An immobilized and highly stabilized self-sufficient monooxygenase as biocatalyst for oxidative biotransformations

Short title: Immobilized and highly stabilized self-sufficient monooxygenase for biocatalysis

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Abstract

BACKGROUND: The implementation of biocatalytic oxidation processes is currently limited by the requirement of expensive cofactors that must be efficiently recycled. In this research, a sustainable approach based on immobilized self-sufficient Baeyer-Villiger monooxygenases is discussed.

RESULTS: A bifunctional biocatalyst composed by an NADPH-dependent cyclohexanone monooxygenase (CHMO) and an $NADP^+$-accepting phosphite dehydrogenase (PTDH) catalyzes ε-caprolactone synthesis from cyclohexanone, using phosphite as a cheap sacrificial substrate for cofactor regeneration. Several immobilized derivatives of the fused enzyme have been prepared with high immobilization yield (97%); the one obtained by affinity adsorption on Co-IDA (IDA: iminodiacetic acid) support has shown to be highly stable conducting to average yields of 80 % after 18 reaction cycles.

CONCLUSIONS: The immobilized self-sufficient monooxygenase has demonstrated to be able to perform Baeyer Villiger oxidation with efficient cofactor recovery and biocatalyst recycling. The proposed biocatalytic process offers access to valuable molecules with high atom economy and limited waste generation.

Keywords: cyclohexanone monooxygenase (CHMO), phosphite dehydrogenase (PTDH), multi-enzymatic reactions, biocatalyst immobilization, biocatalyst reutilization, cofactor recycling

Introduction

Currently, oxygen functionalities represent the most abundant and important functional groups in chemicals and materials. Thus, oxidation reactions are essential in several industries. Conventional chemical oxidations are still widely applied in industrial environments for catalyzing oxidative reactions, even though they entail several disadvantages mainly related to environmental and safety issues.[1] These drawbacks are consequence of the use of i) toxic or hazardous oxidants (organic peracids, bleach, and bromide), ii) organic solvents and iii) heavy metal catalysts. Regarding oxidants, oxygen or air are the preferable compounds but aerating flammable solvents implies a significant safety concern that can only be addressed if water is used as reaction media. In that sense, the use of biocatalysts comes up as a greener and safety alternative since they can catalyze reactions in aqueous media.[1] Nevertheless, industrial scale implementation of biocatalytic processes using oxidoreductases is still scarce.[2]

One of the main hindrances of oxidoreductases to reach industrial environments is their requirement of stoichiometric amounts of expensive cofactors, usually NAD(P)H/NAD(P)$^+$. This cofactor dependence often leads to a non-economically viable process.[2,3] Studies on solving this problem can be found in the literature based on two main approaches: i) the use of whole cells instead of isolated enzymes in order to take advantage of the own cellular metabolism and ii) the development of alternative systems for cofactor regeneration when isolated enzymes are employed. The use of whole cells has several disadvantages such as substrates and/or product diffusional limitations through the cell membrane or the generation of non-desirable by-products.[3] Regarding the use of regenerating systems, several methodologies have been developed such as the chemical, electrochemical and enzymatic regeneration. However, the already available chemical and electrochemical methods do not fulfill all the requirements to be applied at industrial scale, especially the environmental

aspects.[4,5] Thus, the use of enzymes for cofactor regeneration has come up as a more efficient and greener option.[6]

Baeyer-Villiger monooxygenases (BVMO) are flavin-dependent enzymes capable of inserting an oxygen atom between a C-C bond adjacent to a carbonyl group, producing esters or lactones. These oxidized ketones are key building blocks for the synthesis of target molecules in pharma, food, fine and specialty chemical and material sectors.[7,8] Besides, BVMOs have been reported to be able to oxidize C=C bond yielding epoxides, and hetero-atom-containing molecules (e.g. amines or organic sulfides).[9–11]

Herein, an innovative self-sufficient Baeyer-Villiger monooxygenase is studied as an immobilized biocatalyst for such biotransformations. The oxidation of cyclohexanone to obtain ε-caprolactone, a compound of industrial interest for polymer synthesis (e.g. polycaprolactone and nylon 6)[12] is employed as a case study in this paper (Scheme 1). The biocatalyst is a fused enzyme made up of an NADPH-dependent cyclohexanone monooxygenase (CHMO) (EC 1.14.13.22) and an $NADP^+$-accepting phosphite dehydrogenase (PTDH) (EC 1.20.1.1), used for cofactor regeneration.[13,14] This bifunctional biocatalyst allows a multi-enzymatic one-pot reaction that requires a catalytic concentration of the cofactor $NADP^+$, recycling the cofactor simultaneously by using phosphite as a cheap sacrificial substrate. The behavior of the immobilized self-sufficient CHMO/PTDH as a biocatalyst has been studied, looking for stable operation of the oxidative reaction with total cofactor recycling and efficient re-use of the fused enzyme.

Materials and Methods

Materials

High-density metal free agarose resins and 10% cross-linked agarose beads were purchased from Agarose Beads Technologies (Madrid, Spain), Eupergit®CM was acquired from Sigma-Aldrich (St. Louis, MO, USA). $NADP^+$ and NADPH were obtained from BONTAC Bio-

engineering (Shenzhen, Guangdong, China). All other reagents were of analytical grade and obtained from various commercial suppliers.

Enzyme production and purification

Chemically competent *E. coli* NEB10β cells were transformed with the previously described,[14] pBAD derived pCRE-CHMO vector harboring a 6xHis-PTDH-CHMO fusion construct. A 5 mL preculture was grown overnight at 37 °C from a single colony and then transferred to a 5 L flask with 1 L of TB medium containing 0.02% L-arabinose and 50 µg/mL ampicillin. The protein was expressed at 24 °C for approximately 40 hours shaking at 135 rpm. Cells were then harvested by centrifugation (6,000×g for 15 min at 4 °C, JA-10.5 rotor, Beckman Coulter, USA) and resuspended in 5% of the culture volume with 50 mM Tris-HCl buffer pH 7.5. Cell-free extract (CFE) was prepared by sonication and subsequent centrifugation (15,000×g for 45 min at 4 °C, JA-17 rotor, Beckman Coulter). An empty XK 26/20 column (GE Healthcare Life Sciences, Chicago, USA) was packed with approximately 130 mL Ni-Sepharose (GE Healthcare Life Sciences) and connected to a Watson Marlow 120 peristaltic pump. The column was equilibrated with buffer (50 mM Tris-HCl pH 7.5) before the CFE was loaded. The column was washed with 3 column volumes (CV) of buffer and 3 CV of the buffer with 5 mM imidazole. Subsequently, the protein was eluted with buffer containing 500 mM imidazole. 15 mL batches of the purified protein solution were desalted on a HiPrep 26/10 desalting column (GE Healthcare Life Sciences), and then shock frozen in liquid N_2 after addition of 2% (w/v) sucrose for improved retention of enzymatic activity after lyophilisation.[15] The lyophilized protein was stored at -20 °C until further use. Per liter of culture, approximately 180 mg of pure enzyme was yielded.

Enzyme activity assays and protein content analysis

CHMO activity was determined spectrophotometrically by monitoring the decrease of NADPH at 340 nm (ε= 6.22 mM^{-1} cm^{-1}), using cyclohexanone as substrate. The reaction mixture contained 50 mM Tris-HCl (pH 8.5), 0.5 mM cyclohexanone, 0.1 mM NADPH and the appropriate amount of enzyme sample. One unit of CHMO (U) was defined as the amount

of enzyme required to catalyze the conversion of 1 μmol of NADPH to $NADP^+$ per minute at 20 °C and pH 8.5. PTDH activity was measured spectrophotometrically following the formation of NADPH at 340 nm. The reaction mixture contained 50 mM 3-(N-Morpholino) propanesulfonic acid, 4-morpholinepropanesulfonic acid (MOPS) (pH 7.25), 1 mM phosphite, 0.5 mM $NADP^+$ and the appropriate amount of enzyme sample. One unit of PTDH was defined as the amount of enzyme activity required to catalyze the conversion of 1 μmol of $NADP^+$ to NADPH per minute at 25 °C and pH 7.25. All activity assays were carried out using a Cary 50 Bio UV–visible spectrophotometer (Palo Alto, USA). Soluble enzyme measurements were performed using 1.4 mL quartz cuvettes; immobilized enzyme activity was measured using 3.5 mL quartz cuvettes and magnetic stirring.

Total protein content was determined using Bradford Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA), with bovine serum albumin as standard. The percentage of CHMO/PTDH in the lyophilized powder was determined by SDS-PAGE (NuPAGE 12%, Invitrogen, USA) and densitometry with Image Lab™ Software (Bio-Rad, USA).

Influence of pH on enzyme activity and stability

The pH dependence of CHMO and PTDH activity was evaluated by performing enzymatic activity assays replacing Tris-HCl or MOPS buffer by 50 mM acetate buffer (pH 5.0), Tris-maleate buffer (pH 6.0-7.5), Tris-HCl buffer (pH 8.0-9.0), or carbonate-bicarbonate buffer (pH 10.0). Enzyme stability was determined by placing 2 U of CHMO or PTDH on 2 mL of buffer solution and incubating each medium at 25 °C and mild agitation; aliquots were taken at different times and enzyme activity was measured.

Immobilization on agarose based supports

Metal-chelate support charged with Ni^{+2} (Ni-IDA; IDA: Iminodiacetic acid) was prepared by incubating the metal free agarose resin with a 0.2 M solution of $NiSO_4$ (pH 6.0-7.0); Co-IDA was prepared by incubating the agarose resin with 0.2 M solution of $CoCl_2$.[16] Immobilization of CHMO/PTDH on Ni/Co-IDA was carried out by suspending the support on 50 mM Na_2HPO_4, 300 mM NaCl, 20 mM imidazole solution (pH 8.0) in a 1:10 proportion (volume of support/total volume), then the enzyme was added and incubated for 20 minutes at 25 °C

and mild agitation. MANA-agarose (MANA: monoaminoethyl-N-aminoethyl) was prepared by etherification of 10% cross-linked agarose beads with glycidol followed by oxidation with periodate and subsequent modification of the highly activated support with ethylendiamine.[17] CHMO/PTDH was covalently immobilized on MANA-agarose with N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide (EDAC) as an activating agent. For this purpose, the support was suspended in 25 mM MES buffer (pH 6.5) (1:10 volume of support/total volume); CHMO/PTDH was added and left to ionically adsorb to the support for 15 minutes. Then EDAC was added to a final concentration of 25 mM in MES buffer and the mixture was left for 2 hours to allow the activation of the carboxylic groups transforming the ionic adsorption to a covalent one. The immobilization was carried out at 25 °C and mild agitation. Immobilization on Eupergit®CM was carried out by hydrating the resin for 24 hours in distilled water. After the hydration stage the resin was filtered and placed in 1 M potassium phosphate buffer (pH 8.0) in a 1:10 ratio (volume of support/total volume), then CHMO/PTDH was added and left to immobilize for 2 hours at 25 °C and mild agitation. Finally, the free epoxy groups of the resin were blocked with 0.2 M 2-mercaptoethanol for 4 hours at 4 °C. To characterize the time course of the immobilization, samples of suspension and supernatant were periodically withdrawn and CHMO activity was measured. In parallel, CHMO activity in a blank without agarose support was monitored along time.

Immobilization yield was defined as the difference between the total starting activity and the total residual activity in the supernatant related to the total starting activity (Eq. 1).

$$\text{Immobilization yield}\,(\%) = \frac{\text{Initial supernatant activity -Final supernatant activity}}{\text{Initial suspension activity}} \times 100 \quad (1)$$

Retained activity was defined as the difference between the total residual suspension activity and the total residual activity in the supernatant in relation to the total starting activity (Eq. 2).

$$\text{Retained activity}\,(\%) = \frac{\text{Final suspension activity - Final supernatant activity}}{\text{Initial suspension activity}} \times 100 \quad (2)$$

ε-caprolactone synthesis catalyzed by bifunctional CHMO/PTDH

Reactions were performed in a roller mixer at 25 °C at different pH: pH 7.0 (150 mM sodium phosphate buffer), pH 7.5 (150 Mm Tris-maleate buffer) or pH 8.0-8.5 (150Mm Tris-HCl buffer). The reactions were carried out in a medium containing 25 mM cyclohexanone, 1mM $NADP^+$, and 50 mM phosphite. For soluble enzyme, 5 U of the enzyme, based on monooxygenase activity, was added to the medium with a final reaction volume of 5 mL. Reactions with immobilized enzyme were carried out employing 1 mL of support loaded with 10 U of the enzyme, in a final reaction volume of 10 mL.

The atom economy (Eq. 3) and the reaction efficiency (Eq. 4) have been calculated as:

$$\text{Atom economy} = \frac{\text{Molecular mass of } \varepsilon\text{-caprolactone}}{\text{Molecular mass of all the reactants}} \quad (3)$$

$$\text{Reaction efficiency} = \text{Atom economy} \times \text{Product yield} \quad (4)$$

Reusability of immobilized bifunctional CHMO/PTDH

To evaluate the performance of the immobilized biocatalyst on multiple synthesis cycles, 3 mL of support (Co-IDA or MANA-agarose) loaded with 60 U of enzyme were added to the reaction medium with a final volume of 30 mL. CHMO activity and cofactor regeneration were assayed during the first cycle. After each cycle the support was recovered and washed with 150 mM sodium phosphate buffer (pH 7.0), then new reaction medium was added and substrate conversion and product yield were quantified.

Quantification of cyclohexanone and ε-caprolactone

Both ketone and lactone concentrations were analyzed by gas chromatography. Reaction samples were extracted with 20 volumes of ethyl acetate containing methyl benzoate (1 g/L) as internal standard, and the organic phase was analyzed using a 7890A gas chromatograph (Agilent Technologies, USA) equipped with an HP-INNOWAX 19095N-123 column (30 m, 0.53 mm, 1μm, Agilent Technologies). The column temperature was held at 50 °C for 2 minutes and increased to 240 °C at 20 °C/min and held at final temperature for 0.5 minutes. The injector temperature was kept at 250 °C; for the flame ionization detector, the temperature was 300 °C. Helium was used as a carrier gas at a flow rate of 16 mL/min. The side reaction product identity was confirmed by exact mass determination performed in a micrOTOF-Q mass spectrophotometer equipped with an electrospray ion source registering

in negative polarity (Bruker Technologies, USA). Exact mass calibration was performed with sodium formate. MS-ESI −: m/z=131.0712, calcd. for $C_6H_{12}O_3$: 131.0703.

Results and Discussion

Characterization of the biocatalyst: CHMO and PTDH

The bifunctional biocatalyst - cloned and expressed in *E. coli* - was tailored with a hexa-histidine tag to facilitate its purification by metal-chelate affinity chromatography. It was obtained as a single fusion protein made up of CHMO from *Acinetobacter calcoaceticus* NCIMB9871[18] covalently linked, through a short peptide, to a PTDH variant from *Pseudomonas stutzeri* WM88[19] for NADPH regeneration.

Protein content and enzymatic activity of both enzymes were tested, resulting in a specific activity of 3.2 U/mg of protein and 0.8 U/mg of protein for CHMO and PTDH, respectively.

Figure 1 shows the activity and stability at different pH values for both enzymes. CHMO has a maximum activity at pH 9.0; while 7.5 is the optimum pH for PTDH (see Figure 1A, and B). These data are in accordance with previously reported results[18,20] and indicate that the fusion did not affect the pH dependence of the fused enzymes as compared to the non-fused enzymes. The pH stability of the bifunctional biocatalyst has been studied in order to set up the optimum conditions for biocatalyst immobilization and for ε-caprolactone synthesis. CHMO was found to be very unstable at pH lower than 7.0 and higher than 9.0; being more stable in the range 8.0-9.0 (Figure 1C). After 7 hours of incubation at pH 7.0 and 7.5, the enzyme retained more than 40% of the initial activity. Conversely, PTDH demonstrated to be quite stable in the studied range, retaining around 70% of the initial activity at pH 7.0 and 7.5, and above 80% for pH 8.0-9.0 (Figure 1D). More acidic pH media were not studied for PTDH since CHMO already proved to be unstable at pH<7.0.

Coupled oxidation and cofactor regeneration

The evaluation of the bifunctional biocatalyst entailed the selection of reaction conditions compatible for the two enzymes involved in the oxidation of the substrate and the cofactor

regeneration. Taking into account the activity and stability results, four pH values (7.0, 7.5, 8.0 and 8.5) were selected to evaluate the behavior of the self-sufficient monooxygenase. Phosphite and $NADP^+$ were used as substrates, thus forcing the cofactor regeneration to start before the CHMO-catalyzed reaction (see Scheme 1). The oxidation of cyclohexanone with the concurrent regeneration of NADPH catalyzed by soluble CHMO/PTDH took place successfully in the whole selected pH range with complete substrate conversion in 3 hours. Figure 2 shows the time evolution of the reactions performed at pH 7.0 and 8.5. Although complete conversion of the ketone substrate was observed with 1 U of CHMO/mL of reaction, lactone yield was lower than 100% in all cases. As pH increased, lower yields were obtained, moving from 88% at pH 7.0 to 75 % at pH 8.5, and incubation of the reaction mixture for a longer time resulted in a decrease of the synthesized ε-caprolactone concentration in the alkaline range (see Figure 2B for pH 8.5 as an example).The mass imbalance suggested the presence of a side reaction. To confirm this hypothesis, cyclohexanone and ε-caprolactone were incubated separately for 24 hours in the reaction medium at pH 7.0-8.5; initial and final concentration of the compounds were compared (data not shown). Cyclohexanone exhibited very high stability for all the pH tested; on the other hand, ε-caprolactone concentration decreased at alkaline pH values. The product of the decomposition of ε-caprolactone was identified as 6-hydroxyhexanoic acid, produced by lactone hydrolysis. A compound with an exact mass corresponding to the mentioned hydroxyl acid was identified in samples of the reaction medium (see Materials and Methods). In consequence, further reaction experiments were carried out at pH 7.0 to minimize ε-caprolactone ring opening.

Biocatalyst immobilization

The immobilization/stabilization of the bifunctional biocatalyst was intended by affinity adsorption and by covalent attachment. Based on successful immobilization of different poly-His-tagged enzymes by affinity adsorption,[16,21] the immobilization on two metal-chelated supports was performed: Ni-IDA and Co-IDA. As can be seen in Table 1, derivatives on Co-

IDA retained higher activities than the Ni-IDA ones. The process of immobilization on Co-IDA was completed after 20 minutes resulting in an immobilization yield of 97.5% and a retained activity of 90.6% (see Table 1). As an alternative, enzyme immobilization through covalent attachment on different supports (MANA-agarose and Eupergit®CM) was also investigated. The immobilization on MANA-agarose was performed in a two-step procedure: an ionic adsorption followed by covalent bonding. The ionic stage was completed after 15 minutes with a 99% immobilization yield. EDAC was then added as carboxyl activating agent for promoting amide bond formation with the amine groups from the support. Under the best conditions (25mM EDAC for 2 hours), the immobilized biocatalyst showed a final retained activity of 69% (see Table 1). Immobilization on Eupergit®CM was also intended, obtaining high immobilization yield values (>95%) but very low retained activities (16%). The activity loss could be explained because the enzyme is covalently attached to the epoxy groups of the support mainly through their amino residues which are closely located to the catalytic site of CHMO.

Reactions catalyzed by immobilized CHMO/PTDH

The biocatalyst immobilized on Co-IDA was tested in a synthesis reaction at comparable conditions to the reactions with soluble enzyme presented in Figure 2A. An immobilized derivative with 10 CHMO U/mL of support was prepared and the reaction was performed by using 1 U of CHMO/mL of reaction at 25°C and at pH 7.0 to prevent ε-caprolactone hydrolysis. Substrate oxidation and product formation rates were very similar to the ones obtained with soluble enzymes at 1 U of CHMO/ mL of reaction (Figure 3), suggesting the absence of diffusional restrictions. Full ketone conversion was achieved after 3 hours of reaction and more significantly, the immobilized biocatalyst retained 100 % of its activity after all substrate had been consumed. On the contrary, soluble enzymes showed substantial deactivation over time (see Figure 2).The cofactor regeneration efficiency can be emphasized by examining $NADP^+$ and NADPH concentration throughout the reaction time; Figure 4A

and 4B show the cofactor profiles for reactions catalyzed by soluble and immobilized biocatalysts. As already stated, the cofactor was initially added as $NADP^+$ and an almost constant concentration of both oxidized and reduced forms is observed after a short initial reaction time. For this to happen, the rates of formation and consumption of $NADP^+$ and NADPH have to be the same and take on the value of the limiting (slower) reaction. As it can be observed, $NADP^+$ is the most abundant form throughout the reaction and only once cyclohexanone is completely oxidized, the equilibrium shifts completely towards NADPH. This behavior is common for both soluble and immobilized bifunctional catalyst and suggests the cofactor regeneration to be the limiting step in the reaction. Kinetic parameters for soluble CHMO fused to PTDH have been previously reported,[14] being k_{cat} 13 s^{-1} for CHMO and 1.5 s^{-1} [1] for PTDH. As the relative amount of both enzyme activities is maintained in the immobilized enzyme, this substantial difference could explain the relative low $NADP^+$ conversion. After complete cyclohexanone consumption, $NADP^+$ is almost totally converted to NADPH by the PTDH activity.

Reutilization of immobilized enzyme in multiple reaction cycles

Optimization of the reaction conditions has a substantial influence in preparative scale conversions catalyzed by Baeyer-Villiger monooxygenases. On this matter, other authors have dealt with the stabilization of CHMO through immobilization and the consecutive reutilization of the biocatalyst.[22,23]

The substantial improvement in stability by the immobilized bifunctional biocatalyst presents the possibility of its reutilization in successive cycles of oxidation and cofactor regeneration. With that aim, the biocatalysts immobilized on Co-IDA and MANA-agarose were employed, increasing their concentration to 2 U/mL of reaction to reduce the reaction time. The first reaction cycle was performed until 100% conversion was achieved; the reaction profiles are presented in Figure 5A and 5B. Total cyclohexanone conversion was reached after 2 hours for the biocatalyst immobilized on Co-IDA and 2.5 hours for the one immobilized on MANA

agarose, being both fully stable. However, a different course in the evolution of the cofactor concentration over time was observed for the enzyme immobilized on Co-IDA when using 2 U/mL of reaction (Figure 4C). Although a constant level for each reduced and oxidized form of the cofactor was again observed, the concentration of NADPH was higher than the concentration of $NADP^+$ along the reaction time, and there was no accumulation of $NADP^+$ as occurred at 1 U/mL. Aiming to understand these cofactor profile changes, initial rates of CHMO and PDTH-catalyzed reactions were calculated as the rate of ε-caprolactone formation and $NADP^+$ depletion respectively, and are reported in Table 2. As indicated by the initial effectiveness factor η_0 (calculated as the initial reaction rate divided by the initial reaction rate in absence of diffusional restrictions, i.e. soluble enzymes), there are no diffusional restrictions for the Co-IDA derivative at 1 U/mL of reaction. By contrast, at 2 U/mL, the CHMO-catalyzed reaction is diffusion-limited ($\eta_0<1$), while the PTDH-catalyzed reaction remains non-limited. This fact, at longer times, could explain qualitatively the profile shown in Figure 4C in which the cofactor recycling reaction is less limiting. Moreover, according to the data in Table 2, for the MANA-agarose derivative, both reactions are diffusion limited ($\eta_0<1$).

As previously stated, the stability of both immobilized bifunctional biocatalysts has been quantified by employing them in repeated reaction cycles. Each subsequent cycle lasted the same time and substrate conversion and product yield were determined. For the last cycle, the reaction progress was monitored until complete conversion was reached again. The results for CHMO/PTDH immobilized on MANA-agarose are presented in Figure 6A. As already indicated, total cyclohexanone oxidation in the first cycle was reached within 2.5 hours (cycle time) and 80 % ε-caprolactone yield was obtained. The biocatalyst was continuously reused for up to 10 cycles with a sustained decrease of conversion and product yield due to loss of activity of the immobilized bifunctional biocatalyst. On the last reaction cycle, 40% of

cyclohexanone oxidation was reached in 2.5 hours (cycle time) and 8 hours were necessary to achieve 99% conversion of the substrate.

The performance of the biocatalyst immobilized on Co-IDA is depicted in Figure 6B. In the first cycle, full conversion of cyclohexanone and a product yield of 84% were achieved after 2 hours (cycle time); furthermore, the immobilized biocatalyst was successfully reused for up to 18 cycles. In contrast to the results observed with MANA-agarose, almost total conversion of cyclohexanone was achieved for 10 cycles, and in further cycles, the conversion decreased moderately. After 18 cycles, the oxidation reaction catalyzed by the immobilized CHMO/PTDH still reached 74% conversion and 64% of product yield in 2 hours. The reutilization of CHMO/PTDH immobilized on MANA-agarose led to a final biocatalyst yield of 8.5 mg of ε-caprolactone/U of CHMO; for the enzyme immobilized in Co-IDA, this value increased to 20.6 mg of ε-caprolactone/U of CHMO (see Table 3). These values represent a 7.7 and 17 fold increased respectively when compared to the yields for just one cycle.

Conclusions

The CHMO/PTDH bifunctional biocatalyst has shown to be able to catalyze the Baeyer-Villiger oxidation reaction with simultaneous cofactor regeneration employing a cheap sacrificial substrate. The immobilized biocatalyst derivatives have been prepared with high retained activities leading to dramatic increase in stability. The results demonstrate that the biocatalyst immobilized on Co-IDA is very stable, catalyzes the target reaction efficiently, and proper cofactor recycling can be achieved. Moreover, the biocatalyst can be reused for up to 18 reaction cycles. Table 3 summarizes the comparison of the studied alternative with a chemical oxidation with oxygen (air) and acetaldehyde,[24] and a biocatalytic one without cofactor recycling. The atom economy and reaction efficiency of the biocatalytic process with immobilized bifunctional biocatalyst is much higher than the biocatalytic oxidation without cofactor recycling. In addition, the figures are similar to reported data for the chemical

process, with the advantage of milder operation conditions. Furthermore, the high stability of CHMO/PTDH immobilized on Co-IDA allows operation with a sustained high yield (87.7%) and reaction efficiency during 10 cycles and the process can be extended up to 18 cycles with a minor reduction of ε-caprolactone yield (80.5%). Although further case-by-case optimization of substrate concentrations and oxygen transfer rate is necessary, the immobilized self-sufficient oxidase has demonstrated to be a promising biocatalyst for Baeyer-Villiger oxidations, with the advantages of stable operation, efficient cofactor recycling, cheap sacrificial substrate, neutral pH, and biocatalyst recovery.

Acknowledgements

The research for this work has received funding from the European Union project ROBOX (grant agreement n° 635734) under EU's Horizon 2020 Programme Research and Innovation actions H2020-LEIT BIO-2014-1. This document reflects only the author's view and the Agency is not responsible for any use that may be made of the information it contains. The Department of Chemical, Biological and Environmental Engineering of UAB constitutes the Biochemical Engineering Unit of the Reference Network in Biotechnology and the research group 2014 SGR 452, Generalitat de Catalunya. Authors also thank COST Action CM 1303– Systems Biocatalysis for financial support and the Servei d'Anàlisi Química of UAB for their assistance on mass spectrometry analyses. Daniela Valencia acknowledges UAB for funding her Ph.D. grant.

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Tables

Table 1. Immobilization yield and retained activity of CHMO/PTDH immobilized on agarose based supports and Eupergit®CM. For immobilization conditions see Materials and Methods

Support	Enzyme load*	Immobilization yield (%)	Retained activity (%)
Ni-IDA	4 - 5 U of CHMO/mL	98.4	81.5
Co-IDA		97.5	90.6
MANA-agarose		98.5	69.3
Eupergit®CM		99.1	15.8

*No substrate transfer limitations were found at this enzymatic load

Table 2. Initial reaction rate and initial effectiveness factor for ε-caprolactone synthesis catalyzed by soluble and immobilized CHMO/PTDH.

Biocatalyst	Biocatalyst concentration ($U/mL_{reaction}$)	r_0 CHMO ($mM.h^{-1}$)	$r_{0,sp}$ CHMO ($mM.h^{-1}.U^{-1}$)	$\eta_{0,CHMO}$	r_0 PTDH ($mM.h^{-1}$)	$r_{0,sp}$ PTDH ($mM.h^{-1}.U^{-1}$)	$\eta_{0,PTDH}$
Soluble	1	9.1	9.1		0.93	0.93	
Co-IDA derivative	1	9.5	9.5	≈1	0.99	0.99	≈1
Co-IDA derivative	2	12.3	6.1	0.67	2.2	1.1	≈1
MANA derivative	2	10	5	0.55	0.66	0.33	0.35

r_0 CHMO: Initial rate of ε-caprolactone formation; $r_{0,sp}$ CHMO: Specific initial rate of ε-caprolactone formation (per unit of activity); r_0 PTDH: Initial rate of $NADP^+$ consumption; $r_{0,sp}$ PTDH: Specific initial rate of $NADP^+$ consumption (per unit of activity); $\eta_{0,CHMO}$: Effectiveness factor for the CHMO catalyzed reaction at time = 0 ; $\eta_{0,PTDH}$: Effectiveness factor for the PTDH-catalyzed reaction at time = 0. The units of the bifunctional catalyst are referred as CHMO units. Initial concentrations: [cyclohexanone] = 25 mM; [phosphite] = 50 mM; [$NADP^+$] = 1 mM. The reactions were performed at 25 °C and pH 7.0.

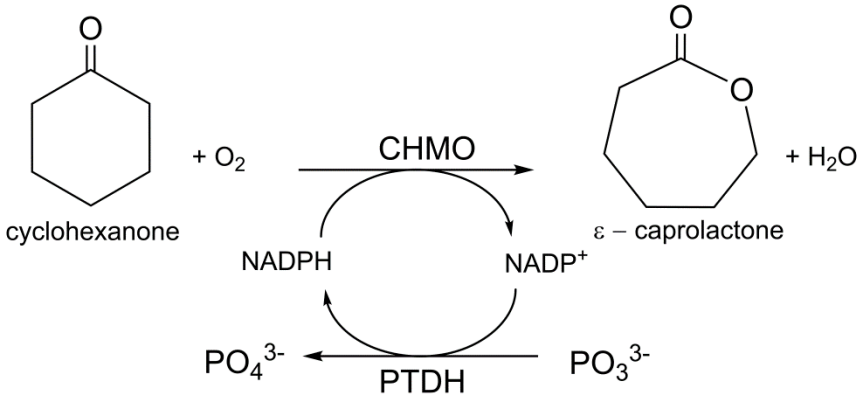
Table 3. Reaction efficiency and product production for ε-caprolactone synthesis catalyzed by enzyme immobilized on Co-IDA in a 30 mL reactor. Reported data of chemical oxidation[24]

and calculated data for a biocatalysis without cofactor recycling are presented for comparison purposes.

* Hypothetical process assumed to render the same yield as 1 cycle with the immobilized enzyme (2 U/mL). ** Cumulative yield. n.a. data not available.

Reaction system	**Biocatalyst**	**Atom economy (%)**	**Product yield (%)**	**Reaction efficiency (%)**	**Product (mg)**	**Specific production ($mg.U_{CHMO}^{-1}$)**
Chemical oxidation	None (metal catalyst)	54.4	90.0	49	n.a	-
No cofactor recycling*	Soluble/immobilized	13	84	10.9	71.6	--
Cofactor recycling	Immobilized (1 cycle)	54.7	84	46	71.6	1.2
	Immobilized (10 cycle)	54.7	87.7**	47.9	750	12.5
	Immobilized (18 cycles)	54.7	80.5**	44	1239	20.6

Schemes



Scheme 1. Oxidation of cyclohexanone to ε-caprolactone catalyzed by CHMO coupled to NADPH regeneration catalyzed by PTDH.

Figures

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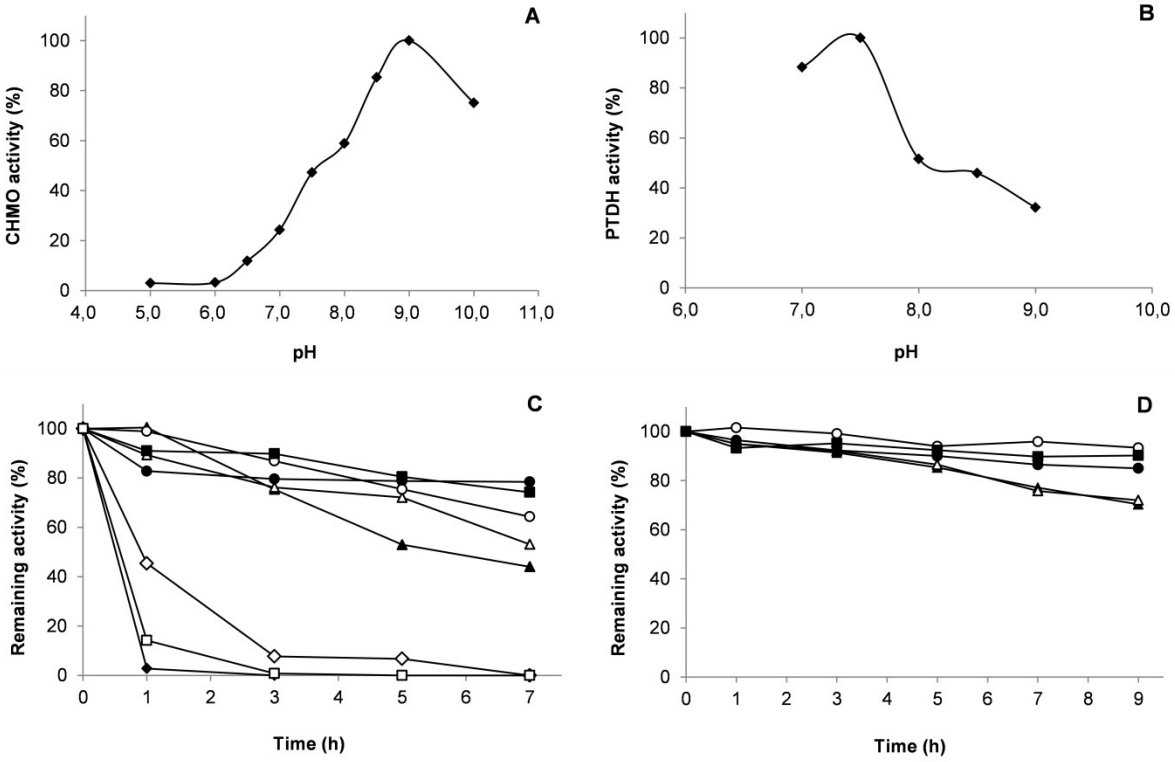

551**Figure 1.** Activity and stability profiles of CHMO and PTDH. (A) CHMO activity was
552measured at pH 5.0-10.0. (B) PTDH activity was measured at pH 7.0-9.0. (C) CHMO and
553(D) PTDH stability towards pH was assayed by incubating the enzyme (1U/mL) in 50 mM
554buffer: (◆) acetate pH 5.0; (•) sodium phosphite pH 6.0; Tris-maleate: (▯) pH 7.0, (△) pH
5557.5; Tris-HCl: (●) pH 8.0, (○) pH 8.5, (■) pH 9.0 and (□) carbonate-bicarbonate pH 10.0.
556Activity and stability assays were carried out at 25 °C.

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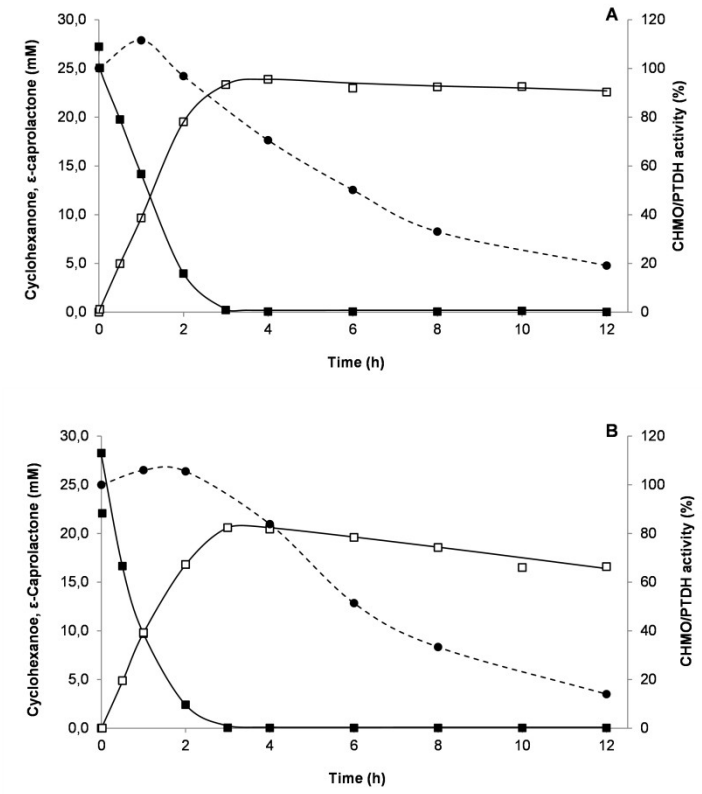

Figure 2. Oxidation of (■) cyclohexanone to (□) ε-caprolactone catalyzed by soluble CHMO/PTDH at pH 7.0 (A) and pH 8.5 (B). (●) Activity of the soluble bifunctional biocatalyst. The reaction medium contained 25 mM cyclohexanone, 50 mM phosphite, 1mM $NADP^+$ and 1 U of CHMO/mL of reaction. Oxidation was performed at 25°C.

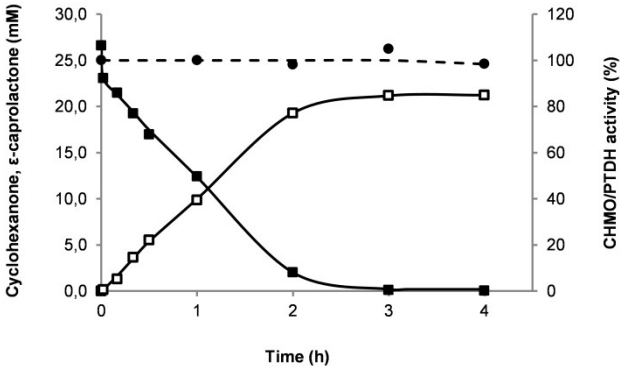

Figure 3. Oxidation of (■) cyclohexanone to (□) ε-caprolactone catalyzed by CHMO/PTDH immobilized on Co-IDA. (●) Activity of the immobilized biocatalyst. The reaction medium contained 25 mM cyclohexanone, 50 mM phosphite, 1mM $NADP^+$ and 1 U of immobilized CHMO/mL of reaction. Oxidation was performed at 25°C and pH 7.0.

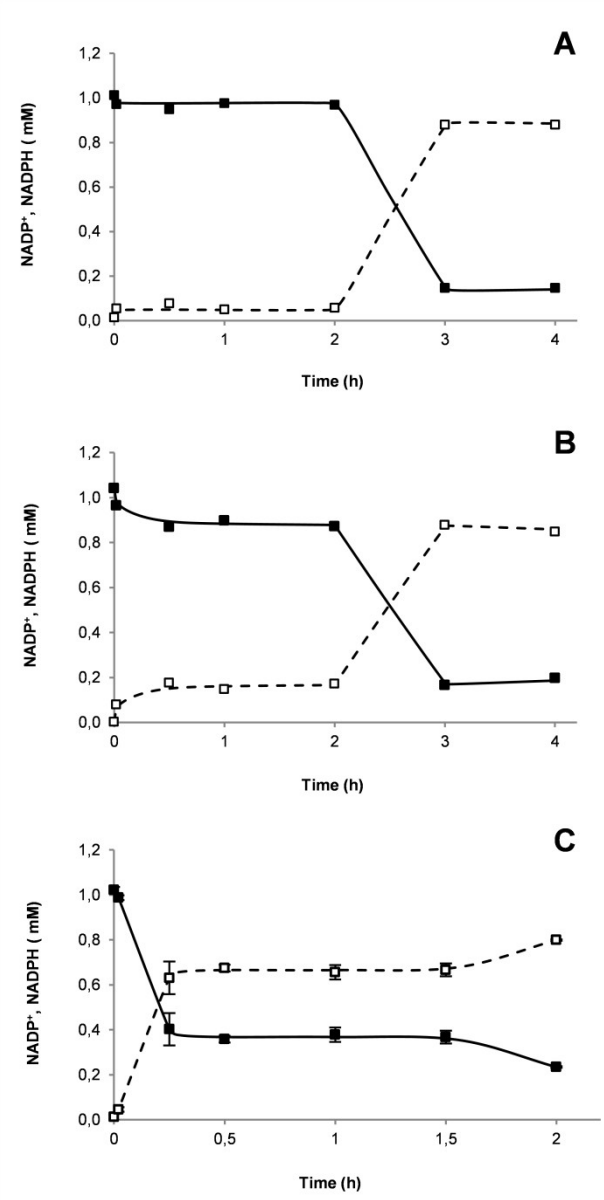

Figure 4. (■) Oxidized and (□) reduced cofactor profiles during cyclohexanone oxidation to ε-caprolactone catalyzed by CHMO/PTDH. A: soluble enzyme (1 U of CHMO/mL of reaction); B: enzyme immobilized on Co-IDA (1 U of CHMO/mL of reaction); C: enzyme immobilized on Co-IDA (2 U of CHMO/mL of reaction). The reaction medium contained 25

620mM cyclohexanone, 50 mM phosphite and 1mM $NADP^+$. Oxidations were carried out at 62125ºC and pH 7.0.

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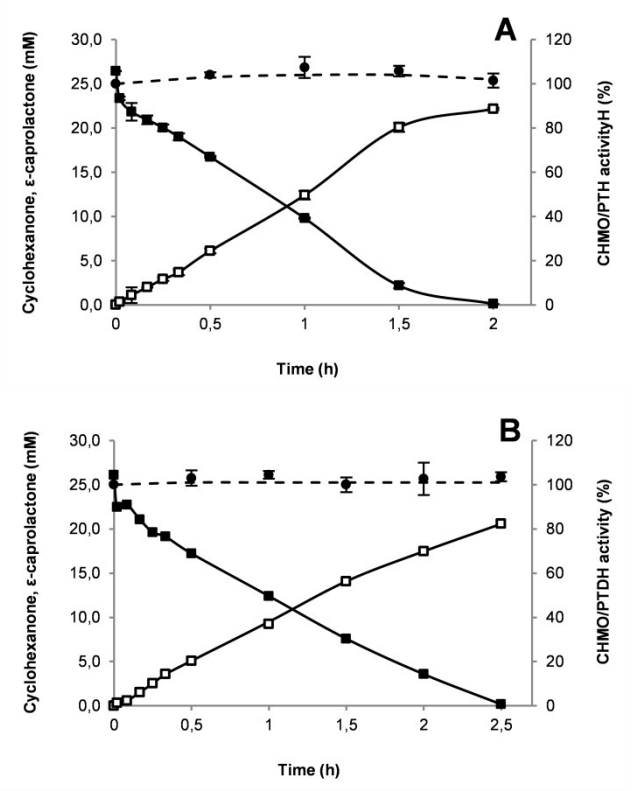

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635**Figure 5.** Oxidation of (■) cyclohexanone to (□) ε-caprolactone catalyzed by 636CHMO/PTDH immobilized on (A) MANA-agarose and (B) Co-IDA. (●) Activity of the 637immobilized biocatalyst. The reaction medium contained 25 mM cyclohexanone, 50 mM 638phosphite, 1mM $NADP^+$ and 2 U of immobilized CHMO/mL of reaction. Oxidations were 639performed at 25 °C and pH 7.0.

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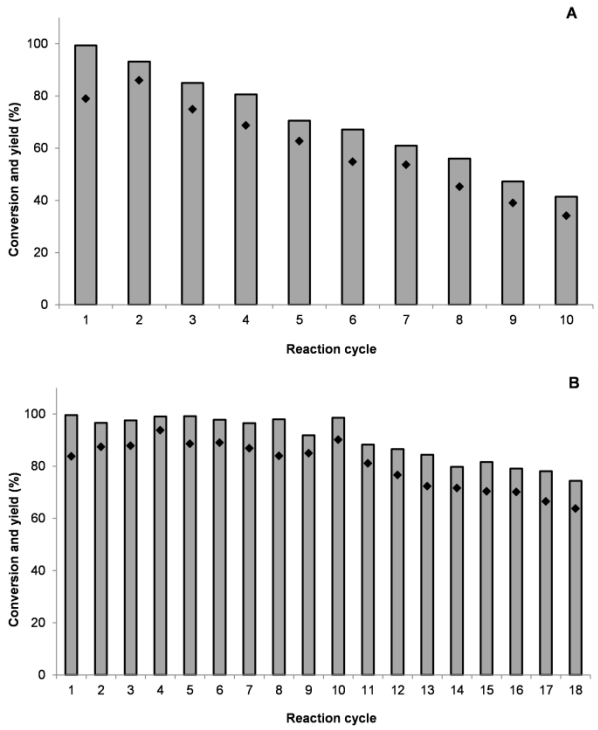

Figure 6. Successive oxidation of cyclohexanone to ε-caprolactone coupled with cofactor regeneration catalyzed by CHMO/PTDH immobilized on (A) MANA-agarose and (B) Co-IDA. Conversion (gray bars) and product yield (◆) after 2.5 or 2 hours (cycle time) are depicted. The reaction medium contained 25 mM cyclohexanone, 50 mM phosphite, 1mM $NADP^+$ and 2 U of immobilized CHMO/mL of reaction. Oxidations were performed at 25 °C and pH 7.0.