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Creating a new biocatalytic complex with extremolipases and biocompatible ionic liquids for improved transesterification reactions

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ABSTRACT

The ongoing energy crisis has spurred increased research into sustainable and more competitive methods for producing biofuels, including biodiesel. In this context, the focus of the current study is to underscore the viability of investing in a novel biocatalytic complex. This complex incorporates extremophilic lipases and biocompatible ionic liquids with the aim of achieving exceptionally high conversions in transesterification reactions without generating glycerol. Through a meticulous screening process encompassing various amino acid and dipeptide-based ionic liquids from the ammonium family, cholinium glycinate turned out to be the optimal choice. This selection was driven not only by its enhanced compatibility with a commercially available *Candida antarctica* lipase B (CaLB) but also with extremophilic enzymes synthesized in-house, derived from halophilic (*Halomonas* spLM1C) and thermophilic (*Thermus thermophilus* HB27) strains. Following rigorous testing of both free and immobilized enzymes, the ideal concentration of the ionic liquid in transesterification reactions was determined to be 1% relative to the sunflower oil content. Comparative analysis of conversion rates between immobilized thermophilic lipase and immobilized CaLB revealed the efficacy of the proposed approach. Maximum conversions were found to increase by 20%, with specific conversion rates soaring by approximately 180% when utilizing the immobilized thermophilic lipase. In conclusion, this research ushers in new prospects for advancing the competitiveness of biocatalytic solutions in glycerol-free transesterification reactions, underscoring its potential to revolutionize the landscape of sustainable energy production.

1. Introduction

The current global energy crisis, stemming from the Ukrainian conflict and subsequent economic sanctions against Russia, compounded by the resurgence of economic activity following the pandemic, has led to an unprecedented decline in diesel reserves, reaching their lowest levels in the past two decades [1]. Diesel fuel serves not only private vehicles but also heating systems and transportation, making the exploration of alternatives to fossil fuels an issue of escalating importance. Beyond economic considerations, environmental factors are also driving the pursuit of more sustainable options, as evidenced by initiatives such as the European Union's commitment to achieve zero emissions by 2050 and the agreement reached at the UN Climate Change Conference (COP26) to reduce greenhouse gas emissions by at least 40 % compared to 1990 levels. Additionally, there is a concerted push to attain a minimum of 32 % of energy consumption from renewable sources [2].

Hence, one of the strategic pathways to address the challenges of climate change involves promoting the adoption of alternative, low-emission, and renewable energy sources in the realm of transportation and cargo hauling, such as biofuels. As a result, decarbonization efforts should encompass not only the advancement of electric systems or green hydrogen but also renewable fuels like biodiesel. Biodiesel is defined as monoalkyl esters of long-chain fatty acids, synthesized through the transesterification of lipid materials (such as animal fats, vegetable oils, algae, and microbial oils) with lower molecular weight alcohols, predominantly methanol [3]. Various approaches have been suggested for obtaining biodiesel from oils, including microemulsification, dilution, or pyrolysis. However, the most prevalent technique is transesterification [4]. The conventional and widely employed biodiesel production process revolves around the transesterification of triglycerides with short-chain alcohols (primarily methanol) at elevated temperatures (approximately 60 °C), with the aid of chemical catalysts. This results in the

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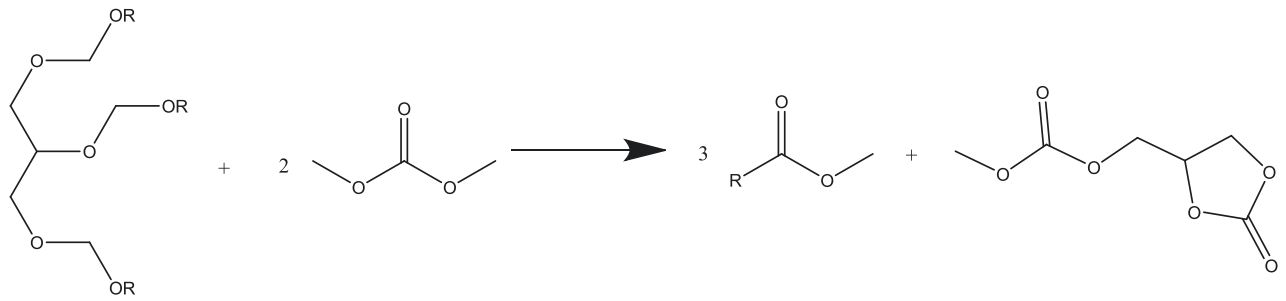

Fig. 1. Schematic reaction of triglycerides and DMC to yield FAME and glycerol dicarbonate.

formation of fatty acid alkyl esters (methyl esters if methanol is used as the acyl acceptor) and glycerin, which exist as two distinct immiscible phases [5–6]. A challenge associated with this method, in addition to separation steps for the two resulting phases, is the substantial generation of glycerol as a co-product (10 kg of glycerol for every 100 kg of biodiesel). This overabundance of glycerol in the market has led to its decreased market value in recent times [7]. In response to this situation, an alternative approach proposes the substitution of alcohols with dimethyl carbonate (DMC) as the acyl acceptor during the synthesis reaction. This modification results in the production of glycerol carbonate (Fig. 1), which is miscible with biodiesel and obviates the need for costly phase separation stages in the process [8].

On the other hand, the biocatalytic approach presents several advantages over the conventional chemical method, including reduced energy consumption and cost due to lower operating temperatures, streamlined processes, and avoidance of wastewater treatment [9,10]. Consequently, lipases (triacylglycerol acylhydrolases E.C. 3.1.1.3) are being explored as substitutes for the current acid or alkaline catalysts. Another intriguing benefit of utilizing these enzymes is their capability to act on non-edible substrates containing over 80 % of free fatty acids (a situation that would necessitate pretreatment in a chemical-based process) [11]. However, the enzyme-based alternative is still undergoing research to enhance the resilience of the biocatalyst (to prevent deactivation) and reduce process duration.

Potential approaches to address these challenges revolve around the incorporation of innovative solvents such as ionic liquids. However, our research group has established that only a limited number of families, centered around the cholinium cation and amino acid anions, exhibit biocompatibility with commercial lipases. This selection mitigates the adverse impacts on biocatalytic efficacy that are typically associated with conventional families, such as imidazolium-based compounds [12–14]. Conversely, the integration of extremophilic enzymes can serve as an additional advantage, contributing to the pursuit of a resilient transesterification catalyst. These enzymes are those produced by organisms thriving in extreme conditions of pH (alcalophiles or acidophiles), temperature (thermophiles or alkalophiles), salinity (halophiles), pressure (piezophiles), humidity (xerophiles) or radiation (radiophiles) [15]. Thus, halophilic and thermophilic lipases could be excellent candidates to withstand the presence of chemicals employed in transesterification reaction (lipid material, acyl acceptor, neoteric solvents, glycerine/glyceril dicarbonate and methyl esters).

In view of the above and given the absence of studies of extremolipase-based glycerol-free transesterification reactions, in-house synthesized extremophilic lipases from *Halomonas* sp. LM1C and *Thermus thermophilus* HB27 [16–18] were selected to be employed together with different amino acid (glycine, leucine, asparagine, glutamine and alanine) and dipeptide-based (glycyl glycine, glycyl leucine, glycyl asparagine, glycyl glutamine and glycyl alanine) ionic liquids belonging to cholinium family.

2. Materials and methods

2.1. Materials

Glycine (purity ≥ 99 %), L-alanine (purity ≥ 99.5 %), L-leucine (purity ≥ 99.5 %), L-aspartic acid (purity ≥ 99 %), L-glutamic acid (purity ≥ 99 %), glycyl-L-glutamic acid, glycyl-L-aspatic acid (purity ≥ 99), choline chloride (purity ≥ 98 %), dimethyl carbonate (purity ≥ 99 %), *p*-nitrophenyl laurate (purity ≥ 98 %), trizma base (purity ≥ 99 %) and 1-ethyl-3-methyl imidazolium ethylsulfate were purchased from Sigma Aldrich. Glycylglycine (purity ≥ 99 %), glycyl-L-alanine (purity ≥ 98 %) and glycyl-L-leucine (purity ≥ 99 %) were delivered by TCI Chemicals. amberlite IRN 78 hydroxide (purity ≥ 95 %) and 1-ethyl-3-methylimidazolium ethyl sulfate were acquired from Alfa Aesar and IoLiTec, respectively. Sodium carbonate (purity ≥ 99 %), calcium chloride (purity ≥ 99 %) were obtained from Scharlau chemicals. Methanol (purity ≥ 99 %) was supplied by Fischer Chemical and hydrochloric acid (purity ≥ 37 %) was sold by Panreac. *Candida antarctica* lipase B (CaLB) was kindly donated by Novozymes.

2.2. Synthesis of amino acid and dipeptide-based ionic liquids

The amino acid based ionic liquids used in this work were synthesized according to the procedure described elsewhere [19] and optimized as follows: ChOH aqueous solution was obtained after passing ChCl through an Amberlite IRN-78 anion exchange resin packed column. ChOH was then neutralized with an equimolar aqueous solution of amino acid by stirring at room temperature for 12 h. After neutralization, water was evaporated under vacuum at 323.15 K. The excess of amino acid was precipitated by adding methanol. The purity of the synthesized ionic liquids was always higher than 95 % and checked by NMR spectra. Karl-Fisher titration allowed determining the water mass fraction of the synthesized ionic liquid was below 4.0 % after having been submitted to vacuum drying for several days.

The structures of cholinium amino acid ionic liquids were confirmed by ^{1}H NMR where each spectra was recorded at 298 K on a Bruker AVANCE spectrometer operating at 400 MHz with D_2O as solvent. The chemical shifts were referenced to it as external standard and the spectra processing was performed with MestRe-C 4.7.0.0 software. The results are displayed below.

[Ch][Gly] δ: 3.17 (9H, s, $(CH_3)_3N$), 3.15 (2H, s, CH_2NH_2), 3.45 (2H, m, CH_2OH), 3.98 (2H, m, CH_2CH_2N). [Ch][Ala] δ: 1.29 (3H, d, CH_3CH), 3.23 (9H, s, $(CH_3)_3N$), 3.36 (1H, q, $CHNH_2$), 3.56 (2H, m, CH_2OH), 4.05 (2H, m, CH_2CH_2N). [Ch][Leu] δ: 1.02 (6H, t, CH_3), 1.46 (2H, m, CH_2), 1.71 (1H, m, CH), 3.24 (9H, s, $(CH_3)_3N$), 3.32 (1H, q, $CHNH_2$), 3.55 (2H, m, CH_2OH), 4.09 (2H, m, CH_2CH_2N). [Ch][Glu] δ: 2.15 (2H, m, CH_2), 2.44 (2H, t, CH_2), 3.23 (9H, s, $(CH_3)_3N$), 3.57 (2H, m, CH_2OH), 3.78 (1H, q, CH-N), 4.10 (2H, m, CH_2CH_2N). [Ch][Asp] δ: 2.73 (1H, q, CH_2), 2.85 (1H, q, CH_2), 3.22 (9H, s, $(CH_3)_3N$), 3.56 (2H, m, CH_2OH), 3.93 (1H, q, CH-N), 4.07 (2H, m, CH_2CH_2N). [Ch][Gly-Gly] δ: 3.18 (9H, s, $(CH_3)_3N$), 3.34 (1H, q, $CHNH_2$), 3.49 (2H, m, CH_2OH), 3.766 (1H, q, CH-N), 4.05 (2H, m, CH_2CH_2N). [Ch][Gly-L-Ala] δ: 1.39 (3H, d, CH_3), 3.24 (9H, s,

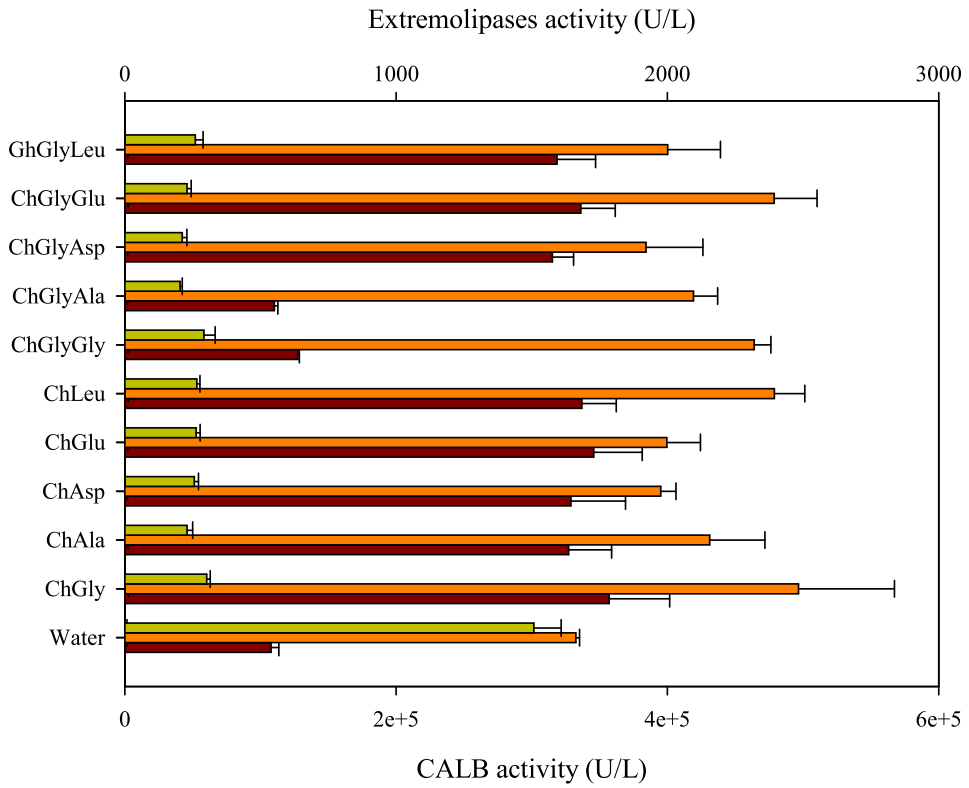

Fig. 2. Lipolytic activity of CaLB ( ), TtHB27L ( ) and HL ( ) in the presence of different amino acid and dipeptide-based ionic liquids belonging to cholinium family. (30 min in 1 M of ionic liquid at the optimum pH and temperature of each enzyme: CALB at 40 °C and pH 8, TtHB27L at 65 °C and pH8, and HL at 29 °C and pH 7).

$(CH_3)_3N$), 3.38 (1H, q, $CHNH_2$), 3.55 (2H, m, CH_2OH), 4.11 (2H, m, CH_2CH_2N), 4.22 (1H, q, CH-N). [Ch][Gly-L-Leu] δ: 0.96 (6H, t, CH_3), 1.64 (2H, m, CH_2), 3.23 (9H, s, $(CH_3)_3N$), 3.39 (1H, q, $CHNH_2$), 3.55 (2H, m, CH_2OH), 4.08 (2H, m, CH_2CH_2N), 4.24 (1H, q, CH-N). [Ch][Gly-L-Glu] δ: 1.95 (1H, m, CH_2), 2.18 (2H, m, CH_2), 2.29 (2H, t, CH_2), 3.242 (9H, s, $(CH_3)_3N$), 3.56 (2H, m, CH_2OH), 3.89 (1H, q, CH-N), 4.10 (2H, m, CH_2CH_2N), 4.24 (1H, q, CH-N). [Ch][Gly-L-Asp] δ: 2.56 (1H, q, CH_2), 2.81(1H, q, CH_2), 3.24 (9H, s, $(CH_3)_3N$), 3.553(2H, s, CH_2), 3.88 (1H, q, CH-N), 4.09 (2H, m, CH_2CH_2N), 4.51(1H, q, CH-N).

2.3. Microorganisms and culture conditions

The bacteria *Halomonas* sp. LM1C and *Thermus thermophilus* HB27 were cultivated as indicated in previous research works of our group [17,18]. In brief, *Halomonas* sp LM1C was cultured in casein peptone (7.5 g/L), yeast extract (10 g/L), $Na_3C_6H_5O_7{\cdot}2H_2O$ (3 g/L), KCl (2 g/L), $MgSO_4{\cdot}7H_2O$ (20 g/L), $MnSO_4{\cdot}H_2O$ (0.25 g/L), $FeSO_4{\cdot}7H_2O$ (0.05 g/L), NaCl (15 % w/v) and Triton X-100 (1 g/L) at pH 6.9 and 21.6 °C. Regarding *Thermus thermophilus* HB27, it was cultured in casein peptone (8 g/L), yeast extract (4 g/L) and NaCl (3 g/L) at pH 8 and 70 °C. All the media compounds were acquired from Merck. Cultures were performed in 250 mL Erlenmeyer flasks containing 100 mL of medium and capped with cellulose stoppers and 3 % of inoculum was added.

2.4. Lipolytic enzymes immobilization

Free *Candida antarctica* lipase and the lipolytic enzymes from *Halomonas* sp. LM1C and *Thermus thermophilus* HB27 were immobilized following the procedure reported previously [20]. Briefly, 1 mL of the enzyme solution (about 100.000 U/L of activity) was diluted in 3 mL of phosphate buffer (25 mmol/L, pH 7.0) and added to 1 g of Immobead®IB150 P epoxy resins. The mixture was kept under stirring in a shaker at 40 °C for 4 h. Vacuum filtration was then used prior to overnight drying at room temperature. Covalent binding was checked by a desorption assay with Triton X-100.

2.5. Obtaining of crude enzymes

After cultivation, the culture broth was centrifuged (10 min, 5000xg) to remove the biomass, and the postincubate concentrated by ultrafiltration with an AMICON cell, equipped with a YM-10 membrane (10 kDa-cutoff). This concentrated liquid constituted the crude enzyme solution.

2.6. Transesterification reaction

The transesterification reaction was carried out in 15 mL glass flasks containing sunflower oil as substrate and dimethyl carbonate (DMC) as acyl acceptor, at a molar ratio 1:6 and 30 % of enzyme (regarding oil mass). The flasks were maintained for 24 h in an orbital shaker operating at 250 rpm and 313.15 K [21].

2.7. Quantification of lipolytic activity

Lipolytic activity was determined through UV spectrophotometry at 600 nm by monitoring *p*-nitrophenyl laurate (2.5 mM) (Sigma-Aldrich) hydrolysis [22]. One activity unit is the amount of enzyme yielding 1 μmol of *p*-nitrophenol per minute at the assay conditions.

2.8. FAME analysis by gas chromatography

FAME quantification was carried out in accordance with EN14103 method. To do that, an Agilent GC-7820A equipment with a flame ionisation detector (GC-FID) and an Agilent HP-88 capillary column (30 m × 0.25 mm I.D. × 0.25 μm film thickness) was employed. The temperature of the injector and detector was kept at 523.15 and 553.15 K,

respectively. Helium was used as carrier gas at a flow rate of 1 mL/min and the split flow was set at 250 mL/min. The temperature was maintained at 393.15 K for 1 min, then increased up to 448.15 K (at a heating rate of 10 K/min) and kept for 10 min, and finally it was enhanced up to 493.15 K at a rate of 3 K/min, and held for 3 min at this value. A mixture (200 mg, 50 % w/w) of sample and internal standard (methyl nonadecanoate C19:0) was introduced in a 15 mL vial with 10 mL of toluene and vortexed. An aliquot was included in a 2 mL vial and the FAME content (%) was obtained as follows:

$$\text{FAME}\ (\%) = \left(\frac{\sum \text{A-A}_{\text{is}}}{\text{A}_{\text{is}}}\right) \bullet \left(\frac{\text{w}_{\text{is}}}{\text{w}}\right) 100, \quad (1)$$

being Σ the sum of the peak areas corresponding to methyl esters (C16:0-C24:1), A_{is} the peak area associated to the internal standard, w_{is} the weight of internal standard and w the sample weight (mg).

Density and viscosity were determined with a Anton Paar DSA-5000 M and Anton Paar Lovis 2000 M, respectively. Viscosity measurement was carried out after a calibration step with a capillary with a diameter of 1.59 mm using standards N-50 and N-80 provided by the manufacturer. Water content after transesterification was calculated by means of Karl-Fisher titration. Cetane number was determined in accordance with international standards [23].

3. Results and discussion

3.1. Influence of synthesized amino acid and dipeptide-based ionic liquids on biocatalytic potential of lipolytic enzymes

In previous research works, we have demonstrated the interesting potential of *Halomonas* sp. LM1C and *Thermus thermophilus* HB27 as sources of robust lipolytic enzymes [16–18]. Hence, these enzymes were chosen for comparative analysis with one of the frequently used lipases in biocatalysis, *Candida antarctica* lipase B [24]. The impact of various amino acid and dipeptide-based ionic liquids featuring the cholinium cation was examined on these free enzymes. This assessment aimed to identify the optimal candidate for serving as a coadjuvant to lipases in glycerol-free transesterification reactions.Therefore, lipase activity was evaluated after half an hour in 1 M aqueous solutions of ChGly, ChGlu, ChLeu, ChAla, ChAsp, ChGlyGly, ChGlyGlu, ChGlyLeu, ChGlyAla and ChGlyAsp, and the results have been compiled in Fig. 2.

It is important to note that no buffer was introduced into the aqueous mixtures to prevent the introduction of additional ions that could disrupt a precise analysis. Furthermore, this decision was influenced by the inherent proton activity of the ionic liquid, which would impede buffering effects [25]. The main difference between the lipase B synthesized by *Candida antarctica* and the lipases from *Thermus thermophilus* HB27 and *Halomonas* sp. LM1C lays in the optimum pH: while the latter is active at acidic and neutral pH values [16], the two formers are more stable at alkaline pHs [26,27]. Consequently, the alkaline properties of the synthesized ionic liquids align better with both commercial and thermophilic lipases. Furthermore, this fact highlights the robustness of thermophilic lipolytic enzymes, as their activity consistently surpasses that of the other enzymes under study. The enhanced performance of thermophilic lipases has been attributed to factors such as the presence of chaperonin proteins, amino acid exchange, and the modulation of electrostatic or hydrophobic interactions. [28,29].

Regarding ionic liquids, the lipolytic activity data reveal that cholinium glycinate is the solvent allowing greater biocompatibility with both commercial and thermophilic enzymes. This behaviour could be attributed to the fact that this particular amino acid is the smallest in the series. Small molecules of this kind have been documented to act as protein stabilizers or "chemical chaperones." This is due to their ability to facilitate accurate refolding in denaturation processes [30,31]. Additionally, glycine is the cheapest and most common amino acid [32] so there are no doubts that this candidate is the one offering greater advantages to be employed as adjuvant in glycerol free transesterification reactions. Therefore, commercial lipase B from *Candida antarctica* and lipolytic enzymes from *Thermus thermophilus* HB27 were selected for further study together with cholinium glycinate ionic liquid.

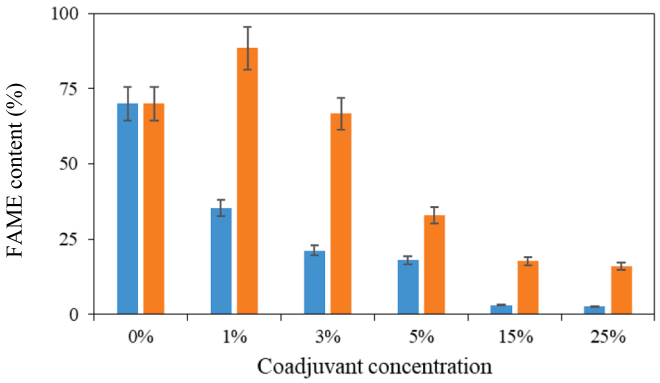

Fig. 3. Influence of coadjuvant concentration on the biodiesel conversion in the presence of CaLB: ( ) $C_2C_1imC_2SO_4$ ( ) ChGly. (Agitation: 250 rpm; volume: 15 mL; temperature: 40 °C; reaction time: 24 h, oil:DMC molar ratio 1:6; enzyme load 30 % (ww regarding oil mass).

3.2. Optimization of ionic liquid content in CaLB-based transesterification reaction

Before examining the effectiveness of ChGly, the capability of these lipases to facilitate glycerol-free transesterification of sunflower oil was initially explored. It was observed that the transesterification conversions achieved using the selected free lipases consistently remained below 1 % (data not presented). This pattern was observed regardless of the molar ratio of oil to acyl acceptor employed. A sensitive point of the process is the biocatalytic potential of the enzymes under investigation, wherein the acyl acceptor could potentially impede their lipolytic activity. In order to substantiate this, the lipolytic activity was tracked throughout the transesterification process, and in all cases, no activity was detected.

To solve this problem, a commercial immobilized CaLB was included in the reaction medium, and the transesterification conversion was measured after 24 h. The reason for betting in this lipase is that the commercial enzyme may offer valuable information prior to carry out the research work in the presence of extremolipases. Conversion values exceeding 70 % substantiate the soundness of the immobilization strategy, aligning with various prior research studies that have highlighted increased lipase robustness following immobilization. This enhancement could be attributed to the augmentation in the number of acidic groups, resulting in heightened polyanionic characteristics [33]. Moreover, immobilization has been associated with bolstered enzyme rigidity stemming from reduced conformational flexibility [34]. This factor could potentially elucidate the superior biocatalytic performance exhibited by immobilized CaLB in sunflower oil transesterification. Correspondingly, other commonly employed commercial immobilized lipases originating from different *Candida* species have demonstrated an expanded optimal temperature range relative to their free counterparts (shifting from 40 to 50 °C for the free lipase and from 20 to 50 °C for the immobilized form) [35].

Therefore, once the robustness of the biocatalyst was assessed, the effect of ChGly concentration in the reaction medium was evaluated for CaLB-based transesterification, considering values comprised between 1 % and 25 % (relative to oil content). Additionally, a conventional ionic liquid based on imidazolium cation was also included for comparative purposes. The results, displayed in Fig. 3, showcase the appropriateness of the suggested ChGly at minimal concentrations. This correlates with a notable enhancement of transesterification conversions, approximately

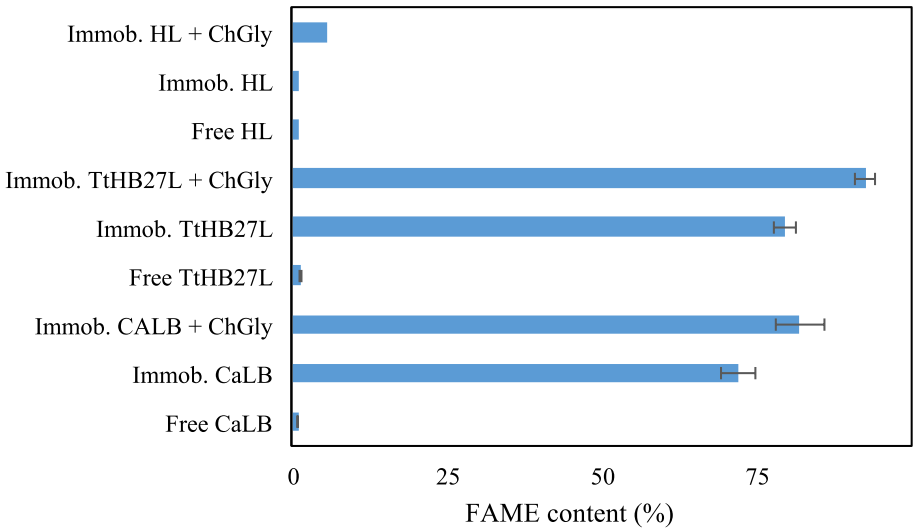

Fig. 4. FAME content for free and immobilized CaLB, TtHB27L and HL in the absence or presence of 1 % ChGly (Agitation: 250 rpm; volume: 15 mL; temperature: 40 °C; reaction time: 24 h, oil:DMC molar ratio 1:6; enzyme load 30 % regarding oil).

20 % higher compared to the control without ionic liquid. Conversely, the conventional imidazolium-based ionic liquid led to a reduction exceeding 50 % at lower concentrations, likely attributed to the well-established adverse impacts associated with this ionic liquid family [12].

Furthermore, it's noteworthy that employing higher concentrations of the ionic liquid leads to a substantial decrease in transesterification conversions. This outcome can likely be attributed to limitations in mass transfer arising from the heightened viscosity of the reaction medium. These findings hold significance as lower concentrations of the ionic liquid would be linked with reduced process costs. In this sense, the optimum results recorded at 1 % are eight times lower than the levels recently reported by Panchal et al [36] for soybean oil transesterification in the presence of methanol as acyl acceptor.

3.3. *Glycerol free transesterification in the presence of extremolipases and ChGly*

With the promising potential of ChGly-supported transesterification in the presence of CaLB established, an in-house immobilization approach was adopted for the studied extremophilic lipases. As depicted in Fig. 4, the results unequivocally demonstrate that this immobilization strategy proves effective in enhancing biocatalytic performance for both the commercial and thermophilic lipases. However, the selected immobilization technique does not yield an enhancement in the biocatalytic activity of the halophilic lipase, as conversions consistently hover near zero. This could potentially stem from the inherently deactivating nature of the transesterification reagents, coupled with the alkaline conditions prevalent in the reaction medium (that might be overly extreme for a lipase that optimally acts under near neutral conditions). In contrast, it is apparent that an immobilized thermoalkalophilic lipase, like the one sourced from *Thermus thermophilus* HB27, surpasses the catalytic potential of the commercial variant, achieving conversion values exceeding 90 % after 24 h of reaction.

In light of the promising results exhibited by TtHB27L, a comprehensive exploration of the glycerol-free transesterification process was conducted. Before tackling larger-scale operations, a pivotal prerequisite is the characterization of process kinetics. Thus, a comprehensive understanding of the evolution of transesterification conversions over time becomes indispensable for a thorough characterization of biodiesel production. Consequently, the conversion was closely monitored over a 24-h period in both the CaLB and TtHB27L-catalyzed reactions, both in the absence and presence of ChGly. This investigation aimed to validate the hypothesis that the pairing of extremophilic lipase with a biocompatible ionic liquid constitutes an effective combination, capable of not only yielding elevated biodiesel levels but also achieving rapid results. Fig. 5 reveals the pertinence of the hypothesis as the conversion values are increased in the order: TtHB27L + ChGly > TtHB27L = CaLB + ChGly > CaLB. In order to quantify these improvements, a logistic model widely described for microbial kinetics [37] was used for the first time to get further insight into the conversion data obtained throughout 24 h of transesterification reaction.

$$X = \frac{X_{\max}}{1 + e^{\left[\ln\left(\frac{X_{\max}}{X_0} - 1\right) - \mu t\right]}} \tag{2}$$

where the conversion percentage (X) at a certain time (t) is presented as a function of the maximum conversion ($X_{\max}$), the initial conversion (X_0) and the specific conversion rates (μ).

These parameters were obtained by means of SOLVER tool in Microsoft Excel, by minimizing the standard deviation (σ) calculated as follows:

$$\sigma = \left(\frac{\sum_i^{n_{DAT}} \left(z_{\exp} - z_{adjust}\right)^2}{n_{DAT}}\right)^{1/2} \tag{3}$$

being n_{DAT}, the number of experimental samples, $z_{\exp}$ and z_{adjust}, the experimental and theoretical data, respectively.

The low standard deviation values and the close-to-unity coefficient of determination (R^2) as listed in Table 1 substantiate the practical relevance of the proposed model in describing experimental data, regardless of the experimental conditions or the biocatalyst employed. This assertion is further validated upon visualizing the transesterification conversions documented throughout the experiments, as depicted in Fig. 5. Furthermore, the $X_{\max}$ values compiled in Table 1 validate the preceding conclusions regarding the enhanced suitability of the immobilized thermophilic lipase in conjunction with ChGly. This combination results in a conversion improvement of over 20 % compared to CaLB. Additionally, it is worth noting that ChGly imparts an additional catalytic effect without inducing any discernible detrimental impact on lipase activity.

Conversely, another vital parameter in terms of industrial viability is the conversion rate, given that the time needed to achieve substantial conversion levels is crucial for positioning the biocatalytic option as a competitive alternative to the conventional chemical catalysts currently utilized on an industrial scale. In this regard, the efficacy of the proposed

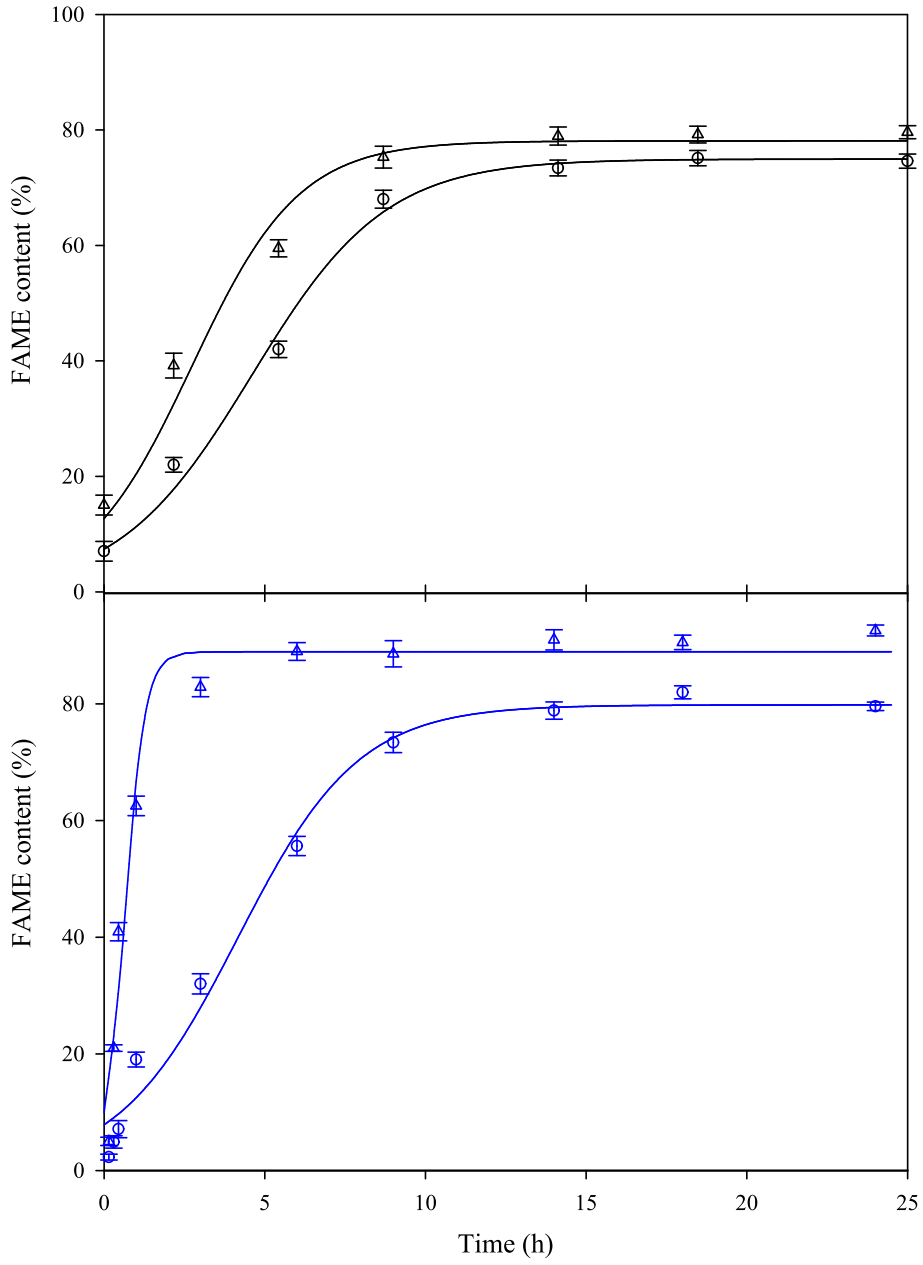

Fig. 5. FAME content in the absence (○) or presence (Δ) of ChGly for immobilized CaLB (black symbols) and TtHB27L (blue symbols). Experimental data are represented by symbols and solid lines refer to theoretical data obtained with the logistic model. (Agitation: 250 rpm; volume: 15 mL; temperature: 40 °C; oil:DMC molar ratio 1:6; enzyme load 30 % regarding oil). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Kinetic parameters for glycerol-free transesterification of sunflower oil in the presence of CaLB and TtHB27L with or without ChGly.

Immob. CaLB		Immob. CalB + ChGly		Immob TtHB27L		Immob. TtHB27L + ChGly	
X_0 (%)	4.5	X_0 (%)	7.8	X_0 (%)	7.1	X_0 (%)	13.1
X_{max} (%)	75.0	X_{max} (%)	79.8	X_{max} (%)	78.2	X_{max} (%)	91.6
μ (h^{-1})	0.523	μ (h^{-1})	0.533	μ (h^{-1})	0.653	μ (h^{-1})	2.828
σ	1.977	σ	3.637	σ	3.683	σ	5.131
R^2	0.996	R^2	0.988	R^2	0.988	R^2	0.989

combination of thermolipase + ChGly can be further affirmed by examining the substantial increase in specific conversion rate values. These values exceed the initial levels by over threefold when compared to the utilization of the commercial CaLB.

Moreover, it is worth noting that the specific conversion rate follows a similar sequence as the recorded conversion levels (TtHB27L + ChGly > TtHB27L = CaLB + ChGly > CaLB). This alignment once again underscores the compatibility of combining the chosen amino acid-based ionic liquid with the thermophilic lipase from *Thermus thermophilus* HB27. This union steers toward the development of a robust biocatalyst that holds potential to supplant prevailing chemical-based alternatives. In this context, it is remarkable that nearly complete conversion levels can be achieved in merely 3 h. This represents an improvement over values reported for a reaction at 50 °C with the same enzyme in triolein transesterification using alcohols (which generates glycerol as a

Table 2
Characterization of the obtained biodiesel.

Property	Value	Limit
FAME content (%)	96.0	96.5 ± 2.5 (min)
Density at 15 °C (kg/m^3)	900.1	860–900 ± 0.3
Kinematic Viscosity (mm^2/s)	4.452	3.5–5.0 ± 0.017
Water content (% ww)	0.039	0.05 (max)
Cetane number	52.3	51.0 ± 1.9 (min)

byproduct) [38]. Finally, in order to get more information on the biodiesel quality, several parameters defined in international standards [39] were checked and these data are presented in Table 2.

As it can be checked, all the studied properties fulfil the conditions required in international standards, so it can be concluded that the combination of extremozymes with biocompatible ionic liquids allows not only synthesizing biodiesel in reduced times (8 times lower than that required with commercial enzymes and other neoteric solvents, as cited elsewhere [21] but also getting high quality biodiesel.

4. Conclusions

This study has showcased the efficacy of amino acid-based ionic liquids in enhancing conversion rates during glycerol-free transesterification reactions, regardless of the immobilized biocatalyst being investigated. Following a preliminary assessment of various ionic liquids - comprising five amino acids and five dipeptides as anions - ChGly was identified as the optimal candidate for use as a supportive agent in immobilized biocatalyzed reactions. Determining the most effective concentration of ChGly (relative to sunflower oil content) preceded an in-depth investigation into its impact on both commercial CaLB and extremophilic lipases. The outcomes revealed significantly heightened conversions and specific conversion rates when employing the thermophilic lipase in conjunction with ChGly, surpassing the performance achieved through the control process (involving CaLB as the biocatalyst). These findings underscore the viability of synergizing biocompatible ionic liquids with robust extremophilic lipases as substitutes for the existing chemical catalysts utilized in industrial biodiesel production.

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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