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Extracting extremophilic lipases from aqueous streams by using biocompatible ionic liquids

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ABSTRACT

In this work, biocompatible ionic liquids based on aminoacids were employed as extractants to separate extremolipases from aqueous streams. First, the influence of aminoacid and dipeptide-based ionic liquids (cholinium glycinate, ChGly, and cholinium glycylglycinate, ChGlygly) on the lipolytic activity of a commercial lipase from *Candida antarctica* (CaLB) and in-house synthesized extremophilic lipases from *Thermus thermophilus* HB27 (TtHB27L) and *Halomonas* sp. LM1C (HL) was investigated. The combination of thermophilic enzyme with ChGly turned out to be the optimum combination for maximizing the biocatalytic performance, clearly improving the levels attained when water was exclusively employed as solvent and also surpassing the activity levels provided for the commercial enzyme CaLB. The salting out capacity of ChGly in aqueous solutions of biodegradable surfactants Tergitol 15S7 and Tergitol 15S9 was discussed, recording immiscibility areas almost covering all the ternary diagrams. The aqueous biphasic systems were experimentally characterized by determining both tie-lines and solubility curves at several temperatures and the data was modelled with relevant equations like Merchuk, Othmer-Tobias and Bancroft ones, as they are the most common ones to describe this kind of equilibrium data. So, ChGly was applied to extract thermophilic and commercial lipases from aqueous solutions at 313.15 K, achieving very high extraction levels (about 100 %) for TtHB27L, which clearly surpasses the maximum extraction values observed for the commercial enzyme (about 80 %). Finally, the process was simulated at real scale through SuperPro Designer v.8.5 for the production of 385 Kg/year of extremolipase.

1. Introduction

The current international exorbitant prices of energy force the academic community to invest efforts in developing new cost-efficient alternatives in the short term. In this sense, lowering the reliance on fossil resources and increasing the use of bio-based chemicals up to 25 % in 2030 is a crucial target to be reached at a European level [1]. Since aircraft and heavy transport combustion engines are far from being replaced by electric ones (that are not economically viable nowadays), biodiesel emerges as a key sustainable fuel to immediately replace fossil alternatives in diesel engines. Based on these premises, the scientific community is actively casting doubt on the much-vaunted competitiveness of chemical-based biodiesel production regarding the biological option [2]. The biocatalysts employed for biodiesel synthesis are lipases (triacylglycerol hydrolases E.C. 3.1.1.3). Although they are enzymes that typically act on triacylglycerols hydrolysis, they are also able to catalyse synthetic reactions like esterifications, transesterifications and interesterifications [3].

These processes (esterifications, transesterifications and interesterifications) often demand the operation at extreme reagents concentrations, pHs or temperatures which may be deleterious for lipase performance. To circumvent this problem, extremophilic microorganisms have emerged as a promising source of more stable lipolytic enzymes. In this context, the existence of thermophiles (those thriving at temperatures over 50 °C), halophiles (those living at high salt concentrations) or acido/alkalophiles (microbes living at extreme pH values) ensure the production of lipases operating at these conditions (high temperatures and extreme pH values and salt concentrations) [4]. However, the low expression levels and the lack of low cost separation strategies makes it difficult the commercialization of extremophilic lipases, and only very few candidates are available [5]. Therefore, it is necessary to research and optimize new economic processes for their production. In this sense, downstream operations usually represent most of the cost for biotechnological plants due to the price

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of chromatography resins or the complexity of the separation strategies.

Although there are many alternatives for lipases separation from culture broths [6], aqueous biphasic systems (ABS) are considered a potential appealing method for enzyme extraction [7] due to a number of advantages like i) the existence of an aqueous environment more biocompatible with enzymes, ii) the possibility of tailor-designing the immiscibility region by an appropriate combination of phase forming compounds, iii) simplicity, iv) quick phase disengagement, v) low energy requirements [8,9]. These systems are usually the result of mixing two hydrophilic polymers, two salts or a polymer and a salt at certain concentrations, although in the last years the emergence of ionic liquids has provided new opportunities for designing task-specific ABS [10]. Ionic liquids are salts which melting point is below 100 °C, and have triggered a great interest due to their negligible volatility, thermal stability and tunability [11]. Notwithstanding the fact that the first generation of ionic liquids were derived from non-renewable sources, natural resources like amino acids are a preferable option due to their greater biocompatibility [12,13].

Amino acids are special green biomolecules as they may act both as cation and as anion, their side chains bear an array of functional groups incorporating chirality and other interesting properties, and they are quite cheap and abundant compounds [14]. Ohno and coworkers [15] were the first to prepare ionic liquids containing amino acids as anion, belonging the cation to imidazolium family. However, it was not since five years later when Moriel et al proposed the use of cholinium cation as a fully biodegradable and non-toxic option [16], so in the last years cholinium-based ionic liquids have been proposed as the epitome of green chemistry [17]. Among the possible candidates, glycine can be highlighted due to it is the smallest one and it is extensively present in proteins [18]. Therefore, cholinium glycinate (ChGly) was already demonstrated to be a biocompatible solvent with lipolytic enzymes [19]. It was selected as model amino acid together with cholinium glycylglycinate (ChGlygly) to act as phase forming compounds in aqueous solutions, as they are the typical matrices where these enzymes are biotechnological synthesized.

In this sense, lipase production often entails the addition of inducers like surfactants [20], so the aqueous solutions of these compounds may be salted out by the addition of the abovementioned ionic liquids, thus creating an immiscibility region easing lipase recovery. Although non-ionic surfactant like Triton family is the one more commonly employed in biotechnological processes, the REACH Annex XIV [21] has banned its use since 2021, so the search of more biodegradable alternatives is welcomed. Thus, two readily biodegradable ethoxylated surfactants, Tergitol 15S7 and Tergitol 15S9, [22] were chosen as models since they represent a more promising alternative.

In view of the above, the effect of ChGly and ChGlygly on the biocatalytic performance of two in-house produced extremophilic lipases has been studied. The most biocompatible one was employed as salting out agent in aqueous solutions of two biodegradable non-ionic surfactants: Tergitol 15S7 and Tergitol 15S9. Then, the ABS was proposed as a platform for extremophilic lipase extraction. Finally, the separation process was simulated after been integrated in a biotechnological process to biosyntehsize lipases by using SuperPro Designer v8.5.

2. Experimental

2.1. Materials

ChGly and ChGlygly were synthesized following previous literature data [19,23], using ChCl (Sigma-Aldrich), glycine (Scharlab) and glycylglycine (Fischer Chemical) as starting raw material (Table S1). The purity of the ionic liquids was checked by ^{1}H NMR and Karl-Fischer titration was employed to determine the water content (<6%).

Novozymes donated *Candida antarctica* lipase B (CaLB) and Dr. J. Berenguer provided *Thermus thermophilus* HB27 (TtHB27L) strain, which was cultivated following the method described elsewhere [24]. *Halomonas* sp. LM1C (HL) was previously isolated after a lipase-producing screening performed in Parque Natural de las Lagunas de La Mata y Torrevieja (Spain) [25] and was cultured in accordance with previous research works of the group [26].

2.2. Biphasic region

The solubility curves of the ternary systems ChGly + Tergitol 15S7 or Tergitol 15S9 + H_2O have been ascertained through the cloud point method at several temperatures (F200ASL digital thermometer, ±0.02 K): 293.15, 303.15, 313.15 and 323.15 K. To do that, water drops were added to binary mixtures of the given surfactant (Tergitol 15S7 or Tergitol 15S9) and ChGly in a thermostatted glass cell equipped with magnetic agitation, until a transparent liquid mixture was attained. The compositions of these experiments were obtained after the individual addition of the mass of each component in ternary mixture (Sartorius Cubis MSA balance 125P- 100-DA ± 10^{-5} g). After the solubility curve was completely defined, the Tie-lines (*TLs*) were inferred as reported elsewhere [27]. In brief, an aqueous mixture with composition belonging to the immiscibility region was prepared under vigorous agitation and left to settle for one day at given temperature. Then, the layer composition was determined by density analysis after a prior calibration step.

2.3. Lipolytic activity in the presence of ChGly, ChGlygly, Tergitol 15S7 and Tergitol 15S9 and quantification

The effect of ChGly and ChGlygly on the biocatalytic potential of CaLB, TtHB27L and HL was ascertained after 15 min in aqueous solutions containing 700 μL of ionic liquid, 200 μL water and 100 μL of enzyme solution at 313.15 K.

The lipolytic activity was monitored through spectrophotometric measurements, using an ethanolic solution of *p*-nitrophenyl laurate as substrate (2.5 mM) [28]. 100 μL of this solution and 800 μL of Tris HCl buffer 50 mM (optimum pH of each enzyme) containing 20 mM $CaCl_2$ were mixed and maintained at 313.15 K for 5 min. Afterwards, a 20 min-reaction time was allowed after sample addition (100 μL) and a 1 M solution of Na_2CO_3 was then added in an ice bath (10 min) to stop the reaction. Then, a centrifugation step (10,000 rpm, 10 min, 277.15 K) was included to remove solids formed in the mixture and the supernatant was introduced in a spectrophotometer (Unicam Helios β, Thermo Electron Corp) to measure the absorbance at 400 nm. These reaction conditions allow defining one activity unit (U/L) as the enzyme able to catalyse the production of 1 μmol of *p*-nitrophenol per min. In all cases, the samples were measured in triplicate, including a blank sample as control.

2.4. Enzyme extraction

Enzyme extraction of CaLB and TtHB27 was evaluated in three different compositions in two *TLs* at 313.15 K. To do that, six different lipase aqueous mixtures containing the selected non-ionic surfactant (Tergitol 15S7) and the ionic liquid (ChGly) were prepared and subjected to a centrifugation stage (3000 rpm, 30 min, and 313.15 K) prior to layer separation. The biocatalytic activity was quantified in each layer following the abovementioned procedure.

3. Results and discussion

3.1. Extremolipase activity in neoteric media

The search of a suitable extraction neoteric solvent demands a preliminary screening to discard possible harmful effects on the biocatalytic performance of lipases. Usually, the biocompatibility of a given solvent is the result of a complex combination of physical properties, including the intrinsic pH, viscosity, hydrogen capacity, hydrophobicity, etc. [29]. Therefore, the first target for evaluating the appropriateness of both ChGly and ChGlygly as extraction compounds is to analyse the lipolytic activity of the extremolipases, TtHB27L and HL in aqueous solutions of these ionic liquids. A commercial lipase (CaLB) was also included as control in the study in order to verify the suitability of the proposed extreme enzymes. The data resulting from these experiments are presented in Fig. 1, and evidence the outstanding biocatalytic potential of the thermophilic lipase (TtHB27L) regarding both the commercial (CaLB) and the halophilic (HL) lipases, no matter the solvent under study.

On the contrary, it becomes clear that both cholinium-based ionic liquids are detrimental for HL. This fact may be due to the intrinsic alkaline pH of the selected amino acid and dipeptide-based ionic liquids, as in accordance with the model proposed elsewhere [25], HL activity at pH values greater than 10 is almost null. Following this rationale, TtHB27L is not altered at the alkaline values provided by the selected ChGly and ChGlygly due to the thermoalkalophilic character of this lipase, as already reported previously [30]. What is more, it is obvious that the alkaline conditions provided by both ionic liquids entail a clear activation of TtHB27L regarding the activity in pure water, as an increase of about 50 % is detected. The data presented also reveal an analogous trend for the commercial lipase (CaLB), which points to the suitability of both ionic liquids to not only avoid the enzyme deactivation, but also improving the biocatalytic potential, in line with the conclusions reported for the behaviour of other commercial lipases in this kind of ionic liquids [19].

On the other hand, since hydrogen bonding capacity has been pointed to be critical for selecting a biocompatible solvent [31], it seems that the proposed ionic liquids do not interplay with the enzyme up to a level that causes a harmful effect on the lipolytic activity, as can be checked with the behaviour of both CaLB and TtHB27L. Given these promising results, ChGly was chosen to be employed as salting out agent in aqueous solutions of the non-ionic surfactants Tergitol 15S7 and Tergitol 15S9.

3.2. Immiscibility region characterization

Lipase production in aqueous culture broths demands the implementation of a downstream stage where ChGly be able to efficiently trigger phase segregation in an aqueous solution containing the selected Tergitol-based surfactants. The solubility data of the aqueous systems composed of ChGly and Tergitol 15S7 or Tergitol 15S9 at 293.15 K, 303.15 K, 313.15 K and 323.15 K were experimentally carried out to appropriately map the immiscibility region (Tables S2 and S3). These data were also correlated with the well-known Merchuk equation [32]:

$$[Tg] = a \cdot \exp\left(b[IL]^{0.5} - c[IL]^3\right) \quad (1)$$

being $[Tg]$ the surfactant mass fraction, $[IL]$ ChGly mass fraction, and a, b, and c, the fitting parameters. The mass fractions were obtained by weighing of each component in the ternary mixture with an analytical balance (c.f. experimental section). These were optimized by means of the standard deviations (σ):

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

where z_{exp}, z_{adjust} and n_{DAT} are the experimental data, the theoretical data and the number of experimental data, respectively. The deviations and the fitting parameters are presented in Table 1, and the experimental and theoretical data can be visualized in Fig. 2. Both the deviation values and the figures evidence the suitability of the proposed model to adequately describe the experimental data, thus confirming the validity of Merchuk equation to model ABS data for systems including a wide range of phase forming compound, from ionic liquids-polymers to polymers-salts and ionic liquids-salts [33–35].

The analysis of the obtained experimental data reveals different sizes of the immiscibility region depending on both the surfactant and temperature of operation. Regarding the first, the only difference between Tergitol 15S7 and Tergitol 15S9 lies in the size of the side ethoxylated chain which alters the non-ionic surfactant

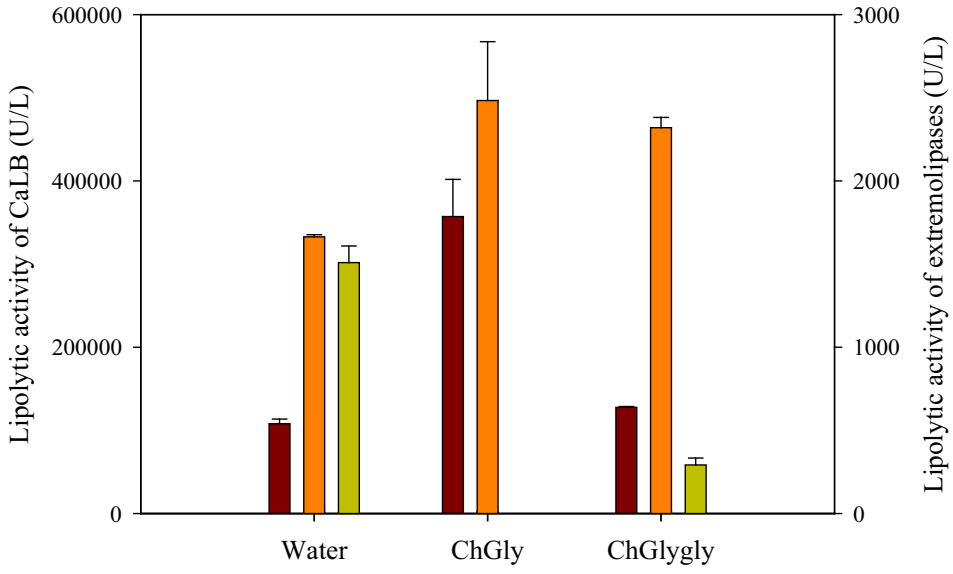

Fig. 1. Effect of synthesized ionic liquids on the activity of CaLB (■), TtHB27L(■) and HL (■) after 15 min in the presence of ionic liquids/water at 313.15 K.

Table 1
Correlation parameters and standard deviation (σ) for the system {Tergitol (1) + ChGly (2) + H_2O (3)} at several temperatures.

T/K	a	b	c	σ
Tergitol 15S7 (1) + ChGly (2) + H_2O (3)				
293.15	1.1082	−1.8200	497.23	0.020
303.15	0.9392	−0.8165	2792.90	0.010
313.15	1.6396	−3.9974	4361.8	0.136
323.15	0.1591	18.189	99,999	0.322
Tergitol 15S9 (1) + ChGly (2) + H_2O (3)				
293.15	0.8840	−0.9184	181.92	0.038
303.15	0.8726	−0.7802	392.96	0.045
313.15	0.6618	1.3543	1391.9	0.069
323.15	0.1246	14.883	20537.9	0.079

hydrophobicity. This has a direct impact in the size of the immiscibility region, as the solubility curves obtained when Tergitol 15S7 is employed as phase forming compound are closer to the water vertex. That means that the greater hydrophobicity of this compound regarding Tergitol 15S9, as can be inferred from the HLB values (12.1 against 13.3, c.f. Table S1), eases a more efficient ChGly interaction with water molecules and a subsequent generation of two aqueous layers, one rich in the non-ionic surfactant and another one rich in the amino acid-based ionic liquid.

On the other hand, it becomes clear that the temperature of operation involves modifications of the biphasic area for both non-ionic surfactants. Thus, it is interesting to note that increased temperature values are translated into a higher salting out capacity of the ionic liquid, in line with the results reported for other systems including conventional imidazolium-based ionic liquids and non-ionic surfactants [27]. Nonetheless, the analysis of literature data reveals an inverse trend when the phase forming components selected for the creation of the immiscibility region are changed, like the combination of ionic liquids and salts [36]. The rationale behind this behaviour may be the different hydrogen bonding capacity of ethoxylate groups at greater temperature values. In this sense, the results reported by Lindman and coworkers [37] highlighting the predominance of conformers displaying very low dipole moments at high temperatures would further surfactant-surfactant interactions instead of surfactant-water ones. The strength of surfactant-surfactant interactions is also evidenced by the clear mitigation of the differences between equilibrium data at several temperatures when Tergitol concentrations are higher than 50 %, in agreement with previous conclusions using other polyethoxylated surfactants [38].

3.3. Extremolipase extraction

The implementation of a suitable lipase extraction strategy involves an in depth characterization of the aqueous biphasic systems composed of Tergitol 15S7 or Tergitol 15S9 and ChGly. Therefore, the tie-lines have been ascertained as described in the experimental section, and useful parameters like the tie-line length (*TLL*) and slope (*S*) were calculated as follows:

$$TLL = \left[\left(w_1^{I} - w_1^{II}\right)^2 + \left(w_2^{I} - w_2^{II}\right)^2\right]^{0.5} \quad (3)$$

$$S = \frac{w_1^{I} - w_1^{II}}{w_2^{I} - w_2^{II}} \quad (4)$$

where w_1 and w_2 are surfactant and ionic liquid mass fraction percentage, respectively and the superscripts I and II refer to the surfactant- and ionic liquid-rich phase, respectively.

All the experimental tie-line data are compiled in Tables 2 and 3, together with the *TLL* and *S* values. Additionally, the tie-line data can be visualized in Figs. S1 and S2. The mass compositions presented in those tables evidenced a proportional relationship between ionic liquid concentration in ChGly-rich phase and surfactant concentration in Tergitol-rich phase, no matter the temperature and surface active compound under study. Again, the chemical explanation may be that the more ionic liquid is present in the mixture the more hydrogen bonds are established with water molecules, so Tergitol molecules are more easily segregated to the upper phase, which in turn results in an increased surfactant purity in this layer. This direct relationship is also confirmed with the trends observed for *TLL* data, which is inverse to the pattern followed by *S* values, in agreement with very recent previous results reported for other aqueous biphasic systems composed of other neoteric solvents and non-ionic surfactants [39].

In addition, the tie-line data were correlated with Othmer-Tobias and Brancroft empirical equations [40,41]:

$$\left(\frac{1 - w_1^{I}}{w_1^{I}}\right) = b\left(\frac{1 - w_2^{II}}{w_2^{II}}\right)^{a} \quad (5)$$

$$\left(\frac{w_3^{II}}{w_I^{II}}\right) = k\left(\frac{w_3^{I}}{w_I^{I}}\right) \quad (6)$$

where 1, 2 and 3 refer to surfactant, ionic liquid and water, respectively, I and II have the same meaning as that mentioned previously, and *a*, *b*, *k* and *r* are the fitting parameters. These values, together with the standard deviations (σ) are listed in Table 4, and they evidenced the higher adequateness of Othmer-Tobias model to correlate the tie-line data, in line with previous conclusions derived from the comparison between both empirical equations [42].

In summary, it becomes evident that Tergitol 15S7 is the surfactant providing not only the greater immiscibility areas, but also the tie-lines reaching higher TLL and S values, so this compound was selected for proposing a suitable lipase extraction platform. Additionally, since the commercial lipase CaLB has its optimum activity at 313.15 K, this temperature was selected to carry out the extraction experiments, which means that the thermophilic enzyme extraction could also be improved when operating at higher temperatures, as its optimum is at values over 353.15 K.

Then, six experiments for lipases extraction were planned including six different feed compositions in two different tie-lines of the ABS composed of ChGly and Tergitol 15S7 at 313.15 K, as can be visualized in Fig. 3. The active lipase extraction capacity (*E*) was defined as follows:

$$E\ (\%) = \frac{A^{II} \cdot V^{II}}{A^{i} \cdot V^{i}} \cdot 100 \quad (7)$$

where *A* and *V* are the lipolytic activity (U/L) and the volume (L), and the superscripts i and II refer to initial conditions and ChGly-rich phase, respectively.

The values of *E* for each feed composition in both CaLB and TtHB27L are presented in Fig. 4, and again confirm the suitability of the proposed biocompatible platform for extracting both lipases,

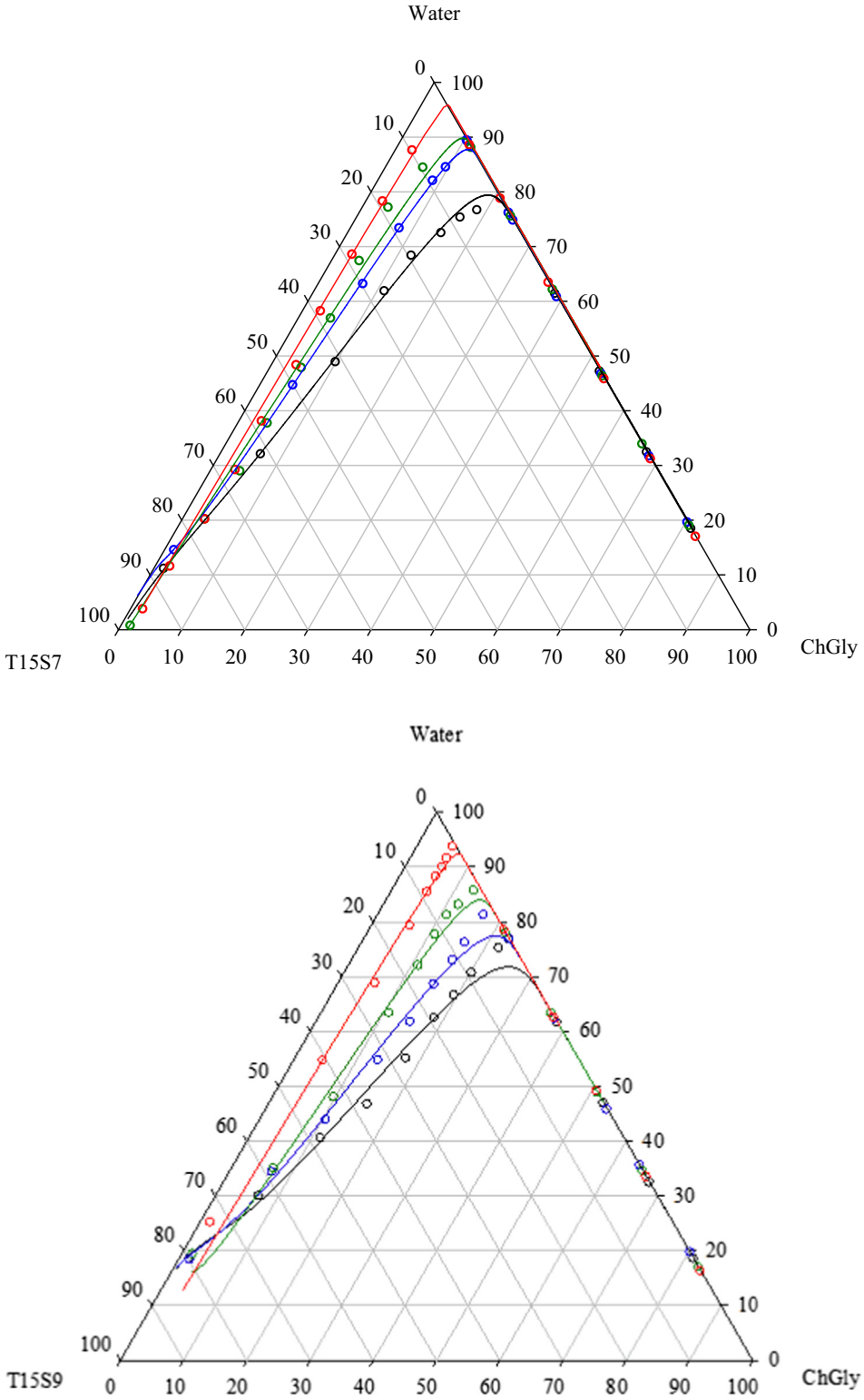

Fig. 2. Solubility data of the ternary systems composed of Tergitol 15S7 (up) or Tergitol 15S9 (down) with ChGly and water at 293.15 K (○), 303.15 K (○), 313.15 K (○) and 323.15 K (○). Experimental data are represented by symbols and the theoretical data are the solid lines.

as levels greater than 80 % can be obtained for both enzymes by tuning the feed composition. Additionally, two different trends are obtained for the commercial and thermophilic lipases: while CaLB reaches a maximum at intermediate water concentrations, extraction levels of TtHB27L are decreased at greater water concentrations.

A plausible explanation may be the structural features of the lipases under study. Although both of them share the characteristic α-β hydrolase fold, the reason for the observed distinct behaviour may lay in their divergences already in the primary structure: while CaLB catalytic triad is composed of Ser105, Asp187 and His224 [43], the amino acids in the active site of TtHB27L are

Table 2
Experimental *TL* in mass percentage, *TLL* and *S* for the system {Tergitol 15S7 (1) + ChGly (2) + H_2O (3)} at several temperatures.[a]

Surfactant-rich phase		IL-rich phase		Feed composition		*TLL*	*S*
100 w_1^I	100 w_2^I	100 w_1^{II}	100 w_2^{II}	100w_1	100w_2		
T = 293.15 K							
97.88	0.97	0.12	81.98	29.89	57.76	126.97	−1.2068
92.79	1.04	0.10	60.75	30.16	42.06	110.25	−1.5522
65.29	8.26	0.09	40.41	30.05	26.14	72.70	−2.0276
T = 303.15 K							
97.87	0.96	0.08	78.61	29.72	55.45	124.87	−1.2594
89.17	1.56	0.13	53.70	29.97	36.82	103.18	−1.7077
69.09	3.03	0.13	33.62	29.92	21.12	75.44	−2.2543
T = 313.15 K							
80.73	3.94	0.13	67.89	29.99	44.93	102.89	−1.2604
71.97	5.06	0.10	46.60	30.07	29.92	83.01	−1.7301
48.54	7.60	0.10	25.99	29.96	14.92	51.81	−2.6334
T = 323.15 K							
82.83	3.52	0.13	68.05	30.07	44.91	104.90	−1.2816
71.80	5.34	0.08	46.66	30.06	29.90	82.77	−1.7357
52.83	5.59	0.11	25.84	29.97	14.97	56.48	−2.6035

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

Table 3
Experimental *TL* in mass percentage, *TLL* and *S* for the system {Tergitol 15S9 (1) + ChGly (2) + H_2O (3)}at several temperatures.[a]

Surfactant-rich phase		IL-rich phase		Feed composition		*TLL*	*S*
100 w_1^I	100 w_2^I	100 w_1^{II}	100 w_2^{II}	100w_1	100w_2		
T = 293.15 K							
96.87	0.61	0.13	76.71	14.93	65.09	123.08	−1.2712
87.91	0.87	0.01	60.09	14.91	49.99	105.99	−1.4843
78.52	1.93	0.09	42.48	15.03	34.91	88.29	−1.9342
T = 303.15 K							
95.64	0.80	0.12	76.37	15.00	64.99	121.80	−1.2640
78.43	2.94	0.14	60.58	15.00	50.03	97.22	−1.3583
59.94	8.31	0.12	42.59	14.97	34.99	68.95	−1.7448
T = 313.15 K							
88.95	2.05	0.19	83.85	14.92	69.98	120.70	−1.0851
77.54	2.82	0.44	66.77	14.97	55.01	100.17	−1.2056
59.57	8.51	0.25	49.48	14.95	39.97	72.09	−1.4479
40.84	10.84	0.15	32.14	15.13	24.98	45.93	−1.9103
T = 323.15 K							
91.41	1.37	0.12	83.85	14.93	69.99	123.03	−1.1068
82.39	1.58	0.26	66.56	14.94	55.01	104.73	−1.2639
71.79	2.16	0.11	49.13	15.01	39.94	85.70	−1.5261
48.74	7.16	0.20	31.58	14.98	24.99	54.34	−1.9877

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

Table 4
Parameters of Othmer-Tobias and Bancroft equations and standard deviation (σ) for the system {Tergitol (1) + ChGly (2) + H_2O (3)} at several temperatures.

T/K	*a*	*b*	σ	*k*	*r*	σ
Tergitol 15S7 (1) + ChGly (2) + H_2O (3)						
293.15	1.1657	0.2285	0.252	0.5385	2.5137	0.070
303.15	1.5233	0.1563	0.019	0.5609	3.1937	0.030
313.15	0.8318	0.4095	0.116	1.1128	3.3862	0.134
323.15	0.8071	0.3716	0.037	1.1509	3.9130	0.060
Tergitol 15S9 (1) + ChGly (2) + H_2O (3)						
293.15	1.4357	0.1986	0.156	0.6332	2.8948	0.123
303.15	1.8266	0.4494	0.208	0.5285	1.6896	0.131
313.15	1.0364	0.6525	0.053	0.9779	1.8311	0.024
323.15	0.9786	0.4386	0.102	1.0049	2.5646	0.069

Ser159, Glu255 and His293 [44]. This fact will obviously impact their hydrogen bonding capacity and may ultimately affect the biocatalytic performance. Additionally, since the commercial preparation of CaLB includes chemical additives to protect the enzyme, this may be also affecting the biocatalytic potential during the extraction process.

In any case, the much higher extraction levels (near to 100 %) recorded for TtHB27L in feed composition 1, allow concluding the suitability of the proposed separation bioplatform for the recovery of this extremophilic enzyme. In the present work, no protective additives have been included in the culture medium, as the extraction process has been implemented with the crude

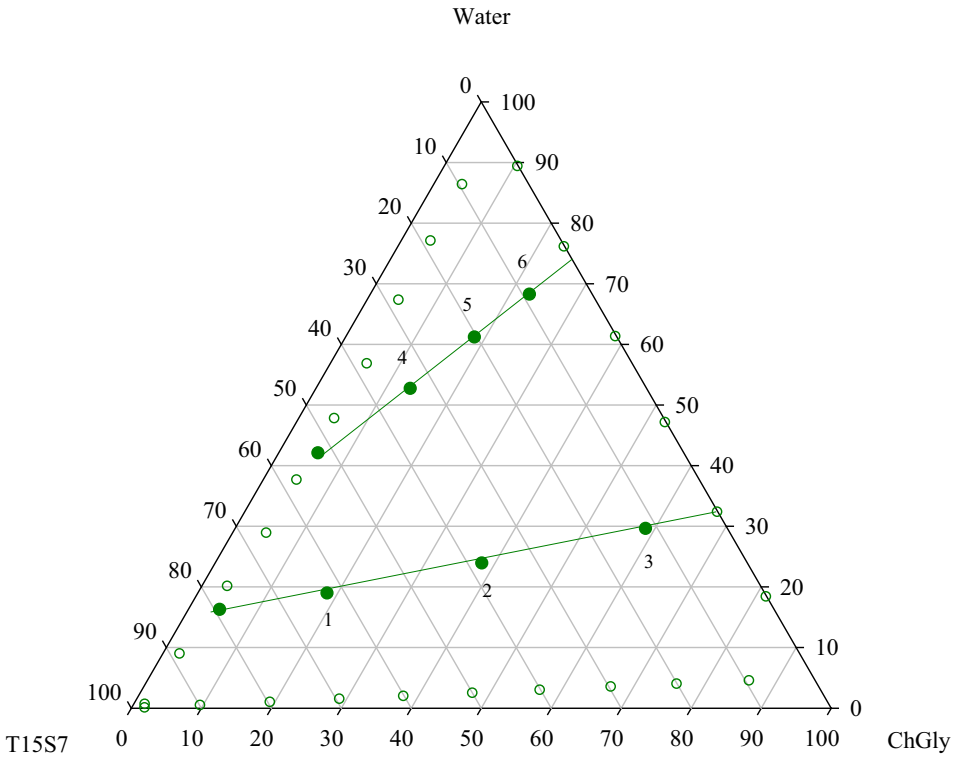

Fig. 3. Tie lines including the different feed concentrations selected for lipase extraction from aqueous solutions at 313.15 K.

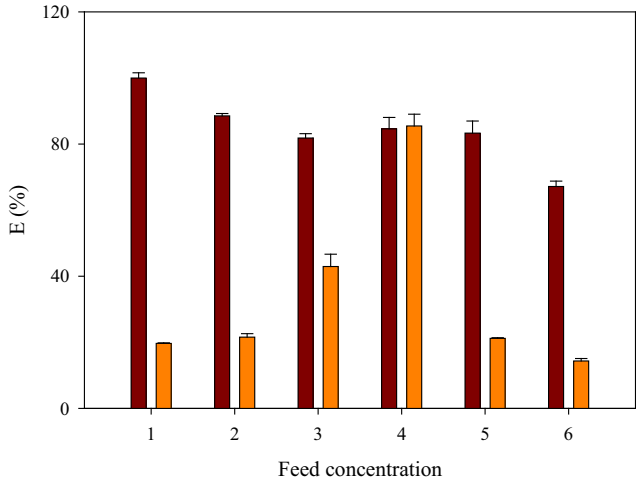

Fig. 4. Effect of feed concentration in active lipase extraction percentage for aqueous solutions of *Thermus* (■) and *Candida antarctica* (■) lipases at 313.15 K.

enzyme at a temperature far from the optimum of TtHB27L. In summary, it becomes clear the interest of using glycinate-based ionic liquids as extraction agents in aqueous solutions of thermophilic lipases as a way to improve both the economics and the environmental sustainability of the biotechnological process.

3.4. Simulation of the process

Simulation can be employed for easing the scaling-up of the current biological process. Since different kind of modern process simulation packages are commercially available like Hysys, SimSci, Prosim, and SuperPro Designer, the last one was chosen due to its optimum performance for biotechnological processes. This software is a flowsheet-driven simulator specifically designed to perform material and energy balances, equipment sizing and costing, economic evaluation, environmental impact assessment, process scheduling, and debottlenecking of batch and continuous processes. Therefore, the simulation of the process was carried out by means of SuperPro Designer v8.5, on the basis of the flowsheet diagram depicted in Fig. 5. In this diagram, all the equipment

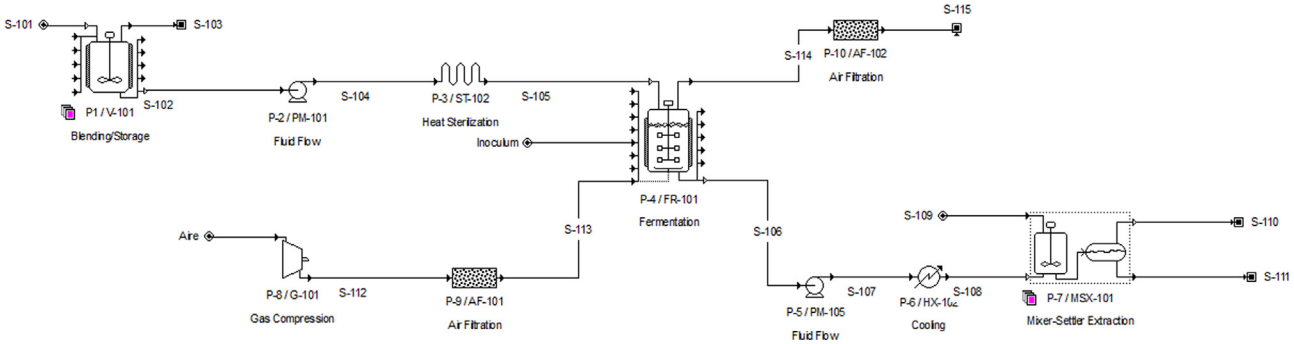

Fig. 5. Flowsheet diagram of the biotechnological process to synthesize lipases.

Table 5
Composition of the main streams per batch in a biotechnological process to biosynthesize extremolipases from *Thermus thermophilus* HB27.

	S-101	Air	Inoculum	S-106	S-109	S-110	S-111
Components	Kg/batch	Kg/batch	Kg/batch	Kg/batch	Kg/batch	Kg/batch	Kg/batch
Casein peptone	800	0	0	462	0	458	4
Yeast extract	400	0	0	240	0	238	2
Water	100,000	0	3000	100,563	0	50,281	50,281
Sodium chloride	300	0	0	146	0	1	146
ChGly	0	0	0	0	111,111	1017	110,094
Tergitol 15S7	0	0	0	0	344,444	340,939	3505
Lipase	0	0	0	0.92	0	0.00	0.92
Biomass	0	0	2.7	91	0	90	1
Nitrogen	0	23,577	0	0	0	0	0
Oxygen	0	7158	0	0	0	0	0

required for both upstream and downstream operations are included, and the results of the simulation can be seen in Table 5. Thus, the separation strategy would be placed after the upstream operations consisting of mixing the culture components in aqueous solutions and sterilizing it prior to proceed to the biological reaction in a fermentor. It was considered to carry out a batch fermentation in a bioreactor containing 100 m^3 of culture medium to allow the thermophilic bacterium *Thermus thermophilus* HB27 to reach the stationary phase in just 12 h at 343.15 K, 0.33 vvm of aeration and 500 rpm of agitation, in accordance with prior results of our group [24]. Process simulation allows concluding the viability of getting 419 batches per year, considering that 28.15 h are required for each batch, as the biological reaction is bottleneck. Taking into account a thermolipase specific activity of 35 U/mg, it is possible to attain about 385 Kg per year in a phase mainly composed of ChGly and water, after a separation in an industrial mixer-settler operating at the compositions leading to near complete extremolipase extraction levels.

4. Conclusions

The present research work has allowed demonstrating the usefulness of ChGly regarding ChGlygly to i) act as segregation agent in aqueous solutions of ethoxylated biodegradable surfactants and ii) to generate a biocompatible milieu avoiding deleterious effects on lipolytic activity of both extremophilic and commercial lipases.

After having thoroughly characterized and modelled the immiscibility region (including both the solubility curves and tie-lines) in aqueous systems composed of ChGly and Tergitol 15S7 or Tergitol 15S9 at four different temperatures, the extraction of extremophilic and commercial lipases was approached. The results obtained revealed an almost complete separation of the thermophilic TtHB27L to the ChGly-rich phase, even higher to those recorded with the commercial CaLB, which demonstrates the potential of the proposed separation method for implementing neoteric downstream stages in biotechnological processes devoted to extremozymes production.

Finally, the simulation with commercial software gave an approximate idea of the sizes of equipment required to produce 385 Kg/year of the extremolipase from *Thermus thermophilus* HB27: two storage tanks of about 80 m^3, one bioreactor of 148 m^3, a mixer of 7 m^3 and a settler of 33 m^3.

In summary, this work opens up new opportunities for the implementation of low cost strategies for the separation of enzymes in biotechnological processes.

Data availability

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

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molliq.2022.120214.

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