

A Bi-enzymatic Convergent Cascade for ϵ -Caprolactone Synthesis Employing 1,6-Hexanediol as a 'Double-Smart Cosubstrate'

Amin Bornadel,^[a] Rajni Hatti-Kaul,^[b] Frank Hollmann,^[c] and Selin Kara^{*[a]}

A bi-enzymatic cascade consisting of a Baeyer–Villiger mono-oxygenase and an alcohol dehydrogenase (ADH) was designed in a convergent fashion to utilise two molar equivalents of cyclohexanone (CHO) and one equivalent of 1,6-hexanediol as a 'double-smart cosubstrate' to produce ϵ -caprolactone (ECL) with water as sole by-product. The convergent enzymatic cascade reaction reported herein, is performed at ambient conditions in water, is self-sufficient with respect to cofactor, and incorporates all starting materials into the desired product, ECL. Among different enzymes explored, the reaction catalysed by cyclohexanone monooxygenase from *Acinetobacter* sp. NCIMB 9871 coupled with ADH from *Thermoanaerobacter ethanolicus* showed the best results, reaching 91% conversion of CHO after 24 h with a product titre of 2 g L⁻¹. Scale-up of the coupled system (50 mL) performed better than the small-scale reactions and >99% conversion of CHO and ECL concentration of 20 mM were achieved within 18 h.

In recent years, biocatalytic cascades and one-pot multi-step enzymatic reactions have attracted growing interest owing to several important advantages: Reduction in the number of steps required for obtaining more complex molecules coupled with the chemo-, regio-, and stereoselectivity of various biocatalysts in such integrated systems potentially result in higher product yields and productivities.^[1] Redox enzymes that usually require expensive cofactors for the electron transfer between molecules, are nowadays mostly coupled in a cascade fashion—allowing simultaneous cofactor regeneration—to perform oxidation and reduction reactions more efficiently and more economically.^[2]

Cofactor regeneration systems proposed earlier to circumvent the use of stoichiometric amounts of the expensive cofactors used surplus amounts of the cosubstrate (e.g. isopropanol, ethanol) and produced stoichiometric amounts of waste.^[3] Using 1,4-butanediol as 'smart cosubstrate' for the cofactor regeneration was the next step towards more sustainable redox biocatalysis,^[4] which has also been validated in non-aqueous media.^[5]

ϵ -Caprolactone (ECL) is a cyclic ester with a substantial market: It is an important monomer for biodegradable, thermoplastic, and elastomeric polymers such as polycaprolactone.^[6] A fully enzymatic approach for ECL synthesis in a linear cascade fashion has been independently reported by the research groups of Harald Gröger and Uwe Bornscheuer,^[7] apparently inspired by the studies reported in the early 90s.^[8] Therein, oxidation of cyclohexanol (CHL) by an ADH was coupled with further oxidation of the formed cyclohexanone (CHO) to ECL by a BVMO. This approach is highly advantageous as it is self-sufficient with respect to the cofactor. Another sustainable feature that this route possesses is maximised incorporation of the starting material that is, CHL into the final product (ECL). More recently, extended reaction systems comprising the ADH-BVMO linear cascade as their core have also been reported. Efforts are now put on re-design and development of either upstream steps before the CHO formation^[9] or downstream steps after the ECL production^[10] in order to minimise the substrate/product inhibition, achieve higher productivities by in situ product removal, and finally obtain polymer precursors for example, 6-aminohexanoic acid^[10a] or oligo-ECL.^[10b]

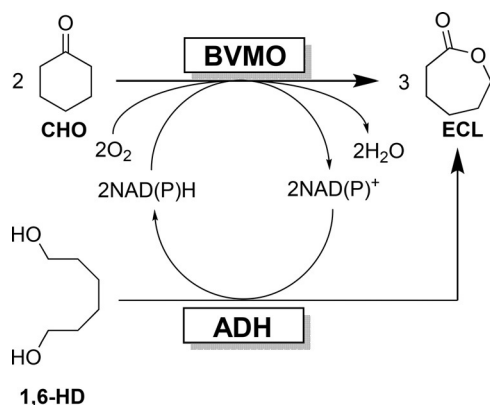
Redox-neutral cascade reactions introduced before this work are classified into two categories: Parallel cascades (i.e. bi-substrate—no intermediate—bi- or tri-product) and linear cascades (i.e. single substrate—one intermediate—single product).^[3b,d] The present study introduces a new class to redox-neutral reactions, herein called a convergent cascade involving bi-substrate and a single product without formation of an intermediate. We report herein on a bi-enzymatic bi-substrate system for production of ECL, consisting of a BVMO for oxidation of CHO and an ADH for oxidation of a 'double-smart cosubstrate' 1,6-hexanediol (1,6-HD) and simultaneous regeneration of NAD(P)H (Scheme 1). On the one hand, the use of a lactone-forming diol as 'smart cosubstrate' leads to a thermodynamically favourable lactone coproduct, which shifts the overall equilibrium towards the desired product, avoiding high surpluses of the cosubstrate and tedious product recovery, and reducing the waste generated. On the other hand, the choice of 1,6-HD as a 'double-smart cosubstrate' allows the coupling

[a] Dr. A. Bornadel, Dr. S. Kara
Institute of Microbiology
Chair of Molecular Biotechnology
Technische Universität Dresden
01062 Dresden (Germany)
E-mail: selin.kara@tu-dresden.de

[b] Prof. Dr. R. Hatti-Kaul
Department of Biotechnology
Center for Chemistry and Chemical Engineering
Lund University
P.O. Box 124, 221 00 Lund (Sweden)

[c] Dr. F. Hollmann
Department of Biotechnology
Delft University of Technology
Julianalaan 136, 2628BL Delft (The Netherlands)

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/cctc.201500511>.



Scheme 1. Synthesis of ϵ -caprolactone (ECL) through a convergent cascade system by coupling a Baeyer–Villiger monooxygenase (BVMO)-catalysed oxidation of cyclohexanone (CHO) to ECL promoted by an alcohol dehydrogenase (ADH)-catalysed oxidation of the ‘double-smart cosubstrate’ 1,6-hexanediol (1,6-HD) for regeneration of NAD(P)H also yielding ECL.

of BVMO-catalysed oxidation of CHO (two equiv) with ADH-catalysed oxidation of 1,6-HD (one equiv) yielding the target compound ECL (three equiv) by using only O₂ and producing only water as by-product.

In the first set of experiments we screened a range of ADHs for activity towards 1,6-HD oxidation. Here, it is worth mentioning that this screening *via* a UV assay might only cover the first step of the lactonisation (i.e. oxidation of the diol to the hydroxy aldehyde). The reaction buffer system was chosen based on a literature survey (Table SI6 in the Supporting Information). Out of seven ADHs (3 NADPH-dependent and 4 NADH-dependent) evaluated, *Thermus* sp. ATN1 ADH (TADH) showed the highest activity (Figure 1). This observation is not surprising as TADH has been successfully applied for the oxidation of primary alcohols or α,ω -diols such as 1,4-butanediol.^[11]

The activities of ADH-A and HLADH did not decrease on increasing 1,6-HD concentrations (Figure SI2 (right) and SI3 (right)). Remarkably, the commercial ADH evo 1.1.200 showed no depletion in its activity up to 800 mM 1,6-HD (Figure SI2(left)). Among the different enzymes tested, the latter ADH also revealed the lowest inhibition due to ECL accumulation (up to 200 mM, Figure SI6). Taking all the enzymes into account, 20 mM CHO was chosen as a safe starting concentration for our proof of concept studies.

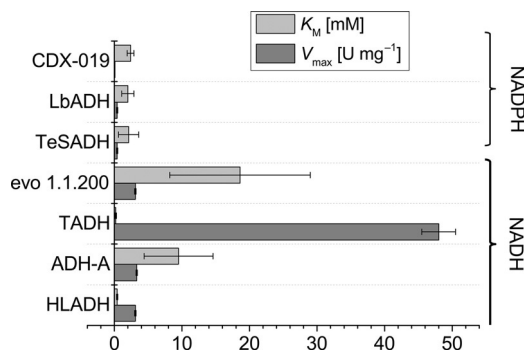


Figure 1. Screening of ADHs for the oxidation of 1,6-HD. Reaction conditions: $c(1,6\text{-HD}) = 0\text{--}800\text{ mM}$, $c(\text{NADP}^+/\text{NAD}^+) = 0.5\text{ mM}$, $c(\text{ADH}) = 0.1\text{ mg mL}^{-1}$, buffer: Tris-HCl (100 mM, pH 8.0), $T = 20^\circ\text{C}$.

Next, by making various combinations of the NADPH-dependent BVMOs and ADHs, and the NADH-dependent BVMOs and ADHs, respectively, 12 and 8 bi-enzymatic systems for the production of ECL were designed. All 20 reactions were carried out using 20 mM (2 g L⁻¹) CHO and 10 mM (1 g L⁻¹) 1,6-HD, and the ECL formed in 48 h is shown in Figure 2. Gratifyingly, in all of the 20 reactions CHO and 1,6-HD were converted to ECL. The highest concentration of ECL was observed when wild-type cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NCIMB 9871 was coupled with *Thermoanaerobacter ethanolicus* ADH (TeSADH) (Figure 2A). Approximately 10 mM of ECL, starting from 20 mM CHO and 10 mM of 1,6-HD, was achieved from the preliminary trials under non-optimised conditions. The second-best combination was the coupling of ECS-Mo01 (commercial crude enzyme preparation of *Acinetobacter calcoaceticus*) with CDX-019 with $\approx 6\text{ mM}$ ECL formed in 48 h. Although TADH showed the highest catalytic activity towards the oxidation of 1,6-HD (Figure 1), lower oxidation activities of the NADH-dependent BVMOs (2,5-DKCMO and 3,6-

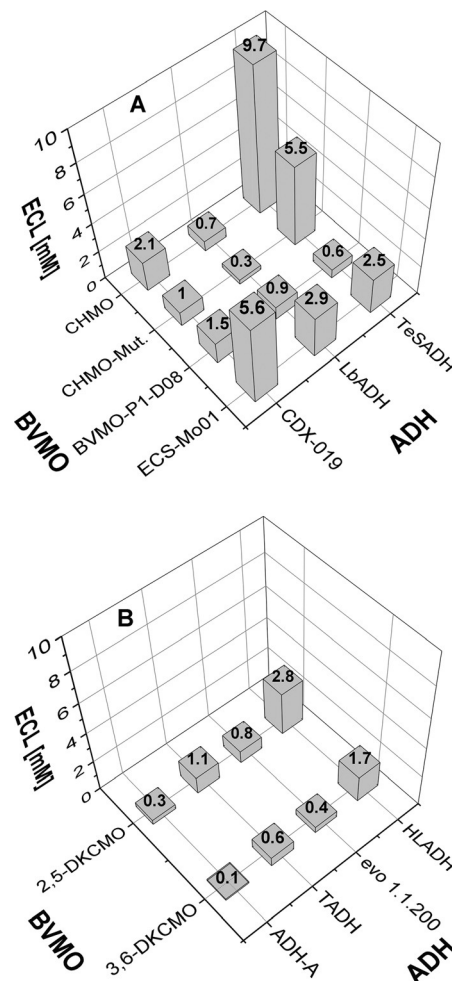


Figure 2. Screening of BVMO and ADH couples for the synthesis of ECL promoted by 1,6-HD as a ‘double-smart cosubstrate’. Reaction conditions: $c(\text{CHO}) = 20\text{ mM}$, $c(1,6\text{-HD}) = 10\text{ mM}$, $c(\text{NADP}^+/\text{NAD}^+) = 1\text{ mM}$, $c(\text{BVMO}) = 0.1\text{ mg mL}^{-1}$; $c(\text{ADH}) = 0.01\text{ mg mL}^{-1}$ (TeSADH, TADH, ADH-A) or 0.1 mg mL^{-1} , buffer: Tris-HCl (100 mM, pH 8.0), 250 rpm, $T = 30^\circ\text{C}$, 48 h. (A) Coupling of NADPH-dependent BVMOs and ADHs, (B) Coupling of NADH-dependent BVMOs and ADHs. Data points are average values of duplicates. Standard deviations are < 7% in Figure 2A and < 5% in Figure 2B.

DKCMO) towards CHO resulted in low ECL formation in those reactions.

Negative control experiments by eliminating one or two of the catalytic components (NADP^+ , CHMO, and TeSADH) were conducted. In these control experiments, the highest ECL formation (6 mM in 72 h) occurred in the absence of TeSADH (Figure S17-C). This observation can be attributed to endogenous ADH activity present in the crude enzyme preparation of CHMO, utilising 1,6-HD and regenerating NADPH. Indeed, 1,6-HD was oxidised by the CHMO preparation, however, with a 12-fold lower activity than TeSADH (Table S110, S111, Figure S18). In addition, tiny amounts of ECL (2 mM in 72 h) were detected when no NADP^+ was externally added (Figure S17-A). This presumably results from NADPH/NADP^+ present in the cell extract preparations (≈ 0.2 mM),^[12] as also reported in the literature.^[13] No ECL formation was observed in the absence of CHMO (Figure S17-B and D).

Incubation of ECL in the reaction buffer (100 mM Tris-HCl, pH 8.0) without or with the cell-free extracts of *Escherichia coli* (strain without expression plasmid) showed ca. 40% depletion in mass (in 72 h, Figure S19). In the screening experiments given above (in 48 h, data not shown) ca. 20% of the total analyte mass was depleted. These mass imbalance issues can to a large extent be the result of an undesired hydrolysis of ECL. Furthermore, evaluation of stability of enzymes without or with their substrates (CHO, BVMOs; 1,6-HD, ADHs) exhibited reduction in their catalytic activities upon incubation, especially for enzymes produced in-house (Figure S110). This is most probably owing to stabilizers (e.g. polyols, salts, sugars etc.) present in commercial enzymes.

Encouraged by the promising results obtained under non-optimised conditions (Figure 2), we aimed for enhancing the productivity of the CHMO-TeSADH coupled system. To this end, higher amounts of the enzymes and substrates were used for the reactions presented in Table 1. As shown for the Reaction No 1 (the data from the CHMO-TeSADH coupled system presented in Figure 2A), the conversion of CHO reached 71% and 90% after 24 and 72 h, respectively. By adding another aliquot of CHMO after 48 h, conversion was increased to 99% after 72 h. By using two-fold higher amounts of enzymes, full conversion was achieved within 48 h (Reaction No 3, Table 1).

However, in the latter reaction, the turnover frequency (TOF) value decreased. By increasing the substrate (i.e. CHO) concentration to 100 mM (hence 1,6-HD to 50 mM), TOF stayed similar (Reaction No 4, Table 1); however, the total conversion of CHO was significantly reduced to 32%. A further increase in the concentration of substrates did not improve the TOF- and conversion values (Reaction No, Table 1), whereas conversion values increased with additional 5-fold higher amounts of enzymes (Reaction No 6, Table 1).

The time course of the reaction revealed the formation of cyclohexanol (CHL) as by-product. This 'back reduction reaction' of CHO to CHL catalysed by the ADH was also reported by Oberleitner et al. (2014),^[9b] during a linear cascade reaction. Indeed, the kinetic analysis of CHO reduction and 1,6-HD oxidation catalysed by TeSADH showed 19-fold higher V_{max} for the reduction reaction, whereas similar K_{M} values for CHO and 1,6-HD were observed (Table S112). The maximum CHL concentration achieved was 7 mM after 24 h (Figure S111, Reaction No 3), which gradually converted to ECL through its re-oxidation to CHO. After 72 h, no CHL was detected.

The practical usefulness of the BVMO-ADH coupled system promoted by 1,6-HD as the 'double-smart cosubstrate' was demonstrated on a semi-preparative scale (50 mL reaction volume) using 98 mg (2 g L^{-1} , 20 mM) CHO and 59 mg (1 g L^{-1} , 10 mM) 1,6-HD. The scaled up reaction progressed better than the small-scale reactions and >99% conversion of CHO and 19.7 mM ECL were achieved within 18 h. This partly results from an efficient O_2 supply (50 mL reaction in 500 mL flask). After 24 h 19 mM ECL and 3.7 mM 1,6-HD were detected (Figure S112, S113). Work-up of the reaction mixture gave 146 mg yellowish oily substance; its ^1H NMR analysis revealed a mixture of three compounds: ECL, poly-ECL and 1,6-HD (1:1.5:0.6) (Figure S114). The turnover numbers (TONs) obtained were 5795, 38000, and 20 for CHMO, TeSADH and for the nicotinamide cofactor, respectively. Through further process optimisation, for example, establishing reactions conditions enabling the use of nicotinamide cofactor at low amounts (<0.1 mM) and continuous production, higher TONs for the nicotinamide cofactor are achievable.

In summary, we have developed a highly atom-efficient synthesis route for ECL, which is an important polymer precursor

Table 1. Conversion of cyclohexanone (CHO) and 1,6-hexanediol (1,6-HD) into ϵ -caprolactone with internal cofactor regeneration.^[a]

| Reaction number | CHO [mM] | 1,6-HD [mM] | CHMO [mg mL ⁻¹] | TeSADH [mg mL ⁻¹] | TOF ^[b] [min ⁻¹] | Conversion over time | | | | | | | |
|------------------|----------|-------------|-----------------------------|-------------------------------|---|----------------------|----------------------------|------|----------------------------|------|----------------------------|------|----------------------------|
| | | | | | | 5 h | | 24 h | | 48 h | | 72 h | |
| | | | | | | [%] | c(ECL) [mM] ^[c] | [%] | c(ECL) [mM] ^[c] | [%] | c(ECL) [mM] ^[c] | [%] | c(ECL) [mM] ^[c] |
| 1 | 20 | 10 | 0.1 | 0.01 | 4.6 | 34.7 | 2.5 | 70.6 | 9.5 | 86.5 | 9.6 | 90.1 | 8.8 |
| 2 ^[d] | 20 | 10 | 0.1 | 0.01 | 4.1 | 27.4 | 2.3 | 57.3 | 6.8 | 72.4 | 9.4 | >99 | 13.3 |
| 3 | 20 | 10 | 0.2 | 0.02 | 2.0 | 36.4 | 2.3 | 90.5 | 9.4 | >99 | 18.3 | >99 | 16.8 |
| 4 ^[e] | 100 | 50 | 0.2 | 0.02 | 1.8 | 6.8 | 2.0 | 20.9 | 7.2 | 26.0 | 11.5 | 32.2 | 13.7 |
| 5 | 200 | 100 | 0.2 | 0.02 | 2.3 | 4.1 | 1.3 | 7.0 | 4.2 | 7.9 | 4.4 | 10.5 | 4.2 |
| 6 | 100 | 50 | 1 | 0.1 | 0.9 | 19.5 | 4.8 | 51.1 | 25.8 | 67.7 | 34.7 | 76.1 | 23.9 |

[a] Reaction conditions: $c(\text{CHO})=20:100:200$ mM, $c(1,6\text{-HD})=10:50:100$ mM, $c(\text{NADP}^+)=1$ mM, $c(\text{CHMO})=0.1\text{--}1$ mg mL⁻¹, $c(\text{TeSADH})=0.01\text{--}0.1$ mg mL⁻¹, Tris-HCl (100 mM, pH 8.0), 250 rpm (orbital shaking). Reactions (1 mL of total volume) run in 4 mL glass-vials. [b] Turnover frequency (TOF) represents $\mu\text{mol}_{\text{ECL}}$ formed in one minute by using $\mu\text{mol}_{\text{BVMO}+\text{ADH}}$ determined over the first five hours (sampling at 0/1/3/5 h). [c] Based on the consumption of CHO determined by GC analysis. [d] The same amount of CHMO was added after 48 h. [e] The data for Reaction No 4 represent average values of duplicates, whereby the standard deviation was <9%. Reaction temperature was 30 °C for the Reaction No 1 whereas 20 °C for the Reactions No 2–6.

with a global demand in the range of multi-kilotons. This is achieved by designing a new redox-neutral convergent cascade, whereby all starting materials incorporate into a single product, ECL. Conversion of CHO to ECL was promoted by ADH-catalysed oxidation of 1,6-HD complementing the BVMO route by regenerating the NAD(P)H and converging to the same product ECL. The CHMO from *Acinetobacter* sp. NCIMB 9871 and ADH from *Thermoanaerobacter ethanolicus* (TeSADH) turned out to be the most suitable enzymes for the coupled system. This study is a first-time report on convergent redox-neutral cascade applying 1,6-HD as a cosubstrate for a BVMO-catalysed oxidation. As such, it represents a proof of concept with absolutely considerable potential for further improvements. Future process development studies for enhancing productivity^[14] will focus on 1) optimisation of biocatalyst concentrations, ratios, etc., 2) the use of high substrate concentrations for achieving enzyme-to-substrate ratios >50 (g g⁻¹)^[15] by biphasic and water-free systems, 3) stabilisation of enzymes by means of immobilisation^[16] or using additives.^[17] Special attention will be devoted to in situ removal of the lactone product and to develop an integrated ECL polymerisation under controlled conditions.

Experimental Section

All chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany), Carl Roth GmbH (Karlsruhe, Germany) or VWR (Dresden, Germany) with a purity ≥98% and were used as received. A detailed description of the experimental procedures as well as the analytical protocols is given in the Supporting Information.

Acknowledgements

S.K. thanks DECHEMA e.V. (Frankfurt am Main, Germany) for financial support in the form of a "Max Buchner" fellowship. A.B. thanks Gesellschaft von Freunden und Förderern der Technische Universität Dresden e.V. for six months of personal funding. Special thanks to Dr. Max Steinhagen and Prof. Dr. Marion B. Ansorge-Schumacher for fruitful discussions on molecular studies. The authors gratefully acknowledge Dr. Jörg H. Schrittwieser for helpful suggestions and proofreading the manuscript. The authors would like to thank (1) Prof. Dr. Wolfgang Kroutil (University of Graz, Austria) for the plasmids hosting alcohol dehydrogenases from *Rhodococcus ruber* (ADH-A), *Thermoanaerobacter ethanolicus* (TeSADH), and from *Lactobacillus brevis* (LbADH), (2) Prof. Dr. Marco W. Fraaije (University of Groningen, The Netherlands) for the plasmids hosting cyclohexanone monooxygenases (CHMOs) from *Acinetobacter* sp. NCIMB 9871 (CRE2-CHMO and CRE2-CHMO L323C-A325C). Crude enzyme preparations were kindly provided by (3) Enzymicals AG, Germany; ECS-Mo01, 2,5-DKCMO and 3,6-DKCMO, (4) evocatol GmbH, Germany; evo 1.1.200 and HLADH, and (5) Codexis, Inc., Belgium; BVMO-P1-D08 and CDX-019.

Keywords: alcohol dehydrogenases • caprolactone • Baeyer–Villiger reaction • monooxygenases • multi-enzymatic cascades • smart cosubstrate

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Received: May 7, 2015

Published online on July 14, 2015