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Cell-free styrene biosynthesis at high titers

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ABSTRACT

Styrene is an important petroleum-derived molecule that is polymerized to make versatile plastics, including disposable silverware and foamed packaging materials. Finding more sustainable methods, such as biosynthesis, for producing styrene is essential due to the increasing severity of climate change as well as the limited supply of fossil fuels. Recent metabolic engineering efforts have enabled the biological production of styrene in *Escherichia coli*, but styrene toxicity and volatility limit biosynthesis in cells. To address these limitations, we have developed a cell-free styrene biosynthesis platform. The cell-free system provides an open reaction environment without cell viability constraints, which allows exquisite control over reaction conditions and greater carbon flux toward product formation rather than cell growth. The two biosynthetic enzymes required for styrene production were generated via cell-free protein synthesis and mixed in defined ratios with supplemented L-phenylalanine and buffer. By altering the time, temperature, pH, and enzyme concentrations in the reaction, this approach increased the cell-free titer of styrene from 5.36 ± 0.63 mM to 40.33 ± 1.03 mM, the highest amount achieved using biosynthesis without process modifications and product removal strategies. Cell-free systems offer a complimentary approach to cellular synthesis of small molecules, which can provide particular benefits for producing toxic molecules.

1. Introduction

Metabolic engineering has enabled the production of commodity chemicals and valuable small molecules by genetically modifying microorganisms and overexpressing heterologous enzymes (Keasling, 2010; Liu and Nielsen, 2019; Nielsen, 2001; Stephanopoulos, 1994; Tyo et al., 2007). Target biochemicals, such as butanol (Shen and Liao, 2008) and artemisinin (Ro et al., 2006), are often selected based on their utility for society. An important, large-volume commodity chemical is styrene, which is produced globally on the scale of 30 million tons per year; 60% of the product is utilized for molded or foamed polystyrene and the remainder contributes to industrially important copolymers, such as styrene-acrylonitrile and styrene-butadiene (James and Castor, 2011). However, styrene production is an entirely petroleum-derived process that requires large excesses of steam and is responsible for over 100 million tons of greenhouse gas emissions each year (Wu et al., 1981; Zheng and Suh, 2019). Through metabolic engineering of *Escherichia coli*, biosynthesis of styrene from glucose via the shikimate pathway was demonstrated as a potential, sustainable alternative to traditional styrene synthesis, albeit at low titers up to ~2.6 mM styrene (McKenna and Nielsen, 2011). More recent efforts utilized genome editing and solvent extraction techniques to increase styrene titers in *E. coli* cultures, but the maximum concentration of styrene was still limited by either the cellular toxicity of styrene (Liu et al., 2018) or the need to use product removal strategies to increase styrene recovery (Lee et al., 2019b). Clearly, the cellular toxicity of styrene as well as the necessity of its removal greatly limit *in vivo* biosynthesis titers and the feasibility of commercial styrene

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biosynthesis (Araya et al., 2000). Circumventing cellular toxicity could prove useful for the biochemical production of styrene.

Recent advances in cell-free technologies have showcased their utility for studying biological processes and engineering biological systems (Bogorad et al., 2013; Chen et al., 2020; Garenne and Noireaux, 2019; Jaroentomeechai et al., 2018; Karim et al., 2019a; Kightlinger et al., 2019; Lee et al., 2019a; Martin et al., 2018; Schwander et al., 2016; Silverman et al., 2019, 2020; Thavarajah et al., 2020). For example, several studies have shown that crude extracts contain native metabolic enzymes and cofactor regeneration responsible for robust cell-free protein synthesis (Caschera and Noireaux, 2014; Des Soye et al., 2019; Jewett et al., 2008; Jewett and Swartz, 2004) and activation of key metabolic reactions in the cell-free environment (Dudley et al., 2015; Jewett and Swartz, 2004; Karim and Jewett, 2018; O'Kane et al., 2019). In fact, cell-free systems have been used for biosynthesis of a wide variety of molecules, including 2,3-butanediol (Kay and Jewett, 2015), mevalonate (Dudley et al., 2016), natural products (Goering et al., 2017; Liu et al., 2019; Zhuang et al., 2020), *n*-butanol (Karim et al., 2019a; Karim and Jewett, 2016; Krutsakorn et al., 2013; Reisse et al., 2016), 3-hydroxybutyrate (Karim et al., 2019a), terpenes (Dudley et al., 2019; Korman et al., 2017), and polyhydroxyalkanoates (Kelwick et al., 2018) using processes with purified enzymes or crude cell extracts (Claassens et al., 2019; Rollin et al., 2018). Cell-free systems provide an open reaction environment and rapid design-build-test cycles to reconstitute biosynthetic pathways *in vitro* to compliment and inform metabolic engineering efforts in cells (Bundy et al., 2018; Dudley et al., 2015; Gregorio et al., 2019; Hodgman and Jewett, 2012; Karim et al., 2019a). Equally important, cell-free systems have shown improved tolerance to toxic small molecules compared to living systems (Kay and Jewett, 2019), providing evidence that cell-free biomanufacturing platforms may be advantageous when cellular systems prove impractical.

In this work, we established a cell-free platform for styrene biosynthesis to increase the achievable titer in biological systems by circumventing the toxicity limits of styrene *in vivo*. We constructed this system in two parts. First, we used cell-free protein synthesis in *E. coli* crude extracts to independently express the two non-native enzymes required to convert L-phenylalanine (L-Phe) to styrene: phenylalanine ammonia lyase 2 (PAL2) from *Arabidopsis thaliana* and ferulic acid decarboxylase 1 (FDC1) from *Saccharomyces cerevisiae* (Liu et al., 2018; McKenna and Nielsen, 2011). Next, we combined these extracts enriched with biosynthetic enzymes with L-Phe and buffer to produce styrene *in vitro* (Fig. 1). We further optimized the cell-free system by tuning enzyme ratios, reaction temperature, and reaction pH to reach styrene titers over 40 mM, more than a 2-fold improvement over previously attempted cellular efforts without the use of elaborate recovery techniques, but less than a reported *in vivo* system utilizing *in situ* product recovery (Lee et al., 2019b). We anticipate this work will expand the application space of cell-free systems and spur new research efforts in the metabolic engineering of toxic chemicals.

2. Materials and methods

2.1. Bacterial strains and plasmids

Plasmid propagation was performed in *E. coli* DH5α (NEB), and *in vitro* enzyme expression was performed in cell extract prepared from *E. coli* BL21 Star (DE3) (Life Technologies). Plasmid pSpal2At, containing the PAL2 gene from *Arabadopsis thanliana*, was a gift from David Nielsen (Addgene plasmid 78286). This gene was cloned into the pJL1 plasmid (Addgene plasmid 69496) using Gibson Assembly after PCR amplification with oligonucleotides from IDT (5'-tttaagaaggagatatacat ATGGATCAAATCGAAGCAATG-3' and 5'-tttgttagcagccggtcgacTTAGCA AATCGGAATCGG-3'). The FDC1 gene from *Saccharomyces cerevisiae* was synthesized and cloned into the pJL1 plasmid for expression by Twist Biosciences. Sequences are provided in Supplementary Table 1. Propagated plasmids were purified using the ZymoPURE Plasmid Midiprep Kit (Zymo Research).

2.2. Cell extract preparation

E. coli extracts were prepared as previously described (Karim and Jewett, 2018; Kwon and Jewett, 2015). In brief, BL21 Star (DE3) cells (Life Technologies) grown in 1 L of 2xYTPG media in full-baffle shake flasks at 37 °C. At an OD_{600} of 0.4, 1 mM of IPTG was added to induce T7 RNA polymerase production. Cells were harvested at an OD_{600} of 3.0. Cells were pelleted via centrifugation at 5,000g for 10 min at 4 °C, washed three times with cold S30 buffer (10 mM tris acetate, pH 8.2; 14 mM magnesium acetate; 60 mM potassium acetate; and 1 mM dithiothreitol), flash-frozen with liquid nitrogen, and stored at −80 °C. For lysis, cells were thawed on ice and resuspended in 1 mL of S30 buffer per gram wet cell mass and then lysed in an EmulsiFlex-B15 homogenizer (Avestin) in a single pass at a pressure of 20,000-25,000 psi. Cellular debris was removed by two rounds of centrifugation at 12,000g for 30 min at 4 °C, and the final supernatant was flash-frozen with liquid nitrogen and stored at −80 °C until use.

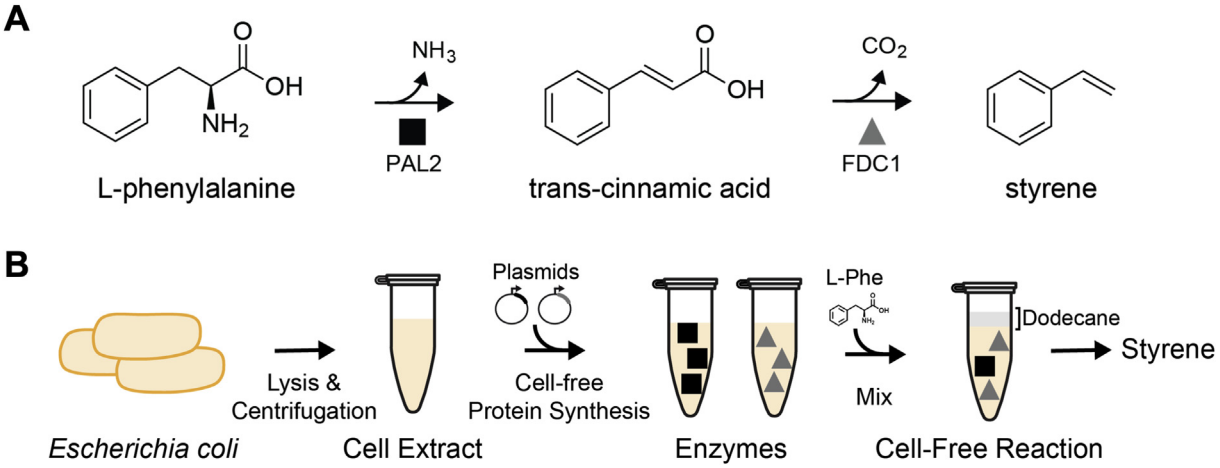

Fig. 1. Enzymatic conversion of L-phenylalanine to styrene. (A) A two-step conversion is catalyzed by phenylalanine ammonia lyase (PAL2) and ferulic acid decarboxylase (FDC1) to produce styrene with ammonia and carbon dioxide as byproducts. **(B)** The enzymes were synthesized with *E. coli* cell extract and mixed with phenylalanine in quantifiable proportions.

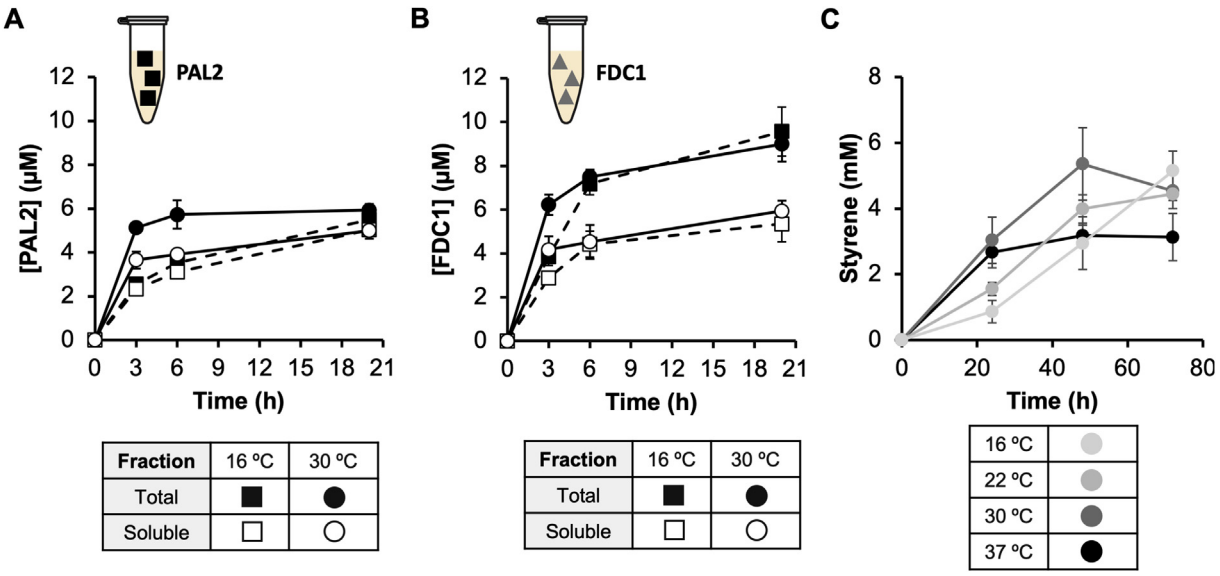

Fig. 2. Expression and activity of the styrene biosynthetic pathway. Expression of PAL2 (A) and FDC1 (B) was assessed by radioactive leucine incorporation, and soluble enzyme fractions were used for subsequent quantification of enzyme concentrations. (C) Combining 0.5 μM PAL2 and FDC1 enabled styrene production, and the maximum titer of 5.36 mM was produced after 48 h at 30 °C. Error bars represent standard deviation of 3 technical replicates.

2.3. Cell-free protein synthesis (CFPS) reactions

CFPS reactions for *in vitro* production of enzymes were assembled with 6 nM template DNA, 10 mg/mL E. *coli* extract, and the cofactors and crowding agents in 57 mM HEPES buffer. These reactions contained 8 mM magnesium glutamate; 10 mM ammonium glutamate; 130 mM potassium glutamate; 1.2 mM adenosine triphosphate; 0.85 mM each of guanosine, uridine, and cytidine triphosphates; 0.034 mg/mL folinic acid; 0.171 mg/mL transfer RNAs; 33.33 mM phosphoenolpyruvate; 2 mM of all 20 canonical amino acids; 0.40 mM nicotinamide adenine dinucleotide; 0.27 mM coenzyme A; 1 mM putrescine; 1.5 mM spermidine (Jewett and Swartz, 2004). The expression level of each enzyme was quantified using radioactive leucine incorporation assays as previously described (Jewett et al., 2008). All reagents and chemicals were purchased from Sigma-Aldrich unless otherwise specified.

2.4. Styrene biosynthesis reactions

Styrene biosynthesis reactions contained 8 mM magnesium glutamate, 10 mM ammonium glutamate, 134 mM potassium glutamate, 100 mM BisTris buffer, 0.5 mM kanamycin, varying concentrations of PAL2 and FDC1 enzymes from CFPS ranging from 0.05 to 1 μM, and 25 or 50 mM L-Phe. A layer of dodecane was placed atop the reaction to capture volatile styrene (Dudley et al., 2019; Lee et al., 2019b).

2.5. Metabolite analysis

Styrene was quantified by diluting 20 μL of dodecane overlay into 200 μL of ethyl acetate containing 0.5 mM *trans*-caryophyllene (Sigma) as an internal standard. 1 μL of this mixture was injected into an Agilent 7890A Gas Chromatograph with 5977A MSD (Agilent, Santa Clara, CA) using an Agilent HP-5MS (30 m length x 0.25 mm i.d. x 0.25 μm film) column with helium carrier gas at constant flow of 1 mL·min-1. The inlet temperature was 70 °C and initial column temperature held at 70 °C for 1 min, increased at 25 °C·min-1 to 250 °C, and maintained at 250 °C for 3 min. The injection volume was 1 μL with a split ratio of 20:1. Extracted ion chromatograms (EIC) for 104 m/z (styrene, peak at 2.97 min) and 133 m/z (caryophyllene, peak at 6.34 min) were integrated using Agilent MassHunter Quantitation Analysis software. Concentrations were determined by use of a standard curve (Supplementary Fig. S1) generated by comparison to styrene (Sigma) standards mixed in dodecane with mock cell-free reactions containing green fluorescent protein in place of the biosynthetic enzymes that were incubated for 24 h (Dudley et al., 2019).

2.6. pH measurements

Samples were analyzed with a Thermo Scientific™ Orion™ ROSS Ultra™ Refillable pH/ATC Triode™. Reactions for which a pH was set prior to reaction start were measured with a mixture of all components except the enzyme-enriched CFPS reactions to avoid premature reaction initiation. Reaction pH was adjusted with glacial acetic acid or 5 N KOH as necessary. Measurements of pH over time were taken after sampling reactions for metabolite analysis.

3. Results & discussion

To establish a cell-free platform for styrene biosynthesis, we took a two-pronged approach: first establishing enzyme synthesis and pathway assembly, and second optimizing physiochemical conditions for improved production.

3.1. Enzyme synthesis and styrene pathway assembly

We first demonstrated the ability to express functional enzymes for the styrene biosynthesis pathway *in vitro* and to reliably capture the volatile styrene product. Cell-free protein synthesis (CFPS) enables rapid production of the enzymes for styrene biosynthesis, PAL2 and FDC1 (Fig. 2A-B). Using CFPS, we produced 4.99 ± 0.36 μM soluble PAL2 and 5.93 ± 0.47 μM soluble FDC1 over the course of a 20-h reaction at 30 °C. In an attempt to express greater soluble fractions of PAL2 and FDC1, we decreased the temperature of the reactions from 30 °C to 16 °C. The decreased temperature increased enzyme solubility at 6 h from ~70% to ~80% for PAL2 and from ~60% to ~67% for FDC1. For both temperatures, a majority (~80%) of the soluble protein made during the reaction is produced by 6 h. Therefore, we chose to run CFPS reactions at 16 °C and stop reactions at 6 h to accelerate the workflow while still obtaining sufficient concentrations of soluble enzymes for all subsequent reactions.

To test for activity, we ran several reactions measuring phenylalanine conversion and styrene production. We confirmed that cell-free

expressed PAL2 alone converts L-Phe to trans-cinnamic acid (Supplementary Fig. S2A) and PAL2 combined with FDC1 produces styrene (Supplementary Fig. S2B) without the addition of typical metabolic cofactors as determined by HPLC. This result also suggests that the extract environment contains or can make sufficient components and cofactors for PAL2 and FDC1 activation (Jun et al., 2018). Additionally, no styrene is produced in cell-free reactions lacking the exogenous enzymes and supplemental L-Phe (Supplementary Fig. S3). Accurate quantification of styrene can be difficult due to its volatility and lack of solubility in aqueous media; therefore, we ran our reactions with a dodecane overlay to capture styrene and detect it by GC-MS. This approach is often used to extract volatile compounds from both *in vivo* (Lee et al., 2019b; Liu et al., 2018) and *in vitro* (Dudley et al., 2019) systems. We found that larger ratios of overlay to reaction volume enabled greater styrene recovery relative to the *trans*-caryophyllene internal standard without inhibiting biosynthesis (Supplementary Fig. S4), likely by reducing styrene evaporation.

After demonstrating enzyme activity and the ability to measure styrene, we investigated the optimal temperature during the biosynthesis segment of the reaction for producing styrene. To do so, we mixed PAL2 and FDC1 after cell-free expression in a second pot reaction at a final concentration of 0.5 μM each. We then incubated reactions containing 25 mM L-Phe at 16, 22, 30, and 37 °C (Fig. 2C). The rate of styrene production was highest at 30 °C and produced a maximum titer of 5.36 mM styrene after 48 h. This titer demonstrates a ~1.5-fold increase over the observed inhibitory concentration of styrene for *E. coli* (Liu et al., 2018), confirming the potential for this cell-free platform to outperform *in vivo* production of toxic compounds.

3.2. Optimization of enzyme ratios and starting substrate concentration

We subsequently stepped through a series of optimizations to improve cell-free styrene synthesis by exploiting the open reaction environment which enables precise control over enzyme concentrations and physiochemical conditions (Karim et al., 2018; Karim and Jewett, 2016; Silverman et al., 2019). Consistent with our previous work, we first normalized the total volume of CFPS added to a biosynthesis reaction by supplementing with CFPS mixtures without plasmid. By doing this, we minimize detrimental effects that additional CFPS volume, specifically the additional small molecules, tends to cause (e.g., decreased final titers of the desired product) (Karim et al., 2018). However, we also wanted to test whether this is universally true or potentially specific to previously studied pathways. We found that styrene biosynthesis reactions with increasing amounts of CFPS fraction result in increased styrene production (Supplementary Fig. S5). Our reactions are less inhibited by the CFPS mixtures likely because we are observing a two-step biosynthesis from L-Phe rather than longer pathways that take advantage of glycolysis that are known to have competition with several pathways branching from pyruvate and acetyl-CoA. Residual L-Phe from added CFPS volume (Supplementary Fig. S3) as well as trace amounts of enzyme factors, such as manganese for FDC1, may contribute to this positive effect. These results suggest that the impact of CFPS reagents should be investigated for each new biosynthetic pathway tested *in vitro*.

We next decided to tune the biosynthetic enzyme ratio by mixing different volumes of cell-free expressed PAL2 and FDC1 in the reactions. We ran 36 unique reaction conditions varying the final PAL2 and FDC1 concentrations from 0 to 1 μM (Fig. 3A). The best condition produced up to 18.03 ± 2.34 mM styrene from 25 mM of added L-Phe. As hypothesized, styrene titer generally increased with increasing enzyme concentrations. However, the best 8 enzyme ratios all produced 16-18 mM styrene, which suggested substrate limitation may prevent higher titers. We doubled the initial concentration of added L-Phe to 50 mM and ran reactions using the top eight enzyme combinations in an attempt to further increase styrene yield. The best reaction condition produced 24.83 ± 0.66 mM styrene with 0.25 μM PAL2 and 1 μM FDC1 (Fig. 3B). Increasing the substrate concentration enabled differentiation between the best conditions with 25 mM L-Phe in Fig. 3A, but the modest 6-7 mM increase in product from 25 mM additional substrate indicated diminishing biosynthetic potential; thus, L-Phe concentrations greater than 50 mM were not examined.

3.3. Optimization of physiochemical conditions

To further optimize the reaction environment for styrene biosynthesis, we explored a range of acidic and alkaline conditions due to the dramatic changes that pH can cause in cell-free systems (Calhoun and Swartz, 2005; Karim et al., 2019b). We tested six different initial pH conditions in reactions for styrene synthesis ranging from pH 5.6 to pH 9.5. Our initial condition began at pH 7.5-7.8 and ended near pH 8 (Fig. 4; dark blue). We observed a strong pH dependence for styrene biosynthesis across the broad pH range, with a maximum titer of 40.33 ± 1.03 mM styrene, indicating ~80% substrate conversion, after 72 h recorded at pH near 7 (Fig. 4A). The most alkaline reaction (pH 9.5) produced less than 1 mM styrene, whereas the most acidic reaction (pH 5.6) produced 3.27 ± 2.85 mM styrene – still greater than the toxicity limit of styrene *in vivo* (Liu et al., 2018; McKenna and Nielsen, 2011). Reaction rates remained steady over 3 full days, which was slower than the reported catalytic rates of purified PAL2 and FDC1 (Fig. 4B) (Cochrane et al., 2004; Payne et al., 2015). Although enzyme behavior can differ *in vitro* between purified systems and our crude cell extracts, the observed pH optimum for styrene biosynthesis lies within a reasonable range based on reported pH optima of 8.4-8.9 and 6.5 for PAL2 and FDC1, respectively (Cochrane et al., 2004; Lin et al., 2015). Additionally, the reaction pH did not significantly change over time when glycolysis was less active (less acetate and lactate), which provided a stable reaction environment without the need for a strong buffer (Fig. 4C).

4. Conclusion

In this study, we demonstrated a substantial increase in styrene titer using a cell-free system compared to *in vivo* biosynthesis through the optimization of reaction temperature, enzyme ratios, and pH. We first determined that PAL2 and FDC1 expressed by *E. coli* CFPS were soluble and active *in vitro* and that, in combination, these enzymes produced the most styrene at 30 °C. Second, we found that cell-free styrene biosynthesis was maximized by combining a low concentration of PAL2 with a high concentration of FDC1, which would maximize conversion of *trans*-cinnamic acid to styrene. Third, we highlighted the pH sensitivity of *in vitro* styrene biosynthesis and found that reactions maintained at a neutral pH produced the highest concentration of monomer. These cell-free reactions achieved a maximum styrene titer of 40.33 ± 1.03 mM (4.20 ± 0.11 g/L). To our knowledge, this represents the highest reported value without process modifications for the continuous removal of accumulated product (Lee et al., 2019b; Liu et al., 2018; McKenna et al., 2015; McKenna and Nielsen, 2011) (Table 1). However, coupling styrene biosynthesis *in vivo* in liter-scale batch reactions with solvent removal processes has led to increased styrene recovery and productivity, achieving ~5 g/L (Lee et al., 2019b). With the demonstrated success of our cell-free styrene biosynthesis platform, implementing more advanced recovery and gas stripping techniques with *in vitro* biosynthesis could result in comparable, or even superior, styrene productivity.

The high titers of the biologically toxic styrene monomer achieved *in vitro* are possible due to the lack of viability constraints in a cell-free system and the ability to finely tune the reaction environment. Although the optimal pH for styrene biosynthesis was within a range consistent with the optima for the PAL2 and FDC1 homologs used, the overall reaction rate appeared much slower than expected (Cochrane et al., 2004; Payne et al., 2015). Despite the apparent decrease in activity of these enzymes *in vitro*, the potential impact of this

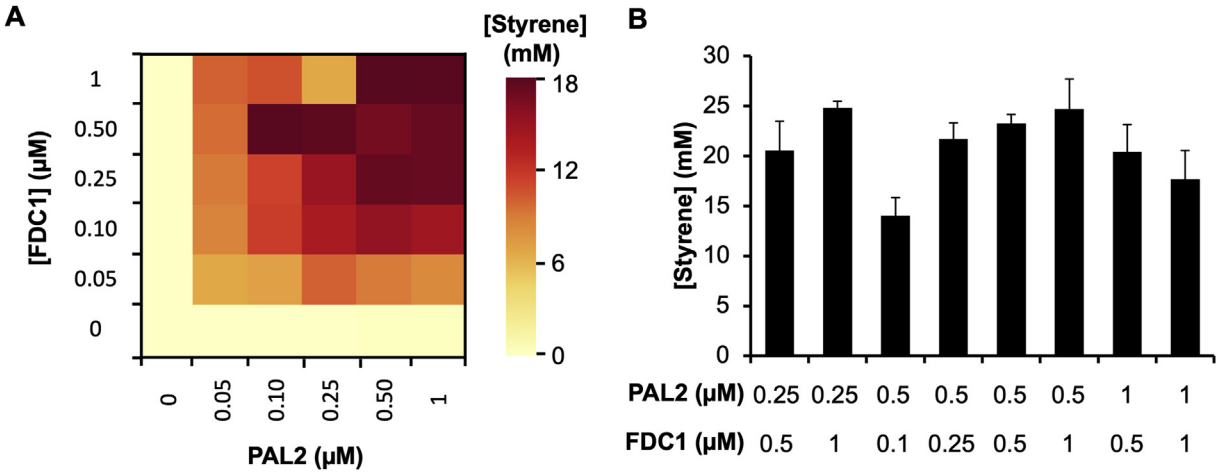

Fig. 3. Modulating enzyme concentrations enhances styrene biosynthesis. (A) Increasing the concentration of each enzyme increased the final titer, as expected. (B) All conditions producing more than 16 mM styrene from 25 mM L-Phe were run with 50 mM L-Phe to identify the optimal enzyme ratio – 0.25 μM PAL2 with 1 μM FDC1. Error bars represent standard deviation of 3 technical replicates.

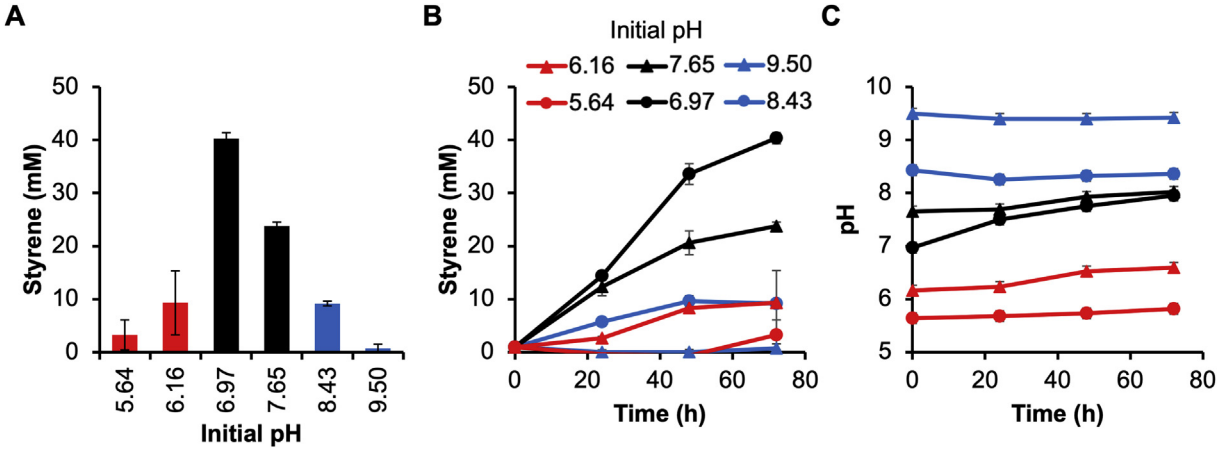

Fig. 4. Styrene biosynthesis is pH dependent. Cell-free reactions containing 0.25 μM PAL2 and 1 μM FDC1 were set with several initial pH values and produced a large range of styrene titers. (A) Endpoint titers after 72 h indicate a clear optimum for reactions starting at neutral pH. (B) The rate of styrene synthesis diminished as the pH deviated from neutral. (C) pH changed little over the course of the reactions. Error bars represent standard deviation of 3 technical replicates.

environmentally friendly, cell-free styrene synthesis approach deserves consideration as an alternative styrene production process. Currently, the primary petroleum-based method of styrene production relies on the catalytic dehydration of ethylbenzene, which requires large amounts of steam and energy (Wu et al., 1981; Zheng and Suh, 2019). While that process itself does not directly produce greenhouse gasses, the process to obtain ethylbenzene requires benzene and ethylene, creating CO_2, CH_4, or other side products during their production from petroleum. We note that CO_2 is released in the biological synthesis pathway as a side-product of FDC1 activity in a 1:1 ratio with styrene. However, the benefits of using much less energy, reduced toxicity of intermediate products, and potentially reduced greenhouse gas emission could make the biochemical production of carbon products like styrene a promising alternative.

Despite the high styrene titer achieved using a cell-free system, the microscale cell-free reactions used in this study cannot feasibly produce styrene in large enough quantities to meet existing needs (James and Castor, 2011). However, with modifications, the high productivity and inherent modularity of this system may make it economically competitive with *in vivo* styrene biosynthesis (Supplemental Fig. S6). Previously developed economic models indicate that styrene can be affordably biosynthesized in a situation where, among other things, (i)

Table 1
Overview of styrene biosynthesis systems. Styrene production based on *E. coli* highlights strategies for overcoming cytotoxicity and product volatility. Studies are listed in order of increasing styrene titer.

Study	System	Modifications	Titer (g/L)	Reaction Length (h)	Productivity (mg/L-h)
McKenna and Nielsen, 2011	*E. coli* batch culture	None	0.260 ± 0.004	29	8.97 ± 0.15
Liu et al. (2018)	*E. coli* batch culture	Isopropyl myristate overlay	0.350	48	7.29
McKenna et al. (2015)	*E. coli* batch culture	Dioctyl phthalate overlay	0.836 ± 0.064	48	17.42 ± 1.33
Lee et al. (2019b)	*E. coli* batch culture	Dodecane overlay	1.7 ± 0.1	50	34 ± 2
This work	Batch *E. coli* cell-free reaction	Dodecane overlay	4.20 ± 0.11	72	58.33 ± 1.53
Lee et al. (2019b)	*E. coli* fed-batch culture	Dodecane overlay and gas stripping	5.3 ± 0.2	60	88.33 ± 3.33

the toxicity limit to *E. coli* is overcome, (ii) small-scale biomanufacturing benefits from an economy of scale, and (iii) the feedstock is glucose (Claypool et al., 2014). At the scale of this method, the cost of a cell-free protein synthesis reaction is currently higher than the cost needed to synthesize proteins *in vivo* (Bundy et al., 2018; Carlson et al., 2012; Silverman et al., 2019). However, larger scales are possible with cell-free protein synthesis scaling linearly up to 100 L (Zawada et al., 2011), and efforts to further reduce the cost of cell-free reactions are ongoing. In addition to the increased styrene productivity as compared to processes without continuous removal of accumulated product reported here, the cost of cell-free styrene biosynthesis could be reduced by lowering the cost of the current feedstock. Alternative approaches could include using native metabolism to convert cheaper and more sustainable substrates such as glucose (McKenna and Nielsen, 2011) or waste streams to the final product, as successfully achieved for limonene (Dudley et al., 2019), or by sourcing the bacterial extract from a strain that overproduces L-phenylalanine. These strategies would reduce the high cost of our current feedstock and potentially increase productivity. Previous work has investigated alternative energy sources for cell-free metabolic pathways (Karim et al., 2018), optimization of energy mix components for cell-free systems (Caschera et al., 2018; Jewett et al., 2008; Jewett and Swartz, 2004), and minimal cell-free energy mixes (Cai et al., 2015) to reduce the cost associated with using phosphoenolpyruvate (PEP) as an energy source. These works, together with future exploration from the cell-free community, will aid in making this and future cell-free systems economically viable.

In summary, the laboratory example of high-titer styrene biosynthesis described here demonstrates the potential of cell-free systems for the production of toxic compounds that are currently produced by petroleum-based processes. Expanding the cell-free approach to producing more value-added chemicals, such as other plastic precursors and biofuels, and increasing the scale of these reactions could spearhead the development of economically viable alternatives to fossil fuel-derived chemicals. Future research utilizing a cell-free approach for styrene biosynthesis should focus on further process modifications to increase product recovery, such as gas stripping or *in situ* product removal, reducing the current cost-per-reaction by investigating alternative feedstocks, and generating usable styrene monomer for future applications like polymerization.

Author statement

W.S.G., B.J.R., and A.K. performed the experiments and analyzed the data. A.S.K. and M.C.J. provided supervisory roles. All authors conceived experiments and wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymben.2020.05.009.

References

Araya, P., Chamy, R., Mota, M., Alves, M., 2000. Biodegradability and toxicity of styrene in the anaerobic digestion process. Biotechnol. Lett. 22, 1477–1481.
Bogorad, I.W., Lin, T.S., Liao, J.C., 2013. Synthetic non-oxidative glycolysis enables complete carbon conservation. Nature 502, 693–697.
Bundy, B.C., Hunt, J.P., Jewett, M.C., Swartz, J.R., Wood, D.W., Frey, D.D., Rao, G., 2018. Cell-free biomanufacturing. Curr. Opin. Chem. Eng. 22, 177–183.
Cai, Q., Hanson, J.A., Steiner, A.R., Tran, C., Masikat, M.R., Chen, R., Zawada, J.F., Sato, A.K., Hallam, T.J., Yin, G., 2015. A simplified and robust protocol for immunoglobulin expression in Escherichia coli cell-free protein synthesis systems. Biotechnol. Prog. 31, 823–831.
Calhoun, K.A., Swartz, J.R., 2005. Energizing cell-free protein synthesis with glucose metabolism. Biotechnol. Bioeng. 90, 606–613.
Carlson, E.D., Gan, R., Hodgman, C.E., Jewett, M.C., 2012. Cell-free protein synthesis: applications come of age. Biotechnol. Adv. 30, 1185–1194.
Caschera, F., Karim, A.S., Gazzola, G., d'Aquino, A.E., Packard, N.H., Jewett, M.C., 2018. High-Throughput optimization cycle of a cell-free ribosome assembly and protein synthesis system. ACS Synth. Biol. 7, 2841–2853.
Caschera, F., Noireaux, V., 2014. Synthesis of 2.3 mg/ml of protein with an all Escherichia coli cell-free transcription-translation system. Biochimie 99, 162–168.
Chen, Z., Kibler, R.D., Hunt, A., Busch, F., Pearl, J., Jia, M., VanAernum, Z., Wicky, B., Dods, G., Liao, H., Wilken, M., Ciarlo, C., Green, S., El-Samad, H., Stamatoyannopoulos, J., Wysocki, V.H., Jewett, M.C., Boyken, S.E., Baker, D., 2020. De novo design of protein logic gates. Science 368, 78–84.
Claassens, N.J., Burgener, S., Vogeli, B., Erb, T.J., Bar-Even, A., 2019. A critical comparison of cellular and cell-free bioproduction systems. Curr. Opin. Biotechnol. 60, 221–229.
Claypool, J.T., Raman, D.R., Jarboe, L.R., Nielsen, D.R., 2014. Technoeconomic evaluation of bio-based styrene production by engineered Escherichia coli. J. Ind. Microbiol. Biotechnol. 41, 1211–1216.
Cochrane, F.C., Davin, L.B., Lewis, N.G., 2004. The Arabidopsis phenylalanine ammonia lyase gene family: kinetic characterization of the four PAL isoforms. Phytochemistry 65, 1557–1564.
Des Soye, B.J., Gerbasi, V.R., Thomas, P.M., Kelleher, N.L., Jewett, M.C., 2019. A highly productive, one-pot cell-free protein synthesis platform based on genomically recoded Escherichia coli. Cell Chem. Biol. 26, 1743–1754 e9.
Dudley, Q.M., Anderson, K.C., Jewett, M.C., 2016. Cell-free mixing of Escherichia coli crude extracts to prototype and rationally engineer high-titer mevalonate synthesis. ACS Synth. Biol. 5, 1578–1588.
Dudley, Q.M., Karim, A.S., Jewett, M.C., 2015. Cell-free metabolic engineering: biomanufacturing beyond the cell. Biotechnol. J. 10, 69–82.
Dudley, Q.M., Nash, C.J., Jewett, M.C., 2019. Cell-free biosynthesis of limonene using enzyme-enriched Escherichia coli lysates. Synth. Biol. 4, ysz003.
Garenne, D., Noireaux, V., 2019. Cell-free transcription-translation: engineering biology from the nanometer to the millimeter scale. Curr. Opin. Biotechnol. 58, 19–27.
Goering, A.W., Li, J., McClure, R.A., Thomson, R.J., Jewett, M.C., Kelleher, N.L., 2017. In vitro reconstruction of nonribosomal peptide biosynthesis directly from DNA using cell-free protein synthesis. ACS Synth. Biol. 6, 39–44.
Gregorio, N.E., Levine, M.Z., Oza, J.P., 2019. A user's guide to cell-free protein synthesis. Methods Protoc. 2.
Hodgman, C.E., Jewett, M.C., 2012. Cell-free synthetic biology: thinking outside the cell. Metab. Eng. 14, 261–269.
James, D.H., Castor, W.M., 2011. Styrene. Ullmann's Encyclopedia of Industrial Chemistry.
Jaroentomeechai, T., Stark, J.C., Natarajan, A., Glasscock, C.J., Yates, L.E., Hsu, K.J., Mrksich, M., Jewett, M.C., DeLisa, M.P., 2018. Single-pot glycoprotein biosynthesis using a cell-free transcription-translation system enriched with glycosylation machinery. Nat. Commun. 9, 2686.
Jewett, M.C., Calhoun, K.A., Voloshin, A., Wuu, J.J., Swartz, J.R., 2008. An integrated cell-free metabolic platform for protein production and synthetic biology. Mol. Syst. Biol. 4, 220.
Jewett, M.C., Swartz, J.R., 2004. Mimicking the Escherichia coli cytoplasmic environment activates long-lived and efficient cell-free protein synthesis. Biotechnol. Bioeng. 86, 19–26.
Jun, S.Y., Sattler, S.A., Cortez, G.S., Vermerris, W., Sattler, S.E., Kang, C., 2018. Biochemical and structural analysis of substrate specificity of a phenylalanine ammonia-lyase. Plant Physiol. 176, 1452–1468.
Karim, A.S., Dudley, Q.M., Juminaga, A., Yuan, Y., Crowe, S.A., Heggestad, J.T., Abdalla, T., Grubbe, W., Rasor, B., Coar, D., Torculas, M., Krein, M., Liew, F., Quattlebaum, A., Jensen, R.O., Stuart, J., Simpson, S.D., Köpke, M., Jewett, M.C., 2019a. In Vitro Prototyping and Rapid Optimization of Biosynthetic Enzymes for Cellular Design. BioRxiv.
Karim, A.S., Heggestad, J.T., Crowe, S.A., Jewett, M.C., 2018. Controlling cell-free metabolism through physiochemical perturbations. Metab. Eng. 45, 86–94.
Karim, A.S., Jewett, M.C., 2016. A cell-free framework for rapid biosynthetic pathway prototyping and enzyme discovery. Metab. Eng. 36, 116–126.
Karim, A.S., Jewett, M.C., 2018. Cell-free synthetic biology for pathway prototyping. Methods Enzymol. 608, 31–57.
Karim, A.S., Rasor, B.J., Jewett, M.C., 2019b. Enhancing control of cell-free metabolism through pH modulation. Syst. Biol. 5.
Kay, J.E., Jewett, M.C., 2015. Lysate of engineered Escherichia coli supports high-level conversion of glucose to 2,3-butanediol. Metab. Eng. 32, 133–142.
Kay, J.E., Jewett, M.C., 2019. A cell-free system for production of 2,3-butanediol is robust to growth-toxic compounds. Metab. Eng. Commun.
Keasling, J.D., 2010. Manufacturing molecules through metabolic engineering. Science 330, 1355–1358.
Kelwick, R., Ricci, L., Chee, S.M., Bell, D., Webb, A.J., Freemont, P.S., 2018. Cell-free prototyping strategies for enhancing the sustainable production of

polyhydroxyalkanoates bioplastics. Syst. Biol. 3.
Kightlinger, W., Duncker, K.E., Ramesh, A., Thames, A.H., Natarajan, A., Stark, J.C., Yang, A., Lin, L., Mrksich, M., DeLisa, M.P., Jewett, M.C., 2019. A cell-free biosynthesis platform for modular construction of protein glycosylation pathways. Nat. Commun. 10, 5404.
Korman, T.P., Opgenorth, P.H., Bowie, J.U., 2017. A synthetic biochemistry platform for cell free production of monoterpenes from glucose. Nat. Commun. 8, 15526.
Krutsakorn, B., Honda, K., Ye, X., Imagawa, T., Bei, X., Okano, K., Ohtake, H., 2013. In vitro production of n-butanol from glucose. Metab. Eng. 20, 84–91.
Kwon, Y.C., Jewett, M.C., 2015. High-throughput preparation methods of crude extract for robust cell-free protein synthesis. Sci. Rep. 5, 8663.
Lee, J., Schwieter, K.E., Watkins, A.M., Kim, D.S., Yu, H., Schwarz, K.J., Lim, J., Coronado, J., Byrom, M., Anslyn, E.V., Ellington, A.D., Moore, J.S., Jewett, M.C., 2019a. Expanding the limits of the second genetic code with ribozymes. Nat. Commun. 10, 5097.
Lee, K., Bang, H.B., Lee, Y.H., Jeong, K.J., 2019b. Enhanced production of styrene by engineered Escherichia coli and in situ product recovery (ISPR) with an organic solvent. Microb. Cell Factories 18, 79.
Lin, F., Ferguson, K.L., Boyer, D.R., Lin, X.N., Marsh, E.N., 2015. Isofunctional enzymes PAD1 and UbiX catalyze formation of a novel cofactor required by ferulic acid decarboxylase and 4-hydroxy-3-polyprenylbenzoic acid decarboxylase. ACS Chem. Biol. 10, 1137–1144.
Liu, C., Men, X., Chen, H., Li, M., Ding, Z., Chen, G., Wang, F., Liu, H., Wang, Q., Zhu, Y., Zhang, H., Xian, M., 2018. A systematic optimization of styrene biosynthesis in Escherichia coli BL21(DE3). Biotechnol. Biofuels 11, 14.
Liu, R., Zhang, Y., Zhai, G., Fu, S., Xia, Y., Hu, B., Cai, X., Zhang, Y., Li, Y., Deng, Z., Liu, T., 2019. A Cell-free Platform Based on Nisin Biosynthesis for Discovering Novel Lanthipeptides and Guiding Their Overproduction in Vivo. bioRxiv.
Liu, Y., Nielsen, J., 2019. Recent trends in metabolic engineering of microbial chemical factories. Curr. Opin. Biotechnol. 60, 188–197.
Martin, R.W., Des Soye, B.J., Kwon, Y.C., Kay, J., Davis, R.G., Thomas, P.M., Majewska, N.I., Chen, C.X., Marcum, R.D., Weiss, M.G., Stoddart, A.E., Amiram, M., Ranji Charna, A.K., Patel, J.R., Isaacs, F.J., Kelleher, N.L., Hong, S.H., Jewett, M.C., 2018. Cell-free protein synthesis from genomically recoded bacteria enables multisite incorporation of noncanonical amino acids. Nat. Commun. 9, 1203.
McKenna, R., Moya, L., McDaniel, M., Nielsen, D.R., 2015. Comparing in situ removal strategies for improving styrene bioproduction. Bioproc. Biosyst. Eng. 38, 165–174.
McKenna, R., Nielsen, D.R., 2011. Styrene biosynthesis from glucose by engineered E. coli. Metab. Eng. 13, 544–554.
Nielsen, J., 2001. Metabolic engineering. Appl. Microbiol. Biotechnol. 55, 263–283.
O'Kane, P.T., Dudley, Q.M., McMillan, A.K., Jewett, M.C., Mrksich, M., 2019. High-throughput mapping of CoA metabolites by SAMDI-MS to optimize the cell-free biosynthesis of HMG-CoA. Sci. Adv. 5.
Payne, K.A., White, M.D., Fisher, K., Khara, B., Bailey, S.S., Parker, D., Rattray, N.J., Trivedi, D.K., Goodacre, R., Beveridge, R., Barran, P., Rigby, S.E., Scrutton, N.S., Hay, S., Leys, D., 2015. New cofactor supports alpha,beta-unsaturated acid decarboxylation via 1,3-dipolar cycloaddition. Nature 522, 497–501.
Reisse, S., Haack, M., Garbe, D., Sommer, B., Steffler, F., Carsten, J., Bohnen, F., Sieber, V., Bruck, T., 2016. In vitro bioconversion of pyruvate to n-butanol with minimized cofactor utilization. Front. Bioeng. Biotechnol. 4, 74.
Ro, D.K., Paradise, E.M., Ouellet, M., Fisher, K.J., Newman, K.L., Ndungu, J.M., Ho, K.A., Eachus, R.A., Ham, T.S., Kirby, J., Chang, M.C., Withers, S.T., Shiba, Y., Sarpong, R., Keasling, J.D., 2006. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440, 940–943.
Rollin, J.A., Bomble, Y.J., St John, P.C., Stark, A.K., 2018. Biochemical production with purified cell-free systems. Biochem. Eng. J.
Schwander, T., Borzyskowski, L.S., Burgener, S., Cortina, N.S., Erb, T.J., 2016. A synthetic pathway for the fixation of carbon dioxide in vitro. Science 354, 900–904.
Shen, C.R., Liao, J.C., 2008. Metabolic engineering of Escherichia coli for 1-butanol and 1-propanol production via the keto-acid pathways. Metab. Eng. 10, 312–320.
Silverman, A.D., Akova, U., Alam, K.K., Jewett, M.C., Lucks, J.B., 2020. Design and optimization of a cell-free atrazine biosensor. ACS Synth. Biol. 9, 671–677.
Silverman, A.D., Karim, A.S., Jewett, M.C., 2019. Cell-free gene expression: an expanded repertoire of applications. Nat. Rev. Genet.
Stephanopoulos, G.N., 1994. Metabolic engineering. Curr. Opin. Biotechnol. 5, 196–200.
Thavarajah, W., Verosloff, M.S., Jung, J.K., Alam, K.K., Miller, J.D., Jewett, M.C., Young, S.L., Lucks, J.B., 2020. A primer on emerging field-deployable synthetic biology tools for global water quality monitoring. npj Clean Water 3.
Tyo, K.E., Alper, H.S., Stephanopoulos, G.N., 2007. Expanding the metabolic engineering toolbox: more options to engineer cells. Trends Biotechnol. 25, 132–137.
Wu, C., Koylinski, T., Bozik, J., 1981. Preparation of styrene from ethylbenzene. US Patent 4 (255), 599.
Zawada, J.F., Yin, G., Steiner, A.R., Yang, J., Naresh, A., Roy, S.M., Gold, D.S., Heinsohn, H.G., Murray, C.J., 2011. Microscale to manufacturing scale-up of cell-free cytokine production–a new approach for shortening protein production development timelines. Biotechnol. Bioeng. 108, 1570–1578.
Zheng, J., Suh, S., 2019. Strategies to reduce the global carbon footprint of plastics. Nat. Clim. Change 9, 374–378.
Zhuang, L., Huang, S., Liu, W.Q., Karim, A.S., Jewett, M.C., Li, J., 2020. Total in vitro biosynthesis of the nonribosomal macrolactone peptide valinomycin. Metab. Eng. 60, 37–44.