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Synthesis of trimethyl-ε-caprolactone with a novel immobilized Glucose dehydrogenase and an immobilized thermostable Cyclohexanone monooxygenase

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21 **Abstract**

22 An often associated drawback with Baeyer-Villiger monooxygenases, that hinders its application in 23 industrial synthesis, is its poor operational stability. Furthermore, these biocatalysts frequently suffer 24 from substrate/product inhibition and require from the costly NADPH cofactor.

25 In this work, a thermostable Cyclohexanone monooxygenase (TmCHMO) was immobilized and used in 26 the synthesis of trimethyl-ε-caprolactone (CHL). As a cofactor regeneration enzyme, a novel and 27 highly active Glucose dehydrogenase (GDH-01) was successfully immobilized for the first time on four 28 different methacrylate supports and on amino-functionalized agarose. This last matrix was chosen to 29 study the recyclability potential of GDH-01 in the target reaction as it presented an immobilization 30 yield of 76.3 ± 0.7% and a retained activity of 62.6 ± 2.3%, the highest metrics among the supports 31 tested.

32 Both immobilized enzymes were studied either separately or together in six reaction cycles (30 mL; 33 [substrate] = 132.5 mM). When both enzymes were used in its immobilized formulation, 2.8 g of CHL 34 could be synthesized. The reaction yield reached almost completion in the first two cycles and slightly 35 dropped from the third cycle reaching 57.2% in the sixth. A biocatalyst yield of 37.3 g CHL g^{-1} of 36 TmCHMO and 474.2 g CHL g^{-1} of GDH-01 were obtained. These values represent a 3.6-fold and 1.9- 37 fold increase respectively, compared with a model reaction where both enzymes were used in its 38 soluble form.

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40 **Keywords**: trimethyl-ε-caprolactone; Baeyer-Villiger monooxygenase; cofactor regeneration; re- 41 cycling; immobilized enzymes biocatalyst yield.

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44## 1. Introduction

45Process chemists have long considered biocatalysis as a good alternative to the conventional routes in 46chemical manufacturing [1]. The use of whole cells, isolated enzymes or immobilized enzymes has 47often proven to be a greener, sustainable and more profitable way to catalyze such chemical reactions 48[2]. The industry has already adopted bioconversions, for example, for the production of amino acids 49[3], lactic acid, succinic acid or 3-hydroxypropionic acid [4] among many others [5,6].

50In the case of the concerned reaction, Baeyer-Villiger oxidations are well known since their discovery 51in 1899 [7]. The chemical route though, usually implies a limited regio-selectivity (to the sterically 52more hindered side), the use of hazardous and pollutant solvents and halogenated oxidants [8–10]. 53At the same time, the transformation of ketones into esters or cyclic ketones into lactones can also be 54accomplished by the so called Baeyer-Villiger monooxygenases (BVMOs) [11–13]. The first evidence 55was provided by Fired *et al.* in 1953 with the conversion of progesterone to testololactone [14]. The 56enzymatic alternative is often associated with milder aqueous conditions, the use of oxygen as 57oxidant and higher selectivity [15]. However, their implementation is hindered by some drawbacks 58that may come with biocatalysts and specially with monooxygenases [16,17]. BVMOs have been 59suffering from low operational stability, substrate and product inhibition and the use of the costly 60NADPH cofactor [11,12,18].

61These limitations can be tackled mainly by three strategies: protein engineering [19], reaction 62engineering [20] and immobilization [21,22]. There are many are the examples of BVMOs that had 63been engineered either by means of directed evolution or rational design [15,23]. For example, a 64recent work by Kathleen *et al.* demonstrated that certain conserved residues in the active site of 65BVMOs, when altered, lead to modified regioselectivity [24,25]. At the same time, the substrate and 66product inhibition can be overcome using a different approach [26,27]. The substrate feeding and 67product removal strategy (SFPR) has been applied for BVMOs processes using resins like Optipore L-68493 or Lewatit VPOC 1163 [28,29]. Finally, immobilization of enzymes is a well-known procedure that

69often confers improved operational and storage stability, allows the possibility to operate in 70continuous mode, facilitates the isolation and purification of the product and allows the re-utilization 71of the biocatalyst [30–32].

72In this sense, one of the first contributions was from Walsh *et al.* who immobilized a cyclohexanone 73monooxygenase (CHMO) together with a glucose dehydrogenase (GDH), as a cofactor regeneration 74enzyme, onto polyacrylamide gel and used it in 1 liter reactions for 10 days [33]. Interestingly, the 75covalent immobilization of BVMOs together with a GDH is one of the most widely used combinations 76in the literature [11]. In this work, this same strategy was applied for the production of the two regio-77isomers of trimethyl-ε-caprolactone (CHL) from 3,3,5-trimethylcyclohexanone (TMCH). Two novel 78enzymes were used for this purpose, the thermostable CHMO from *Thermocrispum municipale* DSM 7944069 (TmCHMO; EC 1.14.13.22) [34] and the highly active GDH-01 (EC 1.1.1.47) (Figure 1).

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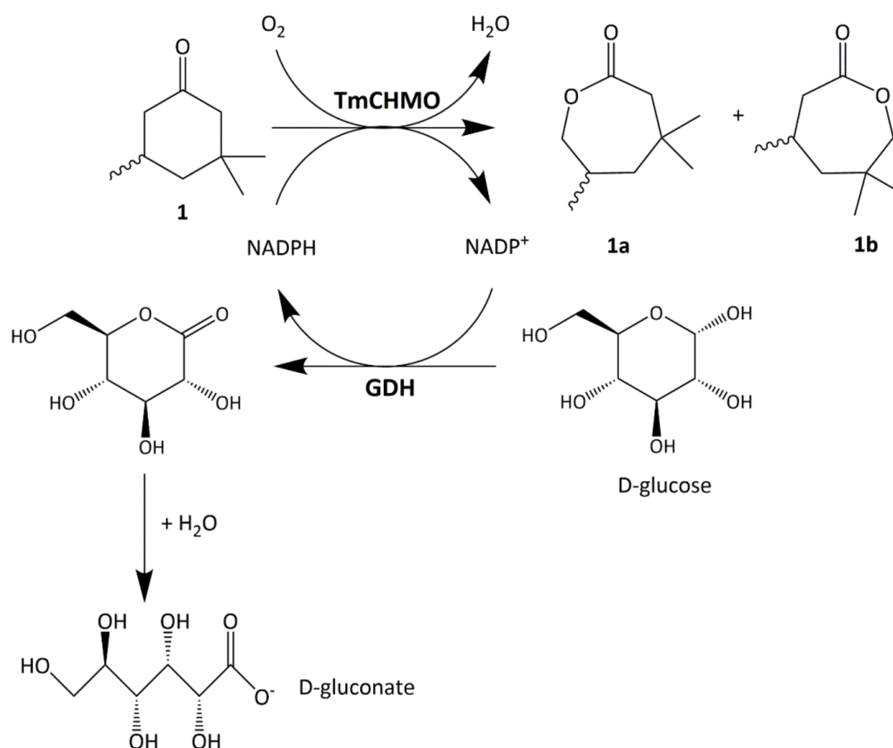

81*Figure 1. Biocatalyzed oxidation of the branched substrate 3,3,5-trimethylcyclohexanone (**1**) (TMCH) to a* 82*mixture of β,β,δ-trimethyl-ε-caprolactone (**1a**) and β,δ,δ-trimethyl-ε-caprolactone (**1b**) (CHL) with a two-*

83*enzyme system using Glucose dehydrogenase (GDH) to regenerate the NADPH using D-(+)-Glucose as a* 84*sacrificial substrate.*

85Immobilization of TmCHMO has been previously conducted by Delgove *et al.* and the derivate was 86also used for the synthesis of CHL [35]. In order to regenerate the cofactor, an immobilized GDH from 87*Thermoplasma acidophilum* was used in that case.

88In the present work, in contrast, the immobilization of the novel GDH-01 has been studied for the first 89time on a broad variety of supports presenting different functional groups and characteristics [36]. 90The immobilized derivate served as biocatalyst together with the immobilized TmCHMO in the target 91reaction and they were re-used for six cycles. The substrate was continuously dosed, in order to avoid 92substrate inhibition, until 132.5 mM were reached. This substrate concentration represents a more 93than 13-fold increase compared with the previous work aforementioned.

94**2. Materials and methods**

95**2.1. Chemicals and supports**

96D-(+)-glucose (> 97.5%) and ethyl acetate (> 99.9%) were purchased from VWR Chemicals (Radnor, 97USA). β-Nicotinamide adenine dinucleotide phosphate disodium salt (> 93%), was obtained from 98SyncoZymes (Pudong Xinqu, China). All the other chemicals and reagents were purchased from 99Sigma-Aldrich and were of analytical grade if not stated otherwise. Buffers, substrate solutions and 100other stocks were prepared freshly and stored at 4 - 6°C for at most 48 h. Commercial Glucose 101dehydrogenase GDH-01 was supplied by InnoSyn B.V. (Geleen, The Netherlands) as liquid enzyme 102formulation (LF). The LF contained 47 ± 1.4 mg protein mL^{-1} with 57.5 ± 4.7% GDH-01 content. The 103specific activity of the GDH-01 resulted in 310.6 ± 28.5 U mg^{-1} enzyme. Metrics obtained from the 104procedures described in section 2.3 and 2.4.

105Methacrylate/styrene resins were kindly donated by Purolite® Life Sciences and stored at 4 - 6°C. High 106density aminoethyl 4BCL agarose (Mana-agarose) was purchased from Agarose Bead Technologies

107(ABT®, Madrid, Spain). Non-functionalized 4BCL agarose was also purchased from ABT® and it was 108further functionalized with epoxy groups following the procedure described by Axarli *et al [37]*.

109**2.2. Recombinant production of TmCHMO and GDH-01 in *E. coli***

110Cyclohexanone monooxygenase from *T. municipale* (TmCHMO) was recombinantly produced in 111*Escherichia coli* in a 10 L scale fed-batch, high cell-density fermentation with glucose as growth 112limiting C-source employing an *E. coli* K12 derivative and a pBR322 derived expression vectors. 500 113mL pre-cultures were used to inoculate 10 kg main culture medium with 100 µg mL^{-1} neomycin. The 114pre-culture was prepared in standard Luria-Bertani (LB) medium supplemented with 100 µg mL^{-1} 115neomycin. The fermentation was performed using mineral medium supplemented with 20 g L^{-1} yeast 116extract. 1.5 days (d) after inoculation of the fermenter as inducer, pre-sterilized L-arabinose was 117added to the fermenter to final concentration 0.02 % (w/w). After about 100 hours (h) the biomass 118was either harvested by centrifugation (wet cells) or the fermentation broth was used as biocatalyst 119as such. The broth contained 412.7 g cell wet weight (cww) per milliliter. Liquid formulation (LF) of 120fermentation was prepared by adding 2 weight equivalents of 100 mM potassium phosphate (KPi) 121buffer (pH 7.0) to 1 equivalent of harvested *E. coli* wet cells (333.3 g_{cww} mL^{-1}) and sonication with an 122ultrasound probe for 20 minutes (10 seconds on, 10 seconds off) with cooling on ice. The LF 123contained 59.4 ± 4.9 mg protein mL^{-1} with 55.2 ± 0.7% TmCHMO content. The specific activity of the 124TmCHMO resulted in 1.76 ± 0.06 U mg^{-1} enzyme. Metrics obtained from the procedures described in 125section 2.3 and 2.4.

126**2.3. Total protein and enzyme content**

127The characterization of the samples was carried out exclusively on liquid formulations which were 128pre-cleared by centrifugation (3220 g for 15 min). The protein concentration was analyzed by means 129of the Bradford Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA) using bovine serum 130albumin (BSA) as standard (0.05 - 0.5 mg mL^{-1}) [38]. The enzyme content was measured using sodium 131dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (NuPage 12%, Invitrogen, USA) run

132in a Mini-PROTEAN II apparatus (BioRad, USA) following the protocol by Laemmli *et al* [39]. Precision 133Plus Protein™ blue prestained protein standards (BioRad, USA) (10 – 250 kDa) were used for 134molecular weight determination. Gels were stained using Coomassie G250 colloidal stain solution 135[34% (v/v) ethanol, 2% (v/v) H_3PO_4, 17% (w/v) $(NH_4)_2SO_4$ and 0.066% Coomassie G250] and the Image 136LAB™ software (BioRad, USA) was used for image processing.

137**2.4. TmCHMO and GDH-01 activity measurements**

138The TmCHMO activity was measured spectrophotometrically following the NADPH (0.1 mM) 139consumption at 340 nm wavelength (ε = 6.22 mM^{-1} cm^{-1}) and using cyclohexanone as substrate (0.5 140mM) [35]. The sample as well as the NADPH and substrate were diluted in 50 mM potassium 141phosphate buffer (KPi) pH 8. One unit of activity (U) was defined as the amount of enzyme required 142to convert 1 µmol of NADPH per minute at 30˚C and pH 8.

143The GDH-01 activity was measured spectrophotometrically following the $NADP^+$ (0.4 mM) 144consumption at 340 nm wavelength (ε = 6.22 mM^{-1} cm^{-1}) and using (+)-D-glucose as substrate (212.5 145mM) [40]. The sample as well as the NADPH and substrate were diluted in 50 mM potassium 146phosphate buffer pH 8. One unit of activity (U) was defined as the amount of enzyme required to 147convert 1 µmol of $NADP^+$ per minute at 30˚C and pH 8.

148Activity of TmCHMO and GDH-01 was measured from liquid formulation samples diluted to an extent 149until the activity measured was in the linear range of the activity test (0.2 to 5 U mL^{-1}).

150**2.5. GDH-01 stability studies**

151The activity decay of four GDH-01 samples (0.8 U mL^{-1}) each one presenting a different pH value (5, 6, 1527 and 8) was measured over time. The GDH-01 LF was 10000-fold diluted in 50 mM phosphate buffer 153solutions and the pH was adjusted using either 1M HCl or 1M NaOH.

154Furthermore, the activity decay of three samples presenting different enzyme concentrations was 155measured after 1 hour incubation at pH 6, 7 or 8 each. The LF containing the over-expressed GDH-01 156was diluted 100-fold, 1000-fold and 10000-fold containing 0.47, 0.047 and 0.0047 mg prot mL^{-1} 157respectively. Each of these solutions was then incubated for 1 hour at 30˚C in 50 mM phosphate 158buffer solutions (pH 6, 7 or 8).

159**2.6. Immobilization of GDH-01 onto Methacrylate/Styrene resins.**

160As a first stage in an immobilization procedure, the supports are loaded with low amounts of enzyme 161so that diffusional limitations are avoided. Methacrylate/Styrene resins from Purolite® were studied 162offering 20 U of GDH-01 per gram of resin which equals to 64.4 µg of enzyme per gram of support.

163A characterization was pursued in order to obtain the retained activity and immobilization yield which 164were calculated as explained elsewhere [40]. Supernatant (precipitated support) and suspension 165(suspended support) were analysed over time until a steady state was reached and in all cases, a 166blank (no support) was also monitored to observe how the enzyme activity was affected by the 167protocol's conditions.

168The Purolite® resins offered a variety of features and functionalities (Table S1) and the immobilization 169in each case was carried out according to the supplier's specifications. The offered enzyme was 170maintained in all cases and the immobilizations proceeded until the supernatant and the suspension 171measured activities reached a steady state. The buffered solution contained 10% (w/v) of the carrier 172in all cases.

173The epoxy functionalized methacrylates (ECR8204F, ECR8215F and ECR8285) were tested using 50 174mM KPi buffer pH 6 mixed with 0.5 M NaCl to increase the ionic strength and favor the attachment. 175The amino functionalized carriers (ECR8309F, ECR8315F, ECR8409F and 8415F) were studied using 50 176mM KPi buffer pH 6. The immobilization with amino functionalized supports is divided in three steps: 177i) ionic adsorption of the enzyme onto the support ii) addition of 10 mM *N*-(3-dimethylaminopropyl)-

178*N*′-ethylcarbodiimide (EDC) and incubation for 1.5 hours to promote the covalent binding and iii) 179addition of 0.5 M NaCl to desorb all the protein attached non-covalently. The non-functionalized 180supports (ECR8806F, ECR1061M and ECR1030M) were tested using 50 mM KPi pH 6. Finally, the 181amino resins were further functionalized with 2% (w/v) glutaraldehyde for 60 min at 25˚C leaving free 182aldehyde groups on the surface of the carrier. The immobilization was carried out using 50 mM KPi 183buffer pH 6.

184A limit was set for both the immobilization yield and the retained activity in order to consider the 185carrier as good candidate, 40% for immobilization yield and 20% for retained activity.

186**2.7. Immobilization of GDH-01 onto functionalized agaroses**

187The immobilization of GDH-01 onto Epoxy-agarose and Mana-agarose was carried out following the 188same procedure as explained above for the epoxy and amino functionalized methacrylate (Purolite®). 18920 U of GDH-01 were offered per gram of support to avoid diffusional limitations. For the Epoxy-190agarose, 50 mM KPi buffer pH 6 with 0.5 M NaCl was used. For the Mana-agarose, 50 mM KPi buffer 191pH 6 was used and the three steps aforementioned were as well followed. In this case, three different 192EDC concentrations were tested: 10, 20 and 30 mM.

193Apart from the characterization stage where low enzyme load is used, the GDH-01 was immobilized 194onto Mana-agarose using high (maximum) loads of LF. The immobilization proceeded as explained 195above with the difference that in this case, the amount of GDH-01 added was higher (11061.6 U g^{-1} 196support). Prior to use the carriers in reaction, they were gently washed with 50 mM KPi buffer pH 6. 197The calculation of the immobilization yield and final activity were calculated following the equations 198published elsewhere [40].

199**2.8. Immobilization of TmCHMO onto Mana-agarose**

200The conditions for the immobilization of TmCHMO onto Mana-agarose were optimized and published 201recently by Delgove *et al.* *[35]*. The characterization of the enzyme and the immobilization was

202obviated in this work and high loads of enzyme were used in the experiments performed (86.5 U g^{-1}

203support offered). After adsorption of the enzyme onto the carrier, the mixture was incubated with 35

204mM EDC for 2 hours. The immobilization yield and final activity were calculated as explained for the

205GDH-01.

206**2.9. Reaction set up and conditions**

207The set up used for either the soluble or the immobilized enzyme reactions, consisted of a sealed

208jacketed glass reactor (30 mL), a pH controller (Metrohm Titrino plus 877) using 1 M NaOH solution

209and it incorporated a propeller stirrer set at 1200 rpm, a thermostat (MGW-LAUDA RC6) set at 30 ˚C, a

210condenser at 6˚C, a compact mass flow regulator (GCR Red-y) to keep a constant air flow of 16 mL

211min^{-1}; and a substrate dosing pump (Harvard Pump11).

212For the reaction with soluble biocatalysts the following conditions were used: an enzyme load of 10%

213(v/v) of TmCHMO broth (57.8 U mL^{-1} of broth) and 0.5% (v/v) of GDH-01 (8408.8 U mL^{-1} of LF); 25 mM

214KPi pH 7; a TMCH dosing rate of 30 mM h^{-1} (240 mM final) together with a methanol dosing rate of

2151.25% (v/v) h^{-1} [10% (v/v) final]; [D-glucose] 375 mM and [NADP$^+$] 0.25 mM.

216For the reactions catalysed by immobilized enzymes the support loaded varied from 1.7% to 10%

217(w/v), the substrate dosing rate was 29 mM h^{-1} (132.5 mM final concentration) and the methanol

218dosing rate was 2.17% (v/v) h^{-1} [10% (v/v) final concentration]. The rest of conditions were the same

219as for the reaction with soluble biocatalysts.

220**2.10. Reaction progress determination by GC-FID**

221Samples (150 µL) were taken periodically from the reactor, weighed and dissolved up to 10 mL with a

222solution of acetonitrile containing 0.5 g L^{-1} of hexadecane that served as Internal Standard (IS). The

223mixture was centrifuged to remove insoluble biomass and the supernatant was analyzed by a gas

224chromatograph (GC) equipped with a flame ionization detector (FID). The concentration of substrate

225and products were determined using calibration curves.

226The centrifuged supernatant samples containing trimethyl-cyclohexanone and trimethyl-ε-227caprolactones were analyzed using a 7890A gas chromatograph (Agilent Technologies, USA) equipped 228with a HP-5 column (30 m, 0.32 mm, 0.25 µm df, Agilent Technologies). The column temperature was 229maintained at 60°C for 2 minutes, increased up to 300°C at 10°C min^{-1} and it was held at final 230temperature for 2 minutes. The injector temperature was kept at 200°C; for the flame ionization 231detector, the temperature was 300°C. Hydrogen was used as a carrier gas at a flow rate of 40 mL min^{-1} 232and air at 450 mL min^{-1}. The retention times observed were: 8.5 min for the substrate **1**, 11.9 min for 233lactone **1b**, 12.1 min for lactone **1a** and 15.8 min for the IS.

234**2.11. Re-usability of immobilized derivates towards the synthesis of trimethyl-ε-caprolactone**

235Reactions performed using either one or both enzymes immobilized, were performed in a similar 236fashion as for the soluble reactions. The difference was that once the reaction was finished, the 237whole reactor content was filtered and the derivate/s were washed gently with 50 mM KPi buffer pH 2387. The immobilized enzyme was placed back into the reactor with no further treatment and the next 239cycle of reaction started.

240**3. Results and discussion**

241**3.1 Stability of GDH-01 in different pHs and concentrations**

242As introduced previously, the aim of this work was the immobilization of the novel GDH-01 and the 243re-utilization of this enzyme together with the TmCHMO in the synthesis of trymethyl-ε-caprolactone. 244As opposite to the TmCHMO, the GDH-01 has never been immobilized before, that is why 245characterization regarding its stability was required. When immobilizing, the media pH must be 246chosen taking into account the support utilized and the activity decay of the enzyme at that certain 247pH. The results obtained are represented in Figure 2 A.

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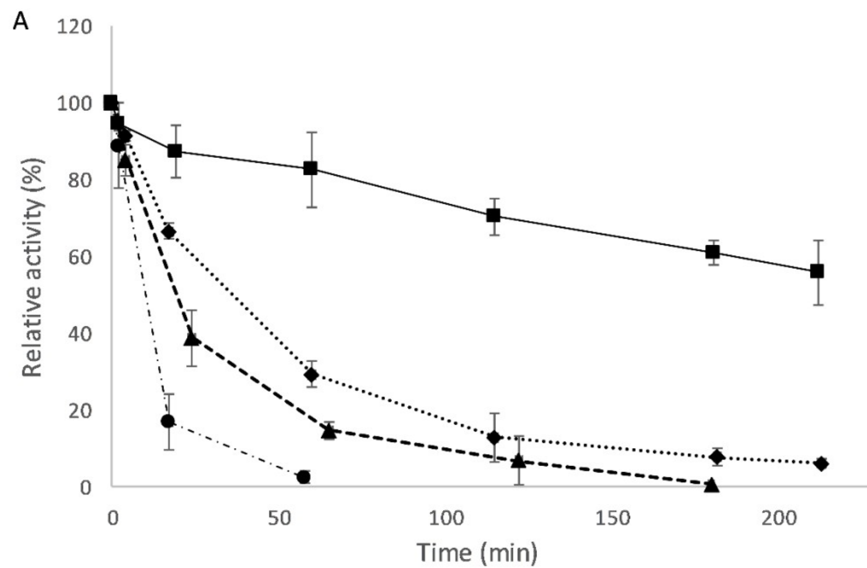

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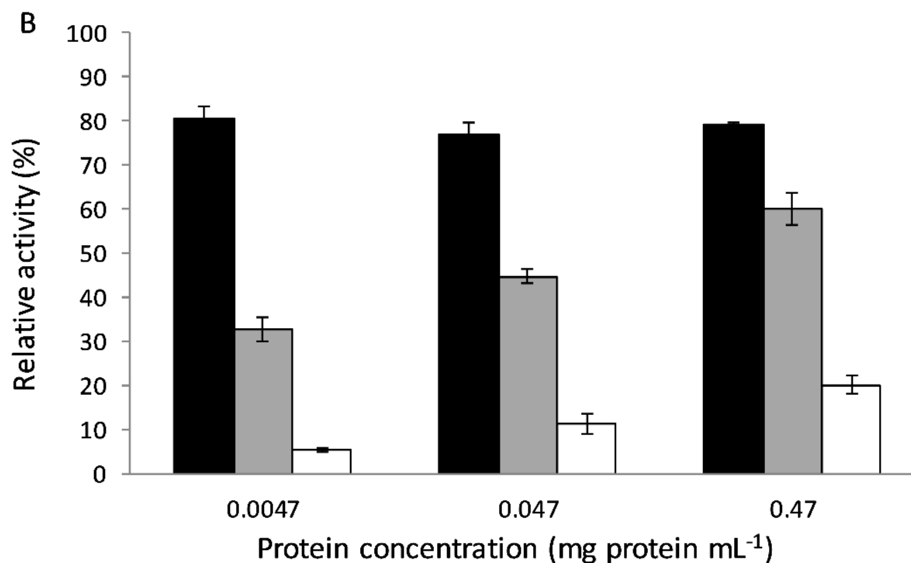


250*Figure 2. GDH-01 stability studies. A) GDH-01 relative activity along time measured at different pH values (of KPi*
251*buffers): pH 5 (black triangles and discontinuous line), pH 6 (black squares and continuous line), pH 7 (black*
252*rhombus and dotted line) and pH 8 (black circles and combined discontinuous spot-line-spot). The initial activity*
253*of the samples was 0.8 U mL^{-1}; 10000-fold dilution of the initial liquid formulation. B) GDH-01 relative activity*
254*after 1 hour incubation at three different pHs: pH 6 (black bars), pH 7 (grey bars) and pH 8 (white bars); and*
255*three different protein concentrations. The initial activities of the samples were 0.8 U mL^{-1}, 8 U mL^{-1} and 80 U*
256*mL^{-1} for the 0.0047, 0.047 and 0.47 mg protein mL^{-1} respectively. The error bars of both figures, A and B,*
257*correspond to the standard error calculated from at least two replicates.*

258As it can be observed in Figure 2 A, when GDH-01 was diluted in pH 8 buffer, it was almost completely 259deactivated after one hour. On the other hand, pH 6 turned out to be the most favorable for this 260enzyme which maintained 50% of the activity after 3.5 hours. In Figure 2 B, the LF containing GDH-01 261was incubated for 1 hour at three different pHs (6, 7 and 8) and three different protein concentrations 262were applied for each pH. The graph shows that, at pH 7 and 8, the enzyme is deactivated to different 263extent depending on the concentration it is in. The lowest concentrated sample suffers the highest 264loss of activity. At pH 6 though, the relative activity after 1 hour is almost the same for the three 265enzyme concentrations. Diluting the GDH-01 LF in a solution containing 5 mg mL^{-1} of Bovine Serum 266Albumin (BSA) or pre-coating the vial with BSA did not improve the GDH-01 stability at pHs different 267from 6.

268The stability dependency on the enzyme concentration has also been observed previously on other 269biocatalysts but not with GDH-01. The dilution of the enzyme below the concentration of the binding 270constant of its subunits or prosthetic groups can provoke the loss of the protein quaternary structure 271or the loss of the essential prosthetic group [41].

272The TmCHMO stability was not measured due to the existence of previous works about its 273immobilization [35].

274**3.2 Immobilization of GDH-01. Characterization of different supports.**

275**3.2.1 Methacrylate/Styrene based supports**

276Glucose dehydrogenase is used in this target reaction as a cofactor regeneration enzyme. In the case 277of the novel GDH-01, the GDH studied in this work, no publications exist regarding its immobilization 278as far as the authors know. Once the stability of the enzyme at different pHs was known, the goal was 279finding a suitable support for immobilization.

280A set of 14 different methacrylate/styrene resins covering a broad range of enzyme carrier features 281were obtained from Purolite® Technologies. The materials supplied had different: pore diameters

282(300 - 1800 Å), enzyme-carrier interactions (ionic, covalent and hydrophobic), functional groups 283(epoxy, amino and aldehyde), linker lengths (C2 - C18), material matrices (methacrylate and styrene) 284and particle sizes (150 – 710 µm). A detailed description of each carrier and the results obtained for 285the immobilization of GDH-01 can be found in the Appendix A (Table A.1).

286The supports that resulted in at least 40% immobilization yield and 20% retained activity are 287presented in the table below (Table 1). As can be seen, only four supports fulfilled the 288aforementioned criteria: one amino functionalized support (ECR8415F) and three aldehyde-289functionalized supports (ECR8315F, ECR8409F and ECR8415F).

290*Table 1. Results regarding the immobilization of GDH-01 onto Methacrylate/Styrene (Purolite®) resins and 291description of the support's features. Only those experiments with at least 40% immobilization yield and 20% 292retained activity are presented. Further information regarding other supports screened can be found in 293Supplementary information, Table S1. The standard error (± %) was calculated from at least two replicates.*

Code	Functional group (Linker)	Matrix	Interaction	Pore diameter (Å)	Particle size (µm)	Immobilization (%)	Retained acitivty (%)
ECR8415F	Amino (C6)	Methacrylate	Ionic/Covalent	1200 - 1800	150 - 300	40.8 ± 2.2	21.3 ± 3.5
ECR8315F	Aldehyde (C7)	Methacrylate	Ionic/Covalent	1200 - 1800	150 - 300	99.9 ± 0.1	23.9 ± 3.4
ECR8409F	Aldehyde (C11)	Methacrylate	Ionic/Covalent	600 - 1200	150 - 300	100 ± 0.0	22.5 ± 2.6
ECR8415F	Aldehyde (C11)	Methacrylate	Ionic/Covalent	1200 - 1800	150 - 300	100±0.0	28.0 ± 5.1

294When working with amino functionalized resins, as is the case of ECR8415F, the immobilization is 295carried out in three steps. First, an ionic interaction between the positively charged amino groups of 296the support and the negatively charged carboxyl groups of the enzyme occurs. In this first binding, 29799.1 ± 0.1% immobilization yield and 60.0 ± 4.1% retained activity were obtained with ECR8415F. 298However, the optimized pH for the target reaction is 7, which changes the positive charge of the 299resin's amino groups, desorbing the enzyme from the carrier. Thus, an agent promoting a covalent 300bond formation is required for its use in the target reaction. *N*-(3-dimethylaminopropyl)-*N*´-

301ethylcarbodiimide (EDC) was chosen due to its high solubility in water. The immobilization yield and
302retained activity obtained were 40.8 ± 2.2% and 21.3 ± 3.5%, respectively. The EDC is often associated
303with enzyme deactivation, however, in this case, the low immobilization yield indicates that the
304amount of EDC added was not enough to covalently bind all the enzyme offered and a major part of
305the initially attached GDH-01 was desorbed when the NaCl was introduced.

306Regarding glutaraldehyde functionalized supports (ECR8315F, ECR8409F and 8415F), the results are
307similar between the three, even though the supports differ in linker lengths and pore diameters. In
308the three cases, the GDH-01 showed immobilization yields close to 100% meaning that the enzyme
309presents high affinity for the carrier. However, either due to miss-orientation, unfolding or stacking,
310the enzyme attached was significantly deactivated (low retained activities) showing less than 30% of
311retained activity in all cases.

312The methacrylate/styrene resins are rather hydrophobic which can contribute to enzyme deactivation
313during immobilization. Taking into account that all the supports tested so far had different
314functionalizations but similar matrices, new materials made out of more hydrophilic materials were
315to be tested. In this sense, agaroses were the first choice for further investigation, aiming to obtain
316immobilized derivatives with higher retained activities [42].

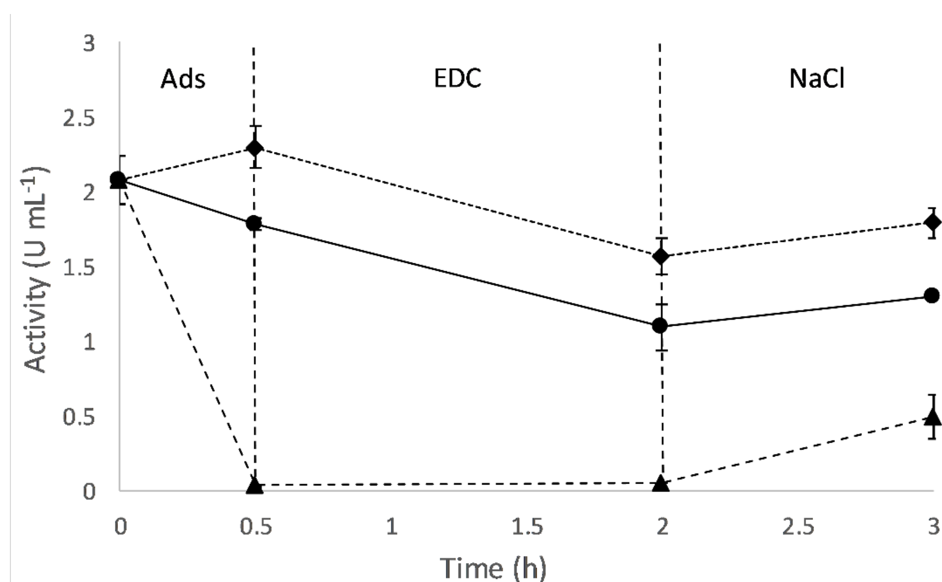
317**3.2.2 Agarose based matrices: Epoxy- and Mana-agarose**

318As mentioned before, more hydrophilic matrices were tested presenting two different
319functionalizations. An epoxy functionalized agarose and an amino functionalized agarose (Mana-
320agarose) were studied as potential immobilization supports for GDH-01.

321The Epoxy-agarose immobilization is usually pursued at alkaline pH. However, the studies performed
322with GDH-01 showed a very poor stability of the enzyme at pH 8 (Figure 2 A). The immobilization was
323therefore conducted at pH 6 and 0.5 M of NaCl were added in order to increase the ionic strength of

324the medium to promote the binding. The results showed low affinity of the enzyme for the support 325and a slight over-activation. At the end, 29.5 ± 7.3% retained activity were obtained.

326The amino functionalized agarose (Mana-agarose) immobilization proceeds like the amino 327functionalized resins from Purolite®. The immobilization takes place in three steps: i) ionic adsorption, 328ii) covalent binding and iii) desorption with 0.5 M NaCl (Figure 3).



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330*Figure 3. Immobilization course of the GDH-01 onto amino functionalized agarose (Mana-agarose) using 10 mM 331EDC concentration and offering 20 units of GDH-01 activity (64.4 µg of enzyme) per gram of agarose. The graph 332shows the activity of the blank (black circles and continuous line), the supernatant (black triangles and 333discontinuous line) and the suspension (black rhombus and dotted line) along time. The immobilization is 334divided in the three different phases: i) ionic adsorption of the enzyme to the carrier (Ads), ii) incubation with 335the covalent bond promoter, EDC (EDC) and iii) desorption of the unattached enzyme with 0.5 M NaCl (NaCl).*

336The results regarding the Mana-agarose immobilization are presented in Table 2. As it can be 337observed in the second and third columns, 98.4 ± 0.2% of the initial GDH-01 bound to the agarose by 338ionic interaction and a slight over-activation occurred (retained activity 105.5 ± 3.8%). As explained 339for the amino-methacrylate supports though, the pH of the reaction (pH 7) does not allow an ionic 340immobilization to be used. Results obtained after covalent binding formation reached 76.3 ± 0.7 % 341and 62.6 ± 2.3% of immobilization yield and retained activity, respectively (Figure 3), which

342represents a high significant improvement compared to the results obtained with the 343methacrylate/styrene matrices.

344*Table 2. Immobilization of GDH-01 onto amino functionalized agarose (Mana-agarose) using three different EDC 345concentrations. 20 units of GDH-01 activity (64.4 µg of enzyme) were offered per gram of agarose. The standard 346error (± %) was calculated from at least two replicates.*

	Ionic Adsorption		**Covalent binding**	
[EDC]	Immobilization Yield (%)	Retained Activity (%)	Immobilization Yield (%)	Retained Activity (%)
10	98.4 ± 0.2	105.5 ± 3.8	76.3 ± 0.7	62.6 ± 2.3
20			94.1 ± 0.1	47.2 ± 3.3
30			98.2 ± 0.1	44.2 ± 0.2

347

348Due to the promising results obtained with Mana-agarose, in addition to the use of 10 mM of EDC, 349two different concentrations were also tested (20 mM and 30 mM) aiming to obtain an immobilized 350derivate with the highest activity possible (Table 2). However, even though when using higher 351concentrations of EDC the immobilization yield increased more than 1.2-fold, the enzyme got 352deactivated and the retained activity dropped to 15.4% and 18.4%, respectively. That is why 10 mM 353of EDC was chosen as the best condition albeit 23.7% of the initial activity remained in the 354supernatant. The immobilization onto Mana-agarose represents a step forward compared to the 355methacrylate/styrene (Purolite®) supports. The retained activity in this case is 2.94-fold higher than 356the amino functionalized methacrylate (ECR8415F) and 2.24-fold higher than the aldehyde 357functionalized methacrylate (ECR8415F), the best candidates from the previous trials. As it happened 358with the other resins, this is the first time that the successful immobilization of GDH-01 onto Mana-359agarose is reported. This support was chosen to study the re-cyclability capacity of GDH-01 in the 360synthesis of trimethyl-ε-caprolactone.

361**3.3 Immobilization of GDH-01 and TmCHMO on Mana-Agarose. Maximum loading capacity**

362The maximum quantity of GDH-01 and TmCHMO that can be attached to Mana-agarose was studied. 363Different amounts of enzyme were immobilized onto the support in order to determine this value.

364Since mass transfer limitation could occur at high enzyme loads, retained activities obtained during 365the immobilization characterization at low loads were used here to calculate the theoretical 366maximum loading capacity in terms of activity units [Table 3, Final activity (U g^{-1} support)].

367*Table 3. Immobilization of GDH-01 onto Mana-agarose using maximum loads of enzyme per gram of support.* 368*The covalent immobilization of GDH-01 was carried out using 10 mM EDC for 1.5 hours while the TmCHMO was* 369*incubated for 2 hours with 35 mM of EDC.*

Enzyme	Activity offered (U g^{-1} support)	Final activity (U g^{-1} support)	Retained protein (mg protein g^{-1} support)
GDH	11061.6	3692.5	23.1
TmCHMO	86.5	53.2	67.7

370

371The immobilization of TmCHMO was carried out using the optimized conditions published recently by 372Delgove *et at.* [35]. The amino-functionalized agarose was used as a carrier and 35 mM of EDC were 373applied as a covalent bond promoter. The obtained retained activity at low loads in that study was 37462.4 ± 2.1%. The results obtained regarding the immobilization of TmCHMO using high loads of 375enzyme, are shown in Table 3. The maximum loading capacity of TmCHMO resulted in 67.7 mg 376protein g^{-1} support and 53.2 U g^{-1} support.

377Regarding the GDH-01, 23.1 mg protein g^{-1} support were immobilized using 10 mM EDC 378concentration. According to the retained activity obtained during the characterization (62.6 ± 2.3%), 3793692.5 U g^{-1} support could be loaded onto Mana-agarose (Table 3).

380**3.4 Synthesis of trimethyl-ε-caprolactone**

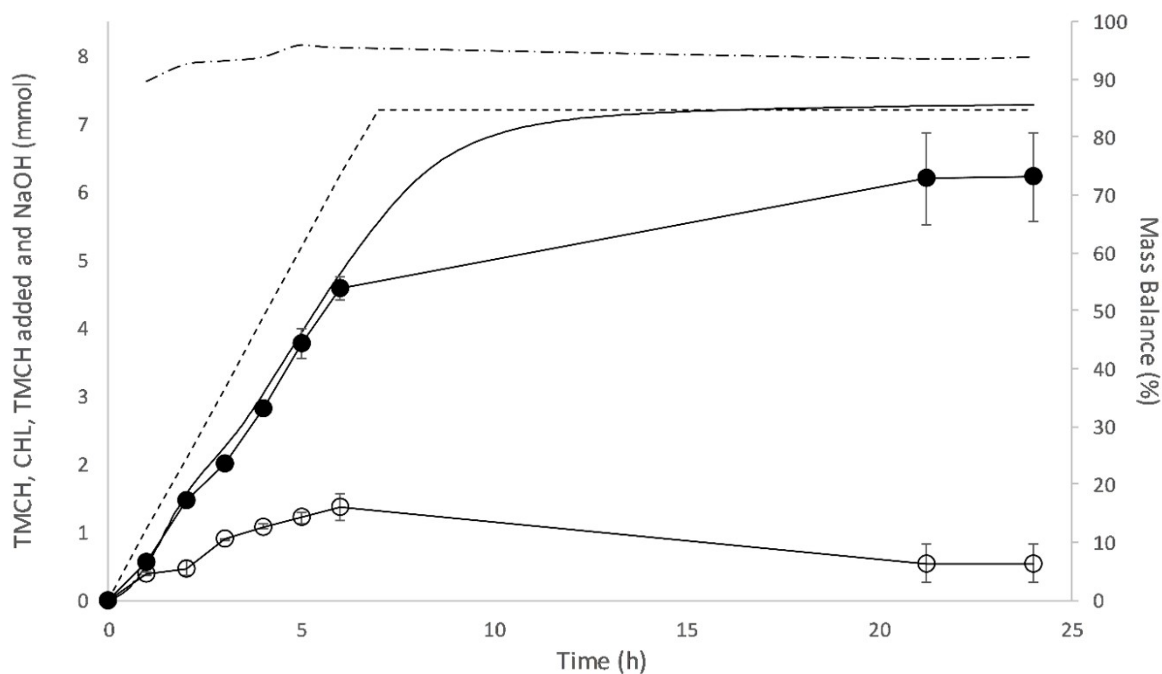
381**3.4.1 Soluble enzymes**

382Aiming to compare the performance of the immobilized derivatives with the soluble enzyme, a 383reaction was run firstly using non-immobilized TmCHMO and GDH-01. Different metrics such as 384biocatalyst yield and total product synthesized were used for comparison. The conditions published

385recently [43] and further optimized by the authors (data not shown) were mimicked in this study at 38630 mL scale using 10% (v/v) load of TmCHMO fermentation broth and 0.5% (v/v) GDH-01 LF.

387The reaction course can be seen in Figure 4. A continuous substrate feeding strategy was used in 388order to avoid substrate inhibition which has been observed for this enzyme [18,43]. Even though the 389substrate was continuously added, certain amount of it was accumulated at the beginning of the 390reaction. At the same time, as it can be seen in the graph, the amount of NaOH added to control the 391pH is well aligned with the product formed and it serves as good indicator of the reaction 392performance. At the end though, after 24 h, a gap exists between the titration and the product 393analyzed. This can be associated with product solubility limitations [43]. In order to obtain reliable 394values for conversion and yield at the end of the reaction the reactor was worked up by adding 395acetonitrile which solubilized the whole substrate and product content.

396



397*Figure 4. Synthesis of trimethyl-ε-caprolactone using the enzymes in its soluble forms: the TmCHMO broth and* 398*GDH-01 LF. The graph shows the reaction course of TMCH (white circles), CHL (black circles), NaOH addition* 399*(black line), substrate dosing (discontinuous line) and mass balance (combined discontinuous spot-line-spot).* 400*Conditions: enzyme load 10% (v/v) of TmCHMO broth (57.8 U mL^{-1} of Broth) and 0.5% (v/v) of GDH-01 LF*

401*(8408.8 U mL^{-1} of LF); temperature 30°C; stirring rate 1200 rpm; air flow 16 mL min^{-1}; pH 7; [TMCH] 30 mM h^{-}*
402*^{1}(240 mM final); Methanol 1.25% (v/v) h^{-1} [10% (v/v) final]; [D-Glucose] 375mM; [NADP+] 0.25mM; titration*
403*solution 1M NaOH.*

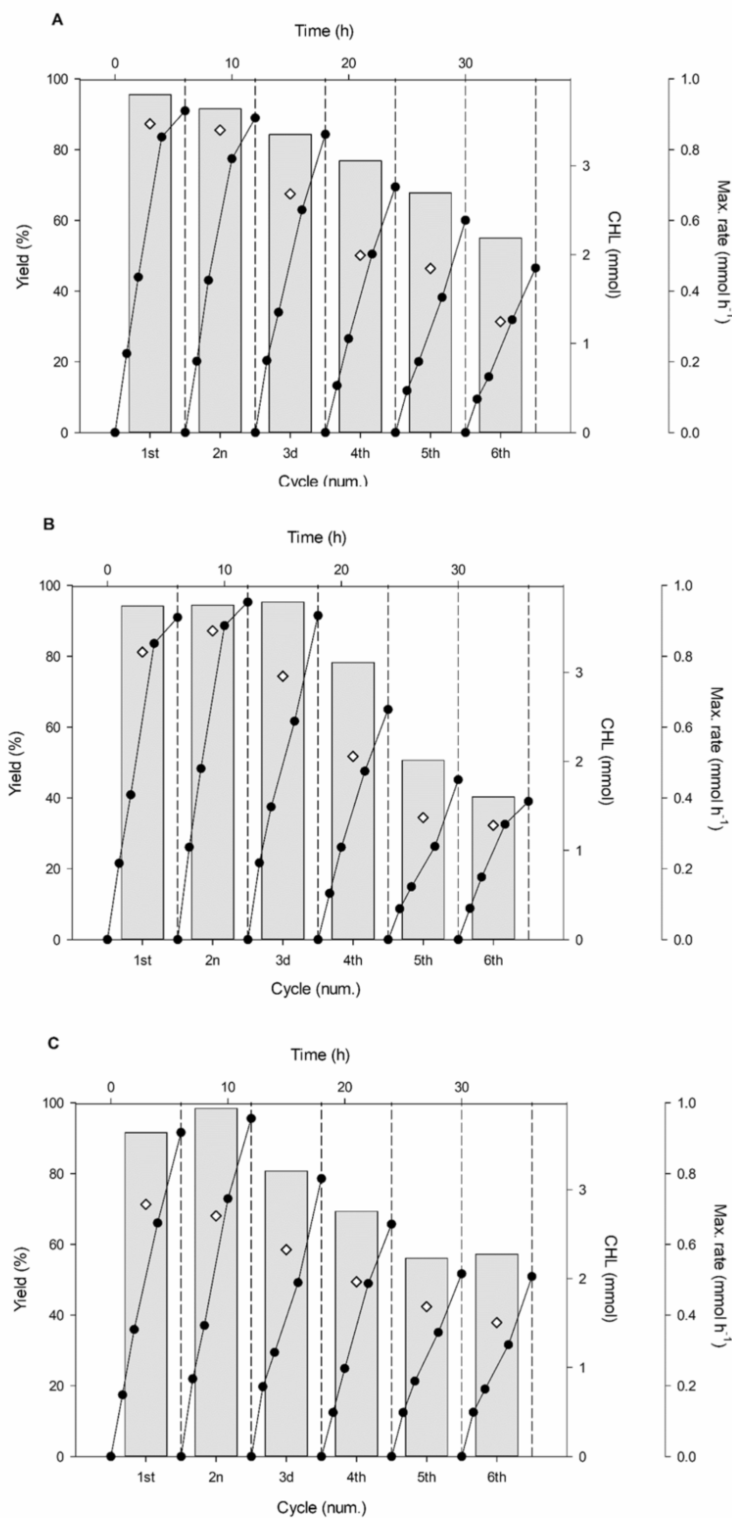
404The conversion and yield of the reaction after 24 h were 92.1 ± 4.5% and 92.0 ± 3.3% respectively.
405The amount of NaOH added was 101.2 ± 1.9% of the final substrate dosed (on molar basis). The
406product concentration reached was 34.5 ± 1.2 g L^{-1} and the biocatalyst yield was 10.5 ± 2 g CHL g^{-1}
407TmCHMO and 255.0 ± 9.2 g CHL g^{-1} GDH-01.

408**3.4.2 Immobilized enzymes**

409From an industrial point of view, the possibility of re-using enzymes in various reaction cycles can
410significantly improve the process throughput. For example, when re-cycling, the required enzyme
411decreases and it facilitates the product isolation and purification among others.

412Three sets of reactions were performed using one of the two enzymes immobilized and the other in
413its soluble form or both enzymes immobilized. Several disadvantages are sometimes observed when
414working with immobilized enzymes: substrates/product mass transfer limitations, lower oxygen
415transfer rates, poor distribution of the carrier in the reactor, enzyme selectivity alterations, etc.

416The reaction time was reduced (6 h), total conversion was prioritized over high product
417concentrations and substrate accumulation was to be avoided. For these reasons, the initial substrate
418concentration was reduced, compared to the reaction with soluble biocatalysts. In each set of
419reactions, re-cycling of the immobilized enzyme/s was intended up to 6 cycles. The results can be
420seen in the graphs below (Figure 5, A, B and C) and a final summary is presented in Table 4.



421

422*Figure 5. Reaction cycles using either one or both enzymes immobilized. Each graph shows the reaction yield*

423*(grey bars), the CHL formation (black circles and continuous line) and the maximum reaction rate (white*

424*rhombus). A) TmCHMO immobilized [3 g of support loaded (53.2 U g^{-1} of support)] and 0.5% (v/v) of GDH-01 LF*

425*(8408.8 U mL^{-1} of LF), B) GDH-01 immobilized [1.5 g of support loaded (1624.9 U g^{-1} of support)] and 10%*
426*TmCHMO LF (46.7 U mL^{-1} of LF) and C) TmCHMO immobilized [2.5 g of support loaded (53.2U g^{-1} of support)]*
427*and GDH-01 immobilized [0.5 g of support loaded (3692.5 U g^{-1} of support)]. Conditions: temperature 30°C;*
428*stirring rate 1200 rpm; Air flow 16mL min^{-1}; pH 7; [TMCH] 29 mM h^{-1}(132.5 mM final); Methanol 2.17% (v/v) h^{-1}*
429*[10% (v/v) final]; [D-Glucose] 375mM; [NADP+] 0.25mM; titration solution 1 M NaOH.*

430 *Table 4. Final summary of the reaction with soluble biocatalysts and the reactions where either one or both of the enzymes were immobilized.*

Figure	Enzyme	Immob	Soluble	Activity in the reactor (U mL^{-1})	Enzyme loaded in the reactor (mg)	CHL formed (mg)	Biocatalyst yield (g CHL g^{-1} enzyme)	Improvement factor
Figure 4.	TmCHMO		X	5.8	98.5	1033.8	10.5	
	GDH-01		X	42.0	4.1		254.6	
Figure 5. A)	**TmCHMO**	**X**		**5.3**	**90.7**	2927.9	**32.3**	**3.1**
	GDH-01		X	42.0	24.4		120.2	0.5
Figure 5. B)	TmCHMO		X	4.7	477.6	2816.5	5.9	0.6
	GDH-01	**X**		**81.2**	**7.8**		**358.9**	**1.4**
Figure 5. C)	**TmCHMO**	**X**		**4.4**	**75.6**	2818.6	**37.3**	**3.6**
	GDH-01	**X**		**61.5**	**5.9**		**474.2**	**1.9**

431

432

433The reaction containing immobilized TmCHMO and soluble GDH-01 LF showed good recyclability 434capacity of the derivate presenting 55% yield in the sixth cycle (Figure 5 A). The maximum rate of the 435reaction decreased from cycle to cycle as did the final yield. The titration, on the other hand, was 436higher than the product formation. In the first cycle 150.3% NaOH was added compared with the final 437added substrate concentration. This over-titration effect decreased along the cycles and even 438reverted in the last two. The titration in the fifth and sixth cycles was 59.5% and 39.4% whilst the 439yield was 67.8% and 55.0%. A possible explanation for this behavior could be found in the support's 440nature. The free and positively charged amino groups present on the surface of the carrier at the end 441of the immobilization, lose their proton when placed in pH 7 medium, causing an extra acidification, 442which was compensated by the auto-titration of NaOH base.

443The reaction where GDH-01 was used in its immobilized form and the TmCHMO was added as LF is 444represented in Figure 5 B. The results show that the yield was maintained during the first three 445cycles (94 - 95%) and then continuously dropped until it reached 40.2% in the sixth. Over-titration 446was also observed in this case (142.6% in the 1st cycle) and the maximum rate decay of the reaction 447was well aligned with the yield, as it happened with the immobilized TmCHMO. It should be noticed 448that the GDH-01 immobilized derivate used in this case was loaded with lower amount of enzyme 449(2765.4 U g^{-1} support) and so it presented lower final activity (1624.9 U g^{-1} support) compared with 450the derivate previously reported (Table 3, 3692.5 U g^{-1} support). At the same time, in order to ensure 451proper recovery of the resin in the filtration and washing operations, 5% (w/v) support load was 452considered to be the minimum required. At the end, the GDH-01 activity offered was higher 453compared to the soluble reaction (42.0 U mL^{-1} compared to 81.2 U mL^{-1}).

454The last reaction was performed with both TmCHMO and GDH-01 immobilized (Figure 5 C). The 455course of the reaction cycles was similar to the previous experiments. Both enzymes could be 456recycled and 57.2% yield was reached in the sixth cycle. The yield in the first two cycles was almost 457complete reaching 91.6 and 98.4%, while in the third cycle the yield dropped to 80.7%. The over-

458titration effect observed in the previous sets of reactions (Figure 5 A and B), was less prominent in 459this one. In the first cycle 107% titration and 91.6% yield were obtained. Regarding the maximum 460rate, no significant differences were observed and the rate decay was well aligned with the yield. The 461activity offered of each enzyme differed from the previous reactions with immobilized enzymes: 17% 462less TmCHMO Units and 24.3% less GDH-01 Units. This was due to the maximum support amount 463that can be loaded to ensure a proper mixing [10% (w/v)] and the GDH-01/TmCHMO activities of the 464immobilized derivatives. Furthermore, when comparing the three reactions, the reaction with 465immobilized GDH-01 and soluble TmCHMO maintained almost full yield for the first three cycles 466(Figure 5 B) while the reaction with immobilized TmCHMO and soluble GDH-01 (Figure 5 A) presented 46791.5% in the second cycle and 84.3% in the third. For this reason, TmCHMO was considered to be, 468most probably, the enzyme limiting the reaction cycles.

469A comparative table with the final metrics is presented (Table 4). Two parameters are shown for 470comparative purposes: the total CHL formed (mg) and the biocatalyst yield (mg CHL mg^{-1} of enzyme). 471Biocatalyst yield was used as the most suitable process metric to compare all the reactions settings 472since it takes into account the grams of enzymes loaded in the reaction which, as already mentioned, 473could not be maintained constant in all experiments performed.

474 The improvement factor refers to the biocatalyst yield obtained in each immobilized set of reactions 475compared with the biocatalyst yield of the soluble reaction. When looking at the three reactions with 476immobilized derivates (Figure 5 A, B and C) the amount of CHL formed almost triples the amount 477obtained in the soluble reaction. At the same time, the amount of immobilized GDH-01 added is also 478higher. As it can be seen, when immobilized, the TmCHMO improves the amount of product that the 479enzyme is able to catalyze by a factor of 3.1 (Figure 5 A) and 3.6 (Figure 5 C). On the other hand, the 480immobilized GDH-01 is able to regenerate the NADPH cofactor until 1.4 and 1.9 times more CHL is 481synthesized by the soluble TmCHMO LF or immobilized one, respectively.

482This work represents a step forward in the utilization of the immobilized TmCHMO compared with a 483previous publication [35]. The amount of CHL synthesized with both enzymes immobilized was 484increased by a factor of 5.2 and the biocatalyst yield obtained was improved by a factor of 2.2 and 14, 485for the TmCHMO and the GDH-01 respectively. In the above mentioned work, a GDH from 486*Thermoplasma acidophilum* (GDH-Tac) was used instead of GDH-01.

487Regarding the cofactor regeneration enzyme, it is the first time that the GDH-01 has been repetitively 488used in six reaction cycles in the synthesis of a product with industrial interest at high substrate and 489product concentrations.

490**4. Conclusions**

491The immobilization of the novel and highly active (310.6 ± 28.5 U g^{-1} enzyme) Glucose dehydrogenase 492GDH-01 has proven successful for the first time. The enzyme presented acceptable retained activities 493(>20%) in four out of the fourteen supports that were tested from Purolite®. Furthermore, the GDH-49401 was adsorbed onto amino functionalized agarose presenting significantly improved metrics, an 495immobilization yield of 98.4 ± 0.2% and a slight over-activation with 105.5 ± 3.8% retained activity. 496When the adsorbed derivate was further treated with a covalent bond promoter (EDC), the 497immobilization yield obtained was 76.3 ± 0.7% and the retained activity 62.6 ± 2.3%. A final derivate 498could be obtained presenting 3692.5 U g^{-1} of support.

499The immobilization of TmCHMO performed previously by Delgove *et al.* [35] could be mimicked in 500this study and highly loaded and active derivates (53.2 U g^{-1} of support) were obtained for its 501application in the synthesis of trimethyl-ε-caprolactone. At the same time, the immobilized GDH-01 502could be used as cofactor regeneration enzyme. A set of five reactions with 6 reaction recycles were 503carried out using either one or both of the enzymes in its immobilized forms. The biocatalyst yield 504obtained in each case for the immobilized enzymes was compared with a model reaction where both 505enzymes were in its soluble form. The best results were obtained with both enzymes immobilized.

506The total CHL produced in 6 different reaction cycles (30 mL, [TMCH] = 132.5 mM each cycle) was 5072818.6 mg. The biocatalyst yields obtained were 3.6 times and 1.9 times higher for the TmCHMO and 508the GDH-01 respectively, compared with the soluble reaction.

509This work represents a step forward compared with the previous research regarding the 510immobilization of TmCHMO and synthesis of CHL. When both enzymes were used immobilized, the 511total product formed was increased 5.2-fold and the TmCHMO biocatalyst yield was increased 2.2-512fold compared with the aforementioned publication [35]. Although the most significant improvement 513was observed in the biocatalyst yield of GDH-01 compared with the GDH used in the previous work, 514GDH-Tac. The biocatalyst yield as gram product obtained per gram (immobilised) enzyme was 515increased 14-fold.

516As it happens with any biochemical process, there are parameters that could be better adjusted and 517higher yields could potentially be achieved. The first parameters that the authors would work on 518would be the variation of the immobilized GDH-01:TmCHMO ratio, and the increase of the final 519substrate concentration. Comparing the soluble and immobilized reactions, the amount of enzyme 520offered does not differ much, however, the final substrate concentration was 1.8 times lower with the 521immobilized derivates.

522In conclusion, this work represents a new input for the potential implementation of TmCHMO and 523GDH-01 in the industrial production of ε-caprolactone derivatives and other lactones.

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533**6. Appendix A**

534*Table A.1. Methacrylate/styrene (Purolite®) screening set of resins and the immobilization results*

535*(immobilization yield and retained activity) for the GDH-01.*

Code	Functional group (Linker)	Matrix	Interaction	Pore diameter (Å)	Particle size (µm)	Immobilization (%)	Retained acitivty (%)
ECR8204F	Epoxy	Methacrylate	Covalent	300-600	150-300	0±2.9	21.9±4.9
ECR8215F	Epoxy	Methacrylate	Covalent	1200-1800	150-300	15.8±2.8	23.6±1.3
ECR8309F	Amino (C2)	Methacrylate	Ionic/Covalent	600-1200	150-300	66.9±1.1	19.1±0.9
ECR8315F	Amino (C2)	Methacrylate	Ionic/Covalent	1200-1800	150-300	76.1±0.7	18.9±0.2
ECR8409F	Amino (C6)	Methacrylate	Ionic/Covalent	600-1200	150-300	36.9±2.1	11.9±4.4
ECR8415F	Amino (C6)	Methacrylate	Ionic/Covalent	1200-1800	150-300	40.8±2.2	21.3±3.5
ECR8285	Epoxy (C4)	Methacrylate	Ionic/Covalent	400-600	300-710	100±0.0	9.7±1.1
ECR8806F	None (C18)	Methacrylate	Hydrophobic	500-700	150-300	99.7±0.1	6.5±0.5
ECR1061M	None	Styrene/Methacrylic	Hydrophobic	600-750	300-710	95.9±0.3	3.1±0.6
ECR1030M	None	Styrene/Methacrylic	Hydrophobic	200-300	300-710	93.7±1.8	3.1±0.8
ECR8309F	Aldehyde (C7)	Methacrylate	Ionic/Covalent	600-1200	150-300	99.5±0.0	16.2±1.1
ECR8315F	Aldehyde (C7)	Methacrylate	Ionic/Covalent	1200-1800	150-300	99.9±0.1	23.9±3.4
ECR8409F	Aldehyde (C11)	Methacrylate	Ionic/Covalent	600-1200	150-300	100±0.0	22.5±2.6
ECR8415F	Aldehyde (C11)	Methacrylate	Ionic/Covalent	1200-1800	150-300	100±0.0	28±5.1

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537**7. References**

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