Rational Engineering of a Multi-Step Biocatalytic Cascade for the Conversion of Cyclohexane to Polycaprolactone Monomers in Pseudomonas taiwanensis

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1. Introduction

In synthetic applications, cascade reactions allow for streamlined product formation via multiple reaction steps with the advantage to avoid the isolation of intermediates, thus saving resources, reagents, and time.[1] Multi-step biocatalysis employing whole cells emerged as a powerful tool for the synthesis of value-added compounds.[2–4,5] Precise and delicate fine-tuning of gene expression is required to balance individual enzyme amounts and activities for the construction of “designer cells.”[1,6,7,8] Especially “artificial cascades” employing heterologous genes of diverse origin constitute a major challenge as they introduce novel enzymatic functions into the host.[4,9] On the one hand, it is crucial to provide sufficient enzyme amounts to sustain reasonable rates. On the other hand, too much overexpression, especially of more than one gene, can severely hamper host metabolism and interfere with its stability.[10,11] Moreover, optimizations regarding the choice of the host strain, substrate uptake, and pathway flux can systematically improve in vivo cascade.[11–13,14] A holistic approach comprising catalyst and reaction engineering allows controlling the product formation patterns.[15,16]

Nowadays, plastics are ubiquitous in human life and cause severe litter problems. Thus, biodegradable polymers such as polycaprolactone (PCL), polylactic acid, and polyhydroxyalkanoate have gained importance.[15] PCL can either be synthesized by the ring-opening polymerization of ε-caprolactone (ε-CL) or by the polycondensation of 6-hydroxyhexanoic acid (6-HA).[16] Industrially, ε-CL is produced from cyclohexane through the Union Carbide Corporation (UCC) process, which suffers from serious environmental issues, a low cyclohexane conversion of 10–12%, and only moderate selectivity of 85–90%.[19,20] General advantages of biocatalysis, such as high selectivities and operation at moderate temperature and ambient pressure, have a big potential to design a more eco-efficient process. Recently, two biocatalytic approaches to synthesize ε-CL from cyclohexane have been published. Pennec et al. demonstrated a one-pot reaction applying purified enzymes,[21] which, however, suffered from a low conversion of 3%. Recently, Au-doped TiO₂ and graphitic carbonitride photocatalysts catalyzing the oxidation of cyclohexane to cyclohexanone were combined with E. coli cells expressing a Baeyer–Villiger monooxygenase in a proof-of-concept study producing 0.4 mM ε-CL from cyclohexane, but with a low conversion (200 mM cyclohexane employed).[22] Karande et al. generated a whole-cell biocatalyst showing superior total turnover numbers (TTN),[23] depending, however, on the expensive growth substrate citrate and the volatile inducer dicyclopentanone. Thereby, they established a 3-step cascade in P. taiwanensis VLB120 by introducing cytochrome P450 monoxygenase (Cyp), cyclohexanol dehydrogenase (CDH), and Baeyer–Villiger cyclohexanone monoxygenase (CHMO) genes from

The current industrial production of polymer building blocks such as ε-caprolactone (ε-CL) and 6-hydroxyhexanoic acid (6HA) is a multi-step process associated with critical environmental issues such as the generation of toxic waste and high energy consumption. Consequently, there is a demand for more eco-efficient and sustainable production routes. This study deals with the generation of a platform organism that converts cyclohexane to such polymer building blocks without the formation of byproducts and under environmentally benign conditions. Based on kinetic and thermodynamic analyses of the individual enzymatic steps, a 4-step enzymatic cascade in Pseudomonas taiwanensis VLB120 is rationally engineered via stepwise biocatalyst improvement on the genetic level. It is found that the intermediate product cyclohexanol severely inhibits the cascade which could be optimized by enhancing the expression level of downstream enzymes. The integration of a lactonase enables exclusive 6HA formation without side products. The resulting biocatalyst shows a high activity of 44.8 ± 0.2 U gCDW⁻¹ and fully converts 5 mM cyclohexane to 6HA within 3 h. This platform organism can now serve as a basis for the development of greener production processes for polycaprolactone and related polymers.
Acidovorax sp. CHX100 (Figure 1). Respective cascade development mainly focused on the monomer ε-CL and gave rise to a maximal activity of 22 $\mu$g CDW$^{-1}$, with the first Cyp-catalyzed step being rate-limiting. The successive enzymes, CDH and CHMO exhibited much higher activities of 80 and 170 $\mu$g CDW$^{-1}$, respectively. In a separate study, the activity of cells containing only the Cyp could be more than doubled by expression system respectively. In a separate study, the activity of cells containing only the Cyp could be more than doubled by expression system engineering.\[24\]

In this study, we set out to amend the latter system with CDH and CHMO genes and thereby achieve high respective activities, utilize glucose instead of citrate as carbon and energy source, prevent the accumulation of intermediates, and keep the expression related metabolic burden reasonably low to allow for stable catalysis. Explicitly, oxygenases such as Cyps or Baeyer-Villiger monooxygenases are prone to form reactive oxygen species via uncoupling reactions, which may hamper the catalytic prowess of the cells.\[23,31,13\] Another point to be considered is that, due to the accumulation of the hydrolysis product 6HA, the approach of Karande et al.\[23\] suffered from restricted cascade selectivity. We aimed to tackle all these points by rational pathway engineering including the characterization of the involved enzymes as the basis for the rational assembly of the expression system. It is thereby crucial to balance enzyme activities without dissipating the cell’s resources.

2. Results

The construction of an efficient biocatalytic in vivo cascade necessitates a balanced expression of the cascade genes to avoid side product accumulation. Besides the well-characterized initiating Cyp,\[24,25\] CDH, and CHMO have been employed for PCL monomer synthesis from cyclohexane,\[23\] but nothing is known about respective reaction kinetics and possible inhibitions by pathway intermediates as they have been observed before, for example, for Baeyer-Villiger monooxygenases.\[26,27\] Consequently, CDH and CHMO were characterized as the first step in this study to support the rational engineering of a productive 3-step cascade based on the optimized Cyp-containing whole-cell biocatalyst.\[24\]

2.1. Characterization of CDH and CHMO

CDH and CHMO were cloned separately into the pSEVA244_T vector\[24\] to characterize their in vivo activity. As CDH catalyzes an equilibrium reaction, the kinetic parameters were assayed for both reaction directions (Table 1). For the reverse reaction with cyclohexanone as substrate, CDH showed a 10 times lower $v_{\text{max}}$ compared to the forward reaction. On the other hand, the $K_v$ values, corresponding to the apparent substrate uptake constant (concentration at which whole cells show half-maximal transformation rates) as it is used in the Monod-kinetics for microbial growth,\[28\] differed by a factor of almost 100 in favor of the reverse reaction (0.05 for cyclohexanone, 3.57 mM for cyclohexanol). The concentration of cyclohexanol and cyclohexanone in the system determine if $v_{\text{max}}$ or $K_v$ prevail. Furthermore, we theoretically and experimentally assessed the cyclohexanol/cyclohexanone concentration ratio at equilibrium. Utilizing the group contribution method\[29\] assuming a physiological intracellular NADH to NAD concentration ratio of 10.6 under aerobic conditions,\[30\] this ratio was determined to be 1.9 (Section S4, Supporting Information). It was confirmed experimentally by applying different initial alcohol and ketone concentrations giving a cyclohexanol/cyclohexanone concentration ratio of 1.95 ± 0.29 after 16 h (Figure S2, Supporting Information). This thermodynamic preference of the backward reaction, together with the low $K_v$ value for cyclohexanone emphasizes the necessity of an efficient cyclohexanone withdrawal by the successive enzyme in the cascade, that is, CHMO.

Substantial research has been conducted with a cyclohexanone monoxygenase originating from Acinetobacter sp.\[31\] Generally, the substrate as well as product toxicity, are features of Baeyer-Villiger monooxygenase-catalyzed reactions.\[26,27\] Substrate toxicity was generally observed at aqueous concentrations in the mm-range, which should thus be avoided during the cascade reaction. Furthermore, CHMO may be inhibited by the cascade intermediate cyclohexanol and its product ε-CL. Acidovorax CHMO indeed was found to be highly prone to inhibition by cyclohexanol (Figure 2A). At a cyclohexanol concentration as low as 0.4 mM, the high initial CHMO activity of 160.3 ± 0.1 $\mu$g CDW$^{-1}$ was found to be reduced to half this rate. Cyclohexanol concentrations ≥ 1.7 mM completely abolished CHMO activity. However, up to an ε-CL concentration of 17 mM, no product inhibition was found for CHMO (Figure 2B).

Table 1. Kinetic parameters of P. taiwanensis VLB120 (pSEVA_CDH).

| Substrate | $v_{\text{max}}$ [Ug CDW$^{-1}$] | $K_v$ [mM] | $k_{\text{cat}}$ [s$^{-1}$] |
|-----------|---------------------------------|------------|-----------------------------|
| Cyclohexanol | 296.6 ± 15.7 | 3.57 ± 0.26 | 2.74 ± 0.14 |
| Cyclohexanone | 29.5 ± 1.3 | 0.05 ± 0.01 | 0.27 ± 0.01 |

$^4$Resting cell bioconversions were performed in 2 mL microcentrifuge tubes with a liquid volume of 1 mL and a biomass concentration of 0.15 g CDW L$^{-1}$ for 3 min; $^5$Apparent substrate constant (concentration at which whole cells show half-maximal transformation rates); $^28$Based on an estimated 5 g CDH in 100 g total cell dry weight (CDW).

Figure 1. Biocatalytic cascade for the synthesis of polycaprolactone (PCL) monomers. The cascade is composed of a cytochrome P450 monooxygenase (Cyp), a cyclohexan dehydrogenase (CDH), and a cyclohexanone monooxygenase (CHMO) for the production of ε-caprolactone (ε-CL).\[23\] Optionally, a lactonase (Lact) catalyzes the ring-opening reaction to yield 6-hydroxyhexanoic acid (6HA).
Summing up, these results emphasize that the produced cyclohexanol needs to be directly converted by CDH to avoid CHMO inhibition. High intracellular CDH and CHMO activities are important to avoid any accumulation of alcohol and ketone intermediates, as already low alcohol amounts can be expected to inherently reinforce such accumulation.

2.2. Assembling Caprolactone-Producing Strains

To assess CDH and CHMO gene expression to different levels, we generated two ε-CL producers based on the platform organism for Cyp gene expression developed recently (24). First, CDH and CHMO genes were placed downstream of the Cyp genes on the same operon in P. taiwanensis VLB120 pSEVA_CL_1 (Figure 3A). Consequently, one mRNA is produced, harboring all 5 genes sequentially. To enhance CDH and CHMO levels, a second strain harboring pSEVA_CL_2 was created. pSEVA_CL_2 contains a second pSEVA_CHMO. The direct comparison of both strains carrying either pSEVA_CL_1 or pSEVA_CL_2 via SDS-PAGE showed that Cyp content decreased by 30%. Interestingly, such decreases in the cells (Table S4, Supporting Information). Concomitantly, the active CHMO content decreased by 30%. Interestingly, such decreases...
Figure 3. Construction and characterization of *P. taiwanensis* VLB120 A–C,F) pSEVA_CL_1 and A,D–F) pSEVA_CL_2. A) Graphical representation of expression units in the plasmids pSEVA_CL_1 and pSEVA_CL_2, respectively, showing bands for Cyp (47.4 kDa), CDH (26.5 kDa), and CHMO (58.8 kDa) after different times of induction and compared with cells containing an empty vector. C,E) Time courses for the production of cyclohexanol, ε-CL, and 6HA and for whole-cell activities for the total product (sum of ε-CL and 6HA) formation in resting cell bioconversions. Cells were cultivated as described in the legend of Figure 2 and induced for 4 h. Resting cell bioconversions were performed with a biomass concentration of 0.5 g CDW L$^{-1}$ in 10 mL KPi-g buffer and started by adding 10 µL of pure cyclohexane (180 µM dissolved in the aqueous phase, 9.2 mM in total concerning the aqueous phase volume). F) Resting cell bioconversions to study cascade inhibition by cyclohexanol. Varying cyclohexanol concentrations were applied in 1 mL KPi-g buffer with a cell concentration of 0.25 g CDW L$^{-1}$. The graphs depict cyclohexanone and ε-CL concentrations as well as the whole-cell activity (ε-CL formation) for an assay time of 10 min. Graphs represent average values and standard deviations of two independent biological replicates. The average experimental errors over all measurements for the concentrations of cyclohexanol, cyclohexanone, ε-CL, and 6HA are 6.9%, 4.7%, 6.7%, and 35.2%, respectively, and 7.2% for the whole-cell activities.
with pSEVA_CL_1. Due to CHMO inhibition and CDH kinetics, cyclohexanol was found to potentially disrupt the cascade in a self-enforcing manner. However, the high CDH and CHMO expression levels in P. taiwanensis VLB 120 (pSEVA_CL_2) efficiently prevented cyclohexanol accumulation. Furthermore, the two-operon approach involved a lower metabolic burden, auguring for stable biocatalytic activities.

### 2.3. Construction and Characterization of a 6HA Producing Strain

Whereas P. taiwanensis VLB 120 (pSEVA_CL_2) showed promising properties regarding cascade activity and stability, the presence of host-intrinsic hydrolases still led to a product mix consisting of e-CL and 6HA (Figure 3C,E). An industrial production process always relies on an efficient DSP, which in turn demands the avoidance of excessive byproduct accumulation. One possibility to prevent e-CL hydrolysis is the knockout of the respective hydrolase(s) in the host strain P. taiwanensis VLB120. However, its genome encodes over 100 enzymes with hydrolytic activity. Consequently, identification and inactivation of the responsible enzyme(s) would be very challenging, especially as several enzymes may be involved in this reaction, possibly even in a cooperative manner. The more promising alternative is to focus on 6HA as the only reaction product, which can also serve as a monomer to produce PCL.\(^{[18]}\) Furthermore, 6HA is significantly less toxic to the cells as compared to e-CL (Figure 4D).

Whereas concentrations of up to 20 mM 6HA did barely affect the growth, 20 mM e-CL reduced the growth rate by \(\approx 50\%\). For 6HA, a half-maximal growth rate was observed at \(\approx 100\mathrm{mm}\), which in turn led to complete growth inhibition in the case of e-CL.

To push the reaction towards 6HA, an additional lactonase was included in the pSEVA_CL_2 construct, originating from the cyclohexane degradation pathway of Acidovorax sp. CHX100 (see Section S5, Supporting Information, for the nucleotide sequence), resulting in pSEVA_6HA_2 (Figure 4A). This construct indeed enabled the exclusive production of 6HA to a concentration of 1.74 ± 0.17 mm after 2 h of reaction (Figure 4C). The high initial specific activity of 52.5 ± 5.0 \(\mathrm{U}\) \(\mathrm{g}\) \(\text{CDW}^{-1}\) (in the first 5 min) dropped by 50% within 30 min and then remained stable. Lactonase gene expression led to a detectable lactonase band and was found to enable a high e-CL hydrolisys activity of 836.6 ± 16.5 \(\mathrm{U}\) \(\mathrm{g}\) \(\text{CDW}^{-1}\), but did not influence Cyp, CDH, or CHMO levels and activities nor the active Cyp concentration (Figure 4B, Table 2 and Table S4, Supporting Information). The growth rate during expression (0.37 ± 0.01 h\(^{-1}\)) also remained comparable to that of the empty vector control (Table S4, Supporting Information). A construct pSEVA_6HA_1 with all genes under the control of only one promoter also was established. It again led to less favorable properties such as slower growth, (transient) cyclohexanol accumulation, and lower initial activities (Table 2 and Table S4, Figure S3, Supporting Information), confirming the superiority of the two-promoter approach.

Finally, the two strains containing two-promoter constructs for the 3- or 4-step pathway were tested for the conversion of 5 mm cyclohexane on a 40 mL scale. Both strains enabled complete conversion within 3 h with the 4-step pathway being superior...
regarding selectivity (100% for 6HA) than the 3-step pathway (80% towards \( \varepsilon \)-CL) (Figure 4E). The initial specific activities of \( P. \) taiwanensis VLB120 harboring pSEVA_CL_2 or pSEVA_6HA_2 were high and in the same range (68.4 ± 6.5 or 61.5 ± 3.2 U g\(_{\text{CDW}}\)\(^{-1}\), respectively). The activities showed a decrease over time, most probably due to the decreasing substrate availability, giving overall activities of 30.8 ± 5.8 and 33.2 ± 0.7 U g\(_{\text{CDW}}\)\(^{-1}\), respectively. Consequently, complete conversion of cyclohexane to 6HA via the in vivo 4-step cascade was found to be feasible and efficient without serious impediments by enzyme kinetics or biocatalyst instability.

Overall, \( P. \) taiwanensis VLB120 (pSEVA_6HA_2) can be considered a highly promising production strain for the conversion of cyclohexane to the PCL monomer 6HA.
3. Discussion

The development of eco-efficient sustainable production processes has been one of the major objectives of biotechnology research over the last decade. Such promise is based on the inherent biodegradability, selectivity, and specificity of biocatalysts. Biotechnological solutions already have replaced chemical processes for the production of biosurfactants, amino acids, and even complex heterocyclic compounds. The research presented in this study aimed to set a basis for the replacement of the highly polluting UCC process by developing a direct route from cyclohexane to the PCL monomer 6HA enabling full conversion.

3.1. Efficient Design of In Vivo Cascades

Various factors including functional expression and stability of the cascade enzymes, toxicity of the reactants and products, equilibrium thermodynamics, cofactor regeneration, and byproduct formation should be considered for the design and construction of efficient whole-cell biotransformation pathways. The final production strain developed in this study for the conversion of cyclohexane to 6HA demonstrated decent activity in the 50–60 U g⁻¹ cell dry weight range for the whole cascade without side product formation. It has been shown that detailed analyses of enzyme kinetics and respective reaction engineering for a 3-step cascade could efficiently enhance the conversion of several unsaturated cyclic alcohols to the corresponding lactones in vitro. Similarly, the CDH investigated in our study, the Kᵥ value of the alcohol dehydrogenase was significantly lower for the reverse reaction, and CHMO was severely inhibited by cyclohexanol. Establishing a kinetic model enabled the setup of an efficient fed-batch process. Increasing the expression and stabilizing the activity of Baeyer–Villiger monooxygenases also led to higher 9-hydroxynonanoic acid and C11 monomer concentrations in 4- and 5-step catalytic cascades, respectively.

Whereas the balancing of enzyme ratios in vitro is a rather straight-forward approach, in case of whole-cell biocatalysis, this requires fine-tuning of expression levels which, furthermore, should not drive the demand of resources beyond cellular capacities. One possibility is the use of different plasmids to adjust the gene copy number, which also can influence plasmid stability. In our previous study, we varied copy number, RBS, and regulatory systems for Cyp gene expression. The best system in terms of activity, stability, and metabolic burden was used in this study to engineer multi-gene operons. The so-called metabolic burden arises from the change in demand for (biomass) building blocks and energy (ATP, NAD(P)H) related to plasmid maintenance, gene expression, and enzyme activity and is system- and condition-dependent. Our results indicate a gradual decrease in the growth rate with increasing operon size (Table S4, Supporting Information). For the cascade investigated, the two-operon- compared to the one-operon approach not only enabled faster growth indicating low metabolic burden, but also led to higher CDH and CHMO expression levels and cascade activities. The relation between gene organization and gene expression is poorly understood. It has been found for E. coli that gene expression increases with the length of the operon resulting in more co-transcriptional translation. Increased translation can result in metabolic burden and misfolded or otherwise non-functional proteins, which was found for the Cyp in our previous study. Also without a terminator after the Cyp genes (Figure 3A). RNA polymerase dissociation may have been promoted by the transcription initiation machinery occupying the downstream promoter region and thereby opening up the DNA. Thus, mRNAs with shorter average length can be expected for the two-promoter- compared to the one-promoter constructs. Shorter mRNAs, in turn, have been found to show increased stability in E. coli cells and to recruit fewer ribosomes, thus decreasing the metabolic burden. In general, the metabolic burden increases with gene and operon size. It is further enhanced by some antibiotics such as kanamycin and thus tends to be high for plasmid-based expression, especially when antibiotic resistance genes are used as selection markers. The two-operon approach may have profited from shorter but more stable mRNAs and can be considered suitable for efficient expression of the designed pathways in P. taiwanensis VLB120. For further optimization, metabolic modeling of cascades and combinatorial pathway engineering taking into account metabolic burden effects may become interesting, although these approaches still suffer from incomplete knowledge.

3.2. Production of PCL Precursors

The biocatalytic production of PCL or its precursors has been heavily investigated over the last years (Table 3). Approaches based on isolated enzymes as well as whole cells have successfully been established. However, most of these approaches relied on cyclohexanol as a substrate which needs to be synthesized from cyclohexane employing an ecologically critical process. Additionally, inhibition of CHMO by cyclohexanol or substrate inhibition necessitated the development of suitable reaction concepts, for example, two-liquid phase or fed-batch systems. The highest productivity of 1.87 g L⁻¹ h⁻¹ was obtained with isolated enzymes by employing an appropriate feeding strategy for the complete conversion of 283 mm cyclohexanol to 6HA. For further optimization, metabolic modeling of cascades and combinatorial pathway engineering taking into account metabolic burden effects may become interesting, although these approaches still suffer from incomplete knowledge.

Compared to cyclohexanol, cyclohexane is an even more challenging substrate due to its high volatility and toxicity. In comparison to solvent-sensitive E. coli employed to convert cyclohexanol to 6HA, we obtained a tenfold higher productivity of 6HA. P. taiwanensis VLB120 is known to tolerate low-logP solvents and can, therefore, be considered suitable for the biotransformation of the more toxic substrate cyclohexane.
Possible prolongation of the reaction with an appropriate substrate feeding and the application of a high-cell density setup hold big potential to further improve the product titer and the volumetric productivity.

This study, for the first time, demonstrates a whole-cell approach directly converting cyclohexane to the PCL precursor 6HA. The biotransformation to \( \epsilon \)-CL presented by Karande et al.[23] could be optimized by enhancing the conversion, yield on biocatalyst, TTN, and specific activity (Table 3). In comparison with this strain, the two strains developed in this study (Table 3, entries 13–15) showed a 3-times higher specific whole-cell activity allowing for lower cell concentrations to achieve a full conversion of 5 mM cyclohexane and thereby higher yields on biocatalyst and TTN. The chemo-biocatalytic approach presented by Li et al. constitutes a proof-of-concept and suffered from low conversion and very low activities.[22] The use of isolated enzymes to convert cyclohexane to \( \epsilon \)-CL suffered from low conversion and TTN, which can be attributed to mass transfer limitations or inherent instability of P450 monoxygenases.[11,21] The cellular environment allows for more stable catalytic activities with superior productivities. As a result, the main limitation for future process development is considered not to lie necessarily in the catalyst itself, but rather in the reaction engineering with cyclohexane feeding/cyclohexanemasstransfer and cell toxification as critical points. This may be solved by cyclohexane feeding potentially via the gas phase. The achieved increase in whole-cell activity and conversion, however, can be considered a huge step forward towards the establishment of an economically viable process.[58]

### Table 3. Comparison of biocatalytic PCL precursor synthesis approaches.

| Substrate | Product | Biocatalyst | Time [h] | Cell concentration [gCDW L\(^{-1}\)] | Conversion [%] | Productivity [g L\(^{-1}\) h\(^{-1}\)] | Maximal concentration [mM] | Yield [g Product g\(^{-1}\) Biocatalyst] | Total turnover number [mol Product mol\(^{-1}\) Biocatalyst] | Specific activity [U mg protein\(^{-1}\)] or [U g CDW\(^{-1}\)] | Reference |
|-----------|---------|-------------|----------|--------------------------------------|---------------|--------------------------------------|-----------------------------|---------------------------------|---------------------------------------------|----------------------------------|-----------------|
| Cyclohexane | \( \epsilon \)-CL | E | 48 | n.a. | 58.2 (48 h) | 0.19 (24 h) | 53 | 12 | 6000 | 0.05 (24h) | [46] |
|           |         | C | 16 | 10 (16 h) | 0.14 (16 h) | 20 | 0.23 | n.c. | 13.3 (2h) | 2.1 (16h) | [51] |
|           |         | C | 20 | 35 | 99.6 | 1.1 | 185 | 0.6 | 69 167 | 38606 | 7 | [52] |
|           |         | E | 24 | n.a. | 94 | 0.27 | 56.4 | 1.29 | 690 | 0.008 | [48] |
|           |         | C | 8 | 1.5 | 100 | 0.244 | 16 | 1.2 | n.c. | 15 | [37] |
| 6HA       |         | C | 70 | 30 | 84 | 0.8 | 168 | 0.67 | n.c. | 3.3 | [53] |
| PCL       |         | E | 20 | n.a. | >99 | 1.87 | 283 | n.c. | n.c. | n.c. | [49] |
|           |         | E | 48 | n.a. | 99 | n.c. | n.c. | n.c. | n.c. | [50] |
| Cyclohexane | \( \epsilon \)-CL | E | 6 | 1.05 | 100 | 0.24 | 5.5 | 0.68 | 47 900 | 28.7 | [51] |
|           |         | C | 3 | 1.05 | 100 | 0.24 | 5.5 | 0.68 | 47 900 | 28.7 | [51] |

\( E = \) isolated enzymes, \( C = \) whole cells; \( \text{b) Not applicable; c) Not calculable; d) Fusion enzyme of ADH and CHMO; e) Calculated referring to Cyp; f) Calculated referring to ADH; g) Calculated referring to CHMO; h) Calculated referring to CHMO containing E. coli.

4. Conclusion

In this study, we developed the strain *P. taiwanensis* VLB120 (pSEVA\(_{6HA}\_2\)) that expresses 6 genes encoding 4 enzymes able to fully convert 5 mM cyclohexane to the PCL monomer 6HA. Accumulation of intermediates and byproducts was successfully prevented, and a high cascade activity was achieved. Our study demonstrated that a balanced expression of pathway encoding genes guided by enzyme characteristics (kinetics, inhibition) allowed for streamlined production of \( \epsilon \)-CL and, especially, 6HA. The constructed orthogonal pathway/cascade also can serve as a template to be amended by additional enzymes to synthesize nylon monomers such as adipic acid and 6-aminohexanoic acid. This in combination with their solvent tolerance[56,59,60] and versatility regarding reactor setups—including biofilm approaches[61–63]—qualify VLB120 strains harboring pSEVA\(_{CL}\_2\) or pSEVA\(_{6HA}\_2\) as a promising platform organism for greener polymer production routes.
5. Experimental Section

**Bacterial Strains, Plasmids, Media, and Chemicals:** Microbial strains and plasmids used in this work are listed in Table S1, Supporting Information. Cells were grown in lysogeny broth (LB) medium [64] or M9 medium [65] with a pH of 7.2 supplemented with 0.5% (w/v) glucose as sole carbon and energy source. Kanamycin (50 µg mL⁻¹) was applied for selection when necessary. Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) or Carl Roth (Karlsruhe, Germany) in the highest purity available and used without further purification. 6HA was acquired from abcr (Karlsruhe, Germany). Molecular biology methods are explained in detail in Section S1, Supporting Information.

**Strain Construction:** The strains and plasmids used in this study are listed in Table S1, Supporting Information. The plasmids pSEVA244_T and pSEVA_Cyp constituted the basis for the constructs engineered in this study and originate from Schäfer et al. [24] The primers used for such engineering are listed in Table S2, Supporting Information.

For the construction of plasmids pSEVA_CDH and pSEVA_Cyp_CDH, the CDH gene was amplified from pCapro [23] using the primers PLS013 and PLS014. The resulting purified fragment was fused either to Knpl-digested pSEVA244_T or pSEVA_Cyp by Gibson Assembly [66] to yield pSEVA_CDH or pSEVA_Cyp_CDH, respectively.

Plasmids pSEVA_CHMO and pSEVA_CL_1 were assembled accordingly. The CHMO gene was amplified from pCapro with primers PLS015A and PLS016 or PLS015B and PLS016. The forward primer was different for the two constructs due to the adjustment of the 5' region (overhang to the plasmid backbone). The resulting purified fragments were fused via Gibson Assembly either to Xbal-digested pSEVA244_T or pSEVA_Cyp by Gibson Assembly [66] to yield pSEVA_CHMO or pSEVA_CL_1, respectively.

**Acidovorax sp.** CHX100 was cultivated for 4 days in nutrient broth (NB) medium [64] for DNA isolation performed with the peqGOLD Bacterial DNA Mini Kit (Peqlab, Erlangen, Germany). The lactonase gene was amplified with the primers PLS017B and PLS018, and Gibson Assembly of the resulting fragment and HindIII-digested pSEVA_CL_1 gave rise to pSEVA_6HA_1.

The P_w promoter was amplified from pSEVA244 [67] with primers PLS021 and PLS022. It was fused to EcoRI-digested pSEVA_CL_1 or pSEVA_6HA_1 via Gibson Assembly to yield pSEVA_CL_2 and pSEVA_6HA_2.

**Growth of Bacterial Cultures:** Cultivations were carried out at 30 °C and 200 rpm in a Multitron shaker (Infors, Bottmingen, Switzerland). Microorganisms were cultivated in an LB pre-culture for ≈20 h, from which an M9 pre-culture (1% v/v) was inoculated and incubated for another 12–16 h. From this culture, an M9 main culture was inoculated to a starting OD₅₅₀ of 0.2. Heterologous gene expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) when the cultures reached an OD₅₅₀ of ≈0.5. Incubation was continued for another 4–6 h, and cells were harvested for SDS-PAGE analyses, SO42⁻ spectra, analyses, and/or activity or toxicity assays (see below). One OD₅₅₀ unit corresponds to a biomass concentration of 0.186 g CDW (grams of cell dry weight) L⁻¹. CO₂ difference spectra (see Section S2, Supporting Information, for details) were recorded to determine the active Cyp concentrations.

**Toxicity Assay:** P. taiwanensis VLB120 was cultivated as described above but without induction. Different concentrations of c-CL or 6HA were added 2 h after inoculation, and the growth rate was determined from this time point on for at least 7 h.

**Resting Cell Bioconversions:** The cells were cultivated as described above, harvested by centrifugation (10 min, 5000 x g, RT), and resuspended to a specific cell concentration in 100 mM potassium phosphate buffer (pH 7.4) supplemented with 1% (v/v) glucose (KPi-g buffer). The cells were transfected to baffled Erlenmeyer flasks (100 mL) or microcentrifuge tubes (2 mL) with liquid volumes of 10 or 1 mL and biomass concentrations of 0.5 or 0.25 gCDW L⁻¹, respectively, equilibrated at 30 °C for 10 min (flasks in a water bath at 250 rpm; microcentrifuge tubes in a Thermomixer C (Eppendorf, Hamburg, 2000 rpm)), then provided with the substrate (as stated in the Table and Figure legends). Biotransformations were stopped by the addition of 0.5 mL ice-cold diethyl ether containing 0.2 mM n-decane as an internal standard to 1 mL sample. After 2 min extraction by vortexing and short centrifugation, the organic phase was dried over water-free Na₂SO₄ before it was transferred to a GC vial for analysis. The aqueous phase was removed with a syringe from the microcentrifuge tube and stored at −20 °C for HPLC analysis. The activity is given in UgCDW⁻¹, where 1 U corresponds to 1 µmol product formed within 1 min reaction time.

For the conversion of 5 mM cyclohexane (Figure 4E), 250 mL screw-capped and baffled Erlenmeyer flasks were used with a liquid volume of 40 mL and a biomass concentration of 1.05 gCDW L⁻¹. The caps contained two septa, a Teflon septum facing the inner side of the flask, and a silicone septum facing outwards. Pure cyclohexane (21.8 µL) was added to start the reaction and the flasks were tightly closed. For each sampling point, 1.5 mL liquid volume was removed through the septa using a syringe. From this sample, 1 mL was extracted with diethyl ether for GC analysis as described above and 0.5 mL was used for HPLC analysis.

For details on analytical methods refer to Section S3, Supporting Information.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

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