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COMMUNICATION

A fed-batch synthetic strategy for a three-step enzymatic synthesis of poly-ε-caprolactone

Christian Scherkus[a], Sandy Schmidt[b,c], Uwe T. Bornscheuer[c], Harald Gröger[d], Selin Kara[a] and Andreas Liese*[a]

Abstract: A three-step enzymatic reaction sequence for the synthesis of poly-ε-caprolactone (PCL) was designed running in a fed-batch operation. The first part of the cascade consists of two oxidation steps starting with an alcohol dehydrogenase catalysed oxidation from cyclohexanol to cyclohexanone and further oxidation to ε-caprolactone (ECL) by means of a Baeyer-Villiger monooxygenase. As a third step a lipase-catalysed hydrolysis of the lactone to 6-hydroxyhexanoic acid (6-HHA) was designed. With this biocatalytic multi-step process reported herein, a severe substrate surplus and product inhibition could be circumvented by a fed-batch operation adding the substrate cyclohexanol and *in situ*-product removal of ECL by hydrolysis, respectively. Up to 283 mM product concentration of 6-HHA could be reached in the fed-batch operated process without loss in productivity within 20 h. After extraction and subsequent polymerisation catalysed by *Candida antarctica* lipase B, the analysis of the unfractionated polymer revealed a bimodal distribution of the polymer population reaching a M_w value of approx. 63,000 g/mol and a dispersity (M_w/M_n) of 1.1 for the higher molecular weight population, thus revealing an alternative route to the conventional synthesis of PCL.

Enzymatic reaction sequences illustrate the toolbox of different enzymatic activities and its versatility to explore smart synthetic process routes in organic synthesis. The synthesis of lactones *via* coupling of an alcohol dehydrogenase (ADH) and a cyclohexanone monooxygenase (CHMO) in a linear fashion was first published by Willetts and coworkers in the early 90s.[1] Later on, Bornscheuer and coworkers demonstrated this strategy for the synthesis of ε-caprolactone (ECL) starting from cyclohexanol (CHL), a readily available compound, while using an engineered polyol dehydrogenase for CHL oxidation.[2] At the same time Gröger and coworkers demonstrated the same linear cascade to ECL while using an ADH from *Lactobacillus kefiri* and elucidated the major kinetic limitations, namely a product inhibition of CHMO by ECL and a decreased stability of CHMO in presence of CHL.[3] Since then research linked to this process concept was extended towards the synthesis of 6-aminohexanoic acid for nylon-6 production[4] or changing the linear to a convergent cascade[5] utilizing a corresponding diol as a 'double-smart cosubstrate' even in a biphasic system.[6] Furthermore, an approach towards ECL-oligomer formation[7] or chiral polyesters[8] by introducing lipase A from *Candida antarctica* (CALA) as an additional biocatalyst to this reaction sequence was followed to prevent the inhibition of CHMO by ECL. However, molecular weight of the desired oligomers obtained from the reaction cascade remained rather low with M_w and M_n values of approximately 1,200 g/mol and 600 g/mol, respectively. The oligomer product of the cascade formed by ring-opening polymerisation (ROP) by CALA still contained 25% of ECL, but could be chemically converted to PCL, which showed identical properties compared to the standard chemically synthesised PCL.[7] This special acyltransferase activity of CALA[9] makes the aforementioned approach attractive, as no enzyme catalysed hydrolysis of ECL occurs despite of the aqueous medium used. Hence, the removal of ECL from the reaction medium, which occurs *via* ROP by CALA, alleviates the inhibition of CHMO yielding the main target compound PCL even as oligomers at the same time.

Polymerisation of ECL is a well investigated process that could be done with a variety of different catalysts either chemically[10] or enzymatically.[11] The advantage of the biocatalytic approach in contrast to the conventional metal-based polymerisation is that residual amounts of metal-based catalysts in the polymer, being problematic from the toxicity point of view[12], are avoided. Additionally, the bio-based concept is ecologically more attractive, since it can already be performed at a significant lower reaction temperature.[13] The enzymatic polymerisation is commonly performed with lipase B from *Candida antarctica* (CALB) preferably as immobilised enzyme in an organic solvent.[14] Due to the versatile applications for ROP and availability of CALB, we focused our attention on extension of the ADH-CHMO linear reaction sequence for the synthesis of ECL with a subsequent polymerisation using this lipase (Scheme 1).

Our initial experiments integrating the CALB-catalysed synthesis of oligomers, which were proposed to precipitate,[15] were not successful. Instead, we observed hydrolysis of ECL to 6-hydroxyhexanoic acid (6-HHA) in the ADH-CHMO-CALB linear reaction sequence (Scheme 1). The observed fast depletion of ECL concentration due to the hydrolysis (proved by pH decrease as well as by HPLC analysis) motivated us to develop our strategy to make use of this hydrolysis activity. Although oligomerisation catalysed by CALA might be the preferred method, as no water needs to be eliminated, lipase-catalysed condensation after extraction of 6-HHA as the hydrolysis product leads to PCL as a reaction being documented in the literature.[16]

The reaction engineering of the demonstrated approach is mainly determined by enzyme kinetics. According to literature data the main limitation is the inhibition of CHMO by the substrate of the cascade CHL and the final product ECL.[2a,3] Inhibition of CHMO by cyclohexanone (CHN) as an intermediate can be neglected as its concentration remains low.

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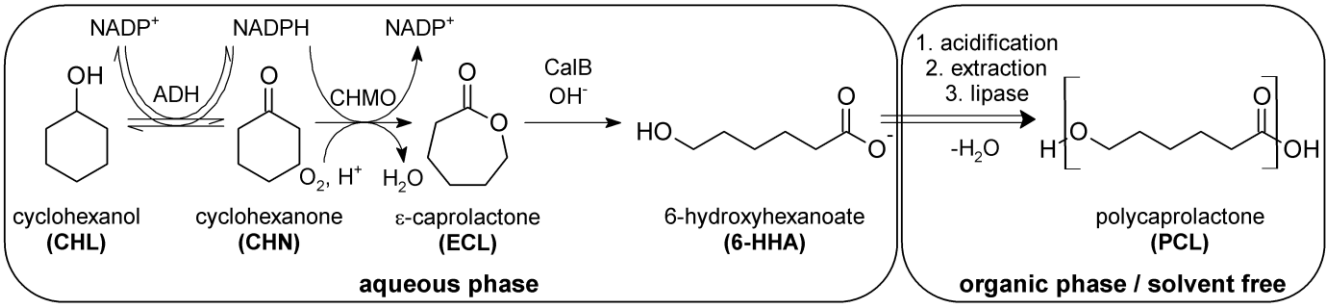


Scheme 1. Reaction scheme of ADH-CHMO linear cascade coupled with CALB-catalysed hydrolysis including isolation of the hydrolysis product of ECL and subsequent CALB-catalysed polymerisation to PCL.

This is also beneficial for the reversible ADH-catalysed reaction step as the undesired reduction of CHN suppressed the oxidation of CHL.

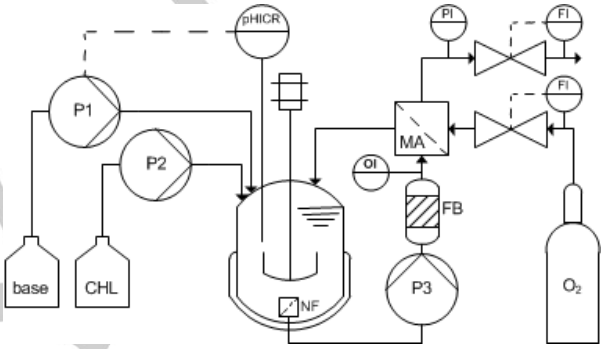
First we evaluated the performance of CALA and CALB in terms of the ECL depletion (Table 1). Two different CALB preparations were tested for different purposes, namely a covalently immobilised CALB on a hydrophilic carrier for hydrolysis of ECL in water and an adsorptively immobilised CALB on a hydrophobic carrier for polymerisation in an organic solvent or in solvent-free conditions. A soluble lyophilised and an immobilised preparation of CALA were tested for comparison with CALB (Table 1 footnote). Herein, our results revealed that CALB (FBL) exhibits approx. 40- to 218-fold higher CLU (caprolactone units) activity compared to the tested CALA preparations being an efficient enzyme for the fast removal of ECL from the aqueous reaction medium.

Table 1. Activities of different lipase preparations for the removal of ECL from the aqueous medium.[a]

Lipase Preparation	PLU/g	CLU/g
CALB (FBL)	3626 ± 300	435 ± 62
CALB (c-LEcta)	3707 ± 148	n. d.
CALA (Sigma)	21 ± 8	2
CALA (c-LEcta)	n. d.	11

[a]PLU: propyl laurate units; CLU: caprolactone units; n. d.: Not determined, CALB (FBL): covalently immobilised lipase on a hydrophilic carrier; CALB (c-LEcta): adsorbtively immobilised lipase on a hydrophobic carrier; CALA (Sigma): adsorbtively immobilised lipase on a hydrophobic carrier; CALA (c-LEcta): lyophilised cell-free extract powder.

To demonstrate the benefit of a fast reactive *in situ*-consumption of ECL and the resulting decrease of the ECL concentration in the reaction medium, it is necessary to compare the reaction system with and without CALB in the presence of increased concentration of ECL and 6-HHA as the hydrolysis product, respectively. The reason for the addition of ECL or 6-HHA at the beginning of a reaction is to focus on the inhibition effect of ECL and its circumvention by hydrolysis at advanced reaction time. Running the process in a fed-batch operation in respect to CHL is a suitable approach to overcome severe substrate inhibition[2a] as its concentration remains low. Therefore, all cascade reactions were performed in a fed-batch mode according to the setup shown in Scheme 2. The immobilised CALB could either be retained using a 200 µm nylon filter mesh in the reaction vessel or packed into a fixed bed. If not mentioned else the nylon filter was used.



Scheme 2. Flow scheme of the experimental setup for fed-batch operation with Silicone tubing placed in an aerated bottle for efficient O_2 supply for membrane aeration (5 m, ID: 2 mm, OD: 3 mm, A/V = 21.75 cm^{-1}) (supporting information). Aeration with O_2 (99.5%) at ambient pressure. FB: CALB (c-LEcta) fixed bed; MA: membrane aeration; NF: 200 µm nylon filter mesh; FI: gas flow indicator; P1: pump autotitration; P2: pump for the supply of CHL; P3: pump for circulating the reaction volume; pHICR: pH indicator, controller and regulator; PI: pressure indicator; OI: oxygen indicator.

Figure 1A describes the result of the fed-batch process whereby 200 mM ECL was present at the beginning, due to the reasons as mentioned above, while pH was kept constant by autotitration (Figure 1B). With a constant feed rate of CHL, ECL concentration increased up to 229 mM in 4.5 hours. However, while the reaction proceeded the rate of ECL formation slightly decreased. The high concentration of ECL present already at the beginning and its further increase affected the activity of CHMO. A decreased activity of ADH resulted from ECL was not observed and remained independent from this (data not shown). The reduced CHMO activity due to the ECL accumulation consequently resulted in the accumulation of CHL (due to the thermodynamically limited ADH-catalysis, thus favoring the reduction of CHN to CHL) which led to a further decrease of CHMO activity. This causes a 'dead-end'-effect if the feed is not stopped and regulated. Addition of CHMO to the reaction mixture can also be a solution to compensate this. However, this strategy would cause a waste of precious potential enzyme activity.

Since molecular oxygen is an essential substrate for the monooxygenase reaction, we focused our attention on monitoring the oxygen concentration during the entire reaction time with the goal to enable sufficient oxygen supply, done *via* membrane aeration (supporting information). Simultaneously, the course of

oxygen concentration can be used as an indicator for the overall 'cascade activity'. An increase in the oxygen concentration indicates that the oxygen consumption rate is lower than the rate of the supply in consequence of either lower CHMO activity due to inhibition (by ECL or by CHL) or full conversion of CHL indicating a completed reaction. After initiating the reaction by starting the substrate feed the oxygen concentration instantly decreased in the first 30 min (Figure 1A). A continuous increase in the oxygen and CHL concentrations (up to more than 25 mM for the latter one) started already after 60 min. In these experiments, due to increased autohydrolysis of ECL to 6-HHA at alkaline conditions, the pH was maintained by autotitration (Figure 1B).

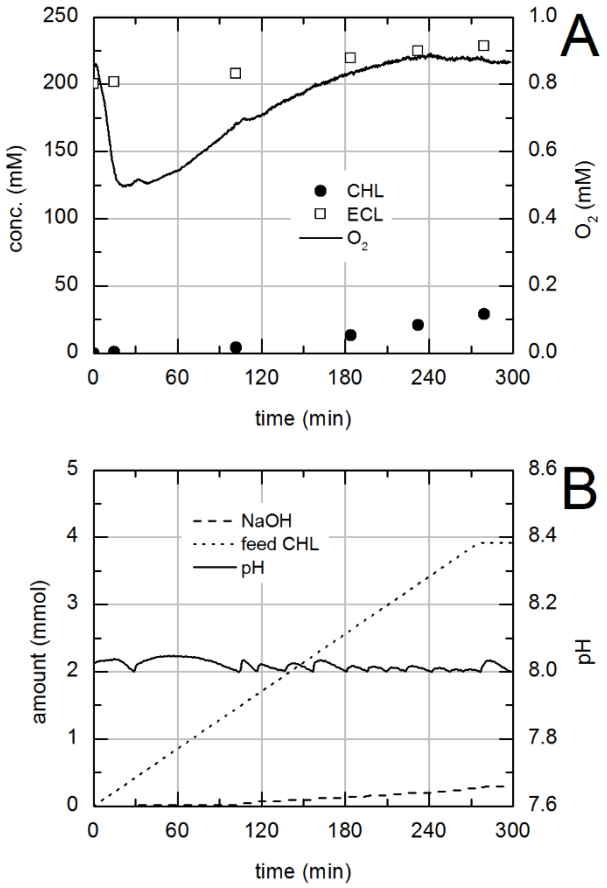


Figure 1. Fed-batch synthesis without lipase in 60 mL phosphate buffer (33 mM, pH 8). Course of concentrations (A) and autotitration (B). Reaction conditions: 30 U CHMO, 33 U ADH, 2 mM $MgCl_2$, 0.2 mM $NADP^+$, 12 mmol (200 mM) ECL, feed rate CHL: 14.23 µmol/min CHL, circulation flow rate: 30 mL/min, titration with NaOH (5 N), 25 °C

An analogous experiment as described above was then conducted by first adding immobilised CALB (FBL, 0.8 g, 348 CLU) leading to the formation of 6-HHA (at 200 mM). As expected, the same amount of ECL (12 mmol, 200 mM) was hydrolysed completely in 40 mL buffer (Figure 2) before the soluble enzymes e.g., ADH, CHMO and water were added to reach the final reaction volume of 60 mL. Herein, additional volume due to the titration was considered.

Subsequently, the fed-batch synthesis started with feeding CHL at a rate of 14.2 µmol/min (Figure 3). Residual concentration of ECL remained less than 4 mM as a result of the lipase activity during the course of reaction. At the same time CHL concentration kept low around 1 mM with a slight increase within 180 min and a stronger increase up to 5 mM after 300 min. This concentration increase is due to the slightly higher CHL feed than the resulting CHMO activity during the course of reaction, which was also observed by a slightly increasing shift of the courses of the titration rate and feed rate (Figure 3B).

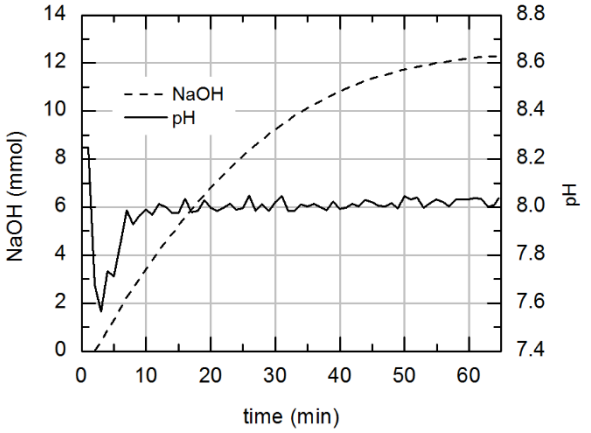


Figure 2. Titration of CALB-catalysed (FBL, 0.8 g, 348 CLU) hydrolysis of 12 mmol ECL to 6-HHA before starting the fed-batch synthesis with lipase in 40 mL phosphate buffer (50 mM, pH 8).

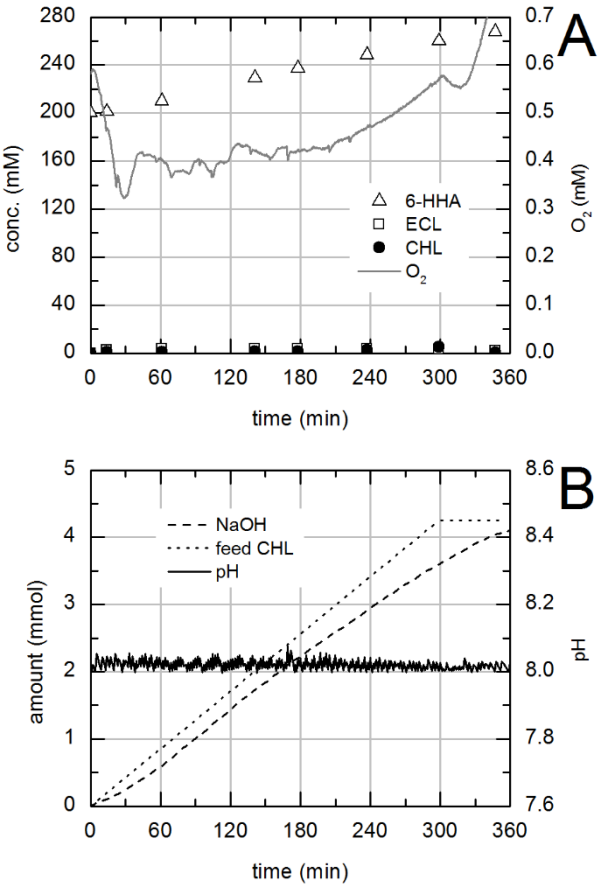


Figure 3. Fed-batch synthesis with lipase in 60 mL phosphate buffer (33 mM, pH 8). Course of concentrations (A) and titration (B). 30 U CHMO, 33 U ADH 0.8 g CALB (FBL, 348 CLU), 2 mM $MgCl_2$, 0.2 mM $NADP^+$, 12 mmol (200 mM) 6-HHA (from previous hydrolysis), feed rate: 14.2 µmoL/min CHL, circulation flow rate: 30 mL/min, titration with NaOH (5 N), 25 °C.

Ideally, the CHL concentration should remained low due to instant hydrolysis of ECL. Consequently, the feed rate and the titration rate should be identical under the prerequisite that the activity of each enzyme was sufficient. Furthermore, each measurement contributed to a loss of reaction volume due to sampling. The withdrawn sample volume was more than that of the fed volume of the substrate CHL and titration with NaOH. In consequence of a constant feed rate the concentration increases while the final reaction volume decreased. Thus, further increase of CHL concentration led to increased inhibition of CHMO.

Comparing both fed-batch experiments with and without lipase the latter leads to an approx. 9-fold higher accumulation of CHL after 240 min (Figure 4). The low CHL accumulation observed is most probably due to the higher remaining CHMO activity resulting from the depletion of ECL by lipase-mediated hydrolysis.

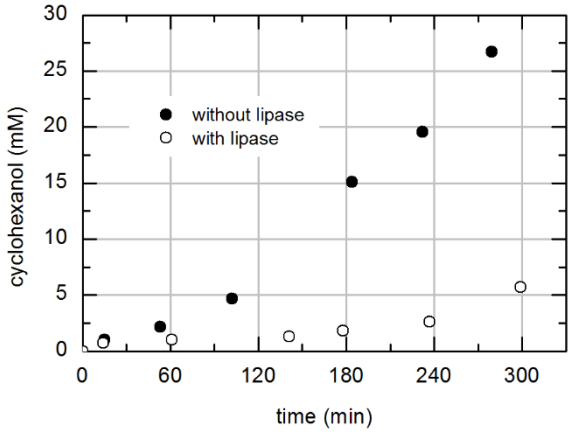


Figure 4. Comparison of the fed-batch experiments without and with CALB concerning the accumulation of cyclohexanol (CHL).

An application of the ADH-CHMO-CALB cascade with a higher volumetric enzyme activity (two-fold) and longer reaction time (20 h) revealed a reaction course without loss in the productivity yielding 283 mM of 6-HHA and a conversion of >99%, whereby the concentrations of CHL and ECL remained at low values of max. 1 and 4 mM, respectively (Figure 5A). One major difference to previous case is that the immobilised lipase was packed into a fixed bed. Similar to the previous case, the titration and feed curves proceed in parallel, whereas with a much better fit in the latter case (Figure 5B). The feed rate was decreased after 7 h to prevent the accumulation of CHL due to posssible loss of enzyme activity while the reaction run further for 13 h without sampling. The loss of activity depends on the stability of the catalysts under the process conditions and limits the time of operation. Hence, as soon as the feed rate exceeds the enzymatic activity the reaction should be stopped or more catalyst should be added or the feed rate should be decreased to make use of residual and valuable activity. If the process is meant to run in a production scale, the half-life times of the catalysts under process conditions are of high interest. Furthermore, the maximum applicable activity is limited by the supply of oxygen.

Since the fed-batch synthesis worked well the isolation of 6-HHA was performed in an analogous experiment where approx. 150 mM of 6-HHA could be reached (supporting information). At the end of the experiment the whole reaction volume was acidified with trifluoroacetic acid until pH 1 was reached prior to the extraction with MTBE. After all organic solvent was removed under reduced pressure a yellow honey-like viscous material could be obtained (supporting information). Since ADH and CHMO are applied as free enzymes, the isolation of 6-HHA by extraction using an organic solvent most probably leads to loss of their activities. Solvent-free polymerisation of 6-HHA was performed under vacuum using CALB immobilised on a hydrophobic carrier (c-LEcta) to prevent the binding of water released from the condensation.

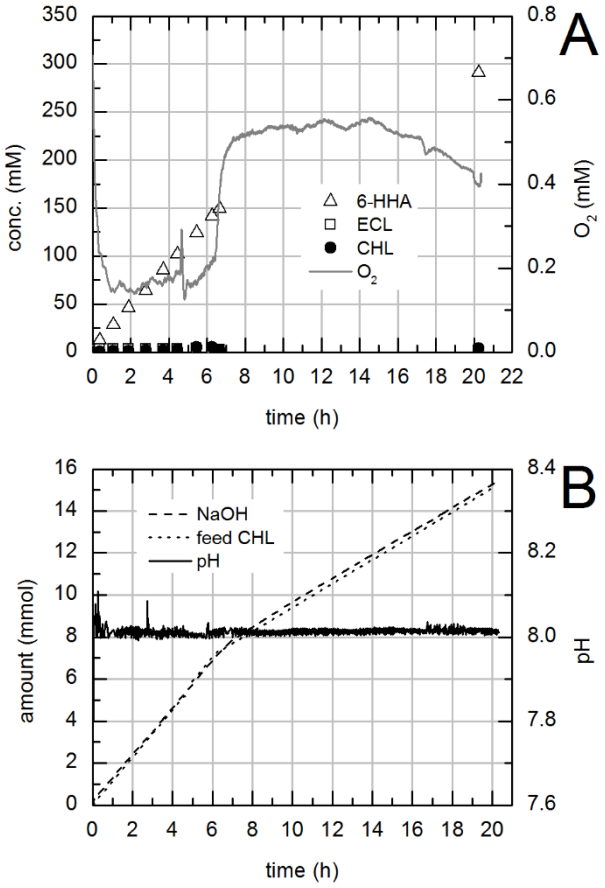


Figure 5. Fed-batch synthesis with lipase in 50 mL sodium phosphate buffer (100 mM, pH 8). Course of concentrations (A) and titration (B). 50 U CHMO, 55 U ADH, 1.5 g CALB (FBL, 653 CLU) in a fixed bed, 5 mM $MgCl_2$, 0.4 mM $NADP^+$, feed rate: 5–38 µmoL/min CHL, circulation flow rate: 10 mL/min, titration with NaOH (5 N), 25 °C.

The 6-HHA used for condensation was obtained from a separate preparative hydrolysis of ECL in water conducted on a larger scale. The obtained polymer was not fractionated and analysed by GPC and revealed a comparable molecular weight with respect to the high molecular weight standards of polystyrene (PS) (Figure 6). The overall signal intensity was similar to 66,000 g/mol PS, which demonstrates the success of the fed-batch process with an integrated lactone hydrolysis. The polymer analytics revealed a bimodal polymer distribution with an overlap of the higher and lower molecular populations. Individual analysis of each population revealed a M_w value of 63,482 g/mol with a distribution (M_w/M_n) of 1.1 for the higher molecular weight population and a M_w value of 24,061 g/mol with a M_w/M_n of 2.1 for the lower molecular weight population (supporting information).

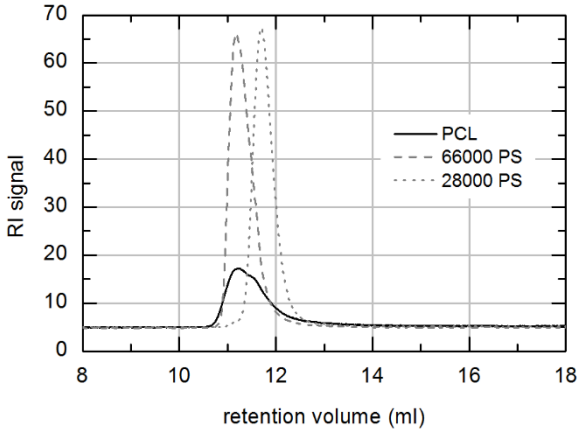


Figure 6. GPC result of solvent-free polymerisation of 6-HHA to PCL in comparison to polystyrene (PS) molecular weight standards (66,000; 28,000 g/mol).

In conclusion, fed-batch processes with and without lipase-catalysed hydrolysis of ECL demonstrated the advantage in this extended ADH-CHMO-lipase cascade reaction. The hydrolysis route enables to maintain the CHMO activity to a significant extent, thus leading to approx. 9-fold less CHL accumulated at advanced reaction time compared to a fed-batch process without lipase. A low catalytic amount of CALB (FBL, 0.8 g, 348 CLU) was sufficient to remove the ECL since the activity with respect to ECL depletion is 40- to 218-fold higher with CALB, depending on the preparation, compared to CALA. This makes the reaction economically more feasible with respect to enzyme consumption. Furthermore, the use of a covalently immobilised enzyme preparation offers (i) no leaching from the carrier, (ii) ease of separation via filtration, and (iii) reuse. In this study, the full enzymatic reaction sequence including downstream processing *via* polymerisation was successfully demonstrated. The non-fractionated PCL obtained from the enzymatic condensation of 6-HHA was comparable to a high molecular weight standard (PS 66,000 g/mol), which shows that this process represents an alternative route for the chemical synthesis of PCL heading to a practical and sustainable approach also for a productive scale. A detailed kinetic study of the linear cascade considering each reaction step individually including the hydrolysis is currently in progress to generate a kinetic model to describe and modify this multi-parameter enzymatic process by simulations.

Experimental Section

Materials

Cyclohexanone (ACS reagent, ≥99%), lauric acid (98%) were purchased from Sigma-Aldrich (Munich, Germany), $MgCl_2$ (≥98.5%), $NADP^+$ disodium salt (≥97%), NADPH tetrasodium salt (≥97%), isopropyl-β-D-thiogalactopyranosid (IPTG, ≥99%), ampicillin (99%), kanamycin sulphate, yeast extract (micro-granulated), LB-medium (Luria/Miller), glycerol (ROTIPURAN, ≥99.5%), tryptone/peptone from casein, 1-propanol (>99.5%) from Carl Roth (Karlsruhe, Germany), ε-caprolactone monomer (99%) from Acros Organics (Geel, Belgium) and used as received. Cyclohexanol (ReagentPlus, 99%) was purchased from Sigma-Aldrich and was used after destillation under reduced pressure to remove impurities of cyclohexanone. *E. coli* BL21 (DE3) competent cells were purchased from New England Biolabs (Ipswich, MA, USA).

Enzymes

CHMO and ADH overexpression: 400 mL of TB prepared in a 2-L baffled shake flask were inoculated with 4 mL overnight culture. Cells were grown at 37 °C until OD_{600} 0.8-1.0 under shaking and the overexpression was then induced with IPTG at a final concentration of 0.1 mM. Cells were further grown under shaking at room temperature (approx. 19-25 °C) for 20–24 h. Afterwards, cells were harvested by centrifugation, washed once with sodium phosphate buffer (100 mM, 50 mM NaCl, pH 7.2) and resuspended in approx. 15 mL of the same sodium phosphate buffer. Next, cells were disrupted by ultrasonication (SONOPULS HD 2070 with sonotrode type KE 76, BANDELIN electronic GmbH & Co. KG, Berlin, Germany) for three times on ice (5 min at 50 % pulse time and 70 % power) Cell debris was separated by centrifugation and cell-free lysate transferred into a new vessel and kept at 4 °C.

Lipases: Lipase B from *Candida antarctica* (CALB) adsorptively immobilised on a hydrophobic carrier was purchased from c-LEcta GmbH (Leipzig, Germany), whereas CALB covalently immobilised on a hydrophilic carrier (FERMASE CALB 10000-K15, 9200 PLU/g) was purchased from Fermenta Biotech Ltd. (*Maharashtra, India*). Lipase A from *Candida antarctica* (CALA) was purchased as immobilised preparation on Immobead 150 from Sigma-Aldrich (Art.#41658, ≥500 TBU/g) and as lyophilized powder (Art.#20605-1, 34 TBU/g) from c-LEcta GmbH.

Enzyme Activity

The activities of ADH and CHMO were determined as initial rate by spectrophotometric measurements with a UVIKON XL spectrophotometer (Goebel Instrumentelle Analytik GmbH, Hallertau, Germany) monitoring the formation or consumption of NADPH at 340 nm for 1 min in a 1 mL cuvette at 25 °C. Sodium phosphate buffer (100 mM, pH 8) was used as reaction medium maintained at 25–27 °C. CHMO activity was measured with 50 µL pre-diluted cell-free crude extract, 0.01 % (v/v) cyclohexanone (0.95 mM) and 0.2 mM NADPH in total 1 mL sodium phosphate buffer (100 mM, pH 8). ADH activity was measured with 50 µL pre-diluted crude extract, 0.01 % (v/v) cyclohexanol (0.95 mM) and 0.6 mM $NADP^+$ in total 1 mL sodium phosphate buffer (100 mM, pH 8).

Lipase activity was determined with two different methods. Activity in ε-caprolactone units (CLU) which is defined as the depletion of one µmol of ε-caprolactone per minute and was determined as initial rate for 10 min and max. 10% conversion of hydrolysing freshly prepared 2 % (v/v) (189 mM) ε-caprolactone in sodium phosphate buffer (100 mM, pH 7) at 30 °C in 1 mL scale and approx. 10 mg immobilised preparation. Samples of 50 µL were taken at 0, 2, 5, 10 min from the reaction and diluted with 950 µL water. The reaction time was prolonged if no conversion could be obtained. The Sample was filtered with a 0.22 µm filter. Conversion was analyzed by HPLC. Herein, autohydrolysis of ε-caprolactone could be neglected. Activity in propyl laurate units (PLU) is defined as the formation of 1 µmol of propyl laurate per minutew and was determined as initial rate after 5 min esterification of lauric acid (400 mg) and 1-propanol (180 µl) with some water (16 µL) using approx. 10–20 mg lipase preparation at 60 °C in a thermostated shaker. Total mass of all reaction compounds was determined to calculate relative amount of lauric acid in the mixture. Ingredients were tempered before the enzyme was added to start the reaction. After 5 min the reaction solution was separated from the catalyst with a syringe capped with a thin capillary and transferred into a glass vessel and weighed to recalculate the initial amount of lauric acid. Ethanol was added to dissolve the precipitated sample with 2–3 drops of 1 % (w/v) phenolphthalein in ethanol as pH indicator. Residual amount of lauric acid was determined by titration with 0.1 M NaOH in 80 % ethanol (aq.). Titration was finished when solution changed from colorless to pink and remained constant for 20 s.

Extraction of 6-Hydroxyhexanoic Acid

The 6-HHA generated from the whole reaction cascade was obtained by acidifying the reaction volume with trifluoroacetic acid and extraction with equal volumes of methyl tert-butyl ether (MTBE) 3–4 times. Extraction volumes were pooled and MTBE removed under reduced pressure. To isolate 6-HHA generated from a preparative hydrolysis reaction the reaction volume was first acidified with hydrochloric acid. The volume was reduced under heat and reduced pressure by evaporating water until sodium chloride precipitated. It was then resuspended in MTBE and filtered to remove insoluble sodium chloride. MTBE was removed under reduced pressure.

Polymersation

Polymerisation of 6-HHA obtained from preparative hydrolysis of ECL (30.61 g, 286 mmol) in water (30.27 g) was performed with 1 g CALB (cLEcta, 3707 PLU) under solvent free conditions in a stirred tank reactor at 70 °C under reduced pressure (80 mbar) to remove the water. Samples were diluted with chloroform and immobilised catalyst separated by filtration through a 0.2 µm nylon filter. Samples were dried under atmospheric conditions.

HPLC Analytics

Samples of ε-caprolactone and 6-hydroxyhexanoic acid for HPLC analytic were performed by taking 50 µL of aqueous sample and added to 950 µL water. In the case of a soluble enzyme (e.g., CALA lyophilisate), samples were diluted in acetonitrile: water (1:1 v/v) mixture. Analytics was performed with an Agilient 1100 Series device on a LiChrospher 100 RP-18 (5 µm) LiChroCART 250-4 column at 30 °C. 50 % (v/v) Methanol in water was used as the eluent with a flow rate of 1 mL/min (isocratic).

GC Analytics

Detection of cyclohexanol, cyclohexanone and ε-caprolactone was performed by extraction of aqueous samples with chloroform. 200 µL of aqueous sample or standard was transferred into a 1 mL tube, 10 µL acetophenone (23.8 mg/mL DMSO) was added as an internal standard and 300 µL chloroform was added. After 1 min of vortex and phase separation by centrifugation 200 µL of chloroform phase was transferred to a GC glass vial with a micro-inlet. Analysis was performed on an Agilent Technologies 7890B (Santa Clara, CA, USA) device with a CP-Chirasil-Dex CB (Agilent), 25 m x 0.25 mm x 0.25 µm column. Oven temperature profile started from 150 °C for 3 min with a ramp to 167 °C (20 °C/min), 190 °C (30 °C/min) and held for 1.4 min. For calibration a mixture of cyclohexanol (204.9 mg, 2.0 mmol), cyclohexanone (215.8 mg, 2.2 mmol) and ε-caprolactone (221.7 mg, 1.9 mmol) in 10 mL water was used for a serial dilution.

GPC Analytics

Dried polymer samples were diluted in 997 µL tetrahydrofurane (THF) and 3 µL toluene as an internal flow standard. GPC was performed with THF and 1 mL/min flow rate and polymers were separated on a GMHHR-L Mixed Bed column (Viscotek, Houston, TX, USA) in series to a PLgel 3 µm MIXED-E column (Agilent Technologies, Santa Clara, CA, USA). A refractive index (RI) detector (K2301, KNAUER Wissenschaftliche Geräte GmbH, Berlin, Germany) was used for monitoring the course of separation. The system was calibrated with molecular weight standards of polystyrene (M_P 66,000; 28,000; 12,600; 9,130; 6,100; 4,920; 3,470; 2,280; 1,250, 162 g/mol) (PSS Polymer Standards Service GmbH, Germany).

A detailed description of bacterial strains, plasmids, cultivation procedure, enzyme activity assays, analytical procedures and experimental setup is given in the supporting information.

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Keywords: ε-Caprolactone • Oxidoreductases • Polymerisation • *Candida antarctica* lipase B • Enzymatic cascade

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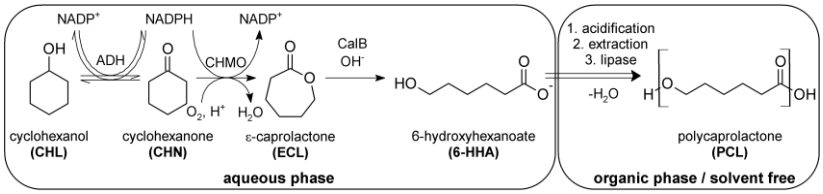

A fed-batch synthesis was applied for a multi-enzymatic reaction sequence to overcome kinetic limitations. Enhanced product concentrations can be achieved by the hydrolysis of ε-caprolactone. Condensation of 6-hydroxyhexanoic acid offers access to poly-ε-caprolactone.

*Christian Scherkus, Sandy Schmidt, Uwe T. Bornscheuer, Harald Gröger Selin Kara and Andreas Liese **

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Hydrolyse and polymerise