Electronic Supplemental Information†

Adipic acid production from lignin

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Materials and Methods

1. Strains, plasmid construction, and gene replacement

Competent NEB (New England Biolabs, Inc., Ipswich, MA) C2925 and Life Technologies (Grand Island, NY) TOP10 was used for plasmid construction of *cis,cis*-muconate producing and phenol utilizing strains, respectively. NEB 5-alpha F'I^q *E. coli* was used for all remaining plasmid constructions and were grown shaking at 225 rpm, 37°C, in LB Broth (Lennox) containing 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl or on LB plates containing 15 g/L agar, with either 10 µg/mL tetracycline or 50 µg/mL kanamycin. *E. coli* was transformed according to the manufacturer's instructions.

Q5® Hot Start High-Fidelity 2X Master Mix (NEB) and primers synthesized by IDT (Integrated DNA Technologies, Inc., Iowa) were used in all PCR amplification for plasmid construction. Primer sequences are shown in Table S1. Plasmids were assembled using Gibson Assembly® Master Mix (NEB) according to the manufacturer's instructions. The sequences of all plasmid inserts were confirmed using Sanger sequencing (GENEWIZ, Inc., South Plainfield, NJ).

Plasmids for gene replacement were constructed in pCM433 (Addgene Inc., Cambridge, MA)[1] or pK18mobsacB from ATCC (American Type Culture Collection, Manassas, VA)[2], both of which are unable to replicate in *P. putida* and contain antibiotic resistance genes to select for integration of the plasmid into the genome by homologous recombination and *sacB* to counter-select for recombination of the plasmid out of the genome.

The pCM433-based integration vector used to replace *catRBCA* with Ptac:*catA* (pMFL22) was constructed by Gibson assembly of 3 PCR products: LP29 and LP33 were used to amplify the homology region upstream from *catA*, LP30 and LP31 were used to amplify the tac promoter from Sigma pFLAG-CTC, LP32 and LP34 were used to amplify the entire coding region of *catA* including its native RBS. After assembly, the 2.2 kb fragment was amplified by PCR using primers LP29 and LP34, and cloned into the pCM433 vector using NotI sites.

The pK18mobsacB-based plasmid for integration of the phenol monooxygenase genes (pMFL56) was constructed by Gibson assembly of 3 PCR fragments using primers LP53 and LP48 to amplify the *catA* homology region, LP49 and LP50 for amplification of six phenol monooxygenase genes, *dmpKLMNOP* using pVI1261 as the template (provided by Dr. Victoria Shingler from the Department of Molecular Biology at Umeå University)[3], and primers LP51 and LP54 for amplification of the homology region downstream from *catA*. Fragments were then cloned into pK18-mob vector using NotI sites. (ESI Fig. S1B†)

In the plasmid for replacement of *pcaHG* with Ptac:*aroY* (pCJ023), the *aroY* gene (ADF69416) from *Enterobacter cloacae* ATCC13047 was optimized for expression in *P. putida* KT2440 using DNA 2.0's Gene Designer software and synthesized in two overlapping gBlock fragments by IDT (ESI Table S2). The first fragment also contained the tac promoter, which was separated from the initiating ATG by a ribosome binding site with the sequence AGAGGAGGGAGA, designed using the Salis Lab RBS Calculator v1.1[4] at https://salis.psu.edu/software/. These fragments were then assembled by Gibson assembly and Ptac:*aroY* was amplified from this assembly with primers oCJ165 and oCJ166. Approximately 1 kb regions upstream and downstream of *pcaHG* were amplified using oCJ100/oCJ101, and oCJ102/oCJ103, respectively. The upstream region of homology, Ptac:*aroY*, and the downstream region of homology were then assembled into pCM433 linearized with restriction enzymes AatII and SacI (NEB) (ESI Fig. S1A†).

Gene replacement plasmids were transformed into *P. putida* strains by electroporation. LB broth was inoculated to an OD_{600} of about 0.02 and incubated shaking at 225 rpm, 30°C, until an OD_{600} of 0.5 – 0.7 was reached. Cells were then centrifuged at 4°C, washed twice in ice-cold water and once in ice-cold 10% glycerol or 3 mM potassium phosphate (KPi), pH 7.0, before being resuspended in 1/100 of the culture's original volume of 10% glycerol (or 3mM KPi). Cells were then stored at -80°C or transformed by electroporation immediately. For transformation, 5 µL (200 ng – 2 µg) of plasmid DNA was added to 50 µL of the electrocompetent cells, transferred to a chilled 0.1-cm electroporation cuvette, and electroporated at 1.6 kV, 25 uF, 200 ohms. 450 µL SOC outgrowth medium (NEB) was added to the cells immediately after electroporation and the resuspended cells were incubated shaking at 225 rpm, 30°C, for one hour. The entire transformation was plated on LB agar plates containing appropriate antibiotics (30 µg/mL tetracycline for pCM433-based plasmids, 50 µg/mL kanamycin for pK18mobsacB-based plasmids) and incubated at 30°C overnight. Transformants were restreaked for single colonies on LB agar and incubated at 30°C overnight to reduce the possibility of untransformed cells being transferred. For sucrose counter-selection, restreaked transformants were streaked for single colonies on YT+20 or 25% sucrose plates (10 g/L yeast extract, 20 g/L tryptone, 250 g/L sucrose, 18 g/L agar) and incubated at 30°C overnight. *P. putida* KT2440 containing the *sacB* gene can grow, although very slowly, on YT+20% or 25% sucrose media. Therefore, colonies presumed to have recombined the *sacB* gene out of the genome

– those colonies that were larger than most – were restreaked on YT+25% sucrose plates and incubated at 30°C overnight to reduce the possibility that cells that had not recombined would be carried along with sucrose resistant isolates. Colonies from the second YT+25% sucrose plates were subjected to colony PCR to check for gene replacement at both junctions. These isolates were also plated on LB plates containing appropriate antibiotics to ensure that they had lost antibiotic resistance and, thus, represented pure gene replacements. The following designations of strains constructed for this publication are as follows: *P putida* KT2440-MLF30 (Δ*catRBCA*::Ptac:*catA*), *P putida* KT2440-MFL59 (Δ*catRBCA*::Ptac:*catA*:*dmpKLMNOP*) *P. putida* KT2440-CJ103 (Δ*catRBCA*::Ptac:*catA*:*dmpKLMNOP* Δ*pcaHG*::Ptac:*aroY*).

2. Analysis of organic substrates and products

Concentrations of glucose, acetate, *cis,cis*-muconate, and remaining aromatic substrates and products were determined from filtered sample supernatants by high performance liquid chromatography (HPLC) on an Agilent1100 series system (Agilent USA, Santa Clara, CA) utilizing a Phenomenex Rezex RFQ-Fast Fruit H+ column (Phenomenex, Torrance, CA) and cation H+ guard cartridge (Bio-Rad Laboratories, Hercules, CA) operating at 85°C. Dilute sulfuric acid (0.01 N) was used as the isocratic mobile phase at a flow rate of 1.0 mL/min. Refractive index and diode array detectors were used for compound detection. By-products were identified by co-elution at the same retention time with pure compounds as well as having matching spectral profiles as that of pure compounds.

Glucose and nitrogen (as the ammonium ion) was monitored using a YSI MBS 7100 Analyzer (YSI Incorporated Life Sciences, Yellow Springs, Ohio).

The cell density of the cultures was determined by measuring the optical density at 600 nm (OD_{600}) of the culture against an LB or M9 blank on a Beckman DU640 spectrophotometer (Beckman Coulter, Brea CA). In cultures that had turned dark during growth, the OD was calculated by measuring the OD_{600} of the culture and subtracting the OD_{600} of the media following centrifugation to pellet cells.

Supplemental Figures & Tables

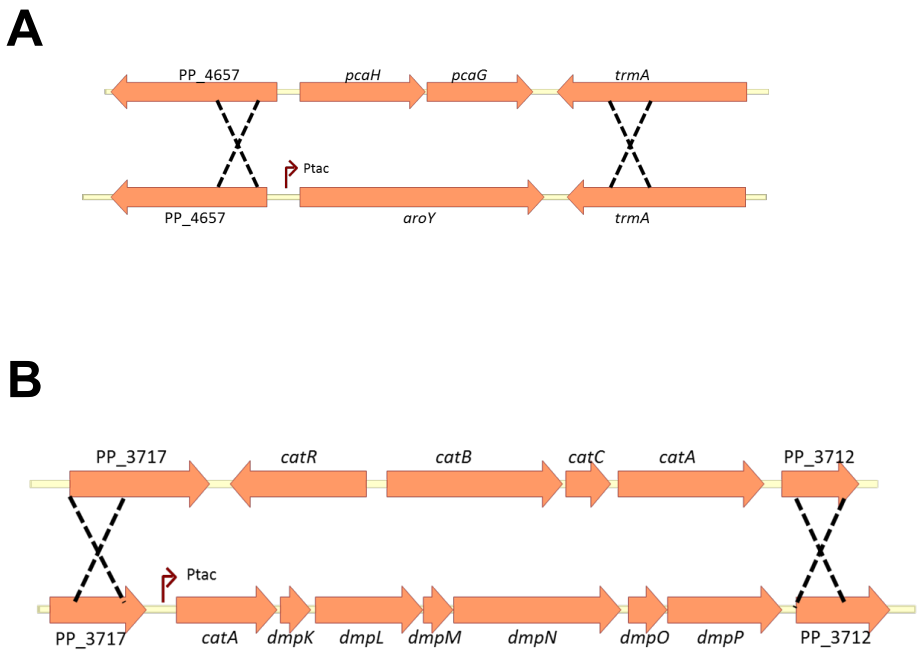

Figure S1. Gene replacement of 3,4 protocatechuate decarboxylase genes (*pcaHG*) with *aroY* from *Enterobacter cloacae* (A) and substitution of *catR* and *catBCA* with *catA* and *dmpKLMNOP* on one operon (B).

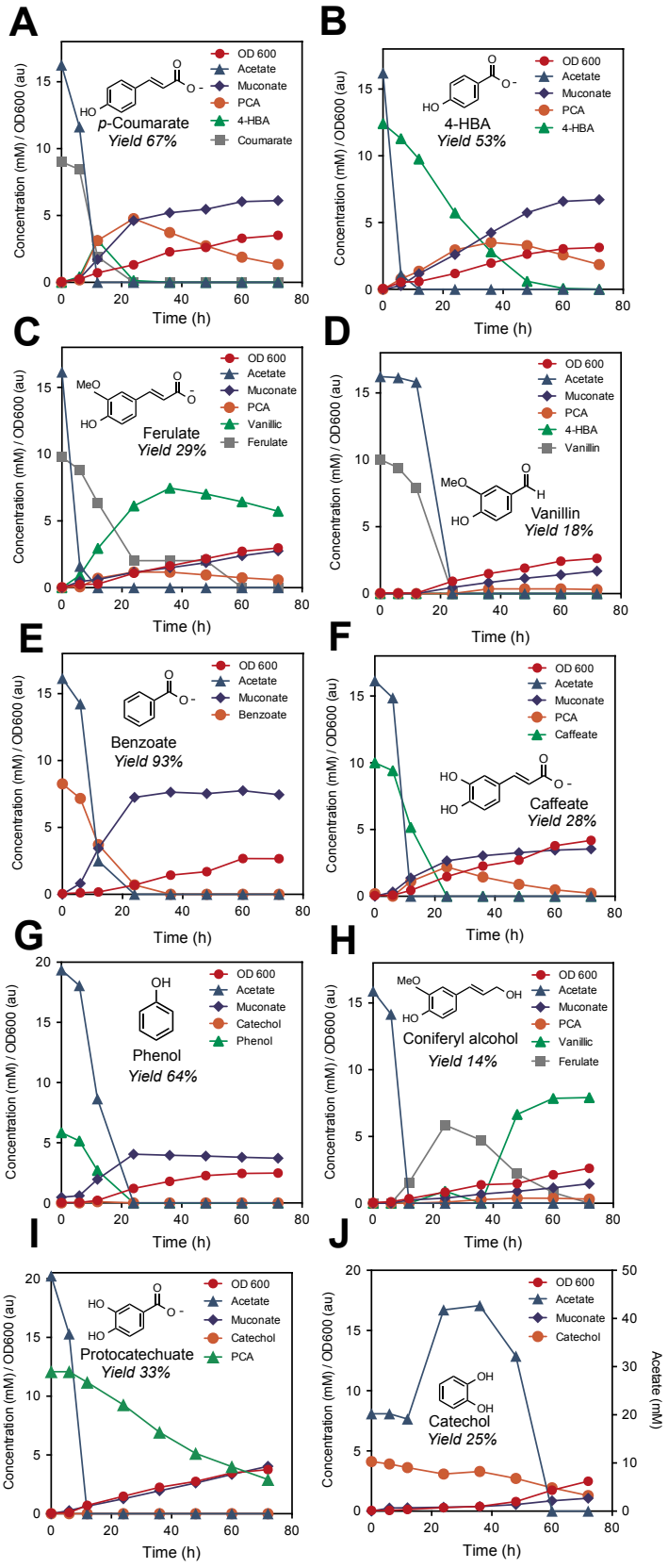

Figure S2. Shake flask experiments with *P. putida* KT2440-CJ103 with model lignin monomers. Experiments were conducted in 125-mL baffled flasks containing 25 mL of M9 media supplemented with 20 mM sodium acetate and 10 mM substrate, with the exception of phenol and catechol, which were added at 5 mM due to growth inhibition at higher concentrations. Additional sodium acetate (20 mM) was added after 12 hours of growth and every 12 hours thereafter. Representative examples of duplicate experiments are shown. Note: Coniferyl alcohol was not monitored by HPLC (panel H).

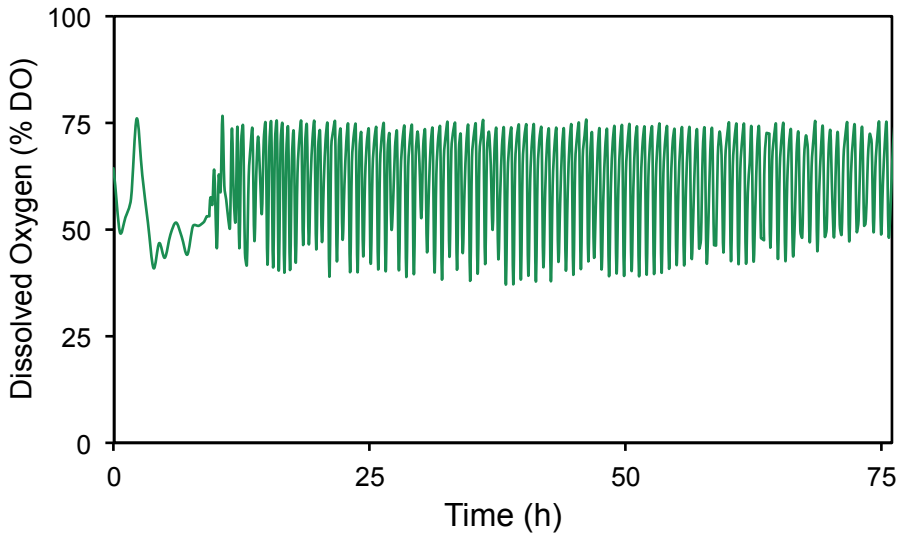

Figure S3. Dissolved oxygen (DO) during course of DO-stat fed-batch cultivation of P. putida KT2440-CJ103. DO oscillation occurred simultaneously with the addition of the glucose:*p*-coumarate:ammonium sulfate feed until process was terminated at 75.5 h and the bioreactor was shut down at 78.5 h.

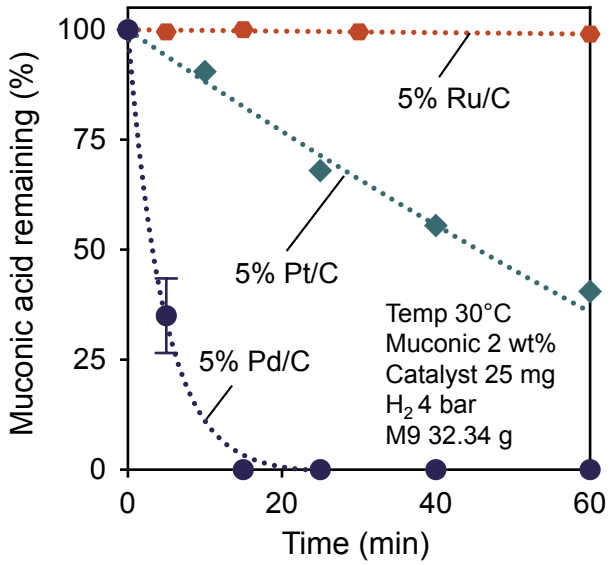

Figure S4. Catalytic screening of muconic acid hydrogenation with noble metal catalysts in M9 media. Hydrogenation reactions were performed using commercial Pd, Pt, and Ru on carbon catalysts (5 wt% metal loading) in M9 media at 30°C. Reaction conditions were as follows: muconic acid 0.66 g, solvent 32.34 g, H_2 pressure 4 bar, catalyst loading 25 mg, stirring 1600 rpm. Reactions were performed in duplicate with error bars indicating conversion standard deviation. Dashed lines indicate pseudo-first order kinetic parameter fits.

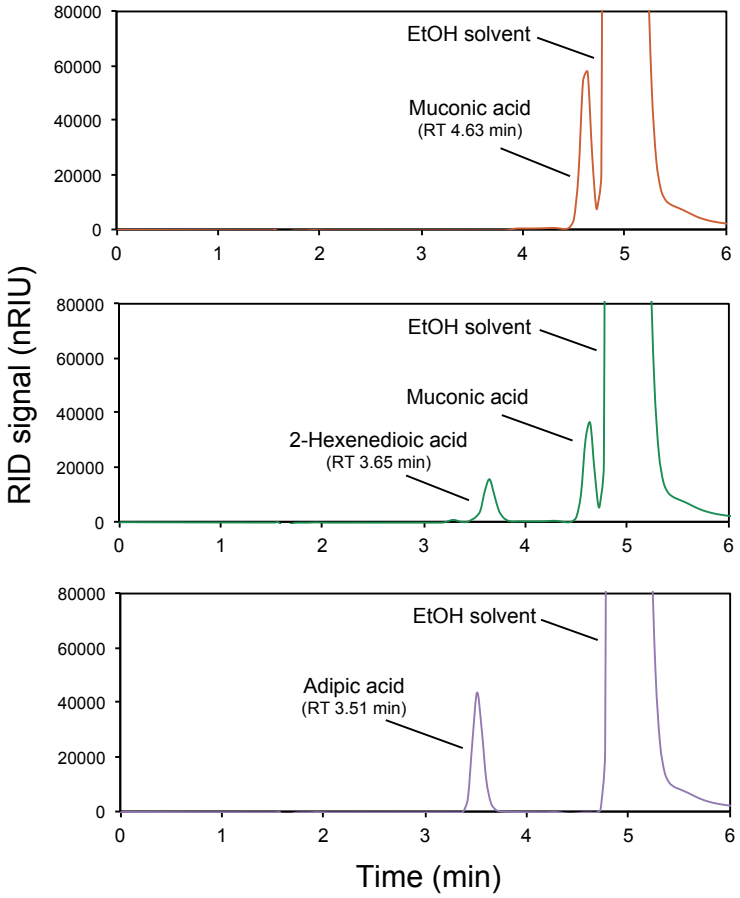

Figure S5. Catalytic conversion of muconic acid to adipic acid, with 2-hexenedioic acid as the primary intermediate. Reaction conditions were as follows: muconic acid 200 mg, ethanol (EtOH) solvent 19.8 g, H_2 pressure 24 bar, catalyst loading 15 mg, stirring 1600 rpm. Product identities were confirmed by GCxGC-TOFMS, as described in Materials and Methods.

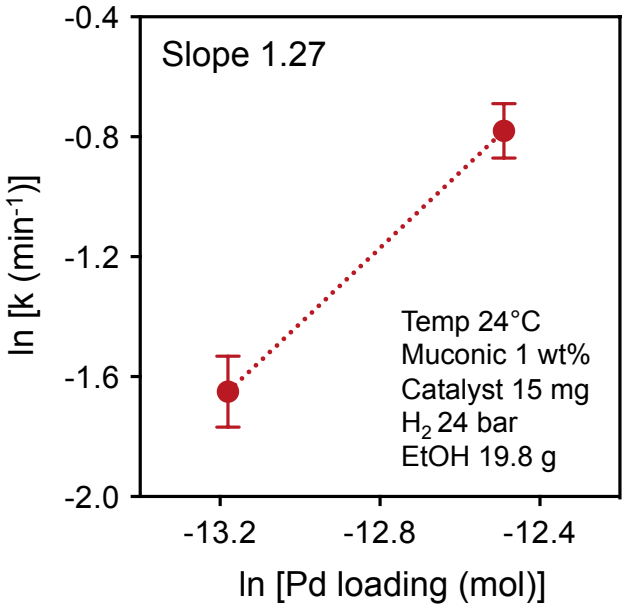

Figure S6. Koros-Nowak criterion to evaluate the influence of mass transfer on muconic acid hydrogenation with Pd/C.[5] A slope of unity supports surface reaction-controlling conditions. Reductions were performed using 1 wt% and 2 wt% Pd/C catalyst synthesized in house. Reaction conditions were as follows: muconic acid 200 mg, ethanol (EtOH) solvent 19.8 g, H_2 pressure 24 bar, catalyst loading 15 mg, stirring 1600 rpm. Rates constants were estimated using pseudo-first order kinetic parameters. Reactions were performed in triplicate, with error bars representing the 95% confidence intervals for the estimated rate constant.

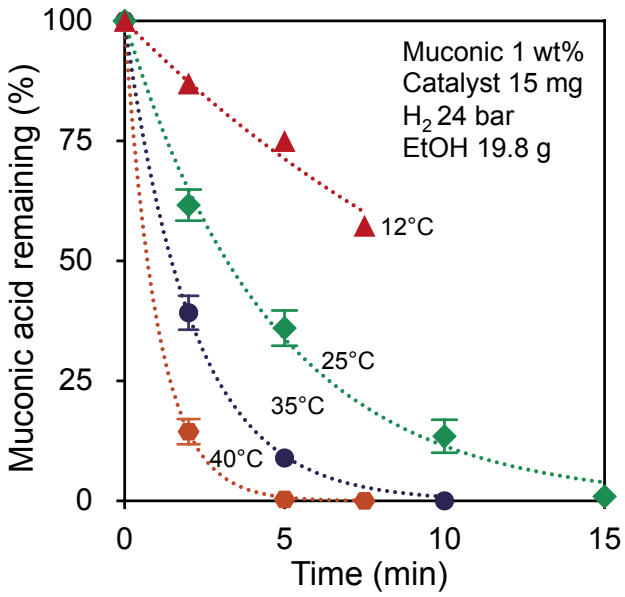

Figure S7. Influence of temperature on the activity of muconic acid hydrogenation with Pd/C. Reductions were performed using a commercial 1 wt% Pd/C catalyst from Alfa Aesar. Reaction conditions were as follows: muconic acid 200 mg, ethanol (EtOH) solvent 19.8 g, H_2 pressure 24 bar, catalyst loading 15 mg, stirring 1600 rpm. Reactions were performed in triplicate with error bars representing conversion standard deviations. Rates constants were estimated using pseudo-first order kinetic parameters, with dashed lines indicating parameter fits.

Table S1. Primers used in construction of gene replacement plasmids	
Primer	**Sequence (5'-3')**
LP29	GCGACACGAAGCTGTATAGCCCTGCCCTATTG
LP30	GCTATACAGCTTCGTGTCGCTCAAGGCG
LP31	ACCTCGTATTGTGTGAAATTGTTATCCGCTCAC
LP32	AATTTCACACAATACGAGGTAAGCACGATG
LP33	CCGCGGCCGCCATCATTGAGACCGCGCG
LP34	CCGCGGCCGCGTGACATAACCTCGAACTCAG
LP48	CAGGACATCATCAGCCCTCCTGCAACGC
LP49	GGAGGGCTGATGATGTCCTGCGCAAGCC
LP50	AACCTCGAACTCAGATGCGCTTGAACAGG
LP51	GCGCATCTGAGTTCGAGGTTATGTCACTGTGATTTTG
LP53	ATCCCCGGGTACCGAGCTCGAATTCATGACCGTGAAAATTTCCCACACTG
LP54	CAGCTATGACCATGATTACGAATTCTTGAATGCCGGCAACCCG
oCJ100	CCGAAAAGTGCCACCTGACGTCGGCCTTGCTGCTGCAG
oCJ101	GCCGCAGCTCGAGATCTGGAATTGTGAGAACGCCTGG
oCJ102	AGATCTCGAGCTGCGGCCGCGGTGAAGCTTGGGGCC
oCJ103	GCTGGATCCTCTAGTGAGCTCACGATTTCCCCATTGCCAG
oCJ165	CCAGGCGTTCTCACAATTCCAGATCTG
oCJ166	GAGCGGCCCCAAGCTTCACCGCGGCCGCTCACTTCTTGTCGCTGAACAGCTCTGG

Table S2. Sequence of synthetic DNA fragments containing the tac promoter and the *E. cloacae aroY* gene optimized for expression in *P. putida* K2440. The start and stop codons of *aroY* are indicated by bold text.

Fragment 1:

CCAGGCGTTCTCACAATTCCAGATCTGAGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAAT
TGTGAGCGGATAACAATTTCACACAGAGGAGGGAGA**ATG**CAGAACCCGATCAACGACCTGCGCTCCG
CGATCGCGCTGCTGCAACGCCATCCGGGTCACTACATCGAAACCGACCACCCGGTCGACCCGAACG
CCGAACTGGCCGGTGTGTACCGCCACATCGGTGCGGGTGGCACCGTGAAACGTCCGACCCGCACCG
GTCCAGCCATGATGTTCAACAGCGTGAAGGGCTACCCAGGCAGCCGCATCCTGGTGGGCATGCACG
CCAGCCGTGAACGTGCCGCCCTGCTGCTGGGCTGCGTGCCAAGCAAACTGGCGCAGCACGTGGGCC
AGGCCGTGAAGAACCCGGTGGCCCCAGTGGTGGTGCCAGCCAGCCAAGCCCCATGCCAAGAACAGG
TGTTCTACGCCGACGACCCGGACTTCGACCTGCGCAAGCTGCTGCCAGCCCCAACCAACACCCCGAT
CGATGCCGGTCCGTTCTTCTGCCTGGGCCTGGTGCTGGCGAGCGACCCGGAAGATACCAGCCTGAC
CGACGTGACCATCCACCGCCTGTGCGTGCAAGAGCGCGACGAGCTGAGCATGTTCCTGGCCGCCGG
TCGCCACATCGAGGTGTTCCGCAAGAAGGCCGAAGCCGCCGGTAAGCCGCTGCCGGTGACCATCAA
CATGGGCCTGGACCCAGCCATCTACATCGGTGCCTGCTTCGAAGCGCCAACCACCCCGTTCGGCTAC
AACGAGCTGGGTGTGGCC

Fragment 2:

ACAACGAGCTGGGTGTGGCCGGTGCCCTGCGTCAGCAACCGGTGGAACTGGTGCAGGGCGTGGCC
GTGAAAGAGAAGGCGATCGCGCGTGCCGAGATCATCATCGAGGGCGAACTGCTGCCAGGCGTGCGC
GTGCGCGAAGATCAGCACACCAACACCGGTCACGCCATGCCGGAATTCCCAGGCTACTGCGGTGAG
GCCAACCCGAGCCTGCCGGTGATCAAGGTGAAGGCCGTGACCATGCGCAACCACGCCATCCTGCAG
ACCCTGGTGGGTCCGGGTGAGGAACACACCACCCTGGCGGGTCTGCCGACCGAAGCCAGCATCCGC
AACGCCGTGGAAGAGGCGATCCCAGGCTTCCTGCAGAACGTGTACGCCCACACCGCCGGTGGCGGT
AAGTTCCTGGGCATCCTGCAGGTCAAGAAGCGCCAGCCGAGCGACGAAGGCCGTCAGGGCCAAGCC
GCCCTGATCGCCCTGGCCACCTACAGCGAGCTGAAGAACATCATCCTGGTGGACGAGGACGTGGAC
ATCTTCGACAGCGACGACATCCTGTGGGCCATGACCACCCGCATGCAGGGCGACGTGAGCATCACCA
CCCTGCCAGGCATCCGTGGCCATCAGCTGGACCCGAGCCAGAGCCCAGACTACAGCACCAGCATCC
GTGGCAACGGCATCAGCTGCAAGACCATCTTCGACTGCACCGTGCCGTGGGCCCTGAAAGCCCGTTT
CGAGCGTGCCCCATTCATGGAAGTGGACCCGACCCCGTGGGCCCCAGAGCTGTTCAGCGACAAGAA
G**TGA**GCGGCCGCGGTGAAGCTTGGGGCCGCTC

Table S3. Shake flask experiments with *P. putida* KT2440-CJ103 using model lignin monomers and supplemental glucose or acetate. Experiments were conducted in 125-mL baffled flasks containing 25 mL of M9 media supplemented with 20 mM sodium acetate or 10 mM of glucose and 10 mM substrate. Muconate yields reported at 72 h.

Substrate	Muconate Molar Yield w/ Glucose (%)	Muconate Molar Yield w/ Acetate (%)
Benzoate	93.4	88.8
Coumarate	90.2	69.2
Ferulate	39.1	29.9

Table S4. Physisorption and chemisorption properties of catalysts evaluated for muconic acid hydrogenation.

Catalyst	Vendor	S_{BET} (m^2/g)	Pore Volume (cc/g)	Dispersion (%)
5% Pt	Sigma	1075	0.71	51
5% Pd	Sigma	750	0.51	22
5% Ru	Sigma	705	0.66	38
1% Pd	Alfa Aesar	825	0.66	19
1% Pd	In-house	781	0.33	13
2% Pd	In-house	760	0.33	12

Table S5. Individual compound molar concentrations and molar closure during catalytic hydrogenation of muconic acid derived from the fed-batch biological conversion.

Time (min)	Muconic (mmol)	2-HDA (mmol)	Adipic (mmol)	Molar Closure (%)
0	57.6	0.0	0.0	100.0
2.5	30.2	45.8	1.2	100.4
5.0	13.0	71.2	5.2	102.8
10.0	2.1	76.1	13.8	103.5
15.0	0.0	61.1	23.5	101.8
25.0	0.0	8.7	50.2	95.8
35.0	0.0	0.0	56.8	98.7

Reaction conditions were as follows: temperature 24°C, muconic acid 200 mg, commercial 1% Pd/C 15 mg, H_2 pressure 24 bar, EtOH solvent 19.8 g. Typical mass closure was >97%, with species molar yields shown in Figure 5.

References

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