

Deep eutectic systems for carbonic anhydrase extraction from microalgae biomass to improve carbon dioxide solubilization

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ABSTRACT

This work is the first proof-of-concept of the use of carbonic anhydrase (CA) enzyme from microalgae biomass, extracted with deep eutectic systems (DES), with the goal of engineering a solution that will lead to a breakthrough in the Carbon Capture and Utilization (CCU) strategy. Three distinct microalgae were processed – *Tisochrysis lutea*, *Chlorella vulgaris*, and *Spirulina sp.* – with three DES – Choline chloride-Urea (ChCl-U), Choline chloride-Poly(ethylene glycol) (ChCl-PEG), and Poly(ethylene glycol)-Urea (PEG-U). To evaluate the most promising microalgae-DES, CA activity was evaluated with a specific enzymatic activity kit and through CO₂ solubility assays. Preliminary results indicate that: DES is a suitable solvent medium for CA extraction from microalgal biomass, preserving its activity (specific CA activity up to 0.70 mU.mg⁻¹); CA extraction efficiency differs between DES and microalgal species, indicating the potential for further research; from the tested DES, the ones containing PEG were favorable to maintain CA activity (CO₂ solubility up to 4 g CO₂.g⁻¹ DES). This work paves the way towards a disruptive CCU approach.

1. Introduction

High levels of carbon dioxide (CO₂) in atmosphere are one of the causes for air pollution, greenhouse effect, climate changes, and acid rain. The concentration of CO₂ in the atmosphere has been increasing due to human induced activities, such as intensive industrialization. Flue gas is known to be one of the biggest sources of atmospheric CO₂ pollution. However, to capture CO₂ from flue gases it must undergo several processes: separation from the other gases, compression, possibly transportation, and conversion into stable products.

It is known that CO₂ is essential for several natural processes like photosynthesis. Photosynthetic organisms, like microalgae, need CO₂ to thrive as they serve as a “carbon sink”. In the presence of sunlight, microalgae capture atmospheric CO₂ through carbonic anhydrase (CA) enzyme, responsible for the interconversion between CO₂ and water and the dissociated ions of carbonic acid. This captured carbon by microalgae is then converted into biomass rich in high-value compounds, such as antioxidant pigments and omega-3 fatty acids [1,2]. Furthermore,

microalgae can capture CO₂ 10–50 times more efficiently than terrestrial plants having also a faster growth [1].

However, the direct use of flue gases from industrial activities to feed microalgae production is not possible since a high accumulation of CO₂ concentration (above 5%) has an inhibitory effect on microalgae growth. Also, flue gases are often a mix of several molecules, such as heavy metals, which can be toxic for microalgae [1]. Therefore, new strategies need to be developed to increase CO₂ capture and purification between the flue gas release and the microalgae consumption. Gas-liquid membrane contactors can be placed in harsh environments for CO₂ capture and purification, and when filled with DES, this CO₂ capture is enhanced [3]. We propose that the CA enzyme from microalgae can be extracted with deep eutectic systems (DES), known to be an enzyme-“friendly” environment [4]. This DES rich in CA enzymes can later be used in gas-liquid membrane contactors and placed in harsh environments for CO₂ capture and purification (Fig. 1). This purified CO₂ has several industrial applications, such as microalgae biomass production, rich in CA and other high-value compounds, providing a

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close loop for CO₂ capture and utilization.

The use of DES as extraction solvents has been extensively explored in the last decade, mainly due to DES's tunable extraction power and to their easy preparation and low cost. Although only a few studies were published using DES as extraction of a biocatalyst, they have been reported as solvents in enzyme reactions (with hydrolases, lipases and proteases) [4,5]. The use of DES as whole-cell biocatalyst was described extensively in bacteria and fungi cells, but not so often in microalgae. To the best of our knowledge, this is the first time that CA extraction directly from different microalgae, using DES, is performed. The possibility of using DES to extract CA from microalgae, and to use this extract directly without back-extraction, lowers the costs in enzyme purification and increases the potential of this technology. DES can be design according to the process' desired properties [4,5].

In this work we aim to answer two research questions:

- 1) Are DES suitable solvents to extract proteins/CA from microalgae biomass?
- 2) Which DES-microalgae combination is more promising in CO₂ scavenging?

The answer to these questions paves the way for the combination of gas-liquid membrane contactors technology coupled with microalgae production, as one engineered solution that may lead to a break-through in all involved scientific and industrial fields. To answer those questions, protein content and CA activity of three distinct microalgae were measured – *Tisochrysis lutea*, *Chlorella vulgaris*, and *Spirulina sp.* – in three DES with different physico-chemical properties and different solubility coefficient towards CO₂ – Choline chloride-Urea (ChCl-U), Choline chloride-Poly(ethylene glycol) (ChCl-PEG), and Poly(ethylene glycol)-Urea (PEG-U).

2. Methods

2.1. Microalgae strains and cultivation

2.1.1. *Tisochrysis lutea*

T. lutea strain was provided by NECTON, S.A. (Olhão, Portugal). *T. lutea* was cultivated in 250 mL Erlenmeyer flasks, with 100 mL of culture, at 25 °C, with an incident light of 90 – 130 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 18:6 day:night cycle, with air enriched with 0.1 – 0.4% CO₂. The cultivation medium consisted of filtered natural sea water (Sartoguard®, PES membrane, 0.1 μm pore size, Sartorius, Germany) with 20 mM HEPES, and supplemented with 3 g L⁻¹ of NaNO₃ and 2 mL L⁻¹ of micronutrients solution (NutriBloom plus, Phytobloom®, Portugal). The pH was adjusted to 8.0.

2.1.2. *Chlorella vulgaris*

C. vulgaris was cultivated in 250 mL Erlenmeyer flasks, with 100 mL

of culture, at 25 °C, with an incident light of 90 – 130 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 18:6 day:night cycle, with air enriched with 0.1 – 0.4% CO₂. The cultivation medium consisted of M8a medium, filtered with 0.22 μm pore size syringe filter, supplemented with 2 g L⁻¹ of NaNO₃. The pH was adjusted to 6.7.

2.1.3. *Spirulina sp*

Spirulina sp. biomass was provided by Algreen, B.V. (Wageningen, Netherlands). *Spirulina* is grown in photobioreactors, at room temperature, with a maximum light intensity of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in day:night cycle. The cultivation medium has already been described in [6–8]. The pH was adjusted to 9.3.

2.2. Deep eutectic systems (DES)

2.2.1. DES preparation

The DES used in this work can be found in Table 1. Both components of the DES were weighed in closed cap vials, according to their molar ratio, and heated to 80 °C while stirring until a homogeneous solution was obtained. Then, DES were cooled to room temperature, and no precipitation was observed. In some experiments, 0.05 and 0.1 mg (± 0.01 mg) of pure CA from bovine erythrocytes (2000 W-A units mg⁻¹, CAS 9001–03–0, Sigma) was added per mL of DES.

2.2.2. DES characterization

Two parameters were determined to establish DES' properties:

- Water content: determined by Karl-Fisher Coulometer 831 (Metrohm) using Coulomat AG (Honeywell) as analyte. This titration method is based on an iodine-iodide reaction, where the water inside

Table 1

Composition of the Deep Eutectic Systems used in this work.

Sample name	DES components		Molar Ratio	[CA] mg/g _{DES}
ChCl-U	Choline Chloride	Urea	1:2	–
ChCl-PEG	Choline Chloride	Polyethylene glycol	1:2	–
PEG-U	Polyethylene glycol	Urea	2:1	–
ChCl-U(CA)	Choline Chloride	Urea	1:2	0.05
ChCl-PEG (CA)	Choline Chloride	Polyethylene glycol	1:2	0.05
PEG-U(CA)	Polyethylene glycol	Urea	2:1	0.05
ChCl-U(CA)	Choline Chloride	Urea	1:2	0.1
ChCl-PEG (CA)	Choline Chloride	Polyethylene glycol	1:2	0.1
PEG-U(CA)	Polyethylene glycol	Urea	2:1	0.1

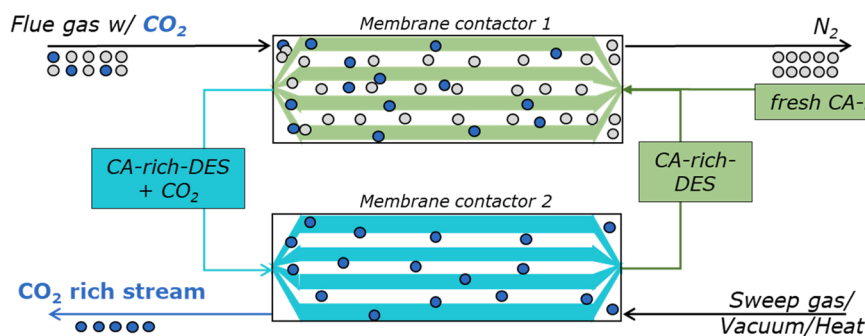


Fig. 1. Gas-liquid membrane contactor process scheme for carbon dioxide (CO₂) separation and purification, using Deep Eutectic Systems (DES) enriched with carbonic anhydrase (CA) extracted from microalgae biomass. A flue gas stream containing CO₂ will enter Membrane contactor 1 outside the fibers. Inside the fibers Membrane contactor 1 is a stream of DES rich in CA. This solvent will capture CO₂ molecules from the flue gas stream resulting in two separate streams, a gas stream rich in N₂, and a liquid stream of DES rich in CA containing the CO₂. This liquid stream will be directed to Membrane contactor 2, where with the help of a sweep gas or vacuum, the CO₂ will be removed from the DES rich in CA. After, DES rich in CA stream can

then be redirected and reused in Membrane contactor 1.

a sample reacts with iodine, and titration ends when all water has reacted. The amount of iodine was determined by measuring the current which is needed to electrochemically generate iodine. The results are an average of at least three independent measurements.

- Density & viscosity: measured in a SVM 3001 viscometer (Anton Paar), from 20° to 80°C, in intervals of 10 °C.

2.3. DES-microalgae extracts

Fresh microalgal biomass was centrifuged (4000 g, 3 min for *T. lutea* and *C. vulgaris*, 15 min for *Spirulina sp.*) and the pellet was resuspended with DES. Due to the presence of cell wall, *C. vulgaris* and *Spirulina sp.* were subjected to cell disruption by bead-beating (4000 rpm, 3 × 60 s, 120 s pause), prior to extraction. Extractions were performed in closed cap vials, for 4 h at room temperature, and extracts were stirred throughout the extraction. A control extraction was performed with 25 mM phosphate buffer at pH 8. Two biomass concentrations were tested, 5 and 10 mg per g DES.

2.4. Analytical measurements

2.4.1. Dialysis

Extracts for Total protein content analysis and CA enzymatic activity were first dialyzed in Spectra-Por® Float-A-Lyzer® G2 membranes, with a molecular weight cut-off (MWCO) of 3.5–5 kDa (Merck). Before use, the membranes were rinsed twice with demineralized water, and once with 25 mM phosphate buffer (pH 8). Then, the extracts – DES-microalgae and DES-pure CA – were loaded (5 mL) and dialyzed for 23 h, using 25 mM phosphate buffer as dialysate. The buffer was refreshed twice. The dialysate conductivity was measured regularly to monitor the transfer of DES to the dialysate. After dialysis, the volume difference was noted (dilution factor).

2.4.2. Total protein content

Total protein content was measured by Lowry colorimetric assay (DC Protein Assay, Bio Rad). Briefly, 10 µL of sample – phosphate buffer (blank) or dialyzed microalgal extracts (undiluted, 2x, and 5x diluted with phosphate buffer) – was mixed with 25 µL of DC Protein Assay Reagent A and 200 µL of Reagent B in a 96-well plate. The plate was incubated for 15 min at room temperature in the dark, and the absorbance was read at 750 nm (Infinite 200 PRO Microplate reader, Tecan). A Bovine Serum Albumin (BSA) standard curve was prepared from a 2 mg/mL BSA stock solution.

2.4.3. Carbonic anhydrase enzymatic activity

CA enzymatic activity was measured using an Activity Assay Kit (PromoKine), based on the esterase activity of CA on an ester substrate, which releases nitrophenol. The specific CA activity is calculated in mU. mL⁻¹, in which 1 U is the amount of CA that converts 1 µmol of substrate per minute.

Briefly, 10 µL of dialyzed extract (DES-algae or CA-DES, undiluted) and 85 µL of Assay Buffer were mixed into a 96-well plate. To assess background activity from the solvents, dialyzed DES and pure phosphate buffer were also loaded in the well plate. A negative control was prepared with 10 µL dialyzed extract, 83 µL Assay Buffer, and 2 µL CA inhibitor (20 mM acetazolamide). A nitrophenol (NP) standard curve was prepared, from 8 to 40 nmol. After 15 min of incubation, 5 µL of ester substrate was added to all wells, except the ones with NP standard. Absorbance was measured at 405 nm every 5 min, within a kinetic cycle of 1 h, at room temperature, in the microplate reader (Infinite 200 PRO Microplate reader, Tecan). The enzymatic activity ($\Delta A/\Delta t$) was calculated from the linear range of the sample plots. From the NP Standard Curve, the slope was obtained ($\Delta A/\text{nmol}$) and used to calculate the released NP per sample (B). The specific CA activity (mU. mL⁻¹) was calculated according to the following, where D is the dilution factor, Δt the reaction time (min), and V the sample volume (µL) (Eq. 1):

$$\text{Specific CA activity} = \frac{B \times D \times 1000}{\Delta t \times V} \quad (1)$$

2.4.4. CO₂ solubility

The solubility of CO₂ in the different DES was measured as previously described by Craveiro et al. [3] (Fig. 2). First, the gas compartment (GC) was purged with CO₂. Then, each DES was placed in the absorption compartment (AC), ensuring that valve 3 was closed. The GC was pressurized and equilibrated at 0.7 bar, and valves 1 and 2 were closed. Finally, valve 3 was opened, allowing the CO₂ expansion into AC chamber, and the pressure decay was followed and registered with a pressure transducer (Druck PCDR 910 model 99166, UK). All the experiments were conducted at constant temperature of 30 °C in a water bath. In the case of DES with enzyme and microalgae, the experiments were conducted in the same manner.

The CO₂ solubility for DES and DES-microalgae systems was then calculated as the solubility under saturation conditions. The presented solubility values were calculated considering that no DES expansion occurs during the experiments. The data collected from the experiments was plotted as pressure vs. time, and from there we can clearly see the pressure decay and the pressure plateau, where no variations of pressure occur anymore. This indicates that the adsorbed CO₂ and the CO₂ present in AC compartment (Fig. 2), are in equilibrium, and we have attained CO₂ saturation of DES. By knowing the pressure variation from the start of adsorption and the value at the plateau, one can easily determine the amount of CO₂ adsorbed by the amount of DES present, being the solubility expressed in terms of $g_{\text{CO}_2} \cdot g_{\text{DES}}^{-1}$.

3. Results and discussion

3.1. Properties of the DES

The physico-chemical properties of the DES affect its extraction capacity and the biological characteristics of the extract, namely protein content and enzymatic activity. Table 2 summarizes some of the measured properties of the DES used in this work.

One of the most important parameters when dealing with DES is their water content. This value should not exceed 50 wt%, since it is hypothesized that above this value there is a disruption of the hydrogen-bond network between DES-components that maintain their supramolecular structure [9,10]. Higher water content (above 50 wt%) leads to an aqueous solution in which DES-components are dissolved and have

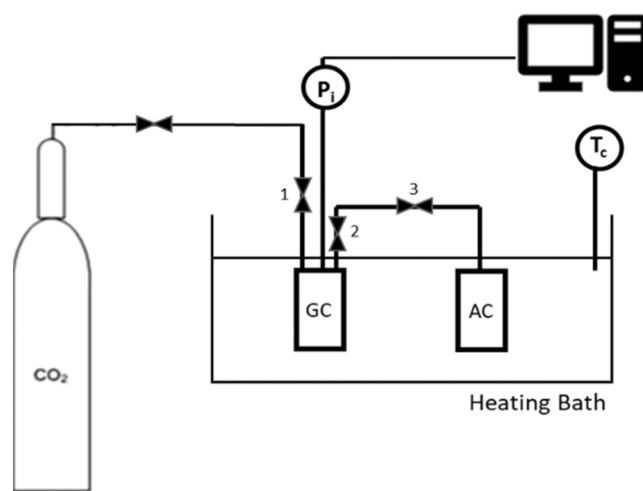


Fig. 2. Experimental set-up for CO₂ absorption experiments. GC refers to gas compartment, AC to the absorption compartment, Pi to pressure indicator and Tc to temperature controller. (adapted from [3]).

Table 2

Deep Eutectic Systems' properties: molecular weight, conductivity ($\mu\text{S}\cdot\text{cm}^{-1}$), water content (wt%), density ($\text{g}\cdot\text{cm}^{-3}$), and viscosity ($\text{mPa}\cdot\text{s}^{-1}$), at 30 °C.

Sample name	Molecular Weight	Conductivity ($\mu\text{S}\cdot\text{cm}^{-1}$)	Water content (wt %)	Density ($\text{g}\cdot\text{cm}^{-3}$)	Viscosity ($\text{mPa}\cdot\text{s}^{-1}$)
ChCl-U	86.58	500	0.264	1.19	544.33
ChCl-PEG	313.21	22	0.354	1.12	74.25
PEG-U	286.58	3	0.188	1.13	137.81

no strong bonding with each other, rather water-water and water-DES-components bonds become dominant [11]. The highest water content is observed in ChCl-PEG, where a few drops of demineralized water were added during its preparation to improve the solubility of the two compounds. The lowest water content was observed in the two DES containing urea, where the hydrogen-bond network is expected to be the strongest [11].

Viscosity and density were measured from 20° to 80°C (Fig. 3). With the increase of temperature, a decrease in viscosity and density is noticeable for all three DES [12]. The highest density and viscosity were measured in ChCl-U. ChCl-based DES are highly hydrophilic, which influences viscosity and density, phenomenon also reported in natural DES containing ChCl [13]. As observed previously [3], the addition of 0.1 $\text{mg}\cdot\text{g}_{\text{DES}}^{-1}$ of CA to ChCl based DES did not influence the viscosity and density of the solvent. Also, the addition of 0.05 and 0.1 $\text{mg}\cdot\text{g}_{\text{DES}}^{-1}$ of CA to the DES in this study did not influence the behavior of viscosity and density.

3.2. Total protein content of the DES-microalgae extracts

Total protein content was measured in dialyzed extracts of DES-microalgae to evaluate the protein extraction capacity of the different DES and with different microalgae, as well as two biomass concentrations: 5 and 10 $\text{mg}\cdot\text{g}_{\text{DES}}^{-1}$ (Fig. 3).

The same protocol was applied to a sample of phosphate buffer with *T. lutea* and *C. vulgaris* biomass, to assess its effect on protein content. Using this methodology, a total protein content of 27.5% ($\pm 1.0\%$) and 28.1% ($\pm 0.7\%$) $\text{g}\cdot\text{g}^{-1}$ biomass was found, for *T. lutea* and *C. vulgaris*. For all samples, standard deviations were lower than 10% between technical replicates, indicating the robustness of the method, therefore it was not repeated for *Spirulina sp.* biomass. These results support the assumption that proteins can be extracted and their functionality can be kept after a dialysis step, where the DES was replaced by phosphate buffer, both protein-“friendly” environments.

When comparing the protein content of DES-*T. lutea* extracts with the total protein content of this microalga, which is around 37% [14], the

maximum extraction yield was 81% with PEG-U. However, extracts with ChCl-U showed a low protein content (2%). This low value can be explained by this DES higher viscosity and/or low water content. ChCl-U has approximately 20-fold higher viscosity than the other DES in this study, and it is known that viscosity hinders mass transfer and the dissolution of molecules, among which proteins [15,16]. Also, proteins in more hydrated ChCl-based DES, with water contents of 25–50 wt%, were shown to have similar structures as when dissolved in an aqueous buffer [17]. This could mean that the hydration of ChCl-U was too low for protein extraction of *T. lutea*. The other two DES (ChCl-PEG and PEG-U) extracted more proteins (22% and 27%), and the protein extraction improved when doubling the biomass (27% and 30%). In contrast with ChCl-U, these DES have lower viscosities which are closer to the viscosity of aqueous buffer [18]. Overall, *T. lutea* extracts with PEG-U, using 10 mg biomass per g DES, resulted in the highest protein content.

For *C. vulgaris*, extracts using 10 mg of biomass revealed a protein content between 11% (PEG-U) to 15% (ChCl-U and ChCl-PEG). When comparing the results obtained using half of the biomass quantity (5 mg), the increase in protein content was not as high as desired. This indicates that the extracts with 10 mg of biomass are most likely saturated, limiting the extraction capacity. A possible saturation of the extracts does not mean that all the proteins were extracted, as the total protein content of *C. vulgaris* can reach up to 50% [19]. This only indicates that the saturation point of the solvent was reached, either by proteins or other microalgal molecules. In conclusion, for *C. vulgaris* biomass, more proteins were extracted by ChCl-U and ChCl-PEG from 10 mg of biomass.

It is reported in literature that *Spirulina sp.* biomass can reach up to 70% of proteins [20], higher than the other species in this study. When using 5 mg of biomass, 12–14% of proteins was extracted. When doubling the biomass concentration, protein content increased 2-fold approximately (between 22% and 24%). Although the extraction yield was lower than the total protein content present in this specie, these results indicate that there's potential for increasing the biomass quantity per extract in further extractions. Overall, ChCl-U and PEG-U extracted more proteins when using *Spirulina sp.*

The results presented in this study indicate that the same DES has different extraction capacity depending on the microalgal specie used. It is possible to notice a trend pointing to the urea-containing DES as the higher protein extraction capacity for the three species of this study. Studies showed that urea causes protein denaturation, because of its interaction with internal hydrogen bonds (chaotropic effect) disrupting the protein structures [21]. However, in the presence of choline chloride and in the form of DES, urea cannot reach the protein's internal structure due to strong hydrogen bonding with choline and chloride [22]. Clearly, the hydrogen bonding of DES components with each other, with

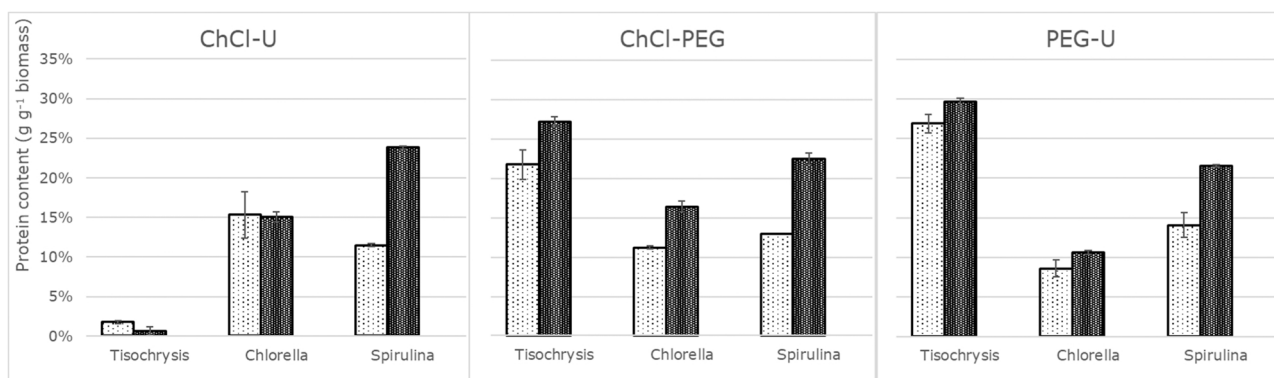


Fig. 3. Total protein content (wt%, $\text{g}\cdot\text{g}^{-1}$ biomass) in dialyzed DES-microalgae extracts using two biomass concentrations: 5 mg (lighter columns) and 10 mg (darker columns). Tisochrysis – Tisochrysis lutea, Chlorella – Chlorella vulgaris, Spirulina – Spirulina sp., ChCl-U: choline chloride – urea, ChCl-PEG: choline chloride – polyethylene glycol, PEG-U: polyethylene glycol – urea.

water, and with proteins, is an important factor to consider in protein extraction from microalgae biomass.

3.3. Carbonic anhydrase

The CA enzymatic activity was determined by two distinct methodologies: an activity Assay Kit performed in pre-dialyzed extracts, and through solubility tests using DES-microalgae extracts.

3.3.1. CA enzymatic activity assay kit

To ensure that only the enzyme's activity was measured, a blank was added, either dialyzed DES or phosphate buffer, without CA standard or biomass. For all samples composed only by solvents – ChCl-U, ChCl-PEG, PEG-U, and phosphate buffer – a negative value of released nitrophenol was recorded, revealing that there's no interference from the solvents.

To understand how the DES affect the activity of the enzyme, pure CA standard was mixed with DES and submitted to the same protocol as microalgal samples, before measuring the specific esterase activity. Results are presented in mU of enzyme per mg standard (Table 3), in which 1 U is the amount of enzyme that catalyses the release of 1 μ mol NP per minute.

All DES-pure CA extracts preserved some of the CA esterase activity, but unfortunately not all activity. The commercial enzyme's activity used in these experiments is around 2000 mU.mg⁻¹, and as it can be observed in Table 3, the activity in the final extracts was lower. This loss of activity could be caused by the dialysis duration (> 27 h) or the extraction protocol, both performed at room temperature (approximately 24 °C). In literature, bovine CA shows an optimal catalytic activity at 35 °C, stable at temperatures up to 50 °C [23]. Contrary to common knowledge about enzymes degrading at a high temperature, Li et al. [24] reported that the CA activity in *C. vulgaris* increased with increasing temperature, 4-fold higher at 30–40 °C than at 20 °C. Due to contradictory information in literature, the temperature effect on CA activity during extraction and dialysis should be further investigated. When comparing the three DES, the lowest CA activity was observed with ChCl-U, where the highest volume increase was observed (dilution factor included in the results). This increase in the volume, due to the hydrophilicity of this DES, could have led to disruption of the DES properties that are known to keep protein activity intact, and therefore preventing CA to keep its initial activity.

Results of the CA activity obtained with the assay kit are presented in Table 4 (with standard deviations of technical replicates). In *T. lutea* and *C. vulgaris* phosphate extracts, CA activity was detected successfully, with standard deviations lower than 10%. This indicates that the assay method is adequate to measure CA extracted from microalgae biomass, and that the phosphate buffer was adequate to keep CA activity.

When comparing the different DES and microalgal species used in this work, the best enzymatic activities were obtained in extracts containing PEG. PEG's compatibility with enzymes can be found in Zhao [25], where this solvent was used for enzyme stabilization. PEG polymer can influence enzyme activity and thermostability, depending on its size and weight. During the interaction between PEG and proteins, a network of hydrogen bonds is formed. This network can cause stabilization or hindrance, which can affect mass transfer [26].

Table 3

Specific Carbonic Anhydrase (CA) activity, and its standard deviation, of dialyzed extracts 0.1 mg pure CA standard per g of Deep Eutectic System. ChCl-U: choline chloride – urea, ChCl-PEG: choline chloride – polyethylene glycol, PEG-U: polyethylene glycol – urea.

DES	Specific CA activity (mU.mg ⁻¹)	Std.dev. (%)
ChCl-U	538.4	< 1
ChCl-PEG	1470.8	29
PEG-U	1306.7	7

Table 4

Specific Carbonic Anhydrase (CA) activity, and its standard deviation, of microalgae dialyzed extracts in Deep Eutectic Systems and phosphate buffer. ChCl-U: choline chloride – urea, ChCl-PEG: choline chloride – polyethylene glycol, PEG-U: polyethylene glycol – urea, Ph. buffer – phosphate buffer, no activity: no activity was detected either in 5 or 10 mg of biomass per g of DES.

		Biomass conc. (mg.g ⁻¹ DES)	Specific CA activity (mU.mg ⁻¹)	Std. dev. (%)
<i>T. lutea</i>	Ph. buffer	5	1.57	7
	ChCl-U	10	0.42	11
	ChCl-PEG	5	0.66	30
	PEG-U	no activity		
<i>C. vulgaris</i>	Ph. buffer	5	1.34	2
	ChCl-U	no activity		
	ChCl-PEG	no activity		
	PEG-U	10	0.70	18
<i>Spirulina sp.</i>	ChCl-U	10	0.17	2
	ChCl-PEG	5	0.36	86
	PEG			
	PEG-U	10	0.18	53

When working with extracts from any biological source, three phenomena can occur: i) the enzyme was not extracted; ii) the enzyme was extracted but the enzymatic activity was affected or lost; or iii) the enzyme was extracted and remained active. For *T. lutea*, CA activity was detected in ChCl-U (when using 10 mg of biomass) and in ChCl-PEG (using only 5 mg of biomass). Although PEG-U-*T. lutea* showed the highest protein content, no CA activity was detected in this extract. This shows that a high protein content does not necessarily mean that CA was extracted, or that its activity is preserved. Considering that ChCl-PEG extracted most of *T. lutea* proteins, and that showed the highest enzymatic activity both for pure CA (Table 3) and in the DES extract, this should be considered the best DES to extract and keep CA activity for this microalga biomass.

For *C. vulgaris*, CA activity was only detected in PEG-U (for 10 mg of biomass). This extract revealed the lowest protein content from the tested DES, indicating that higher protein extraction doesn't directly translates into higher enzymatic activity. Since no CA activity was detected in extracts using lower biomass, but it is detected with the phosphate buffer, we can conclude that these DES have higher affinity for other biomass molecules, decreasing the extraction capacity for the enzyme.

For *Spirulina sp.*, the results show that CA was detected in all three DES, although in lower values than the other DES-microalgae extracts. ChCl-PEG showed the lowest protein content, but the highest CA activity. ChCl-U and PEG-U had similar CA activity, also with higher protein content.

Regarding DES properties (Table 2), PEG-U has the lowest conductivity, water content, density, and viscosity, of the three DES used in this work. On the contrary, most of these values are the highest for ChCl-U. From previous research on the effect of viscosity on enzyme activity in yeast cells, it was reported that viscosity leads to higher protein friction which causes inhibition of enzyme' catalysis rate [16]. These findings are supported by Kramer's rate theory, that describes that the reaction-rate of chemical reactions in solution is highly dependent of the friction [15]. Since ChCl-U has the highest viscosity, this could explain the low enzymatic activity detected in extracts using this DES, due to more difficult mass transfer of substrate and products. However, Craveiro et al. [3], observed that when CA was dissolved in different DES, highest solubilities were achieved in ChCl-U and that the presence of CA enhanced this solubility. This would indicate that pure CA can be active in ChCl-U, which agrees with the results from this work. As reported by

de Castro et al. [27], water activity is an important parameter regarding the enzyme's ability to capture CO₂. In Craveiro et al. work [3], water content of ChCl-U was 2.0 wt%, which is higher than the water content of the DES in this work, explaining the difference in the obtained results.

An aspect that is left out of this discussion is the extracted quantity of CA. As the aim of the performed experiments was to extract and detect the CA activity, results were assessed from a qualitative perspective. However, comparing the activity of a known concentration of CA (0.1 mg per g DES) to the biomass extracts, the enzyme activities were higher for pure CA. Knowing that microalgae biomass consists of several compounds, of which CA only represents a small fraction, this difference in activity is expected. CA enzymatic activity of different microalgae species, of which *C. vulgaris*, was reported to reach up to 17 mU mg⁻¹ biomass [28], however the extraction was performed with Tris-HCL buffer. The use of DES to extract CA keeping its activity is a novel approach, and therefore this work represents pioneer steps in a field with high potential, not only from the point of CO₂ capture and purification, but also enhancing the microalgal biorefinery possibilities.

3.3.2. CO₂ solubility

From the experiments described in 2.4.4 it was possible to obtain solubility values for the three DES, for the DES-microalgae extracts, and for the DES-pure CA (Fig. 4). From these results it is clear that ChCl-PEG showed higher solubility values, while ChCl-U showed the lowest. The solubility of a gas through a liquid is highly dependent of the affinity of the gas to the liquid, which according to the obtained results, is higher between CO₂ and ChCl-PEG. This DES is also the one that presents a higher water content, which can enhance CO₂ solubility in the DES. This is more noticeable in the DES ChCl-PEG that has a viscosity at least one order of magnitude lower, when compared with ChCl-U (Table 2).

Additionally, it is observed that viscosity is inversely proportional to CO₂ solubility, and that viscosity values of ChCl-PEG and PEG-U are in the same range. DES containing PEG showed higher CA activity, which is also observed from the solubility values. *T. lutea* showed the best activity in all three DES, especially in ChCl-PEG, which is in accordance with previous results. The authors hypothesize that this higher CA activity is due to *T. lutea* cell wall absence, facilitating CA extraction and stabilization. The CO₂ solubilizing capacity of ChCl-PEG combined with this microalga is similar to the one obtained with pure CA (0.1 mg/g_{DES}). This result reveals the efficiency of using microalgae whole-biomass instead of pure CA, which can be a big economic advantage for the purpose of CO₂ purification from flue gases.

Previous studies reported solubility values of CO₂ in ChCl-based DES to be in the order of 4.36×10^{-1} , for the case of ChCl:urea, and

increasing to 6.27×10^{-1} when adding 0.1 mg of CA (per g_{DES}) [3]. As previously mentioned, the higher solubility values obtained in ChCl-U are related with the higher amount of water in this DES. In this work, the water content in ChCl-U was 0.2 wt%, against the 2 wt% cited by Craveiro et al. [3], which can account for the differences in CO₂ solubility. It is well known that, when a certain amount of water is present in the medium where the enzyme is dispersed, the enzyme has more conformational liberty and its activity is enhanced [29]. Furthermore, CA catalyzes the reaction between CO₂ and water to form bicarbonate [30], so it is clear that a higher amount of water also resulted in higher CO₂ transformation, and consequent solubility. Also, from Fig. 4, and considering pure DES and pure CA, higher amounts of CA result in higher solubility, and both 0.05 and 0.1 mg_{CA}/g_{DES} resulted in close solubility values. However, smaller amounts of CA do not enhance CO₂ solubility in the case of ChCl-PEG. Therefore, it can be hypothesized that a low amount of enzyme coupled with a low amount of water content might not be ideal for the CO₂ conversion reaction.

4. Conclusions

In this work, first steps were made to produce a solvent for enhanced CO₂ capture and purification to be used in gas-liquid membrane contactor systems, by extracting CA from microalgae biomass with DES. Three different microalgae species – *T. lutea*, *C. vulgaris*, and *Spirulina sp.* – and three different DES – ChCl-U, ChCl-PEG, and PEG-U – were used to uncover the best DES-microalgae combination. The results showed that more proteins were extracted when using *T. lutea* and DES containing PEG (between 20% and 30% g_{protein}/g_{biomass}). In particular, ChCl-PEG revealed higher CA enzymatic activities for both for *T. lutea* and *Spirulina sp.*, and PEG-U for *C. vulgaris*. Additionally, ChCl-PEG provided the best CO₂ solubility for all the three microalgae in this study, with results similar of using 0.1 mg of pure CA enzyme (per g of DES). These results cement the idea of using microalgae biomass as CA enzyme source for Carbon Capture and Utilization (CCU) strategies.

CRedit authorship contribution statement

All authors approved the submitted version and agreed with their contributions to the work. Rita Craveiro – data acquisition and interpretation, draft of the manuscript Fleur Dusschooten – data acquisition and analysis, revision of the manuscript. Rita Nabais – data acquisition. Iulian Boboescu – data interpretation, revision of the manuscript. Calvin Lo – conception and design of the work, revision of the manuscript. Luisa Neves – conception and design of the work, data interpretation, draft

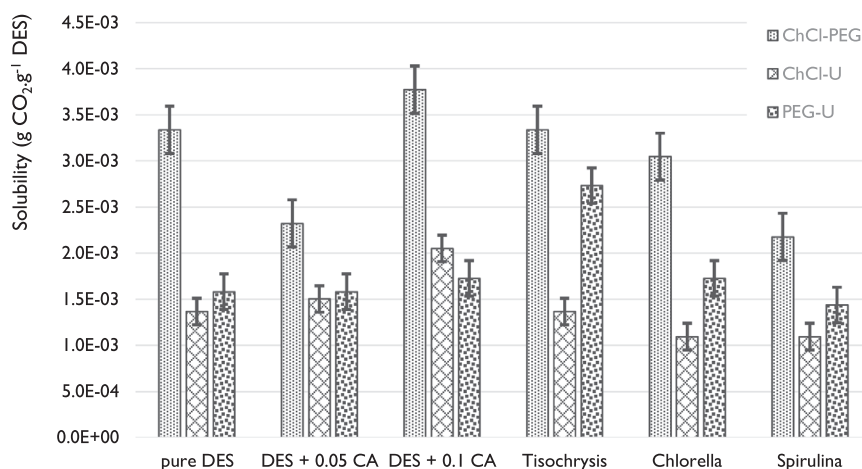


Fig. 4. Solubility values (g CO₂/g DES) for the three Deep Eutectic Systems (DES): pure DES, 0.05 and 0.1 mg of pure Carbonic Anhydrase enzyme dissolved per g of DES, and DES-microalgae extracts. ChCl-U: choline chloride – urea, ChCl-PEG: choline chloride – polyethylene glycol, PEG-U: polyethylene glycol – urea. Tisochrysis – Tisochrysis lutea, Chlorella – Chlorella vulgaris, Spirulina – Spirulina sp. The values were determined as solubility under saturation conditions.

and revision of the manuscript. Marta Sá – conception and design of the work, data interpretation, draft and revision of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The datasets generated and analyzed during the current study will be available in the eLab Journal repository, after publication.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jcou.2022.102225](https://doi.org/10.1016/j.jcou.2022.102225).

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