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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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Abstract

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

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

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

Keywords: trimethyl-ε-caprolactone; Baeyer-Villiger monooxygenase; cofactor regeneration; recycling; immobilized enzymes biocatalyst yield.
1. Introduction

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

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

These limitations can be tackled mainly by three strategies: protein engineering [19], reaction engineering [20] and immobilization [21,22]. There are many are the examples of BVMOs that had been engineered either by means of directed evolution or rational design [15,23]. For example, a recent work by Kathleen et al. demonstrated that certain conserved residues in the active site of BVMOs, when altered, lead to modified regioselectivity [24,25]. At the same time, the substrate and product inhibition can be overcome using a different approach [26,27]. The substrate feeding and product 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...
often confers improved operational and storage stability, allows the possibility to operate in continuous mode, facilitates the isolation and purification of the product and allows the re-utilization of the biocatalyst [30–32].

In this sense, one of the first contributions was from Walsh et al. who immobilized a cyclohexanone monooxygenase (CHMO) together with a glucose dehydrogenase (GDH), as a cofactor regeneration enzyme, onto polyacrylamide gel and used it in 1 liter reactions for 10 days [33]. Interestingly, the covalent immobilization of BVMOs together with a GDH is one of the most widely used combinations in the literature [11]. In this work, this same strategy was applied for the production of the two regioisomers of trimethyl-ε-caprolactone (CHL) from 3,3,5-trimethylcyclohexanone (TMCH). Two novel enzymes 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).

Figure 1. Biocatalyzed oxidation of the branched substrate 3,3,5-trimethylcyclohexanone (1) (TMCH) to a mixture of β,β,δ-trimethyl-ε-caprolactone (1a) and β,δ,δ-trimethyl-ε-caprolactone (1b) (CHL) with a two-
Enzyme system using Glucose dehydrogenase (GDH) to regenerate the NADPH using D-(+)-Glucose as a sacrificial substrate.

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

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

2. Materials and methods

2.1. Chemicals and supports

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

Methacrylate/styrene resins were kindly donated by Purolite® Life Sciences and stored at 4 - 6°C. High density 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 further functionalized with epoxy groups following the procedure described by Axarli et al [37].

1092.2. Recombinant production of TmCHMO and GDH-01 in E. coli

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

1262.3. Total protein and enzyme content

127 The characterization of the samples was carried out exclusively on liquid formulations which were pre-cleared by centrifugation (3220 g for 15 min). The protein concentration was analyzed by means of the Bradford Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA) using bovine serum albumin (BSA) as standard (0.05 - 0.5 mg mL⁻¹) [38]. The enzyme content was measured using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (NuPage 12%, Invitrogen, USA) run
in a Mini-PROTEAN II apparatus (BioRad, USA) following the protocol by Laemmli et al [39]. Precision Plus Protein™ blue prestained protein standards (BioRad, USA) (10 – 250 kDa) were used for molecular weight determination. Gels were stained using Coomassie G250 colloidal stain solution [34% (v/v) ethanol, 2% (v/v) H$_3$PO$_4$, 17% (w/v) (NH$_4$)$_2$SO$_4$ and 0.066% Coomassie G250] and the Image LAB™ software (BioRad, USA) was used for image processing.

2.4. TmCHMO and GDH-01 activity measurements

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

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

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

2.5. GDH-01 stability studies

The activity decay of four GDH-01 samples (0.8 U mL$^{-1}$) each one presenting a different pH value (5, 6, 7 and 8) was measured over time. The GDH-01 LF was 10000-fold diluted in 50 mM phosphate buffer solutions and the pH was adjusted using either 1M HCl or 1M NaOH.
Furthermore, the activity decay of three samples presenting different enzyme concentrations was measured after 1 hour incubation at pH 6, 7 or 8 each. The LF containing the over-expressed GDH-01 was diluted 100-fold, 1000-fold and 10000-fold containing 0.47, 0.047 and 0.0047 mg prot mL\(^{-1}\) respectively. Each of these solutions was then incubated for 1 hour at 30°C in 50 mM phosphate buffer solutions (pH 6, 7 or 8).

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

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

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

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

The epoxy functionalized methacrylates (ECR8204F, ECR8215F and ECR8285) were tested using 50 mM KPi buffer pH 6 mixed with 0.5 M NaCl to increase the ionic strength and favor the attachment. The amino functionalized carriers (ECR8309F, ECR8315F, ECR8409F and 8415F) were studied using 50 mM KPi buffer pH 6. The immobilization with amino functionalized supports is divided in three steps: i) ionic adsorption of the enzyme onto the support ii) addition of 10 mM \textit{N}-\textit{3-dimethylaminopropyl}-
178′-ethylcarbodiimide (EDC) and incubation for 1.5 hours to promote the covalent binding and iii) addition of 0.5 M NaCl to desorb all the protein attached non-covalently. The non-functionalized supports (ECR8806F, ECR1061M and ECR1030M) were tested using 50 mM KPi pH 6. Finally, the amino resins were further functionalized with 2% (w/v) glutaraldehyde for 60 min at 25°C leaving free aldehyde groups on the surface of the carrier. The immobilization was carried out using 50 mM KPi buffer pH 6.

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

2.7. Immobilization of GDH-01 onto functionalized agaroses

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

Apart from the characterization stage where low enzyme load is used, the GDH-01 was immobilized onto Mana-agarose using high (maximum) loads of LF. The immobilization proceeded as explained above with the difference that in this case, the amount of GDH-01 added was higher (11061.6 U g⁻¹ support). Prior to use the carriers in reaction, they were gently washed with 50 mM KPi buffer pH 6.

The calculation of the immobilization yield and final activity were calculated following the equations published elsewhere [40].

2.8. Immobilization of TmCHMO onto Mana-agarose

The conditions for the immobilization of TmCHMO onto Mana-agarose were optimized and published recently by Delgove et al. [35]. The characterization of the enzyme and the immobilization was...
obviated in this work and high loads of enzyme were used in the experiments performed (86.5 U g⁻¹ support 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.

2.9. Reaction set up and conditions

The set up used for either the soluble or the immobilized enzyme reactions, consisted of a sealed 207jacketed glass reactor (30 mL), a pH controller (Metrohm Titrino plus 877) using 1 M NaOH solution 208and it incorporated a propeller stirrer set at 1200 rpm, a thermostat (MGW-LAUDA RC6) set at 30°C, a 209condenser at 6°C, a compact mass flow regulator (GCR Red-y) to keep a constant air flow of 16 mL 210min⁻¹; and a substrate dosing pump (Harvard Pump11).

For the reaction with soluble biocatalysts the following conditions were used: an enzyme load of 10% 212(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 213KPi pH 7; a TMCH dosing rate of 30 mM h⁻¹ (240 mM final) together with a methanol dosing rate of 21425.25% (v/v) h⁻¹ [10% (v/v) final]; [D-glucose] 375 mM and [NADP⁺] 0.25 mM.

For the reactions catalysed by immobilized enzymes the support loaded varied from 1.7% to 10% 216(w/v), the substrate dosing rate was 29 mM h⁻¹ (132.5 mM final concentration) and the methanol 217dosing rate was 2.17% (v/v) h⁻¹ [10% (v/v) final concentration]. The rest of conditions were the same 218as for the reaction with soluble biocatalysts.

2.10. Reaction progress determination by GC-FID

Samples (150 µL) were taken periodically from the reactor, weighed and dissolved up to 10 mL with a 221solution of acetonitrile containing 0.5 g L⁻¹ of hexadecane that served as Internal Standard (IS). The 222mixture was centrifuged to remove insoluble biomass and the supernatant was analyzed by a gas 223chromatograph (GC) equipped with a flame ionization detector (FID). The concentration of substrate 224and products were determined using calibration curves.
The centrifuged supernatant samples containing trimethyl-cyclohexanone and trimethyl-ε-caprolactones were analyzed using a 7890A gas chromatograph (Agilent Technologies, USA) equipped with a HP-5 column (30 m, 0.32 mm, 0.25 µm df, Agilent Technologies). The column temperature was maintained at 60°C for 2 minutes, increased up to 300°C at 10°C min⁻¹ and it was held at final temperature for 2 minutes. The injector temperature was kept at 200°C; for the flame ionization detector, the temperature was 300°C. Hydrogen was used as a carrier gas at a flow rate of 40 mL min⁻¹ and air at 450 mL min⁻¹. The retention times observed were: 8.5 min for the substrate 1, 11.9 min for lactone 1b, 12.1 min for lactone 1a and 15.8 min for the IS.

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

Reactions performed using either one or both enzymes immobilized, were performed in a similar fashion as for the soluble reactions. The difference was that once the reaction was finished, the whole reactor content was filtered and the derivate/s were washed gently with 50 mM KPi buffer pH 7. The immobilized enzyme was placed back into the reactor with no further treatment and the next cycle of reaction started.

3. Results and discussion

3.1 Stability of GDH-01 in different pHs and concentrations

As introduced previously, the aim of this work was the immobilization of the novel GDH-01 and the re-utilization of this enzyme together with the TmCHMO in the synthesis of trimethyl-ε-caprolactone. As opposite to the TmCHMO, the GDH-01 has never been immobilized before, that is why characterization regarding its stability was required. When immobilizing, the media pH must be chosen taking into account the support utilized and the activity decay of the enzyme at that certain pH. The results obtained are represented in Figure 2 A.
Figure 2. GDH-01 stability studies. A) GDH-01 relative activity along time measured at different pH values (of KPi buffers): pH 5 (black triangles and discontinuous line), pH 6 (black squares and continuous line), pH 7 (black rhombus and dotted line) and pH 8 (black circles and combined discontinuous spot-line-spot). The initial activity of the samples was 0.8 U mL$^{-1}$; 10000-fold dilution of the initial liquid formulation. B) GDH-01 relative activity after 1 hour incubation at three different pHs: pH 6 (black bars), pH 7 (grey bars) and pH 8 (white bars); and three different protein concentrations. The initial activities of the samples were 0.8 U mL$^{-1}$, 8 U mL$^{-1}$ and 80 U 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, correspond to the standard error calculated from at least two replicates.
As it can be observed in Figure 2 A, when GDH-01 was diluted in pH 8 buffer, it was almost completely deactivated after one hour. On the other hand, pH 6 turned out to be the most favorable for this enzyme which maintained 50% of the activity after 3.5 hours. In Figure 2 B, the LF containing GDH-01 was incubated for 1 hour at three different pHs (6, 7 and 8) and three different protein concentrations were applied for each pH. The graph shows that, at pH 7 and 8, the enzyme is deactivated to different extent depending on the concentration it is in. The lowest concentrated sample suffers the highest loss of activity. At pH 6 though, the relative activity after 1 hour is almost the same for the three enzyme concentrations. Diluting the GDH-01 LF in a solution containing 5 mg mL$^{-1}$ of Bovine Serum Albumin (BSA) or pre-coating the vial with BSA did not improve the GDH-01 stability at pHs different from 6.

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

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

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

3.2.1 Methacrylate/Styrene based supports

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

A set of 14 different methacrylate/styrene resins covering a broad range of enzyme carrier features were obtained from Purolite® Technologies. The materials supplied had different: pore diameters
(300 - 1800 Å), enzyme-carrier interactions (ionic, covalent and hydrophobic), functional groups (epoxy, amino and aldehyde), linker lengths (C2 - C18), material matrices (methacrylate and styrene) and particle sizes (150 – 710 µm). A detailed description of each carrier and the results obtained for the immobilization of GDH-01 can be found in the Appendix A (Table A.1).

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

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

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

When working with amino functionalized resins, as is the case of ECR8415F, the immobilization is carried out in three steps. First, an ionic interaction between the positively charged amino groups of the support and the negatively charged carboxyl groups of the enzyme occurs. In this first binding, 99.1 ± 0.1% immobilization yield and 60.0 ± 4.1% retained activity were obtained with ECR8415F. However, the optimized pH for the target reaction is 7, which changes the positive charge of the resin’s amino groups, desorbing the enzyme from the carrier. Thus, an agent promoting a covalent bond formation is required for its use in the target reaction. \(N\)-(3-dimethylaminopropyl)-\(N\)’-
ethylcarbodiimide (EDC) was chosen due to its high solubility in water. The immobilization yield and
retained activity obtained were 40.8 ± 2.2% and 21.3 ± 3.5%, respectively. The EDC is often associated
with enzyme deactivation, however, in this case, the low immobilization yield indicates that the
amount of EDC added was not enough to covalently bind all the enzyme offered and a major part of
the initially attached GDH-01 was desorbed when the NaCl was introduced.

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

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

3.2.2 Agarose based matrices: Epoxy- and Mana-agarose

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

The Epoxy-agarose immobilization is usually pursued at alkaline pH. However, the studies performed
with GDH-01 showed a very poor stability of the enzyme at pH 8 (Figure 2 A). The immobilization was
therefore conducted at pH 6 and 0.5 M of NaCl were added in order to increase the ionic strength of
the medium to promote the binding. The results showed low affinity of the enzyme for the support and a slight over-activation. At the end, 29.5 ± 7.3% retained activity were obtained.

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

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

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

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

<table>
<thead>
<tr>
<th>[EDC]</th>
<th>Ionic Adsorption</th>
<th>Covalent binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immobilization Yield (%)</td>
<td>Retained Activity (%)</td>
</tr>
<tr>
<td>10</td>
<td>98.4 ± 0.2</td>
<td>105.5 ± 3.8</td>
</tr>
<tr>
<td>20</td>
<td>94.1 ± 0.1</td>
<td>47.2 ± 3.3</td>
</tr>
<tr>
<td>30</td>
<td>98.2 ± 0.1</td>
<td>44.2 ± 0.2</td>
</tr>
</tbody>
</table>

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

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

The maximum quantity of GDH-01 and TmCHMO that can be attached to Mana-agarose was studied. Different amounts of enzyme were immobilized onto the support in order to determine this value.
Since mass transfer limitation could occur at high enzyme loads, retained activities obtained during the immobilization characterization at low loads were used here to calculate the theoretical maximum loading capacity in terms of activity units [Table 3, Final activity (U g⁻¹ support)].

Table 3. Immobilization of GDH-01 onto Mana-agarose using maximum loads of enzyme per gram of support.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity offered (U g⁻¹ support)</th>
<th>Final activity (U g⁻¹ support)</th>
<th>Retained protein (mg protein g⁻¹ support)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDH</td>
<td>11061.6</td>
<td>3692.5</td>
<td>23.1</td>
</tr>
<tr>
<td>TmCHMO</td>
<td>86.5</td>
<td>53.2</td>
<td>67.7</td>
</tr>
</tbody>
</table>

The immobilization of TmCHMO was carried out using the optimized conditions published recently by Delgove et al. [35]. The amino-functionalized agarose was used as a carrier and 35 mM of EDC were applied as a covalent bond promoter. The obtained retained activity at low loads in that study was 62.4 ± 2.1%. The results obtained regarding the immobilization of TmCHMO using high loads of enzyme, are shown in Table 3. The maximum loading capacity of TmCHMO resulted in 67.7 mg protein g⁻¹ support and 53.2 U g⁻¹ support.

Regarding the GDH-01, 23.1 mg protein g⁻¹ support were immobilized using 10 mM EDC concentration. According to the retained activity obtained during the characterization (62.6 ± 2.3%), 3692.5 U g⁻¹ support could be loaded onto Mana-agarose (Table 3).

3.4 Synthesis of trimethyl-ε-caprolactone

3.4.1 Soluble enzymes

Aiming to compare the performance of the immobilized derivatives with the soluble enzyme, a reaction was run firstly using non-immobilized TmCHMO and GDH-01. Different metrics such as biocatalyst yield and total product synthesized were used for comparison. The conditions published...
recently [43] and further optimized by the authors (data not shown) were mimicked in this study at 30 mL scale using 10% (v/v) load of TmCHMO fermentation broth and 0.5% (v/v) GDH-01 LF.

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

Figure 4. Synthesis of trimethyl-ε-caprolactone using the enzymes in its soluble forms: the TmCHMO broth and GDH-01 LF. The graph shows the reaction course of TMCH (white circles), CHL (black circles), NaOH addition (black line), substrate dosing (discontinuous line) and mass balance (combined discontinuous spot-line-spot). 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
The conversion and yield of the reaction after 24 h were 92.1 ± 4.5% and 92.0 ± 3.3% respectively. The amount of NaOH added was 101.2 ± 1.9% of the final substrate dosed (on molar basis). The product concentration reached was 34.5 ± 1.2 g L⁻¹ and the biocatalyst yield was 10.5 ± 2 g CHL g⁻¹ TmCHMO and 255.0 ± 9.2 g CHL g⁻¹ GDH-01.

3.4.2 Immobilized enzymes

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

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

The reaction time was reduced (6 h), total conversion was prioritized over high product concentrations and substrate accumulation was to be avoided. For these reasons, the initial substrate concentration was reduced, compared to the reaction with soluble biocatalysts. In each set of reactions, re-cycling of the immobilized enzyme/s was intended up to 6 cycles. The results can be seen in the graphs below (Figure 5, A, B and C) and a final summary is presented in Table 4.
Figure 5. Reaction cycles using either one or both enzymes immobilized. Each graph shows the reaction yield (grey bars), the CHL formation (black circles and continuous line) and the maximum reaction rate (white 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%
426TmCHMO LF (46.7 U mL⁻¹ of LF) and C) TmCHMO immobilized [2.5 g of support loaded (53.2 U g⁻¹ of support)]
427and GDH-01 immobilized [0.5 g of support loaded (3692.5 U g⁻¹ of support)]. Conditions: temperature 30°C;
428stirring rate 1200 rpm; Air flow 16 mL 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] 375 mM; [NADP⁺] 0.25 mM; titration solution 1 M NaOH.
Table 4. Final summary of the reaction with soluble biocatalysts and the reactions where either one or both of the enzymes were immobilized.

<table>
<thead>
<tr>
<th>Figure</th>
<th>Enzyme</th>
<th>Immob</th>
<th>Soluble</th>
<th>Activity in the reactor (U mL⁻¹)</th>
<th>Enzyme loaded in the reactor (mg)</th>
<th>CHL formed (mg)</th>
<th>Biocatalyst yield (g CHL g⁻¹ enzyme)</th>
<th>Improvement factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 4.</td>
<td>TmCHMO</td>
<td>X</td>
<td></td>
<td>5.8</td>
<td>98.5</td>
<td>1033.8</td>
<td>10.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GDH-01</td>
<td>X</td>
<td></td>
<td>42.0</td>
<td>4.1</td>
<td></td>
<td></td>
<td>254.6</td>
</tr>
<tr>
<td>Figure 5. A)</td>
<td>TmCHMO</td>
<td>X</td>
<td></td>
<td>5.3</td>
<td>90.7</td>
<td>2927.9</td>
<td>32.3</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>GDH-01</td>
<td>X</td>
<td></td>
<td>42.0</td>
<td>24.4</td>
<td></td>
<td></td>
<td>120.2</td>
</tr>
<tr>
<td>Figure 5. B)</td>
<td>TmCHMO</td>
<td>X</td>
<td></td>
<td>4.7</td>
<td>477.6</td>
<td>2816.5</td>
<td>5.9</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>GDH-01</td>
<td>X</td>
<td></td>
<td>81.2</td>
<td>7.8</td>
<td></td>
<td></td>
<td>358.9</td>
</tr>
<tr>
<td>Figure 5. C)</td>
<td>TmCHMO</td>
<td>X</td>
<td></td>
<td>4.4</td>
<td>75.6</td>
<td>2818.6</td>
<td>37.3</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>GDH-01</td>
<td>X</td>
<td></td>
<td>61.5</td>
<td>5.9</td>
<td></td>
<td></td>
<td>474.2</td>
</tr>
</tbody>
</table>
The reaction containing immobilized TmCHMO and soluble GDH-01 LF showed good recyclability capacity of the derivate presenting 55% yield in the sixth cycle (Figure 5 A). The maximum rate of the reaction decreased from cycle to cycle as did the final yield. The titration, on the other hand, was higher than the product formation. In the first cycle 150.3% NaOH was added compared with the final added substrate concentration. This over-titration effect decreased along the cycles and even reverted in the last two. The titration in the fifth and sixth cycles was 59.5% and 39.4% whilst the yield was 67.8% and 55.0%. A possible explanation for this behavior could be found in the support’s nature. The free and positively charged amino groups present on the surface of the carrier at the end of the immobilization, lose their proton when placed in pH 7 medium, causing an extra acidification, which was compensated by the auto-titration of NaOH base.

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

The last reaction was performed with both TmCHMO and GDH-01 immobilized (Figure 5 C). The course of the reaction cycles was similar to the previous experiments. Both enzymes could be recycled and 57.2% yield was reached in the sixth cycle. The yield in the first two cycles was almost complete reaching 91.6 and 98.4%, while in the third cycle the yield dropped to 80.7%. The over-
titration effect observed in the previous sets of reactions (Figure 5 A and B), was less prominent in this one. In the first cycle 107% titration and 91.6% yield were obtained. Regarding the maximum rate, no significant differences were observed and the rate decay was well aligned with the yield. The activity offered of each enzyme differed from the previous reactions with immobilized enzymes: 17% less TmCHMO Units and 24.3% less GDH-01 Units. This was due to the maximum support amount that can be loaded to ensure a proper mixing [10% (w/v)] and the GDH-01/TmCHMO activities of the immobilized derivatives. Furthermore, when comparing the three reactions, the reaction with immobilized GDH-01 and soluble TmCHMO maintained almost full yield for the first three cycles (Figure 5 B) while the reaction with immobilized TmCHMO and soluble GDH-01 (Figure 5 A) presented 91.5% in the second cycle and 84.3% in the third. For this reason, TmCHMO was considered to be, most probably, the enzyme limiting the reaction cycles.

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

The improvement factor refers to the biocatalyst yield obtained in each immobilized set of reactions compared with the biocatalyst yield of the soluble reaction. When looking at the three reactions with immobilized derivates (Figure 5 A, B and C) the amount of CHL formed almost triples the amount obtained in the soluble reaction. At the same time, the amount of immobilized GDH-01 added is also higher. As it can be seen, when immobilized, the TmCHMO improves the amount of product that the enzyme is able to catalyze by a factor of 3.1 (Figure 5 A) and 3.6 (Figure 5 C). On the other hand, the immobilized GDH-01 is able to regenerate the NADPH cofactor until 1.4 and 1.9 times more CHL is synthesized by the soluble TmCHMO LF or immobilized one, respectively.
This work represents a step forward in the utilization of the immobilized TmCHMO compared with a previous publication [35]. The amount of CHL synthesized with both enzymes immobilized was increased by a factor of 5.2 and the biocatalyst yield obtained was improved by a factor of 2.2 and 14, for the TmCHMO and the GDH-01 respectively. In the above mentioned work, a GDH from Thermoplasma acidophilum (GDH-Tac) was used instead of GDH-01.

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

4. Conclusions

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

The immobilization of TmCHMO performed previously by Delgove et al. [35] could be mimicked in this study and highly loaded and active derivates (53.2 U g\(^{-1}\) of support) were obtained for its application in the synthesis of trimethyl-ε-caprolactone. At the same time, the immobilized GDH-01 could be used as cofactor regeneration enzyme. A set of five reactions with 6 reaction recycles were carried out using either one or both of the enzymes in its immobilized forms. The biocatalyst yield obtained in each case for the immobilized enzymes was compared with a model reaction where both enzymes were in its soluble form. The best results were obtained with both enzymes immobilized.
The total CHL produced in 6 different reaction cycles (30 mL, [TMCH] = 132.5 mM each cycle) was 2818.6 mg. The biocatalyst yields obtained were 3.6 times and 1.9 times higher for the TmCHMO and GDH-01 respectively, compared with the soluble reaction.

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

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

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

### 5. Acknowledgments

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### 6. Appendix A

#### Table A.1. Methacrylate/styrene (Purolite®) screening set of resins and the immobilization results (immobilization yield and retained activity) for the GDH-01.

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


