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# **Enhancing the productivity of the bi-enzymatic convergent cascade for $\epsilon$ -caprolactone synthesis through design of experiments and a biphasic system**

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## **Abstract**

A two-step Design of Experiments (DoE) strategy followed by a two-liquid-phase system (2LPS) was applied to enhance the  $\epsilon$ -caprolactone yield in the cyclohexanone monooxygenase (CHMO)-alcohol dehydrogenase (ADH) convergent cascade system. The key reaction parameters were identified and optimized for the determination of an optimal operational window for the aqueous media. In the 2LPS system, high partitioning of the lactone product was observed in 2-MeTHF and in toluene; however, these solvents led to drastically reduced enzymatic activity. Dodecane was chosen as the non-miscible organic phase owing to the enzymes' high residual activity, despite the low partitioning of the lactone. Cyclohexanone concentrations up to 75 mM were applied in the aqueous media. The turnover numbers for the nicotinamide cofactor and for the ADH reached up to 980 and 392000, respectively whereas a turnover number value of 5600 was achieved for the CHMO. By employing a 2LPS, whereby 91 mM of cyclohexanone was applied in the second phase, turnover numbers were slightly increased.

## **Keywords**

Caprolactone; Enzymatic Cascades; Oxidoreductases; Design of Experiments; Two-Liquid-Phase System

## 1 Introduction

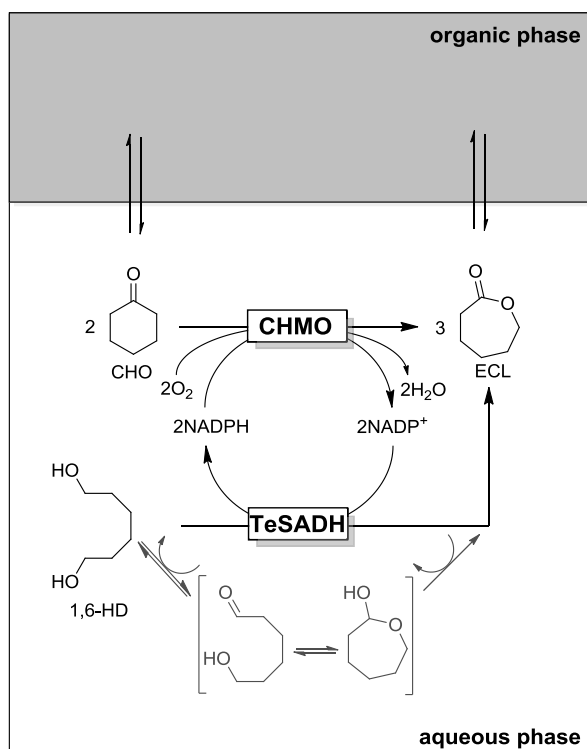
Nowadays, the use of biocatalysts has been widely recognized as a resource efficient, energy saving, safe, economical and an environmentally benign way for the synthesis of high value-added products in chemical transformations.<sup>1</sup> A technically relevant example where the above-given advantages play a major role can be found in the synthesis of bulk lactones.<sup>2</sup> Bulk lactones have a broad range of industrial applications and are of huge commercial importance. In particular,  $\epsilon$ -caprolactone (ECL), with a global annual production of multi-kilotons, is a key precursor in the production of biodegradable, thermoplastic, and elastomeric polymers.<sup>3</sup>

A fully enzymatic approach for the synthesis of lactones in a linear cascade fashion (i.e. single substrate – one intermediate – single product) was first reported in the early 90s.<sup>4</sup> Nowadays, this cascade approach is used for ECL synthesis, whereby oxidation of cyclohexanol (CHL) by an alcohol dehydrogenase (ADH) (or a designed polyol dehydrogenase) is coupled with the further oxidation of cyclohexanone (CHO) to ECL by a cyclohexanone monooxygenase (CHMO).<sup>5</sup> Recently, we reported a new, fully enzymatic system for the synthesis of ECL running in a convergent cascade fashion (i.e. bi-substrate – no intermediate – single product).<sup>6</sup> The bi-enzymatic bi-substrate convergent system consists of a CHMO for oxidation of CHO and an ADH for two-step oxidation of a so-called ‘double-smart cosubstrate’ 1,6-hexanediol (1,6-HD) for the *in situ* regeneration of the nicotinamide cofactor. The enzymes employed in this reaction cascade were wild-type CHMO from *Acinetobacter sp.* NCIMB 9871<sup>7</sup> and ADH from *Thermoanaerobacter ethanolicus* (TeSADH)<sup>8</sup> (Scheme 1), as their coupling showed the highest product yield in our proof-of-concept study. Our previous study revealed the formation of CHL from the ADH-catalyzed reduction of CHO, which however gradually converted to ECL during the course of reactions.

The characterization of the bi-enzymatic cascade system described above and the identification of its optimal operational window necessitate a detailed analysis of interactions between the various reaction parameters. To achieve this, design of experiments (DoE)<sup>9</sup>, an organized and systematic approach for studying multi-parameter systems, is of a substantial usefulness. Herein, a suitable DoE model to obtain a maximum amount of information from a minimum number of experiments is used to map the reaction system in terms of different responses, with respect to different parameters and their interactions. The DoE data are used for statistical modelling and analysis of the reaction to understand the parameters’ impacts on target responses e.g. substrate conversion, by-product formation, product yield, turnover number (TON) for the cofactor and for the enzymes etc. The bi-enzymatic convergent system described herein is influenced by several parameters such as (1) temperature, (2) pH, (3) available O<sub>2</sub> amount, (4) substrates concentrations, (5) cofactor concentration and (6) the enzymes amounts. Each of these parameters can directly and interactively influence the reaction profile and the product (analytical) yield.

For optimization purposes, the so-called two-liquid-phase system (2LPS) is an established method to overcome a range of challenges encountered especially in oxidoreductase-catalyzed reactions.<sup>10</sup> Substrate toxicity and product inhibition issues can be overcome through the use of an organic phase as a substrate reservoir and a product sink. By doing so, high concentrations of hydrophobic

substrates can be applied to achieve high space-time yields. Furthermore, the reaction can be shifted to the product side by selectively removing the (co)product. In order to alleviate these limitations mentioned above other strategies for example fed-batch synthesis<sup>11</sup> and the use of resins for substrate feeding and product removal (SFPR)<sup>12</sup> have been documented in the literature. In addition to those, in order to overcome the production inhibition due to ECL, the use of CAL-A for *in situ* oligomerization has been shown to be useful.<sup>5d</sup>



**Scheme 1.** The two-liquid-phase system (2LPS) applied for the removal of  $\epsilon$ -caprolactone (ECL) synthesized in a CHMO-TeSADH convergent cascade coupled for the oxidation of cyclohexanone (CHO) and for the two-step oxidation of 1,6-hexanediol (1,6-HD) for simultaneous regeneration of NADPH.

Based on our previous experience with the recently developed CHMO-ADH convergent cascade, we became interested in enhancing its productivity. Therefore, the aim of the present study was to first identify an optimal operational window for the CHMO-ADH convergent cascade through a two-step DoE approach. Thereafter, in order to further enhance the productivity, we established a two-liquid-phase (2LPS) system (Scheme 1) for the selective extraction of the lactone product.

## 2 Results and discussion

### 2.1 DoE for screening of reaction parameters

A two-step DoE approach was used to screen and optimize the CHMO-ADH convergent cascade with several key factors involved. For screening purposes, eight reaction parameters (i.e. temperature,  $O_2$ , pH,  $c(\text{CHO})$ ,  $c(\text{CHMO})$ ,  $c(\text{TeSADH})$ ,  $c(\text{NADP}^+)$ , and time) were evaluated for their impacts on four target responses chosen (i.e. conversion of CHO,  $c(\text{ECL})$ ,  $c(\text{CHL})$ , TON for  $\text{NADP}^+$ ). The concentration of 1,6-HD was always kept at a half molar equivalent of CHO, hence it was not further

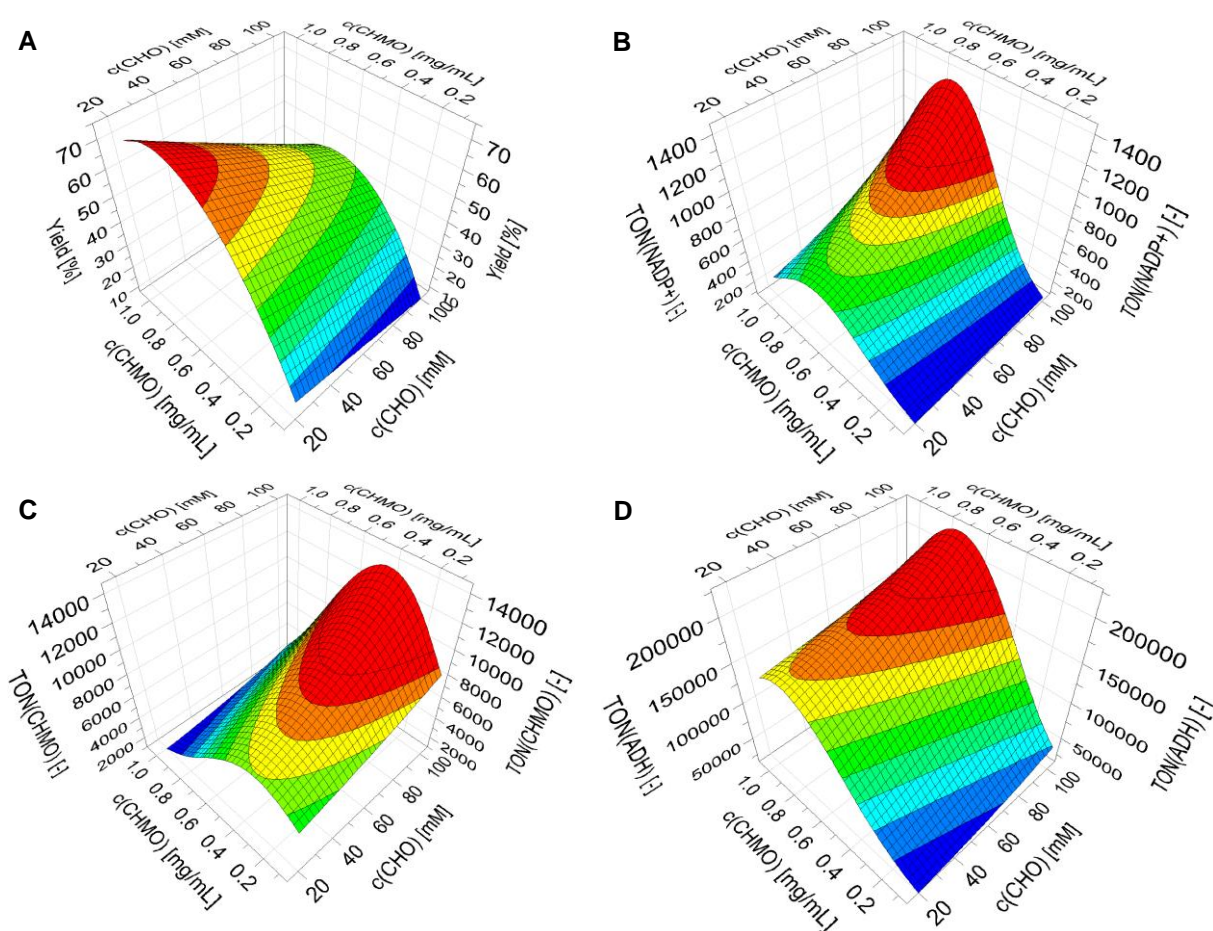
included in the reaction parameters. It is worth mentioning here that the kinetic analysis of TeSADH for CHO reduction and for 1,6-HD oxidation revealed that the ADH exhibits 19-fold higher  $V_{\max}$  for the reduction compared to the oxidation, while similar  $K_M$  values for CHO and 1,6-HD were detected.<sup>6</sup> With respect to the cofactor recycling, total turnover numbers (TTNs) at least 100,000 are aimed for the technical scale applications, whereas TTN values of 1,000-10,000 are sufficient for the laboratory scale.<sup>13</sup>

A total of 19 experiments (16 individual experiments and three replicate center points, Table SI1 and SI2) based on a 'fractional factorial design' were evaluated, instead of all 256 potential experiments. A partial least squares (PLS) model was well fitted ( $R^2$  in the range of 0.9 to 1.0) to the data for the aforementioned four target response values shown in Table SI3 by the DoE software (MODDE 8.0, Umetrics, Umeå, Sweden). The summary of the PLS model gave high predictabilities ( $Q^2 > 0.8$ ) for  $\alpha(\text{ECL})$  and TON ( $\text{NADP}^+$ ), whereas moderate predictabilities ( $Q^2 \geq 0.5$ ) were obtained for the conversion of CHO and for the formation of CHL (Table SI4). Moreover, the summary of the PLS model showed very high reproducibility values ( $>0.99$ ) (Table SI4).

According to the variable importance plot (VIP, Fig. SI1) representing the normalized values corresponded to the different model parameters (the VIP of 1.0 represents an average importance), four of the parameters, namely: pH, temperature,  $\text{O}_2$ , and time, were found to have lower impacts on the CHMO-ADH convergent cascade, compared to the other parameters. Through understanding some of the influences of these four parameters (i.e. pH, temperature,  $\text{O}_2$  and time) on the responses (Fig. SI2), the values for these factors were fixed in such a way as to improve the reactions the most. Therefore, a slightly alkaline pH value of 8.0, the highest  $\text{O}_2$  amount by having the maximal headspace ratio ( $\frac{\text{Headspace volume (mL)}}{\text{Volume of the reaction (mL)}}$ ), and room temperature ( $\sim 20^\circ\text{C}$ ), were chosen for the optimization step, in order to obtain high product (analytical) yields within 24 to 48 hours.

## 2.2 DoE for optimization of the reaction parameters identified in the screening step

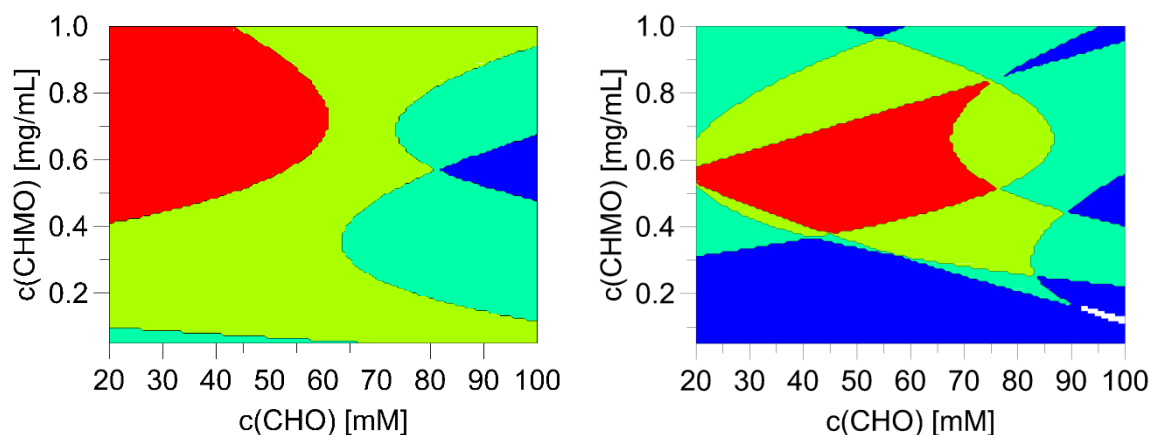
Based on the results of the screening study, we focused our attention on four reaction parameters i.e.  $\alpha(\text{CHO})$ ,  $\alpha(\text{CHMO})$ ,  $\alpha(\text{TeSADH})$ , and  $\alpha(\text{NADP}^+)$  in the optimization step. Based on a 'central composite face-centered design', in total 27 reactions (24 individual experiments and three replicate center points) were run (Table SI5). Herein, we considered it worthwhile to change the target responses for optimization purposes. The responses for the screening study were (1) conversion of CHO, (2)  $\alpha(\text{ECL})$ , (3)  $\alpha(\text{CHL})$ , and (4) TON ( $\text{NADP}^+$ ), whereas for the optimization step we chose the responses as (1) analytical ECL yield, (2) TON ( $\text{NADP}^+$ ), (3) TON (CHMO), and (4) TON (ADH). These target responses were chosen mainly because the monitoring of ECL formation represents the performance of the convergent cascade better compared to the conversion of CHO, as the consumed amount of CHO cannot be directly correlated to the formation of ECL, owing to the ADH-catalyzed reduction of CHO yielding the by-product CHL. In addition, we aimed at optimizing the TON values for the enzymes. The response values (data obtained in 24 hours) used for the optimization via response surface modeling (RSM) are given in the Supporting Information (Table SI6). The predicted surface plots for the four target responses used in the RSM are shown in Figure 1.



**Figure 1.** Response surface plots predicted based on the DoE for optimization (for 24 hours) indicating the impacts of the reaction parameters (e.g.  $c(\text{CHO})$ ,  $c(\text{CHMO})$ ,  $c(\text{TeSADH})$ , and  $c(\text{NADP}^+)$ ) on the target responses (A) analytical ECL yield, (B) TON ( $\text{NADP}^+$ ), (C) TON (CHMO), (D) TON (ADH), when the concentrations of TeSADH and  $\text{NADP}^+$  are at lower levels of 0.005 mg/mL (0.002 U/mL) and 0.05 mM, respectively.

During the optimization step, we observed a decrease in the analytical yield of ECL upon incubation after 24 hours (Reactions No. 3, 11, 15 and 17, Table SI6 and Table SI9). This decrease can be attributed either to lactone hydrolysis<sup>14</sup> or to uncontrolled oligomerization/polymerization<sup>6</sup>.

In order to identify an optimal operational window based on the DoE optimization results, the criteria were set as ECL yield >60% (seen in the proof-of-concept study), TON ( $\text{NADP}^+$ ) >100, TON (CHMO) >1000, and TON (ADH) >10,000, with TeSADH at 0.005 mg/mL (0.002 U/mL) and  $\text{NADP}^+$  at 0.05 mM. The sweet spot analysis showed that all the criteria are met within 24 hours when CHO concentration is in the range of 20 to 60 mM and the CHMO concentration between 0.4 and 1 mg/mL (1.3 and 3.3 U/mL) (Figure 2, left). For the 48-hour process, an optimal operational region for meeting all the criteria is obtained when CHO and CHMO concentrations are in the range of 20–75 mM and 0.4–0.8 mg/mL (1.3–2.6 U/mL), respectively (Figure 2, right).



**Figure 2.** Sweet spot plots according to the PLS models (left for 24 hours and right for 48 hours), predicting the optimal operational window for obtaining analytical ECL yield >60%, TON (NADP<sup>+</sup>) >100, TON (CHMO) >1000 and TON (ADH) >10,000, by using TeSADH and NADP<sup>+</sup> of 0.005 mg/mL (0.002 U/mL) and 0.05 mM, respectively. **■** Sweet spot (four criteria met), **■** three criteria met, **■** two criteria met, **■** one criterion met.

Subsequently, in order to verify the optimization results obtained through the PLS models we ran two reactions to compare the predicted data with the empirical results. Conditions for both reactions were chosen in such a way to meet the sweet spots shown in Figure 2. Table 1 represents the comparison between the model-based predicted data and the empirical data (average of duplicate runs). In both reactions, the empirical data were in very good agreement with the data predicted by the models developed for both reactions after 24 and 48 hours (Table 1). The response surface plots predicted by the PLS model developed for the reaction after 48 hours are given in the Supporting Information (Fig. SI6).

In our previous study, the highest analytical ECL yield was 60% under the non-optimized conditions with low to good TONs for the cofactor and enzymes.<sup>6</sup> Following a two-step DoE approach as shown here, TON values were increased 50-fold in case of the nicotinamide cofactor and 10-fold in case of the ADH. TON for CHMO remained similar (approx. 5800 in the previous study) and analytical yield of ECL increased to 71% (Table 1). The full conversion of CHO was achieved in the reaction given in Entry 1 in 24 hours, whereas 83% of conversion was achieved in 24 hours in the reaction shown in Entry 2 (Table 1).

With respect to the autohydrolysis of ECL, a hydrolysis rate of 0.006  $\mu\text{mol ECL}/\text{min}$  was detected under the reaction conditions applied (100 mM Tris-HCl, pH 8.0 and 20°C, Supporting Information). On the other hand, the formation of oligomer products might occur owing to the hydrolases present in the crude CHMO preparation. In order to monitor the formation of the 6-hydroxyhexanoic acid and oligomer/polymer products an HPLC method was developed. We separately ran the reaction given in Entry 1-Table 1 (in duplicates) and collected the chromatograms up to 40 hours. After 40 hours, CAL-A<sup>5d</sup> or CAL-B was added to enhance the formation of the oligomer products or the hydroxy acid, respectively. The formation of the by-products could be successfully followed (Supporting Information).



**Table 1**

Verification table comparing the results predicted by the PLS model developed based on the DoE for optimization and empirical results (lower and upper limits based on 95% confidence interval) obtained by running the reactions under the same conditions

Entry	Time [h]	Data	ECL Yield <sup>c</sup> [%]	Lower Limit	Upper Limit	TON (NADP <sup>+</sup> ) [-]	Lower Limit	Upper Limit	TON (CHMO) [-]	Lower Limit	Upper Limit	TON (ADH) [-]	Lower Limit	Upper Limit
1 <sup>a</sup>	24 <sup>d</sup>	Predicted	61.5	49.3	73.6	981	661	1454	10,117	6830	14,986	370,144	258,184	530,654
		Empirical	63.6	61.5	65.7	829	802	856	5056	4894	5219	331,568	320,921	342,214
	48 <sup>d</sup>	Predicted	60.5	42.1	78.9	632	361	1106	6693	4596	9749	224,902	123,618	409,172
		Empirical	70.5	49.9	91.1	915	706	1123	5579	4307	6850	365,824	282,454	449,193
2 <sup>b</sup>	24 <sup>e</sup>	Predicted	59.2	47.6	70.7	1478	1017	2150	8701	5989	12,642	550,435	390,828	775,221
		Empirical	45.2	42.8	47.6	962	921	1002	4190	4013	4367	384,637	368,373	400,900
	48 <sup>e</sup>	Predicted	59.0	41.9	76.1	1011	602	1699	6127	4321	8687	357,630	205,157	623,420
		Empirical	46.1	44.4	47.8	980	955	1005	4271	4162	4381	392,130	382,105	402,155

<sup>a</sup> c(CHO) = 50 mM, c(1,6-HD) = 25 mM, c(CHMO) = 0.5 mg/mL (1.7 U/mL), c(ADH) = 0.005 mg/mL (0.002 U/mL), c(NADP<sup>+</sup>) = 0.05 mM, T = 20°C, 180 rpm, headspace 34 mL.

<sup>b</sup> c(CHO) = 75 mM, c(1,6-HD) = 37.5 mM, c(CHMO) = 0.7 mg/mL (2.3 U/mL), c(ADH) = 0.005 mg/mL (0.002 U/mL), c(NADP<sup>+</sup>) = 0.05 mM, T = 20°C, 180 rpm, headspace 34 mL.

<sup>c</sup> Analytical yield of ECL [%] =  $\frac{ECL [mM]}{CHO [mM] + 1,6-HD [mM]}$  determined via GC.

<sup>d</sup> Conversion of CHO = 100% (24 h), 100% (48 h).

<sup>e</sup> Conversion of CHO = 83% (24 h), 91% (48 h).

### 2.3 The two-liquid-phase system applied for the removal of ECL

After identifying optimal reaction conditions for the aqueous medium, we focused our attention on a two-liquid-phase system (2LPS) for the selective removal of ECL. In a first set of experiments, we evaluated the partition coefficients of reaction components in seven organic solvents, namely: 2-methyltetrahydrofuran (2-MeTHF), methyl *tert*-butyl ether (MTBE), cyclopentyl methyl ether (CPME), toluene, cyclohexane, heptane, and dodecane. These organic solvents have a wide range of  $\log P^{15}$  (octanol/water partition coefficient) values (between 0.7 and 6.8, Table SI12) and have been successfully applied in dehydrogenase<sup>14, 16</sup> and monooxygenase<sup>17,18</sup> catalyzed reactions. Among the solvents evaluated, although the highest partition coefficient for ECL was obtained in 2-MeTHF and in toluene (Table SI13), we decided against them due to the loss in the enzymes' activity in a 2LPS (Fig. SI7). Using 2-MeTHF or toluene, no residual activity was detected after 1 hour and 24 hours, respectively, whereas 44% of residual activity was observed in the case of dodecane after 24 hours. The decrease in the activity (calculated as the formation rate of ECL from the total amount of enzymes) can be mostly attributed to the reduced activity of CHMO under these reaction conditions. Protein-engineering studies performed to increase the stability of CHMO from *Acinetobacter* sp. NCIMB 9871 were documented in the literature.<sup>19</sup>

Although  $\log P$  cannot be a direct criterion<sup>16</sup> for enzymes' stability, higher residual activities were detected using hydrophobic solvents. This observation might account for lower 'molecular toxicity' effect of a hydrophobic solvent compared to an hydrophilic one.<sup>20</sup> For subsequent experiments, we focused on dodecane, as it did not impair the biocatalysts' activity/stability significantly, when compared to the other solvents. In addition, its high boiling point (216°C) would minimize solvent evaporation over the course of the reaction while agitating.

We applied the conditions given in Entry 1-Table 1 and ran the reaction in a 2LPS ( $V_{\text{organic}}/V_{\text{aqueous}} = 1$ ). By doing so, ECL concentration reached 34 mM in 24 hours but decreased to 28 mM upon incubation in the following 24 hours (Entry 1-Table 2, ● Figure 3). This is most probably due to the autohydrolysis and/or oligomerization of ECL in the aqueous medium, as mentioned above. It is worth mentioning here that the initial concentration of CHO in the aqueous medium was not 50 mM in the above-given reaction, as 45% of it diffuses to the dodecane phase (partitioning = 0.82 (org.: aq.), Table SI13).

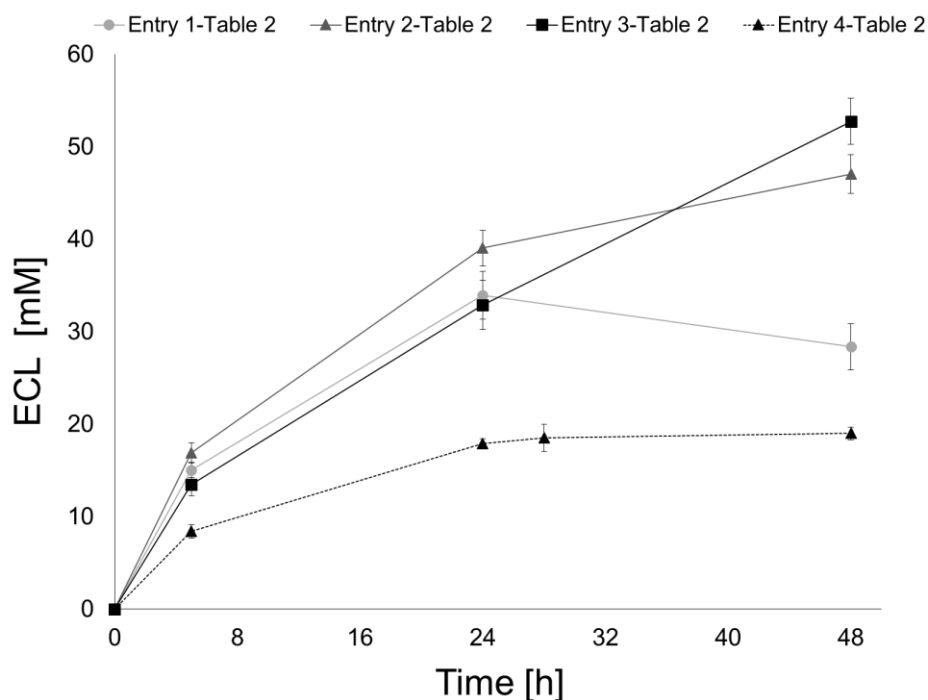
**Table 2** Experiments performed for the CHMO-ADH convergent cascade applied for the synthesis of ECL using the 2LPS approach

Entry	Organic Phase	Partition Coefficient of CHO [-]	CHO <sub>initially added to the aqueous phase</sub> [mM]	CHO <sub>initially added to the organic phase</sub> [mM]	1,6-HD <sub>initially added to the aqueous phase</sub> [mM]
1	Dodecane (5 mM toluene)	0.82	50	–	25
2	Dodecane (5 mM toluene)	0.82	91	–	25
3	Dodecane (5 mM toluene)	0.82	–	91	25
4	Toluene (5 mM dodecane)	4.56	–	278	25

Reaction conditions: Aqueous phase (0.5 mL 100 mM Tris-HCl at pH 8.0):  $c(\text{CHO}) = 50$  mM (Entry 1), 91 mM (Entry 2), 0 mM (Entry 3), 0 mM (Entry 4);  $c(1,6\text{-HD}) = 25$  mM;  $c(\text{CHMO}) = 0.5$  mg/mL (1.7 U/mL);  $c(\text{ADH}) = 0.005$  mg/mL (0.002 U/mL);  $c(\text{NADP}^+) = 0.05$  mM;  $T = 20^\circ\text{C}$ , 180 rpm, headspace 34 mL. Organic phase (0.5 mL dodecane (5 mM toluene) or 0.5 mL toluene (5 mM dodecane):  $c(\text{CHO}) = 0$  mM (Entry 1), 0 mM (Entry 2), 91 mM (Entry 3), 278 mM (Entry 4).

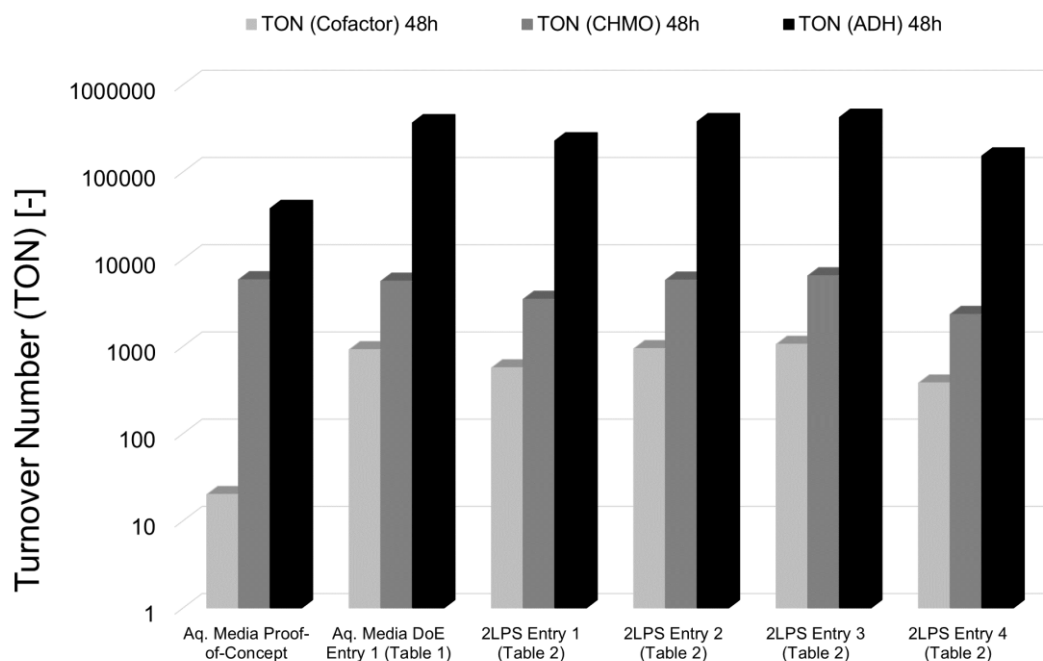
Next, taking the partitioning of CHO into account, we ran the above-given reaction in a 2LPS while keeping the molar ratio of CHO to 1,6-HD as 2:1 in the aqueous medium (Entry 2-Table 2, ▲ Figure 3). Under these conditions, the ECL concentration increased to 39 mM and further increased to 47 mM in 48 hours. The reason why higher ECL concentrations were not achieved was attributed to the formation of CHL (2.4 mM in 5 hours, 4.8 mM in 24 hours) and to the incomplete conversion of CHO over 48 hours. In the aqueous medium, CHL as the reduction product from CHO reached to its maximum concentration in 5 hours (1.6 mM), which gradually converted to ECL through its re-oxidation to CHO. The time courses of reactions in the aqueous medium and in the 2LPS are given in the Supporting Information (Fig. SI8). This difference in the progress of the reactions in the 2LPS compared to the aqueous medium is attributed to the ‘molecular toxicity’ effect of the organic phase, which to a larger extent decreased the rate of the monooxygenase reaction.

Thereafter, we applied CHO to the organic phase while keeping the concentration at 50 mM in the aqueous medium based on its partitioning (Entry 3-Table 2, ■ Figure 3). In that reaction, ECL concentration reached 33 mM in 24 hours and 53 mM in 48 hours. Finally, to satisfy our curiosity, we also ran a 2LPS in toluene for comparison (Entry 4-Table 2, ▲ Figure 3). In that case, 18 mM of ECL was observed in 24 hours, without any further increase up to 48 hours, as also confirmed by the analysis of enzymes’ activity upon incubation with toluene in a 2LPS.



**Figure 3.** CHMO-ADH Convergent cascade for the synthesis of ECL using the 2LPS approach (Entry 1-Table 2 (●), Entry 2-Table 2 (▲), Entry 3-Table 2 (■), Entry 4-Table 2 (▼)). Data points are average values of duplicates. Aqueous phase (0.5 mL 100 mM Tris-HCl at pH 8.0):  $c(\text{CHO}) = 50 \text{ mM}$  (Entry 1),  $91 \text{ mM}$  (Entry 2),  $0 \text{ mM}$  (Entry 3),  $0 \text{ mM}$  (Entry 4);  $c(1,6\text{-HD}) = 25 \text{ mM}$ ;  $c(\text{CHMO}) = 0.5 \text{ mg/mL}$  ( $1.7 \text{ U/mL}$ );  $c(\text{ADH}) = 0.005 \text{ mg/mL}$  ( $0.002 \text{ U/mL}$ );  $c(\text{NADP}^+) = 0.05 \text{ mM}$ ;  $T = 20^\circ\text{C}$ ,  $180 \text{ rpm}$ , headspace  $34 \text{ mL}$ . Organic phase (0.5 mL dodecane (5 mM toluene) or 0.5 mL toluene (5 mM dodecane)):  $c(\text{CHO}) = 0 \text{ mM}$  (Entry 1),  $0 \text{ mM}$  (Entry 2),  $91 \text{ mM}$  (Entry 3),  $278 \text{ mM}$  (Entry 4).

In summary, the TON values slightly increased with the 2LPS approach, albeit no significant enhancement was observed compared to the aqueous medium. The increment in TONs in the optimization steps is shown in Figure 4. In our studies, toluene was shown to be unsuitable as a solvent for the CHMO-ADH convergent cascade. In contrast to toluene, dodecane was a less abrasive solvent, although it led to no significant enhancement in the productivity owing to the low partition coefficient for ECL. The productivity of the cascade system considering the ADH reached to  $1210 \text{ kg}_{\text{product}}/\text{kg}_{\text{ADH}}$  whereas for CHMO the value was  $12 \text{ kg}_{\text{product}}/\text{kg}_{\text{CHMO}}$ . Admittedly, the productivities obtained in this study require further optimizations to fulfill the requirements reported for bulk chemicals ( $>5000 \text{ kg}_{\text{product}}/\text{kg}_{\text{free enzyme}}$ ).<sup>21</sup> The instability of the CHMO due to the long-term storage may also play a significant role. In general, CHMO variants designed for higher oxidative and long-term stability<sup>19</sup> might be useful for the convergent cascade system presented here.



**Figure 4.** Turnover numbers (TONs) for the cofactor and enzymes obtained in CHMO-ADH convergent cascade applied for the synthesis of ECL using the 2LPS approach in comparison to the aqueous medium. TON values of the proof-of-concept<sup>6</sup> study represent the data after 18 hours. Information on the entries are shown in Table 1 and Table 2. Data are average values of duplicates.

### 3 Conclusion

Overall, this work establishes a step-wise optimization strategy to enhance the productivity of the recently reported redox-neutral CHMO-ADH convergent cascade. Identification of the key factors involved in the bi-enzymatic system and their further optimizations promoted a higher ECL yield and TONs in the aqueous medium. TON values for NADPH (up to 1,000) and for the ADH (up to 400,000) increased 50-fold and 10-fold, respectively. Due to the decrease in the lactone yield observed over time, a 2LPS was implemented for the selective removal of ECL, which showed a slight increase in TON values.

Presently, the TONs for the cofactor and for the monooxygenase need further optimizations. Therefore, ongoing research in our laboratories focuses on establishing a (semi)continuously operated 2LPS<sup>22</sup> to extract ECL and thus to further increase the TONs (to reach at least 10,000). Our future experiments will also focus on finding an alternative organic solvent with a better partition coefficient for ECL with only minimal influence on the enzymes' activity. Recently, Groeger and coworkers reported on the use of polydimethylsiloxane (PDMS) thimbles<sup>23</sup> for the compartmentalization of chemo- and biocatalytic reactions to avoid the undesired interaction between the enzyme and chemical agents.<sup>24</sup> With respect to the CHMO-ADH convergent system presented here, the use of PDMS thimbles might be a useful approach for the separation of aqueous- and organic phase to alleviate the enzyme deactivation by the organic solvent. Furthermore, von

Langermann and coworkers recently reported biocatalytic active static emulsions (BASE)<sup>25</sup> as a useful strategy for compartmentalization of enzymatic reactions. This approach might be also useful for the CHMO-ADH convergent cascade system while using a second organic phase.

## 4 Experimental

### 4.1 General

The chemicals used in this study were purchased from different commercial sources (Sigma-Aldrich, Carl Roth GmbH and Acros Organics) and used as received. Heterologous expression and purification of TeSADH and heterologous expression of CHMO were performed as described previously<sup>6</sup> and both enzymes were used as lyophilized stocks. Protein concentrations in the lyophilized enzyme preparations were determined using the BCA protein quantification kit (Pierce™) according to manufacturer's (Thermo Scientific, Carlsbad, United States of America) instructions, using BSA as the standard. A detailed description of the designed experiments for two-step DoE approach, experimental procedures as well as the analytical protocols is given in the Supporting Information.

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### Supplementary data

Supplementary data related to this article can be found online at <http://dx.doi.org/XXXXXXXXXXXXX>.

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