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Assessing biocatalysis using dihydrolevoglucosenone (Cyrene™) as versatile bio-based (co)solvent

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ABSTRACT

An emerging biogenic aprotic solvent is the cellulose-derived dihydrolevoglucosenone, or 6,8-dioxabicyclooctanone (Cyrene™). This paper explores the use of Cyrene in lipase-catalyzed biotransformations, both in aqueous solutions – as co-solvent – as well as non-conventional media for synthesis. Cyrene is useful as organic solvent for lipophilizations using benzoic acid and glycerol as a model system for substrates with unpaired solubilities. The immobilized lipase B from *Candida antarctica* is active in Cyrene, yielding up to ~ 10 g product L^{-1}. Interestingly, crosslinked aggregates immobilized lipases (CLEA) remain significantly stable in Cyrene, enabling its use along several catalytic cycles. Cyrene is highly hygroscopic and forms geminal-diol structures with water, leading to solvent mixtures with a (tailored) gradient of polarities, what may be promising for biocatalysis in aqueous solutions. CALB displays hydrolytic reactions with different proportions of Cyrene as cosolvent, and CLEA derivatives remain fully active over 6 cycles at 30 % v/v Cyrene.

1. Introduction

The quest of novel environment-friendly solvents is currently an important trend in Sustainable Chemistry, as solvents impact significantly in the ecological footprint of chemical processes [1–4]. An increasing number of promising solvents are becoming available, like 2-methyltetrahydrofuran (2-MeTHF) [5–8] cyclopentyl-methyl ether (CPME) [9–11], other biogenic alternatives (e.g. *p*-Cymene, etc.) [12,13], or even solvent-free processes. Solvents that may be versatile, tuneable, and able to dissolve chemicals with unpaired solubilities are particularly appreciated, as they may be adapted to many challenging synthetic processes. Herein, Deep Eutectic Solvents (DES) are prototypical examples, as the combination of hydrogen bond donor and acceptors may lead to solvents with different physical-chemical properties [14–16]. Connected to solvents, the use of enzymes as catalysts represents a valuable alternative for (sustainable) organic synthesis, with remarkable successful examples at industrial scale [17–19]. Combining environment-friendly solvents with biocatalytic reactions may become quite synergistic, in particular when continuous processes are implemented [20]. Identifying novel enzyme-compatible biogenic solvents is thus of high interest.

A recent promising alternative is the use of dihydrolevoglucosenone, or 6,8-dioxabicyclooctanone (Cyrene™) as dipolar aprotic solvent which could replace traditional aprotic solvents such as *N*,*N*-dimethylformamide (DMF) and *N*-methyl-2-pyrrolidone (NMP) [21,22]. Cyrene can be derived from cellulose through pyrolysis [23] and hydrogenation [24], or through a combination of both processes [25]. Very recently, alkene reductases have been proposed for the final step of the synthesis (double bond reduction) [26]. Cyrene has already shown interesting applications in different areas of (synthetic) chemistry, as recently reviewed [21]. Importantly, a promising feature of Cyrene is the fact that subsequent chemical derivatizations can be envisioned (*e.g.* Grignard chemistry on the ketone group), leading to a new family of (tuned) Cyrene-based solvents, with tailored properties [27]. Furthermore, Cyrene is highly water-miscible, presumably due to the formation of an equilibrium with its hydrate, forming a ketal, the Cyrene geminal-diol (Fig. 1). This phenomenon occurs also with 1,2-diols, allowing the formation of novel solvent types (the so-called Cygnets solvents family) [27,28]. Remarkably, these *in-situ* created derivatives may display a gradient of polarities depending on their proportions, rendering tailored solvents that may dissolve a broad range of organic molecules [28].

The potential of the versatility of Cyrene to create a novel family of biogenic and designed solvents – together with its high water-miscibility – may be of high relevance for biocatalysis, as mixtures of Cyrene-water (*e.g.* as co-solvent) with adapted properties can be

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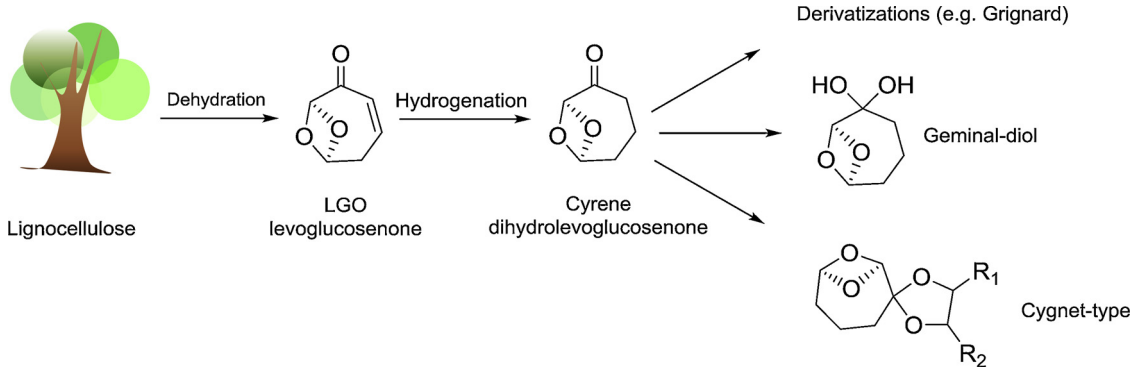

Fig. 1. Cyrene™ synthesis and some possibilities for derivatization to form a family of novel biogenic and tunable solvents [27,28].

envisaged. Moreover, Cyrene may be chosen in enzymatic synthetic reactions as well – using it as organic solvent to create a non-conventional media –, in which substrates with unpaired solubilities are employed. However, examples of using Cyrene in biocatalysis are still scarce and rather discouraging [12,29]. The use of Cyrene as organic media in the lipase-catalyzed esterification of 2-phenylpropionic acid with ethanol did not lead to conversion, while more apolar solvents (*e.g.* hexane or biogenic *p*-Cymene) did result in effective esterification [12]. Albeit, arguably, the low LogP of Cyrene (-1.52) [12] may become a hurdle for lipase-catalyzed synthetic reactions, the poor synthetic results obtained might also be partly explained by the enzyme inactivation at high acidic concentrations (200 mM of phenylpropionic acid) [12], which may have a more dramatic effect in (highly hygroscopic) polar media than in apolar ones. Herein, a derivatization of the solvent to create a more apolar system (see some potential options for Cyrene in Fig. 1), [27,28] while keeping efficient unpaired solubilities, would be a promising research line. A new family of biogenic (co)solvents would be thus available for biotransformations in aqueous and in non-conventional media.

This paper explores the use of Cyrene in biocatalysis, using it in hydrolytic reactions (as co-solvent), as well as in non-conventional media for lipase-catalyzed lipohilizations. Herein, benzoic acid and glycerol are used as prototypical substrates with unpaired solubilities, to render α-monobenzoate glycerol α-MBG (Fig. 2). Glycerol and benzoic acid are esterified by using immobilized lipase B from *Candida antarctica* (CAL-B). The α-MBG has applications as emulsifier agent [30], plasticizer [31], as well as building block in pharmaceuticals [32–34]. Due to the unpaired polarities of the substrates, many solvents have already been evaluated, leading to moderate-to-high conversions (over 60 %) [30,35,36]. A particularly relevant example is the use of Deep Eutectic Solvents (DES), which are efficient media for these enzymatic reactions [37–40].

2. Materials and methods

2.1. Chemicals and materials

Cyrene™, benzoic acid and glycerol were purchased from Sigma and the biocatalyst Novozym 435 was kindly donated by Blumos S.A (Chile). All reagents were used without further purification.

2.2. Protocol for CLEAs preparation

Crosslinked aggregates of CALB (CALB-CLEA) were prepared by mixing the liquid enzyme (2.5 mL) from the original preparation with 25 mM phosphate buffer pH 7 (2.5 mL; 1:1 v/v). This enzyme solution was enriched by BSA in a mass ratio of enzyme/BSA = 2.5 until the BSA dissolved completely by shaking gently to avoid generating foam. To this enzyme/albumin solution, 10 mL of a saturated solution of ammonium sulfate was added dropwise in a cold bath at 4 °C. To achieve a complete precipitation of the proteins present, the solution was kept for 30 min. Then glutaraldehyde (25 % v/v) was added dropwise in a molar ratio of glutaraldehyde/enzyme = 0.027 (2 mL) and was allowed to react with constant stirring at 300 rpm for 1.5 h at 4 °C. The resulting suspension containing the CLEA was centrifuged at 5 °C for 10 min at 10,000 *g* and the supernatant was discarded. Later on, the CLEA was re-suspended in 100 mM phosphate buffer pH 7 and was re-centrifuged at 10,000 *g* for 5 min, this operation was repeated three to four times until the biocatalyst was washed. The resulting biocatalyst was stored at 4 °C [39].

2.3. Enzyme activity determination

Enzymatic activity was determined by measuring the absorbance at 348 nm, produced by the release of *p*-Nitrophenol (p-NP) resulting from

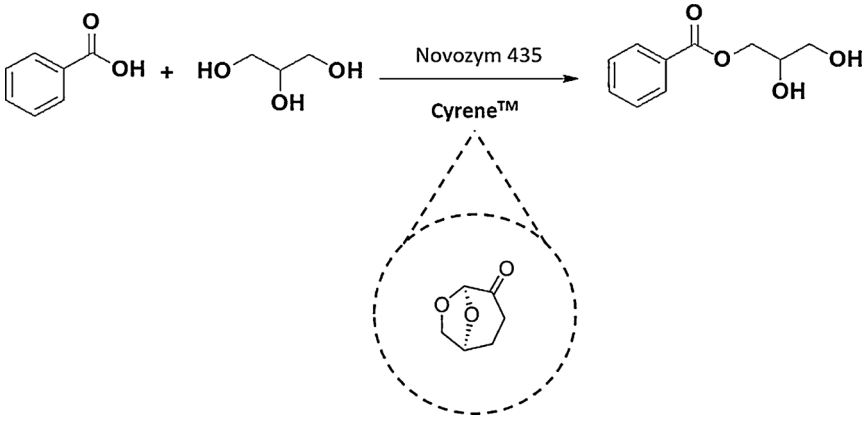

Fig. 2. Lipase-catalyzed esterification of benzoic acid and glycerol to obtain α-MBG using Cyrene as reaction media.

the hydrolysis of 0.3 mM p-NPB (ε = 5.8 [Abs x L x mmol-1]) in 25 mM phosphate buffer pH 7 at 30 °C. An international unit of activity (IU) was defined as the amount of enzyme that hydrolyzes 1 μmol of *p*-NPB per minute [39].

2.4. General procedure for esterification

The esterification was performed in a sealed reactor, and the mixture (substrates and solvent) was shaken at 165 rpm at 60 °C. The reactor was loaded with the biocatalyst, and then the reaction started when the reaction media was added. The media was prepared by mixing the substrates (glycerol and benzoic acid) with the Cyrene™ at 60 °C for 40 min. The conversion was calculated according to Equation (1):

$$Conversion(\%) = \left[\frac{n_p - n_{po}}{n_{so}} \times \frac{v_s}{v_p}\right] \times 100$$

Where P = product; n_{Po} = amount of product P at the start of the reaction (mol); n_{So} = amount of substrate S at the start of the reaction (mol); n_P = amount of product P at the end of the reaction (mol); v_S = stoichiometric factor for substrate S; and v_P = stoichiometric factor for product P.

2.5. Operational stability

To determine the operational stability of the biocatalysts under reaction conditions, recycling of the biocatalysts was performed in sequential batch reactions with the same amount of biocatalyst per volume of reaction media in each batch. After each batch, the biocatalyst was recovered from the reaction medium by filtration for Novozym 435 and centrifugation at 10,000 rpm for CALB-CLEA and was washed three times with 25 mM phosphate buffer pH 7. The biocatalysts were then washed three times with 25 mM phosphate buffer pH 7.

2.6. Analytic method

The reaction product was quantified by UHPLC (Altus A-30, PerkinElmer with a diode array detector) with a Kromasil Eternity-2.5-C_{18} UHPLC column (100 × 4,6 mm I.D) at 273 nm, 30 °C and 0.5 mL/min. Mobile phase A = H_3PO_4 0.1 % and mobile phase B = Methanol. The gradients used are detailed in Table 1:

3. Results and discussion

3.1. Effect of operational conditions in synthesis reaction

The solubility of benzoic acid and glycerol in Cyrene was firstly assessed. In both cases, efficient dissolutions were observed up to 1 M of each substrate, demonstrating the potential of Cyrene to serve as solvent for challenging compounds with different polarities. It must be noted that the intended enzymatic reaction had been successfully assessed in dioxane as solvent as well, with excellent enantiomeric excess ((*R*)-enantiomer, > 99 %) [35]. It can be expected, thus, that dioxane may share some analogous properties with Cyrene (highly water miscibility, low LogP, polarity, etc.). Once the solubilities of benzoic acid and glycerol were demonstrated in Cyrene, the enzymatic reaction was firstly studied by using different conditions of temperature and biocatalyst loadings (Fig. 3). As observed, CALB can perform the lipophilization in Cyrene, reaching full conversion when relatively high enzyme loadings are added. The reaction reached higher rates when temperature increased up to 60 °C, as expected from an enzymatic process using thermostable CALB as catalyst.

Table 1
UHPLC gradients.

Time (min)	Mobile phase B (%)
0	30
14	44
14.2	30
20	30

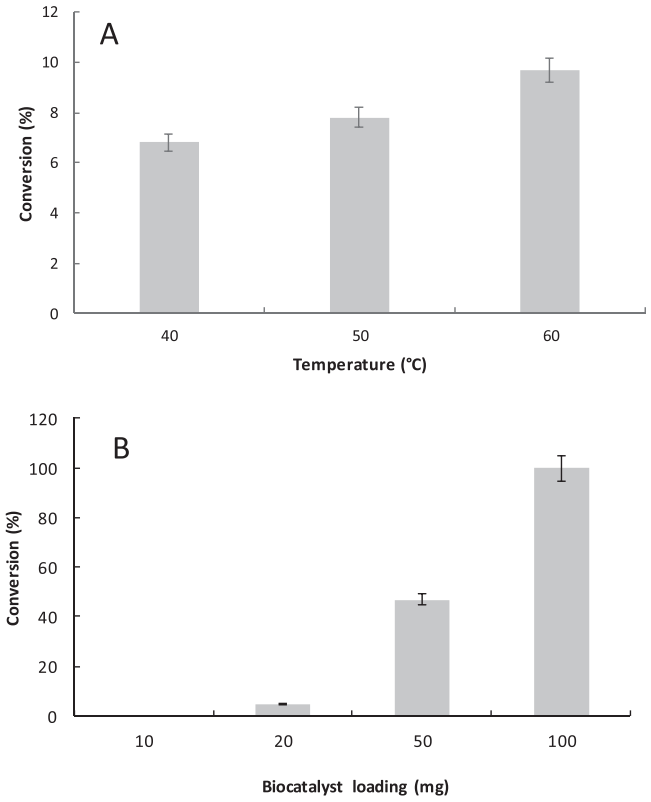

Fig. 3. Effect of temperature (A) and biocatalyst loadings (B) on the esterification of benzoic acid and glycerol in Cyrene. Reaction conditions: Figure A: 6 h of reaction, 100 mg of biocatalyst (Nov-435), 3 mL of reaction volume, 50 mM benzoic acid, 50 mM glycerol, different temperatures; Figure B: 24 h of reaction, 3 mL of reaction volume, benzoic acid 10 mM, glycerol 200 mM, different biocatalyst loadings (Nov-435). With 10 mg CAL-B no conversion was observed in the timeframe of the experiments.

In a subsequent set of experiments, the substrate ratio was evaluated, by performing the reaction at different concentrations of benzoic acid and glycerol (Fig. 4). To keep the acidic conditions low (up to 50 mM), increasing concentrations of glycerol were added to the reaction system. As depicted in Fig. 4 at a ratio of 50/400 mM (benzoic acid

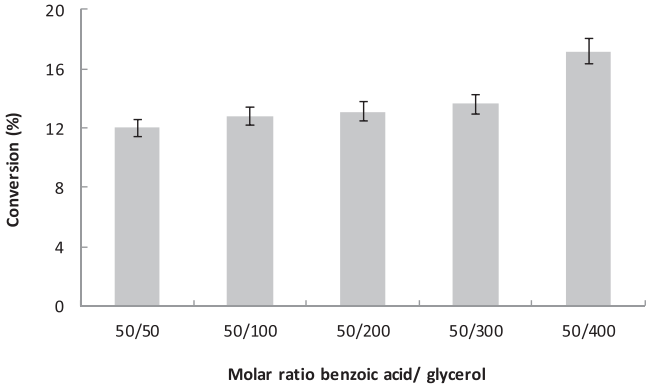

Fig. 4. Effect of molar ratio of substrates on conversion in the esterification to obtain α-MBG. Reaction conditions: 60 °C, 6 h of reaction, 100 mg of biocatalyst, 3 mL of reaction volume.

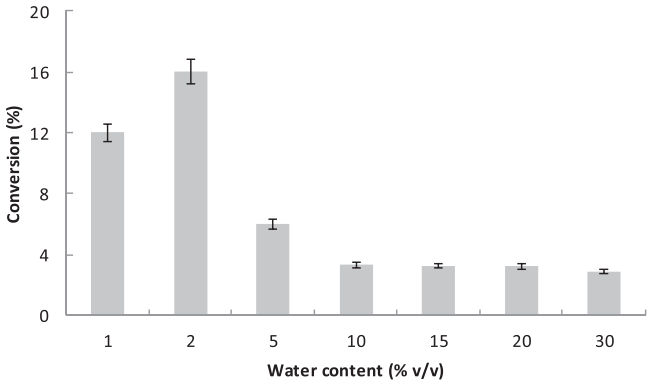

Fig. 5. Effect of water content on the conversion in the esterification to obtain α-MBG. Reaction conditions: 60 °C, 6 h of reaction, 100 mg of biocatalyst (Nov-435), 3 mL of reaction volume, 50 mM benzoic acid and 50 mM glycerol.

/ glycerol) the conversion increased by approximately 6 % with respect to the 50/50 mM ratio (benzoic acid / glycerol). Following classic premises of an esterification, this may be due to the hygroscopic effect of glycerol, which might reduce the available water, shifting the esterification to the product formation [29]. Nonetheless, in this case another aspect that may play a role is the (partial) formation of a Cygnet-type solvent between Cyrene and glycerol (see Fig. 1), which might modify the apparent LogP of the newly solvent phase (Cyrene, Cyrene geminal diol, Cygnet-glycerol), and would then create a more hydrophobic media, which could be beneficial for the lipase synthetic performance. The gradient-like distribution was evidenced by Costa Pacheco et al. [27], with studies conducted with Cyrene-water mixtures. The enzymatic reactive system of our work appears to be more complicated, as glycerol and benzoic acid are also present.

Triggered by the changes observed in the conversion, the effect of water was subsequently assessed in the reaction. To this end, different amounts of phosphate buffer (pH 7) were added to the Cyrene solvent, in the range of 0–30 % (v/v) (Fig. 5). As depicted in Fig. 5, the reaction displayed its optimum performance at an addition of 0–2 % phosphate buffer (v/v) and decreased significantly at higher amounts. Several aspects may be playing a role in these results. Firstly, the lipase may need some water to keep its active structure, which would explain why some water addition is beneficial (Fig. 4). Secondly, Cyrene is hygroscopic, and may probably absorb some of the added water, leading to some geminal-diol solvent system, which may exert some beneficial effect for the lipase (Fig. 1). Thirdly, a higher amount of water (> 2 % v/v) may probably influence the esterification equilibrium, shifting it back to the substrate side (glycerol and benzoic acid). The reaction mechanism is an enzymatic hydrolysis reaction, in which the enzyme catalyzes the breakdown of the linkages generating glycerol, benzoic acid and water. With respect to Cyrene when forming derivatives with water, it would act as water sink, suppressing (partly) the hydrolysis reaction.

In a subsequent set of experiments, the reaction was studied in terms of productivities, using different amounts of benzoic acid (10 and 50 mM). Results are depicted in Fig. 6. At low concentrations of benzoic acid (10 mM), full conversion was achieved in less than 48 h, accounting for a (still suboptimal) productivity of ~3 g product L^{-1} d^{-1}. Remarkably, the enzyme resulted active in the solvent system and with 50 mM of benzoic acid, and the enzymatic performance remained at a constant rate for at least 50 h, accumulating 10 g product L^{-1} in that case.

To further assess the operational stability in Cyrene, the enzymatic esterification was carried out in sequential batch reactors, using the commercially available, immobilized CAL-B (Nov-435), as well as crosslinked aggregates of Lipase B from *Candida antarctica* (CALB-CLEA) (Fig. 7). Operational stability was carried out in sequential batch

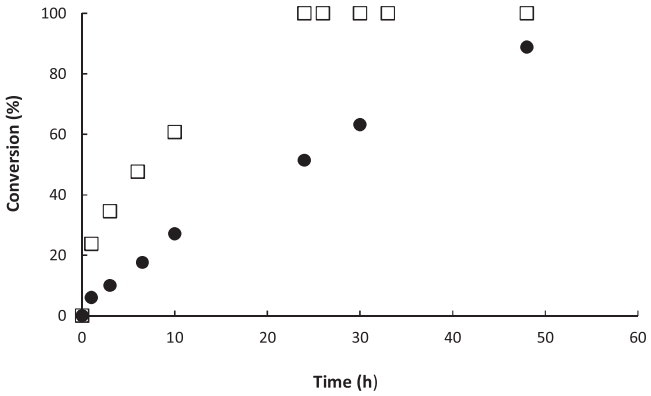

Fig. 6. Kinetics of enzymatic esterification to produce α-MBG using Cyrene™as a reaction media. Reaction conditions: 48 h of reaction, 333 mg of biocatalyst (Nov-435), 10 mL of reaction volume, molar ratio benzoic acid/ glycerol = 1/ 20 and 60 °C. (●) 50 mM benzoic acid; (□) 10 mM benzoic acid.

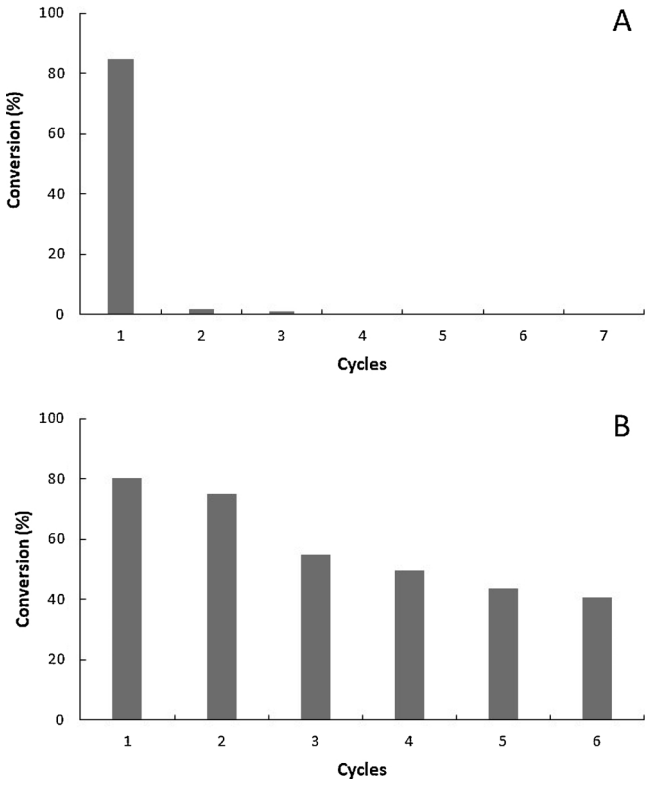

Fig. 7. Operational stability of the biocatalysts A) Nov-435 and B) CALB-CLEA. Reaction conditions: 24 h reaction, 67 mg of biocatalyst, reaction volume 2 mL, 10 mM benzoic acid, 200 mM glycerol molar ratio substrates 1:20.

reactors keeping the same amount of biocatalyst by volume of reaction media. Before starting the next batch, the biocatalysts were washed with 25 mM phosphate buffer pH 7. Remarkably, the Nov-435 derivative loses its operational stability after the first cycle, whereas the CALB-CLEA remain active over several cycles, in which activity is gradually lost (ca. half of it after 6 cycles). Apart from the expected desorption of the enzyme and support dissolution in the Nov-435, the low LogP of Cyrene may play a deleterious role [41]. Interestingly, the CLEA derivatives seem to be more resistant to the solvent, presumably due to their covalent bonding [37].

3.2. Cyrene™ as cosolvent in hydrolysis reaction

Once the potential of Cyrene as solvent for non-conventional media

Table 2
Hydrolytic activity of Nov-435 and CALB-CLEA biocatalysts at different concentrations of Cyrene™.

Biocatalyst	Cyrene™ concentration % (v/v)	Enzymatic activity (IU/$g_{Biocat.}$)
Nov-435	0*	73,2
	20	66,1
	30	60,3
	40	15,7
CALB-CLEA	0*	67
	20	50,7
	30	47,6
	40	28,8

* The hydrolysis activity was determined in 25 mM of phosphate buffer pH 7.

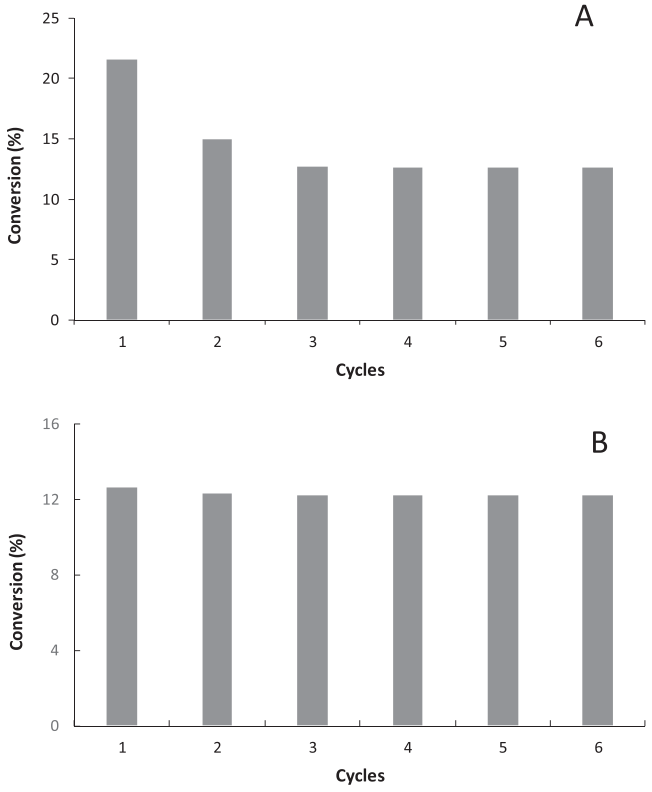

Fig. 8. Operational stability of the Nov-435 and CALB-CLEA biocatalysts in the hydrolysis reaction of *p*-NPB in Cyrene 30 % (v /v). Reaction conditions: 0.3 mM of *p*-NPB, 10 min, and 30 °C.

in biocatalysis was demonstrated, its assessment as co-solvent in aqueous media for hydrolytic reactions was conducted. To that end, both Nov-435 and CALB-CLEA biocatalysts were assessed in the hydrolysis of *p*-nitrophenol butyrate (*p*-NPB) using different proportions of Cyrene™ as co-solvent (0–40 % v/v) (Table 2).

Apparently (Table 2), the enzymatic activity decreased at higher proportions of Cyrene™, presumably due to deactivation of the biocatalyst. However, it may also be that the hydrolysis kinetics change when water activity decreases (at more proportions of co-solvent). To clarify in-depth this point, the operational stability of the biocatalysts in the hydrolysis was performed into sequential batch reactors (Fig. 8). As observed, both biocatalysts (Nov-435 and CALB-CLEA) retained activity over cycles. Remarkably, the CALB-CLEA derivative was totally stable over 6 cycles, clearly demonstrating that the combination of an adequate biocatalyst design with media engineering can be very powerful to set biocatalytic reactions. Further optimizations may involve stepwise additions of substrates, continuous reactors, etc. Moreover, the design of more robust biocatalyst variants that can be adapted for the solvent may become a promising research line, as recently shown for other solvents in a screening of transaminases [42]. Likewise, a thorough study on Cyrene, and how its tunability may change the LogP, and make it more adaptable to lipase-catalyzed reactions, may be a further line of research as well.

4. Conclusions

In summary, the use of Cyrene in biocatalysis, both as co-solvent for hydrolytic reactions, as well as non-conventional media for biocatalytic reactions has been assessed, using lipase-catalyzed lipophilization reactions involving substrates with unpaired solubility. Some lipase derivatives (CLEA) display promising operational activities in both reaction media. For instance, under reaction conditions of low benzoic acid loadings, some buffer addition (up to 2 % v/v), full conversion in the reaction is achieved. The achieved productivities are lower than those observed in other systems (e.g. Deep Eutectic Solvents), but further optimizations may be implemented, related to genetic design, enzyme immobilization, substrate dose, and reactor set-up. Beyond this proof-of-concept, Cyrene may become a very promising media for enzymatic processes, not only related to lipases, but also extendable to other enzymatic systems. Actually, based on its outstanding versatility – enabling the formation of tailored solvents –, combined with its high miscibility in water, what may allow its use as co-solvent as well, the use of Cyrene in other enzymatic systems using ketoreductases, transaminases, whole cells, etc., should be assessed. The fact that different Cygnet-type solvents can be formed and create a gradient-like of solubility properties of the system may in fact open exciting opportunities in biocatalysis, introducing a new palette of bio-based solvents for enzymatic reactions.

CRediT authorship contribution statement

Nadia Guajardo: Conceptualization, Methodology, Data curation, Investigation, Writing - original draft. **Pablo Domínguez de María:** Conceptualization, Methodology, Data curation, Writing - review & editing.

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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