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Designing biodegradable aqueous biphasic systems for the selective separation of enzymes

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ABSTRACT

In this work, the salting-out strength of reline eutectic mixture in aqueous solution of the non-ionic surfactant Triton X-102 has been demonstrated at temperatures between 298.15 and 323.15 K as a preliminary step to be employed in downstream operations of a biotechnological process. The phase diagrams data of reline-based Aqueous Two Phase Systems (ATPS) have been correlated using empirical models based on exponential and polynomial equations. The tie-lines have been empirically ascertained through physical properties characterization and Othmer-Tobias equation was proposed to correlate those data. After having studied the effect of the proposed reline eutectic mixture on two model enzymes (protease and lipase), the performance of this ATPS for selectively separating them was evaluated for three different feed compositions on the same tie-line, demonstrating their pertinence to segregate the lipase to one of the phases (yield about 95 %) and the protease to the other phase (yield about 98 %). These promising results are interesting for application in biotechnological reactions where proteases have been proved to be deleterious for lipases activity, as the biocompatibility of the proposed platform does not involve drastic negative effects on any of the enzymes.

1. Introduction

Ever since the adoption of the 2030 Agenda for Sustainable Development Goals (SDGs) by all United Nations Member States in 2015, there has been a notable increase in attempts to advocate for more sustainable production processes. The concept of sustainable development emphasizes the need for innovation to build a more equitable, resilient, and sustainable world for present and future generations. Within this framework, sustainable development and biotechnology are intricately intertwined, since biotechnology plays a pivotal role in advancing SDGs by providing innovative solutions across various sectors like agriculture [1], medical field [2] or renewable energy [3]. Furthermore, in industrial settings, biotechnology contributes by producing biochemicals, enzymes, and fermentation products [4].

Recent advancements in industrial biotechnology have revolutionized the production of chemical products, offering cost-effective alternatives to traditional petrochemicals and enabling the creation of unique chemicals for commercial use [5]. Particularly, the commercial utilization of enzymes, such as lipases and proteases, has surged globally across various industrial sectors due to their exceptional catalytic activity, selectivity, and substrate specificity [6]. Lipases (triacylglycerol ester hydrolases EC 3.1.1.3) belong to the group of serine hydrolases and stand out for being able to exert their biocatalytic effect in the absence of cofactors, whereas proteases (EC 3.4.21) are involved in the hydrolysis of peptide bonds for protein catabolism and the generation of amino acids. Both are involved in a plethora of bio and chemical reactions like hydrolysis, transesterification or waste management [7,8].

With recent advancements in upstream processing, the bottleneck in enzyme production now lies in downstream processing, encompassing extraction, purification, and preconcentration steps. These processes are integral to biological product development and significantly influence the final product cost. Therefore, there is a pressing need to devise straightforward and efficient methods for biomolecule recovery, ensuring flexibility for continuous operation and commercial viability. Among the options, Aqueous Two Phase Systems (ATPS) have been highlighted, as they are constituted by water soluble components, which become immiscible by the action of a salting out agent (such as salts, polymers or electrolytes). Owing to their water-rich environment, ATPS processes fulfil the requisites regarding green chemistry principles and gain potential for the research of industrial applications [9]. ATPS offers several advantages, including simple equipment requirements, mild operating conditions, low energy consumption, and typically high

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separation yields [10]. Consequently, this alternative liquid–liquid equilibrium methodology has become increasingly significant in biomolecule extraction, including vitamins [11,12], proteins [13–15], and hormones [16,17], as well as in environmental remediation [18] and purification processes [19,20]. However, large-scale application of ATPS often faces challenges such as high viscosity, toxicity linked to certain phase-forming agents, or instability of biomolecules [21]. Therefore, it's essential to develop biobased ATPS that not only guarantee compatibility with biomolecules but also adhere to current environmental regulations.

Since the beginning of the 21st century, the so-called green solvents like Ionic liquids (ILs) and Deep Eutectic Solvents (DESs) have emerged as effective substitutes for traditional organic solvents in liquid–liquid extraction and have attracted significant interest in ATPS formation [22,23]. DESs, in particular, have garnered attention due to their unique properties compared to ILs, including excellent biodegradability, low toxicity, high biocompatibility, and cost-effective synthesis without the need for further purification. DESs are defined as eutectic mixtures formed by the interaction of two or more components, typically a hydrogen bond donor (HBDs) like metal chloride, alcohols, carboxylic acids, among others and a hydrogen bond acceptor (HBAs) mainly quaternary ammonium salts and metal chloride hydrates under a certain molar ratio, resulting in a liquid state with unusual solvent properties [24]. Moreover, they have demonstrated exceptional performance across a diverse array of processes, including catalysis [25], electrochemistry [26], environmental applications [27], as cryoprotective agents [28,29] or as separation agents [30,31].

In view of the above, the main goal of this work was to assess the performance of the ATPS reline eutectic mixture (composed of choline chloride and urea (molar ratio 1:2), respectively) in aqueous solutions of non-ionic surfactant polyoxyethylene octylphenol (Triton X-102) to outline a biomolecules recovery process, since the pH of both phase forming compounds falls into the optimum pH range of the enzymes under study (7.8 for DES and 7.5 for surfactant). Firstly, the experimental solubility curves were analysed at various temperatures under atmospheric pressure, and the equilibrium binodal and tie-line data were fitted to empirical equations to comprehensively delineate the immiscibility region. Then, the possible deactivating effect of the selected DES (pure and in aqueous solution) was analysed on two model biomolecules (among the top-three most important enzymes in terms of sales), *Candida antarctica* lipase B and *Bacillus licheniformis* protease, prior to employ ATPS as a means to selectively separate them from aqueous solutions.

2. Experimental section

2.1. Materials

Urea (CH_4N_2O, CAS 57-13-6, purity 99 wt%) was supplied by Panreac. Choline chloride ($C_5H_{14}ClNO$, CAS 67-48-1, purity 98 wt%) was purchased from Merck and subjected to vacuum drying ($2\cdot10^{-1}$ Pa) at room temperature for 2 days to remove residual solvents and moisture. Ethanol (C_2H_6O, CAS 64-17-5, purity 99 wt%), *p*-nitrophenyl laurate ($C_{18}H_{27}NO_4$, CAS 1956-11-2, purity 98 wt%), Trizma base ($C_4H_{11}NO_3$, CAS 77-86-1, purity 99 wt%), calcium chloride ($CaCl_2$, CAS 10043-52-4, purity 99 wt%), sodium carbonate (Na_2CO_3, CAS 497-19-8, purity 99 wt %), azocasein protease substrate (CAS 102110-74-7), Triton X-102 ($C_{14}H_{22}O(C_2H_4O)_{12}$, CAS 9036-19-5, purity 98 wt%), alkaline protease from *Bacillus licheniformis* (optimum pH 7.5) (CAS 9014-01-1, 7–14 U/mg) were all acquired from Merck. *Candida antarctica* lipase B (optimum pH between 7 and 9) was generously provided by Novozymes. All solutions were consistently prepared using double-distilled deionized water.

2.2. Preparation of reline eutectic mixture

Reline eutectic mixture was synthesized by the heating method. In brief, choline chloride as HBA was mixed with urea as HBD in a molar ratio of 1:2, respectively, and stored in a round-bottom flask. Later, the flask was immersed in an oil bath set to a constant temperature (≈373.15 K) and the contents were stirred until the mixture reached a stable state, characterized by the appearance of a colourless liquid. A water mass fraction of $2.5\cdot10^{-3}$ was determined by Karl-Fisher titration after vacuum drying ($2\cdot10^{-1}$ Pa) and this value was considered for the experiments. The eutectic mixture was stored at 298.15 K and the solution remained stable over time.

2.3. Determination of phase diagrams.

The binodal curves were carried out using a jacketed glass vessel equipped with a magnetic stirrer at temperatures ranging from 298.15 to 323.15 K, monitored by a F200 ASL digital thermometer with an uncertainty of ±0.01 K. The mass composition of all mixtures was determined gravimetrically using a Sartorius Cubis MSA 125P-100-DA analytical balance, (uncertainty $\pm10^{-5}$ g). Solubility data were obtained using the cloud point titration method, a widely recognized technique described previously [32].

After mapping the biphasic area, the tie-lines (TLs) were established by introducing a measured quantity of Triton X-102, reline DES and water into separate glass ampoules within the immiscibility region. The mixture was vigorously stirred for 1 h and then allowed to settle for 24 h to ensure phase separation at the specified temperature. Every layer was carefully extracted using a syringe and subjected to density and refractive index measurements. For this purpose, both an Anton Paar DSA 5000 M digital vibrating tube densimeter (uncertainty of $\pm2\cdot10^{-5}$ $g\cdot cm^{-3}$) and a Dr. Kernchen ABBEMAT WR refractometer (uncertainty of $\pm4\cdot10^{-5}$) were utilized, after calibration with Milli-Q water and tetrachloroethylene as per the manufacturer's recommendations.

2.4. Enzyme partition

Reline eutectic solvent was added to a mixture containing an aqueous solution of the enzyme and surfactant up to a total volume of 3 mL at a given concentration. The mixture was vigorously stirred and then left to settle by centrifugation at 1,560 g for 15 min at 298.15 K. The partitioning of enzymes was assessed for various feed compositions, expressed as percentages, comprising (65, 20), (44, 44), and (75, 9) (reline DES, Triton X 102), respectively. Samples from the surfactant- and DES-rich phases were taken with a syringe and the lipolytic activity was determined by UV spectrophotometric assay.

2.5. Enzymatic activity quantification

Protease and lipolytic activity were determined through UV spectrophotometry (Unicam Helios β, Thermo Electron Corp spectrophotometer). For lipolytic activity, the standard assay based on the hydrolysis of *p*-nitrophenyl laurate was utilised and the procedure previously outlined in our research work [33]. As brief guidelines, 100 μL of each sample were added to a mixture of 100 μL of *p*-nitrophenyl laurate (2.5 mM in ethanol) and 800 μL of Tris HCl buffer 50 mM (pH 8) containing $CaCl_2$ 20 mM and incubated at 313.15 K for 5 min. The enzymatic reaction was stopped after 20 min by adding a $CaCO_3$ solution (1 M) in a water ice bath. Subsequently, the sample was centrifuged at 17,360 g for 10 min at 277.15 K and the supernatant was measured at 400 nm. One activity unit (U/L) was defined as the amount of enzyme biocatalyzing the release of 1 μmol of *p*-nitrophenol per minute under standard assay conditions.

Protease activity was determined following the protocol proposed by Ginther [34] using azocasein as substrate. In brief, 120 μL of the sample to be analyzed were added to Eppendorf tubes containing 480 μL of 1 %

Table 1
Solubility data for {Triton X-102 (1) + reline (2) + H_2O (3)} from T = (298.15–323.15) K and 0.1MPa[a].

T = 298.15 K		T = 303.15 K		T = 313.15 K		T = 323.15 K	
100 m_2	100 m_1	100 m_2	100 m_1	100 m_2	100 m_1	100 m_2	100 m_1
1.92	87.44	0.96	83.17	0.91	79.10	0.50	76.82
8.20	78.96	7.51	75.04	8.34	71.17	7.76	67.94
17.72	66.97	16.48	64.63	15.63	62.57	14.66	57.56
25.16	57.17	24.80	55.14	21.93	53.34	21.15	49.60
33.65	46.64	30.49	47.51	29.84	41.53	27.46	39.48
39.37	39.40	37.56	37.27	36.45	33.75	31.93	31.26
46.15	30.47	44.69	27.14	40.95	26.52	36.02	25.95
53.07	22.38	49.55	20.31	44.50	19.05	40.41	18.17
58.63	13.91	53.85	14.72	50.53	12.85	46.26	11.88
64.32	6.84	60.82	7.03	55.61	7.07	50.12	5.45
68.62	1.55	63.49	3.41	60.23	2.10	52.20	2.19
2.08	94.58	1.09	94.51	1.08	94.54	0.62	96.03
9.04	87.00	8.71	87.02	10.04	85.66	9.75	85.34
20.05	75.77	19.58	76.79	19.02	76.15	19.09	74.96
29.01	65.90	29.33	65.20	27.54	67.00	27.73	65.02
39.21	54.35	36.96	57.60	39.65	55.20	38.15	54.84
46.78	46.82	47.26	46.89	48.53	44.94	46.27	45.30
56.35	37.20	58.84	35.74	56.71	36.73	53.23	38.36
65.45	27.60	67.07	27.49	66.22	28.35	63.18	28.41
75.13	17.82	73.33	20.05	73.81	18.77	71.76	18.43
83.95	8.92	83.33	9.63	81.53	10.37	78.38	8.53
89.24	3.25	82.32	4.42	81.42	2.84	82.37	3.46
83.71	0.25	82.57	0.20	77.81	0.23	80.39	0.22
79.80	0.25	77.08	0.22	75.90	0.25	74.00	0.22
76.97	0.24	69.73	0.48	72.64	0.20	68.91	0.21
72.89	0.23	67.82	0.23	67.82	0.23	62.93	0.16
				64.01	0.21	57.01	0.24

[a] Standard uncertainties are ur(w) = ± 0.02, u(T) = ± 0.01 K; u(P) = ± 2 kPa.

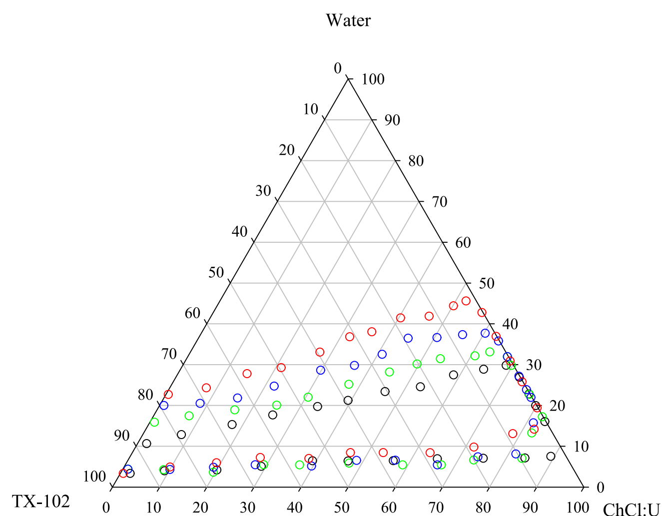

Fig. 1. Solubility data for Triton X-102 (1) + reline (2) + H_2O (3) at 298.15 K (◯), 303.15 K (◯), 313.15 K (◯), 323.15 K (◯) and 0.1 MPa.

azocasein in an appropriate pH buffer with 2 mM $CaCl_2$. The tubes were left for one hour at 313.15 K and 600 μL of 10 % trichloroacetic acid (TCA) were added and the tubes were centrifuged at 1,560 g for 5 min. Then 800 μL of the supernatant were mixed with 200 μL of 1.8 N NaOH, and the absorbance was read at 420 nm. Blanks were prepared by adding the sample after TCA. Enzymatic activity is defined as the amount of protease that produces an increase of 0.1 absorbance units in one hour of reaction per mL of sample.

In all cases, enzymatic activity was assessed in triplicate, and the reported results represent the mean values with the standard deviation derived from the three measurements. Moreover, the absence of interference from both reline and Triton X-102 was confirmed by including controls without enzyme but containing the same concentration present in the corresponding phase (upper and lower).

Table 2
Correlation parameters and standard deviation for the system {Triton X-102 (1) + reline (2) + H_2O (3)}[a].

T/K	α	β	γ	δ	ε	σ
Eq. (1)	Triton X-102 (1) + reline (2) + H_2O (3)					
298.15	0.9807	−0.8016	6.8589			0.0151
303.15	0.8887	−0.6552	8.9451			0.0129
313.15	0.8487	−0.6666	11.661			0.0123
323.15	0.8121	−0.7085	16.486			0.0125
Eq. (2)	Triton X-102 (1) + reline (2) + H_2O (3)					
298.15	0.7995	0.8472	−2.8923	1.1670		0.0190
303.15	0.7378	1.1042	−3.4248	1.5457		0.0242
313.15	0.6942	1.2497	−3.8675	1.9780		0.0198
323.15	0.6956	1.1365	−4.0105	2.3398		0.0209
Eq. (3)	Triton X-100 (1) + reline (2) + H_2O (3)					
298.15	0.1570	−2.6803	4.8382	−8.6230		0.0210
303.15	−0.0033	−2.2269	4.6510	−9.8928		0.0189
313.15	−0.0745	−2.0435	4.4810	−11.205		0.0170
323.15	−0.1372	−2.0764	4.9202	−14.076		0.0163
Eq. (4)	Triton X-102 (1) + reline (2) + H_2O (3)					
298.15	0.8878	−2.4554	24.435	1.2422	6.9879	0.0052
303.15	0.8358	−2.2562	22.156	1.2055	5.5570	0.0059
313.15	0.7938	−3.1712	32.517	1.3781	5.7661	0.0066
323.15	0.7696	−3.4555	67.722	1.3080	5.7936	0.0078

[a] Standard deviation (σ) was calculated by means of Eq. (5).

3. Results and discussion

3.1. Aqueous biphasic systems characterization and modelling

Before implementing a strategy for biomolecule recovery following a biotechnological process, it is essential to assess the effectiveness of the deep eutectic solvent reline in inducing phase separation. This evaluation involves examining its ability to promote phase disengagement in model aqueous solutions of Triton X-102 which serves as a representative example of a non-ionic surfactant and was previously explored by our group in combination with different salting-out agents like salts, ionic liquids or natural deep eutectic solvents [10,33,35–38]. Thus, the binodal curves were characterised for the systems {Triton X-102 + reline + H_2O} at T = (298.15, 303.15, 313.15. 323.15) K and the experimental solubility data are compiled in mass fraction in Table 1, and they are plotted in Fig. 1. The experimental data of the mass fraction of system components were successfully fitted by using empirical nonlinear expressions, widely used in ATPS studies [39,40].

$$[Y] = \alpha \cdot \exp(\beta X^{0.5} - \gamma X^3) \quad (1)$$

$$[Y] = \alpha + \beta X^{0.5} + \gamma X + \delta X^2 \quad (2)$$

$$[Y] = \exp(\alpha + \beta X^{0.5} + \gamma X + \delta X^2) \quad (3)$$

$$[Y] = \alpha \cdot \exp(\beta X^{\delta} + \gamma X^{\varepsilon}) \quad (4)$$

where Y and X are, respectively, the non-ionic surfactant Triton X-102 and reline eutectic mixture weight mass fraction of binodal curves. Therefore, α, β, γ, δ and ε represent the fitting parameters, which were obtained after minimizing the standard deviation (σ):

$$\sigma = \left(\frac{\sum_i^{nDAT} \left(z_{\exp} - z_{adjust} \right)^2}{n_{DAT}} \right)^{1/2} \quad (5)$$

Table 3
Experimental tie–lines in mass percentage for the system Triton X-102 (1) + reline (2) + H_2O (3) from 298.15 K to 323.15 K and 0.1 MPa[a].

Triton X-102-rich phase		reline-rich phase		Feed composition		*TLL*	*S*
$100w_1^I$	$100w_2^I$	$100w_1^{II}$	$100w_2^{II}$	$100w_1$	$100w_2$		
298.15 K							
92.04	1.31	0.26	86.26	27.69	60.02	125.06	−1.0804
87.96	1.5	0.2	77.26	24.99	54.75	115.94	−1.1584
82.87	5.27	0.47	74.37	26.02	58.37	107.54	−1.1925
303.15 K							
93.08	1.43	0.19	83.86	15.06	71.54	124.19	−1.1269
88.41	0.9	0.2	76.93	14.98	64.64	116.45	−1.1602
84.41	0.9	0.22	69.63	15	57.71	108.69	−1.2249
313.15 K							
93.34	0.93	0.2	87.16	15.03	72.5	126.93	−1.0801
90.28	0.57	0.21	78.62	14.93	66.69	119.18	−1.1539
87.73	0.72	0.19	71.94	15	60.66	112.36	−1.2203
84.73	0.7	0.21	64.69	15.01	54.7	106.01	−1.3209
323.15 K							
94.56	0.86	0.2	80.59	15.05	69.55	123.54	−1.1833
92.77	0.54	0.18	76.96	15.06	64.65	120.05	−1.2116
90.25	0.54	0.22	71.46	15.01	59.7	114.61	−1.2694
87.39	0.45	0.2	63.1	15.06	53.68	107.39	−1.3916
82.37	0.71	0.19	57.03	15.05	47.7	99.62	−1.4593

[a] Standard uncertainties are u_r (w) = ±0.02.

where z_{exp} and z_{adjust} depict the experimental and the theoretical values, respectively, and n_{DAT} matches the number of data. SOLVER tool in Microsoft Excel was employed to optimize the coefficients, which are presented in Table 2. A visual inspection of the data indicates that the proposed equations serve our goal to describe the segregation behaviour of Triton X-102 in the presence of the reline eutectic mixture. More specifically, the analysis in terms of standard deviations reveals that a five-parameter equation (number 4) is the one that better correlates the experimental data. The same behaviour was observed by the authors in previous works where non-ionic surfactants belonging to the polyoxyethylene t-octylphenol family were involved [10].

The thorough analysis conducted on the immiscibility regions brings to light the suitability of reline DES as a salting-out agent within aqueous solutions of Triton X-102. Notably, although different salting out agents have been employed in the past to salt-out Triton-based surfactants [35–38], the use of reline does not entail any problem with phase formation, and the size of the biphasic area is even greater than that reported for conventional ionic liquids [35]. This observation holds considerable interest as it suggests the potential for enhanced applicability of these systems in the context of the ongoing research. Specifically, the expanded biphasic region size offers promise for easing the implementation of these systems, a critical consideration given the importance of precise concentration selection to mitigate the risk of enzyme activity loss. Such findings underscore the potential utility of reline DES and warrant further exploration to fully harness its salting-out capabilities in various aqueous environments. In this sense, the effect of including an aqueous culture medium composed of casein peptone (8 g/L), yeast extract (4 g/L) and sodium chloride (3 g/L) on the phase equilibrium was evaluated by replacing the water vertex by this solution, and no appreciable changes were detected. Analogously, the absence of changes in binodal curves was checked after replacing water by a centrifuged culture broth (after microbial cellular growth), which confirms the suitability and stability of the proposed systems for the selective separation of enzymes.

Concerning the influence of temperature, the binodal curves were studied in the range from 298.15 K to 323.15 K, as it corresponds to the optimum for most commercial enzymes. The data presented in Fig. 1 reveal a clear correlation between higher temperatures and larger biphasic areas. This phenomenon is likely attributed to the attenuation of hydrogen bonds between the surfactant and water molecules at elevated temperatures. Lindman et al. [41] proposed that at higher temperatures, conformers with reduced dipole moments predominate, rendering the ethoxylated chains of the surfactant more apolar. Consequently, this promotes interactions between surfactant molecules while impeding interactions between surfactant and water molecules. Analogous temperature-dependent trends have been documented for other choline-based DES, which act as segregation agents in aqueous polymer solutions [42]. These findings highlight the importance of temperature control in modulating the phase behaviour of surfactant-water systems, offering valuable insights into the underlying molecular interactions that govern phase separation phenomena. Such insights contribute to the broader understanding of surfactant behaviour in aqueous environments and hold implications for various industrial and research applications.

3.2. Tie-lines determination

Having established the efficacy of the proposed DES as a phase promoter in non-ionic surfactants, the next step involved experimental determination of tie-lines (*TLs*) across the same temperature range to comprehensively characterize the systems. Additionally, tie-line length (*TLL*) and the slope of the tie-lines (S) were calculated using Eqs. (6) and (7) respectively.

$$TLL = \left[\left(w_1^{I} - w_1^{II}\right)^2 + \left(w_2^{I} - w_2^{II}\right)^2\right]^{0.5} \quad (6)$$

$$S = \frac{w_1^{I} - w_1^{II}}{w_2^{I} - w_2^{II}} \quad (7)$$

being w_1 and w_2 the mass fraction of non-ionic surfactant and DES, respectively. The superscripts I and II denote the surfactant- and DES-rich phase, respectively. These calculated values, along with the tie-lines, are tabulated in Table 3 and graphically represented in Fig. 2. This meticulous characterization provides valuable insights into the phase behaviour of the systems under study, offering a quantitative understanding of the distribution of phases and the interfacial tensions governing their equilibrium. Such detailed analysis is crucial for

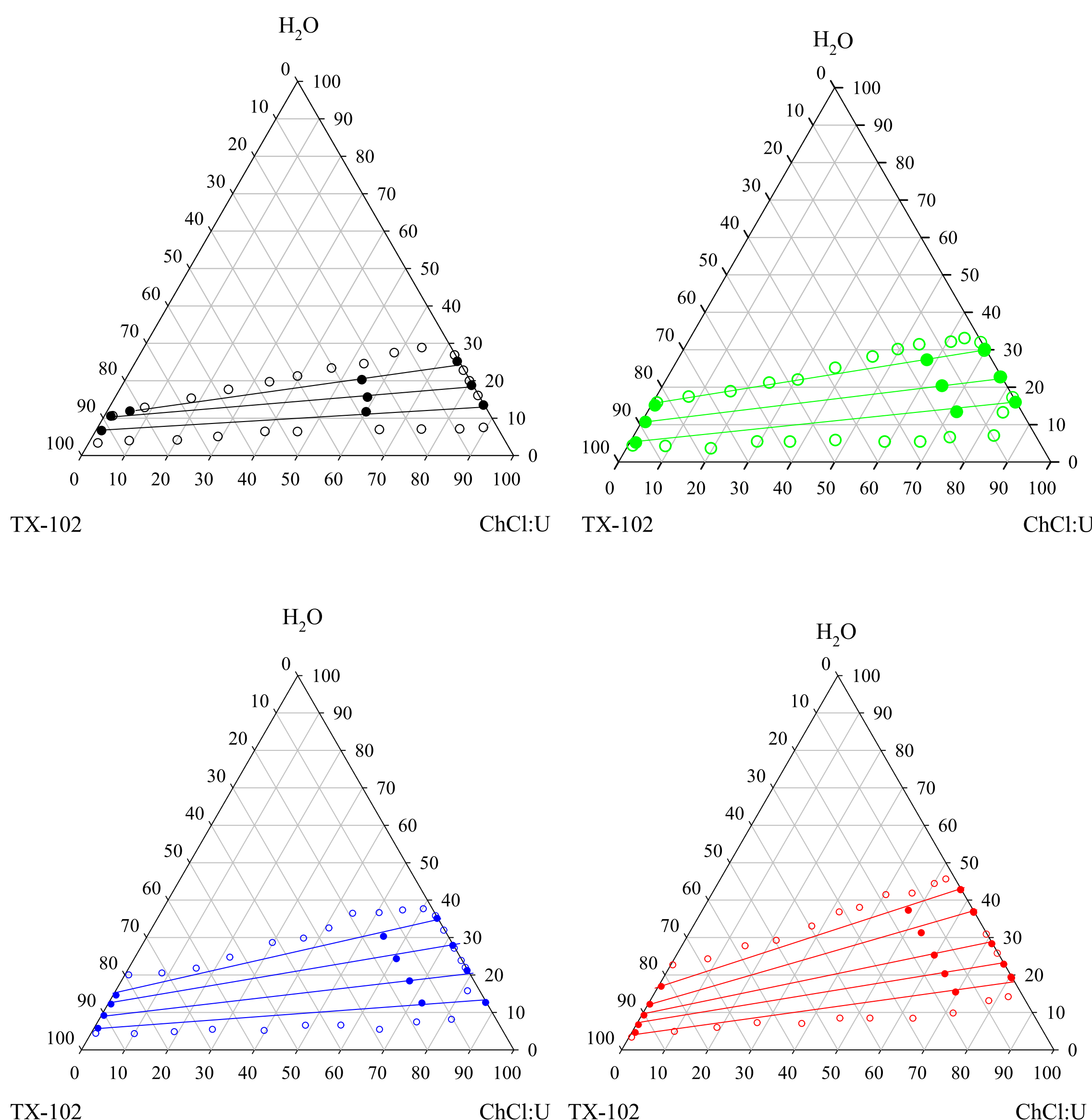

Fig. 2. Tie-line data for Triton X-102 (1) + reline (2) + H_2O (3) at 298.15 K (◯), 303.15 K (◯), 313.15 K (◯), 323.15 K (◯) and 0.1 MPa. Solid lines are a guide to the eye.

elucidating the underlying mechanisms driving phase separation and facilitating informed decision-making in various industrial and research applications.

The analysis conducted on the data presented in Table 3 reveals a significant correlation between the concentration of reline in the heavy DES-rich phase and the abovementioned key parameters. Specifically, an increase in DES concentration corresponds to elevated levels of Triton X-102 in the light phase and larger tie-line length (TLL) values. This phenomenon can be understood in terms of the competition between the DES and Triton X-102 for hydrogen bond formation with water molecules. Essentially, the presence of more salting-out agent promotes the migration of non-ionic surfactant to the upper phase, a trend consistent with previous research findings [32]. On the other hand, the values of the slope of the tie-lines (S) are notably higher when tie-lines are in proximity to the water vertex, as can be visualized in Fig. 2 and Table 3. However, it is noteworthy that regardless of the specific conditions, tie-lines consistently lead to the equilibrium of two aqueous phases, each containing approximately 10 % to 40 % of water content. This equilibrium configuration holds practical significance, particularly in applications involving biomolecule extraction, as it mitigates potential detrimental effects. The analysis underscores the intricate interplay between DES concentration, surfactant distribution, and tie-line characteristics. These insights enhance our understanding of phase behaviour in these systems, offering valuable guidance for optimizing processes involving biomolecule extraction and other related applications. Othmer-Tobias equation [43] was employed to relate the tie line mass concentration of the top phase with that of the bottom phase to obtain a linear function, as follows:

Table 4
Othmer-Tobias parameters for the system Triton X-102 (1) + reline (2) + H_2O (3) from 298.15 K to 323.15 K and 0.1 MPa[a].

T/K	m	n	R^2
298.15	1.1224	0.6847	0.999
303.15	1.0723	0.456	0.991
313.15	0.7158	0.2763	0.999
323.15	1.099	0.2827	0.985

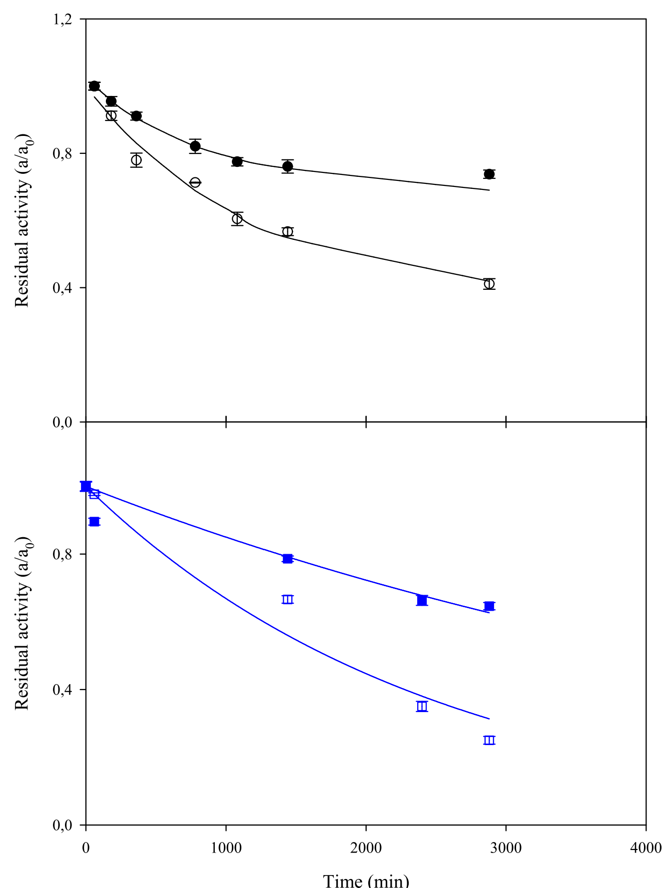

Fig. 3. Deactivation curves for *Bacillus licheniformis* alkaline protease (blue) and *Candida antarctica* lipase B (black) at 313.15 K in the presence of neat reline (full symbols) and its aqueous solutions (void symbols) at 65 % concentration (w/w). Experimental data are represented by symbols and theoretical data corresponding to the proposed 2-steps deactivation model are represented by solid line.

$$\left(\frac{1-w_1^{I}}{w_1^{I}}\right) = n\left(\frac{1-w_2^{II}}{w_2^{II}}\right)^{m} \tag{8}$$

being w_1 and w_2 Triton X-102 and reline mass fraction, respectively, the superscripts I and II referring to the top (mostly constituted by Triton X-102) and bottom (containing high reline concentrations) phases, respectively. The parameters n and m have been obtained through minimization of deviation as explained before.

Upon a conscious examination of the data presented in Table 4 in conjunction with the regression coefficients, it becomes apparent that the equation effectively characterizes the liquid–liquid equilibrium data. The consistently high R^2 values, all surpassing the 0.9 threshold, stress the robustness and reliability of this mathematical expression. This observation is in accordance with previous research findings concerning non-ionic surfactant-based ATPS [44,45],

3.3. Selective separation of Bacillus licheniformis alkaline protease and Candida antarctica lipase B

Once the suitability of the proposed system was demonstrated it was applied to the separation of lipases from proteases. In prior investigations on microbial lipolytic enzymes production, we have established that nutrient depletion in microbial cultures may result in a sudden decline in lipase activity, potentially attributed to the enzymatic action of proteases [46]. These proteases may hydrolyze the available proteins within the culture medium to fulfil microbial nutritional requirements. A potential remedy for this issue involves the prompt and selective separation of lipases and proteases once this situation occurs. Leveraging Triton surfactants, commonly integrated into microbial culture formulations for lipase production, presents a viable approach to address this challenge [47]. In the case of DES, their biological effects often differ significantly from those of its individual components, which may contribute to its toxicity and cytotoxicity. However, there is considerable controversy regarding the toxicity of this class of alternative solvents. This controversy primarily arises from the lack of specific studies. The parameters used to classify these eutectic mixtures as having high or low toxicity include the nature of the individual components, their viscosity, density, and pH values. Reline DES, composed of biodegradable molecules such as choline and urea, is expected to have low toxicity, with only its slightly basic pH potentially contributing to any problems with enzymes with optimum pH different from this value.

The initial phase involved assessing the deactivation potential of the proposed reline DES concerning the model enzymes selected for this study: *Bacillus licheniformis* alkaline protease and *Candida antarctica* lipase B. Deactivation curves were experimentally determined at 313.15 K for both enzymes in pure reline and aqueous solutions over a two-day period, with the results presented in Fig. 3. It is evident that reline induces less deactivation of both lipase and protease, as their activity after 48 h is twice as high in the pure DES compared to its aqueous solution. This finding aligns with recent research comparing enzyme stability in water versus DES, particularly cellulases and pectinases [48].

To further elucidate the deactivation process, the experimental data were suitably fitted to a series-type deactivation model involving two first-order steps, incorporating an intermediate enzyme form (E_1) and a final state (E_2), with relative specific activities (α_1 and α_2) [49].

$$E \xrightarrow{k_1} E_1^{\alpha_1} \xrightarrow{k_2} E_2^{\alpha_2} \tag{9}$$

The residual activity of the enzyme (a/a_0) was delineated by the subsequent equation, contingent upon time (t):

$$\frac{a}{a_0} = \left[1 + \frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_2}{k_2 - k_1}\right]\exp(-k_1 t) - \left(\frac{k_1}{k_2 - k_1}\right)(\alpha_1 - \alpha_2)\exp(-k_2 t) + \alpha_2 \tag{10}$$

The values of the first order deactivation constants k_1 and k_2, the specific relative activities, α_1 α_2, and the determination coefficients obtained are shown in Table 5. It becomes evident that the proposed model suitably describes the deactivation data, as the determination coefficients are always higher than 0.9. Thus, taking into account the values of the parameters obtained after the fitting, the free energy (ΔG) of the deactivation process was also ascertained through Eq. (11), where h, k_B and R are the Planck's constant (J min), the Boltzmann's constant

Table 5
Kinetic and thermodynamic parameters of *C. antarctica* lipase B and *Bacillus licheniformis* alkaline protease deactivation in pure reline and aqueous solutions (Concentration = 65 % w/w).

Compound	α_1	α_2	k_1 (min^{-1})	k_2 (min^{-1})	ΔG^0 (kJmol^{-1})	RMSD	R^2
Lipase + DES	1.8905	0	$1.62\cdot10^{-4}$	9.999	182.2	0.0438	0.945
Lipase + DES + water	1.7701	0	$4.03\cdot10^{-5}$	8.899	180.2	0.0569	0.968
Protease + DES	4.995	0.7085	$1.33\cdot10^{-3}$	0.2303	186.3	0.0048	0.978
Protease + DES + water	5.023	0.3676	$8.69\cdot10^{-4}$	19.999	176.3	0.0257	0.982

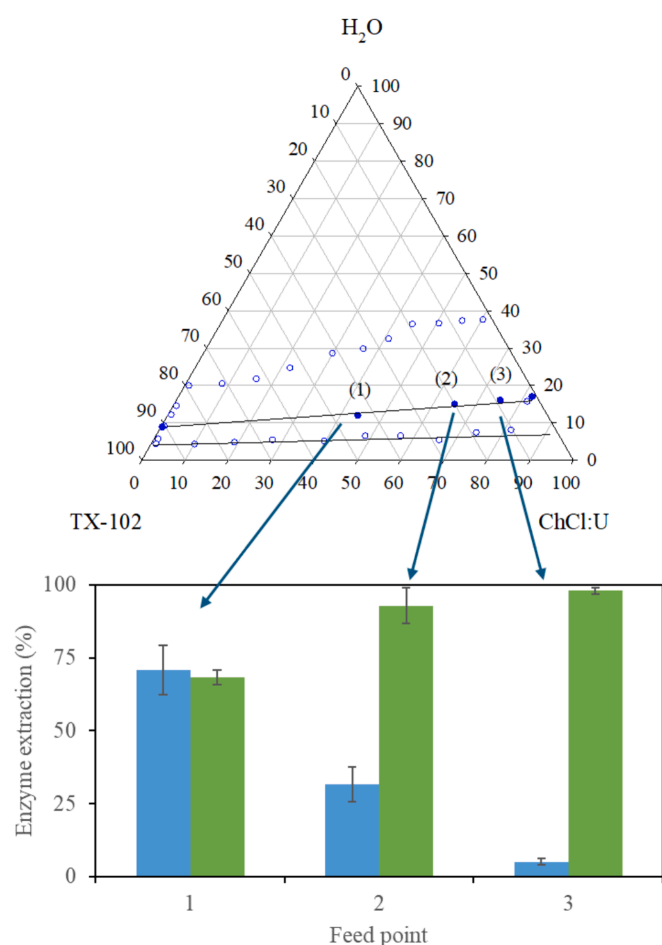

Fig. 4. Extraction of *Candida antarctica* lipase B (blue) and alkaline protease from *Bacillus licheniformis* (green) at different feed compositions at 313.15 K. In all compositions protease was extracted in the reline-rich phase and lipase in Triton X-102-rich phase.

(J K^{-1}), and the gas constant (J mol^{-1} K^{-1}), respectively, and T is temperature (K).

$$-\Delta G = RT\ln\left(\frac{k_1 h}{k_B T}\right) \quad (11)$$

The Gibbs free energy of deactivation values at 313.15 K are also compiled in Table 5, and they are in the same order of magnitude as those reported for other hydrolytic enzymes [50]. Further, the results allow to confirm on a thermodynamical basis the previous observations regarding the destabilizing effect of water addition to DES, as both with lipase and protease it involves a reduction in its values (from 182.2 kJ/mol to 180.2 kJ/mol for lipase and from 186.3 kJ/mol to 176.3 kJ/mol for protease).

Bearing in mind this information, the final step of this work was thus checking the ability of the proposed ATPS at 313.15 K for selectively separating the lipase B from *Candida antarctica* and the alkaline protease from *Bacillus licheniformis*. Three points with different feed concentrations in the same tie-line were employed to evaluate the partition of both enzymes. The evaluation of the enzyme distribution between phases was carried out through the enzyme extraction percentage (E^n) of a given n phase, calculated as follows (%):

$$E^{II} = EA^{II} \cdot 100 / \left(EA^{I} + EA^{II}\right) \quad (12)$$

where EA^{I} and EA^{II} are the enzyme lipolytic activity in Triton X-102 (top) and reline (bottom) −rich phases, respectively.

A tie-line further from the water vertex was chosen on the basis of the results obtained from the stability study, as the operation at low water concentrations was desired to avoid high losses of lipolytic activity. The obtained experimental data are presented in Fig. 4, and allow confirming that feed concentration is crucial to achieve a high selectivity of the proposed ATPS. Hence, the increase of reline concentration in the system from 44 to 75 % led to a drastic enhancement of enzyme separation up to 98 % of protease in the reline-rich phase (purification factor of 1.8) and 95 % of lipase in Triton X-102-rich phase (purification factor of 7.7). It seems also that the benign chemical environment existing in feed concentration (3) is not detrimental for the biocatalytic activity of both enzymes, which also remarks the suitability of the proposed neoteric solvent to be employed in downstream operations in biotechnological processes.

4. Conclusion

This study explored reline/Triton X-102 ATPS at various temperatures for biotechnological purposes. Phase diagrams and tie-lines were determined and correlated using several empirical models, prior to implement these systems for the selective separation of a lipase from *Candida antarctica* and a protease from *Bacillus licheniformis*. Once the stability in the presence of pure reline and aqueous solutions was evaluated and successfully fitted to a two-step deactivation model, the performance of the system was analyzed to separate both enzymes. The evaluation at three different feed compositions revealed the importance of the ternary compositions in the extraction yields, and the mixtures with higher DES concentration allowed an efficient segregation of lipase to the top phase (yield ∼ 95 %) and protease to the bottom phase (yield ∼ 98 %), which is promising for biotechnological processes where proteases are jointly produced with lipases, causing them severe negative effects.

CRediT authorship contribution statement

María S. Álvarez: Writing – review & editing, Writing – original draft, Methodology, Investigation. **Francisco J. Deive:** Writing – original draft, Validation, Conceptualization. **Ana Rodríguez:** Formal analysis. **María A. Longo:** Visualization, Validation, Conceptualization.

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

No data was used for the research described in the article.

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