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Biocatalytic conversion of cycloalkanes to lactones using an in-vivo cascade in *Pseudomonas taiwanensis* VLB120†

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Abstract

Chemical synthesis of lactones from cycloalkanes is a multi-step process challenged by limitations in reaction efficiency (conversion and yield), atom economy (by-products) and environmental performance. A heterologous pathway comprising novel enzymes with compatible kinetics was designed in *Pseudomonas taiwanensis* VLB120 enabling in-vivo cascade for synthesizing lactones from cycloalkanes. The respective pathway included cytochrome P450 monooxygenase (CHX), cyclohexanol dehydrogenase (CDH), and cyclohexanone monooxygenase (CHXON) from *Acidovorax* sp. CHX100. Resting (non-growing) cells of the recombinant host *P. taiwanensis* VLB120 converted cyclohexane, cyclohexanol, and cyclohexanone to ε-caprolactone at 22 U g_{CDW}^{-1}, 80-100 U g_{CDW}^{-1}, and 170 U g_{CDW}^{-1}, respectively. Cyclohexane was completely converted with a selectivity of 65% for ε-caprolactone formation in 2h without accumulation of intermediate products. Promiscuity of the whole-cell biocatalyst gave access to analogous lactones from cyclooctane and cyclodecane. A total product concentration of 2.3 g L^{-1} and a total turnover number of 36720 was achieved over 5h with a biocatalyst concentration of 6.8 g_{CDW} L^{-1}. This article is protected by copyright. All rights reserved

Introduction

The oxidation of cyclohexane to cyclohexanol and cyclohexanone (Ketone-Alcohol: KA-oil) is an important commercial reaction with an industrial history of 75 years (Weissermel and Arpe, 2003). This reaction is the main route for the production of monomers, such as ε-caprolactone, ε-caprolactam, and adipic acid for the polymer industry (Weissermel and Arpe, 2003). However, the high reactivity of KA-oil towards oxygen limits the cyclohexane per-pass conversion to 10-12% and KA-oil selectivity to 75-80% (Schuchardt et al., 1992; Schuchardt et al., 2001). In course of the process, pure cyclohexanone separated from the KA-oil is further reacted with a peracetic acid to synthesize ε-caprolactone (UCC process) on the multi-thousand tons per annum scale (Weissermel and Arpe, 2003). This chemical route involves multiple steps (Figure 1A), with several unit operations including numerous heat and pressure variations. Such process complexity very often results in high capital expenditures (CapEx) and operational expenditures (OpEx).

Realizing multi-step reactions in one-pot circumvents several purification steps and provides opportunities to reduce waste, time, and costs (Muschiol et al., 2015; Oberleitner et al., 2017; Schrittwieser et al., 2017). In this context, several biocatalytic cascades have been designed to synthesize ε-caprolactone starting from cyclohexanol, 2-cyclohexen-1-ol or hexane-1,6-diol (Bornadel et al., 2016; Mallin et al., 2013; Oberleitner et al., 2014; Scherkus et al., 2016; Scherkus et al., 2017; Staudt et al., 2013). Recently, a biocatalytic reaction concept that mimicked chemical reaction route to synthesize lactones from cycloalkanes was designed by utilizing an in-vitro cascade of four isolated enzymes (Pennec et al., 2015). However, this elegant reaction concept resulted in a low total turnover number (TTN) of 822 at approx. 2.5% cyclohexane conversion. Although such in-vitro cascade allows the highest flexibility to balance

enzyme ratios for enhancing reaction performance (Muschiol et al., 2015), the necessity of several unit operations for the production of isolated enzymes and cofactors leads to high CapEx and OpEx costs. In this context, in-vivo cascade offers economic perspectives by combining production and regeneration of enzymes and cofactors necessary to conduct cascade reactions in one single unit operation (de Carvalho, 2016; Schrewe et al., 2013; Schrittwieser et al., 2017; Bayer et al., 2015). However, for the efficient turnover of the in-vivo cascade, the catalytic efficiency of the individual enzymes needs to be compatible to streamline substrate conversion towards the main product without accumulation of intermediate compounds.

In a previous study, we introduced CHX (cytochrome P450 cyclohexane monooxygenase) for the selective oxidation of cyclohexane to cyclohexanol (Karande et al., 2016). The genes encoding this enzyme system were isolated from *Acidovorax* sp. CHX100, a bacterial strain capable of utilizing cyclohexane as the sole source of carbon and energy (Salamanca and Engesser, 2014). This organism shows high growth rates with cyclohexane indicating the presence of naturally efficient enzymes for conversion of cycloalkanes. This work now aims at designing in-vivo cascade in *P. taiwanensis* VLB120 by assembling and exploiting catalytic efficiency of enzymes evolved in *Acidovorax* sp. CHX100 to transform cycloalkanes to lactones.

Initially the identification, isolation, and functional expression of novel genes from *Acidovorax* sp. CHX100 encoding cyclohexanol dehydrogenase (CDH) and cyclohexanone monooxygenase (CHXON) and their functional coupling to cytochrome P450 (CHX) in recombinant *Pseudomonas taiwanensis* VLB120. This synthetic pathway allowed the transformation of cyclohexane to ε-caprolactone at overall rates of 22 U g_{CDW}^{-1}, using resting cells of *P. taiwanensis* VLB120 pCom10_capro. Complete conversion of 5 mM cyclohexane was achieved with a yield of 65% for ε-caprolactone. By continuous supply of cyclohexane through the gas

phase allowed total product concentration of 2.3 g L^{-1} and a total turnover number of 36720 over 5h.

Experimental Section

Chemicals: All chemicals and primers used were purchased from Sigma-Aldrich (Steinheim, Germany) or Carl-Roth (Karlsruhe, Germany) with the highest purity available, and used without further purification. Reagents for molecular biology have been obtained from Life Technologies GmbH, Darmstadt, Germany.

Microbial strains, plasmids, cultivation and transformation methods: All organisms (Table S1, supporting information) used have been cultivated in Luria-Bertani (LB) medium (DSMZ_medium 381) or M9*-medium (DSMZ_medium 382) supplemented with 0.5% (w/v) citrate as a carbon source, US* trace elements (Emmerling et al., 2002), and kanamycin 50 µg mL^{-1}. Pre-cultures were incubated overnight in 5-10 mL LB-medium or M9*-medium in a horizontal shaker (30°C and 200 rpm 25 mm amplitude), Multitron, Infors HT, Bottmingen, Switzerland). Plasmids were introduced into *P. taiwanensis* VLB120 by electroporation (2500 V, Equibio EasyjecT Prima, Kent, UK) as described in Sambrook and Russell, 2001 (Sambrook and Russell, 2001).

Construction of pCom10_capro: The plasmid pCom10_capro was constructed in three consecutive steps. First, the genes for cyclohexanol dehydrogenase (*cdh*) and cyclohexanone monooxygenase (*chxon*) have been coupled on plasmid pCom10_chxon_cdh according to the following procedure: Respective genes have been amplified from the genomic DNA of *Acidovorax* CHX100 by standard polymerase chain reaction (PCR) (Sambrook and Russell, 2001). Primers are listed in Table S1 (supporting information). The resulting PCR products were cloned into the pCom10 backbone via the restriction sites NdeI/AscI and AscI/HindIII,

respectively, yielding plasmid pCom10_chxon_cdh. This was introduced into competent *P. taiwanensis* VLB120 via electroporation and after overnight growth on LB agar plates containing kanamycin (50 µg mL^{-1}), pCom10_chxon_cdh was isolated from the obtained transformants. Correct insertion of the genes was verified using restriction digest analysis and PCR. Second, restriction sites were adapted for coupling the chxon_cdh cassette to pCom10_CHX (Karande et al., 2016) via PCR using degenerated primers carrying the respective sequences for restriction enzyme recognition (Table S1). Finally, *chxon_cdh* encoding the dehydrogenase and the genes for the BVMO were coupled to *chx* located on pCom10_CHX (Karande et al., 2016) using standard cloning procedures. The resulting plasmid pCom10_capro was introduced into *P. taiwanensis* VLB120 and after overnight growth on LB agar plates containing 50 µg mL^{-1} kanamycin, the desired plasmid was isolated from obtained transformants. Correct insertion of all genes was verified using restriction digest analysis and PCR. The plasmid map of pCom10_capro is given in the supporting information (Figure S1).

Analytical methods: Proteins were detected using SDS-PAGE according to Laemmli 1970 loading 10 µg of total protein per lane (Laemmli, 1970). Active CYP was quantified in whole cells via CO (carbon monoxide) difference spectra done according to Cornelissen et al., 2011 (Cornelissen et al., 2011).

Concentrations of the different lactones were measured by GC (Trace 1310, Thermo Scientific, Waltham, USA) equipped with a flame ionization detector and a TR-5MS GC Column (dimensions: 15 m length, 0.25 mm inner diameter (Thermo Scientific, Waltham, USA)) with molecular nitrogen as carrier gas and 1 µl injection volume in splitless injection mode. The temperature profile setting was as follows: 40°C (3 min), 40-170°C (15°C min^{-1}), 170-300 (100°C min^{-1}). Products were quantified based on calibration curves from commercially

available standards. For commercially non-available standards (2-oxonanone and oxacydoundecan-2-one), compound concentrations were estimated from GC/FID signals based on the comparison of carbon content in the lactone with the available standards (delta-valerolactone, ε-caprolactone and oxacyclododecan-2-one). Additionally, the standard curve estimated from the carbon content of lactones was verified by comparing the standard curve of 2-oxonanone isolated from the reaction mixture. Both standard curves showed similar results (data not shown here). The head-space measurements were performed on the same GC-column and detector as described above. The oven temperature was set to 35°C for 3 min with 100 µl injection volume in split injection mode (split flow 11 mL min^{-1}, and a split ratio of 7). The cyclohexane standard curve in the head space was estimated by evaporating pure cyclohexane in 120 mL serum bottles sealed with a rubber cap. Gas-tight syringe (Hamilton GASTIGHT Syringe, 100 µL) was used to take 100 µL of headspace cyclohexane vapor and injected on GC. Cyclohexane evaporation in the presence of 10 mL aqueous phase (100 mM Kpi buffer) was determined in the serum bottle (120 mL) by adding pure cyclohexane (0.15-20 µL) and incubating the bottle in a rotary shaker at 30°C and 180 rpm, 25 mm amplitude. After 1 minute of incubation, 100 µL of headspace cyclohexane vapor was taken by using a gas-tight syringe (Hamilton GASTIGHT Syringe, 100 µL) and sample injected on GC (Figure S2, supporting information).

Lactones were identified with GC-MS (Figure S4, S5, S6, supporting information) using a Thermo ISQ LT single Quadrupole MS (Thermo Scientific, Waltham, USA) coupled to a GC (Trace 1310, Thermo Scientific, Waltham, USA). Concentrations of 6-hydroxyhexanoic acid, 8-hydroxyoctanoic acid, and 10-hydroxydecanoic acid were determined by HPLC on a Dionex Ultimate 3000 (Thermo Scientific, Waltham, USA) equipped with an Acclaim® OA column

(Thermo Scientific, Waltham, USA). The mobile phase A consisted of 10 mM sodium sulphate containing 0.1% (v/v) formic acid. Acetonitrile was used as mobile phase B. 10-20 µL of sample was injected, while the flow and the column temperature were kept constant at 0.34 mL min^{-1} and 30°C, respectively. The flow profile was as follows: 5%B for 3 min, 5 to 80%B in 17 min, 80-5%B in 5 min and 5%B for 5 min. Detection was accomplished via a UV spectrometer (210 nm).

Biotransformation of cycloalkanes: Cells were grown in M9* mineral media supplemented with 0.5% citrate and kanamycin (50 µg mL^{-1}), to an OD_{450} of 0.9 to 1.0 (0.17-0.19 g_{CDW} L^{-1}) and then induced by the addition of (0.025% v/v) dicyclopropylketone (DCPK). During growth, cells were sampled every 60 min for SDS-PAGE. Cells were harvested 5-6 h after induction by centrifugation (Thermo Electron Corporation, Langenselbold, Germany) at 20°C, 3000 g, 15 min, washed and re-suspended in 100 mM Kpi buffer pH 7.4 supplemented with 1% of citrate (hereafter referred to as 'reaction buffer') to a cell concentration between 3.6-3.8 g_{CDW} L^{-1}. This cell stock (20 mL) was transferred to baffled Erlenmeyer flasks (volume 100-110 mL) tightly closed with Teflon sealed screw caps and incubated in a rotary shaker at 30°C, 180 rpm (25 mm amplitude) for 10 min. The screw caps contained a small hole (2 mm), embedded within the silicone to inject a syringe needle for sampling. The biotransformation was initiated by adding 5 mM of the respective cycloalkane to the flasks. Samples were taken every 30 minutes for 2h and analyzed by gas chromatography (GC) and high-pressure liquid chromatography (HPLC).

For evaluating whole-cell based biocatalytic performance, varying cell concentrations between 1.7-6.8 g_{CDW} L^{-1} in 20 mL were transferred to baffled Erlenmeyer flasks (volume 1000 mL) and incubated in a rotary shaker (30°C; 180 rpm, 25 mm amplitude)). The biotransformation was started by adding 200 mM of cyclohexane to the gas container which allowed a continuous

supply of cyclohexane to the aqueous phase via the gas phase. Samples were analyzed by GC and HPLC.

For GC analysis, the reaction was stopped by adding 0.9 mL of ice cold diethyl ether containing 0.2 mM decane as an internal standard to an equal amount of the reaction mixture phase. Both phases were mixed for 2 min on a vortex at room temperature for product extraction and then centrifuged (17000 rcf, 2 min; Thermo Electron Corporation, Langenselbold, Germany) to separate the phases. The ether phase was dried over anhydrous Na_2SO_4 and analyzed by gas chromatography. For high-pressure liquid chromatography (HPLC) analysis, the samples were centrifuged for 10 min at 13000 g, RT (Thermo Electron Corporation, Langenselbold, Germany). Supernatants were used for HPLC analysis while cell pellets were discarded. Specific activities were calculated as U g_{CDW}^{-1}, where 1 U is equal to 1 µmol reaction product synthesized per minute.

Biocatalyst characterization (kinetics study): Cells were grown, induced, harvested and resuspended in reaction buffer as described above to a cell density of 0.5 g_{CDW} L^{-1} cells. This cell suspension was distributed 10 mL each to baffled Erlenmeyer flasks (volume 100 mL) tightly closed with Teflon-sealed screw caps and incubated in a rotary shaker at 30°C and 180 rpm, 25 mm amplitude) for 10 min. The biotransformation was initiated by adding 0.1 µL to 20 µL pure cyclohexane to the respective flasks. The reaction was terminated after 10 minutes and ε-caprolactone formation was quantified using gas chromatography. Pure cyclohexane added to the aqueous phase was evaporated in the head space within a minute (Figure S2 supplementary information) due to the high cyclohexane vapor pressure (121.721 mm Hg at 30°C, Henry's law constant $K_{air/water}$ 0.15 atm m^3 $mole^{-1}$) and very low water solubility (55 mg L^{-1}).

The determination of reaction kinetics for cyclohexanol and cyclohexanone transformation was conducted in 1 mL reaction buffer containing 0.25 g_{CDW} L^{-1} cells. This cell suspension was transferred to 2 mL Eppendorf tubes and incubated in an Eppendorf Thermomixer® (30°C; 2000 rpm; 10 min). The biotransformation was initiated by adding cyclohexanol or cyclohexanone to the respective tube. The reaction was terminated after 10 minutes as described above and reactants and ε-caprolactone concentrations were determined using gas chromatography.

Results

Biocatalyst development for in-vivo production of lactones from cycloalkanes: In our previous study, cytochrome P450 monooxygenase (CHX) originating from *Acidovorax* sp. CHX100 was evaluated for the oxidation of cyclohexane to cyclohexanol using recombinant *P. taiwanensis* VLB120 as the host organism (Karande et al., 2016). Additional genes complementing the reaction sequence from cyclohexane to ε-caprolactone have been identified in the genome of *Acidovorax* sp. CHX100 via sequence comparison. These are *cdh* and *chxon* which encode a cyclohexanol dehydrogenase (CDH) and a cyclohexanone monooxygenase (CHXON), correspondingly. Respective genes showed 99% homology to cyclopentanol dehydrogenase and 83% homology to cyclohexanone-1,2-monooxygenase from *Acidovorax* sp. JS42 (Genbank submission ID 1959986).

The genes *cdh* and *chxon* were assembled into one operon with *chx* on the plasmid pCom10_CHX resulting in pCom10_capro. This new plasmid encoded the three enzymes necessary to yield ε-caprolactone from cyclohexane. It was inserted into *P. taiwanensis* VLB120, which served as a host organism for further studies. Synthesis of the three enzymes CHX, CDH and CHXON in *P. taiwanensis* VLB120 pCom10_capro was monitored by visualizing the

respective protein bands using SDS-PAGE (Figure 2A). Additionally, the functional expression of *chx* was verified via CO difference spectra measurements (Figure 2B).

Whole cell kinetics reveal that the CHX reaction rate is controlling lactone synthesis: The application of a whole-cell biocatalyst harboring an enzyme cascade necessitates matching enzyme kinetics to prevent accumulation of intermediate compounds and subsequently maximize final product yield. Therefore apparent whole-cell kinetic parameters, such as maximum whole-cell specific reaction rates (V_{max}) and substrate uptake constants (K_S), were determined for resting *P. taiwanensis* VLB120 pCom10_capro.

Due to the high vapor pressure (121.721 mm Hg at 30°C) and very low water solubility (55 mg L^{-1}) of cyclohexane, the substrate evaporated immediately and accumulated in the head space within a minute upon addition to the aqueous phase (Figure S2). Therefore, the *in-vivo* kinetics was evaluated based on the cyclohexane concentration in the gas phase as it acted as a substrate reservoir to supply of cyclohexane continuously to the aqueous phase. For the transformation of cyclohexane to ε-caprolactone *P. taiwanensis* VLB120 pCom10_capro showed a Michaelis-Menten type kinetic behavior (Figure 3A). The maximal reaction velocity of 22 U g_{CDW}^{-1} and the apparent cyclohexane uptake constant $K_{S(air)}$ of 0.082 mM ($K_{S(aqueous)}$ of 0.013 mM) were deduced from the Hanes plot given in the supporting information (Figure S3A; Table 1). No accumulation of intermediate products could be detected, indicating that the individual enzyme reaction kinetics was compatible for the transformation of cyclohexane to ε-caprolactone in a single step. Our previous study based on cyclohexane oxidation to cyclohexanol showed the maximal specific activity of 20-22 U g_{CDW}^{-1} (Karande et al., 2016). A similar activity was observed for the concerted reaction of cyclohexane to ε-caprolactone which indicates that the first reaction step, the cyclohexane oxidation to cyclohexanol, is the rate governing reaction for

caprolactone synthesis. To verify this hypothesis, whole-cell kinetics with the intermediate compounds such as cyclohexanol and cyclohexanone were investigated.

In comparison to cyclohexane, higher substrate uptake constants (K_S) and specific activities (V_{max}) were observed for the substrates cyclohexanol and cyclohexanone (Table 1). The maximal specific activities for the conversion of cyclohexanol and cyclohexanone to ε-caprolactone were 80-100 U g_{CDW}^{-1} and 170 U g_{CDW}^{-1}, respectively (Table 1; Figure 3B and 3C). In addition, cyclohexanol concentrations above 0.4 mM inhibited CDH activity. The high rates determined for the CDH and CHXON catalyzed reactions compared to the cytochrome P450 monooxygenase (CHX) and no accumulation of intermediate compounds demonstrates that the transformation of cyclohexane to caprolactone is governed by the CHX step. Fine tuning gene expression for increasing the ratio of CHX to CDH and CHXON and thus maximizing the hydroxylation rate of cyclohexane may be one of the strategies to improve lactone synthesis rate above 22 U g_{CDW}^{-1}.

In-vivo biotransformation of cycloalkanes to corresponding lactones by P. taiwanensis VLB120 pCom10_capro: The substrate scope of *P. taiwanensis* VLB120 pCom10_capro was evaluated using cyclohexane, cyclooctane, and cyclodecane as model compounds. A complete conversion (100%) of cyclohexane was observed with 65% selectivity for ε-caprolactone (Figure 4A). 6-hydroxyhexanoic acid was detected as a by-product and consequently, the ε-caprolactone yield dropped. This hydrolysis activity might arise from host intrinsic hydrolase(s). Although the conversion of cyclooctane was lower (83% in 2h) compared to cyclohexane, the selectivity for 2-oxonanone was surprisingly high (97%, Figure 4B). This suggests that the host intrinsic hydrolase system is very specific for ε-caprolactone. A further increase in substrate size to cyclodecane resulted in a decreased conversion of 2.5% and a product concentration of only 0.15

mM oxacydoundecan-2-one (Figure 4C). The low conversion might be due to a lower catalytic efficiency of CHX for these substrates. Other possible reasons include the low solubility of cyclodecane in water or difficulties of the bulk substrates in overcoming the membrane barrier of the cell. Both could limit substrate uptake and consequently decrease the reaction performance. Apart from the aforementioned compounds also cyclopentane, cycloheptane, and cycloundecane have been identified as possible substrates, and the corresponding lactones were identified (data not shown). Overall, *P. taiwanensi*s VLB120 pCom10_capro resting cells showed 100% conversion of cyclohexane and 81% conversion of cyclooctane with selectivity for the lactone of 65%, and 97%, respectively.

Whole-cell based biocatalytic performance for ε-caprolactone synthesis: The whole-cell biocatalytic performance was evaluated based on the product concentration, volumetric productivity, and TTN (Total Turnover Number) at three different cell densities (6.8, 3.4, 1.7 g_{CDW} L^{-1}). The biotransformation was started by adding 200 mM of cyclohexane to the gas container which allowed a continuous supply of cyclohexane to the aqueous phase via the gas phase. By averaging two independent experiments, maximum product concentrations of 17 mM ε-caprolactone (1.94 g L^{-1}) and 3.1 mM 6-hydroxyhexanoic acid (0.41 g L^{-1}) were achieved in 5h utilizing 6.8 g_{CDW} L^{-1} of a biocatalyst (Figure 5). The cells showed the lower specific activity of 8.5-10 U g_{CDW}^{-1} at a higher cell density of 6.8 g_{CDW} L^{-1} compared to 15-18 U g_{CDW}^{-1} at lower cell densities (3.4 to 1.7 g_{CDW} L^{-1}). The difference in initial volumetric productivity with biomass concentration may be explained by the substrates (cyclohexane and oxygen) mass transfer limitation. Additionally, a fast decrease in activity was observed at lower biomass concentrations (1.7 and 3.4 g_{CDW} L^{-1}) which might result from the toxic nature of cyclohexane. This is in accordance with our previous results (Karande et al., 2016). If the rate of cyclohexane

conversion in the aqueous phase is lower than the cyclohexane mass transfer to the aqueous phase, then cyclohexane accumulates in the aqueous phase until toxic concentrations, leading to a complete loss in the whole-cell activity. At higher biomass concentration, activity is decreasing slowly suggesting less or no cyclohexane accumulation due to a high conversion rate.

Discussion

Selective oxyfunctionalization of inert C-H bonds in cycloalkanes is a key challenge in designing biocatalytic reaction cascades for lactone synthesis from cycloalkanes. Therefore, mainly molecules such as cyclohexanol, 2-cyclohexen-1-ol or hexane-1,6-diol containing a functional group were used as model compounds for the development of biocatalytic one-pot synthesis of ε-caprolactone (Bornadel et al., 2016; Mallin et al., 2013; Oberleitner et al., 2014; Scherkus et al., 2016; Scherkus et al., 2017; Staudt et al., 2013). In this context, lactone synthesis has been predominantly studied starting from the substrate cyclohexanol while using isolated alcohol dehydrogenase from *L. kefir* and cyclohexanone monooxygenase from *Acinetobacter* sp. (Mallin et al., 2013b; Scherkus et al., 2016; Scherkus et al., 2017a; Schrewe et al., 2013; Staudt et al., 2013). The substrate and product inhibition constitute a major limitation for the development of such *in-vitro* cascade. Such shortcomings have been overcome by reaction engineering. Integrating a fed-batch concept circumvented substrate inhibition (Scherkus et al., 2016) whereas implementing lipase A from *Candida antarctica* into the process relieved product inhibition by enabling *in situ* oligomerization of the lactone (Schmidt et al., 2015).

Reports on three-step cascade starting directly from cycloalkanes are scarce and only limited to in-vitro cascade (Pennec et al., 2015). Reaction performance is in general much worse than for the two-step reaction as demonstrated by Pennec et al., 2014, who reported a ε-caprolactone

cyclohexane conversion 2.5% and total turnover number (TTN) of 822 (Table 2). Typically, such *in-vitro* cascades are assembled by combining enzymes from different organisms which might lack optimal concerted kinetics necessary to achieve a high turnover. The imbalance in the enzyme kinetics very often results in accumulation of intermediate products as shown for the synthesis of lactone from 2-cyclohexenol (Oberleitner et al., 2013). Although intermediate compounds were successfully converted to the main product after 8 h of reaction time (Oberleitner et al., 2013), such *in-vivo* cascades possess additional constraints to reactor operations, due to reaction time necessary for complete conversion, and possess economic challenge for the production of high volume low price compounds.

In this context, Nature based solutions for hydrocarbon mineralization may provide an elegant concept to assemble efficient enzyme cascades for lactone synthesis from cyclohexane. *Acidovorax* sp. CHX100 is a bacterial strain able to grow with cyclohexane as a sole source of carbon and energy (Salamanca and Engesser, 2014). High growth rates of 0.19 h^{-1}, 0.075 h^{-1}, and 0.2 h^{-1} on cyclohexane, cyclohexanol, and cyclohexanone respectively, suggest naturally efficient enzymes with high catalytic turnover for cyclohexane degradation to ε-caprolactone (Salamanca and Engesser, 2014). By assembling enzymes involved in hydrocarbon degradation from *Acidovorax* sp. CHX100 high resting cell activities of 22 U g_{CDW}^{-1} have been achieved by the concerted action of all three enzymes CHX, CDH, and CHXON, and without accumulation of intermediate products in *P. taiwanensis* VLB120 (Figure 3). Thus, Nature's solutions demonstrated to be ideal for biocatalysis and enabled high conversion rates of cycloalkanes without accumulation of intermediate products.

Key challenges in designing an in-vivo *cascade for lactone synthesis from cyclohexane*: Hydrolysis of ε-caprolactone to 6-hydroxyhexanoic acid by host intrinsic hydrolase(s) reduced ε-

caprolactone selectivity to 65% (Figure 4A). This hydrolase enzyme seems to be very specific for ε-caprolactone as only low conversion rates were observed for 2-oxonanone (2.6%) and oxacycloundecan-2-one (0.4%). To avoid ε-caprolactone hydrolysis and to achieve 100% selectivity, common strategies such as knock-out of the hydrolase or *in-situ* product removal based on a second organic or solid phase should be investigated (Hilker et al., 2004; Hilker et al., 2005). Alternatively, one could benefit from this activity and produce other value added compounds from ε-caprolactone. An interesting *in-vitro* approach has been reported for the transformation of cyclohexanol to ε-caprolactone and subsequently, by the addition of lipase (CalA) to the aqueous phase, to poly-caprolactone (Schmidt et al., 2015). The here presented synthetic pathway could be extended in a similar fashion for transforming ε-caprolactone to ε-aminohexanoic acid (Sattler et al., 2014).

Cyclohexane toxicity is another key bottleneck to solve for an efficient *in-vivo* reaction cascade synthesizing ε-caprolactone. A rapid decrease in volumetric activity for cyclohexane conversion was observed at 1.7 and 3.4 g_{CDW} L^{-1} compared to 6.8 g_{CDW} L^{-1} (Figure 5), indicating severe cell toxification by cyclohexane. An elegant approach to overcome cycloalkane toxicity would be the up-regulation of RND-type efflux pumps in *P. taiwanensis* VLB120. This enhances solvent tolerance of the host and thereby provides a stable reaction environment for the respective recombinant enzymes (Volmer et al., 2014). Another approach to overcome cyclohexane toxicity is a fine balance between the cyclohexane conversion rate to the mass transfer rate by using a segmented flow biofilm membrane reactor (Karande et al., 2016). Overall, we consider that an integrated and concerted approach combining novel biological and reactor concepts is necessary for the successful development of an *in-vivo* caprolactone process from cyclohexane.

Conclusion

This study reports the design of a heterologous pathway involving cytochrome P450 monooxygenase, cyclohexanol dehydrogenase, and cyclohexanone monooxygenase in *P. taiwanensis* VLB120 for the *in-vivo* production of lactones from cycloalkanes. Characterization of the whole-cell biocatalyst revealed an apparent maximum reaction rate of 22 U g_{CDW}^{-1} for caprolactone synthesis directly from cyclohexane. The performance of the heterologous pathway was evaluated in shake flasks ensuring a constant cyclohexane supply via the air phase. Thereby a maximum product concentration of 2.3 g L^{-1} with 36720 TTN was achieved over 5h applying 6.8 g_{CDW} L^{-1} of the whole-cell biocatalyst. This *in-vivo* cascade utilizing naturally evolved synchronized consecutive enzymes is a powerful strategy for producing interesting monomers from cycloalkanes. This broadens the toolbox to overcome contemporary issues connected with cyclohexane oxidation chemistry for the biotechnological production of ε-caprolactone with the potential to reach metrics amenable to technical scale operation.

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Figure List:

Figure 1. Comparison between chemical and biocatalytic route(s) for ε-caprolactone synthesis from cyclohexane (Berezin et al., 1966; Weissermel and Arpe, 2003). **(A)** Three-step chemical process and **(B)** In-vivo biocatalytic process to synthesize ε-caprolactone from cyclohexane. The respective enzyme cascade is located in *P taiwanensis* VLB120 pCom10_capro (magnified figure). The cascade comprises cytochrome P450 monooxygenase (CHX), cyclohexanol dehydrogenase (CDH), and cyclohexanone monooxygenase (CHXON). The cofactor NAD^+ is regenerated either via CDH or via central carbon metabolism of the host.

Figure 2. A) SDS PAGE analysis of *P. taiwanensis* VLB120 harboring pCom10_capro at 0 to 6h after induction. CHXON: cyclohexanone monooxygenase (58.8 kDa), CHX: cytochrome P450 (47.4 kDa), and CDH: cyclohexanol dehydrogenase (26.5 kDa) are indicated by arrows. **B)** CO difference spectra to verify functional incorporation of the heme group into P450 monooxygenase CHX of *P. taiwanensis* VLB120 pCom10_capro 2h, 3h, 4h and 5h after induction. The peak at 450 nm indicates active CypP450 whereas a peak at 420nm is associated with inactive CypP450 (Guengerich et al., 2009). Organisms were cultivated in M9* medium containing 0.5% citrate; induction at OD450 0.9 to 1 with DCPK

Figure 3. Whole cell kinetics of *P. taiwanensis* VLB120 pCom10_capro for different substrates. **A)** Cyclohexane conversion to ε-caprolactone (three-step reaction). Substrate concentrations are given in the head space due to the high vapor pressure of cyclohexane. **B)** Cyclohexanol conversion to ε-caprolactone (two-step reaction). **C)** Cyclohexanone conversion to ε-caprolactone (single-step reaction). Whole-cell activities were deduced from resting cell assays performed for 10 minutes.

Figure 4. Time course of lactone and ω-hydroxyacid formation with resting *P. taiwanensis* VLB120 pCom10_capro cells for different starting compounds. **A)** Cyclohexane conversion to ε-caprolactone and 6-hydroxyhexanoic acid. **B)** Cyclooctane conversion to 2-oxonanone and 8-hydroxyoctanoic acid. **C)** Cyclodecane conversion to oxacycloundecan-2-one. 10-hydroxydecanoic acid was not detected

Figure 5. Time course of ε-caprolactone (capro) and 6-hydroxyacid (6HXA) formation with resting *P. taiwanensis* VLB120 pCom10_capro cells at different biomass concentrations
Abbrevations: **H** = high cell densities of 6.8 g_{CDW} L^{-1}, **M** = medium cell densities of 3.4 g_{CDW} L^{-1}, **L** = low cell densities of 1.7 g_{CDW} L^{-1}, **act** = specific activity

Table Caption:

Table 1. Kinetic data from *P. taiwanensis* VLB120 pCom10_capro determined via resting cells assays for the transformation of cyclohexane, cyclohexanol, and cyclohexanone to caprolactone.

Table 2. Comparison between *in-vivo* and *in-vitro* multi-enzyme cascades for the transformation of cyclohexane to ε-caprolactone

Table 1: Kinetic data from *P. taiwanensis* VLB120 pCom10_capro determined via resting cells assays for the transformation of cyclohexane, cyclohexanol, and cyclohexanone to caprolactone.

Substrate	V_{max} U/g_{CDW}	Apparent substrate uptake constant (mM)	Apparent substrate inhibition constant (mM)
Cyclohexane to caprolactone[a]	22	0.082[c] (0.013)[d]	NA
Cyclohexanol to caprolactone[b]	80-100	0.2	0.4
Cyclohexanone to caprolactone[a]	170	0.23	NA

Data fitting: a) Michaelis-Menten kinetics v=Vmax [S]/(K_S+[S]), K_S is the substrate concentration at which the reaction velocity is running at the half of its maximal reaction velocity. Michaelis-Menten kinetics was determined using the Hanes plot for cyclohexane and cyclohexanone. For details refer to the supporting information. b) The kinetic data were extracted from experimental measurements. c) Due to the high volatility of cyclohexane, the substrate uptake constant $K_{S(air)}$was measured based on the cyclohexane concentration in the head space (Figure S3A, supporting information). The high evaporation of cyclohexane was also experimentally confirmed by measuring head-space cyclohexane concentration (Figure S2, supporting information). d) The substrate uptake constant $K_{S(aqueous)}$ was estimated based on the partition coefficient of cyclohexane in the air to aqueous phase ($K_{air/water}$= 6.18 mM mM^{-1}).

Table 2: Comparison between *in-vivo* and *in-vitro* multi-enzyme cascades for the transformation of cyclohexane to ε-caprolactone

Catalyst	ε-caprolactone ($g\ L^{-1}$)	6-hydroxyacid ($g\ L^{-1}$)	Vol. productivity ($g\ L^{-1}\ h^{-1}$)	TTN	References
Cell (6.8 $g_{CDW}\ L^{-1}$)	1.94	0.41	0.46	36720	This study
Cell (3.4 $g_{CDW}\ L^{-1}$)	1.22	0.23	0.28	45585	This study
Cell (1.7 $g_{CDW}\ L^{-1}$)	0.54	0.09	0.13	40265	This study
Enzymes	0.59	ND	0.05	822	Pennec et al., 2014

Experimental data obtained in Figure 5 are used to calculate TTN in whole-cell based catalysis. TTN: mol product/mol catalyst. TTN calculations are based on a CHX concentration of 80 nmol g_{CDW}^{-1}.

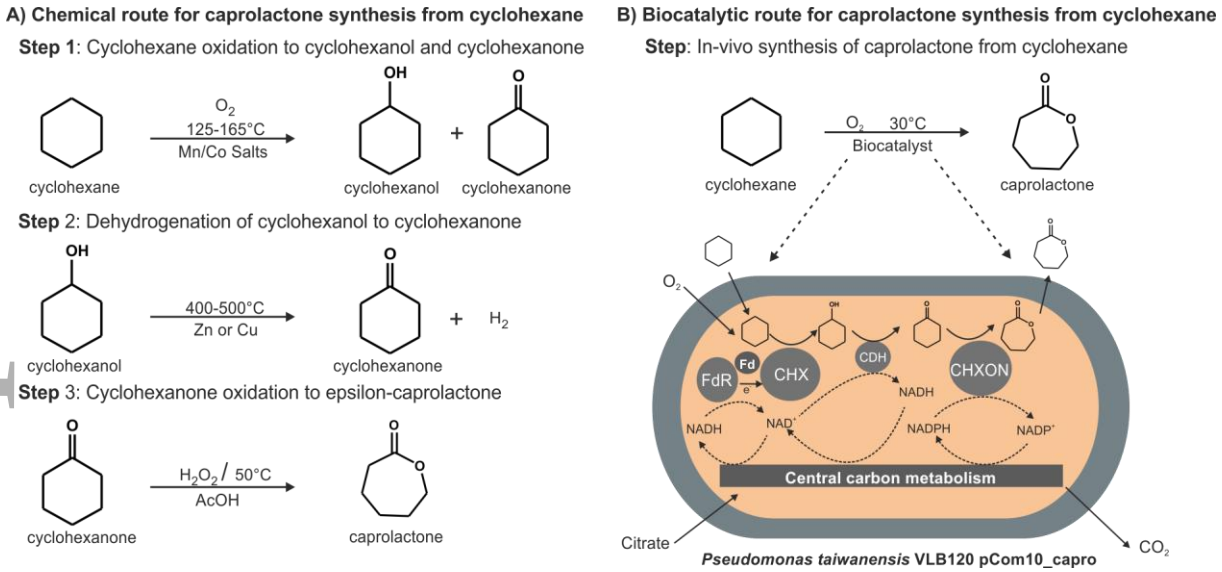

Figure 1

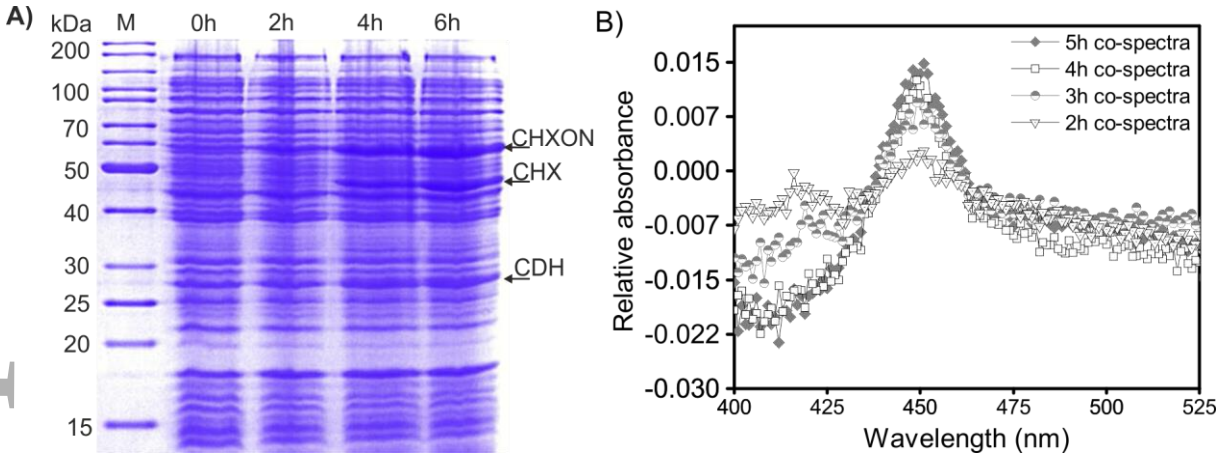

Figure 2

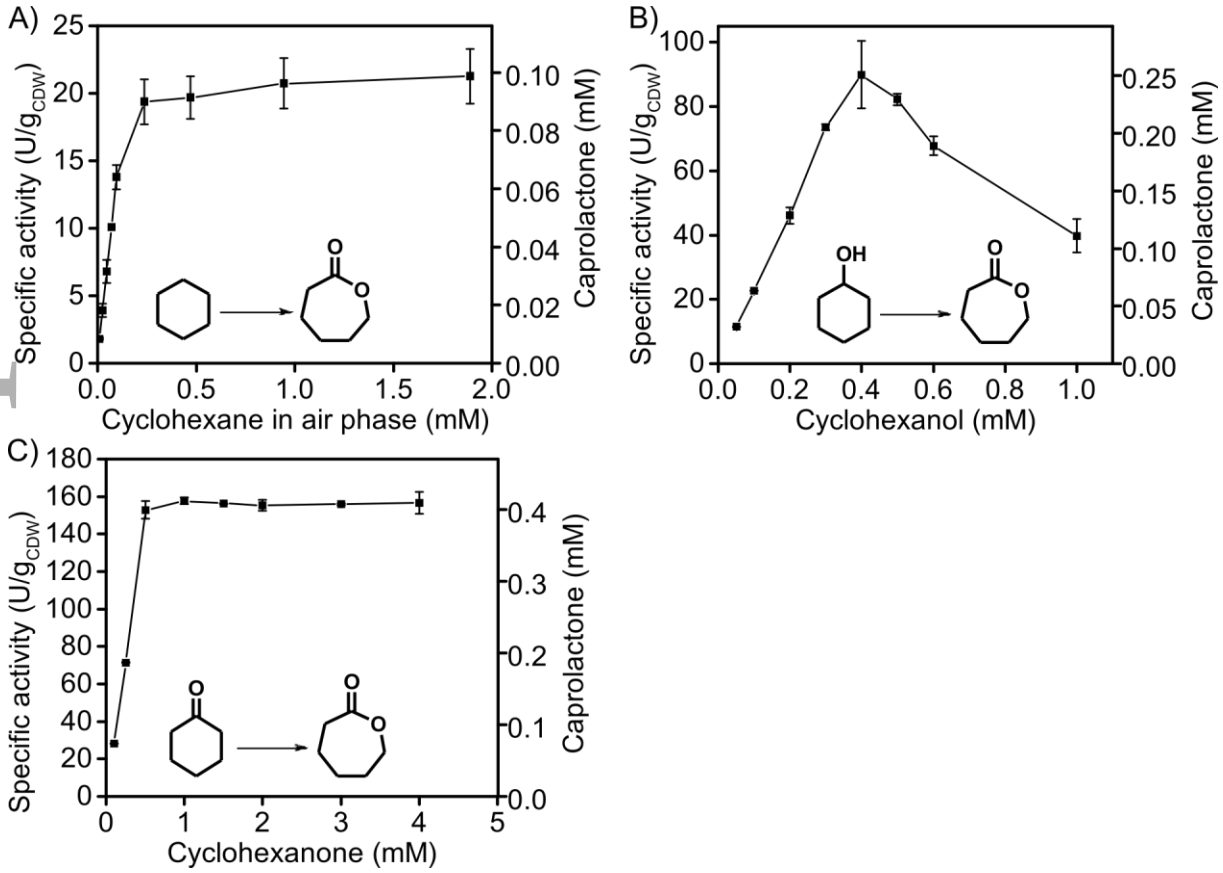

Figure 3

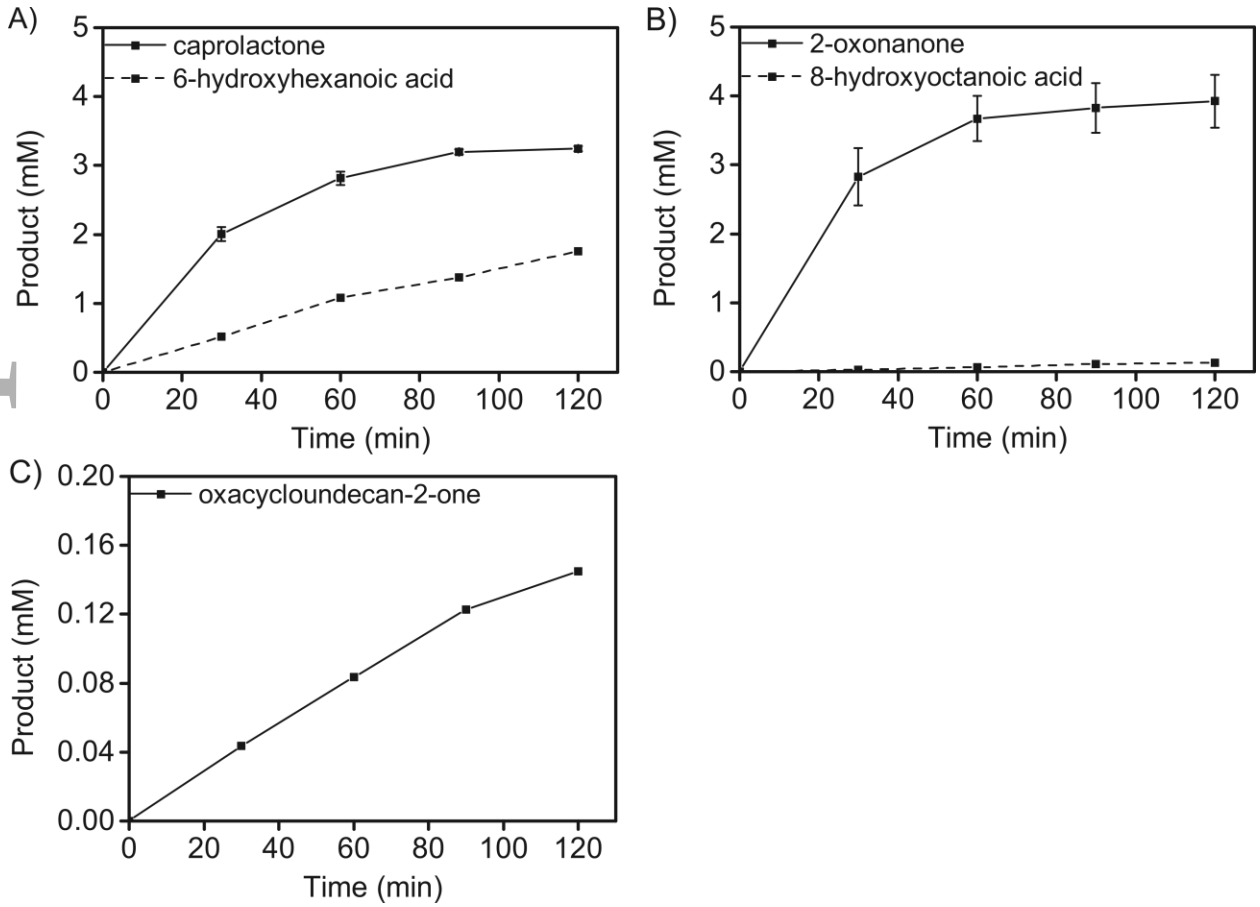

Figure 4

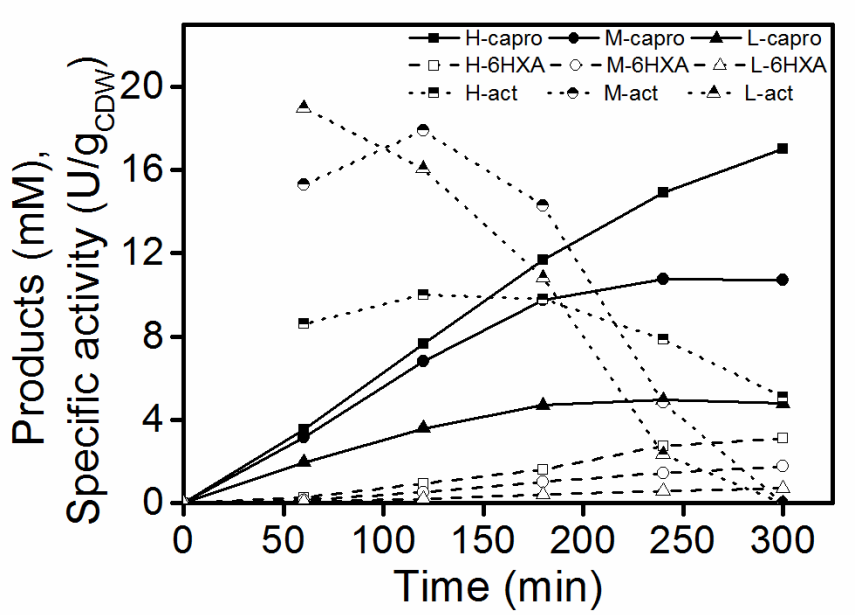

Figure 5