Rational Design of Artificial Biofilms as Sustainable Supports for Whole-Cell Catalysis Through Integrating Extra- and Intracellular Catalysis

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Biofilms are promising candidates for sustainable bioprocessing applications. This work presents a rational design of biofilm catalysts by integrating extra- and intracellular catalysis systems with optimized substrate channeling to realize efficient multistep biosynthesis. An assembly of four enzymes in a “three-in-one” structure was achieved by rationally placing the enzymes on curli nanofibers, the cell surface, and inside cells. The catalytic efficiency of the biofilm catalysts was over 2.8 folds higher than that of the control whole-cell catalysis when the substrate benzaldehyde was fed at 100 mM. The highest yield of o-phenyllactic acid catalyzed by biofilm catalysts under optimized conditions was 102.19 mM, also much higher than that of the control catalysis test (52.29 mM). The results demonstrate that engineered biofilms are greatly promising in integrating extra- and intracellular catalysis, illustrating great potentials of rational design in constructing biofilm catalysts as sustainable supports for whole-cell catalysis.

Introduction

Complicated biotransformations rely on the synergy of multiple enzymes. However, coordinating multiple enzymes to achieve highly efficient transformation is highly challenging. Intra-cellular catalysis is limited by the biological environment that is highly regulated for the living system and not necessarily optimized for the reaction efficiency of a specific reaction pathway segment. On the other hand, extracellular catalysis can be engineered to realize intensified reactions but is often challenged with a narrow product scope and harsh conditions that are not compatible with biocatalysts. In line with this, hybrid biocatalytic systems that promise close interactions between extracellular enzymes and intracellular components may provide a platform that can best integrate the advantages of both systems, which further maximizes the efficiency of complicated biotransformations. However, closely integrating extra- and intracellular catalysis to minimize the mass transfer distance is still a challenge. Especially, substrate channeling formation is a key factor to achieve high catalytic efficiency, which is very different from the traditional combination of free enzymes and whole-cell catalysts. Inspiration can be found in Nature, where biofilms stand out as key solutions.

Engineered Escherichia coli biofilms, as living bioscaffolds, have attracted attention in the biocatalysis research community because of the programmability and site-specific assembly of these biofilms. E. coli biofilms are ideal platforms for extra-/intracellular catalysis because the functionalized curli nanofibers enable precise control of the number and orientation of enzymes at the nanometer scale, and they allow for close interaction with intracellular environments by binding to the E. coli cell surface. The cell surface provides a place for enzyme localization and acts as an interposition connecting extra- and intracellular environments. Furthermore, the intracellular environment provides a natural place for enzyme catalysis and regeneration of co-factors. Although E. coli biofilms have been used for extra-/intracellular catalysis, their use as hybrid systems to maximize the catalytic efficiency of complicated biotransformation goes far beyond that, especially when the spatial arrangement of multienzymes is optimized based on substrate channeling using a rational design strategy.

Engineered E. coli biofilms provide distinct and sequential extra-/intracellular positions for site-specific co-localization of multiple enzymes, which means that enzymes can be rationally positioned at curli nanofibers, cell surfaces, or intracellular environments. Based on the reaction types and enzymatic properties, enzymes requiring high loading or improved stability can be positioned at the curli nanofibers. Enzymes...
involved in the intermediate reactions can also be placed on the cell surface with improved stability. Furthermore, reactions involving co-factors must be positioned in an intracellular environment. More importantly, the precise integration of extra- and intracellular catalysis at the nanometer scale in *E. coli* biofilms should follow the direction of mass transfer to guarantee the formation of substrate channeling, thereby maximizing the total catalytic performance.

To optimize the synergistic catalysis of multiple enzymes, we investigated a rational design method, which integrates extra- and intracellular catalysis using engineered *E. coli* biofilms. A “three-in-one” structure was constructed based on substrate channeling, where four enzymes were spatially placed on the curli nanofibers, on the cell surface, and inside the cells in a layer-by-layer manner. The spatial placement of the enzymes within the assembly was designed to follow the substrate channeling required by the reaction cascade and the direction of mass transfer from the bulk liquid phase to the intracellular reactions. This could minimize the mass transfer distance, which was driven by the local concentration gradients. Moreover, the prepared *E. coli* biofilm catalysts were far more advantageous than traditional whole-cell biocatalysts in terms of catalytic efficiency, illustrating the potential of rational design for maximizing the synergistic catalysis of multiple enzymes, which can be chosen as sustainable supports for whole-cell catalysis.

**Results and Discussion**

**Cultivation and verification of functional biofilms formed by the EX10 strain**

To test the feasibility and potential of the designed *E. coli* biofilm catalysts as sustainable supports for whole-cell catalysis, the route for D-phenyllactic acid (D-PLA) synthesis reported by Song et al. was used. D-PLA is an organic acid widely distributed in honey and fermented foods. D-PLA has also shown outstanding antimicrobial activity towards fungi and gram-positive and gram-negative bacteria by destroying their cell membranes. Moreover, they can also be used as chiral monomers for the production of biodegradable poly-PLA or pharmaceuticals. Among the synthesis approaches of D-PLA, enzymatic cascade biocatalyst is considered as a “green” alternative. Dehydrogenases have been successfully used for the synthesis of D-PLA using phenylpyruvic acid as a substrate, including D-lactate dehydrogenase. Furthermore, researchers have also developed a cascade synthetic route using L-α-amino acids as substrates, where L-amino acid deaminase (L-AAD), D-hydroxyisocaproate dehydrogenase (D-Hic), and formate dehydrogenase (FDH) were involved. To further reduce the cost of substrates, Song et al. reported a chiral-group-resetting process catalyzed by using four enzymes in cells, applying benzaldehyde and glycine as substrates. This route was considered as a promising alternative for D-PLA synthesis. In the form of catalysts used, purified enzymes, cell-free extracts, or whole-cell catalysts have all been examined for cascade synthetic reactions. Especially, whole-cell catalysis has been attractive because it can provide a natural environment for enzymes and can efficiently regenerate co-factors. However, whole-cell catalysis system cannot always guarantee synergy among the enzymes in a living cell. For example, in the work of Song et al., the catalytic efficiency and yield of whole-cell catalysts appeared to be low, which was attributed to the existence of bottleneck enzymes and un-optimized substrate channeling. The catalytic performance can be improved by rationally engineering the bottleneck enzymes and substrate channeling based on engineered *E. coli* biofilms, especially by coordinating the advantages of extra- and intracellular catalysis.

As shown in Figure 1b, the synthesis of D-PLA through the chiral-group-resetting process is as follows: (1) C–C bond formation catalyzed by PaTA (threonine aldolase from *Pseudomonas aeruginosa*), (2) atypical deamination catalysed by CgTA (3), and (3) D-PLA formation and nicotinamide adenine dinucleotide (NADH) regeneration catalyzed by D-hydroxyisocaproate dehydrogenase (D-HicDH) and an FDH from *Granulicella mallensis* (GraFDH2), respectively. In our rational design (Figure 1), PaTA was assembled on curli nanofibers with high enzyme loading properties because increasing the loading of PaTA could guarantee a high yield of D-PLA. This process is achieved through the specific recognition of SpyTag/SpyCatcher conjugation pairs. CgTA is the main rate-limiting enzyme, which is displayed on the *E. coli* cell surface to improve its catalytic stability. The surface display of CgTA was then achieved using a truncated ice-nucleation protein, InaK. Furthermore, D-HicDH and GraFDH2 are co-localized inside the cells because they reduce the incompatibility among reactions and facilitate the co-factor regeneration. More importantly, this design follows the substrate channeling required by the reaction cascade, which follows the direction of mass transfer from the bulk liquid phase to the intracellular reactions. Thus, this design is highly promising for improving the catalytic efficiency and final yield of D-PLA by engineering bottleneck enzymes and substrate channeling, as well as maintaining the advantages of extra- and intracellular catalysis.

We aim to construct and cultivate functional biofilms formed by EX10 strains that contain curli nanofibers, surface-displayed CgTA, and intracellular D-HicDH and GraFDH2 (Figure 1). These fractions must be verified through biochemical or microscopic analyses. Furthermore, functional biofilms formed by EX10 can catalyze the synthesis of D-PLA (C5) from β-hydroxy-α-amino acids (C3).

The formation of curli nanofibers in *E. coli* biofilms is regulated by the csgBAC and csgDEFG operons, where CsgA is the main structural subunit and CsgB acts as an anchoring protein. In our previous work, we developed a biofilm display platform based on the csgA-deletion strain BL21:ΔCsgA with a chloramphenicol resistance gene. For this study, we first eliminated the chloramphenicol resistance gene from the genome, resulting in the BL21:ΔCsgA strain with no csgA and...
any resistance genes (Figure S1, Supporting Note 1). After the transformation of the two recombinant plasmids into BL21:ΔCsgA, the EX10 strain was obtained (Figure 1a). Furthermore, after cultivating the recombinant strain EX10 into functional biofilms, the dyeing process [Congo red (CR) and crystal violet (CV)] and microscopic analysis were used to characterize their biochemical and morphological features.

As shown in Figure 2a–d, the binding amount of EX10 biofilms towards CR and CV was much higher than that of BL21:ΔCsgA biofilms, which was attributed to the secretion and assembly of CsgA(SpyTag). Compared to the field-emission scanning electron microscopy (FESEM) results for BL21:ΔCsgA (Figure 2e) and EX10 biofilms (Figure 2f), obvious extracellular masses were observed in the EX10 biofilms. Furthermore, curli nanofibers were imaged in the EX10 biofilms using transmission electron microscopy.

Figure 1. Rational design and engineering of biofilm catalyst through layer-by-layer assembly for d-PLA production. (a) Construction of recombinant plasmids for the preparation of biofilm catalysts. The pET21a-PtA-SpyCatcher was transformed into BL21(DE3) to obtain EX02, and the pET21a-CsgB-CsgA(SpyTag)-InaK-CgTD and pACYCDuet-GraFDH2-d-HicDH were transformed into BL21:ΔCsgA to obtain EX10. (b) Schematic diagram of the d-PLA production from benzaldehyde and glycine catalyzed by the four enzymes. (c) Illustration of the layer-by-layer assembly of the four enzymes on biofilms and their application in d-PLA production. PtA-SpyCatcher was assembled on the curli nanofibers through the specific recognition between SpyCatcher and SpyTag; InaK-CgTD was displayed on the cell surface; GraFDH2/d-HicDH were expressed in cells to achieve the regeneration of NADH and the synthesis of d-PLA, respectively.
microscopy (TEM) (Figure 2h), where no nanofibers were bound to the cell surface in BL21:ΔCsgA:ΔCsgA biofilms (Figure 2g).

These results are consistent with our previous work, which indicated that SpyTag-modified curli nanofibers were formed in the EX10 biofilms. The appreciable curli nanofibers formed in the functional biofilms (EX10) could contribute to the assembly of PaTα-SpyCatcher with high enzyme loading.

FESEM, TEM, and confocal laser scanning microscopy (CLSM) were used to verify the surface display of the InaK-CgTD. We first synthesized Ni\textsuperscript{2+}-functionalized SiO\textsubscript{2} nanoparticles (NiSiO\textsubscript{3}) that could specifically bind to His-tagged proteins (Figures S2–S6, Supporting Note 2). After co-incubation of the functional biofilms and NiSiO\textsubscript{3} nanoparticles, FESEM results of the EX11 biofilms after incubation with NiSiO\textsubscript{3} nanoparticles. Some nanoparticles were tightly immobilized on the cell surface. The EX11 strains were obtained by transforming pET21a-CsgB-CsgA(SpyTag)-InaK-CgTD(His) and pACYCDuet-GraFDH2-d-HicDH plasmids into BL21:ΔCsgA. (k) TEM results of the EX10 biofilms after incubation with NiSiO\textsubscript{3} nanoparticles. No nanoparticles were immobilized on the cell surface. (l) TEM result of the EX11 biofilms after incubation with NiSiO\textsubscript{3} nanoparticles. Some nanoparticles were tightly immobilized on the cell surface. (m) Confocal laser scanning microscopy (CLSM) results of the EX12 biofilms after incubation with mCherry solutions. No red fluorescence was observed. The EX12 strains were obtained by transforming pET21a-CsgB-CsgA(SpyTag)-InaK-CgTD(EFCA) and pACYCDuet-GraFDH2-d-HicDH plasmids into BL21:ΔCsgA. (n) CLSM results of the EX12 biofilms after incubation with mCherry-InaD solutions. Red fluorescence with high intensity was observed. (o) SDS-PAGE analysis of the cell lysis of EX10 biofilms: 0, protein marker; 1, the supernatant of cell lysis; 2, the precipitation of cell lysis.

Figure 2. Preparation, determination, morphological analysis, and function verification of the functional biofilms formed by the EX10 strain. (a) CV staining of the BL21:ΔCsgA (left) and EX10 (right) biofilms. (b) CR staining of the BL21:ΔCsgA (left) and EX10 (right) biofilms. (c) Total biomass determination by CV staining of the BL21:ΔCsgA (1) and EX10 (2) biofilms. (d) Curli nanofibers determination by CR staining of the BL21:ΔCsgA (1) and EX10 (2) biofilms. (e) FESEM results of BL21:ΔCsgA biofilms with no extracellular masses. (f) FESEM results of the EX10 biofilms with clear extracellular masses. (g) TEM results of the BL21:ΔCsgA biofilms with no curli nanofibers. (h) TEM results of EX10 biofilms with clear curli nanofibers. (i) FESEM result of the EX10 biofilms after incubation with NiSiO\textsubscript{3} nanoparticles. No nanoparticles were immobilized on the cell surface. (j) FESEM results of the EX11 biofilms after incubation with NiSiO\textsubscript{3} nanoparticles. Some nanoparticles were tightly immobilized on the cell surface. The EX11 strains were obtained by transforming pET21a-CsgB-CsgA(SpyTag)-InaK-CgTD(His) and pACYCDuet-GraFDH2-d-HicDH plasmids into BL21:ΔCsgA. (k) TEM results of the EX10 biofilms after incubation with NiSiO\textsubscript{3} nanoparticles. No nanoparticles were immobilized on the cell surface. (l) TEM result of the EX11 biofilms after incubation with NiSiO\textsubscript{3} nanoparticles. Some nanoparticles were tightly immobilized on the cell surface. (m) Confocal laser scanning microscopy (CLSM) results of the EX12 biofilms after incubation with mCherry solutions. No red fluorescence was observed. The EX12 strains were obtained by transforming pET21a-CsgB-CsgA(SpyTag)-InaK-CgTD(EFCA) and pACYCDuet-GraFDH2-d-HicDH plasmids into BL21:ΔCsgA. (n) CLSM results of the EX12 biofilms after incubation with mCherry-InaD solutions. Red fluorescence with high intensity was observed. (o) SDS-PAGE analysis of the cell lysis of EX10 biofilms: 0, protein marker; 1, the supernatant of cell lysis; 2, the precipitation of cell lysis.
(Figure S7) and the samples were imaged using CLSM.\textsuperscript{[21]} Red fluorescence was detected in Figure 2n, but not in Figure 2m, illustrating the successful display of InaK-CgTD onto the cell surface, which was ascribed to the specific binding between EFCA and InaD.\textsuperscript{[20]} From the FESEM, TEM, and CLSM results, we concluded that InaK-CgTD was successfully displayed on the cell surfaces of the EX10 biofilms. Whole-cell structures can also protect enzymes from inactivation and provide an environment for co-factor regeneration.\textsuperscript{[14b]} The SDS-PAGE analysis was used to determine the expression of GraFDH2 and d-HicDH in the cells, and the results are shown in Figure 2o. Clear bands with predicted molecular weights were detected, and the expression of GraFDH2 and d-HicDH was further confirmed by purification (Figure S8). The above results indicated that functional EX10 biofilms were successfully prepared, as expected, where InaK-CgTD was displayed on the cell surface and GraFDH2/d-HicDH was over-expressed in cells.

Immisciblization of PaTA-SpyCatcher onto EX10 biofilms

After immobilizing PaTA-SpyCatcher (produced by EX02 strains) onto the EX10 biofilms by means of affinity binding between SpyTag and SpyCatcher, the prepared biofilm catalysts could use benzaldehyde (C1) and glycine (C2) as substrates to synthesize C5 (Figure 1).\textsuperscript{[6c,8]} We first determined the function of curli nanofibers in the EX10 biofilms, which meant it was to verify whether the SpyTag-modified curli nanofibers could specifically bind to the SpyCatcher-modified proteins. As shown in Figure 3b, high-intensity emission from green fluorescence proteins (GFP) was observed under CLSM, indicating the tight binding of GFP-SpyCatcher (Figure S9b) onto the SpyTag-modified curli nanofibers.\textsuperscript{[22]} However, GFP (Figure S9a) could not bind to the curli nanofibers because of the lack of biological conjugation pairs (Figure 3a). The results indicated that EX10 biofilms had the function to specifically immobilized SpyCatcher-modified proteins in cell lysis solutions. In other words, EX10 biofilms could directly immobilize and purify PaTA-SpyCatcher.

Figure 3. Function analysis of the curli nanofibers in EX10 biofilms and immnobilization of PaTA-SpyCatcher onto the EX03 biofilms. (a) CLSM results of the EX10 biofilms after incubation with GFP solutions. Only weak green fluorescence was observed. (b) CLSM results of the EX10 biofilms after incubation with GFP-SpyCatcher solutions. Green fluorescence with high intensity were determined. (c) Influence of the immobilization time on the enzyme loading of EX03 biofilms. (d) Influence of the concentration of PaTA-SpyCatcher solutions on the enzyme loading of EX03 biofilms.
from EX02 cell lysis solutions without being significantly influenced by other unwanted proteins.

Increasing the concentration of PaTA could also improve the reaction rate.8 Thus, the highest loading of PaTA-SpyCatcher (Figures S10 and S11) onto the EX10 biofilms was investigated in this section. To avoid the influence of other enzymes on PaTA-SpyCatcher loading and activity, EX03 biofilms without InaK-CgTD, GraFDH2 and d-HicDH were cultivated and used as an alternative to EX10 biofilms. As shown in Figure 3c, from 0 to 9 h, the amount of immobilized PaTA-SpyCatcher increased gradually and remained unchanged after 9 h. However, the highest activity was observed at 6 h, and a significant decrease in activity was observed after 6 h. This phenomenon may be ascribed to the blockage of active sites caused by the immobilization of surplus PaTA-SpyCatcher. Thus, the optimal immobilization time was set to 6 h. In Figure 3d, as the concentration of PaTA-SpyCatcher solutions increased from 0.5 to 10 mg mL\(^{-1}\), the activity gradually increased. However, the highest protein loading was determined as 24.42 mg g\(^{-1}\) EX03 biofilms. The immobilization amount of PaTA-SpyCatcher was appealing, which was attributed to the high content of SpyTag-modified curli nanofibers in the E. coli biofilms (Figure 2b,d,f,h). The high loading of PaTA-SpyCatcher contributed to an improvement in the reaction rate. In 8.0 mg mL\(^{-1}\) solutions, the immobilized activity was 96 % of the highest activity (in 10 mg mL\(^{-1}\) solutions), which was approximately 63.54 U g\(^{-1}\) EX03 biofilms. Finally, in 8.0 mg mL\(^{-1}\) solutions, EX10 biofilms were used to immobilize PaTA-SpyCatcher from the solutions for 6 h, resulting in the final biofilm catalysts used for the D-PLA synthesis. However, considering that EX10 biofilms contain fewer curli nanofibers than EX03 biofilms, the actual immobilization amount and activity of PaTA-SpyCatcher in the EX10 biofilms would be lower than that in EX03 biofilms.

**Optimization of reaction conditions**

Reaction conditions are key factors for the enzyme catalysis, especially for improving or balancing the overall activity of cascade reactions. After immobilization of the PaTA-SpyCatcher onto the EX10 biofilms, the prepared biofilm catalysts were used to optimize the reaction conditions, where the addition amount of benzaldehyde was maintained at 100 mM. CgTD is a PLP-dependent aldolase, where PLP acts as an activator by binding to the active site of CgTD.23 As shown in Figure 4a, the addition of PLP had no significant effect on the production of D-PLA, and the highest yield of D-PLA was obtained when 140 μM of PLP was added. Furthermore, HCOONH\(_4\) acted as a hydrogen donor for the NADH regeneration, which had a significant effect on the synthesis of D-PLA (Figure 4b). The highest yield of D-PLA at 24 h was observed when the concentration of HCOONH\(_4\) was 60 mM, and higher concentrations of HCOONH\(_4\) decreased the total transformation speed. However, although the yield of D-PLA at 100 mM HCOONH\(_4\) was lower (88.76 %) than that at 60 mM HCOONH\(_4\) at 24 h, we maintained the optimal concentration of HCOONH\(_4\) at 100 mM to guarantee the complete transformation of substrates, that is, the ratio of HCOONH\(_4\) to benzaldehyde was maintained at 1:1.

![Figure 4](image-url)

*Figure 4. Optimization of reaction conditions. (a) Influence of the PLP concentration. (b) Influence of the HCOONH\(_4\) concentration. (c) Influence of the addition of NAD\(^+\). (d) Influence of the ratio of glycine to benzaldehyde. (e) Influence of the concentration of reaction buffers. (f) Influence of the addition of Tween 80.*
Figure 4c shows that the addition of NAD\(^+\) facilitated the synthesis of d-PLA, and a higher concentration of NAD\(^+\) contributed to a higher transformation speed. However, for economic considerations and further comparison, the final concentration of NAD\(^+\) was set to 1 mM, as previously reported.\(^{30}\) As shown in Figure 4d, when the ratio of glycine to benzaldehyde was lower than 4:1, the yield of d-PLA increased as the ratio increased. However, when the concentration was higher than 4:1, d-PLA production was limited. Thus, the optimal ratio of glycine to benzaldehyde was determined to be 4:1. Moreover, reaction buffers with higher concentrations provide a more stable reaction environment, and their influence on the d-PLA production is shown in Figure 4e. The highest yield of d-PLA (100\%) was observed when the concentration of potassium phosphate buffer (KPB) was 50 mM and increasing the concentration of KPB decreased the production of d-PLA. We finally set the concentration of KPB to 100 mM (99.78\%) to improve its buffering ability and make it more suitable for the transformation of substrates with higher concentrations. Tween 80 often acts as an emulsifier for substrate dissolution, and its influence on d-PLA production is shown in Figure 4f. Without the addition of Tween 80, the production of d-PLA was the lowest, which was attributed to inadequate contact between the enzymes and substrates. The optimal addition of Tween 80 was determined to be 0.05\% for significant improvement in d-PLA production. It is worth pointing out that we used Tween 80 (0.05\%) to replace DMSO (10\%) in the reaction system, because organic solvents with high concentrations easily influence the catalytic activity and stability of enzymes, thus further reducing the yield of products.\(^{30}\)

The optimal reaction conditions were as follows: 140 μM PLP, 1:1 ratio of HCOONH\(_4\)/benzaldehyde, 1 mM NAD\(^+\), 4:1 ratio of glycine/benzaldehyde, 0.05\% Tween 80, and KPB (pH 8.0, 100 mM).

**Synthesis of d-PLA**

In this section, biofilm catalysts were compared with whole-cell catalysts to investigate whether the rational design of biofilm catalysts could obtain higher transformation efficiency and yield while maintaining the advantages of whole-cell catalysts.

The prepared biofilm catalysts were used to synthesize d-PLA under optimal reaction conditions. The conversion curves of benzaldehyde with the addition of 50 mg mL\(^{-1}\) wet biofilm catalysts (approximately 10 mg mL\(^{-1}\) dried biofilm catalysts) are shown in Figure 5a. When the benzaldehyde concentration was 10 mM, the conversion rate of benzaldehyde was as high as 99.9\% in 9 h, which was far more efficient than the 85\% in 36 h reported by Song et al.\(^{30}\) Even when the concentration of benzaldehyde was increased to 100 mM, the conversion rate of benzaldehyde reached 98.84\% in 36 h. However, in the report by Song et al., 90 mM benzaldehyde was added to the reaction system in two batches, which was attributed to the inhibitory effect of the substrates. Their results differed from how our prepared

![Figure 5. d-PLA synthesis catalyzed by the biofilm and whole-cell catalysts. (a) Conversion curve of benzaldehyde catalyzed by 50 mg mL\(^{-1}\) biofilm catalysts. (b) Comparison of the yield of d-PLA catalyzed by biofilm and whole-cell catalysts (50 mg mL\(^{-1}\)). (c) Change in the d-PLA yield after increasing the addition of whole-cell catalysts. An improvement of 31.1\% was determined when the addition of whole-cell catalysts increased from 50 to 100 mg mL\(^{-1}\). (d) Conversion curve of benzaldehyde catalyzed by 150 mg mL\(^{-1}\) biofilm catalysts. (e) Comparison of the yield of d-PLA catalyzed by biofilm and whole-cell catalysts (150 mg mL\(^{-1}\)). (f) Change in the d-PLA yield after increasing the addition of whole-cell catalysts.](image-url)
biofilm catalysts had a higher tolerance towards substrates with high concentrations. When the concentration of benzaldehyde was more than 100 mM, the highest conversion rates were only 88.91 and 68.13% at concentrations of 120 and 140 mM, respectively. We also prepared whole-cell catalysts for d-PLA production, and the yield of d-PLA with 100 mM benzaldehyde is shown in Figure 5b. This result showed that the highest yield of whole-cell biocatalysts was only 29.67% in 60 h, while the highest yield was identified as 78.25% in 48 h using the biofilm catalysts. Furthermore, the transformation efficiency of the biofilm catalysts (0.0594 μmol h⁻¹ mg⁻¹) was 2.80-fold higher than that of the whole-cell catalysts (0.0212 μmol h⁻¹ mg⁻¹), which might be ascribed to the formation of substrate channeling through the layer-by-layer assembly (Figure S12a). Moreover, we increased the addition of whole-cell biocatalysts to investigate whether d-PLA production could be improved, as shown in Figure 5c. The addition of 100 mg mL⁻¹ of whole-cell catalysts improved the yield of d-PLA to 45.65%. However, the transformation efficiency and yield were still far lower than those of biofilm catalysts, illustrating the advantages of using biofilms to assemble multiple enzymes.

To further investigate the potential of biofilm catalysts in d-PLA production, 150 mg mL⁻¹ of wet biofilm catalysts were added to the reaction system with an improved concentration of benzaldehyde (Figure 5d). Increasing the amount of biofilm catalysts significantly increased the transformation efficiency with the addition of 100 mM benzaldehyde. Even when the concentration of benzaldehyde was increased to 140 mM, the conversion rate of benzaldehyde was still up to 97.3%. However, when the concentration of benzaldehyde was increased above 200 mM, the conversion of benzaldehyde was highly inhibited, with a final conversion rate of no more than 5% (Figure 5d). As shown in Figure 5e, with the addition of 140 mM benzaldehyde, the highest yield of d-PLA was 90.66% using the biofilm catalysts at 36 h. Compared with biofilm catalysts, the yield of d-PLA catalyzed by the whole-cell catalysts was only 47.89% at 36 h. It was worth pointing out that improving the addition of whole-cell catalysts would not contribute to the production of d-PLA (Figure 5f). More importantly, the catalytic efficiency of the biofilm catalyst (0.0228 μmol h⁻¹ mg⁻¹) was still about 1.33-fold higher than that of the whole-cell catalyst (0.0172 μmol h⁻¹ mg⁻¹, Figure S12b). For the determination of d-PLA, we only determined the content of d-PLA in the supernatant of the reaction system. However, some products may be adsorbed onto the biofilms or kept inside the cells without external secretion, leading to a low d-PLA yield. Thus, ethyl acetate was used to extract d-PLA in biofilm or whole-cell catalysts with the determined d-PLA contents of 11.53 and 4.40 mg mL⁻¹, respectively. The real yield of the d-PLA catalyzed by biofilm and whole-cell catalysts was at least 102.19 and 52.29 mg mL⁻¹, respectively. However, this still cannot lead to complete conversion, which is attributed to the metabolic background of E. coli. E. coli strains can convert aldehydes into carboxylic acids or their corresponding alcohols, thus causing loss of the starting substrates.[8] The produced d-PLA was identified by using high-performance liquid chromatography (HPLC) mass spectrometry (MS) (Figure S13a). A strong quasi-molecular ion can be observed at m/z 165, which was corresponded to [M–H]⁻ of d-PLA. Furthermore, benzoic acid has been identified as a by-product through this analysis, which was believed resulted from the oxidation of benzaldehyde (Figure S13b).

From the results in Figure 5e, the production of d-PLA catalyzed by whole-cell catalysts did not increase further after 24 h. Combined with the results from Figure 5ef, this phenomenon might be ascribed to the effect of product inhibition, thus illustrating a higher product tolerance of biofilm catalysts compared with that of whole-cell catalysts. For the biofilm catalysts, the transformation efficiency increased as the concentration of benzaldehyde increased from 100 to 140 mM (Figure S14). However, the real-time yield of d-PLA decreased when the concentration of benzaldehyde was increased to more than 140 mM, which might be ascribed to the effect of substrate inhibition (Figure S14). Song et al. reported that when the concentration of benzaldehyde was greater than 50 mM, the catalytic activity of CgTD was significantly inhibited due to substrate inhibition.[8] However, this phenomenon occurred only when the concentration of benzaldehyde was increased to 140 mM using biofilm catalysts, which might be ascribed to the substrate tolerance improvement of InaK-CgTD when displayed on the cell surface. Furthermore, when PaTA-SpyCatcher was assembled onto the curli nanofibers, its catalytic stability significantly improved, which might be another factor that resulted in the high yield of d-PLA (Figure S15).

The above results clearly indicate that the prepared biofilm catalysts are far more advantageous than whole-cell catalysts in terms of catalytic efficiency and yield, illustrating the success of rational design in constructing hybrid biocatalytic systems by combining bottleneck enzyme engineering and substrate channeling. The reasons for the improved transformation efficiency and yield were as follows: (1) the substrate channeling formation via layer-by-layer assembly to improve the catalytic efficiency; (2) the high content of PaTA-SpyCatcher assembled onto the curli nanofibers; (3) the improved catalytic stability of PaTA-SpyCatcher after assembly onto the curli nanofibers; (4) the tolerance of InaK-CgTD towards substrates and improved products when displayed on the cell surface; and (5) the layer-by-layer assembly enabled the division of reactions to reduce the incompatibility among reactions. Furthermore, we observed that the results obtained by whole-cell catalysts in our study were more appealing than those in the report by Song et al., which might be ascribed to the changes in reaction conditions.[8] In particular, Tween 80 (0.05%) was used to replace DMSO (10%) to protect the enzymes from organic solvents.

**Conclusion**

We developed a rational design strategy based on substrate channeling to construct biofilm catalysts, which was achieved by integrating extra- and intracellular catalysis systems to optimize the synergistic catalysis of multiple enzymes. An
assembly of four enzymes (PoTA, CgTD, GrafDH2, and d-HicDH) in a “three-in-one” structure is achieved by rationally placing the enzymes on curli nanofibers, on the cell surface, and inside the cells. The catalytic efficiency of the biofilm catalysts was 2.80 and 1.33-fold higher than that of the whole-cell catalysts with the addition of 100 and 140 mM benzaldehyde as substrates, respectively. Furthermore, the highest yield of d-PLA catalyzed by biofilm catalysts was 102.19 mM, whereas that of the whole-cell catalysts was 52.29 mM. The improvement in the catalytic efficiency was attributed to the optimized substrate channeling, which followed the direction of mass transfer from the bulk liquid phase to the intracellular reactions, with extra-cellular enzymes placed on surface of the cells thereby minimizing the mass transfer resistance. It appeared the prepared biofilm catalysts are far more advantageous than traditional whole-cell catalysts, indicating the potential of rational design in constructing biofilm catalysts as sustainable supports for whole-cell catalysis. This work promises a powerful strategy in maximizing catalytic efficiency of complicated multi-step biotransformations.

**Experimental Section**

**Construction of biofilm catalysts**

To construct “three-in-one” biofilm catalysts, EX10 strains were induced and cultivated into functional biofilms. Furthermore, EX02 strains were induced to express PoTA-SpyCatcher. Induced EX02 cells were then ruptured by sonication, followed by centrifugation at 10000 g for 15 min to remove the insoluble substances. The supernatant was used as an enzyme solution to be incubated with the EX10 biofilms, and the initial immobilization system contained 50 mg of EX10 biofilms in 1 mL of enzyme solution. The mixture was then incubated at 20 °C with rotary shaking (150 rpm). The incubation time and concentration of the PoTA-SpyCatcher solution were optimized.

**Synthesis of d-PLA**

After the immobilization of PoTA-SpyCatcher, the prepared biofilm catalysts were used to synthesize d-PLA. The standard reaction system contained 1 M glycine, 100 mM benzaldehyde, 100 μM PLP, 1 mM NAD⁺, 100 mM HCOONa, 0.1% Tween-80, and 1 mL potassium phosphate buffer (KPB, pH 8.0, 50 mM). After the addition of 50 mg mL⁻¹ of wet biofilm catalysts, the mixture was incubated at 25 °C with rotary shaking (200 rpm) for 24 h. After the reaction, the liquid was centrifuged at 10000 g for 15 min to remove the insoluble substances. Then, 500 μL supernatant was added to 500 μL pretreatment liquid (ultrapure water/acetonitrile = 1:1), and after mixing, the mixture was statically maintained for 5 min before centrifugation. The supernatant was injected into the HPLC system to determine the synthesis of d-PLA.[1]

Under standard reaction conditions, the amount of PLP, HCOONa, NAD⁺, and Tween 80, the ratio of glycine to benzaldehyde, and the concentration of reaction buffer were separately changed to investigate their effects on d-PLA synthesis. Under the optimal reaction conditions, 50 and 150 mg biofilm catalysts were separately used to investigate their potential in the synthesis of d-PLA with whole-cell catalysts as control.

**Acknowledgements**

This work was sponsored by the National Natural Science Foundation of China (21636003, 21672065, and 22077032), the International S&T Innovation Cooperation Key Project (2017YFE0129600), the Fundamental Research Funds for the Central Universities (22221818014), and the 111 Project (B18022).

**Conflict of Interest**

The authors declare no conflict of interest.

**Data Availability Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Keywords**: biofilm catalysts · biotransformations · enzymes · immobilization · rational design

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