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

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22 **ABSTRACT**

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

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

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

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

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44 ## 1. INTRODUCTION

45Enzymatic reactions have been identified as a sustainable technology since they usually 46follow the rules of green chemistry.[1, 2] Oxidative biocatalysis, and Baeyer-Villiger 47monooxygenases (BVMOs) in particular, is an alternative of lesser toxicity compared to 48chemical oxidation.[3] BVMOs are biocatalysts capable of catalyzing the oxidation of 49(cyclic) ketones by inserting one atom of oxygen in a C-C bond, therefore generating water as 50by-product. BVMOs can catalyze the oxidation of a wide range of cyclic ketones of various 51ring sizes including alkyl substituted ketones, as well as perform enantioselective 52sulfoxidation.[4-6] These oxidative enzymes have been applied to the synthesis of 53intermediates for the pharmaceutical industry,[7-9] and chiral molecules for fine chemical 54and fragrances.[10] Additionally, several BVMOs have been identified as relevant 55biocatalysts for the synthesis of lactones as monomers for polymeric materials, for example, 56ε-caprolactone, either from whole-cell[11] or via a cascade reaction,[12] lauryl lactone,[13] 57which is a nitrile-substituted ε-caprolactone used as precursor for polyamide,[14] and β,δ-58trimethyl-ε-caprolactone (TMCL).[15, 16] Alkyl substituted lactones are particularly 59interesting for the synthesis of polyesters with low glass transition temperature (T_g < 0 °C in 60general).[17] This property enables applications such as biodegradable plasticizers[18] or 61encapsulating agents for coating formulations[19] with polymers from TMCL for example.

62The applicability of BVMOs is however hindered by their lack of robustness, either due to 63thermolability or to limited stability in the presence of organic solvents. Using protein 64engineering, several mutants of cyclohexanone monooxygenase with improved 65thermostability were created.[20-22] The discovery of new thermostable BVMOs contributes 66to the development of their applicability in biotransformations.[23-26] Recently, a 67cyclohexanone monooxygenase from *Thermocrispum municipale* DSM 44069 (TmCHMO;

68EC 1.14.13.22) was identified as being particularly relevant for the preparation of lactones as
69polymeric building blocks due to its high thermostability, good resistance to organic solvents,
70and broad substrate scope towards cyclic ketones.[27, 28]

71Although TmCHMO has already been applied for the synthesis of ε-caprolactone derivatives
72from 3,3,5-trimethylcyclohexane, using either a self-sufficient fused biocatalyst[15] or a
73glucose dehydrogenase to regenerate the NADPH co-factor,[16] this enzyme has not yet been
74immobilized. Immobilization of whole-cells or isolated enzymes is indeed known to increase
75the operational stability of enzymes. Additionally, immobilization has several advantages
76including facilitating the recovery of the biocatalyst, decreasing the costs of downstream
77processing, and potentially decreasing the enzyme cost per kilogram of product, provided that
78the immobilized biocatalysts maintain their activity throughout the reuses.[29, 30]

79So far, isolated BVMOs have mostly been immobilized to polymeric supports by covalent
80binding.[31] For example, a cyclohexanone monooxygenase from *Acinetobacter*
81*calcoaceticus* (AcCHMO) was immobilized on Eupergit (polyacrylamide based supported
82beads) *via* covalent binding with a glucose 6-phosphate dehydrogenase for the synthesis of
83chiral lactone building blocks.[32] Fusions of AcCHMO with a polyol dehydrogenase were
84similarly immobilized for the synthesis of ε-caprolactone.[33] The immobilized biocatalyst,
85however, displayed a low stability on the support and a poor operational stability. Recently,
86MANA-agarose (monoaminoethyl-*N*-aminoethyl)-agarose was identified as a suitable
87support for the immobilization of a fused AcCHMO-phosphite dehydrogenase (AcCHMO-
88PTDH).[34] For this enzyme, a higher retained activity was achieved with metal-chelate
89supports such as Ni-iminodiacetic acid (Ni-IDA) and Co-IDA.[34]

90In this article, our goal is to expand the use of immobilized BVMOs and evaluate them for
91the 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. In total, three biocatalyst formats were examined for TmCHMO and GDH-Tac: soluble enzymes, enzymes immobilized separately, and co-immobilized enzymes. The performances of the immobilized enzymes were evaluated in over 15 repeated biooxidation cycles, in which the immobilized biocatalysts are re-used each time, 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 (ε_{NADPH} = 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]

116GDH-Tac activity was determined spectrophotometrically at 340 nm following the NADPH
117(ε_{NADPH} = 6.22 mM^{-1} cm^{-1}, 400 µM) formation using D-Glucose (200 mM) as substrate and
118sodium phosphate buffer 100 mM pH 8.0.[35] The basal production of NADPH by unspecific
119enzymes present in the lysate was determined by this same test but avoiding the addition of
120substrate and adding buffer instead of the amount of enzyme required. This production rate is
121subtracted from the measurement with D-glucose. One unit of activity (U) was defined as the
122enzyme required to convert 1 µmol of $NADP^+$ per min at those given conditions (30 ºC, pH
1238.0). The absorbance was recorded using a spectrophotometer Cary 50 Bio UV-visible (Palo
124Alto, USA).

125 2.3. Preparation of immobilized TmCHMO and immobilized GDH-Tac

126The general procedure for the covalent immobilization of the enzymes on MANA-agarose
127(density: 1.07 g mL^{-1}) comprised three main steps: i) the ionic adsorption of the enzyme to
128the support, ii) the addition of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) as an
129activating agent to promote amide bond formation between the amino groups of the support
130and the carboxyl groups of the enzyme (glutamic and aspartic residues), and iii) the addition
131of NaCl to desorb all the enzyme that was not covalently bound to the support. After the
132immobilization, the derivatives were washed carefully.

133The immobilization of TmCHMO was carried out by suspending the support in 25 mM MES
134buffer (pH 6.0); then the enzyme was added to the suspension and left to adsorb ionically to
135the support for 0.25 h. After that time, EDC was added to final concentrations of 25 or 35
136mM and left for 2 h. Finally, NaCl was added to a final concentration of 1 M and incubated
137for 1 h. The immobilized derivative was washed with distilled water and filtered.

138The immobilization of GDH-Tac was performed in 50 mM sodium phosphate buffer (pH
1396.0). The ionic adsorption step was completed after 0.5 h. A 200 mM stock solution of EDC

140was prepared, the pH was adjusted to 6.0 with HCl; different volumes were added to get final 141concentrations of 1, 3, 5, 10 or 15 mM and incubated for 1h. Afterwards, NaCl was added to 142a final concentration of 0.5 M and incubated for 0.5 h. Lastly, the support was washed gently 143with 100 mM sodium phosphate buffer (pH 8.0) and filtered.

144For the co-immobilization of TmCHMO and GDH-Tac, the support was suspended in 50 mM 145sodium phosphate buffer (pH 6.0); both enzymes were added and incubated 0.25 h. After the 146ionic step was completed, EDC was added to final concentrations of 10 or 20 mM and 147incubated 1 h. NaCl was added to a final concentration of 1M. The derivative was washed 148with distilled water and filtered.

149The characterization of the immobilization was carried out by measuring the activity of the 150supernatant and the suspension throughout the entire process, in order to determine the 151retained activity (Equation (1)) and immobilization yield (Equation (2)). TmCHMO and 152GDH-Tac immobilized on MANA-agarose were stored at 4 °C prior to use.

153 $$\text{Retained activity } (\%) = \frac{\text{Final suspension activity - Final supernatant activity}}{\text{Initial supernatant activity}} \times 100 \quad (1)$$

154 $$\text{Immobilization yield } (\%) = \frac{\text{Initial supernatant activity -Final supernatant activity}}{\text{Initial supernatant activity}} \times 100 \quad (2)$$

155 **2.4. Determination of enzyme content**

156The cell lysate was pre-clarified by centrifugation (3220 g for 15 min.), and the total protein 157content was determined by means of a Bradford Protein Assay Kit (Thermo Fisher Scientific, 158Waltham, USA) using bovine serum albumin as standard.

159Enzyme content was assessed using sodium dodecyl sulphate polyacrylamide gel 160electrophoresis (SDS-PAGE) (NuPage 12%, Invitrogen, USA) ran in a Mini-PROTEAN II

161apparatus (BioRad, USA) following the protocol of Laemmli *et al*.[36] Low range protein 162markers were used for subunit molecular mass determination. Gels were stained using 163Coomassie G250 colloidal stain solution (34% v v^{-1} ethanol, 2% v v^{-1} H_3PO_4, 17% w v^{-1} 164NH_4SO_4 and 0.066% Coomassie G250) and Image LAB™ software (BioRad, USA) was used 165for image processing.

166 2.5. Determination of the reaction progress for biocatalyzed reactions using 167 GC-FID

168The substrate and product concentration were determined by GC-FID analysis in triplicate. 169Aliquots of the reaction mixture (50 µL) were taken and diluted in acetonitrile (950 µL). The 170sample was centrifuged using an Eppendorf centrifuge 5424 to remove precipitated protein 171and analyzed by gas chromatography (GC-FID). The concentrations of substrate and lactones 172were determined using calibration curves. GC-FID analyses were performed using a 173Shimadzu GC-2010 Plus Gas Chromatograph with a hydrogen flame-ionization detector and 174an SPB-1 capillary column (30 m × 0.25 µm × 0.25 mm inner diameter). For kinetics, the 175following program was used: starting temperature of 60 ˚C maintained for 2 minutes, 176temperature increased to 200 ˚C at a heating rate of 15 ˚C min^{-1} and then maintained at 200 177˚C for 2 minutes, and temperature finally increased to 320 ˚C at a heating rate of 20 ˚C min^{-1} 178and maintained at 320 ˚C for 2 minutes (sample injected at 250 ˚C, with a split ratio of 10, 1792 µL injection volume). The following retention times were observed for kinetic samples 180measured from the reaction mixture: 6.83 min for substrate **1**, 9.25 min and 9.36 min for 181lactones **1b** and **1a** (Figure 1).

182 2.6. Reaction set-up and reaction conditions

183The enzymatic reaction was controlled with a Metrohm 887 Titrino Plus titration apparatus. 184The pH was monitored and adjusted to pH 8.0 by automatic addition of a solution of NaOH

185(1 M). The reaction was performed in a double walled-glass and the temperature was
186maintained to 30 °C. The reactions were performed in potassium phosphate buffer (25 mM),
187at pH 8.0. The reaction was stirred at 500 rpm, and air was bubbled in the reaction volume at
188a rate of 8 mL min^{-1}.

189 **2.7. Bioreaction with soluble TmCHMO and GDH-Tac biocatalysts**

190The reaction vessel was loaded with 10 mM of 3,3,5-trimethylcyclohexanone (47.4 µL), 250
191µM of $NADP^+$ (5.9 mg), 350 mM of glucose, and 10% v v^{-1} of methanol (3 mL) for a total
192reaction volume of 30 mL. The reaction was started by the addition of a 3.07% v v^{-1} of
193soluble TmCHMO (0.921 mL of CFE containing 32.1 mg TmCHMO) and 4.87% v v^{-1}
194soluble GDH-Tac (1.422 mL of CFE containing 65.8 mg soluble GDH-Tac). Protein content
195and enzyme content of cell lysates was evaluated by means of a Bradford Protein assay kit
196and SDS-PAGE, respectively, as described in section 2.4.

197An additional 10 mM of substrate (47.4 µL) was added every hour until a total of 140 mM of
198substrate.

199 **2.8. Reusability of immobilized TmCHMO and GDH-Tac biocatalysts**

200The reaction vessel was loaded with 10 mM of 3,3,5-trimethylcyclohexanone (47.4 µL), 250
201µM of $NADP^+$ (5.9 mg), 30 mM of glucose, and 10% v v^{-1} of methanol (3 mL) for a total
202reaction volume of 30 mL. The reaction was started by the addition of 5% v v^{-1} of
203immobilized TmCHMO (20 mg TmCHMO g^{-1} support, 1.605 g of supported enzyme
204corresponding to 32.1 mg TmCHMO) and 5% v v^{-1} of immobilized GDH-Tac (29 mg GDH-
205Tac g^{-1} support, 1.605 g supported enzyme corresponding to 46.5 mg GDH-Tac). The
206substrate and product concentration were determined by GC-FID analysis in triplicate. At the
207end of the reaction, the immobilized TmCHMO and immobilized GDH-Tac were filtered and

208washed with buffer. New reaction medium containing 10 mM 3,3,5-trimethylcyclohexanone, 209250 μM $NADP^{+}$, 30 mM glucose and 10% v v^{-1} of methanol was prepared; to which the 210immobilized TmCHMO and immobilized GDH-Tac rinsed with buffer were added to start the 211reaction. The supported enzymes were stored at 4 °C overnight after cycles 5 and 10.

212 **2.9. Reusability of the co-immobilized TmCHMO and GDH-Tac biocatalysts**

213The reactions were performed in a similar fashion as for the immobilized TmCHMO and 214GDH-Tac biocatalyst. The biocatalyst concentration was 5.4% v v^{-1} (18.4 mg TmCHMO and 2159.1 mg GDH-Tac g^{-1} support, 1.74 g of supported co-immobilized enzymes corresponding to 21632.1 mg of TmCHMO and 15.83 mg of GDH-Tac).

217 **3. RESULTS AND DISCUSSION**

218 **3.1. Biocatalyst immobilization on MANA-agarose support**

219The main goal was the oxidation of 3,3,5-trimethylcyclohexanone using the thermostable 220TmCHMO (Figure 1). The NADPH cofactor was regenerated by applying GDH-Tac, which 221uses glucose as a sacrificial cosubstrate. For this, both enzymes were evaluated in their 222soluble form as well as immobilized on MANA-agarose (separately or co-immobilized).

223Firstly, the immobilization of TmCHMO and GDH-Tac on MANA-agarose was studied 224aiming to define the best conditions for the immobilization of the biocatalysts following two 225approaches: separate enzyme immobilization and co-immobilization. Aiming to characterize 226the immobilization processes these studies were performed at low activity loads to ensure no 227mass transfer limitations once the enzyme is immobilized in the support (Table 1).

228In order to obtain the highest immobilization yield and retained activity, the immobilization 229of TmCHMO on MANA-agarose was studied. For covalent immobilization in MANA-

230agarose, EDC has to be added once the protein is ionically adsorbed to promote its covalent
231binding to the support which occurs between the amino groups of the support and the
232carboxyl groups of the protein. In previous reported studies on immobilization of CHMO
233from *Acinetobacter calcoaceticus* on MANA-agarose it was shown that 25 mM of EDC led to
234high immobilization yields (98.5 ± 1.1 %) and retained activities (69.3 ± 7.3 %).[34]
235Therefore, 25 mM was selected as EDC concentration to perform the immobilization of
236TmCHMO. The results showed that the BVMO was completely adsorbed after 0.25 h.
237However, lower values of immobilization yield and retained activity as expected were
238obtained after EDC incubation (77.0 ± 0.7 % and 59.7 ± 0.1 %, respectively). The low
239immobilization yield could indicate that the EDC concentration used was not enough to
240ensure a complete covalent attachment of TmCHMO. Thus, the concentration of EDC was
241increased up to 35 mM allowing an immobilization yield of 93.0 ± 0.4 % and a retained
242activity of 62.4 ± 2.1 % (Table 1). Therefore, 35 mM was selected as the most appropriate
243concentration of EDC for TmCHMO immobilization in MANA-agarose.

244Regarding the immobilization of GDH-Tac, from previous reported results it was shown that
245the enzyme was ionically adsorbed onto MANA-agarose after 0.5 h and 10 mM of EDC was
246a suitable concentration for its covalent immobilization.[35] However, in the present work,
247the GDH-Tac immobilization was studied at different concentrations of EDC trying to find
248compatible conditions for the subsequent co-immobilization of GDH-Tac and TmCHMO.
24910mM of EDC led to 78.7 ± 3 % of immobilization yield and 57.1 ± 2 % of retained activity.
250Nevertheless, when higher concentrations EDC were tested (15 and 20 mM) the obtained
251retained activities were 16 % and 17 % lower, respectively, indicating that GDH-Tac could be
252deactivated at concentration higher that 10 mM of EDC. This negative effect of EDC on
253enzyme activity has been already reported for other enzymes.[37] Therefore, the optimum
254EDC concentration found for TmCHMO immobilization (35 mM) was not tested according

255to the negative results obtained at 15 and 20 mM. Lower concentration of EDC (1, 3 and 5 256mM) were also tested. Lower immobilization yields (37.0 ± 5.0, 44 ± 6.0 and 53 ± 1.0 %, 257respectively) and lower retained activities (37.0 ± 4.0, 25.0 ± 4.0 and 56.0 ± 3.0 %, 258respectively) were obtained probably due to an uncomplete covalent attachment of GDH-Tac. 259Therefore, 10 mM was selected as the most appropriate concentration of EDC for this 260enzyme immobilization in MANA-agarose (Table 1).

261For the co-immobilization of TmCHMO and GDH-Tac, two EDC concentrations were tested, 26210 and 20 mM. These values were selected taking into account the results obtained in the 263previous immobilization studies carried out with the enzymes separately: concentration lower 264than 10 mM were discarded due to the low expected retained activity of TmCHMO and 265concentrations higher than 20 mM were not tested taking into account the loss of activity that 266GDH-Tac suffers at high concentrations of EDC. Using 20 mM of EDC, the retained activity 267of GDH-Tac was 19 % lower (38.9 ± 2.3 %) compared to the immobilization carried out at 10 268mM of EDC and similar immobilization yield (98.7 ± 0.4 %) were obtained. Regarding the 269TmCHMO, an increase from 10 to 20 mM did not improve significantly the obtained 270immobilization yield. Finally, an EDC concentration of 10 mM was selected (Table 1) since, 271as expected, GDH-Tac retained activity was significantly affected by high EDC 272concentration.

273 3.2. Reutilization of the immobilized biocatalysts and comparison with the 274 soluble enzymes

275The soluble and immobilized biocatalysts were applied to the oxidation of 3,3,5-276trimethylcyclohexanone. Similarly to our previous studies with TmCHMO and this substrate, 277[15] it was necessary to control the pH during the reaction since each molecule of the 278substrate that was converted resulted in the formation of one molecule of *D*-gluconolactone

279which spontaneously hydrolyzed to gluconic acid and consequently increased the acidity of 280the reaction (Figure 1). Auto-titration of the reaction by addition of NaOH at 1 M ensured a 281constant pH throughout the reaction course. A co-solvent (10% v v^{-1} methanol) was added to 282aid the solubility of the substrate, which is rather limited in water. This co-solvent was 283selected based on our previous results showing that this co-solvent results in the fastest 284reaction rate compared to other tested organic co-solvents.[15]

285The oxidation of 3,3,5-trimethylcyclohexanone was first performed with both soluble 286TmCHMO and GDH-Tac using a TmCHMO load of 1.07 mg mL^{-1} of reaction medium and 287an enzyme ratio of 1:2.0 (mg TmCHMO mg GDH-Tac^{-1}). These soluble enzymes are cell-free 288extracts containing 34 mg of TmCHMO mL^{-1} and 46 mg of GDH-Tac mL^{-1}. The enzyme 289concentrations were determined by means of Bradford assay kit and SDS-PAGE according to 290the procedure described in section 2.4. Total conversion of the initial substrate (10mM) was 291achieved in 1 h. Once the initial substrate was completely consumed, a fed-batch strategy was 292applied by supplying an additional 10 mM of substrate to the reaction mixture every hour up 293to a total of 150 mM of 3,3,5-trimethylcyclohexanone. The results showed that, while the 294ketone was fully converted in 1 h for the first 3 substrate additions, the accumulation of 295unreacted substrate was observed for the rest of the reaction until a final substrate 296concentration of about 100 mM (Figure 2a). This change in the enzymatic reaction rate was 297directly correlated to the amount of base needed to maintain the pH of the reaction, which is 298related to the amount of gluconic acid co-product formed and substrate converted (Figure 2b).

299The conversion for each addition was calculated and the obtained results are depicted in 300Figure 3. A sharp decrease in conversion per substrate addition was observed until an average 301conversion of about 10% was observed. This was attributed to the loss of enzymatic activity 302during the reaction, but substrate inhibition of TmCHMO as a consequence of substrate

303accumulation in the reaction mixture probably also played a role. Product accumulation in the 304reaction media could also contribute to a decrease in subsequent conversions since BVMOs 305often exhibit product inhibition, as has been previously reported by other authors.[31, 38, 39]

306Process metrics were analyzed for the fed-batch strategy using soluble enzymes (Table 2). 307The total process time after 14 additions was 14.4 h with a final product amount of 0.308 g 308and a final unreacted substrate amount of 0.423 g. The biocatalyst yields reached 9.6 and 4.7 309mg of product mg^{-1} of TmCHMO and GDH-Tac, respectively.

310The performance of the TmCHMO and GDH-Tac which were separately immobilized at high 311enzymatic loads was also studied. The TmCHMO immobilized derivative contained 20 mg of 312monooxygenase g^{-1} of support, while the GDH-Tac derivative contained 29 mg of GDH-Tac 313g^{-1} of support. Aiming to compare the results with the soluble enzymes, reactions with 314separately immobilized enzymes were carried out using the same load of TmCHMO (1.07 mg 315TmCHMO per mL of reaction). The ratio of TmCHMO/GDH-Tac was slightly lower (1:1.5) 316since it is determined by i) the maximum immobilized derivative that can be used (10% v v^{-1}) 317to ensure a proper suspension and mixing and, ii) the enzymes load per mg of support 318obtained during the immobilization processes.

319Separately immobilized derivatives were used in the biooxidation reaction, where the first 320cycle took about 1.33 hour to total substrate conversion (Figure 4a). The increase in reaction 321time for a total conversion of the substrate during the first cycle could be related to i) the 322lower amount of loaded GDH-Tac with the immobilized enzymes which could lead to the 323cofactor regeneration reaction being the limiting step or/and ii) diffusion limitations of the 324NADP(H) co-factor between the bead particles containing TmCHMO and GDH-Tac or /and 325iii) oxygen, glucose or 3,3,5-trimethylcyclohexanone mass transfer limitations due to the 326diffusional restriction of these molecules in the support particles.

327The operational stability of the biocatalysts was studied by re-using the same immobilized 328enzymes over several reactions or cycles. At the end of the reaction, both immobilized 329enzymes were recovered by filtrating the reaction mixture. These enzymes were reused in a 330new reaction for conversion of the substrate, using the same reaction conditions for all cycles. 331In total, the immobilized enzymes were reused up to 15 times aiming to compare the results 332with the data obtained using soluble enzymes where 14 additions were carried out (Figure 3334b). Full conversion was obtained for the first 5 cycles, after which the conversion started to 334decrease slowly.

335The process metrics obtained using separately immobilized biocatalysts are shown in table 2. 336Even though the total reaction time of the process was 1.4-fold higher, the average final 337product amount (0.422 g) increased by 37 %. Moreover, the use of separately immobilized 338enzymes also improves the process performance by reducing in 2.1-fold the final unreacted 339substrate amount (0.199 g) and increasing the TmCHMO biocatalyst yield by 36%. The 340overall biocatalyst yield is increased by 74% due to the better performances obtained with the 341separately immobilized biocatalysts, despite the lower GDH-Tac biocatalyst loading (70% of 342the GDH-Tac loading of the reaction with the soluble enzymes).

343The performance of the enzymes that were co-immobilized at high loads was also studied 344(TmCHMO: 18.4 mg g^{-1} of support; GDH-Tac: 9.1 mg g^{-1} of support). In order to compare 345the performance of the co-immobilized catalysts with the biocatalysts immobilized separately 346and the soluble enzymes, the amount of co-immobilized support used in the oxidation 347reaction was calculated so that the same amount of TmCHMO was applied in all cases (1.07 348mg mL^{-1}). The ratio TmCHMO/GDH-Tac in this case (1:0.5) was determined by the ratio 349obtained during the co-immobilization process, where both enzymes compete for the same 350support.

351For this bioconversion, the reaction time was 1.17 h until the full conversion of the substrate, 35217% higher compared to the soluble enzymes (Figure 5a). The higher reaction time compared 353to the soluble enzymes could be due to the lower GDH-Tac load or to mass diffusional 354restrictions, as already mentioned with the separately immobilized enzymes. However, even 355though lower TmCHMO/GDH-Tac ratio was used when co-immobilized derivatives were 356used (1:0.5) compared to the separately immobilized enzymes (1:1.5), the reaction time was 35712% lower. Thus, the reduction of the reaction time of the co-immobilized derivative 358compared to the separately immobilized biocatalyst probably indicates that NADP(H) 359cofactor diffusional restrictions between bead particles is likely the main cause of reaction 360time increase when separately immobilized derivatives are used.

361The operational stability studies were also carried out with the co-immobilized derivative 362during 15 cycles (Figure 5b). Compared to the biocatalysts immobilized separately, the co-363immobilized biocatalysts performed much better with the re-uses. A substrate conversion of 36458% was achieved for the last cycle (15) compared to 39% substrate conversion obtained for 365the same cycle with the biocatalysts immobilized separately.

366Regarding the process metrics (Table 2), co-immobilization, in particular, proved to be the 367best option of this biotransformation with higher average conversion over all re-utilization 368cycles (83%) despite the lower concentration of GDH-Tac in the reaction. The highest 369biocatalyst yields and final average product amounts were achieved with the co-immobilized 370biocatalysts. Comparing to the separately immobilized enzymes, all process metric analyzed 371were improved: 1.14-fold decrease in total process time, a 1.3-fold increase in final average 372product amount, a 1.4-fold decrease in the unreacted substrate, a 1.1-fold increase in average 373conversion, and a 1.3-fold increase in TmCHMO biocatalyst yield. The GDH-Tac biocatalyst

374yield was improved by 3.7-fold because the experiment with the co-immobilized enzymes 375achieved the best performances with the lowest GDH-Tac loading.

376Compared to the soluble enzymes for which a fed-batch strategy was applied, even though 377the total process time was slightly increased, the final average product formed was improved 378in 1.7-fold, the unreacted substrate amount decreased in 3-fold, the average final conversion 379was increased in 1.6-fold, and the total biocatalyst yield was 3.6-fold higher. These values 380prove the better performance of the co-immobilized enzymes in the target reaction studied 381compared to separately immobilized enzymes.

382 4. CONCLUSIONS

383TmCHMO was successfully immobilized on a MANA-agarose support with the co-enzyme 384GDH-Tac to ensure co-factor regeneration. Both the enzymes immobilized separately and co-385immobilized displayed good retention of activity in repeated re-utilization for the oxidation of 3863,3,5-trimethylcyclohexanone. Co-immobilization proved to give the most efficient 387biocatalyst format, achieving the highest average conversion over 15 re-utilization cycles 388(83%) and a high significant improvement of 3.6-fold of the total biocatalyst yield compared 389to the soluble enzymes. Compared to the biocatalysts which were separately immobilized, a 390highest reaction rate was observed which was attributed to more efficient diffusion of the 391NADP(H) co-factor between the two enzymes immobilized on the same support. 392Immobilization of TmCHMO with GDH-Tac was thus identified as being a relevant 393biocatalyst format for lactone synthesis. This work demonstrates that immobilized 394thermostable BVMOs are promising biocatalysts for the synthesis of lactones, and in 395particular polymeric building blocks. Future work will aim at improving the process 396performance metrics such as the volume of the reaction and the product concentration, which 397are required for large-scale production of enzymatic reactions.

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482**Figure 1.** Biocatalyzed oxidation of 3,3,5-trimethylcyclohexanone ***1*** with TmCHMO and 483GDH-Tac to give the regio-isomeric lactones ***1a*** and ***1b*** which can be polymerized by ring 484opening polymerization. The enzymes were either immobilized on a MANA-agarose or 485soluble.

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

495 on MANA-agarose under optimum conditions.

Enzyme	Offered enzyme load*	Immobilization yield (%)	Retained activity (%)	U g support $^{-1}$
TmCHMO	5 U g^{-1} of support (8 mg TmCHMO g^{-1} of support)	93.0 ± 0.4	62.4 ± 2.1	3.1 ± 0.1
GDH-Tac	5 U g^{-1} of support (3.7 mg GDH-Tac g^{-1} of support)	78.7 ± 3.0	57.1 ± 2.0	2.9 ± 0.14
Co-immobilized TmCHMO and GDH-Tac	5 U g^{-1} of support of each enzyme	79.4 ± 2.3 (TmCHMO) 96.5 ± 0.4 (GDH-Tac)	12.9 ± 1.6 (TmCHMO) 48.2 ± 6.5 (GDH-Tac)	0.7± 0.1 (TmCHMO) 2.4± 0.5 (GDH-Tac)

496 Error values correspond to standard deviation (n=2)

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

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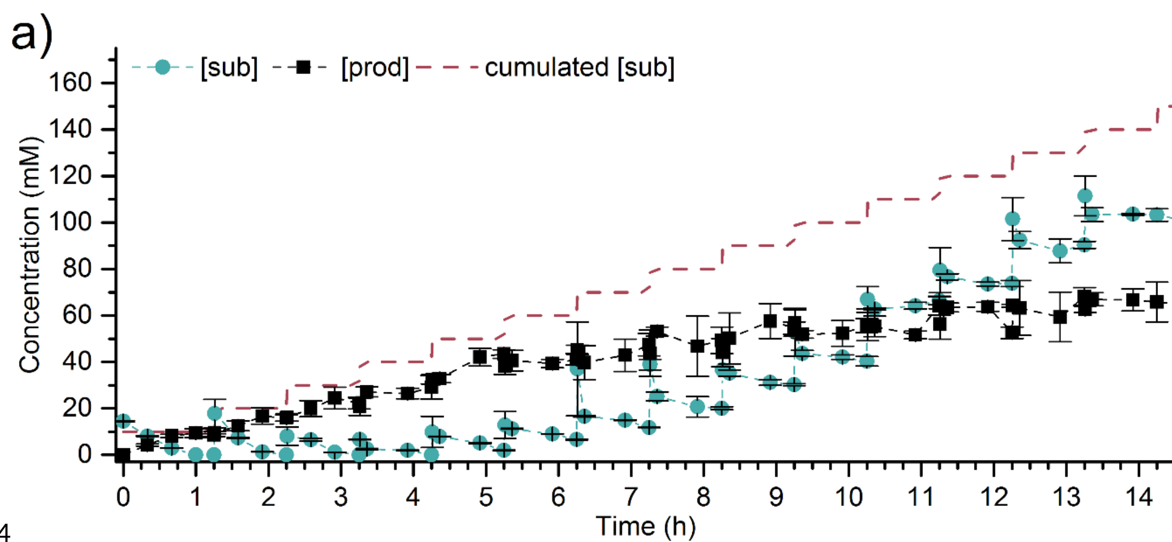

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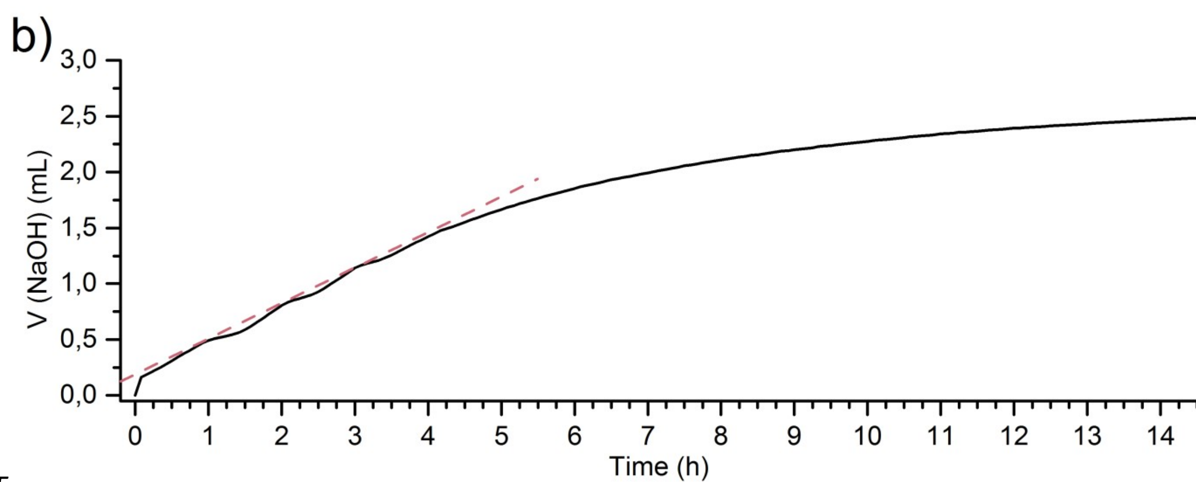

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506**Figure 2**. a) Reaction course of the conversion of 3,3,5-trimethylcyclohexanone with soluble 507TmCHMO and soluble GDH-Tac (TmCHMO/GDH-Tac 1:2.0) with the concentration of 508substrate (blue circles) and product (black squares). The total amount of substrate 509accumulated is shown with a pink dotted line. b) Profile of the volume of NaOH (1M) added

510during the course of the reaction. The pink dotted line indicates the initiation rate of NaOH 511addition. Reaction conditions: 10 mM of substrate initially + 10 mM every hour, 10% v v^{-1} 512methanol, 3.07% v v^{-1} soluble TmCHMO (1.07 mg mL^{-1}), 4.87% v v^{-1} soluble GDH-Tac (2.19 513mg mL^{-1}), 350 mM glucose, 250 µM $NADP^+$. Error values correspond to standard deviation 514(n=3).

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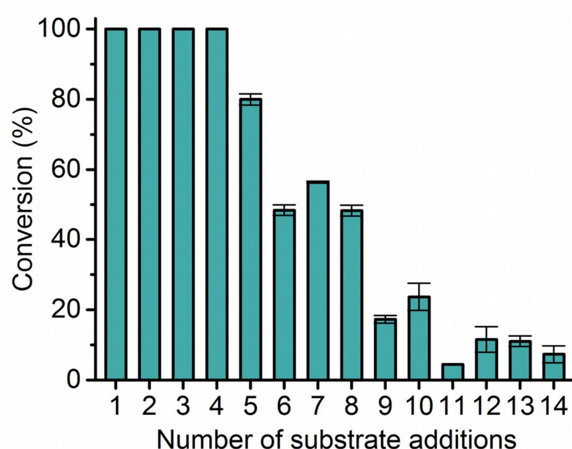

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517**Figure 3.** Sequential additions of substrate for the reaction with soluble TmCHMO and 518soluble GDH-Tac (TmCHMO/GDH-Tac 1:2.0) with conversion as a function of the number 519of substrate additions (conversion = 1-($[sub]_f$/$[sub]_i$) with $[sub]_f$ the substrate concentration 520before the next addition of substrate and $[sub]_i$ the substrate concentration after the last 521addition of substrate). Error values correspond to standard deviation (n=3).

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531**Table 2.** Overview of the performances of TmCHMO and GDH-Tac biocatalysts for the

532oxidation of 3,3,5-trimethylcyclohexanone

Biocatalyst format	Ratio TmCHMO :GDH-Tac (mg:mg)	Total reaction time (h)	Product formed[a] (g)	Unreacted substrate[a] (g)	Average conv[b] (%)	Biocatalyst yield[c] (mg product mg biocatalysts^{-1})		
						TmCHMO	GDH-Tac	Total
Soluble	1:2.0	14.4	0.308 ± 0.040	0.423 ± 0.007	51 ± 3	9.6 ± 1.2	4.7 ± 0.6	3.1 ± 0.4
Immobilized	1:1.5	20.0	0.422 ± 0.034	0.199 ± 0.010	73 ± 2	13.1 ± 1.0	9.1 ± 0.7	5.4 ± 0.4
Co-immobilized	1:0.5	17.5	0.538 ± 0.039	0.138 ± 0.005	83 ± 1	16.8 ± 1.2	34.0 ± 2.5	11.2 ± 0.8

533[a] Cumulated amount of product and unreacted substrate (sum of each cycle for the

534immobilized enzymes and value measured at the end of the reaction for the soluble enzymes)

535[b]Average conversion calculated for 15 cycles for the immobilized enzymes and for 14

536additions for the soluble enzymes.[c] Biocatalyst yield = total mg of product mg of biocatalyst^{-1}

537(TmCHMO, GDH-Tac or TmCHMO + GDH-Tac). Error values correspond to standard

538deviation (n=3).

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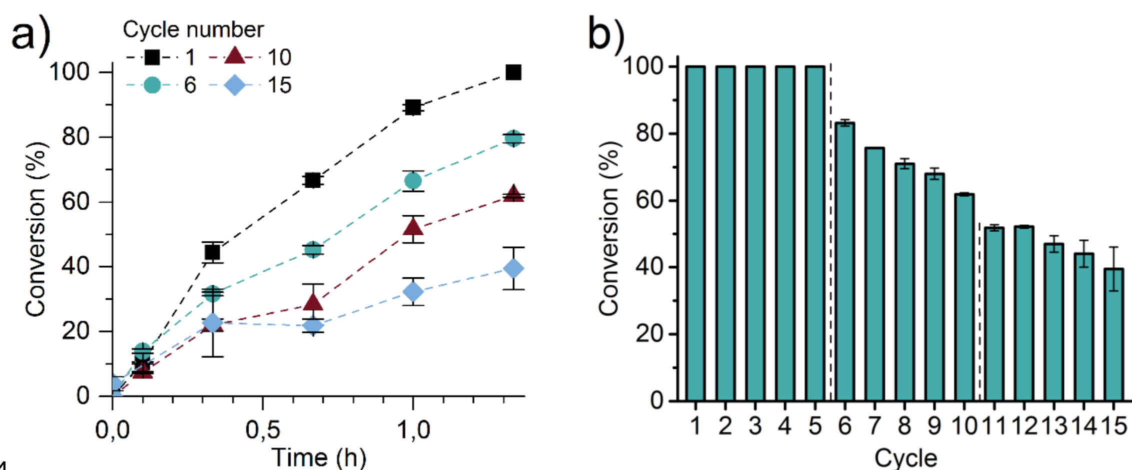

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545 **Figure 4.** Re-uses of TmCHMO and GDH-Tac immobilized on separate supports 546 (TmCHMO/GDH-Tac 1:1.5) with a) reaction profile for cycles 1, 6, 10, 15; and b) substrate 547 conversion after 1.33 hour for all cycles. The vertical dotted lines indicate overnight storage 548 of the immobilized enzymes in buffer solution. Reaction conditions: 10 mM of substrate, 549 10% v v^{-1} methanol, 5% v v^{-1} immobilized TmCHMO, 5% v v^{-1} immobilized GDH-Tac, 30 550 mM glucose, 250 µM $NADP^{+}$, 1.33 h reaction time. Error values correspond to standard 551 deviation (n=3).

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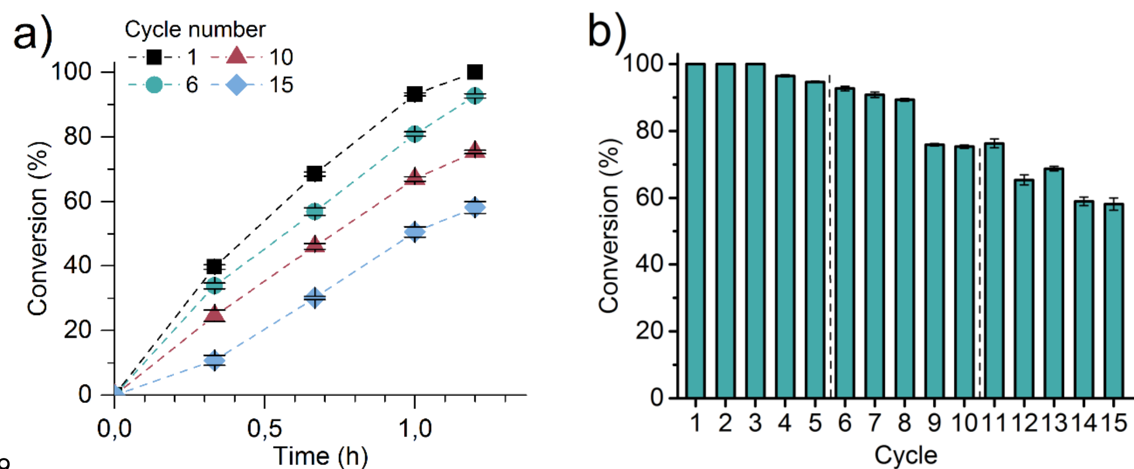

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560 **Figure 5.** Re-uses of co-immobilized TmCHMO and GDH-Tac (TmCHMO/GDH-Tac 1:2.0) 561 with a) reaction profile for cycles 1, 6, 10, 15; and b) substrate conversion after 1.17 hour for 562 all cycles. The vertical dotted lines indicate overnight storage of the immobilized enzymes in 563 buffer solution. Reaction conditions: 10 mM of substrate, 10% v v^{-1} methanol, 5.4% v v^{-1} co-564 immobilized TmCHMO and GDH-Tac, 30 mM glucose, 250 µM $NADP^+$, 1.17 h reaction 565 time. Error values correspond to standard deviation (n=3).