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1 **High performing immobilized Baeyer-Villiger monooxygenase and glucose**
2 **dehydrogenase for the synthesis of ϵ -caprolactone derivative**

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22 **ABSTRACT**

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

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

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

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

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43

44 1. INTRODUCTION

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

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

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

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

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

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

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

98 **2. EXPERIMENTAL**

99 **2.1.Chemicals.**

100 3,3,5-Trimethylcyclohexanone (98%, Sigma-Aldrich), methanol (Biosolve), (+)-glucose
101 (>99%, Alfa Aesar) were used as received. High-density aminoethyl 4BCL agarose (MANA-
102 agarose, Agarose Beads Technologies) was stored at 4 °C. β -Nicotinamide adenine
103 dinucleotide phosphate disodium salt (NADP⁺, 97%, Alfa Aesar), and N-(3-
104 dimethylaminopropyl)-N'-ethylcarbodiimide (EDC, \geq 97%, Sigma-Aldrich) were stored at -
105 20 °C. TmCHMO and GDH-Tac were produced and supplied by InnoSyn BV (Geleen, The
106 Netherlands).

107 **2.2.TmCHMO and GDH-Tac activity assays**

108 TmCHMO activity in the CFE was determined spectrophotometrically following NADPH
109 consumption at 340 nm ($\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) with cyclohexanone as a substrate. The mixture
110 contained Tris-HCl (50 mM, pH 8.5), cyclohexanone (0.5 mM), NADPH (0.1 mM). One unit
111 of TmCHMO (U) was defined as the amount of enzyme required to catalyze the conversion of
112 1 μmol of NADPH to NADP⁺ per min at 20 °C and pH 8.5.[34]

113 GDH-Tac activity was determined spectrophotometrically at 340 nm following the NADP⁺ (ϵ
114 = $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$, 400 μM) consumption using D-Glucose (200 mM) as substrate and sodium
115 phosphate buffer 100 mM pH 8.0.[35] The basal production of NADPH by unspecific enzymes

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

121 **2.3.Preparation of immobilized TmCHMO and immobilized GDH-Tac**

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

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

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

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

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

$$148 \text{ Retained activity (\%)} = \frac{\text{Final suspension activity} - \text{Final supernatant activity}}{\text{Initial supernatant activity}} \times 100 \quad (1)$$

$$149 \text{ Immobilization yield (\%)} = \frac{\text{Initial supernatant activity} - \text{Final supernatant activity}}{\text{Initial supernatant activity}} \times 100 \quad (2)$$

150 **2.4.Determination of enzyme content**

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

154 Enzyme content was assessed using sodium dodecyl sulphate polyacrylamide gel
155 electrophoresis (SDS-PAGE) (NuPage 12%, Invitrogen, USA) ran in a Mini-PROTEAN II
156 apparatus (BioRad, USA) following the protocol of Laemmli *et al.*[36] Low range protein
157 markers were used for molecular weight determination. Gels were stained using Coomassie
158 G250 colloidal stain solution (34% v v⁻¹ ethanol, 2% v v⁻¹ H₃PO₄, 17% w v⁻¹ NH₄SO₄ and
159 0.066% Coomassie G250) and Image LABTM software (BioRad, USA) was used for image
160 processing.

161 **2.5. Determination of the reaction progress for biocatalyzed reactions using GC-FID**

162 The substrate and product concentration were determined by GC-FID analysis in triplicate.
163 Aliquots of the reaction mixture (50 μL) were taken and diluted in acetonitrile (950 μL). The
164 sample was centrifuged using an Eppendorf centrifuge 5424 to remove precipitated protein and
165 analyzed by gas chromatography (GC-FID). The concentration of substrate and lactones were
166 determined using calibration curves. GC-FID analyses were performed using a Shimadzu GC-
167 2010 Plus Gas Chromatograph with a hydrogen flame-ionization detector and an SPB-1
168 capillary column (30 m \times 0.25 μm \times 0.25 mm inner diameter). For kinetics, the following
169 program was used: starting temperature of 60 $^{\circ}\text{C}$ maintained for 2 minutes, temperature
170 increased to 200 $^{\circ}\text{C}$ at a heating rate of 15 $^{\circ}\text{C min}^{-1}$ and then maintained at 200 $^{\circ}\text{C}$ for 2 minutes,
171 and temperature finally increased to 320 $^{\circ}\text{C}$ at a heating rate of 20 $^{\circ}\text{C min}^{-1}$ and maintained at
172 320 $^{\circ}\text{C}$ for 2 minutes (sample injected at 250 $^{\circ}\text{C}$, with a split ratio of 10, 2 μL injection volume).
173 The following retention times were observed for kinetic samples measured from the reaction
174 mixture: 6.83 min for the substrate **1**, 9.25 min and 9.36 min for the lactones **1b** and **1a** (Figure
175 1).

176 **2.6. Reaction set-up and reaction conditions**

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

182 **2.7. Bioreaction with soluble TmCHMO and GDH-Tac biocatalysts**

183 The reaction vessel was loaded with 10 mM of 3,3,5-trimethylcyclohexanone (47.4 μ L), 250
184 μ M of NADP⁺ (5.9 mg), 350 mM of glucose, and 10% v v⁻¹ of methanol (3 mL) for a total
185 reaction volume of 30 mL. The reaction was started by the addition of a 3.07% v v⁻¹ of soluble
186 TmCHMO (0.921 mL of CFE containing 32.1 mg TmCHMO) and 4.87% v v⁻¹ soluble GDH-
187 Tac (1.422 mL of CFE containing 65.8 mg soluble GDH-Tac). An additional 10 mM of
188 substrate (47.4 μ L) was added every hour until a total of 140 mM of substrate.

189 **2.8.Reusability of immobilized TmCHMO and GDH-Tac biocatalysts**

190 The reaction vessel was loaded with 10 mM of 3,3,5-trimethylcyclohexanone (47.4 μ L), 250
191 μ M of NADP⁺ (5.9 mg), 30 mM of glucose, and 10% v v⁻¹ of methanol (3 mL) for a total
192 reaction volume of 30 mL. The reaction was started by the addition of 5% v v⁻¹ of immobilized
193 TmCHMO (20 mg TmCHMO g⁻¹ support, 1.605 g of supported enzyme corresponding to 32.1
194 mg TmCHMO) and 5% v v⁻¹ of immobilized GDH-Tac (29 mg GDH-Tac g⁻¹ support, 1.605 g
195 supported enzyme corresponding to 46.5 mg GDH-Tac). The substrate and product
196 concentration were determined by GC-FID analysis in triplicate. At the end of the reaction, the
197 immobilized TmCHMO and immobilized GDH-Tac were filtered and washed with buffer.
198 New reaction medium containing 10 mM 3,3,5-trimethylcyclohexanone, 250 μ M NADP⁺, 30
199 mM glucose and 10% v v⁻¹ of methanol was prepared; to which the immobilized TmCHMO
200 and immobilized GDH-Tac rinsed with buffer were added to start the reaction. The supported
201 enzymes were stored at 4 °C overnight after cycles 5 and 10.

202 **2.9.Reusability of the co-immobilized TmCHMO and GDH-Tac biocatalysts**

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

207 **3. RESULTS AND DISCUSSION**

208 **3.1. Biocatalyst immobilization on MANA-agarose support**

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

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

218 In order to obtain the highest immobilization yield and retained activity, the immobilization of
219 TmCHMO on MANA-agarose was assayed testing two different N-(3-dimethylaminopropyl)-
220 N'-ethylcarbodiimide (EDC) concentrations (25 and 35 mM). EDC is added once the protein
221 is ionically adsorbed to promote its covalent binding to the support. The results showed that
222 TmCHMO was completely adsorbed after 0.25 h and 35 mM of EDC was selected as the most
223 appropriate concentration allowing an immobilization yield of 93.0% and a retained activity of
224 62.4% (Table 1).

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

230 For the co-immobilization of TmCHMO and GDH-Tac, two EDC concentrations were tested,
231 10 and 20 mM. These values were selected taking into account the results obtained in the
232 previous immobilization studies. An EDC concentration of 10 mM was selected since GDH-
233 Tac retained activity was significantly affected by high EDC concentration.

234 **3.2.Reutilization of the immobilized biocatalysts and comparison with the soluble** 235 **enzymes**

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

246 The oxidation of 3,3,5-trimethylcyclohexanone was first performed with both soluble
247 TmCHMO and GDH-Tac using a TmCHMO load of 1.07 mg mL⁻¹ of reaction medium and an
248 enzyme ratio of 1:2.0 (mg TmCHMO mg GDH-Tac⁻¹). Total conversion of the initial substrate
249 (10mM) was achieved in 1 h. Once the initial substrate was completely consumed, a fed-batch
250 strategy was applied by supplying an additional 10 mM of substrate to the reaction mixture
251 every hour up to a total of 150 mM of 3,3,5-trimethylcyclohexanone. The results showed that,
252 while the ketone was fully converted in 1 h for the first 3 substrate additions, the accumulation
253 of unreacted substrate was observed for the rest of the reaction until a final substrate

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

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

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

268 The performance of the TmCHMO and GDH-Tac which were separately immobilized at high
269 enzymatic loads was also studied. The TmCHMO immobilized derivative contained 20 mg of
270 monooxygenase g^{-1} of support, while the GDH-Tac derivative contained 29 mg of GDH-Tac
271 g^{-1} of support. Aiming to compare the results with the soluble enzymes, the reactions were
272 carried out using the same load of TmCHMO (1.07 mg TmCHMO per mL of reaction). The
273 ratio of TmCHMO/GDH-Tac was slightly lower (1:1.5) since it is determined by i) the
274 maximum immobilized derivative that can be used (10% v v⁻¹) to ensure a proper suspension
275 and mixing and, ii) the enzymes load per mg of support obtained during the immobilization
276 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.

325 Comparing to the separately immobilized enzymes, all process metric analyzed were improved:
326 1.14-fold decrease in total process time, a 1.3-fold increase in final average product amount, a
327 1.4-fold decrease in the unreacted substrate, a 1.1-fold increase in average conversion, and a
328 1.3-fold increase in TmCHMO biocatalyst yield. The GDH-Tac biocatalyst yield was improved
329 by 3.7-fold because the experiment with the co-immobilized enzymes achieved the best
330 performances with the lowest GDH-Tac loading.

331 Compared to the soluble enzymes for which a fed-batch strategy was applied, even though the
332 total process time was slightly increased, the final average product formed was improved in
333 1.7-fold, the unreacted substrate amount decreased in 3-fold, the average final conversion was
334 increased in 1.6-fold, and the total biocatalyst yield was 3.6-fold higher. These values prove
335 the better performance of the co-immobilized enzymes in the target reaction studied compared
336 to separately immobilized enzymes.

337 **4. CONCLUSIONS**

338 TmCHMO was successfully immobilized on a MANA-agarose support with the co-enzyme
339 GDH-Tac to ensure co-factor regeneration. Both the enzymes immobilized separately and co-
340 immobilized displayed good retention of activity in repeated re-utilization for the oxidation of
341 3,3,5-trimethylcyclohexanone. Co-immobilized proved to give the most efficient biocatalyst
342 format, achieving the highest average conversion over 15 re-utilization cycles (83%) and a high
343 significant improvement of 3.6-fold of the total biocatalyst yield compared to the soluble
344 enzymes. Compared to the biocatalysts which were separately immobilized, a highest reaction
345 rate was observed which was attributed to more efficient diffusion of the NADP(H) co-factor
346 between the two enzymes immobilized on the same support. This work demonstrates that
347 immobilized BVMOs are promising biocatalysts for the synthesis of lactones, and in particular
348 polymeric building blocks.

349 **ACKNOWLEDGMENTS.**

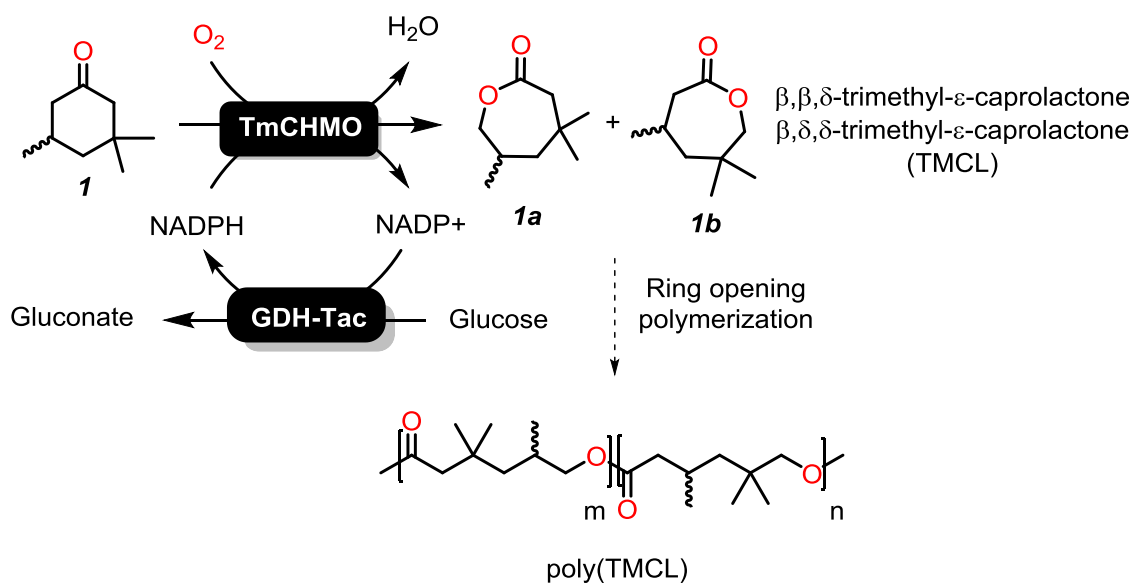
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423 **Figure 1.** Biocatalyzed oxidation of 3,3,5-trimethylcyclohexanone **1** with TmCHMO and
 424 GDH-Tac to give the regio-isomeric lactones **1a** and **1b** which can be polymerized by ring
 425 opening polymerization. The enzymes were either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

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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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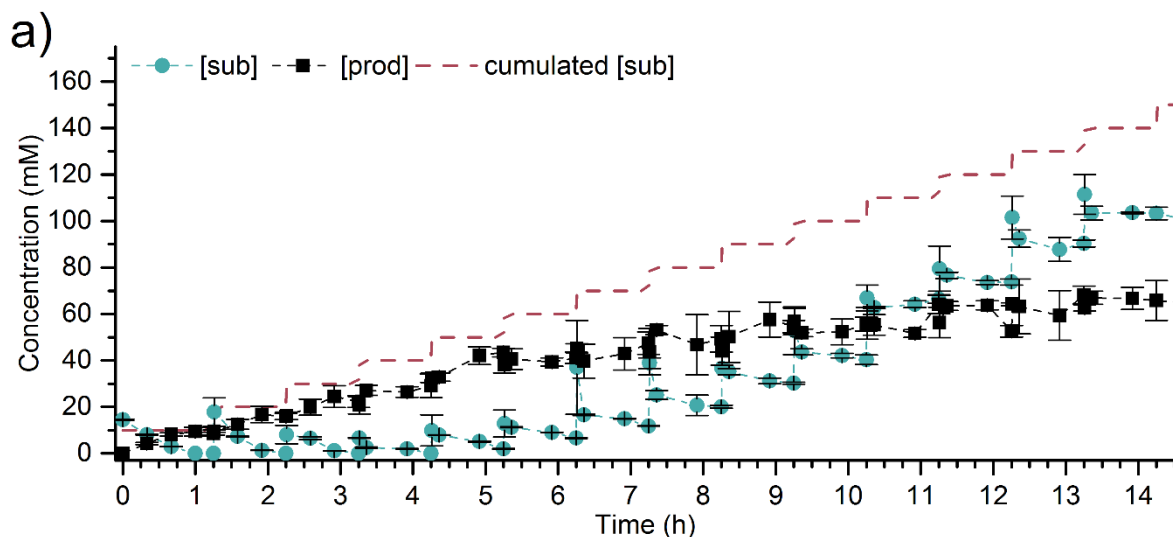
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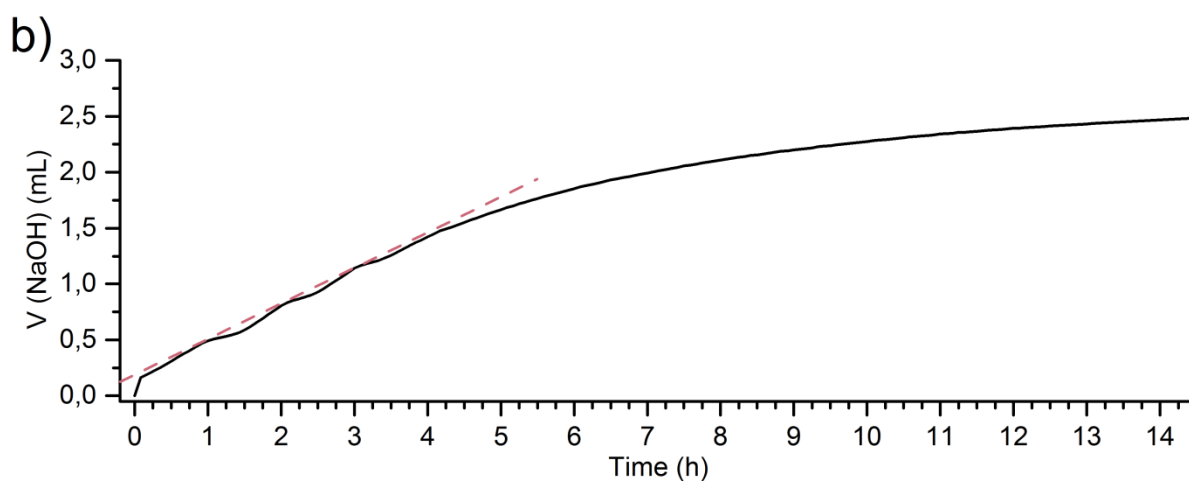
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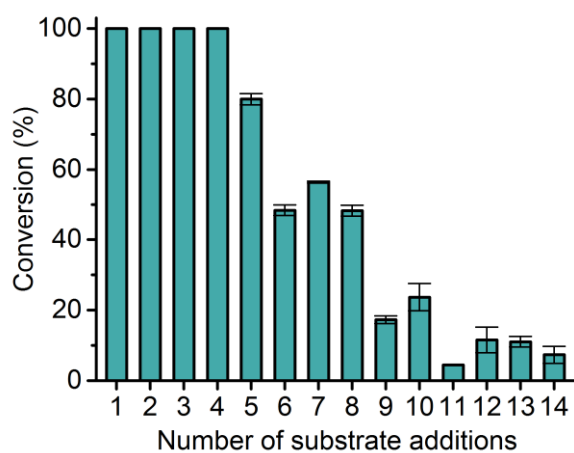


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446 **Figure 2.** a) Reaction course of the conversion of 3,3,5-trimethylcyclohexanone with soluble
 447 TmCHMO and soluble GDH-Tac (TmCHMO/GDH-Tac 1:2.0) with the concentration of
 448 substrate (blue circles) and product (black squares). The total amount of substrate accumulated
 449 is shown with a pink dotted line. b) Profile of the volume of NaOH (1M) added during the
 450 course of the reaction. The pink dotted line indicates the initiation rate of NaOH addition.
 451 Reaction conditions: 10 mM of substrate initially + 10 mM every hour, 10% v v⁻¹ methanol,
 452 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),
 453 350 mM glucose, 250 μM NADP⁺.

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

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471 **Table 2.** Overview of the performances of TmCHMO and GDH-Tac biocatalysts for the
 472 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

473 ^a Cumulated amount of product and unreacted substrate (sum of each cycle for the
 474 immobilized enzymes and value measured at the end of the reaction for the soluble enzymes)

475 ^b Average conversion calculated for 15 cycles for the immobilized enzymes and for 14
 476 additions for the soluble enzymes. ^c Biocatalyst yield = total mg of product/mg of biocatalyst
 477 (TmCHMO, GDH-Tac or TmCHMO + GDH-Tac).

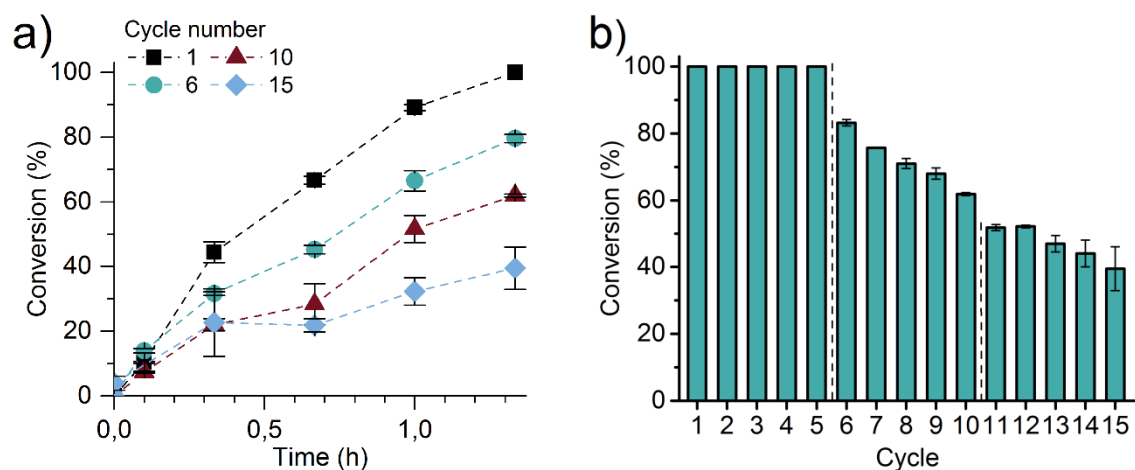
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484 **Figure 4.** Re-uses of TmCHMO and GDH-Tac immobilized on separate supports
 485 (TmCHMO/GDH-Tac 1:1.5) with a) reaction profile for cycles 1, 6, 10, 15; and b) substrate
 486 conversion after 1.33 hour for all cycles. The vertical dotted lines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

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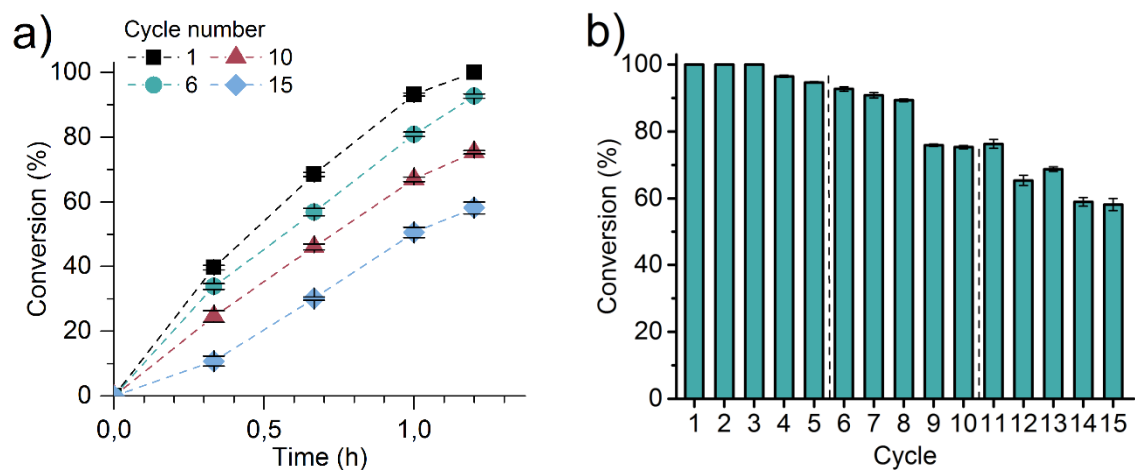
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499 **Figure 5.** Re-uses of co-immobilized TmCHMO and GDH-Tac (TmCHMO/GDH-Tac 1:2.0)
500 with a) reaction profile for cycles 1, 6, 10, 15; and b) substrate conversion after 1.17 hour for
501 all cycles. The vertical dotted lines indicate overnight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