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High performing immobilized Baeyer-Villiger monooxygenase and glucose dehydrogenase for the synthesis of Ɛ-caprolactone derivative

Marie A. F. Delgove[a], Daniela Valencia[b], Jordi Solé[b], Katrien V. Bernaerts[a], Stefaan M. A. De Wildeman[a], Marina Guillén[b], Gregorio Álvaro[b]

[a] Maastricht University, Aachen-Maastricht Institute for Biobased Materials (AMIBM), Brightlands Chemelot campus, Urmonderbaan 22, 6167 RD Geleen, The Netherlands

[b] Bioprocess Engineering and Applied Biocatalysis Group, Department of Chemical, Biological and Environmental Engineering, Universitat Autònoma de Barcelona, 08193 Bellaterra, Catalonia, Spain

Corresponding author:

Gregorio Álvaro

Department of Chemical, Biological and Environmental Engineering, Universitat Autònoma de Barcelona, 08193 Bellaterra, Catalonia, Spain

Telephone: +34 93 581 2791

Fax: +34 93 581 2013

gregorio.alvaro@uab.cat

Authors' e-mail addresses

Marie A. F. Delgove[a]: marie.delgove@maastrichtuniversity.nl; Daniela Valencia: danielapatricia.valencia@uab.cat; Jordi Solé[b]: jordi.jole.jerre@uab.cat; Katrien V. Bernaerts[a]: katrien.bernaerts@maastrichtuniversity.nl; Stefaan M. A. De Wildeman[a]: s.dewildeman@maastrichtuniversity.nl; Marina Guillén[b]: marina.guillen@uab.cat

ABSTRACT

The industrial application of Baeyer-Villiger monooxygenases is typically hindered by stability and cofactor regeneration considerations. Stability of biocatalysts can be improved by immobilization. The goal of this study was to evaluate the (co)-immobilization of a thermostable cyclohexanone monooxygenase from *Thermocrispum municipale* (TmCHMO) with a glucose dehydrogenase (GDH) from *Thermoplasma acidophilum* for NADPH cofactor regeneration.

Both enzymes were immobilized on an amino-functionalized agarose-based support (MANA-agarose). They were applied to the synthesis of 3,3,5-trimethylcyclohexanone for the synthesis of ε-caprolactone derivatives which are precursors of polyesters. The performances of the immobilized biocatalysts were evaluated in reutilization reactions with up to 15 cycles and compared to the corresponding soluble enzymes. Co-immobilization proved to provide the most efficient biocatalyst with an average conversion of 83% over 15 reutilization cycles leading to a 50-fold increase of the biocatalyst yield compared to the use of soluble enzymes which were applied in a fed-batch strategy.

TmCHMO was immobilized for the first time, with very good retention of the activity throughout reutilization cycles. This immobilized biocatalyst contributes to the application of BVMOs in up-scaled biooxidation processes.

Keywords: biocatalyst immobilization, Baeyer-Villiger monooxygenase, lactone monomer, cofactor recycling, glucose dehydrogenase

1. INTRODUCTION

Enzymatic reactions have been identified as a sustainable technology since they usually follow the rules of green chemistry.[1,2] Oxidative biocatalysis, and Baeyer-Villiger monooxygenases (BVMOs) in particular, is an alternative of lesser toxicity compared to chemical oxidation.[3] BVMOs are biocatalysts capable of catalyzing the oxidation of (cyclic) ketones by inserting one atom of oxygen in a C-C bond, therefore generating water as by-product. BVMOs can catalyze the oxidation of a wide range of cyclic ketones of various ring size including alkyl substituted ketones, as well as perform enantioselective sulfoxidation.[4-6] These oxidative enzymes have been applied to the synthesis of intermediates for the pharmaceutical industry,[7-9] and chiral molecules for fine chemical and fragrances.[10] Additionally, several BVMOs have been identified as relevant biocatalyst for the synthesis of lactone as monomers for polymeric materials, for example, ε-caprolactone, either from whole-cell[11] or via a cascade reaction,[12] lauryl lactone,[13] a nitrile-substituted ε-caprolactone as precursor for polyamide,[14] and β,δ-trimethyl-ε-caprolactone (TMCL).[15,16] Alkyl substituted lactones are particularly interesting for the synthesis of polyesters with low glass transition temperature (T_g < 0 °C in general).[17] This property enables applications such as biodegradable plasticizers[18] or encapsulating agents for coating formulations[19] with polymers from TMCL for example.

The applicability of BVMOs is however hindered by their lack of robustness, either due to thermostability or to limited stability in the presence of organic solvents. Using protein engineering, several mutants of cyclohexanone monooxygenase with improved thermostability were created.[20-22] The discovery of new thermostable BVMOs contributes to the development of their applicability in biotransformations.[23-26] Recently, a cyclohexanone monooxygenase from *Thermocrispum municipale* DSM 44069 (TmCHMO; EC 1.14.13.22)

was identified as being particularly relevant for the preparation of lactones as polymeric building blocks due to its high thermostability, good resistance to organic solvents, and broad substrate scope towards cyclic ketones.[27,28]

Although TmCHMO has already been applied for the synthesis of ε-caprolactone derivatives from 3,3,5-trimethylcyclohexane, using either a self-sufficient fused biocatalyst[15] or a glucose dehydrogenase to regenerate the NADPH co-factor,[16] this enzyme has not yet been immobilized. Immobilization of whole-cells or isolated enzymes is indeed known to increase the operational stability of enzymes. Additionally, immobilization has several advantages including facilitating the recovery of the biocatalyst, decreasing the costs of downstream processing, and potentially decreasing the enzyme cost per kilogram of product, provided that the immobilized biocatalysts maintain their activity throughout the reuses.[29,30]

So far, isolated BVMOs have mostly been immobilized to polymeric supports by covalent binding.[31] For example, a cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* (AcCHMO) was immobilized on Eupergit (polyacrylamide based supported beads) *via* covalent binding with a glucose 6-phosphate dehydrogenase for the synthesis of chiral lactone building blocks.[32] Fusions of AcCHMO with a polyol dehydrogenase were similarly immobilized for the synthesis of ε-caprolactone.[33] The immobilized biocatalyst, however, displayed a low stability on the support and a poor operational stability. Recently, MANA-agarose (monoaminoethyl-*N*-aminoethyl)-agarose was identified as a suitable support for the immobilization of a fused AcCHMO-phosphite dehydrogenase (AcCHMO-PTDH).[34] For this enzyme, a higher retained activity was achieved with metal-chelate supports such as Ni-iminodiacetic acid (Ni-IDA) and Co-IDA.[34]

In this article, our goal is to expand the use of immobilized BVMOs and evaluate them for the synthesis of lactones as polymeric building blocks. The immobilization of TmCHMO and a

glucose dehydrogenase from *Thermoplasma acidophilum* (EC 1.1.1.47) (GDH-Tac) are described with the aim of oxidizing 3,3,5-trimethylcyclohexanone to alkyl substituted ε-caprolactone derivatives (Figure 1). The enzymes were immobilized on a MANA-agarose support, either separately or co-immobilized on the same support, by covalent bonding. The performances of the immobilized enzymes were evaluated in over 15 repeated biooxidation cycles and compared to the corresponding soluble enzymes.

2. EXPERIMENTAL

2.1.Chemicals.

3,3,5-Trimethylcyclohexanone (98%, Sigma-Aldrich), methanol (Biosolve), (+)-glucose (>99%, Alfa Aesar) were used as received. High-density aminoethyl 4BCL agarose (MANA-agarose, Agarose Beads Technologies) was stored at 4 ˚C. β-Nicotinamide adenine dinucleotide phosphate disodium salt ($NADP^+$, 97%, Alfa Aesar), and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC, ≥97%, Sigma-Aldrich) were stored at -20 ˚C. TmCHMO and GDH-Tac were produced and supplied by InnoSyn BV (Geleen, The Netherlands).

2.2.TmCHMO and GDH-Tac activity assays

TmCHMO activity in the CFE was determined spectrophotometrically following NAPDH consumption at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) with cyclohexanone as a substrate. The mixture contained Tris-HCl (50 mM, pH 8.5), cyclohexanone (0.5 mM), NADPH (0.1 mM). One unit of TmCHMO (U) was defined as the amount of enzyme required to catalyze the conversion of 1 µmol of NADPH to $NADP^+$ per min at 20 °C and pH 8.5.[34]

GDH-Tac activity was determined spectrophotometrically at 340 nm following the $NADP^+$ ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$, 400 µM) consumption using D-Glucose (200 mM) as substrate and sodium phosphate buffer 100 mM pH 8.0.[35] The basal production of NADPH by unspecific enzymes

present in the lysate was determined by this same test but avoiding the addition of substrate and adding buffer instead. This production rate is subtracted from the measurement with D-glucose. One unit of activity (U) was defined as the enzyme required to convert 1 µmol of $NADP^+$ per minute at those given conditions (30 ºC, pH 8.0). The absorbance was recorded using a spectrophotometer Cary 50 Bio UV-visible (Palo Alto, USA).

2.3.Preparation of immobilized TmCHMO and immobilized GDH-Tac

The general procedure for the covalent immobilization of the enzymes on MANA-agarose (density: 1.07 g mL^{-1}) comprised three main steps: i) the ionic adsorption of the enzyme to the support, ii) the addition of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) as an activating agent to promote amide bond formation between the support and the enzyme, and iii) the addition of NaCl to desorb all the enzyme that was not covalently bound to the support. After the immobilization, the derivatives were washed carefully.

The immobilization of TmCHMO was carried out by suspending the support in 25 mM MES buffer (pH 6.0); then the enzyme was added to the suspension and left to adsorb ionically to the support for 0.25 h. After that time, EDC was added to final concentrations of 25 or 35 mM and left for 2 h. Finally, NaCl was added to a final concentration of 1 M and incubated for 1 h. The immobilized derivative was washed with distilled water and filtered.

The immobilization of GDH-Tac was performed in 50 mM sodium phosphate buffer (pH 6.0). The ionic adsorption step was completed after 0.5 h. A 200mM stock solution of EDC was prepared, the pH was adjusted to 6.0 with HCl; different volumes were added to get final concentrations of 1, 3, 5, 10 or 15 mM and incubated for 1h. Afterwards, NaCl was added to a final concentration of 0.5 M and incubated for 0.5 h. Lastly, the support was washed gently with 100 mM sodium phosphate buffer (pH 8.0) and filtered.

For the co-immobilization of TmCHMO and GDH-Tac, the support was suspended in 50 mM sodium phosphate buffer (pH 6.0); both enzymes were added and incubated 0.25 h. After the ionic step was completed, EDC was added to final concentrations of 10 or 20 mM and incubated 1 h. NaCl was added to a final concentration of 1M. The derivative was washed with distilled water and filtered.

The characterization of the immobilization was carried out by measuring the activity of the supernatant and the suspension throughout the entire process, in order to determine the retained activity (Equation (1)) and immobilization yield (Equation (2)). TmCHMO and GDH-Tac immobilized on MANA-agarose were stored at 4 °C prior to use.

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

$$\text{Immobilization yield (\%)} = \frac{\text{Initial supernatant activity -Final supernatant activity}}{\text{Initial supernatant activity}} \times 100 \quad (2)$$

2.4.Determination of enzyme content

The cell lysate was pre-clarified by centrifugation (3220 g for 15 min.), and the total protein content was determined by means of a Bradford Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA) using bovine serum albumin as standard.

Enzyme content was assessed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (NuPage 12%, Invitrogen, USA) ran in a Mini-PROTEAN II apparatus (BioRad, USA) following the protocol of Laemmli *et al.*[36] Low range protein markers were used for molecular weight determination. Gels were stained using Coomassie G250 colloidal stain solution (34% v v^{-1} ethanol, 2% v v^{-1} H_3PO_4, 17% w v^{-1} NH_4SO_4 and 0.066% Coomassie G250) and Image LAB™ software (BioRad, USA) was used for image processing.

2.5. Determination of the reaction progress for biocatalyzed reactions using GC-FID

The substrate and product concentration were determined by GC-FID analysis in triplicate. Aliquots of the reaction mixture (50 µL) were taken and diluted in acetonitrile (950 µL). The sample was centrifuged using an Eppendorf centrifuge 5424 to remove precipitated protein and analyzed by gas chromatography (GC-FID). The concentration of substrate and lactones were determined using calibration curves. GC-FID analyses were performed using a Shimadzu GC-2010 Plus Gas Chromatograph with a hydrogen flame-ionization detector and an SPB-1 capillary column (30 m × 0.25 µm × 0.25 mm inner diameter). For kinetics, the following program was used: starting temperature of 60 ˚C maintained for 2 minutes, temperature increased to 200 ˚C at a heating rate of 15 ˚C min^{-1} and then maintained at 200 ˚C for 2 minutes, and temperature finally increased to 320 ˚C at a heating rate of 20 ˚C min^{-1} and maintained at 320 ˚C for 2 minutes (sample injected at 250 ˚C, with a split ratio of 10, 2 µL injection volume). The following retention times were observed for kinetic samples measured from the reaction mixture: 6.83 min for the substrate **1**, 9.25 min and 9.36 min for the lactones **1b** and **1a** (Figure 1).

2.6.Reaction set-up and reaction conditions

The reactions were performed with a Metrohm 887 Titrino Plus titration apparatus. The pH was monitored and adjusted to pH 8.0 by automatic addition of a solution of NaOH (1 M). The reaction was performed in a double walled-glass and the temperature was maintained to 30 °C. The reactions were performed in potassium phosphate buffer (25 mM), at pH 8.0. The reaction was stirred at 500 rpm, and air was bubbled in the reaction volume at a rate of 8 mL min^{-1}.

2.7.Bioreaction with soluble TmCHMO and GDH-Tac biocatalysts

The reaction vessel was loaded with 10 mM of 3,3,5-trimethylcyclohexanone (47.4 µL), 250 µM of $NADP^+$ (5.9 mg), 350 mM of glucose, and 10% v v^{-1} of methanol (3 mL) for a total reaction volume of 30 mL. The reaction was started by the addition of a 3.07% v v^{-1} of soluble TmCHMO (0.921 mL of CFE containing 32.1 mg TmCHMO) and 4.87% v v^{-1} soluble GDH-Tac (1.422 mL of CFE containing 65.8 mg soluble GDH-Tac). An additional 10 mM of substrate (47.4 µL) was added every hour until a total of 140 mM of substrate.

2.8.Reusability of immobilized TmCHMO and GDH-Tac biocatalysts

The reaction vessel was loaded with 10 mM of 3,3,5-trimethylcyclohexanone (47.4 µL), 250 µM of $NADP^+$ (5.9 mg), 30 mM of glucose, and 10% v v^{-1} of methanol (3 mL) for a total reaction volume of 30 mL. The reaction was started by the addition of 5% v v^{-1} of immobilized TmCHMO (20 mg TmCHMO g^{-1} support, 1.605 g of supported enzyme corresponding to 32.1 mg TmCHMO) and 5% v v^{-1} of immobilized GDH-Tac (29 mg GDH-Tac g^{-1} support, 1.605 g supported enzyme corresponding to 46.5 mg GDH-Tac). The substrate and product concentration were determined by GC-FID analysis in triplicate. At the end of the reaction, the immobilized TmCHMO and immobilized GDH-Tac were filtered and washed with buffer. New reaction medium containing 10 mM 3,3,5-trimethylcyclohexanone, 250 µM $NADP^+$, 30 mM glucose and 10% v v^{-1} of methanol was prepared; to which the immobilized TmCHMO and immobilized GDH-Tac rinsed with buffer were added to start the reaction. The supported enzymes were stored at 4 °C overnight after cycles 5 and 10.

2.9.Reusability of the co-immobilized TmCHMO and GDH-Tac biocatalysts

The reactions were performed in a similar fashion as for the immobilized TmCHMO and GDH-Tac biocatalyst. The biocatalyst concentration was 5.4% v v^{-1} (18.4 mg TmCHMO and 9.1 mg GDH-Tac g^{-1} support, 1.74 g of supported co-immobilized enzymes corresponding to 32.1 mg of TmCHMO and 15.83 mg of GDH-Tac).

3. RESULTS AND DISCUSSION

3.1.Biocatalyst immobilization on MANA-agarose support

Our goal was the oxidation of 3,3,5-trimethylcyclohexanone using the thermostable TmCHMO (Figure 1). The NADPH cofactor was regenerated by applying GDH-Tac, which uses glucose as a sacrificial cosubstrate. For this, both enzymes were evaluated in their soluble form as well as immobilized on MANA-agarose (separately or co-immobilized).

Firstly, the immobilization of TmCHMO and GDH-Tac on MANA-agarose was studied aiming to define the best conditions for the immobilization of the biocatalysts following two approaches: separate enzyme immobilization and co-immobilization. Aiming to characterize the immobilization processes these studies were performed at low activity loads to ensure no mass transfer limitations once the enzyme is immobilized in the support (Table 1).

In order to obtain the highest immobilization yield and retained activity, the immobilization of TmCHMO on MANA-agarose was assayed testing two different N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) concentrations (25 and 35 mM). EDC is added once the protein is ionically adsorbed to promote its covalent binding to the support. The results showed that TmCHMO was completely adsorbed after 0.25 h and 35 mM of EDC was selected as the most appropriate concentration allowing an immobilization yield of 93.0% and a retained activity of 62.4% (Table 1).

Regarding the immobilization of GDH-Tac, it was ionically adsorbed onto MANA-agarose after 0.5 h. In the second phase of the immobilization, EDC was introduced at different concentrations (1, 3, 5, 10, 15 and 20 mM) to promote the covalent binding of the enzyme to the support. Among the EDC concentrations tested, 10 mM was chosen as the optimum as it presented 78.7% immobilization yield and 57.1% retained activity (Table 1).

For the co-immobilization of TmCHMO and GDH-Tac, two EDC concentrations were tested, 10 and 20 mM. These values were selected taking into account the results obtained in the previous immobilization studies. An EDC concentration of 10 mM was selected since GDH-Tac retained activity was significantly affected by high EDC concentration.

3.2.Reutilization of the immobilized biocatalysts and comparison with the soluble enzymes

The soluble and immobilized biocatalysts were applied to the oxidation of 3,3,5-trimethylcyclohexanone. Similarly to our previous studies with TmCHMO and this substrate,[15] it was necessary to control the pH during the reaction since each molecule of the substrate that was converted resulted in the formation of one molecule of *D*-gluconolactone which was hydrolyzed to gluconic acid and consequently increased the acidity of the reaction (Figure 1). Auto-titration of the reaction by addition of NaOH at 1 M ensured a constant pH throughout the reaction course. A co-solvent (10% v v^{-1} methanol) was added to aid the solubility of the substrate, which is rather limited in water. This co-solvent was selected based on our previous results showing that this co-solvent results in the fastest reaction rate compared to other tested organic co-solvents.[15]

The oxidation of 3,3,5-trimethylcyclohexanone was first performed with both soluble TmCHMO and GDH-Tac using a TmCHMO load of 1.07 mg mL^{-1} of reaction medium and an enzyme ratio of 1:2.0 (mg TmCHMO mg GDH-Tac^{-1}). Total conversion of the initial substrate (10mM) was achieved in 1 h. Once the initial substrate was completely consumed, a fed-batch strategy was applied by supplying an additional 10 mM of substrate to the reaction mixture every hour up to a total of 150 mM of 3,3,5-trimethylcyclohexanone. The results showed that, while the ketone was fully converted in 1 h for the first 3 substrate additions, the accumulation of unreacted substrate was observed for the rest of the reaction until a final substrate

concentration of about 100 mM (Figure 2a). This change in the enzymatic reaction rate was directly correlated to the amount of base needed to maintain the pH of the reaction, which is related to the amount of gluconic acid co-product formed and substrate converted (Figure 2b).

The conversion for each addition was calculated and the obtained results are depicted in Figure 3. A sharp decrease in conversion per substrate addition was observed until an average conversion of about 10% was observed. This was attributed to the loss of enzymatic activity during the reaction, but substrate inhibition of TmCHMO as a consequence of substrate accumulation in the reaction mixture probably also played a role. Product accumulation in the reaction media could also contribute to a decrease in subsequent conversions since BVMOs often exhibit product inhibition, as has been previously reported by other authors.[31,37,38]

Process metrics were analyzed for the fed-batch strategy using soluble enzymes (Table 2). The total process time after 14 additions was 14.4 h with a final product amount of 0.308 g and a final unreacted substrate amount of 0.423 g. The biocatalyst yields reached 9.6 and 4.7 mg of product mg^{-1} of TmCHMO and GDH-Tac, respectively.

The performance of the TmCHMO and GDH-Tac which were separately immobilized at high enzymatic loads was also studied. The TmCHMO immobilized derivative contained 20 mg of monooxygenase g^{-1} of support, while the GDH-Tac derivative contained 29 mg of GDH-Tac g^{-1} of support. Aiming to compare the results with the soluble enzymes, the reactions were carried out using the same load of TmCHMO (1.07 mg TmCHMO per mL of reaction). The ratio of TmCHMO/GDH-Tac was slightly lower (1:1.5) since it is determined by i) the maximum immobilized derivative that can be used (10% v v^{-1}) to ensure a proper suspension and mixing and, ii) the enzymes load per mg of support obtained during the immobilization processes.

277 Separately immobilized derivatives were used in the biooxidation reaction, where the first cycle
278 took about 1.33 hour to total substrate conversion (Figure 4a). The increase in reaction time for
279 a total conversion of the substrate during the first cycle could be related to i) the lower amount
280 of loaded GDH-Tac with the immobilized enzymes which could lead to the cofactor
281 regeneration reaction being the limiting step or/and ii) diffusion limitations of the NADP(H)
282 co-factor between the bead particles containing TmCHMO and GDH-Tac or /and iii) oxygen,
283 glucose or 3,3,5-trimethylcyclohexanone mass transfer limitations due to the diffusional
284 restriction of these molecules in the support particles.

285 The operational stability of the biocatalysts was studied. At the end of the reaction, both
286 immobilized enzymes were recovered and reused for conversion of additional substrate in the
287 same reaction conditions. In total, the immobilized enzymes were reused up to 15 times aiming
288 to compare the results with the data obtained using soluble enzymes where 14 additions were
289 carried out (Figure 4b). Full conversion was obtained for the first 5 cycles, after which the
290 conversion started to decrease slowly.

291 The process metrics obtained using separately immobilized biocatalysts are shown in table 2.
292 Even though the total reaction time of the process was 1.4-fold higher, the average final product
293 amount (0.422 g) increased by 37 %. Moreover, the use of separately immobilized enzymes
294 also improves the process performance by reducing in 2.1-fold the final unreacted substrate
295 amount (0.199 g) and increasing the TmCHMO biocatalyst yield by 36%. The overall
296 biocatalyst yield is increased by 74% due to the better performances obtained with the
297 separately immobilized biocatalysts, despite the lower GDH-Tac biocatalyst loading (70% of
298 the GDH-Tac loading of the reaction with the soluble enzymes).

299 The performance of the enzymes that were co-immobilized at high loads was also studied
300 (TmCHMO: 18.4 mg g^{-1} of support; GDH-Tac: 9.1 mg g^{-1} of support). In order to compare the

301 performance of the co-immobilized catalysts with the biocatalysts immobilized separately and
302 the soluble enzymes, the amount of co-immobilized support used in the oxidation reaction was
303 calculated so that the same amount of TmCHMO was applied in all cases (1.07 mg mL^{-1}). The
304 ratio TmCHMO/GDH-Tac in this case (1:0.5) was determined by the ratio obtained during the
305 co-immobilization process, where both enzymes compete for the same support.

306 For this bioconversion, the reaction time was 1.17 h until the full conversion of the substrate,
307 17% higher compared to the soluble enzymes (Figure 5a). The higher reaction time compared
308 to the soluble enzymes could be due to the lower GDH-Tac load or to mass diffusional
309 restrictions, as already mentioned with the separately immobilized enzymes. However, even
310 though lower TmCHMO/GDH-Tac ratio was used when co-immobilized derivatives were used
311 (1:0.5) compared to the separately immobilized enzymes (1:1.5), the reaction time was 12%
312 lower. Thus, the reduction of the reaction time of the co-immobilized derivative compared to
313 the separately immobilized biocatalyst probably indicates that NADP(H) cofactor diffusional
314 restrictions between bead particles is likely the main cause of reaction time increase when
315 separately immobilized derivatives are used.

316 The operational stability studies were also carried out with the co-immobilized derivative
317 during 15 cycles (Figure 5b). Compared to the biocatalysts immobilized separately, the co-
318 immobilized biocatalysts performed much better with the re-uses. A substrate conversion of
319 58% was achieved for the last cycle (15) compared to 39% substrate conversion obtained for
320 the same cycle with the biocatalysts immobilized separately.

321 Regarding the process metrics (Table 2), co-immobilization, in particular, proved to be the best
322 option of this biotransformation with higher average conversion over all re-utilization cycles
323 (83%) despite the lower concentration of GDH-Tac in the reaction. The highest biocatalyst
324 yields and final average product amounts were achieved with the co-immobilized biocatalysts.

Comparing to the separately immobilized enzymes, all process metric analyzed were improved: 1.14-fold decrease in total process time, a 1.3-fold increase in final average product amount, a 1.4-fold decrease in the unreacted substrate, a 1.1-fold increase in average conversion, and a 1.3-fold increase in TmCHMO biocatalyst yield. The GDH-Tac biocatalyst yield was improved by 3.7-fold because the experiment with the co-immobilized enzymes achieved the best performances with the lowest GDH-Tac loading.

Compared to the soluble enzymes for which a fed-batch strategy was applied, even though the total process time was slightly increased, the final average product formed was improved in 1.7-fold, the unreacted substrate amount decreased in 3-fold, the average final conversion was increased in 1.6-fold, and the total biocatalyst yield was 3.6-fold higher. These values prove the better performance of the co-immobilized enzymes in the target reaction studied compared to separately immobilized enzymes.

4. CONCLUSIONS

TmCHMO was successfully immobilized on a MANA-agarose support with the co-enzyme GDH-Tac to ensure co-factor regeneration. Both the enzymes immobilized separately and co-immobilized displayed good retention of activity in repeated re-utilization for the oxidation of 3,3,5-trimethylcyclohexanone. Co-immobilized proved to give the most efficient biocatalyst format, achieving the highest average conversion over 15 re-utilization cycles (83%) and a high significant improvement of 3.6-fold of the total biocatalyst yield compared to the soluble enzymes. Compared to the biocatalysts which were separately immobilized, a highest reaction rate was observed which was attributed to more efficient diffusion of the NADP(H) co-factor between the two enzymes immobilized on the same support. This work demonstrates that immobilized BVMOs are promising biocatalysts for the synthesis of lactones, and in particular polymeric building blocks.

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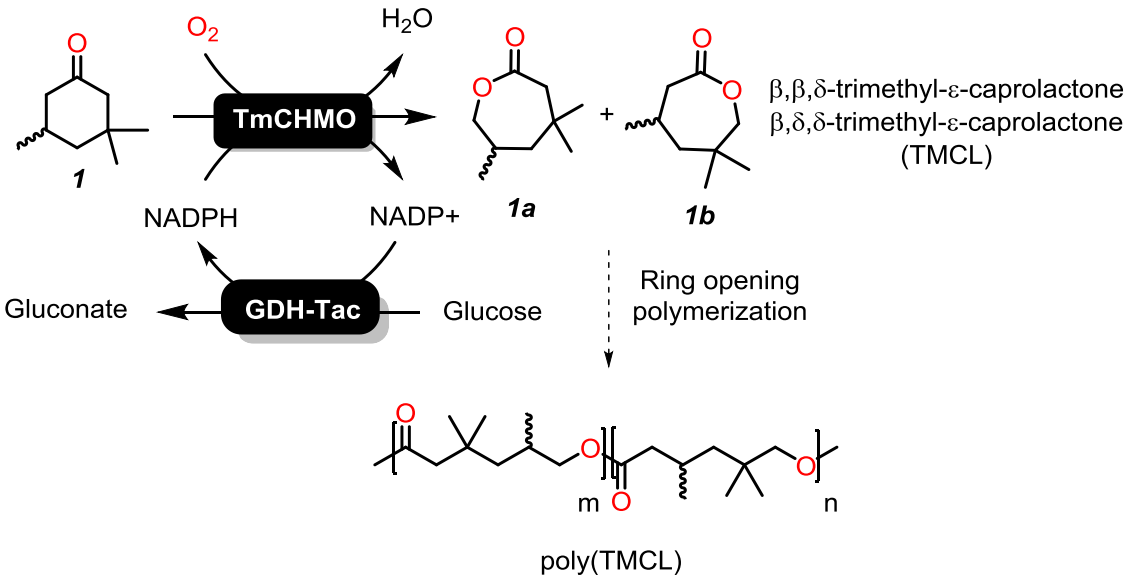

Figure 1. Biocatalyzed oxidation of 3,3,5-trimethylcyclohexanone ***1*** with TmCHMO and GDH-Tac to give the regio-isomeric lactones ***1a*** and ***1b*** which can be polymerized by ring opening polymerization. The enzymes were either immobilized on a MANA-agarose or soluble.

435 **Table 1.** Overview of the characterization of the immobilization of TmCHMO and GDH-Tac

436 on MANA-agarose under optimum conditions.

Enzyme	Offered enzyme load*	Immobilization yield (%)	Retained activity (%)
TmCHMO	5 U g^{-1} of support (8 mg TmCHMO g^{-1} of support)	93.0	62.4
GDH-Tac	5 U g^{-1} of support (3.7 mg GDH-Tac g^{-1} of support)	78.7	57.1
Co-immobilized TmCHMO and GDH-Tac	5 U g^{-1} of support of each enzyme	79.4 (TmCHMO) 96.5 (GDH-Tac)	12.9 (TmCHMO) 48.2 (GDH-Tac)

437 *No substrate transfer limitations were found at this enzymatic load

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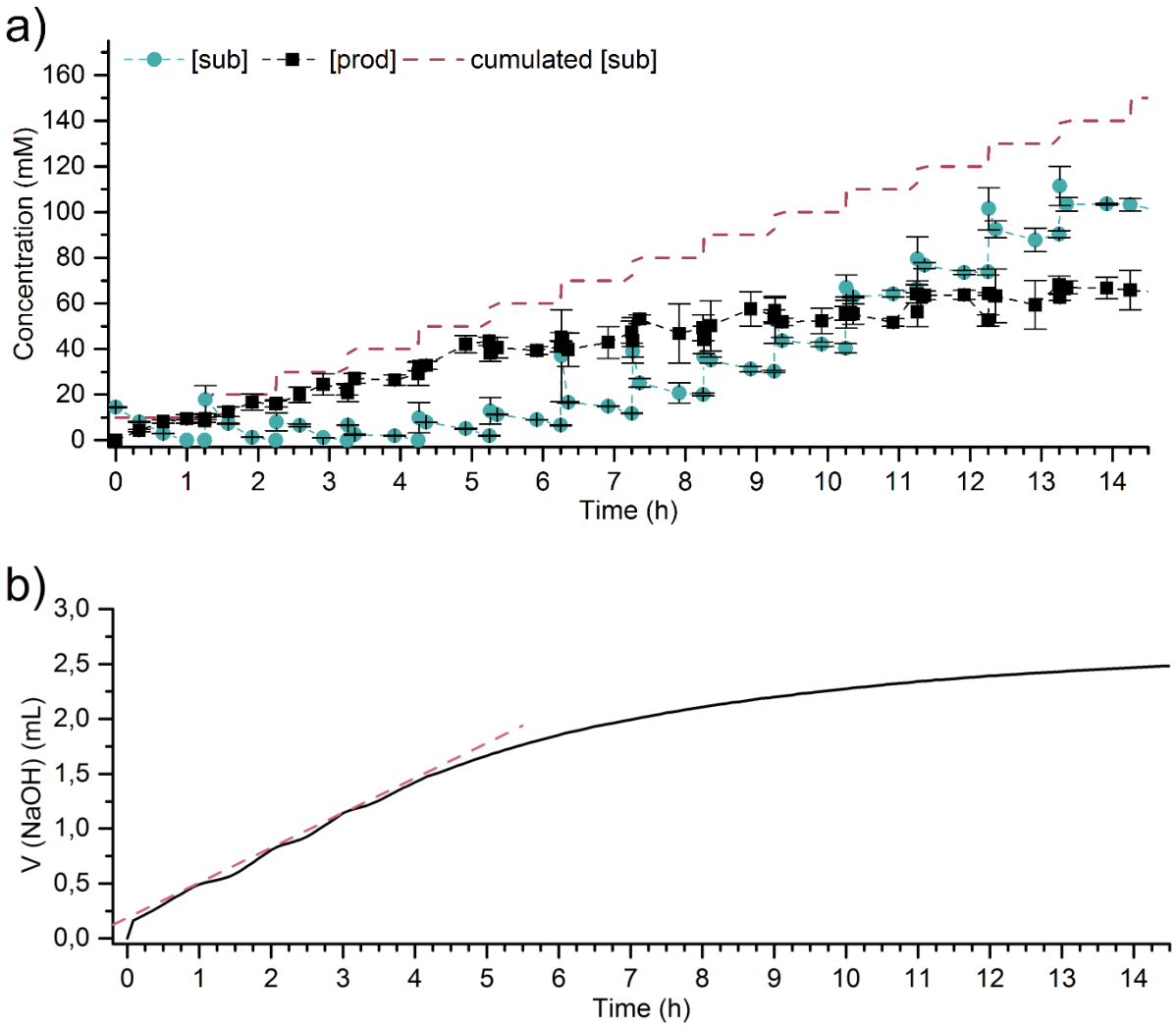

Figure 2. a) Reaction course of the conversion of 3,3,5-trimethylcyclohexanone with soluble TmCHMO and soluble GDH-Tac (TmCHMO/GDH-Tac 1:2.0) with the concentration of substrate (blue circles) and product (black squares). The total amount of substrate accumulated is shown with a pink dotted line. b) Profile of the volume of NaOH (1M) added during the course of the reaction. The pink dotted line indicates the initiation rate of NaOH addition. Reaction conditions: 10 mM of substrate initially + 10 mM every hour, 10% v v^{-1} methanol, 3.07% v v^{-1} soluble TmCHMO (1.07 mg mL^{-1}), 4.87% v v^{-1} soluble GDH-Tac (2.19 mg mL^{-1}), 350 mM glucose, 250 µM $NADP^{+}$.

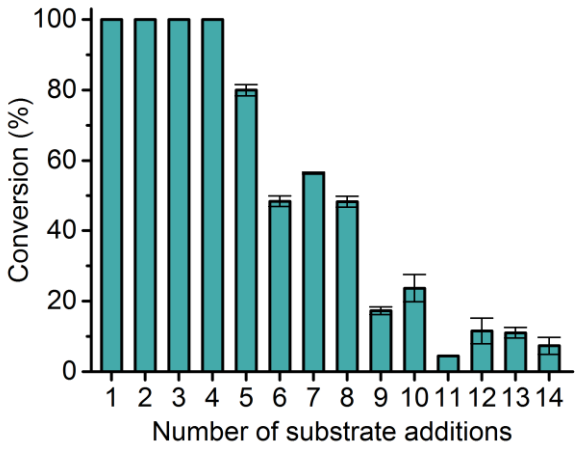

Figure 3. Sequential additions of substrate for the reaction with soluble TmCHMO and soluble GDH-Tac (TmCHMO/GDH-Tac 1:2.0) with conversion as a function of the number of substrate additions (conversion = $1-([sub]_f/[sub]_i)$ with $[sub]_f$ the substrate concentration before the next addition of substrate and $[sub]_i$ the substrate concentration after the last addition of substrate).

Table 2. Overview of the performances of TmCHMO and GDH-Tac biocatalysts for the oxidation of 3,3,5-trimethylcyclohexanone

Biocatalyst format	Ratio TmCHMO :GDH-Tac	Total reaction time (h)	Product formed[a] (g)	Unreacted substrate[a] (g)	Average conv[b] (%)	Biocatalyst yield[c] (mg product/mg biocatalysts)		
						TmCHMO	GDH-Tac	Total
Soluble	1:2.0	14.4	0.308	0.423	51	9.6	4.7	3.1
Immobilized	1:1.5	20.0	0.422	0.199	73	13.1	9.1	5.4
Co-immobilized	1:0.5	17.5	0.538	0.138	83	16.8	34.0	11.2

[a] Cumulated amount of product and unreacted substrate (sum of each cycle for the immobilized enzymes and value measured at the end of the reaction for the soluble enzymes) [b]Average conversion calculated for 15 cycles for the immobilized enzymes and for 14 additions for the soluble enzymes. [c] Biocatalyst yield = total mg of product/mg of biocatalyst (TmCHMO, GDH-Tac or TmCHMO + GDH-Tac).

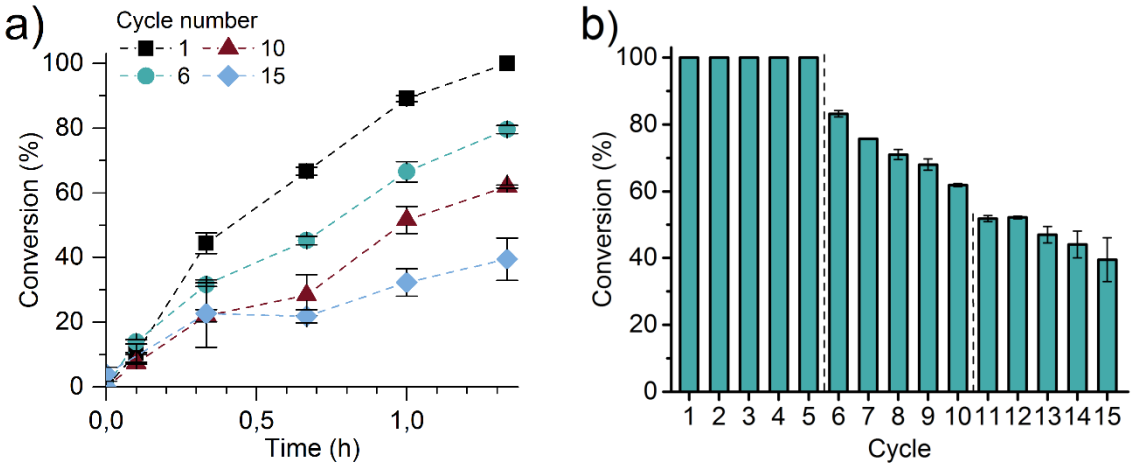

Figure 4. Re-uses of TmCHMO and GDH-Tac immobilized on separate supports (TmCHMO/GDH-Tac 1:1.5) with a) reaction profile for cycles 1, 6, 10, 15; and b) substrate conversion after 1.33 hour for all cycles. The vertical dotted lines indicate overnight storage of the immobilized enzymes in buffer solution. Reaction conditions: 10 mM of substrate, 10% v v^{-1} methanol, 5% v v^{-1} immobilized TmCHMO, 5% v v^{-1} immobilized GDH-Tac, 30 mM glucose, 250 µM $NADP^+$, 1.33 h reaction time.

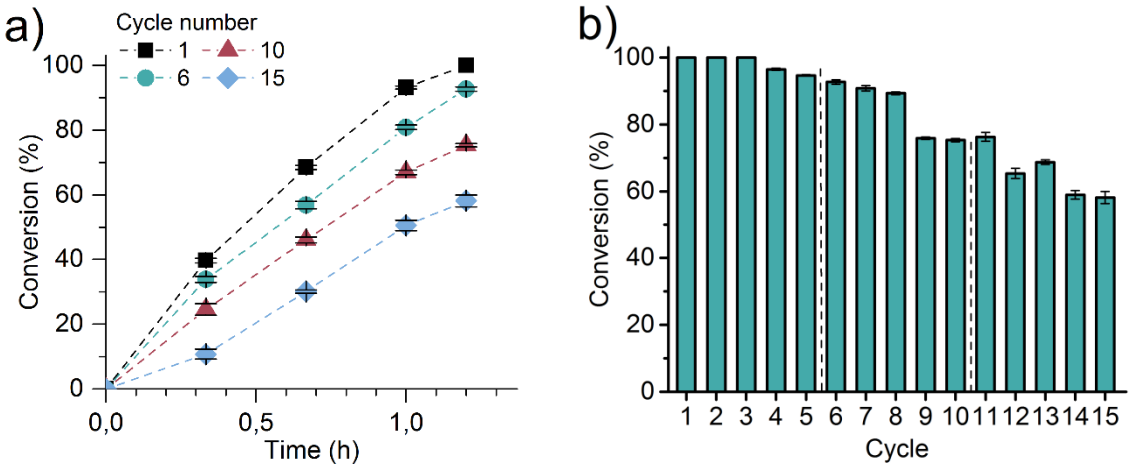

Figure 5. Re-uses of co-immobilized TmCHMO and GDH-Tac (TmCHMO/GDH-Tac 1:2.0) with a) reaction profile for cycles 1, 6, 10, 15; and b) substrate conversion after 1.17 hour for all cycles. The vertical dotted lines indicate overnight storage of the immobilized enzymes in buffer solution. Reaction conditions: 10 mM of substrate, 10% v v^{-1} methanol, 5.4% v v^{-1} co-immobilized TmCHMO and GDH-Tac, 30 mM glucose, 250 µM $NADP^+$, 1.17 h reaction time.