigated and it is considered a promising industrial strain due to its versatility, high growth rate, and biomass composition [25]. *T. suecica* has been traditionally used in aquaculture [26] and recently it has been highlighted due to the techno-functional properties of its proteins [27]. In addition, the Non Random Two Liquids (NRTL) model is used to describe equilibrium and partition coefficients. The NRTL model was selected because of its flexibility to describe systems of different chemical nature, including electrolyte solutions and IL [13], and because of its simplicity compared with models like UNIQUAC or UNIFAC [28]. To our knowledge, this is the first attempt to describe the partitioning of crude microalgae proteins in ATPS containing ILs using thermodynamic models.

2. Experimental section

2.1. Materials

The Ionic liquid Iolilyte 221PG (> 95%) was purchased from Iolitec®. Citric acid monohydrate (> 99.0%) was purchased from Merck Millipore®. Bovine serum albumin (BSA, > 96%, 66.4 kDa), potassium citrate tribasic monohydrate (> 99.0%) and D-Ribulose 1,5-diphosphate carboxylase (Rubisco, ~ 540 kDa), a partially purified protein from spinach, were purchased from Sigma-Aldrich®. Potassium citrate buffer stock solution was prepared by weighing and mixing 60% (w/w) citric acid monohydrate with 60% (w/w) potassium citrate tribasic until pH 7 was reached.

2.2. Microalgae cultivation and harvesting

Two microalgal strains were used for this study: *Neochloris oleoabundans and Tetraselmis suecica*. *N. oleoabundans* (UTEX 1185, University of Texas Culture Collection of Algae) was cultivated in fresh water using a fully automated 1400 L vertically stacked tubular photobioreactor supplied with Bold's basal medium [29]. *T. suecica* (UTEX LB2286, University of Texas Culture Collection of Algae, USA) was cultivated in 25 L flat panel photobioreactors in sea water supplied with Walne medium. Cultivation details are given elsewhere [29]. Both photo-bioreactor systems were located in AlgaePARC (Wageningen, The Netherlands). After harvesting, biomass was stored at 4 °C until further use.

2.3. Fractionation process

The harvested microalgae were suspended in MilliQ® water to obtain a biomass concentration of ~90 g L^{-1}. The microalgae suspension was disrupted in a horizontal stirred bead mill (Dyno-Mill Research Lab, Willy A. Bachofen AF Maschinenfabrik, Switzerland) using 0.5 mm beads as described by Postma et al. [29]. The milled suspension was then centrifuged at 14000 rpm and 20 °C for 30 min in a Sorval® LYNX 6000® centrifuge (ThermoFisher Scientific®). The supernatant was recovered and subjected to a two steps filtration process. First, ultrafiltration was conducted on a laboratory scale tangential flow filtration (TFF) system (Millipore®, Billerica, MA) fitted with a membrane cassette with a filtration area of 50 cm^2 and a cut-off of 1000 kDa (Pellicon® XL Ultrafiltration Biomax®). The process was run at constant transmembrane pressure until a 5× concentration factor was achieved. The resulting permeate was then filtered three times over a 3 kDa Ultracel® Amicon® Ultra centrifugal filter (Millipore®, Tullagreen, IRL). Each run was performed for 20 min at 4000*g* and 20 °C; MilliQ® water was used as feed for the second and third run. The resulting retentate, regarded as crude protein (CP), was stored at −20 °C until further use.

2.4. Characterization of the crude protein extract

The crude protein (CP) extract was characterized based on proteins, carbohydrates, lipids and ash content. Soluble proteins were quantified using the DC Protein assay (Bio-Rad), which is based on the Lowry assay [30]. Bovine serum albumin (Sigma–Aldrich) was used as protein standard and absorbance was measured at 750 nm using a microplate reader (Infinite M200, Tecan, Switzerland).

Total carbohydrates content was determined by the method of Dubois [31], which is based on a colorimetric reaction in phenol-sulfuric acid which is measured at 483 nm. Glucose (Sigma–Aldrich) was used as standard. Total lipids were analysed following the method of Folch [32]. Lipids were extracted three times with chloroform/methanol/Phosphate buffer saline (1:2:0.8 v/v). After extraction, the excess of solvent was removed in a vacuum concentrator (RVC 2-25 CD +, Christ GmbH) and total lipid content was determined gravimetrically.