Biotransformation of $p$-xylene into terephthalic acid by engineered *Escherichia coli*

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Terephthalic acid (TPA) is an important industrial chemical currently produced by energy intensive and potentially hazardous $p$-xylene ($p$X) oxidation process. Here we report the development of metabolically engineered *Escherichia coli* system for biological transformation of $p$X into TPA. The engineered *E. coli* strain harbours a synthetic TPA pathway optimized through manipulation of expression levels of upstream and downstream modules. The upstream pathway converts $p$X to $p$-toluic acid ($p$TA) and the downstream pathway transforms $p$TA to TPA. In a two-phase partitioning fermentation, the engineered strain converts 8.8 g $p$X into 13.3 g TPA, which corresponds to a conversion yield of 96.7 mol%. These results suggest that the *E. coli* system presented here might be a promising alternative for the large-scale biotechnological production of TPA and lays the foundations for the future development of sustainable approaches for TPA production.

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Terephthalic acid (TPA) is a large-volume commodity chemical consumed principally as a monomer precursor in the manufacture of polyethylene terephthalate, which finds a broad range of applications in making beverage containers, fibres, films and other applications. Currently, TPA is chemically synthesized through a series of oxidation reactions from p-xylene (pX), a petroleum-derived raw material. Among various chemical methods for TPA synthesis (Supplementary Table 1), the most notable commercial process, known as the Amoco process, employs a liquid-phase aerobic oxidation scheme. Briefly, pX is diluted with glacial acetic acid, and manganese and cobalt salts (for example, acetates) are used as catalysts together with a bromide compound (for example, sodium bromide) added as a promoter. With a typical TPA yield of over 95 mol%, this technology now accounts for nearly all of the TPA produced worldwide.

There are, however, several drawbacks associated with this petroleum-based process, for example, high energy requirement at high temperature (175-225 °C) and pressure (15-30 bar), acetic acid loss by solvent burning, requirement of reactor coating with special alloy material, potential hazard to environment and stratospheric ozone by heavy metal catalysts and bromide derivatives, and formation of 4-carboxybenzaldehyde (4-CBA) as a byproduct. In comparison with chemical processes, biotransformation processes for chemical production offer several advantages such as reaction operation under milder conditions (for example, ambient temperature and pressure), use of fewer and less toxic chemicals and no need to use expensive and often toxic heavy metal catalysts. Given these evident process advantages, we were motivated to produce TPA through whole-cell biotransformation of pX. Our motivation for bio-based TPA was further enhanced by recent development of several processes for biomass-derived pX production (Supplementary Table 2), such as Gevo’s renewable pX from isobutanol, fructose to pX through 2,5-dimethylfuran and bio-based ethylene to pX. Thus, if a microbial strain that can biotransform pX to TPA is developed, bio-based renewable production of TPA can be realized.

There have been several attempts to discover and isolate wild-type bacterial strains from nature that are able to convert pX to TPA (Supplementary Table 3). In addition, a recombinant Escherichia coli harbouring a xylene monooxygenase (XMO) was developed to convert pX to 4-carboxybenzyl alcohol (4-CBAL), followed by a chemical process to oxidize 4-CBAL to TPA. However, the conversion efficiencies reported in the above studies were extremely low. Furthermore, in the case of wild-type bacterial isolates, the underlying catalytic mechanisms, that is, genes and encoded enzymes and pathways responsible for each conversion steps, have not yet been elucidated. Thus, it is necessary to first elucidate and validate each reaction steps of the bioconversion process, which can subsequently be implemented for pathway design, strain development and process optimization towards achieving high TPA yield. Here we report the development of a metabolically engineered E. coli strain capable of oxidizing pX into TPA, based on detailed studies on pathway reactions, and reconstruction of synthetic pathway for TPA production. In addition, TPA production was improved through manipulation of enzyme expression levels for increasing the metabolic flux towards TPA biosynthesis, while reducing byproducts formation, and developing a two-phase partitioning fermentation process.

Results
Designing a synthetic pX to TPA biotransformation pathway.

We first designed a synthetic metabolic pathway converting pX to TPA (Fig. 1), which can be divided into two parts: upstream pathway responsible for converting pX to p-toluic acid (pTA) and downstream pathway responsible for converting pTA to TPA. The upstream pathway is found in the initial three steps of natural degradation of pX (Supplementary Fig. 1a), which are the sequential oxidation of one of the two methyl groups of pX to its corresponding alcohol (p-toluinalcohol, pTALC), aldehyde (p-tolualdehyde, pTALD) and pTA catalysed by XMO, benzyl alcohol dehydrogenase (BADH) and benzaldehyde dehydrogenase (BZDH), respectively. Activities of these enzymes have been identified in a variety of organisms (Supplementary Table 4).

In particular, Pseudomonas putida is a well-known natural degrader of various aromatic chemicals including toluene and xylenes (m- and p-xylene). For the TOL plasmid pWW0 in *P. putida* mt-2 harbours an operon containing the *xylMA*, *xylB* and *xylC* genes encoding XMO, BADH and BZDH, respectively, for initiating the degradation of toluene and xylenes. It has been reported that the *xylMA*-encoded XMO is capable of multi-step oxidation of pX to pTALC and also to pTALD and pTA at less efficiencies. In addition, the *xylB*-encoded BADH was shown to contribute to the backward conversion from benzaldehyde to benzyl alcohol under the physiological condition.

Based on these observations, a synthetic upstream pathway was designed in this study as follows. The *P. putida* F1 *xylMA* genes encoding XMO and *P. putida* KT2440 *xylC* gene encoding BZDH were chosen for the conversion of pX to pTALD and pTALD to pTA, respectively. The reasons why we did not choose *xylB*-encoded BADH are as follows: first, it can result in the backward formation of pTALC from pTALD, which compromises the overall flux for TPA production; second, *xylMA*-encoded XMO can consecutively oxidize pX to pTALD by consuming two molecules of cofactor NADH (together with molecular oxygen), which would allow the whole pX to TPA pathway to be redox neutral (Fig. 1); it is noteworthy that BADH would use NAD+ for the oxidation reaction converting pTALC to pTALD.

The downstream pathway from pTA to TPA can be found from the reactions involved in natural degradation of p-toluene sulfonate and pTA (Supplementary Fig. 1b). The three steps of the downstream pathway are the sequential oxidation of the remaining methyl group of pTA to the corresponding alcohol (4-CBAL), aldehyde (4-CBA) and acid (TPA), catalysed by p-toluene sulfonate monooxygenase (TsaMB), 4-CBAL dehydrogenase (TsaC) and 4-CBA dehydrogenase (TsaD), respectively. These three enzymes are encoded by *tsaMB*, *tscA* and *tscD*, respectively, and are present as an operon in the plasmid pTSA of *Comamonas testosteroni* T-2 (refs 14,15,16). Thus, the downstream pathway converting pTA to TPA was established by employing these three genes. As will be described below, the upstream and downstream pathways were combined to establish a synthetic pathway for the conversion of pX to TPA.

A synthetic pathway for pX to TPA biotransformation.

All the upstream and downstream pathway genes in the synthetically designed TPA pathway were separately cloned and their expression in *E. coli* DH5x were confirmed by SDS–polyacrylamide gel electrophoresis (PAGE) (Supplementary Fig. 2), except for XylA subunit of XMO and TsaMB, whose bands could not be seen in the SDS–PAGE analysis. Thus, enzyme functions were examined by whole-cell bioconversion assays using the resting cells expressing each gene or two genes in the operon. Cells expressing XMO (DH5x harbouring the plasmid pTrcMA) converted pX not only to pTALC but also to pTALD (Supplementary Fig. 3a), which agrees with previous finding. In addition, the result suggested production of pTA, but this observation was not reconfirmed. It is interesting to observe fluctuations of pTALC.
and pTALD concentrations. This phenomenon seems to be due to the interconversion between pTALC and pTALD caused by the combined activities of recombinant XMO and endogenous aldehyde-reducing enzymes17. Cells expressing BZDH (DH5α harbouring the plasmid pTrcC) converted pTALD to pTA, but interestingly, pTALC was formed much more than pTA (Supplementary Fig. 3b). This backward conversion of pTALD to pTALC revealed the existence of the activities of endogenous aldehyde reduction enzymes in E. coli17. Cells expressing TsaMB (DH5α harbouring the plasmid pTrcMB) converted pTA to 4-CBAL (Supplementary Fig. 3c). TsaC and TsaD were deliberately co-expressed by mimicking the natural tsAMBCD operon and the resultant cells harbouring the plasmid pTacCD successfully converted 4-CBAL to TPA (Supplementary Fig. 3d). Having confirmed the correct enzyme functionalities of each catalytic step, the upstream and downstream pathways were separately assembled by constructing plasmids pTrcMAC and pTacMBCD, respectively, and examined for bioconversion of pX to pTA and pTALC to TPA, respectively. Both pathways were confirmed to be functional as we designed (Supplementary Fig. 3e,f).

After validating that both the upstream and downstream pathways of pX to TPA conversion were functional, the whole combined pathway was assembled into E. coli DH5α by transforming both pTrcMAC and pTacMBCD (Fig. 2a; gene expression configuration i, designated as Pi). Then, shake flask cultures were performed to examine conversion of pX to TPA. As pX is nearly insoluble in aqueous medium, highly volatile and toxic to cells18, a specialized scheme for flask cultivation was designed as follows. A screw-cap flask (250 ml) containing 50 ml of culture medium was connected with a small attached vial into which pX was loaded and came into contact with culture medium by evaporation (Supplementary Fig. 4). Cultivation of an engineered DH5α strain harbouring plasmids corresponding to Pi in a flask designed as above produced 34.6 mg l⁻¹ of TPA (a total of 1.7 mg TPA) from 200 μl pX (173.2 mg pX) supplied from the attached vial (Fig. 2b,c). On the other hand, pTA was accumulated to as high as 129.2 mg l⁻¹ (Fig. 2d), which indicates an imbalanced flux through the whole synthetic pathway; obviously, the downstream pathway is a bottleneck.

**Figure 1 | Biotransformation pathway from pX to TPA and the central carbon metabolism of E. coli.** Glucose and pX are transported into the cell from the aqueous and organic (OA) phases, respectively. The synthetic biotransformation pX to TPA is shown on the right side of the cell. The synthetic pathway was established in E. coli through the introduction of two plasmids system shown at the bottom. TPA produced is excreted from cells into the aqueous phase in the form of TPA di-salts at the culture pH of 7.0 employed during the two-phase fermentation. The redox cofactor-related reactions are also indicated. BADH, benzyl alcohol dehydrogenase; BZDH, benzaldehyde dehydrogenase; 4-CBA, 4-carboxybenzaldehyde; 4-CBAL, 4-carboxybenzyl alcohol; NAD⁺, nicotinamide adenine dinucleotide oxidized form; NADH, nicotinamide adenine dinucleotide reduced form; pTA, p-toluic acid; pTALC, p-toluic acid; pTALD, p-toluicdehyde; pX, p-xylene; TPA, terephthalic acid; TsaC, 4-CBAL dehydrogenase; TsaD, 4-CBA dehydrogenase; TsaMB, toluate methylmonooxygenase; XMO, xylene monooxygenase.
transcriptase–quantitative PCR (RT–qPCR) (Supplementary Fig. 5) and the sums of transcript abundance for upstream and downstream pathway genes in each gene expression configuration were illustrated (Fig. 2a, left panel). When plasmids corresponding to \( \Pi \), \( \Pi_2 \) and \( \Pi_3 \) were introduced into \( E. coli \) DH5a, both cell growth and TPA production were variable (Fig. 2b–d). DH5a harbouring plasmids corresponding to \( \Pi_2 \), in which the upstream pathway expression level was enhanced compared with \( \Pi \), produced similar amount of TPA (34.3 mg l\(^{-1}\)) and more pTA (211.5 mg l\(^{-1}\)). When DH5a harbouring plasmids corresponding to \( \Pi_3 \), in which the expression levels of both upstream and downstream pathways were elevated beyond \( \Pi_2 \), was cultured, TPA was produced up to 145.3 mg l\(^{-1}\) without any accumulation of pTA. In DH5a harbouring plasmids corresponding to \( \Pi_4 \), which showed the least expression levels of upstream pathway genes, the TPA titre was only 77.5 mg l\(^{-1}\), despite the highest cell density reached among the four constructed expression systems. Furthermore, it was found that the ratio of downstream pathway expression level to upstream (in short, pathway expression ratio) was inversely correlated to the formation of pTA. For example, no pTA was produced in DH5a harbouring plasmids corresponding to \( \Pi_4 \), which had the highest pathway expression ratio of 1.90. DH5a

Figure 2 | Pathway assembly with modular optimization and transformation of \( pX \) into TPA in shake-flask cultures. (a) Schematic illustration of the constructed plasmids for pathway genes expression (right panel) and the sums of transcript levels of upstream and downstream pathway genes in the four pathway configurations, \( \Pi \), \( \Pi_2 \), \( \Pi_3 \) and \( \Pi_4 \) (left panel). Trc and Tac, IPTG-inducible promoters; pBR322 and p15A, replication origins; half-circle box, ribosome-binding site (RBS); T-shape box, terminator. (b) Cell growth, (c) TPA production and (d) byproduct pTA formation profiles of \( E. coli \) DH5x harbouring \( \Pi \) (red diamond), \( \Pi_2 \) (blue rectangle), \( \Pi_3 \) (green circle) and \( \Pi_4 \) (purple triangle) by flask cultures. Values and error bars represent the mean and s.d. of duplicate experiments.

Figure 3 | Tolerance of \( E. coli \) to various concentrations of TPA disodium salt. The symbols represent the concentrations of TPA disodium salt tested as follows: filled diamond, 0 g l\(^{-1}\); filled rectangle, 5 g l\(^{-1}\); filled triangle, 10 g l\(^{-1}\); filled circle, 20 g l\(^{-1}\); open diamond, 30 g l\(^{-1}\); open rectangle, 40 g l\(^{-1}\); open triangle, 50 g l\(^{-1}\); and open circle, 60 g l\(^{-1}\). Values and error bars represent the mean and s.d. of repeated experiments.
harbouring plasmids corresponding to PiII had the pathway expression ratio of 0.34, leading to pTA detection during the culture but ultimately no pTA remained. However, there was significant pTA formation in DH5α harbouring plasmids corresponding to Pi and PiII because of the relatively lower pathway expression ratios (0.25 and 0.12, respectively). Overall, DH5α harbouring plasmids corresponding to PiII produced TPA to the highest titre of 145.3 mg l⁻¹ (a total of 7.3 mg TPA), a 3.3-fold increase compared with that obtained with a strain harbouring Pi. In addition, accumulation of the major byproduct pTA was eliminated. Using this *E. coli* DH5α strain harbouring plasmids corresponding to PiII, fermentation experiments were performed as described below.

**TPA toxicity to *E. coli***. Before performing fermentations, the TPA toxicity to *E. coli* was first examined, because it has not been evaluated. As TPA solubility is very low in aqueous solution (for example, solubility in water at 25 °C and 1 atm is 0.0017 g per 100 ml)¹⁹, TPA disodium salt (solubility in water at 25 °C and 1 atm is 14 wt%)²⁰ was used to prepare various concentrations of TPA salt solutions, which is the main form of TPA (pKₐ of 3.52 and pKₐ₂ of 4.46) present in the culture medium (pH 7.0). Exposing cells to various concentrations of TPA disodium salts up to 10 g l⁻¹ (equivalent to TPA concentration of 7.9 g l⁻¹) showed only slight reduction in the final optical density (Fig. 3). *E. coli* cells could tolerate up to 40 g l⁻¹ of TPA disodium salts (equivalent to TPA concentration of 31.6 g l⁻¹). At this TPA disodium salt concentration, the final optical density was a half that of the control without TPA disodium salts (Fig. 3). This relatively high TPA tolerance is particularly important, as *E. coli* can potentially be metabolically engineered to overproduce TPA to a high concentration suitable for industrial applications.

**Two-phase fermentation for TPA production from pX**. To overcome the problems (such as volatility, insolubility and toxicity) of feeding pX into the fermentor, where strong agitation and aeration are needed to grow cells, a modified organic/aqueous two-phase partitioning bioreactor system¹⁸ was adopted in this study. Oleyl alcohol (OA; 60 wt%, industrial grade) was selected as an immiscible organic carrier phase for two-phase reactor system, owing to its several advantageous characteristics including biocompatibility with a number of microorganisms including *E. coli*, no degradation or utilization by *E. coli*, good phase stability and low cost²¹. Thus, pX was dissolved in OA and allowed to partition into the aqueous phase with a partition coefficient of 524 as determined previously¹⁸. For example, when 2.1 g pX is dissolved in 0.51 OA, the resulting concentration of pX is 8 mg l⁻¹ in aqueous phase (1)¹¹. All two-phase partitioning fermentations in this study were conducted in 21 MR medium as the aqueous phase and 0.51 OA as the organic phase in which pX was dissolved. Using this two-phase fermentation system, *E. coli* DH5α harbouring plasmids corresponding to PiII was examined for TPA production by feeding 10 and 20 g pX, respectively. Cells were induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at the OD₆₀₀ of ca. 5. OA (0.51) containing pX was started to be fed into the fermentor at the OD₆₀₀ of ca. 10 at a feeding rate of 1.4 ml min⁻¹. When 10 g pX (in 0.51 OA) was used, 2.7 g l⁻¹ of TPA was produced and 1.7 g l⁻¹ of pTA was also accumulated (Supplementary Fig. 6a); the other performance data are summarized (Table 1). In both cases, significant amounts of pX (3.8 and 14.3 g, respectively) were lost by evaporation based on the simple pX mass balance calculations: the total amount of pX provided – (the amount of pX converted to TPA and pTA) – (residual pX in the organic phase).

Next, the two-phase fermentation process was further improved to produce TPA to a higher concentration with higher productivity and conversion yield, while minimizing the formation of byproduct pTA. The two-phase fermentation process was divided into two stages: biomass production stage and pX biotransformation stage. In this manner, batch culture was performed to grow cells to the OD₆₀₀ of ca. 30 and then the nutrient feeding for the DO-stat fed-batch culture was started. At the starting point of nutrient feeding, cells were induced with 1 mM IPTG. For the bioconversion of pX to TPA, pX was started to be fed at the OD₆₀₀ of ca. 40. When 10 g pX (in 0.51 OA) was fed at the same feeding rate as above, 6.7 g l⁻¹ of TPA was produced and only 0.2 g l⁻¹ of pTA was accumulated, giving the overall TPA productivity of 0.278 g l⁻¹ h⁻¹ and conversion yield of 96.7 mol% (Fig. 4). However, when 20 g pX (in 0.51 OA) was fed at the same feeding rate, 5.8 g l⁻¹ of TPA was produced with accumulation of 6.0 g l⁻¹ of pTA, giving the overall TPA productivity of 0.126 g l⁻¹ h⁻¹ and conversion yield of 44.4 mol% (Supplementary Fig. 7). These results indicated that improved TPA production can be achieved by increasing the

| Strain | Condition | TPA (g l⁻¹) | Byproduct* pTA (g l⁻¹) | Overall productivity (g l⁻¹ h⁻¹) | Maximum productivity (g l⁻¹ h⁻¹) | Yield (mol%) |
|--------|-----------|-------------|------------------------|---------------------------------|---------------------------------|-------------|
| DH5α/PiI | 10°, 5°, 10°; Manual 1:4 | 2.743 | 1.688 | 0.043 | 0.226 | 57.1 |
| DH5α/PiI | 20°, 5°, 10°; Manual 1:4 | 1.240 | 2.185 | 0.020 | 0.113 | 31.7 |
| DH5α/PiI | 20°, 30°, 10°; DO-stat 1:4 | 6.662 | 0.184 | 0.278 | 0.748 | 96.7 |
| DH5α/PiII | 20°, 30°, 40°; DO-stat 1:4 | 5.844 | 6.007 | 0.126 | 0.861 | 44.4 |
| DH5α/PiII | 20°, 30°, 40°; DO-stat 0.4 | 6.905 | 1.902 | 0.150 | 0.546 | 74.8 |

OA, oleyl alcohol; pTA, p-tolualic acid; pX, p-xylene; TPA, terephthalic acid.
*Only pTA was detected as the byproduct without other pathway intermediates.
†The amount of pX (g) that was fed into the fermenter.
‡The OD₆₀₀ value at which point cell culture was induced.
§The OD₆₀₀ value at which point pX feeding was started.
||The feeding rate of the OA containing dissolved pX (mL min⁻¹).
OD<sub>600</sub> value before induction and pX feeding. It can also be reasoned that a lower pX feeding rate appears to be more beneficial for pX to TPA conversion. To this end, another two-phase DO-stat fed-batch fermentation was carried out with 20 g pX under the same condition, except for using a reduced pX feeding rate of 0.4 ml min<sup>-1</sup>. This fermentation resulted in the production of 6.9 g<sub>T</sub> of TPA and 1.9 g<sub>T</sub> of pTA, giving the TPA productivity of 0.150 g l<sup>-1</sup> h<sup>-1</sup> and conversion yield of 74.8 mol% (Supplementary Fig. 8). As expected, the TPA titre, yield and productivity were all increased compared with that at a feeding rate of 1.4 ml min<sup>-1</sup>. The conversion yield and overall productivity were both reduced. It can be anticipated that further optimization will allow more efficient production of TPA from pX.

**Discussion**

In this study, we report a biocatalytic process for the oxidation of pX to TPA by developing a metabolically engineered *E. coli* strain harbouring a synthetically designed pathway. Through designing the synthetic upstream and downstream pathways that convert pX to pTA and pTA to TPA, respectively, by optimizing the gene expression levels, TPA could be successfully produced from pX. The proof-of-concept two-phase fermentation results described above suggest that it is possible to efficiently produce TPA from pX with high conversion yield by employing the engineered *E. coli* strain developed in this study. As pX can be derived from biomass<sup>8-10</sup>, TPA can now be produced from biomass rather than fossil resources using the system reported here. Although microbial production of TPA directly from renewable biomass feedstock such as glucose is still not possible, this study provides a hint to future studies on designing a *de novo* biosynthesis pathway from glucose to TPA. Development of an engineered strain that can produce TPA by direct one-step fermentation from carbohydrates will be an interesting next research topic to study.

**Methods**

**Chemicals and bacterial strains.** pX, pTA and 4-CRL were purchased from Sigma-Aldrich. pTALC, pTALD, 4-CRA and TPA were from Acros Organics. OA (60 wt%, industrial grade) was from Tokyo Chemical Industry. *E. coli* DH5α was used as host strain for both gene cloning and pX to TPA conversion. For plasmid construction and propagation, *E. coli* DH5α was routinely grown in Luria–Bertani (LB) broth or on LB plates (1.5%, w/v, agar) supplemented with 50 µg ml<sup>-1</sup> of ampicillin.

**Plasmid construction.** All the DNA manipulations and cloning for pathway construction were according to the standard protocols<sup>12</sup>. The plasmids and primers used in this study are listed in Supplementary Tables 5 and 6, respectively. To construct pTacMA, the xylCMA genes were amplified from the genome of *P. putida* KT2440 using xylC(xylC)-f and xylC(xylC)-r primers, and cloned into pTrc99A vector amplified using pTrc(xylCMA)-f and pTrc(xylCMA)-r primers by Gibson assembly<sup>12</sup>. To construct pTrecC, the xylC gene was amplified from the genome of *P. putida* KT2440 using xylC(xylC)-f and xylC(xylC)-r primers, and cloned into pTrc99A vector amplified using pTrc(xylC)-f and pTrc(xylC)-r primers by Gibson assembly. To construct pTrecMAC, the xylC gene was amplified from pTrc(xylC(xylC))-f and xylC(xylC(xylC(xylC))-r primers, and assembled with pTrecMA linearized by enzyme digest at BamHI site by Gibson assembly. To construct pTrecMB, the tsAMB genes were amplified from the genomic DNA extract of *C. testosteroni* T-2 using the primers tsAMB-f and tsAMB-r, and cloned into pTrc99A vector at EcoRI and BamHI sites. To construct pTacC, the trcC genes were amplified from the genomic DNA extract of *C. testosteroni* T-2 using the primers trcC-f and trcC-r, and cloned into pTrc99A vector at EcoRI and XbaI sites. To construct pTacCMBD, the trcC-tdsMB-terminator cassette was amplified from pTrecMB using trcC-tdsMB-f and trcC-tdsMB-r primers and assembled with pTacC linearized by enzyme digest at BamHI site by Gibson assembly. To construct pTrecMAC-MBD, the RBS-tsAMC cassette amplified from pTrecMB using RBS-tsAMC-f and RBS-tsAMC-r primers, and the RBS-tsAMB cassette amplified from pTrecMB using RBS-tsAMB-f and RBS-tsAMB-r primers were assembled with pTrcCMBD linearized by enzyme digest at EcoRI site by Gibson assembly. To construct pTrecMBD, the RBS-tsAMC cassette was amplified from pTrecMB using RBS-tsAMC-f and RBS-tsAMC-r primers, and assembled with pTrecCMBD linearized by enzyme digest at EcoRI site by Gibson assembly. All constructed vectors were confirmed by colony PCR and DNA sequencing.

**SDS-PAGE analysis.** To identify the overexpression of heterologous pathway enzymes XylA, XylC, TsAM, TsAc and TsdA (Supplementary Fig. 2), *E. coli* DH5α cells harbouring the respective recombinant plasmids were collected (OD<sub>600</sub> 1.0 × 3 ml) from the 10 ml test tube culture with LB medium induced with 1 mM IPTG. Cell pellets were washed with 1 ml of 10 mM Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.2), centrifuged at 16,000 g for 2 min at 4 °C and resuspended in 0.3 ml of the same buffer. Cell lysates of recombinant *E. coli* were obtained by sonication (High-Intensity Ultrasonic Liquid Processors, Sonics & Material Inc., Newtown, CT). Partially disrupted cells were removed by centrifuging the sonicated samples at 16,000 g for 5 min at 4 °C. The supernatants were used for SDS–PAGE analysis. For identification of the membrane-bound subunit XylM, membrane protein fraction was isolated by further centrifugation at 16,000 g for 30 min at 4 °C, followed by resuspension in 0.5 ml of 10 mM Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.2) containing 0.5% (wt/vol) sarcosyl. After incubation at 37 °C for 30 min, insoluble fraction containing membrane proteins was gained by centrifugation at 16,000 g for 30 min at 4 °C. Membrane proteins were finally prepared by washing the insoluble pellet with 10 mM Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.2) followed by resuspending in 30 µl of TE buffer (pH 8.0).

**RT-qPCR analysis.** The relative abundance of heterologous messenger RNAs was determined using RT–qPCR (Supplementary Fig. 5). Total RNA was extracted from the log-phase culture cells using the Fast HQ RNA Extraction Kit (iNtRON Biotechnology). The preparation of complementary DNA and qPCR were performed in a single step by using the One Step SYBR PrimeScript RT–PCR Kit (TaKaRa, Clontech) and on a LightCycler 96 Instrument (Roche Diagnostics), according to the manufacturer’s instructions. The primers used for the genes xylC, xylA, xylC, tsAm, tsAc, tsaB and tsAc were listed in Supplementary Table 6. The 16S ribosomal RNA was used as the reference gene for relative quantification.

**Whole-cell activity assay.** The whole-cell activity assay using *E. coli* resting cells was conducted as follows<sup>11</sup>. Recombinant *E. coli* cells were incubated in 50 ml of LB medium in the presence of antibiotics as described above. At the OD<sub>600</sub> of 0.6, cells were induced by the addition of 1 mM IPTG. The incubation was continued for 4–6 h. When the OD<sub>600</sub> increased to around 2.0, cells were harvested and resuspended in 7 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 1% w/v glucose (Supplementary Fig. 3). Aliquots of 1 ml cell suspension were distributed into 1.5 ml Eppendorf microcentrifuge tubes and horizontally incubated in a shaker incubator at 200 r.p.m. and 30 °C. After 5 min, each respective substrate, pX (100 mg ml<sup>-1</sup>), pTA (200 mg ml<sup>-1</sup>), pTA (200 mg ml<sup>-1</sup>), pTA (200 mg ml<sup>-1</sup>) and 4-CRL were added, and incubated for 30 min at 30 °C. The absorbance was measured at 405 nm.

**Whole-cell activity assay:** The whole-cell activity assay using *E. coli* resting cells was conducted as follows<sup>11</sup>. Recombinant *E. coli* cells were incubated in 50 ml of LB medium in the presence of antibiotics as described above. At the OD<sub>600</sub> of 0.6, cells were induced by the addition of 1 mM IPTG. The incubation was continued for 4–6 h. When the OD<sub>600</sub> increased to around 2.0, cells were harvested and resuspended in 7 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 1% w/v glucose (Supplementary Fig. 3). Aliquots of 1 ml cell suspension were distributed into 1.5 ml Eppendorf microcentrifuge tubes and horizontally incubated in a shaker incubator at 200 r.p.m. and 30 °C. After 5 min, each respective substrate, pX (100 mg ml<sup>-1</sup>), pTA (200 mg ml<sup>-1</sup>), pTA (200 mg ml<sup>-1</sup>), pTA (200 mg ml<sup>-1</sup>) and 4-CRL were added, and incubated for 30 min at 30 °C. The absorbance was measured at 405 nm.

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(280 mg l⁻¹), were added from a stock solution, where PX and pTALD were dissolved in ethanol and pTA and 4-CBAl were dissolved in the assay buffer. The reaction tubes containing the desired concentrations of (280 mg l⁻¹) were transferred into a 300 ml baffled flask containing 50 ml of fermentation medium supplemented with TPA disodium salt at various concentrations and cultivated under 37 °C in a shaken incubator at 200 r.p.m. The medium was prepared from a TPA disodium salt stock solution of 100 g l⁻¹. Control experiments were conducted under the same conditions, except for the TPA disodium salt supplementation. Samples were periodically withdrawn to monitor the cell growth (OD₆₀₀) and the time profiles of cell growth were compared.

TPA toxicity test. The potentially toxic effect of TPA on microbial cell growth was examined using wild-type E. coli strain W3110 in flask culture containing the same medium as described below for TPA production (Fig. 3). Seed cultures were grown in test tubes containing 15 ml of LB medium and transferred into a 250 ml baffled flask containing 10 ml of LB medium and cultivated in a rotary shaker at 200 r.p.m. at 37 °C. After 12 h, 1 ml of seed culture was transferred into 50 ml MR medium in the designed flask at 37 °C under 300 r.p.m. When cells grew up to the OD₆₀₀ of around 0.6, the cell culture was induced with 1 mM IPTG and transferred to a rotary shaker at 30 °C, 200 r.p.m. When necessary, antibiotics were added to the culture medium as described above.

Two-phase partitioning fermentations were carried out in a 6.6 l fermentor (Bioflo 3000; New Brunswick Scientific Co., Edison, NJ) containing 1.8 l of MR medium supplemented with 20 g l⁻¹ of glucose and 10 mg l⁻¹ of thiamine HCl. The MR medium (pH 7.0) contained per litre: 6.67 g KH₂PO₄, 4 g (NH₄)₂SO₄, 1.0 g MgSO₄·7H₂O, 0.8 g citric acid and 5 ml trace metal solution. Glucose, thiamine HCl, MR salt medium, MgSO₄·7H₂O and trace metal solution were sterilized separately. Cells were inoculated from glycerol stock into 25 ml test tubes containing 10 ml LB medium and cultivated in a rotary shaker at 200 r.p.m. at 37 °C. After overnight cultivation at 37 °C and 200 r.p.m., 4 ml of cell culture was transferred into a 500 ml baffled flask containing 200 ml MR medium supplemented with 10 g l⁻¹ of glucose and 10 mg l⁻¹ of thiamine HCl. Cells were cultured for 12 h and inoculated into the fermentor. The culture pH was controlled at 7.0 using 50% ammonia solution. The dissolved oxygen was controlled at 40% (v/v) by flowing 21 min⁻¹ of air and automatically changing the agitation speed from 600 to 1,000 r.p.m. in the preliminary runs. Cells were induced with 1 mM IPTG when the culture pH was close to 7.0. When the OD₆₀₀ value reached ca. 5. When the OD₆₀₀ value reached ca. 10, OA (0.5 l) containing 10 or 20 g PX were fed into the fermentor via a peristaltic pump at indicated rates. When the glucose concentration of the culture broth decreased below 5 g l⁻¹, 50 ml of nutrient feeding solution was manually added to adjust the glucose concentration in the culture broth above 3 g l⁻¹. The feeding medium contained 10 g l⁻¹ of glucose, 10 g MgSO₄·7H₂O, 10 mg thiamine HCl and 5 ml trace metal solution. In the latter runs, batch culture was performed to grow cells to the OD₆₀₀ of ca. 30 and then the nutrient feeding for the DO-stat fed-batch culture was started. At the starting point of nutrient feeding, cells were induced with 1 mM IPTG. When the OD₆₀₀ value reached ca. 40, OA (0.5 l) containing 10 or 20 g PX were fed into the fermentor via a peristaltic pump at indicated rates. The nutrient feeding solution was the same as above.

Analytical procedures. Cell growth was monitored by measuring the optical density at 600 nm (OD₆₀₀) with Ultraspec 3100 spectrophotometer (Amerham Biosciences, Uppsala, Sweden). The concentration of glucose and organic acids (acetate, lactate and formate) were measured by highHPLC (Waters 1515/2414/2797, USA). The following standards were used: glucose, 100 mg l⁻¹; formic acid, 10 mg l⁻¹; acetic acid, 5 mg l⁻¹. TAL and pTALD were dissolved in acetonitrile. The concentration of residual PX in OA during two-phase cultivations was quantified by gas chromatography (7890, Agilent Technologies) equipped with 80/120 Carbopack B AW packed glass column (Supelco, Bellefonte, PA) and a flame ionization detector (Agilent Technologies).

Data availability. The data supporting the findings of this study are available from the authors upon reasonable request.

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Author contributions
S.Y.L. generated the idea. Z.W.L. and S.Y.L. designed the project. Z.W.L. performed experiments and analysed the data. Z.W.L. and S.Y.L. wrote the manuscript.

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Competing interests: Authors declare that they have conflict of interest as the technology described here is patent filed (KR-10-2015-0066686) for potential commercialization.

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