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Deep eutectic solvent-based protein recovery from microalgal biomass grown in piggery wastewater☆

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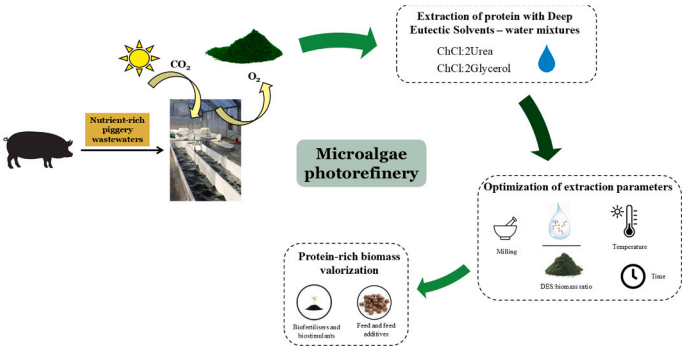
HIGHLIGHTS

- Protein-rich biomass grown in photobioreactors treating wastewater was valorized.
- Non-toxic natural deep eutectic solvents extract protein from microalgal biomass.
- Addition of 81.6 % water to DES ChCl:2 Urea improves protein recovery.
- Maximum yield with milled microalgae, 12:1 DES:biomass ratio at 30 °C for 0.5 h.
- Taguchi design facilitated evaluation and optimization of extraction parameters.

GRAPHICAL ABSTRACT



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ABSTRACT

Microalgae are a promising alternative to address wastewater treatment while converting wastewater nutrients into valuable protein-rich biomass, providing a dual-purpose solution. This study investigates protein recovery from microalgal biomass based on *Scenedesmus almeriensis* cultivated in swine wastewater using deep eutectic solvents (DESs) and their water mixtures. The behavior of two DESs (choline chloride:urea and choline chloride: glycerol at a 1:2 molar ratio) was evaluated with varying amounts of water. Both DESs outperformed water in protein extraction, and adding water to the DES:biomass mixture improved recoveries. The impact of the experimental factors was evaluated using a factorial design. The highest protein yield (16.8 %) was obtained with DES ChCl:2Urea with 81.6 % of water, by milling discs, a 12:1 DES:biomass ratio and extraction at 30 °C by 0.5 h. Despite moderate yields, this work underscores the potential of aqueous solutions of DESs as green solvents for protein recovery from microalgae.

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1. Introduction

The increasing concern about the environmental impact of food and goods production and the scarcity of raw materials has led to the implementation of the circular economy philosophy, in which the revalorization of the waste generated together with the design of more environmentally friendly processes has become a priority (D'Amato et al., 2017). Livestock farming is a polluting source of concern that affects water, soil, and atmosphere and is intensifying due to the protein needs of a growing population (Smith et al., 2024). The discharge of untreated livestock wastewater, rich in nutrients and organic matter (Shim et al., 2021), can result in eutrophication and contamination of water resources (Hu et al., 2017). Biological treatment of nutrient rich wastewater generates large amounts of biomass that can serve as a source of biomolecules and energy (Zhang et al., 2018).

Microalgae are photosynthetic microorganisms capable of growing symbiotically with bacteria in wastewater, offering a sustainable and efficient alternative for treating nutrient-rich effluents, such as swine wastewater (López-Sánchez et al., 2022). Species like *Scenedesmus obliquus* have been successfully employed for this purpose, leveraging the microalgae-bacteria consortium to reduce pollutants while generating valuable biocompounds (Tan et al., 2024). Consequently, applying the photobiorefinery concept to economically revalorize livestock wastewater into high value products is an emerging and increasingly important area of research (Dixon and Wilken, 2018).

Among the compounds that accumulate in microalgae, proteins are particularly noteworthy. Some species of microalgae have a protein content ranging from 20 to 60 % (dry weight), comparable to traditional protein sources like meat, fish or pulses (Kumar et al., 2022). This protein content can also vary significantly depending on the culture conditions. For example, He et al. cultured *Scenedesmus obliquus* in swine wastewater, achieving a protein content of 39.3 % in the biomass, above the content in the same microalgae cultured in domestic sewage with lower nutrients concentration (He et al., 2023). These protein fractions are valuable as they have interesting applications as functional polypeptides in industry, biostimulants in agriculture, or amino acids for animal feed (Sathasivam et al., 2019). Processes used in protein extraction are often based on cell disruption methods, exhibiting some drawbacks, such as the use of large amounts of organic solvents, long extraction times, chemically aggressive conditions and high temperatures, which can contribute to protein degradation (Dixon and Wilken, 2018). Thus, more environmentally and economically sustainable protein recovery methods are needed.

Deep eutectic solvents (DESs) are a promising alternative and have recently been investigated for protein recovery from other substrates such as flour (Svigelj et al., 2017) or oat (Yue et al., 2021). DESs consist of one or more hydrogen bond donors (HBDs) and one or more hydrogen bond acceptors (HBAs) that, when mixed in the proper molar ratio, present a significant decrease in melting point compared to pure initial compounds and their ideal mixture, resulting in liquid solvents at room temperature (Smith et al., 2014). A key characteristic of DESs is their easy preparation by mixing the individual components in an adequate molar ratio and applying a slight heating without further purification steps (Santana-Mayor et al., 2020). Furthermore, the most widely used precursors are nontoxic and biodegradable, such as choline chloride (ChCl) or betaine as HBA, and urea or glycerol (Gly) as HBD (Cannavacciuolo et al., 2023). However, despite their advantages, research on the use of DES for the extraction of protein from microalgal biomass is still scarce (Cicci et al., 2017).

The aim of this work is to perform a soft and sustainable protein extraction from microalgal biomass grown in swine wastewater using DES-based solid–liquid extraction. To achieve this goal, the properties and extractive capacity of solvents prepared from two DESs (ChCl:2Gly and ChCl:2Urea) with different amounts of water are investigated and compared with the extractive capacity of pure water. After selecting the best combination of DES-added water content, the influence of four extraction parameters (type of milling methods used as pretreatment to facilitate cell wall break-up, DES-to-biomass ratio, temperature and contact time) is evaluated by applying an orthogonal fractional factorial experimental design (Taguchi parameter design). Protein and carbohydrate recoveries are measured to assess the efficiency of the extraction process, not only in terms of protein yield but also considering the valorization of other biomass fractions. This research aims to broaden the understanding of DES applications in the extraction of microalgal protein, contributing to the development of the biorefinery concept, aiming for a more holistic utilization of microalgal biomass and contributing to the development of sustainable and efficient extraction processes.

2. Materials and methods

2.1. Biomass cultivation and characterization

Biomass formed by a consortium of microalgae *Scenedesmus almeriensis* and bacteria grown in a photobioreactor treating piggery wastewater was provided by the University of Almeria (Almería, Spain) and used as raw material in this work. A fiberglass thin-layer cascade photobioreactor of 1200 L (surface, 30 m^2; depth, 4 cm) was inoculated with *Scenedesmus almeriensis* and fed with pig slurry diluted at 10 %. The photobioreactor operated with a dilution rate of 0.33 d^{-1}, until constant biomass concentration and composition were achieved. *Scenedesmus almeriensis* was the most abundant species in the consortium, comprising 96 % of the total microalgae, identified and quantified by optical microscopy (Lorenzo-Hernando et al., 2019). Different bacteria phyla such as *Verrucomicrobium*, *Firmicutes*, *Cyanobacteria*, and *Proteobacteria*, originating from the piggery wastewater, were previously identified in biomass grown in 10 % diluted piggery wastewater in the same photobioreactor using similar conditions (Rojo et al., 2021). The biomass was freeze-dried and stored at 4 °C until further use to prevent degradation and to assess a constant biomass composition throughout this research. Standard methods were used to quantify the main components of the biomass, including water and ash content (AOAC, 2023), proteins (Rhee, 2001), carbohydrates (Van Wychen and Laurens, 2020), and lipids (Folch et al., 1957). The chemical composition of the biomass was 44.5 % protein, 20.4 % carbohydrates, 11.9 % lipids, 15.1 % ash and 5.6 % water.

2.2. Protein extraction with deep eutectic solvents

Two deep eutectic solvents (DESs) prepared from choline chloride as HBA and urea or glycerol as HBD were evaluated to extract proteins from microalgal biomass. These two DESs were selected because they are among the most widely studied hydrophilic DESs, are composed of natural and biodegradable components, and have previously shown good performance in protein extraction from various biological matrices (Yadav and Venkatesu, 2022). The DESs were prepared and characterized, and the effect of the addition of water to the pure solvent was investigated to assess its impact on recovery efficiency and sample handling, since the pure DESs are viscous and difficult to mix with the dry biomass. The effect of additional factors on the extraction process was then studied using the most suitable pure solvent.

2.2.1. Deep eutectic solvents preparation and characterization

The appropriate amounts of HBA and HBD were accurately weighed to achieve a molar ratio of 1:2 (HBA:HBD), mixed in a flask, heated at 60 ± 0.1 °C, and stirred in an orbital incubator (Optic Ivymen System, Logroño, Spain). When a clear, colorless liquid was obtained with no solid particles, the mixture was allowed to cool to room temperature. Choline chloride (ChCl), glycerol (Gly), and urea were purchased from Fisher Scientific (Waltham, United States) and used without further purification.

The DESs ChCl:2Gly and ChCl:2Urea were characterized to check

their proper formation and to see if their physicochemical properties (like viscosity or density) could explain their extraction performance. The density and viscosity of the DESs were measured at different temperatures (20, 25, 30 and 40 °C) and atmospheric pressure, using an Anton Paar SVM 3000 Stabinger densimeter-viscosimeter (Graz, Austria) with a high-precision thermostat. The water content was determined using the Karl-Fischer method with an automatic titrator (Mettler Toledo C20 KF Coulometer, Barcelona, Spain). The IR spectra of the two pure DESs, their corresponding starter materials, and the DES-water mixtures were recorded by attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR) using a Bruker Tensor 27 FTIR spectrometer (Karlsruhe, Germany).

2.2.2. Influence of solvent composition on protein recovery

The effect of two factors on the extraction process was studied: i) the pure solvent at three levels: ChCl:2Gly, ChCl:2Urea and pure water, used as a reference solvent to evaluate the improvement in protein recovery yield due to the use of DES; and ii) the influence of the addition of water to the pure solvent:biomass mixture, assayed at seven levels. The addition of water was studied to check whether, by reducing the viscosity and density of the solvent, the extraction capabilities of the DESs can be improved. Furthermore, the addition of water was investigated to test if protein extraction with DES could be applied to fresh biomass, which contains up to 80 % water and thus inherently introduces water into the process. The levels of this factor, defined as the mass percentage of water added compared to the total solvent mass of the mixture, were 0, 40.0, 57.1, 69.0, 81.6, 87.0, and 90.9 %. A two-factor full factorial design was implemented by combining all factor levels to study the effect of the assayed factors and their interaction. The experimental design was completely randomized and each treatment was performed in duplicate.

Solid-liquid extraction experiments were carried out by mixing the pure solvent (ChCl:2Gly, ChCl:2Urea or water) and freeze-dried biomass in Erlenmeyer flasks in a 9:1 mass ratio (4.5 g of solvent per 0.5 g of biomass). This ratio was selected based on preliminary experiments, in which 9:1 provided a good balance between extraction efficiency and solvent use compared to 18:1 mass ratio. This is also in line with values reported in previous studies using DESs for protein extraction from other biomasses (Chen et al., 2021; Grudniewska et al., 2018). Then, known amounts of extra water were added to the pure solvent:biomass mixture to achieve the different levels of added water. Subsequently, the samples were stirred vigorously in an orbital shaker incubator at 25 °C for 1 h at 500 rpm. Afterwards, the solid and liquid phases were separated by centrifugation at 5000 rpm for 5 min (ALC PK120 centrifuge, Winchester, USA). The liquid phase was transferred to a 100 mL volumetric flask and made up to the mark with deionized water. Finally, 40 mL aliquots were filtered using 45 μm pore-size nylon filters and stored in the dark at 4 °C, to avoid degradation, until protein and carbohydrate quantification (Section 2.3).

2.2.3. Effect of operational parameters

With the optimal DES and added water selected from the previous experiments, the effect of four operational factors on the protein and carbohydrates recovery yields was evaluated using a $L_9(3_4)$ Taguchi orthogonal fractional experimental design (Stufken and Peace, 1994). Control factors and their levels were selected to elucidate the conditions providing maximum protein recovery yield whereas minimizing coextracted carbohydrates: biomass milling method as a pretreatment step (P), DES:biomass ratio (R), extraction temperature (T), and contact time (t). The experimental design matrix with the combination of factor levels to be assayed in each trial is depicted in Table 1. All experiments were performed in duplicate for better estimation of the residual error.

The experimental procedure was as follows: the biomass used was first milled using one of three methods (none, mortar or disc mill). Manual grinding was carried out in an agate mortar grinding the biomass for 10 min, and disc grinding was performed using a Retsch vibratory disc mill Type RS-1 (Düsseldorf, Germany) during 1 min at 700 rpm. When no milling was applied, the freeze-dried biomass was directly used. Then, DES and biomass were mixed in Erlenmeyer flasks at DES:biomass ratios of 3:1, 6:1, or 12:1, and water was added to the mixture to achieve the optimal value previously found. Subsequently, the samples were stirred vigorously in an orbital shaker incubator at the necessary temperature (20, 30 or 40 °C) and time (0.5, 1 or 2 h) at 500 rpm. After extraction, the liquid fraction was treated and stored as described in Section 2.2.2.

Table 1
Taguchi's $L_9(3^4)$ orthogonal design with the factors and levels to be assayed and the mean results and standard deviations of two replicates for protein and carbohydrates recovery yields, PRY and CRY.

Trial	P	R ($g \cdot g^{-1}$)	T (°C)	t (h)	PRY (%)	CRY (%)
1	None	3	20	0.5	7.1 ± 0.1	13.1 ± 0.2
2	Mortar	6	20	1	10.0 ± 0.2	14.5 ± 0.2
3	Discs mill	12	20	2	16.2 ± 0.4	21.0 ± 0.2
4	Discs mill	6	30	0.5	15.8 ± 0.2	20.6 ± 0.3
5	None	12	30	1	10.1 ± 0.3	13.0 ± 0.3
6	Mortar	3	30	2	10.0 ± 0.3	14.8 ± 0.6
7	Mortar	12	40	0.5	11.6 ± 0.7	16.9 ± 0.7
8	Discs mill	3	40	1	14.8 ± 0.1	22.4 ± 0.1
9	None	6	40	2	10.2 ± 1.0	15.1 ± 1.4

2.3. Quantification of proteins and carbohydrates

Spectrophotometric methods were used to quantify proteins and carbohydrates in the filtered liquid extracts: the bicinchoninic acid assay for proteins (Smith et al., 1985), and the phenol–sulfuric acid method for carbohydrates (Taghavijeloudar et al., 2022). These methods are widely used in these types of analysis due to their simplicity, speed, and good accuracy. Proteins were determined using the BCA protein assay kit (Fisher Scientific, Hampton, United States) according to the manufacturer's instructions. The standard curve was built with bovine serum albumin (BSA). Total carbohydrates were estimated as glucose equivalents through the phenol–sulfuric acid method, using D-(+)-glucose to prepare the standard curve (Nielsen, 2010). Sulfuric acid and D-glucose were acquired from Sigma Aldrich (Burlington, United States), while phenol was provided by Merck (Darmstadt, Germany). All chemicals used were of analytical grade and were used as received without further purification. The equipment used for the absorbance measurements was a Spectronic Genesys 5 (Waltham, United States). All measurements were made in duplicate. The extraction results were evaluated in terms of two response variables denoted as protein recovery yield (PRY) and carbohydrate recovery yield (CRY), both expressed as a percentage of dry weight respect to the bio-compounds in the untreated biomass and calculated using Eqs. (1) and (2):

$$PRY = \frac{V_T \cdot c_P}{m_0 \cdot P} \cdot 100 \quad (1)$$

$$CRY = \frac{V_T \cdot c_C}{m_0 \cdot C} \cdot 100 \quad (2)$$

where m_0 is the mass of untreated dry biomass (in g), V_T is the total volume of the extract (in L), c_P and c_C are the measured concentrations of proteins and carbohydrates in the liquid fraction, respectively (in $g \cdot L^{-1}$) and P and C are the mass fraction of proteins and carbohydrates in the untreated dry biomass, respectively.

2.4. Statistical analysis

The experimental results were evaluated using analysis of variance (ANOVA) to discern factors that significantly affect the recovery yields of protein and carbohydrates. The least significant difference (LSD) test was used to study statistically significant differences between the levels of each factor. To concisely illustrate the results of multiple

comparisons, the compact letter display methodology was adopted (Piepho, 2018). Statistical analysis and graph generation were conducted using R software (version 4.3.2). A significance level of 5 % was applied to all statistical tests.

Experimental data from the Taguchi design were fitted following a multiple linear regression model. However, as the milling factor P is categorical, it had to be converted into two dummy variables, M and D, which stand for 'mortar' and 'discs' milling, respectively. The multiple linear regression model equation is shown in Eq. (3):

$$y = b_0 + b_T \cdot T + b_t \cdot t + b_R \cdot R + b_M \cdot M + b_D \cdot D \quad (3)$$

where y stands for the measured response variable (PRY or CRY), b_0 represents the constant term and b_i the terms for linear effects of each factor. Although T, t, and R could theoretically take any continuous value, M and D can only be 1 or 0. Both will be 0 if no grinding was applied, M = 1 and D = 0 if mortar was applied, and D = 1 and M = 0 if disc milling was used. Using the generalized linear model fitting (GLM), the model explaining the maximum amount of variance (highest R^2) was retained, and the model coefficients and their standard deviations were estimated. Only factors with significant influence on the response variable were considered. Finally, verification experiments were performed to validate the models.

3. Results and discussion

3.1. Deep eutectic solvents characterization

The experimental values of density and viscosity with their corresponding fit are shown in Figs. 1a and 1b, respectively. Both DESs had similar densities, although slightly lower values were found for the glycerol-based DES. Regarding its viscosity values, ChCl:2Urea exhibited a higher viscosity than ChCl:2Gly, making its handling more difficult. The values obtained (see supplementary material) agree with those reported in the literature (Xie et al., 2014; Yadav et al., 2014; Yadav and Pandey, 2014). The water content of both pure DES was below 1 %, in mass percentage.

DESs are defined by forming a hydrogen bond structure that is intended to be responsible for their unexpectedly low eutectic point (Smith et al., 2014). However, DESs generally have high viscosities, so water is usually added to reduce their viscosity to favor the mass-transfer diffusion process, disrupting the DES structure as a result of their hydrogen-bond donor behavior. To analyze how water influences the structure of DES, the IR spectra of pure DES and their mixtures with different percentages of water added were compared with literature (see supplementary material) (Delgado-Mellado et al., 2018; Ghaedi et al., 2017; Jurić et al., 2021). IR spectra showed that as the water content increases, the O—H stretch band (3400–3200 cm^{-1}) is broadened. The effect is less acute in the case of ChCl:2Gly as it already presents hydrogen bonds due to glycerol, but is more visible for ChCl:2Urea, for which its two N—H stretch bands are almost lost after the addition of 81.6 % and 90.9 % of water. Following with ChCl:2Gly, the two peaks at 3000–2800 cm^{-1} vanished for the samples with a water content greater than 57.1 %. In the fingerprint region, the C═O stretch band (1661.11 cm^{-1}) and the H—N—H scissor bending (1600 cm^{-1}) of the urea finally become the H—O—H scissors band of the water when its concentration is higher than 81.6 % in the mixture. Furthermore, the bending of CH_2 and C—OH (1500–1400 cm^{-1}) fades when water levels are above 81.6–90.9 %. Similarly, the symmetric stretching of C—N^+ (866–864 cm^{-1}) and the bending of N—H (786.53 cm^{-1}) also disappear when high water levels are reached. These spectral changes indicate significant disruption of the hydrogen bonding network that defines the deep eutectic nature of the solvent. Hammond et al. checked this by characterizing DES-water interactions through neutron total scattering and empirical potential structure, concluding that for ChCl:2Urea with a water content ≥51 %, the DES-water and water-water interactions dominate over the DES-DES interactions) (Hammond et al., 2017). In view of these results, it seems that water contents above 40–50 % lead to a transition from a deep eutectic mixture to an aqueous solution of the DES components. As such, while systems with up to 40 % of water may still retain DES-like interactions, solvents with higher water content should be more accurately described as aqueous solutions of DES, rather than DESs themselves.

3.2. Influence of solvent composition on protein and carbohydrates recoveries

The PRY and CRY values of the extraction experiments performed on non-milled freeze-dry biomass at 25 °C for 1 h using both DES and their combination with water are depicted in Fig. 2. As can be seen, the recovery yields for carbohydrates were slightly higher than those for proteins. However, since the amount of protein is more than twice that of carbohydrates in the initial biomass, the amount of proteins is greater than that of carbohydrates in the extracts. The experimental repeatability was good, ranging from 0.1 to 6 % as a relative standard deviation (% RSD).

ANOVA of the experimental results (see supplementary material) indicated that both factors and their interaction significantly influence protein and carbohydrates recovery (p-values <0.05). Nevertheless, for PRY, the pure solvent has a stronger effect than the added water (62.8 % vs. 27.3 % contribution to the total variance of the results), whereas for CRY, the added water contributed more significantly than the pure solvent (60.5 % vs. 6.4 % contribution). The LSD test for comparing

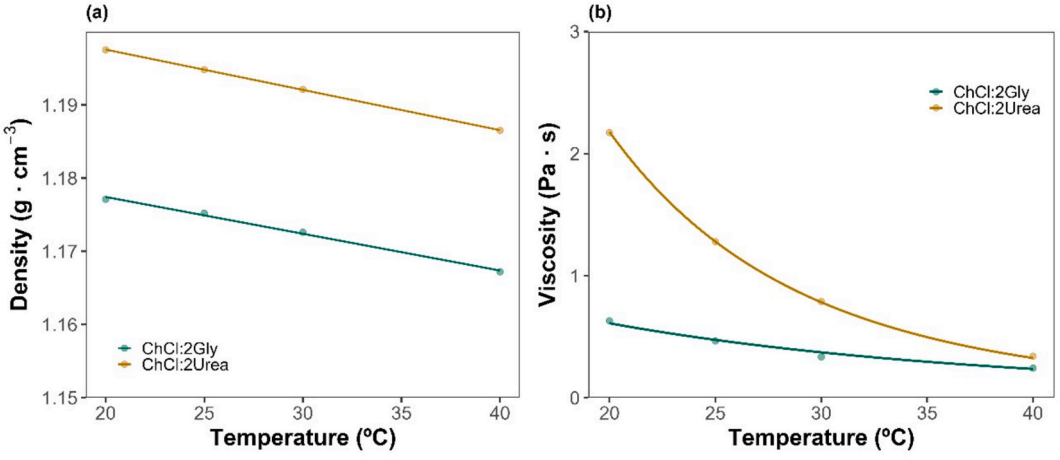

Fig. 1. Density (a) and viscosity (b) at different temperatures and atmospheric pressure for the pure DES.

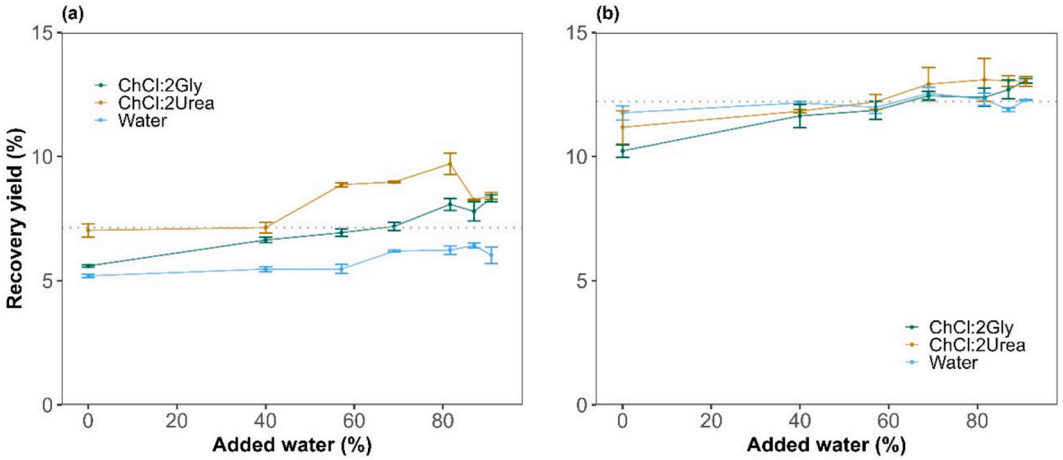

Fig. 2. Influence of added water (x-axis) and solvents tested (in different colors) on the recovery yield of proteins (a) and carbohydrates (b). The vertical error bars represent the standard deviation of the means. The dotted line indicates the great mean for proteins or carbohydrates, respectively.

means detected significant differences among most of the factor levels assessed. However, the behavior varied according to the response variable studied. The mean plots are shown in Fig. 3.

Focusing on the pure solvent factor, all levels were statistically different for PRY, while for CRY, ChCl:2Gly and water did not differ and gave significantly lower yields than ChCl:2Urea. ChCl:2Urea DES provided the highest yields for both PRY and CRY, with a stronger effect observed for PRY. This outcome agrees with the well-known ability of urea to solubilize proteins and peptides that are not covalently bound to large complexes (Karan et al., 2019). Hence, the extractant behavior of urea-based solvents appears to maintain the good solubilization properties of urea. This is consistent with the solvent polarity Dimroth Reichardt 30 scale, $E_T(30)$, in which the increasing polarity order is water (63.1 kcal·mol^{-1}) > ChCl:2Gly (58.3 kcal·mol^{-1}) > ChCl:2Urea (57.0 kcal·mol^{-1}) (Martín et al., 2023; Ruesgas-Ramón et al., 2017). However, while solvent polarity may partially explain these results (proteins are generally less polar than carbohydrates) the role of specific interactions such as hydrogen bonding must also be considered.

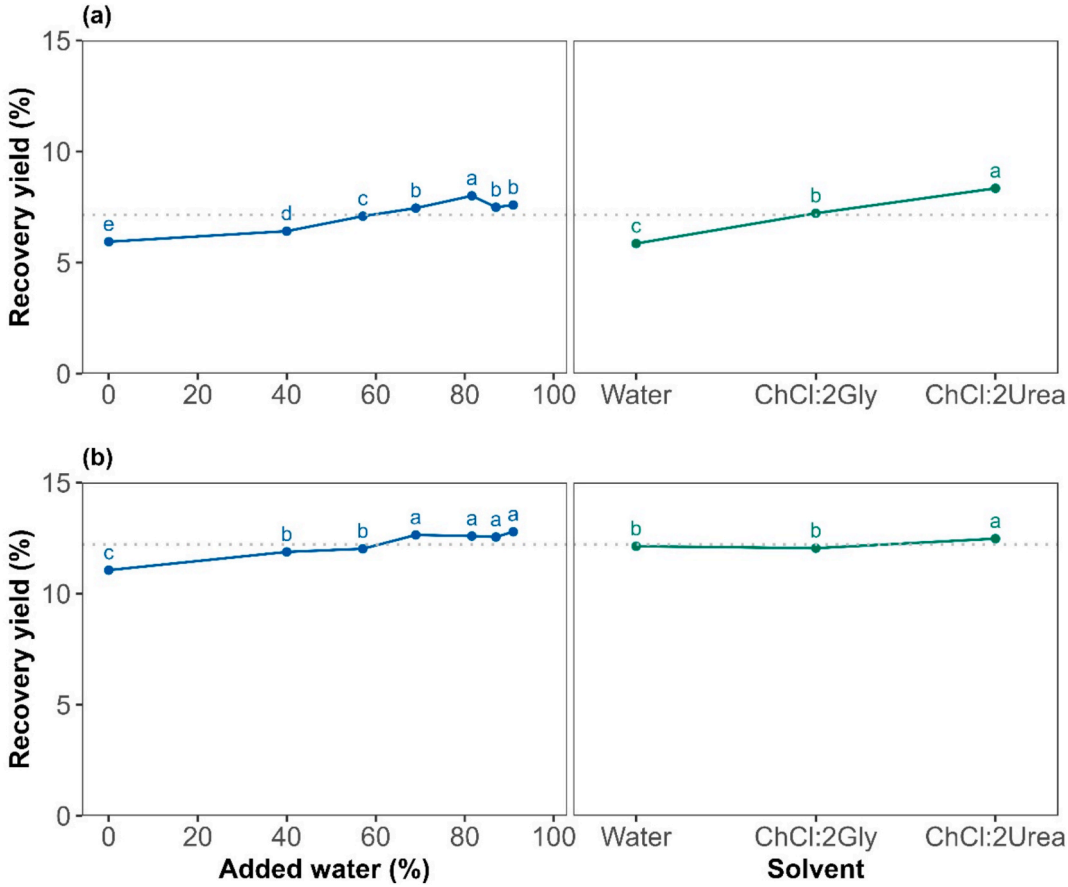

Fig. 3. Mean plots for the different levels of added water and solvent for recovery of proteins (a) and carbohydrates (b). The letters on top of each point are displayed according to the compact letter display methodology using LSD post hoc test. Means of the levels of a factor with a common letter are not significantly different. The horizontal dotted line indicates the great mean of the response.

Carbohydrates, especially polysaccharides, often form extensive hydrogen-bonding networks, making them insoluble even in highly polar solvents (Singh et al., 2021). This may explain the limited carbohydrate recovery from the microalgal biomass in this study. Therefore, both polarity and hydrogen-bonding capacity of the solvents contribute to the observed extraction selectivity.

Regarding added water, maximum PRY was found at around 80 % for the two DESs studied, but for CRY there were no significant differences between added water levels ranging from 69 to 90.9 %, although values of 0–57.1 % provided lower yields. These results suggest that added water improves both PRY and CRY up to a certain value (81.6 % for PRY and 69.0 % for CRY), beyond which the amount of water causes a decrease in PRY while keeping CRY constant; therefore, reducing selectivity. Moreover, the influence of the added water depends on the pure solvent used, as shown by the significant interaction between factors. This can be partly attributed to the high viscosity of ChCl:2Gly and ChCl:2Urea compared to water, which may hinder effective mixing and limit contact with the biomass, thereby reducing extraction efficiency. In such cases, the addition of water reduces viscosity and can enhance the mass transfer coefficient, improving extraction yields (Vilková et al., 2020). Nevertheless, viscosity adjustment alone does not fully explain the observed effects. Water addition can also alter the polarity, hydrogen bonding network, and overall solvation properties of the solvent system, which may influence the selectivity and efficiency of solute extraction (Negi et al., 2024).

However, as discussed in Section 3.1, high dilutions of DES might lead to loss of the supramolecular structure of DES, and the mixture will begin to behave as an aqueous solution of the DES components, therefore solubilizing higher amounts of carbohydrates since the main component of the mixture would be water and not DES (García-Roldán et al., 2023). Furthermore, this could also account for the differences observed between the PRY and CRY values in the different DES-water mixtures compared to pure water. Interestingly, when CRY was studied, a flatter trend was observed in comparison to PRY for both pure solvent and added water factors. This could be attributed to the fact that carbohydrates are more polar and hydrophilic than proteins, so they are more likely to be solubilized in water than in non-aqueous solvents (Mena-García et al., 2019), thus reaching the saturation point with less water. Therefore, ChCl:2Urea with a water content of 81.6 % was selected for further process optimization experiments because it maximizes PRY without significantly increasing CRY and is easy to handle due to its low viscosity. The physical properties of this mixture were also determined, leading to a 100-fold decrease in viscosity compared to that obtained for pure DES (see supplementary material). In addition, it was checked that this DES-water mixture, despite losing the hydrogen bond network that forms the DES, possesses properties and characteristics for protein extraction that are distinct (and better) from those of pure DES and water separately. Under the above conditions, the maximum value obtained for PRY was 9.71 %, recovering 13.1 % of carbohydrates.

3.3. Optimization of operational parameters

To improve extraction efficiency of proteins while minimizing carbohydrate recovery, another experimental design was carried out to optimize additional extraction parameters. Using ChCl:2Urea with 81.6 % added water as solvent, the effect of the control factors milling method, DES:biomass ratio, temperature and extraction time was evaluated. The study of milling method as pretreatment (P) could provide information on whether an additional step of cell disruption is needed to promote the release of compounds from the cells. With respect to DES:biomass ratio (R), higher ratios generally favor the mass transfer of the analytes to the solvent, but they also entail increased process costs, so a compromise solution must be found. Finally, since temperature (T) and time (t) are two critical parameters in an extraction process (Walas, 2013), they also need to be evaluated.

Three levels for each factor were studied to identify potential quadratic effects. As pretreatment, the use of different biomass milling methods of increasing cell disruption potential (none, manual, and disc mills) was investigated. Different DES:biomass ratios were assayed to determine whether more proteins are solubilized or, alternatively, if a saturation point is reached. As 9:1 ratio had already been tested, two lower ratios (3:1 and 6:1) were tested to check if a lower amount of DES could lead to similar protein yields, and a higher ratio (12:1) was applied to determine whether the increase of the DES used may significantly improve recovery. The levels of T (20, 30 and 40 °C) and t (0.5, 1 and 2 h) during the extraction process were chosen around the values employed in the preliminary experiments (25 °C and 1 h). Temperatures greater than 40 °C were not tested to avoid protein degradation. Increasing the contact time usually improves extraction yields; however, longer times were not considered as they would increase energy and time costs.

Studying the four factors at three levels through a complete factorial design becomes less practical in terms of time and resources due to the exponential increase in the number of trials required ($3^4 = 81$ trials). To study the main effects of each factor level with reduced experimental work, fractional factorial designs are preferred. The $L_9(3^4)$ Taguchi orthogonal fractional design (Stufken and Peace, 1994) was selected, reducing the number of experiments to 9 (18 trials as the 9 experiments were performed in duplicate). The experimental design matrix, the mean results and standard deviations of PRY and CRY for the nine treatments are shown in Table 1.

The PRY values obtained ranged from 7.1 % to 16.2 %, while the CRY values ranged between 13.0 % and 22.4 %. For both response variables, the precision of duplicate measurements remained below 10 % as a relative standard deviation (RSD). An ANOVA of the experimental results was performed to determine the most influential factors (see supplementary material). As the experimental design has only 17 degrees of freedom, the factor interactions could not be explored. Within the studied range of contact time (0.5 to 2 h), this parameter was not statistically significant for PRY and CRY (p-value > 0.05). For CRY, the DES:biomass ratio (6 to 12 $g \cdot g^{-1}$) was also not significant, while it was for PRY, where it had a significant influence. In terms of the contribution of the factor to the total variance, the type of milling was the most significant factor, accounting for 87.5 % and 91.1 % for PRY and CRY, respectively. This is justified because when milling is applied, it facilitates the disruption of the cell wall and the release of biocompounds, such as proteins and carbohydrates. Then, for PRY, the DES:biomass ratio emerged as the second most relevant factor (7.9 %), followed by the temperature (2.6 %). For CRY, only temperature proved to be significant in addition to milling, accounting for a contribution of 7.3 % of the total variance. The main effects plot combined with the results of the LSD test is depicted in Fig. 4 and was used to determine the levels of factors that maximize PRY while minimizing CRY.

Regarding the milling factor, the mortar provided higher yields than the non-milling application, but the use of the disc mill improved both PRY and CRY more significantly. With respect to the DES:biomass ratio, the 12:1 level also exceeded the other PRY-tested ratios, although the increase was not as acute as the milling factor. Concerning temperature, higher levels increased both PRY and CRY, but no significant improvements were found for PRY when using 40 °C instead of 30 °C. In contrast, for CRY, 40 °C was the temperature that significantly produced higher performances, with no differences observed between 20 °C and 30 °C. This is consistent with the general knowledge that higher temperatures may aid in the mass transfer of the analyte from the biomass to the solvent phase (Hou et al., 2017). However, when the temperature exceeds a certain limit, proteins may partially denature (breaking down into smaller peptides). In summary, these results suggest that the optimal extraction conditions that maximize protein recovery were found to be the inclusion of a pretreatment step of intense biomass grinding in a disc mill, a 12:1 DES:biomass ratio and a temperature of 30 °C, as higher temperatures would only mean higher energy costs without a substantial improvement in protein extraction efficiency.

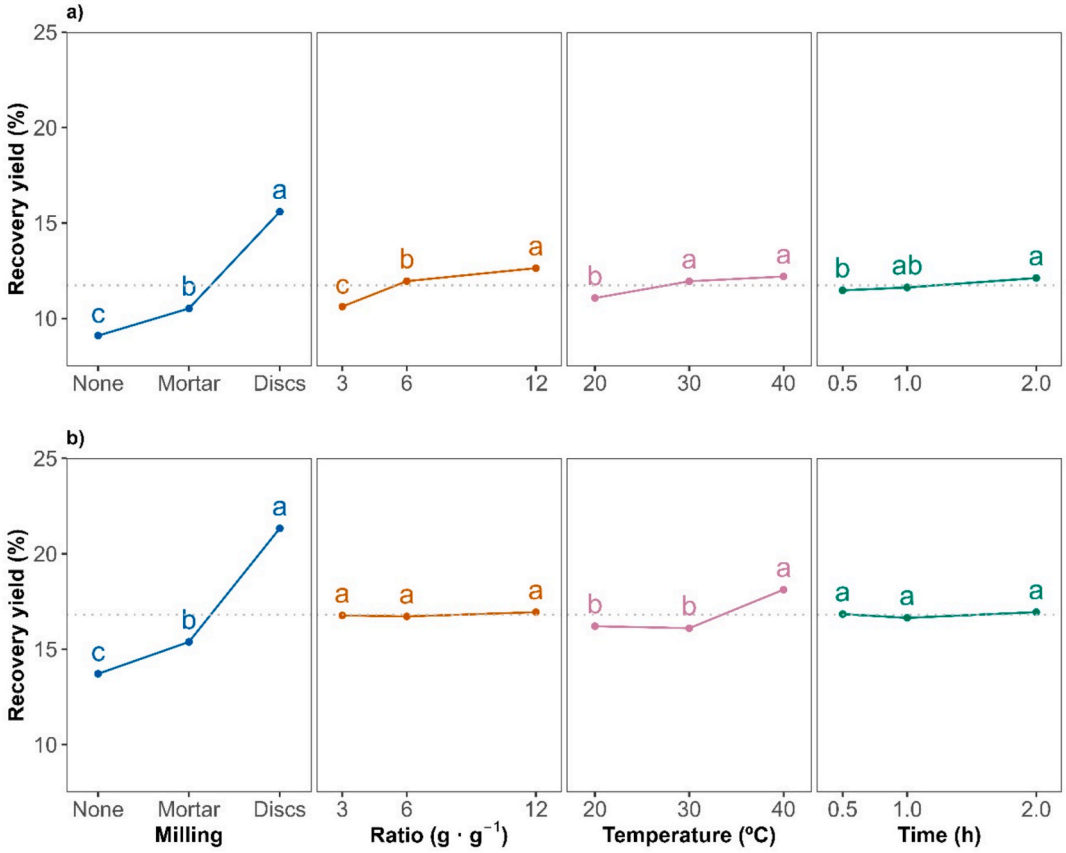

Fig. 4. Main effect plots for the different levels of milling method, DES:biomass ratio, temperature and contact time for the recovery yield of proteins (a) and carbohydrates (b). The letters on top of each point are displayed according to the compact letter display methodology using the LSD test. Means with a common letter within the same factor are not significantly different. The horizontal dotted line indicates the great mean of the response studied.

Since ANOVA demonstrated that contact time is a nonsignificant factor, 0.5 h was chosen as the optimal factor level. Finally, while higher DES ratios could have been tested to improve protein recovery, increased solvent consumption and associated process costs would likely result in only modest improvements in PRY values. Using experimental data, linear regression models for the response variables PRY and CRY with their respective significant factors were built. The model equations with their corresponding factor coefficients and standard error for each variable and their explained variances (R^2) are depicted in Eq. (4) and Eq. (5):

$$\mathrm{PRY} = (5.9 \pm 0.7) + (0.06 \pm 0.02)\cdot \mathrm{T} + (0.21 \pm 0.04)\cdot \mathrm{R} + (1.4 \pm 0.4)\cdot \mathrm{M} + (6.5 \pm 0.4)\cdot \mathrm{D} \quad R^2 = 0.957 \tag{4}$$

$$\mathrm{CRY} = (10.8 \pm 0.7) + (0.10 \pm 0.02)\cdot \mathrm{T} + (1.7 \pm 0.4)\cdot \mathrm{M} + (7.6 \pm 0.4)\cdot \mathrm{D} \quad R^2 = 0.955 \tag{5}$$

According to its model, the optimal factor levels that maximized PRY were the use of disc mill as biomass grinding pretreatment, and extraction at 30 °C for a contact time of 0.5 h and a 12:1 DES:biomass ratio. As this combination of factor levels was not included in the Taguchi experimental design, it was assayed in duplicate to confirm the optimal levels of the extraction parameters selected and to validate the predictive models. The PRY obtained was 16.8 %, which was two-fold the amount obtained in the preliminary experiments in Section 3.2, before optimization. Regarding CRY, the achieved value was 21.3 %, higher than those obtained in Section 3.2, but the increase was less acute than for PRY and thus optimization has improved extraction selectivity towards proteins. The relative errors of the experimental results obtained under these optimal conditions compared to the predicted value of the model were 1.2 % for PRY and −12.7 % for CRY.

To our knowledge, only one study has directly applied solid–liquid protein extraction from microalgae using deep eutectic solvents. Cicci et al. examined different biomass milling methods to study the extraction of proteins, carbohydrates, lipids, chlorophylls and carotenoids from pure *Scenedesmus dimorphus* (without bacteria, UTEX 1237) grown in synthetic media, using DES prepared with propane-1,2-diol, choline chloride and water in a 1:1:1 molar ratio. The highest protein extraction yield was achieved by applying the solvent combined with ultrasounds, attaining an extraction efficiency of 27 %. However, when only using ball milling (a harsher technique than the mortar or disc milling used in this work), the recoveries were comparatively lower than the maximum obtained in this study (10 % vs. 16.8 %) (Cicci et al., 2017). Other works have employed chemical hydrolysis strategies for the recovery of proteins from microalgae and bacteria consortiums based on *Scenedesmus almeriensis*. For instance, Rojo et al. obtained 90 % PRY, with also high CRY (80 %) when using alkaline conditions at high temperatures (120 °C, 2 M NaOH). Nonetheless, in the experiments carried out at 40 °C (the same temperature used in the current work) they achieved PRY lower than 20 % using acidic hydrolysis conditions (coextracting 60 % of carbohydrates), while for the alkaline experiments at that temperature the yields achieved were in the range of 35 % for PRY and almost 70 % for CRY (Rojo et al., 2023). Lorenzo et al. also attempted alkaline hydrolysis protein solubilization, but under their optimal conditions (55 °C, 5 h and 0.5 M NaOH) the PRY obtained was 16.9 %, which is comparable to the values achieved in this work (Lorenzo-

Hernando et al., 2019). Finally, Amiri et al. recovered 21.4 % of proteins using cellulase and up to 27.9 % using a multi-enzyme complex from pure microalgae *Scenedesmus obliquus*. However, alkaline treatment yielded 19.1 % of proteins, a value comparable to that presented in this paper (Amiri et al., 2024).

Overall, this study has shown that the use of DESs based on natural biodegradable compounds at low temperatures and treatment times provides results comparable to those of methods that employ harsh conditions and are time-consuming and energy-demanding. However, further efforts are needed to enhance protein yields and increase the selectivity for proteins while minimizing carbohydrate extraction. A promising approach would be to design a nontoxic DES-based solvent with a higher affinity for proteins. In this sense, the combination of enzymes with aqueous solutions of DES could be beneficial due to their potential cooperative effects. Furthermore, other green pretreatment techniques, such as ultrasound or microwave, are recommended, as these methods can potentially disrupt the cell wall of microalgae, facilitating the interaction of DES with the biocompounds of interest. Finally, alternative techniques such as liquid–liquid extraction, specifically aqueous two-phase systems (ATPS) based on DES, should also be explored.

4. Conclusions

The recovery of protein from microalgae biomass based on *Scenedesmus almeriensis* cultivated in piggery wastewater was investigated. Two different DESs combined with water were examined. ChCl:2Urea with 81.6 % added water yielded the best protein recovery (9.7 %), highlighting distinct behaviors of DES-water mixtures. Using a Taguchi experimental design, optimal protein recovery (16.8 %) was achieved using disc milling pretreatment, and extraction with 12:1 DES:biomass ratio at 30 °C, although carbohydrate recovery remained high. Extraction time was not significant. Further research is required to enhance protein extraction selectivity and yield, exploring ultrasound or microwave pretreatment, novel DES, enzyme combinations, or aqueous two-phase systems.

CRediT authorship contribution statement

David Moldes: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Patricia F. Requejo:** Writing – review & editing, Supervision. **Marisol Vega:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Silvia Bolado:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: As SILVIA BOLADO RODRIGUEZ, a [co-]author on this paper, is an associate editor of *Bioresource Technology*, she was blinded to this paper during review, and the paper was independently handled by Samir Kumar Khanal as an EiC.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2025.133446.

Data availability

Data will be made available on request.

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