Supporting information

Multi-enzymatic cascade reactions with *Escherichia coli*-based modules for synthesizing various bioplastic monomers from fatty acid methyl esters

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. †Hee-Wang Yoo and †Hyunsang Jung equally contributed to this work.

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1. Experimental

1.1 Bacterial strains and materials.
E. coli DH5α was used for genomic manipulations, and BW25113 (ΔfadD, DE3) was used for protein expression and whole-cell reaction to reduce the loss of substrate. Chloroform was obtained from Junsei (Tokyo, Japan), N,N-bis-(trimethylsilyl) trifluoracetamide (BSTFA) and N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), Pyridine, every aliphatic substrate or products without DAME, 8-amino-1-octanol, 10-amino-1-decanol were purchased from Sigma-Aldrich Chemical Co.(St. Louis, MO, USA). Bacteriological agar, Luria Bertani (LB) broth, and terrific broth (TB) media were purchased from BD Difco (Franklin Lakes, NJ, USA). DAME, 8-amino-1-octanol, 10-amino-1-decanol was purchased from Tokyo Chemical Industry (Tokyo, Japan).

1.2 Cultivation and protein expression
For expression of each module system, plasmids harboring the corresponding genes were transformed to E. coli BW25113 (DE3) ΔfadD and the transformants were grown overnight in Luria-Bertani medium containing 100 µg/mL of ampicillin or 50 µg/mL streptomycin for cell-Hm and 100 µg/mL of ampicillin or 50 µg/mL kanamycin for cell-Am and cell-Rm at 37 °C. This seed culture was used to inoculate the Terrific-Broth and the cells were cultivated at 37 °C until the cell concentration reached an optical density with 1 cm of path length, at 600 nm (OD600) of 0.6. UV absorbance at 600 nm was measured with Multiskan spectrum (Thermo scientific, USA). The protein expression was induced by adding 0.05 mM isopropyl-thio-β-D-galactopyranoside (IPTG) for cell-Hm and 0.1 mM for cell-Am and cell-Rm at 18 °C for 18 hours.

1.3 Concurrent reaction system in shake flask.
For concurrent reaction, cells were harvested by centrifugation, washed with potassium phosphate buffer (pH 7.5, 100 mM) or Tris buffer (pH 8.0, 100 mM). The concurrent reaction was initiated by adding appropriate concentration of substrate from 1M stock in ethanol and glucose to 10mL of cell resuspension in a 100 mL flask. In the case of α,ω-diamine compounds, amino donor was supplied and 10 mM MgCl₂ was supplied for the biosynthesis of α,ω-diols. All reactions were carried out at 30°C and 200 rpm, and pH was titrated through 5M NaOH every 6 hours.

1.4 Carbon source optimization for 1,12-diol biosynthesis in shake flask.
For the optimization of carbon source glucose, glycerol and buffer+TB, the cells were diluted in potassium phosphate buffer (pH 7.5, 100 mM) with 30 OD₆₀₀ of cell-Hm and cell-Rm, respectively. The corresponding concentration of glucose, 1%(w/v) glycerol and 1%(w/v) TB were added and reaction was carried out at 30°C, 200rpm for 24 hours. For the comparison of complex media, the cells were diluted in LB, TB and RB respectively for the biosynthesis of 1,12-diol. The reaction conditions to produce 1,12-diol were same with that of concurrent reaction.

1.5 Sequential reaction system in shake flask
In order to produce α,ω-diamines and ω-amino alcohols by sequential reaction, α,ω-diol was produced by cell-Hm and cell-Rm beforehand with 100 mM DAME with 10mL of reaction solution in 100 mL shake flask. After 24 hours, 120 OD₆₀₀ cell-Am, 400 mM benzyl amine and 1% DMSO were added to the reaction media, and the total volume was adjusted to 15 mL. The reaction was conducted at 30°C and 200 rpm for 24 hours.

1.6 pH-controlled preparative scale whole-cell biotransformations
Whole-cell biotransformation in the reactor with constant pH control was performed in 916 Ti-Touch with magnetic stirrer (Metrohm, Switzerland) pH controller. The reaction media for the biosynthesis of each product was the same condition with that of in the shake flask, and the total volume was 40 mL. In the case of Sequential reaction final volume of the reaction solution was 45 mL. The pH was kept constant at 7.5 or 8.0 through a pH
controller, and the pH was adjusted by 2M NaOH. The reaction was carried out at the same reaction conditions as the above experiments.

1.7 Analysis by gas chromatography

The quantitative analysis of products were determined as previously described.\textsuperscript{2-3} Quantitative analysis was performed using a gas chromatography instrument with a flame ionization detector (GC/FID) fitted with an AOC-20i series auto sampler injector (GC 2010 plus Series, Shimadzu Scientific Instruments, Kyoto 604-8511, Japan). A nonpolar capillary column (Agilent J&W HP-5 column 30 m×320 µm i.d. 0.25 µm film thickness) was used for the analyses. To analyze \( \omega \)-HFAs and \( \alpha,\omega \)-diols, the products were extracted with double volume of CHCl\(_3\) after vigorous vortexing for 1 min. After centrifugation, the organic phase was transferred to an Eppendorf tube and samples converted to their trimethylsilyl derivatives by incubation at 50 °C for 20 min with an excess of BSTFA. We used Injector 260°C; flow 1.5 mL/min; Temperature program 50°C/hold 1 min. and 10°C per min to 250 °C / hold 0 min and 5°C per min to 280 °C / hold 5 min respectively. For analysis of \( \omega \)-amino fatty acid and \( \alpha,\omega \)-diamines, samples were evaporated and pyridine was used for dilution of the samples. After 10 min of sonication, the samples were then mixed with an equal volume of MSTFA by vigorous vortexing for 1 min and converted to the trimethylsilyl derivatives by incubation at 50 °C for 20 min. After centrifugation for 3 min, supernatant was injected to GC/FID with Injector 230°C; flow 1.5 mL/min; Temperature program 90°C/hold 0 min; 15°C per min to 200 °C / hold 0 min and 5°C per min to 280°C / hold 5 min respectively. Each peak in GC chromatogram was identified by comparison with that of an authentic sample. In the case of 10-amino decanoic acid, standard curve of 12-amino dodecanoic acid (12-ADA) was used for quantification due to unavailability of the authentic sample. The qualitative analysis of products was determined by GC/MS analysis. GC/MS analysis was carried out using a Finnigan MAT system (gas chromatograph model AG3000 Trace GC Ultra ITQ1100) connected to an ion trap mass detector (Fig. S13†). Each sample preparation and analysis conditions were carried out under the same conditions as GC/FID.

1.8 Purification of 12-ADA and 1,12-diol

The 12-ADA as model compound was isolated and purified by established protocol. After production of 12-ADA in the pH controller with 40 mL reaction, the reaction media were heated at 80°C for 30 minutes to stop the reaction. To remove organic compound such as excess benzyl amine and remained substrate, the heated reaction media was extracted with ethyl acetate (2 \times 40mL) and the aqueous layer was collected. Since 12-ADA was insoluble in ethyl acetate and water, it precipitated and separated together with the aqueous layer. Next, equal volume of tert-butanol, NaOH (1.1 eq) and Di-tert-butyl dicarbonate (Boc-anhydride 2.0 eq) was added to the aqueous layer and stirred at room temperature for 18 hours. After the resulting reaction solution was extracted with ethyl acetate and washed with brine, and dried with MgSO\(_4\). To enhance purity of the purified product, the resulting solution was purified with silica column chromatography with Hexane and ethyl acetate. Finally, the purified Boc-protected 12-ADA was concentrated in vacuum to obtain desired product. The isolation yield was 66.5% (390mg of Boc protected 12-ADA). The purified product was confirmed by \(^1\text{H NMR with CDCl}_3\) (Fig. S14†).

For the 1,12-diol purification, after production of 1,12-diol in the pH controller with 40 mL reaction, the reaction media were heated at 80°C for 30 minutes to stop the reaction. Unlike 12-ADA, 1,12-diol is soluble in ethyl acetate, 1,12-diol was extracted with ethyl acetate three times (3 \times 40mL) and organic layer was collected. After collect the organic layer, silica column chromatography with Hexane and ethyl acetate was performed to obtained 1,12-diol. To increase the purity of 1,12-diol, we gave a gradient to the composition of Hexane : Ethyl acetate, and confirmed 1,12-diol through TLC with KMnO\(_4\) as staining solution. e
## Supporting Table

### Table S1 E-factor calculations for biotransformation

<table>
<thead>
<tr>
<th></th>
<th>12-ADA</th>
<th>1,12-diol</th>
<th>12-aminol</th>
<th>1,12-diamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (g)</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>45</td>
</tr>
<tr>
<td>Cells (g)</td>
<td>0.72</td>
<td>1.08</td>
<td>2.52</td>
<td>2.835</td>
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<tr>
<td>Glucose (g)</td>
<td>9.984</td>
<td>12.480</td>
<td>24.960</td>
<td>12.480</td>
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<tr>
<td>Buffer (g)</td>
<td>4.844</td>
<td>0.656</td>
<td>0.656</td>
<td>0.738</td>
</tr>
<tr>
<td>Substrate (g)</td>
<td>0.852</td>
<td>0.852</td>
<td>0.852</td>
<td>0.852</td>
</tr>
<tr>
<td>Amino donor(g)</td>
<td>0.857</td>
<td>0</td>
<td>1.714</td>
<td>1.929</td>
</tr>
<tr>
<td>([\text{By-products}]_{\text{Max}}) (g) (^a)</td>
<td>0.452</td>
<td>0.634</td>
<td>0.680</td>
<td>0.588</td>
</tr>
<tr>
<td>Sum of waste (g)</td>
<td>46.873</td>
<td>42.37</td>
<td>45.57</td>
<td>51.09</td>
</tr>
<tr>
<td>Product (g)</td>
<td>0.400</td>
<td>0.2180</td>
<td>0.172</td>
<td>0.264</td>
</tr>
<tr>
<td>E-factor</td>
<td>117.18</td>
<td>194.358</td>
<td>264.942</td>
<td>193.523</td>
</tr>
<tr>
<td>E-factor (w/o)</td>
<td>17.183</td>
<td>10.872</td>
<td>32.384</td>
<td>23.068</td>
</tr>
</tbody>
</table>

\(^a\) Since substrate might be degraded or remained as it is (unreactive). So \([\text{By-products}]_{\text{Max}}\)=substrate (g) - product(g)

\(^b\) It is assumed that all the glucose was consumed for generating energy and converted into CO\(_2\) and H\(_2\)O

\(^c\) The amino donor (benzylamine, MW 107) will be converted into benzaldehyde (MW 106) and benzyl alcohol (MW 108) or remained as it is. Molecular weight of benzylamine, benzaldehyde and benzyl alcohol are very similar. Therefore, it is assumed that the sum of them would not be changed during the reaction and is same with initially added amino donor (g)

Therefore, Sum of waste= Water (g) + Cell(g) + Buffer(g) +Initially added amino donor (g) + \([\text{By-products}]_{\text{Max}}\)
Supporting Figures

Fig. S1 Screening of various monooxygenases and ω-TAs in Cell-Hm and Cell-Am, respectively for the concurrent reaction producing 12-ADA: (A) Screening of monooxygenases for the production of 12-ADA using concurrent reaction system employing SpTA as ω-Transaminase in Cell-Am (B) Screening of ω-Transaminases for the production of 12-ADA with PpAlkB as monooxygenase in Cell-Hm. The reaction was performed in potassium phosphate buffer (100 mM, pH 7.5) supplemented with 30 OD_{600} of each cell-module, 1% (w/v) glucose, 20 mM S-(α)-MBA, 10 mM DAME at 30 °C with 200 rpm for 12 hours; Abbreviations: AlkB : monooxygenase from Pseudomonas putida Gpo1; PpAlkB : monooxygenase from Pseudomonas pelgia; CuAlkB : monooxygenase from Curvibacter sp. PAE-UM, CiAlkB : monooxygenase from Citreicella sp. 357; ToAlkB : monooxygenase from Thalassolituus oleivorans; LaAlkB : monooxygenase from Leptospira alstonii; SpTA : ω-transaminase from Silicibacter pomeroyi; VfTA : ω-transaminase from Vibrio fluvialis JS17; CvTA : ω-transaminase from Chromobacterium violaceum; AtTA : ω-transaminase from Agrobacterium tumefaciens. Data represent the means of three experiments and error bars represent the standard deviation.
Fig. S2 Optimization of reaction conditions for the biosynthesis of 12-ADA (A) optimization of cell mass ratio of Cell-H<sup>m</sup> and Cell-A<sup>m</sup> (Total OD<sub>600</sub> of Cell-H<sup>m</sup> and Cell-A<sup>m</sup> was fixed as 60); (B) Optimization of glucose concentration. Each concentration of glucose was fed to the reaction media every 6 hours; (C) Optimization of amino donor; (D) Optimization of reaction buffer system. Data represent the means of three experiments and error bars represent the standard deviation.
Fig. S3 Inhibitory effect of 12-HDA on monooxygenase activity in the biotransformation of DAME. The reaction was performed with 10 mM of DAME substrate and different initial concentrations (0, 5, 10 mM) of 12-HDA in 100 mM potassium phosphate buffer (pH 7.5). Data represent the means of three experiments and error bars represent the standard deviation.

Fig. S4 Production of 1,12-diol using 100 mM of DMAE substrate. The reaction was performed in potassium phosphate buffer (100 mM, pH 7.5) with 30 OD_{600} each of Cell H” and Cell R”, 100 mM DAME, 10 mM MgCl₂, 1% (w/v) glucose at 30 °C with 200 rpm for 24 hours. Green circles indicate 1,12-diol, red circles indicate 12-HDA, purple circle indicates DAME and yellow circle indicates DDDA. Data represent the means of three experiments and error bars represent the standard deviation.
Fig. S5 Optimization of carbon source for the production of 1,12-diol. Comparison of the various glucose concentrations, glycerol and TB+buffer, and potassium phosphate buffer (100 mM, pH 7.5) supplemented with 30 OD$_{600}$ in Cell-H$_m$ and Cell-R$_m$ to 100 mM DAME, 10 mM MgCl$_2$ with 1, 5, 10% (w/v) glucose, 1% (w/v) glycerol, 1% (w/v) TB at 30°C for 24 h. To compare complex media, cells were diluted in each complex media with 100 mM 30 OD$_{600}$ of Cell-H$_m$ and Cell-R$_m$, 100 mM DAME, and 10 mM MgCl$_2$ and the reaction was conducted at 30°C for 24 h. Abbreviations: LB: Luria-Bertani broth, TB: Terrific broth, RB: Riesenberg media. Data represent the means of three experiments, and error bars represent the standard deviation.
Fig. S6 Optimization of glucose concentration for the biosynthesis of 1,12-diol using a biocatalytic cascade employing Cell-H<sup>m</sup> and Cell-A<sup>m</sup>. Optimization of glucose concentration supplied every 6 hours. Each glucose concentration indicates % (w/v) value (glucose was supplied every 6 hours). Glucose optimization was performed in potassium phosphate buffer (100 mM, pH 7.5) with 100 mM DAME substrate at 30 °C with agitation of 200 rpm for 24 hours. Data represent the means of three experiments and error bars represent the standard deviation.

Fig. S7 Optimization of cellmass concentration of Cell-H<sup>m</sup> for the biosynthesis of 1,12-diol. The cell concentration of Cell-R<sup>m</sup> was fixed to 30 OD<sub>600</sub> and only the concentration of Cell-H<sup>m</sup> was changed to OD<sub>600</sub> of 30, 60, 90 and 120. The optimization of cell concentration was performed in potassium phosphate buffer (100 mM, pH 7.5) with 100 mM DAME, 10 mM MgCl₂ at 30 °C with 200 rpm for 24 hours. Data represent the means of three experiments and error bars represent the standard deviation.
**Fig. S8** Sequential reaction to produce 1,12-diamine without DMSO. 100 mM DAME, 10 mM of MgCl$_2$, 30 OD$_{600}$ of Cell-R$^m$, 60 OD$_{600}$ of Cell-H$^m$ potassium phosphate buffer (100 mM, pH 7.5). The reactions were conducted at 30°C and 200 rpm for 24 hours beforehand and 400 mM of benzyl amine, 120 OD$_{600}$ of Cell-A$^m$ was added to the reaction solution after 24 h. The graph depicts the production of fatty acid derivatives after 24 h of addition of Cell-A$^m$. Data represent the means of three experiments and error bars represent the standard deviation.

**Fig. S9** Biosynthesis of 12-aminol and 1,12-diamine from DAME by Cell-H$^m$, Cell-R$^m$, and Cell-A$^m$ in one-pot sequential and concurrent reaction systems. 12-aminol and 1,12-diamine production in concurrent (24 hours after start) or sequential reaction system (24 hours after the addition of amino donor and Cell A$^m$).
Fig. S10 Concurrent reaction for the biosynthesis of 12-aminol and 1,12-diamine with varying carbon chain lengths (C8-C12) using 50 mM benzyl amine in shake flask. Orange square indicates 12-aminol and grey square indicates 1,12-diamine. Data represent the means of three experiments, and error bars represent the standard deviation.

Fig. S11 Subsequent reaction for the biosynthesis 12-aminol and 1,12-diamine with varying carbon chain lengths (C8-C12) using 400 mM benzyl amine in shake flask. The reaction was performed in potassium phosphate buffer (100 mM, pH 7.5) supplemented with 60 OD$_{600}$ Cell-H$^m$ and 30 OD$_{600}$ Cell-R$^m$, 5% (w/v) glucose, 100 mM FAME substrate, 10 mM of MgCl$_2$ at 30°C and 200 rpm 24 hours for 1,12-diol production. After 24 hours, 120 OD$_{600}$ Cell-A$^m$ and 400 mM benzyl amine, 1% DMSO were added to the reaction media for the production of 1,12-diamine. Grey square indicates 1,12-diamine and orange square indicates 12-aminol. Data represent the means of three experiments and error bars represent the standard deviation.
**Fig. S12** pH-controlled preparative scale biosynthesis of fatty acid derivatives. (a) Biosynthesis of 12-ADA in a reactor with pH-stat system. The reaction was performed in Tris buffer (100 mM, pH 8.0) supplemented with 40 OD$_{600}$ of Cell-H, 20 OD$_{600}$ of Cell-A, 100 mM DAME, 200 mM benzyl amine, 0.5 mM of PLP, and 2% (w/v) glucose for every 6 hours at 30°C for 48 hours. (b) Biosynthesis of 1,12-diol in a reactor with pH-stat system. The reaction was conducted in potassium phosphate buffer (100 mM, pH 7.5) supplemented with 60 OD$_{600}$ of Cell-H, 30 OD$_{600}$ of Cell-R, 100 mM DAME, 10 mM MgCl$_2$, and 2.5% (w/v) glucose for every 6 hours at 30°C for 48 hours. Data represent the means of three experiments, and error bars represent the standard deviation.

**Fig. S13** pH-controlled concurrent reaction for the biosynthesis of 12-aminol and 1,12-diamine using 50 mM of benzyl amine. 100 mM DAME, 50 mM of benzyl amine, 0.5 mM of PLP, 10 mM of MgCl$_2$, 1% DMSO, 30 OD$_{600}$ of Cell-R, 60 OD$_{600}$ of Cell H, 120 OD$_{600}$ of Cell A in potassium phosphate buffer (100 mM pH 7.5.). The reactions were conducted at 30°C and 200 rpm. Red circle indicates 12-aminol and black circle indicates 1,12-diamine. Data represent the means of three experiments and error bars represent the standard deviation.
Fig. S14 GC/MS data
Isolation and Purification of 12-ADA from the reaction medium

12-ADA, as a model compound, was isolated and purified by the established protocol. The reaction medium (40 mL) from the pH-stat system was heated at 80°C for 30 minutes to stop the reaction. To remove organic compound such as excess benzylic amine and remained substrate, the heated reaction medium was extracted with ethyl acetate (2 x 40mL) and the aqueous layer was collected. Since 12-ADA is insoluble in ethyl acetate and water, it precipitated and separated together with the aqueous layer.

Next, equal volume of tert-butanol, NaOH (1.1 equiv.) and Di-tert-butyl dicarbonate (Boc-anhydride 2.0 equiv.) was added to the aqueous layer and stirred at room temperature for 18 hours. The resultant solution was extracted with ethyl acetate, washed with brine, and dried with MgSO₄. The resulting solution was purified with silica column chromatography using Hexane and ethyl acetate solvent system. Finally, the purified Boc-protected 12-ADA was concentrated in vacuum to obtain desired product with the isolated yield of 66.5% (390 mg of Boc-protected 12-ADA). The purified product was confirmed by ¹H NMR (Fig. S15†).

¹H NMR spectrum of Boc-protected 12-ADA (400 MHz, CDCl₃): 1.26 (m, 16H), 1.48 (s, 9H), 1.64 (m, 2H), 2.35 (t, J = 12, 2H), 4.17 (t, J = 8, 2H), 4.88 (s, 1H), 10.05 (s, 1H)

Fig. S15 NMR Data of the purified Boc-protected 12-ADA
Isolation and Purification of 1,12-diol from the reaction medium

After successful purification of 12-ADA, we further tried to isolate 1,12-diol. The reaction medium (40 mL) from the pH-stat system was heated at 80°C for 30 minutes to stop the reaction. Since 1,12-diol is soluble in ethyl acetate, 1,12-diol was extracted with ethyl acetate three times (3 x 40mL) and organic layer was collected. The resultant organic layer was further subjected to silica column chromatography using a gradient of Hexane and ethyl acetate solvent system. Finally, the purified 1,12-diol was concentrated in vacuum to obtain a desired product with the isolation yield of 52.5% (114.6 mg of 1,12-diol). The purified product was confirmed by $^1$H NMR (Fig. S16†).

$^1$H NMR spectrum of 1,12-diol (400 MHz, CDCls): 1.24 (s, 16H), 1.37-1.41 (m, 4H), 3.35-3.38 (t, J = 13.04 4H), 4.34 (s, OH)

Fig. S16 NMR Data of the purified 1,12-diol
Reference


