One-pot biocatalytic route from cycloalkanes to α,ω-dicarboxylic acids by designed *Escherichia coli* consortia

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Aliphatic α,ω-dicarboxylic acids (DCAs) are a class of useful chemicals that are currently produced by energy-intensive, multistage chemical oxidations that are hazardous to the environment. Therefore, the development of environmentally friendly, safe, neutral routes to DCAs is important. We report an in vivo artificially designed biocatalytic cascade process for biotransformation of cycloalkanes to DCAs. To reduce protein expression burden and redox constraints caused by multi-enzyme expression in a single microbe, the biocatalytic pathway is divided into three basic *Escherichia coli* cell modules. The modules possess either redox-neutral or redox-regeneration systems and are combined to form *E. coli* consortia for use in biotransformations. The designed consortia of *E. coli* containing the modules efficiently convert cycloalkanes or cycloalkanols to DCAs without addition of exogenous coenzymes. Thus, this developed biocatalytic process provides a promising alternative to the current industrial process for manufacturing DCAs.
lipidic α,ω-dicarboxylic acids (DCAs) are a class of chemicals extensively used in the preparation of perfumes, polymers, adhesives, and macrolide antibiotics. Currently, the majority of industrial DCAs are synthesized by energy-intensive, hazardous multistage oxidations. For example, adipic acid (AA) is the most industrially important DCA, with a global market of about $6.3 billion per year. The current industrial production process for AA relies on a two-step chemical oxidation under harsh conditions using cyclohexane (CH) as starting material (Fig. 1a). This industrial process has low efficiency with formation of succinic acid and glutaric acid as byproducts. In addition, this process generates almost 10% of global anthropogenic nitrous oxide N₂O emissions because it uses a large amount of nitric acid. These emissions cause serious environmental problems such as global warming and ozone depletion. The alternative routes of directed oxidation of CH to AA with hydrogen peroxide and carbonylation of 1,3-butadiene to adipate diester by a designed palladium catalyst produce no nitrous oxide waste and are more environmentally friendly. However, substrate prices and technical challenges (such as catalyst stability, preparation of the catalyst, and heavy metal recovery) limit their implementation.

To address these challenges, environmentally friendly, biobased approaches for DCA production have been recently developed. Biobased routes from renewable feedstocks such as glucose have been attempted. However, these routes require complex engineering of production microbes by metabolic engineering and synthetic biology techniques, and many challenges remain including redox constraints, enzyme optimization, selection of suitable production hosts and metabolic pathways, and potential harm to cell growth. In addition, these methods generate only a limited range of DCA products.

Artificially designed multi-enzyme cascades are a useful tool for accomplishing challenging reactions that cannot be achieved by one-pot chemical catalysis. Among them, the construction of an in vivo multi-enzymatic cascade offers many advantages over in vitro approaches since the costly steps (e.g., enzyme purification, addition of expensive cofactors) can be avoided. For these reasons, the de novo design of in vivo cascade reactions has gained attention with successful examples such as the oxy- and amino-functionalization of alkenes and synthesis of α-functionalized organic acids from glycine and aldehydes.

Nevertheless, protein expression burden and redox balance issues often arise when attempting to express multiple enzymes in a single microbe for the construction of in vivo whole-cell catalysts. To solve these problems, and inspired by the metabolic engineering concept of using microbial consortia through co-culture of engineered organisms to improve production of targeted compounds, we envisioned the design of microbial-consortia-mediated in vivo biocatalytic cascades with these following advantages: (i) protein expression burden and redox constraints can be reduced by distributing the biocatalytic pathway among different cell modules; (ii) each expression system or cell module can be constructed and optimized in parallel, substantially reducing development time; and (iii) the catalyst loading of each cell module can be adjusted, allowing beneficial interactions among cell modules to enhance productivity. In addition, to eliminate redox constraints and cross-contamination, cofactor self-sufficiency-based modularization can be employed to ensure that each cell module is either redox neutral or coupled to redox regeneration. This strategy of modularization is rarely used.

Here, we develop an in vivo, artificially designed biocatalytic cascade for the oxidation of cycloalkanes or cycloalkanols to DCAs using a designed biocatalytic Escherichia coli consortia system modularized for redox self-sufficiency. With the oxidation of CH to AA as a model reaction, each basic cell module with assigned functions are engineered and optimized in parallel, followed by combinatorial optimization to achieve efficient production of AA from CH (Fig. 1b). Finally, the substrates are expanded to cycloalkanes with different carbon numbers to demonstrate the generality of the developed biocatalytic system.

### Results

#### Design and modularization of a biocatalytic cascade

To implement the targeted production of different DCAs from cycloalkanes, we designed an artificial biosynthetic route based on biocatalytic retrosynthesis (Fig. 2). The cascade had six enzymatic reactions and eight enzymes (Fig. 2). The ideal in vivo cascade would be a single-cell biocatalyst harboring all necessary enzymes. Therefore, we first tried to construct E. coli cells expressing all enzymes needed to produce AA from cyclohexanol (CHOL) or CH. We designed one-pot biocatalytic route for synthesis of AA from CH using an Escherichia coli consortium, composed of three E. coli cell modules.

![Fig. 1 Industrial chemical and designed biocatalytic processes for adipic acid (AA) production.](image)

a) Current industrial process for synthesis of AA by multistage chemical oxidation from cyclohexane (CH). b) Designed one-pot biocatalytic route for synthesis of AA from CH using an Escherichia coli consortium, composed of three E. coli cell modules.
Fig. 2 Design and modularization of an artificial biocatalytic cascade. Modularization was developed based on the rule that each module would be either redox neutral or coupled to a redox-regeneration system: Module 1 involves P450-catalyzed hydroxylation of cycloalkanes 1a-d coupled to a glucose dehydrogenase (GDH) involved cofactor NAD(P)H regeneration. Module 2 comprises of a redox neutral system consisting of an alcohol dehydrogenase (ADH1) and a Baeyer–Villiger monooxygenase (BVMO), which catalyzes oxidation of cycloalkanols 2a-d to corresponding lactones 3a-d. Module 3 contains lactonase-catalyzed hydrolysis of lactones to hydroxyl acids 4a-d, followed by consecutive oxidations to DCA 5a-d with an alcohol dehydrogenase (ADH2) and an aldehyde dehydrogenase (ALDH), in which NAD^+ regeneration was achieved in presence of NADH oxidase (NOX).

However, none showed satisfactory productivity with only trace amounts of product AA (2-4 mM) detected (Supplementary Figs. 1b, 2b). We hypothesized that this could be caused by expression burden from attempting to express multiple enzymes in a single E. coli cell, as well as redox imbalances from performing multiple redox-mediated oxidation reactions in the same reaction vessel (cell)28.

To address these issues, the biocatalytic cascade was divided into three modules based on the rule that each would be either redox neutral or coupled to redox regeneration (Fig. 2). Module 1 includes a P450-catalyzed hydroxylation of cycloalkanes 1a-d to corresponding alcohols 2a-d coupled to a glucose dehydrogenase (GDH) involved cofactor NAD(P)H regeneration. Module 2 is a redox neutral system30 that consists of an alcohol dehydrogenase (ADH1) catalyzes the oxidation of the alcohols 2a-d to ketones 3a-d, and a Baeyer–Villiger monooxygenase (BVMO) mediates the conversion of cycloalkanones to lactones 4a-d. Module 3 comprises the lactonase-catalyzed hydrolysis of lactones to hydroxyl acids 5a-d, and an alcohol dehydrogenase (ADH2) and an aldehyde dehydrogenase (ALDH) for consecutive oxidations to DCA products 7a-d with NADH oxidase (NOX)-mediated NAD^+ regeneration. Each module was expressed in E. coli, resulting in three cell module catalysts designated as cell modules 1, 2, and 3. This potentially reduced the protein expression burden and avoided cofactor cross-contamination by confining the cofactor NAD(P)H/NAD(P)^+ inside the cells.

As a first attempt, the constructed cell modules were combined (2 and 3; or 1, 2, and 3) without optimization to form an E. coli consortium (EC) for AA 7b production. Interestingly, AA 7b production increased by 9-fold (to 18 mM) and 6-fold (to 13 mM) when using, respectively, CHOL 2b and CH 1b as substrates (Supplementary Figs. 1b, 2b), supporting our hypothesis. Therefore, the EC system was further investigated systematically for DCA production from either cycloalkanols or cycloalkanes.

Engineering of basic cell module catalysts. The three basic cell module catalysts were constructed and optimized in parallel, then combined to form an EC for use in the conversion of cycloalkanes or cycloalkanols to DCAs. As a proof of concept, we used transformation of CHOL 2b or CH 1b to AA 7b as a model reaction.

Engineering modular cell 3, responsible for conversion of ε-caprolactone (CL) 4b to AA 7b, we used lactonase from Rhodococcus sp. HI-3131 and alcohol dehydrogenase (ADH2) and aldehyde dehydrogenase (ALDH) from Acinetobacter sp. NCIMB987132,33. To provide sufficient NAD^+ cofactor, NADH oxidase (NOX) originating from Lactobacillus brevis DSM 20054 was employed44. As shown in Fig. 3a, module 3 cells were constructed as different plasmid configurations, for a total of eight recombinant E. coli cells. These cells were individually examined and compared as catalysts for the conversion of 50 mM CL 4b to AA 7b (Fig. 3b). All cells produced AA 7b (16–42 mM), with E. coli (M3b_M3E) exhibiting the highest productivity of 42 mM AA 7b (84% yield) in 22 h with little intermediate accumulation (Fig. 3b). Thus, this whole-cell biocatalyst was chosen for subsequent applications. The reaction process of this biotransformation was monitored using 100 mM CL 4b (Fig. 3c). Within 2 h, the substrate was hydrolyzed to 6-hydroxyhexanoic acid (6-HHA) 5b, which was completely oxidized to AA 7b after 6 h. When the substrate concentration was increased to >100 mM, however, the intermediate 6-HHA 5b was not completely converted, even with prolonged reaction time. A possible reason for this was the reduced pH caused by AA in the reaction solution. Therefore, to further improve productivity, the reaction pH was maintained by adjustment and substrate was added in fed-batch mode. As shown in Fig. 3d, a total of 500 mM substrate 4b was added in three portions at predetermined times. Product AA 7b amounted to 433 mM (87% yield) within 26 h with only 5 mM 6-HHA 5b intermediate. From a practical viewpoint, the 1% side product was negligible.
HHA from ε was used to catalyze methyl esterification, a recent report indicates that CL from biobased fructose36. Thus, our strains could also provide a stearothermophilus by the alcohol dehydrogenases tested (prim-ADH from intermediate 6-HHA 5b
of AA 7b. However, the dead-end intermediate 6-HHA 5b was formed, which cannot be oxidized by the alcohol dehydrogenases tested (prim-ADH from Bacillus steaetherophilus). Instead, the esterase 008-SD from Bacillus subtilis was used to catalyze methyl esterification of the precursor ε-caprolactone in the presence of methanol. In contrast, ADH2 from Acinetobacter sp. NCIMB9871 used in our system accepts 6-HHA 5b as a good substrate, preventing the dead-end. In addition, a recent report indicates that CL 4b can be produced from biobased fructose36. Thus, our strains could also provide a route (Supplementary Fig. 4) for biobased renewable production of AA 7b at ca. 66 g L$^{-1}$, comparable to the highest value (68 g L$^{-1}$) reported using engineered E. coli cells with glucose as a carbon source13.

To engineer cell module 2 for converting CHOL 2b to CL 4b, we used an alcohol dehydrogenase (ADH1) from Lactobacillus brevis ATCC 1486937 and a Baeyer-Villiger monooxygenase (BVMO) originating from Acinetobacter sp. NCIMB9871 with two mutations (C376L/M400I)38 conferring higher oxidative stability compared to the wild-type. The two enzymes formed a redox-neutral system in which NADPH was recycled by a hydrogen-borrowing process30. Similarly, six recombinant E. coli host cells were obtained with different combinations of ADH1 and BVMO (Fig. 4a). The resulting E. coli strains were tested for conversion of 50 mM CHOL 2b to CL 4b with cyclohexanone (CHONE) 3b as an intermediate (Fig. 4b). Cells with one-plasmid systems showed much higher productivity than cells with two-plasmid systems. Among one-plasmid systems, E. coli (M2E) had the highest catalytic performance, producing 32 mM CL 4b (64% yield) in 3 h. We found that product concentration was lower than expected due to autohydrolysis of the product CL 4b to 6-HHA 5b in the reaction buffer system. The whole-cell catalyst E. coli (M2E) was chosen for subsequent reactions.

For the construction of cell module 2, we attempted to express both enzymes (BVMO and ADH1) from the same T7 promoter in one plasmid with a ribosome binding site (RBS) in between. A more balanced, strong protein expression was achieved when the ADH1 gene was placed next to the T7 promoter. Using the best cell module 2 as catalyst, as much as 50 mM CHOL 2b was converted to products without addition of exogenous cofactors and cosubstrates, which was much higher than production from
an E. coli cell catalyst (20 mM) expressing both enzymes with two individual T7 promoters in the presence of glucose and acetone as cosubstrates.

The activation of an inert C-H bond of cycloalkanes for formation of the corresponding alcohol is challenging. Thus far, only a few reports have used P450 monooxygenase for the hydroxylation of CH 1b to CHOL 2b. A common issue is low activity. In this study, the self-sufficient cytochrome P450BM3, a long-chain fatty acids monooxygenase from Bacillus megaterium, was considered. It exhibits the highest reported monooxygenase activity among P450 enzymes towards its natural substrates. However, the activity is essentially lost for small molecules such as propane, cyclohexane or benzene. In our earlier study, P450BM3 mutants were generated with high activity towards structurally small substrates (CH 1b and CHONE 3b) by introducing the relatively large hydrophobic phenylalanine to reduce the size of the binding pocket. The two most active mutants, P450BM3 A82F and A82F/A328F were employed in cell module 1. In addition, the previously engineered mutant P450BM3 19A12, which contains 20 mutations and has relatively high activity for CH 1b, was also tested and compared for biotransformation of CH 1b to CHOL 2b. As shown in Fig. 5, three recombinant E. coli cells expressing three different P450BM3 variants were tested for transformation at a CH concentration of 50 mM (Fig. 5b). E. coli (M1C) containing P450BM3 mutant 19A12 showed the highest activity, producing ca. 9.5 mM CHOL 2b within 3 h. This result was much higher than with the other P450s (CYP_CHX and CYP153A6), which catalyzed the same reactions and had a product concentration ranging from 10 μM to 4 mM. Subsequently, a cofactor regeneration system was constructed by introducing GDH based on P450 BM3 mutant 19A12 (Fig. 5a, Entry 4), which further improved the productivity to 11.5 mM CHOL 2b and 2.5 mM CHONE 3b. The resulting E. coli (M1D) as cell module 1 was employed in cascade reactions for AA production. However, cell module 1 was still less efficient than the constructed cell modules 2 and 3. We surmised that the problem would be solved to a certain extent after coupling to the cell modules 2 and 3 because the constant removal of CHOL would reduce product inhibition.

Construction of E. coli consortia for AA 7b production. After generating the three selected cell modules, western blot was carried out to examine the enzyme expression of cells containing the modules: E. coli (M3B_M3E), E. coli (M2E) and E. coli (M1D). For comparison, the protein expression of all needed enzymes (modules 1 + 2 or modules 2 + 3) in a single E. coli cell was also conducted. Five of eight enzymes (P450, ADH1, ADH2, ALDH and lactonase) were expressed with His-tag, while the others (GDH, BVMO and NOX) were expressed with Flag-tag (Supplementary Table 1). A general trend can be found that the expressions of almost all the recombinant enzymes in individual
module system were comparable or higher than those in single E. coli strain composed of multiple modules. For instance, the expression levels of GDH in cell module 1, ADH1 and BVMO in cell module 2 were much higher compared to cells containing multiple modules (Supplementary Fig. 7). P450 was detectable in the cell module 1, but not in cell expressing enzymes modules 1, 2, and 3. Meanwhile, we also determined P450 concentrations based on CO-binding difference spectra. The result showed that P450 concentration in cell module 1 was 2.20 μM, but P450 was undetectable in the cell containing enzyme modules 1, 2, and 3 (Supplementary Table 2), which is in accordance with the western blot results. Therefore, we expected much higher productivity with the E. coli consortia of a combination of several modules.

Next, we tested the one-pot conversion of CHOL to AA with the E. coli consortium 2_3 (EC2_3) composed of selected cell modules 2 and 3. Optimization of the EC2_3-catalyzed one-pot conversion was conducted by adjusting the ratio and total amount (cell density) of the two modules in the consortium. As shown in Fig. 6a, AA formation was detected under different conditions. Product concentration increased with increased catalyst loading (8–32 g cell dry weight [CDW] L\(^{-1}\)) and a 1:1 ratio of cell catalysts showed the highest catalytic performance. Conversion of CHOL to AA was investigated with this E. coli consortium of modules 2 and 3 under optimized conditions. CHOL was converted to provide 46 mM AA after 24 h reaction (Fig. 6b), which was more than 10-fold higher than production by a single strain containing modules 2 and 3 (2–3 mM AA). Although some examples exist of in vitro biocatalytic cascades starting from CHOL and ending with 6-aminohexanoic acid and oligomers of ε-caprolactone as products in the presence of expensive cofactors and cosubstrates, an artificially designed in vivo biocatalytic route to AA from CHOL has not been reported. CHOL-degrading bacteria such as Arthrobacter sp. and Rhodococcus sp. have been found that naturally degrade the cyclohexanol via a similar route, but they further metabolized AA and thus it does not accumulate.

To examine the scalability of the developed E. coli consortia system EC2_3 for AA production from CHOL, the reaction was conducted in 1-L fermenter with 400 mL reaction mixture containing 50 mM CHOL. Without further optimizations, 44 mM of AA was produced after 36 h reaction (Supplementary Figs. 8, 9), which was comparable to that obtained in shaking flasks (Fig. 6b).

Finally, the cell modules 1, 2 and 3 with optimal functions were combined to form the E. coli consortium 1_2_3 (EC1_2_3) for the conversion of CHOL to CHOL. The reaction conditions including the ratios of modular cells and cell loading were likewise investigated. As shown in Fig. 6c, the highest product concentration was obtained at a cell loading of 12 g CDW L\(^{-1}\), which then decreased when the cell density was above it. A possible reason for this could be sensitivity of P450 to poor oxygen transfer and limited hydrophobic substrate availability caused by increased viscosity at high cell density, leading to reduced catalytic efficiency. This hypothesis needs to be addressed in a further study. Optimization of the ratio of whole-cell catalysts showed that 2:1:2 (modules 1:2:3) had the highest catalytic performance. Consortium EC1_2_3 catalyzed the conversion of CHOL to AA under optimized conditions with 100 mM substrate, reaching a maximum of 31 mM AA in 20 h without intermediate.

Considering the importance of the stability of the developed E. coli consortia, we determined the catalytic performance of each cell module after preincubation under reaction conditions. It was shown that both cell module 1 and 3 retained ~81% of their catalytic ability, while the percentage for module 2 was significantly reduced to only 47% after 24 h preincubation (Supplementary Fig. 11), suggesting the poor stability of module 2. In addition, the viability of E. coli cells during the EC1_2_3-catalyzed reaction was tested using the LIVE/DEAD BacLight™ Viability kit. The results showed that the percentage of viable cells with undamaged membrane dropped to ~50% just after adding the substrate CHOL, then further reduced to only 13% at 3 h reaction (Supplementary Fig. 12). The rapid reduction of live cell percentage might be due to the membrane damage caused by the strong hydrophobic nature of substrate CHOL. However, we would like to stress that, the non-viable cells may still have the desired enzymatic activities, leading to the difficulty in the accurate measurement of cells with enzymatic activities especially in a continuous and dynamic manner. Furthermore, the increased membrane permeability of E. coli cells may benefit the access of substrate and product molecules, accelerating the microbial consortia-based cascade reactions.

The aforementioned difference in stability of each cell module and reduced viability of cells during the reactions could be addressed by some solutions: (a) intermittent supplementation
of underdog subpopulations to elongate the modular reaction; (b) cell immobilization and enzyme engineering to improve the robustness of cell catalysts; (d) establishment of the biocompatible biphasic system (e.g., ionic-water or organic-water system) with the substrate deposited in ionic or organic phase, to prevent the cells in the aqueous phase from being damaged by the system; (e) with the substrate deposited in ionic or organic phase, to establish the cell catalysts; (d) dermination of the substrate scope and generality of the developed E. coli consortia system as well as its advantages due to the flexible operational mode for improving the productivity.

Next, the scalability of the developed E. coli consortia EC1_2_3 was likewise examined for converting CH 1b in 1-L fermenters with 400 mL reaction mixture. A ratio of 2:1:2 (final CDW was 12 g L$^{-1}$, module 1: module 2: module 3) was first tested for conversion of 200 mM CH 1b. The reaction system still works but with low efficiency, the titer of AA 7b after 36 h reaction was around 4 mM (Supplementary Fig. 13a). The possible reasons could be the different conditions (e.g., agitation and oxygen mass transfer) between shaking flasks and fermenters, which still needs further study. Considering the successful scaled-up of CHOL 2b to AA 7b with EC2_3 (Supplementary Fig. 8), a two-step addition of catalysts strategy was attempted, in which cell module 1 was first used to convert CH 1b to CHOL 2b with a certain accumulation, followed by addition of module 2 and module 3 to convert CHOL 2b to AA 7b. Consequently, AA 7b production was boosted to 22 mM after 36 h reaction (Supplementary Fig. 13b), demonstrating the potential of scalability of the developed E. coli consortia system as well as its advantages due to the flexible operational mode for improving the productivity.

Fig. 6 Construction of E. coli consortia for the production of AA 7b. a Optimization of conditions for E. coli consortium 2_3 (EC2_3)-catalyzed conversion of CHOL 2b to AA 7b with a reaction time of 6 h. b Time course for EC2_3 catalyzed conversion of CHOL 2b to AA 7b under optimized conditions at 50 mM substrate. Blue line: AA; Orange line: 6-HHA; green line: CL; yellow line: CHONE; purple line: CHOL. c Optimization of conditions for E. coli consortium 1_2_3 (EC1_2_3)-catalyzed conversion of CH 1b to AA 7b with a reaction time of 6 h. d Time course for EC1_2_3-catalyzed conversion of CH 1b to AA 7b under optimized conditions at 100 mM substrate. Blue line: AA; Orange line: 6-HHA; green line: CL; yellow line: CHONE; purple line: CHOL. The data shown in a and c are presented as mean value of two biological replicates. The data shown in b and d are presented as mean value ± SD (standard deviations) of three biological replicates. Source data are provided as a Source Data file.
yields of 13–45%. This demonstrated the generality of the E. coli consortia biocatalytic system.

Compared with other biobased fermentation methods with engineered E. coli for DCAs production, our biocatalytic route provides general access to DCAs with varying chain length (C5 to C8) from different starting chemicals (e.g., cycloalkanes, cycloalkanols, or lactones). In contrast, metabolic engineering strategies require individual engineering of metabolic pathways for each product. In addition, with cycloalkanes or cycloalkanols as substrates, our approach gave much higher product titers than fermentation methods for production of glutaric acid 7a (1.6–6.3 g L⁻¹ vs. 0.82 g L⁻¹) and suberic acid 7d (1.1–7.3 g L⁻¹ vs. 0.254 g L⁻¹), and for pimelic acid 7c, which has not been realized by metabolic pathway engineering in E. coli. For AA 7b, product titer was as high as 66.6 g L⁻¹ using biobased CL as substrate, which is comparable to the highest value reported (68 g L⁻¹). For downstream processing, our biocatalytic process offers an easier product purification procedure because it uses resting cells as catalysts in a buffered system. In fermentations with engineered host cells, costly, complicated multistep processes (e.g., extraction, chromatography and recrystallization) are often required because of impurities in the culture medium and metabolites or byproducts from growing cells. A comparison of our approach to other biobased methods is summarized in Supplementary Table 3.

In summary, the biocatalytic process we developed is a general approach for one-pot synthesis of DCAs using either cycloalkanes or cycloalkanols as starting materials. This process is an ideal solution to the problems encountered in the industrial chemical processes. The easier product isolation and substrate recovery procedure of our biocatalytic process shows great advantages over chemical and fermentation methods. The concept of microbial-consortia-mediated biocatalytic pathway reconstruction and reduct self-sufficiency-based modularization provides solutions and guidance for further development of in vivo artificial biocatalytic cascades for challenging transformations. To further improve the efficiency of this biocatalytic system, future work will focus on engineering rate-limiting enzymes such as P450 and BVMO, fine-tuning protein expression by promoter and RBS engineering, and scale-up of the bioprocess in bioreactors with precisely controlled parameters.

### Methods

**Construction of recombinant E. coli.** DNA fragments of genes encoding enzymes and the linear plasmid backbone were amplified by PCR using primers with 15- to 20-bp homologous arms that enabled the subsequent recombination. Genes for enzymes were assembled via overlap PCR and cloned into linear vector in the presence of T5 exonuclease to generate 15-bp or 20-bp sticky ends to promote recombination efficiency. Reaction mixtures were 5 µL containing linear vector, enzyme genes, buffer 4.0 (New England Biolabs) and T5 exonuclease, incubated in ice-water for 5 min, followed by quick addition of 50 µL competent cells (E. coli DH5a) for transformation and plating on LB agar containing appropriate antibiotics. Resulting transformants were picked and DNA sequence for confirmation. Plasmids containing targeted enzyme genes were transformed into E. coli BL21 cells for protein expression and whole-cell biocatalyst preparation. The detailed information of the strain and plasmids, primers and synthetic gene sequences was listed in Supplementary Tables 4-6.

**Protein expression and preparation of cell module biocatalysts.** Constructed E. coli cells were inoculated into 5 mL LB medium containing antibiotics (50 µg mL⁻¹ kanamycin, 100 µg mL⁻¹ ampicillin or both), and cultured at 37 °C, 220 rpm for 6 h. Precultures (1 mL) were transferred into 50 mL TB medium with appropriate antibiotics in 250-mL shaking flasks and cultured at 37 °C, 220 rpm for 2–3 h until OD₆₀₀ 0.6–0.8, then IPTG was added to a final concentration of 0.2 mM. The temperature was shifted to 25 °C for 14–16 h. For E. coli cells containing enzyme module 3, protein expression conditions were modified to (a) 0.1 mM IPTG, and (2) protein expression at 20 °C for 20 h after IPTG addition. Cells were harvested by centrifugation at 3040 × g, 15 °C for 10 min, washed with 200 mM potassium phosphate buffer (pH 8.0) and used as whole-cell biocatalysts.

**Typical procedure for cell module 3 converting CL 4b to AA 7b.** The substrate 4b was added to a 4-mL suspension of E. coli cells expressing module 3 (final CDW was 16 g L⁻¹) in potassium phosphate buffer (0.2 M, pH 8.0). Reactions were started at 30 °C, 200 rpm in 100 mL shaking flasks with screw caps with substrate addition in fed-batch mode (200 mM 4b, followed by 200 mM and 100 mM 4b after 6 h and 10 h). The pH was maintained around 8.0 by adding 10 M NaOH. Samples were taken at appropriate intervals and prepared for GC analysis. Sample preparation for GC to determine AA 7b and 6-HHA 5b was 450 µL water, 50 µL HCl (4 M) and 500 µL ethyl acetate (EtOAc) added to each 50-µL reaction sample with vortexing and centrifuging (13,680 × g, 1 min). The organic phase was collected and dried over anhydrous Na₂SO₄ for derivatization and GC analysis with a SH-Rtx-WAX column by adding 450 µL water and 500 µL EtOAc containing 2 mM n-decane (internal standard) to 50-µL reaction sample, followed by vortexing and centrifuging (13,680 × g, 1 min). The organic phase was dried over anhydrous Na₂SO₄ and directly used for gas chromatography (GC). The result of cell module
Typical procedure for cell module 2 converting CHOL 2b to CL 4b. For substrate concentration (19.5 µL 7b (44 µL, 0.05 g mL−1), ratio of modular cell 2 and modular cell 3 was 2:1) in potassium phosphate buffer (0.2 M, pH 8.0) was added to 10 mL NaOH. Samples were taken at appropriate intervals and prepared for GC analysis as described for cell module 2 converting CHOL 2b to CL 4b at varying substrate concentrations is presented in Supplementary Fig. 14c.

Typical procedure for cell module 1 converting CH 1b to CHOL 2b. The 22 µL substrate CH 1b (final concentration 50 mM) was added to a 4 mL suspension of modular E. coli cells expressing enzymes of module 1 (final CDW was 8 g L−1) in potassium phosphate buffer (0.1 M, pH 8.0). Reactions were at 25 °C, 200 rpm in 100-mL shaking flasks with screw caps. The pH was maintained around 8.0 by adding 10 M NaOH. Samples were taken at appropriate intervals and prepared for GC analysis as described for cell module 2 converting CHOL 2b to CL 4b (see GC chromatograms in Supplementary Figs. 15, 16).

Typical procedure for E. coli consortium 1_2_3 converting 1a-d to 7a-d. Cyclohexane 1b (44 µL, final concentration was 100 mM) was added to a 4 mL suspension of E. coli consortium 1_2_3 (1.5 g L−1 in potassium phosphate buffer (0.2 M, pH 8.0). We added 0.05 g mL−1 glucose initially to facilitate NADPH regeneration and 50 mM final substrate concentration (19.5 µL, 22 µL, 24.7 µL, or 27.5 µL 1d) was used for substrate scope examination. Reactions were at 25 °C, 200 rpm in 100-mL shaking flasks for 24 h. For 1d, to ensure complete conversion of intermediate products 5d to corresponding α,ω-dicarboxylic acids 7d, a 2 mL-suspension of E. coli cell module 3 (32 g CDW L−1) in potassium phosphate buffer (0.2 M, pH 8.0) was added after 24 h. During the reaction, the pH was maintained around 8.0 by adding 10 M NaOH. After reaction, mixtures were extracted three times with 30 mL EtOAc, and the organic phase was evaporated for substrate recovery. The water phase was acidified to below pH 2.0 with 4 M HCl followed by three extractions with 50 mL EtOAc. The organic phase was dried over anhydrous Na2SO4. The solvent was removed using a rotary evaporator, and white solids were dissolved in 30 µL N-methyl-N-(trimethylsilyl) trifluoroacetamide and 60 µL pyridine. Derivatization reactions were at 65 °C for 1 h and mixtures were used for GC analysis with an SH-Rtx-1 column.

GC analysis. For GC analysis with an SH-Rtx-1 column: 90 µL EtOAc containing an internal standard (25 mM n-decanne) was added to derivative mixtures. Samples were analyzed using a SHIMADZU Nexi GC-2030 system equipped with a flame-ionization detector and SH-Rtx-1 column (30 m x 0.25 mm, 0.25 µm). Temperatures of injector and detector were 250 °C and 280 °C, respectively. Temperature program was 5 °C per min from 50 °C to 120 °C, 40 °C per min to 240 °C, and held at 240 °C for 1 min.

For GC analysis with an SH-Rtx-WAX column (30 m x 0.25 mm, 0.25 µm): Obtained mixtures were analyzed with the temperature program: 5 °C per min from 50 °C to 120 °C, 40 °C per min to 240 °C, and held at 240 °C for 3 min.

Typical procedure for cell module 1 converting CH 1b to CHOL 2b. The 22 µL substrate CH 1b (final concentration 50 mM) was added to a 4 mL suspension of modular E. coli cells expressing enzymes of module 1 (final CDW was 8 g L−1) in potassium phosphate buffer (0.1 M, pH 8.0) containing 0.05 g mL−1 glucose for 24 h. During the reaction, the pH was maintained at 7.5, 5.7, and 5.2 for 4 h. For 1d, the pH was maintained around 8.0 by adding 10 M NaOH. Samples were taken at appropriate intervals and prepared for GC analysis as described for cell module 2 converting CHOL 2b to CL 4b (see GC chromatograms in Supplementary Figs. 14, 15).

Preparative procedure for α,ω-dicarboxylic acids 7a-d. The cycloalkanols (final concentration 50 mM; 18.3 µL cyclopetanol 2a, 21.9 µL cyclohexanol 2b, 24.8 µL cycloheptanol 2c, or 28.4 µL cyclooctanol 2d) was added to a 4 mL suspension of E. coli cells expressing enzymes 1_2_3 (1.5 g L−1) in potassium phosphate buffer (0.2 M, pH 8.0). Reactions were at 25 °C, 200 rpm in 100-mL shaking flasks with screw caps. The pH was maintained around 8.0 by adding 10 M NaOH. Samples were taken at appropriate intervals and prepared for GC analysis as described for cell module 2 converting CHOL 2b to CL 4b at varying substrate concentrations is presented in Supplementary Fig. 14c.

3 catalyzed CL 4b to AA 7b at varying substrate concentrations is presented in Supplementary Fig. 14a.

**Data availability**

The data supporting the findings of this study are available within the article and its Supplementary Information Files or from the corresponding author on reasonable request. Source data are provided with this paper.

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Author contributions

A.L conceived and supervised the project, F.W., J.Z., and Q.L. performed the experiments and analyzed the data; J.Y., R.L., J.M., X.Y., G.W.Z., H.L.Y., C.Z., C.G.A., and L.M. analyzed the data; A.L. wrote the manuscript; all authors checked and modified the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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