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Cell-free protein synthesis enables one-pot cascade biotransformation in an aqueous-organic biphasic system

Wan-Qiu Liu[1] | Changzhu Wu[2] | Michael C. Jewett[3] | Jian Li[1]

[1]School of Physical Science and Technology, ShanghaiTech University, Shanghai, China

[2]Department of Physics, Chemistry and Pharmacy, Danish Institute for Advanced Study (DIAS), University of Southern Denmark, Odense, Denmark

[3]Department of Chemical and Biological Engineering, Center for Synthetic Biology, Northwestern University, Evanston, Illinois

Correspondence
Changzhu Wu, Department of Physics, Chemistry and Pharmacy, Danish Institute for Advanced Study (DIAS), University of Southern Denmark, Odense 5230, Denmark.
Email: wu@sdu.dk

Jian Li, School of Physical Science and Technology, ShanghaiTech University, 201210 Shanghai, China.
Email: lijian@shanghaitech.edu.cn

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Abstract

Biocatalytic cascade reactions have become increasingly important and useful for chemical synthesis. However, biocatalysts are often incompatible with organic solvents, which prohibits many cascade reactions involving nonpolar substrates. In this study, we used cell-free protein synthesis (CFPS) to express enzymes in an aqueous-organic biphasic system for the construction of an artificial enzymatic pathway. CFPS-expressed enzymes without purification performed efficiently to convert styrene (below 20 mM) to (*S*)-1-phenyl-1,2-ethanediol (two steps in one pot) with 100% conversion. In addition, our CFPS system showed great tolerance to different organic solvents, and, importantly, the entire biocatalytic system can be consistently scaled up without a reduction of the substrate conversion rate. We, therefore, anticipate that our cell-free approach will make a possible cost-effective, high-yielding synthesis of valuable chemicals.

KEYWORDS

biocatalysis, biotransformation, biphasic system, cascade reaction, cell-free protein synthesis

Enzymatic cascades, which have become a green and sustainable method for organic synthesis, provide an interesting approach for manufacturing value-added chemicals and advanced pharmaceutical intermediates (Bornscheuer et al., 2012; Reetz, 2013; Schmermund et al., 2019; Sheldon & Brady, 2019; Sheldon & Woodley, 2018). Enzymes offer high enantioselectivity, fast conversion rates, and mild reaction conditions. In addition, many natural enzymes have similar reaction conditions in cellular aqueous environments, which are often at nearly neutral pH and ambient temperature. Therefore, enzymes sourced from diverse organisms can be rationally selected to construct natural or artificial enzymatic pathways to conduct chemical transformations amenable for manufacturing (Wu & Li, 2018). However, the natural environment for enzymatic biotransformations is in aqueous conditions, which is unfavorable to many industrial reactions with water-insoluble substrates (Klibanov, 2001). To improve the solubility of these types of substrates, water is often replaced with organic solvents as the reaction media but this can denature or reduce catalytic activities of enzymes (Klibanov, 1997). As a result, this made the use of enzymes less attractive for industrial applications. To tackle this problem, aqueous-organic biphasic systems, such as emulsions (Wang, van Oers, Rutjes, & van Hest, 2012; Wiese, Spiess, & Richtering, 2013; Wu, Bai, Ansorge-Schumacher, & Wang, 2011) and membrane bioreactors (Boontawan, & Stuckey, 2006; Doig, Boam, Leak, Livingston, & Stuckey, 1998; Molinari, Aragozzini, Cabral, & Prazeres, 1997), have been developed for efficient biotransformations. These systems are designed to separate enzymes in a favorable aqueous environment from the organic phase containing the substrate, increasing the aqueous/organic interface, and thereby enhancing biocatalytic productivity.

Biotransformations using enzymatic cascades are typically implemented with purified enzymes (in vitro), whole-cells (in vivo), or even hybrid systems (in vivo/in vitro; France, Hepworth, Turner, &

Flitsch, 2017). However, each system has its own limitations. For in vitro systems, the enzyme purification process is laborious, time-consuming, and costly. For whole-cell biocatalysis, the cell membrane often poses a serious barrier to substrate uptake and product export (Ladkau, Schmid, & Bühler, 2014). Thus, developing new methods to circumvent these obstacles is highly desirable. Recently, cell-free systems have emerged as an attractive and powerful platform for biomanufacturing (Bundy et al., 2018; Carlson, Gan, Hodgman, & Jewett, 2012; Dudley, Karim, & Jewett, 2015; Li, Zhang, & Liu, 2018; Liu, Zhang, Chen, & Li, 2019; Silverman, Karim, & Jewett, 2020; Swartz, 2018). In this context, cell-free protein synthesis (CFPS) plays a core role in the production of various proteins in vitro (Jaroentomeechai et al., 2018; Kwon et al., 2013; Li et al., 2016; Lu, Welsh, & Swartz, 2014; Martin et al., 2018; McNerney et al., 2019; O'Kane, Dudley, McMillan, Jewett, & Mrksich, 2019; Wilding et al., 2019). Because of the absence of cell walls, cell-free systems bypass mass transfer limitations and are more tolerant of toxic substrates, intermediates, and products than living microbial cells. In addition, these open cell-free systems allow for easy upstream (e.g., reaction control and optimization) and downstream (e.g., sampling and purification) operations. Furthermore, enzymatic reactions can be directly driven via in situ CFPS-expressed enzymes (biocatalysts) without purification (Goering et al., 2017; Zhuang et al., 2020). However, the potential of CFPS based cell-free systems have not been fully exploited as an efficient approach to carry out cascade biotransformations with water-insoluble substrates.

Here, we use CFPS to enable the one-pot production of (*S*)-1-phenyl-1,2-ethanediol from the nonpolar substrate, styrene, in an aqueous-organic biphasic system (Figure 1). We assembled a two-step, artificial enzymatic cascade composed of three enzymes co-expressed in a high-yielding *Escherichia coli*-based CFPS system (Li et al., 2016). In the first step, the substrate styrene is converted to an intermediate styrene oxide by a styrene monooxygenase (SMO, which consists of two subunit enzymes StyA and StyB) from *Pseudomonas* sp. strain VLB120 (Panke, Witholt, Schmid, & Wubbolts, 1998). In the second step, epoxide hydrolase (SpEH) from *Sphingomonas* sp. HXN-200 (Wu, Li, Chin, & Li, 2013) catalyzes the hydrolysis of styrene oxide to form (*S*)-1-phenyl-1,2-ethanediol, which is a valuable chiral pharmaceutical building block (Bala & Chimni, 2010; Renat, Koenigs, Claus, David, & Magnus, 2009). These biocatalytic reactions are carried out in a compartmentalized aqueous-organic emulsion system prepared with a simple block copolymer, poly(ethylene glycol)-block-poly(ε-caprolactone (PEG-b-PCL), to provide an exceptionally large interfacial area for efficient biocatalysis (Zhao, Ansorge-Schumacher, Haag, & Wu, 2018).

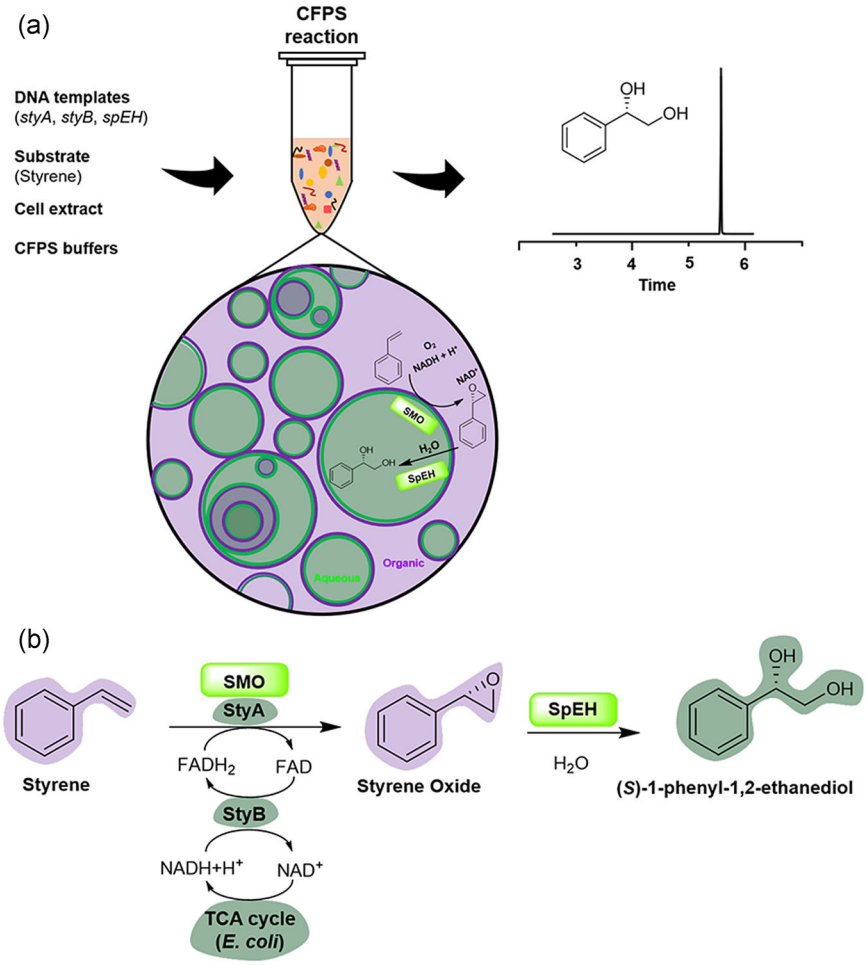

FIGURE 1 (a) Schematic diagram of one-pot cascade biotransformation from styrene to (*S*)-1-phenyl-1,2-ethanediol in a CFPS/emulsion system. (b) Overall two-step enzymatic cascade reactions. CFPS, cell-free protein synthesis; SMO, styrene monooxygenase; SpEH, epoxide hydrolase; TCA cycle, tricarboxylic acid cycle [Color figure can be viewed at wileyonlinelibrary.com]

We first determined how active an *E. coli*-based CFPS system is in our aqueous-organic emulsions by expressing super-folder green fluorescent protein (sfGFP). The aqueous CFPS reaction mixture was prepared as reported previously (Li et al., 2016). The organic phase was generated by dissolving 20 mg/ml water-insoluble triblock co-polymer (PCL_{21}-PEG_{45}-PCL_{21}) in toluene (Zhao et al., 2018). After mixing the aqueous and organic components at a ratio of 1:1 (vol/vol), the CFPS reaction was carried out at 30°C and 150 rpm in a shaker for 10 hr and, subsequently, the emulsion structure was identified by a confocal laser scanning microscopy. As shown in Figure 2, both single and multiple compartmentalized structures were observed and sfGFP was synthesized in the aqueous phase with a bright green color. The yield of sfGFP in the aqueous phase reached 1.09 ± 0.01 mg/ml, which is comparable to that in a positive control CFPS reaction (1.33 ± 0.03 mg/ml) without the organic phase. These results indicate that our CFPS system is active for protein expression in the aqueous-organic emulsion.

We next constructed the artificial enzymatic cascade with StyA (46.3 kDa), StyB (18.4 kDa), and SpEH (42.9 kDa). Before one-pot coexpression of these three enzymes, each enzyme was separately expressed by adding 200 ng of plasmid per 15 μl CFPS reaction. Although each enzyme was successfully expressed, their expression levels varied (Figure 3a, left). To balance the expression level of each enzyme during coexpression, we adjusted plasmid concentrations for each enzyme in CFPS reactions. We found that the best combination of three plasmids was 200 (StyA), 50 (StyB), and 200 ng (SpEH), according to the enzyme expression levels and enzyme solubility (Figure 3a, right). In addition, each enzyme was purified (Figure S1) and the molar ratio of StyA:StyB:SpEH was about 1:2:1.5 (15.95:33.70:23.78 μM). Previous studies reported that the maximal styrene conversion rate was obtained when the molar amount of StyB was equal to or higher than that of StyA (Otto, Hofstetter, Röthlisberger, Witholt, & Schmid, 2004; Panke et al., 1998). Therefore, our CFPS expression system could provide a reasonable enzyme stoichiometry for the enzymatic reactions. Using these conditions, we moved CFPS reactions to the aqueous-organic system for one-pot cascade reactions. To construct the whole biocatalytic pathway, the catalytic ability of each enzyme was initially investigated. The data are shown in Figure 3b and Table S1. Cell-free expression of StyA was sufficient to oxidize styrene (the conversion was not complete, 17%) in the absence of StyB, whereas expression of StyB alone did not yield any detectable product of styrene oxide. Interestingly, when StyA and StyB were coexpressed, the activity of StyA for the epoxidation of styrene into styrene oxide was notably restored (30% conversion rate). Our results agree with previous reports that StyA plays an indispensable role in the catalytic activity of SMO and StyB helps maximize the epoxidation (Otto et al., 2004; Panke et al., 1998). SpEH in our system was highly active, which can convert styrene oxide to (*S*)-1-phenyl-1,2-ethanediol with a 100% conversion rate. While coexpression of StyA and SpEH allows the formation of (*S*)-1-phenyl-1,2-ethanediol from styrene at low levels (17% conversion), coexpressing all three enzymes together in one pot enables a higher conversion (37%) of styrene to (*S*)-1-phenyl-1,2-ethanediol with no detectable styrene oxide intermediate remaining.

Next, we set out to optimize our system to enhance the cascade productivity. We began our optimization by investigating the concentrations of three plasmids used per 15 μl CFPS reaction. As shown in Figure 3a, the optimal plasmid ratio was StyA:StyB:SpEH = 4:1:4 (200–50–200 ng/15 μl), which gives rise to the maximum soluble expression of each enzyme in one pot. Therefore, we chose to add different plasmid concentrations to reach the same plasmid ratio. The results indicate that the highest yield of (*S*)-1-phenyl-1,2-ethanediol was achieved with the addition of more plasmids at 200–50–200 ng (Figure 4a). However, a further increase of plasmids (300–75–300 ng) obviously reduced the product yield. The cell-free reaction temperature is often a key parameter for optimization because it affects enzyme expression and activity (Li, Wang, Kwon, & Jewett, 2017). We, therefore, compared the product yields at different reaction temperatures ranging from 20°C to 35°C. Our data suggest that productivity at 30°C reached the highest level, which is 2–4 times higher than that at other temperatures (Figure 4b). Since the optimum reaction temperature in

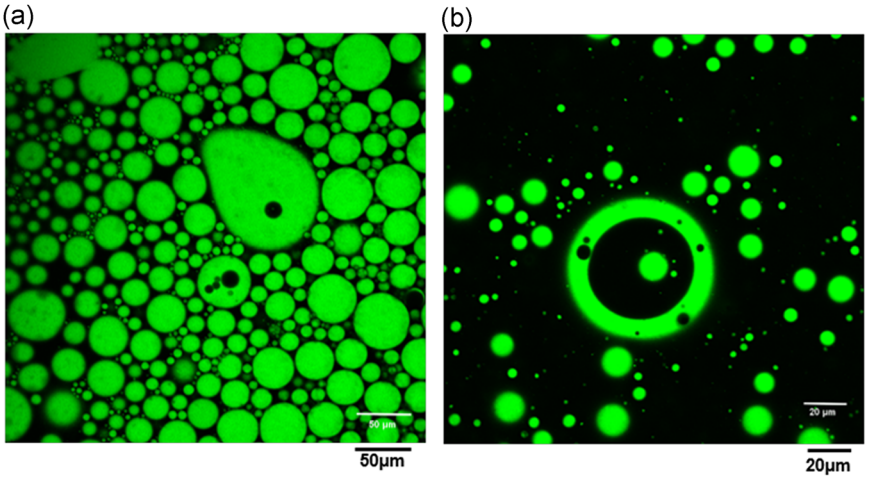

FIGURE 2 Cell-free synthesis of sfGFP in the CFPS/emulsion system and the single-/multi-compartmentalized structures of the emulsion detected by confocal laser scanning microscopy (A: ×20 and B: ×40). CFPS, cell-free protein synthesis; sfGFP, super-folder green fluorescent protein [Color figure can be viewed at wileyonlinelibrary.com]

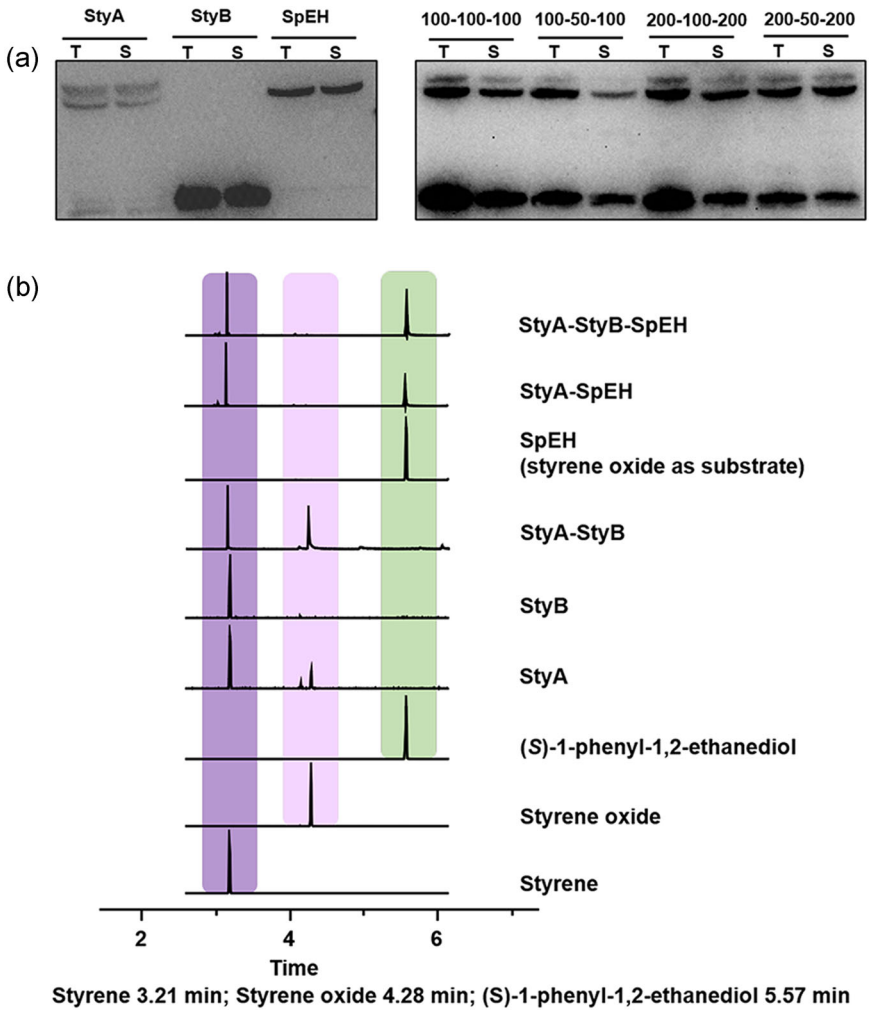

FIGURE 3 (a) Western-blot analysis of individual expression (left) and coexpression (right) of StyA (46.3 kDa), StyB (18.4 kDa), and SpEH (42.9 kDa) labeled with the anti-His antibody. Numbers on the top of the right panel indicate three plasmid concentrations (StyA–StyB–SpEH) per 15 μl CFPS reaction. Note that the below band of StyA (left) is a truncated protein. (b) GC–MS detection of substrate (styrene), intermediate (styrene oxide), and product ((*S*)-1-phenyl-1,2-ethanediol). The concentrations of styrene and styrene oxide were 10 mM when they were used as substrates in the reaction. Three independent reactions were performed for each condition, and an example trace is shown. CFPS, cell-free protein synthesis; GC–MS, gas chromatography–mass spectrometry; S, soluble protein; SpEH, epoxide hydrolase; T, total protein [Color figure can be viewed at wileyonlinelibrary.com]

the *E. coli*-based CFPS system is 30°C as reported previously (Goering et al., 2017; Li et al., 2016), we decided to use this temperature in our following cascade biotransformation. After the above primary optimization, although the final product (*S*)-1-phenyl-1,2-ethanediol was produced, the conversion rates based on the substrate (10 mM) were still low (maximally about 40%; Figure 4b). We hypothesized that the low expression of StyA in CFPS (Figure 3a) likely limited the conversion rate because StyA predominantly contributes to the catalytic activity of SMO (Otto et al., 2004; Panke et al., 1998).

To test this hypothesis, we increased StyA in our biocatalytic system by overexpressing StyA in *E. coli* (see protein gel in Figure S2), followed by preparing StyA-enriched cell extracts to coexpress StyB and SpEH in CFPS reactions (final StyA concentration per reaction: 20.15 μM). By doing this, the conversion rate was notably increased up to 72% after an 8 hr reaction with the (*S*)-1-phenyl-1,2-ethanediol concentration of 28.9 mM (Figure 4c). By contrast, without StyA-enriched cell extract in CFPS, the conversion rate was only about 10% in the presence of 40 mM styrene. Therefore, StyA-enriched cell extract was applied to our following CFPS reactions. Previous studies have shown that the activity of StyB depends on NADH consumption, which acts as an electron donor (Otto et al., 2004). We therefore next sought to supply additional NADH to the CFPS system and investigate the effect of NADH concentration on product synthesis. As shown in Figure 4d, with the addition of NADH, the product yields obviously increased and 2 mM NADH gave rise to the highest yield of 20 mM (100% conversion based on 20 mM styrene). We next attempted to test a broad range of the substrate concentrations from 10 to 400 mM to see if our cell-free system was robust for the cascade biotransformation. Notably, 100% conversion was achieved with low-substrate concentrations (<20 mM). However, further increase of styrene concentrations from 40 to 400 mM reduced conversion rates from 93% to 11%, respectively (Figure 4e; see the group of "CE + StyA 2 mM NADH"), perhaps as a result of toxicity and inhibition of styrene on the enzymes. Overall, in our current optimized cell-free system, we obtained the highest yield of ~45 mM, which is 113-fold higher than the initial yield (0.4 mM) of control using only the aqueous phase (see Figure S3 for initial data). While our maximal yield is lower than a previous report by using whole-cell biotransformation (143 mM final product from 1 M styrene), the conversion rate of our system is higher (e.g., 93% with 40 mM styrene; Figure 4e) than that of the whole-cell system (14.3%; Gao, Wu,

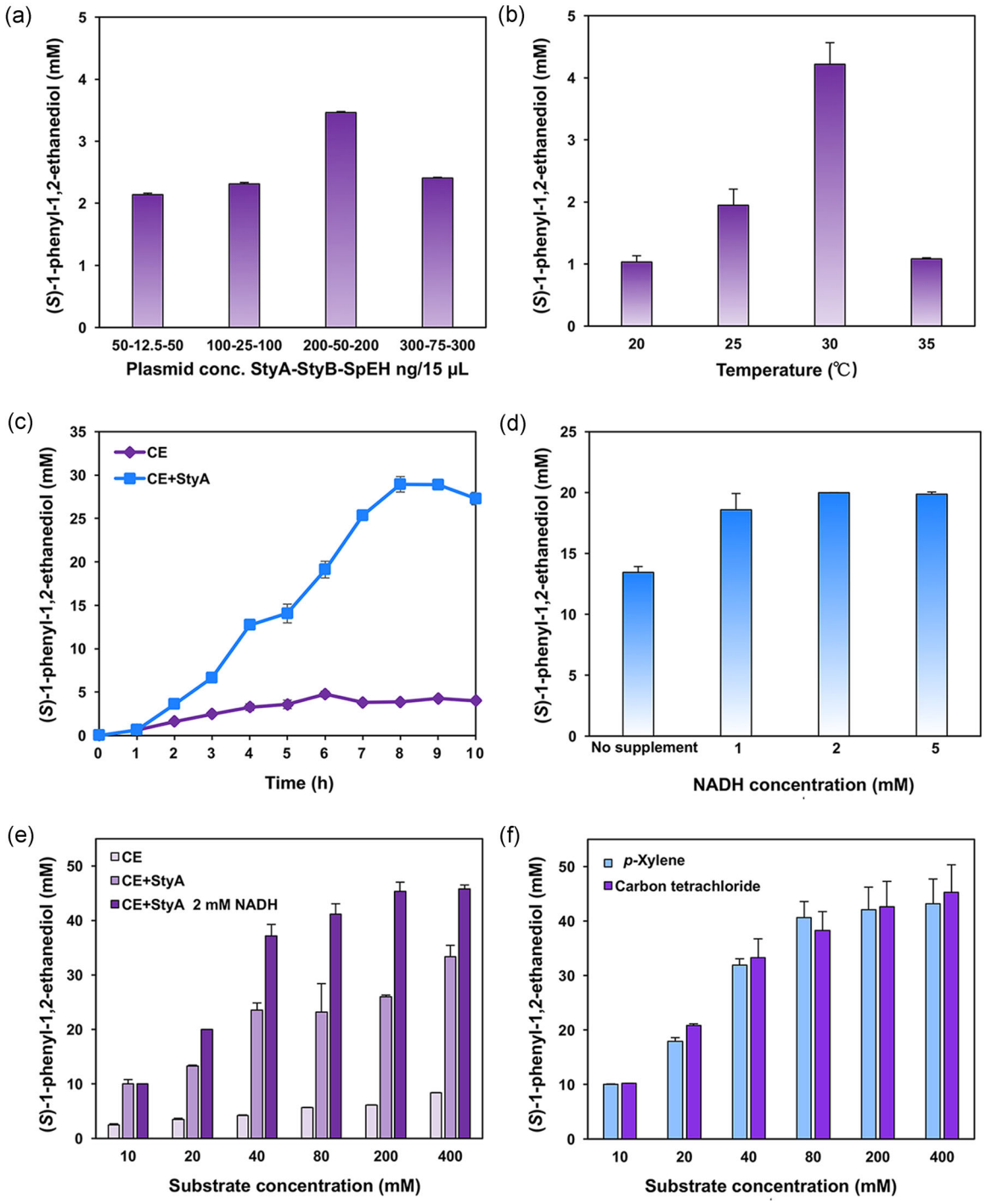

FIGURE 4 Optimization of (*S*)-1-phenyl-1,2-ethanediol production in the CFPS/emulsion system. (a) Optimization of plasmid concentration. Other conditions: substrate = 10 mM, reaction time = 10 hr, reaction temperature = 30°C. (b) Optimization of reaction temperature. Other conditions: substrate = 10 mM, reaction time = 10 hr, plasmid (StyA–StyB–SpEH) concentration = 200–50–200 ng/15 µl. (c) Time courses of (*S*)-1-phenyl-1,2-ethanediol production with 40 mM styrene. (d) Effect of the additional supplement of NADH on (*S*)-1-phenyl-1,2-ethanediol formation with 20 mM styrene. (e) Production of (*S*)-1-phenyl-1,2-ethanediol with different styrene concentrations in three reaction systems. (f) Production of (*S*)-1-phenyl-1,2-ethanediol with different styrene concentrations in two CFPS/emulsion systems (*p*-xylene and carbon tetrachloride). Three independent CFPS reactions were performed for each condition, and one standard deviation is shown. CE, cell extract without overexpressed StyA; CE + StyA, StyA-enriched cell extract; CE + StyA 2 mM NADH, StyA-enriched cell extract plus 2 mM NADH in the reaction; CFPS, cell-free protein synthesis; SpEH, epoxide hydrolase [Color figure can be viewed at wileyonlinelibrary.com]

Praveen, Loh, & Li, 2017). Moreover, our cell-free system enables complete conversion (100%) of styrene at low concentrations (10 and 20 mM), which is higher than previously reported whole-cell biotransformation in a two-liquid-phase system (92% conversion, 20 mM styrene; Wu et al., 2014). The higher conversion rate in our CFPS system is likely in part due to the faster mass transfer without cell walls or other membrane boundaries.

To demonstrate our system for potential applications, we were curious to know if CFPS might work with other organic solvents, since there might be some benefits being able to use different solvents to achieve other cascade biotransformations. We, therefore, investigated five other solvents (i.e., ethyl acetate, tetrahydrofuran, carbon tetrachloride, methylene chloride, and *p*-xylene) that can dissolve the copolymer PCL_{21}-PEG_{45}-PCL_{21}. We initially expressed sfGFP in different solvent systems. Our data indicate that sfGFP can be synthesized in high yields (>1 mg/ml) from carbon tetrachloride and *p*-xylene but substantially low yields from ethyl acetate, methylene chloride, and tetrahydrofuran (Figure S4). This protein yield difference is because the increase of solvent polarity leads to a decrease of emulsion stability (Davis & Smith, 1976). In our case, carbon tetrachloride and *p*-xylene are less polar than the other three, thus resulting in more stable emulsions for protein synthesis. Therefore, we next used carbon tetrachloride and *p*-xylene to prepare aqueous-organic emulsions for cascade reactions. As shown in Figure 4f, both systems worked like the toluene system that 100% conversion was observed with a low styrene concentration (10 mM), but it also gradually reduced to around 10% at 400 mM substrate. This success suggests that cell-free expression of many other enzymes, being able to catalyze broad organic soluble substrates, might be also possible in our emulsions. On the other hand, more choice of organic solvents demonstrates the robustness, flexibility, and applicability of our CFPS system in different aqueous-organic emulsions.

Finally, we scaled up our reaction system to 3 ml (1.5 ml CFPS + 1.5 ml emulsion) performed in a 50-ml tube. When using a moderate concentration of styrene (80 mM) as a starting substrate, we observed a yield of 21 mM (26% conversion). This is comparable to the small-scale reaction (180 μl), which had a yield of 23 mM (29% conversion). Our consistent results between an order of magnitude increase in reaction scale suggests the possibility for using cell-free systems to meet the demand for future synthesis of chemicals. In addition, we investigated the reusability of our system and found that the relative activity of the third cycle still retained 62% of the initial activity (Figure S5). While our system can be reused, more work needs to be done in the future to keep the system's high activity in terms of reusability.

In summary, we demonstrated the ability to use CFPS in a biphasic system, which we anticipate will provide a new, potential platform approach for chemists and biologists to synthesize valuable chemicals when water-insoluble substrates, bioconversion rates, mass transfer, and cellular toxicity limit the feasibility of whole-cell cultivation/fermentation. This platform has several key features. First, the enzymes necessary for the biotransformation were expressed by CFPS without purification and these enzymes performed biocatalytic reactions in situ at the aqueous/organic interface. Second, our optimized system was highly efficient, achieving 100% conversion of styrene to (*S*)-1-phenyl-1,2-ethanediol with substrate concentrations below 20 mM. Surprisingly, this system was only possible when StyA was enriched in the extract source strain by overexpression and StyB, which is almost insoluble when expressed in cells, was made by CFPS (Figure S6). Third, we discovered that the CFPS system was tolerant of a variety of organic solvents, including toluene, carbon tetrachloride, *p*-xylene, ethyl acetate, and methylene chloride. Fourth, we showed linear scalability of product titers and conversion yields over an order of magnitude range in volume. Fifth, we demonstrated the reusability of our system with several operational cycles. Taken together, these features show the robustness and scalability of the CFPS/emulsion system for biotransformation efforts. Looking forward, we envision that our cell-free system will enable new directions in developing biocatalytic cascades for defined biotransformations, providing a new and feasible avenue for efficiently synthesizing and manufacturing chemicals of pharmaceutical and industrial importance.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

J. L., C. W., and W. Q. L. designed the experiments. W. Q. L. performed the experiments, analyzed the data, and drafted the manuscript. J. L., C. W., and M. C. J. revised and edited the manuscript. J. L. conceived and supervised the study. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

ORCID

Jian Li http://orcid.org/0000-0003-2359-238X

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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