Electronic Supplementary Material (ESI) for Green Chemistry.

Supporting Information

Integrated electrosynthesis and biosynthesis for the production of adipic acid from lignin-derived phenols

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1. Experimental section

All potentials refer to Ag/AgCl sat. KCl reference electrode (0.197 V vs. standard hydrogen electrode (SHE)).

Chemicals and electrode materials

All chemicals were of at least analytical grade. All solutions were prepared with de-ionized water (Milli-Q IQ 7000, Merck KgaA, Darmstadt, Germany). H_2SO_4 and phenol were supplied by Merck KGaA (Germany). Cyclohexanol (99%) was purchased from Alfa Aesar Th. Geyer GmbH, 2-methoxycyclohexanol (97%) from Thermo Scientific Th. Geyer GmbH, 2-methoxycyclohexanone (97%) from Sigma-Aldrich® Th. Geyer GmbH, methoxycyclohexane (98%) from abcr GmbH, and (1S, 1S)-*trans*-1,2-cyclohexandiol (99%) and *cis*-1,2-cyclohexandiol (99%) were purchased from Th. Geyer GmbH. 2-methoxyphenol (98+%, guaiacol) was supplied by Thermo Scientific Th. Geyer GmbH, and 2,6-dimethoxyphenol (≥98%, syringol) and pyrocatechol (≥99%) were purchased from Sigma-Aldrich® (Germany). LB-media (Carl Roth, Germany), kanamycin (Carl Roth, Germany), gentamycin (Carl Roth, Germany) and streptomycin (Merck, Germany).

Supported catalysts Pt/C (10wt%), Pt/C (20wt%), Pt(0) ENCAT(TM) 40, Pd/C (10wt%), Ru/C (5wt%), Rh/C (5wt%), Pt/Al_2O_3 (5wt%), Pd/Al_2O_3 (10wt%), Ru/Al_2O_3 (5wt%), and Nickel on Silica Gel/Alumina (~65% Ni) were purchased from Sigma-Aldrich® Th. Geyer GmbH (Germany). Lead foil 0.25 mm (99.95%), silver foil 0.1 mm (99.99%), and platinum foil 0.1 mm (99.99%) were supplied by (Goodfellow, UK). Platinized titanium was purchased from Umicore, Schwaebisch Gmuend, Germany. Al oxide and Ti oxide were purchased from Thermo Fisher Scientific Inc, CA, USA.

Media preparation

For the agar plates and the liquid complex-media, LB-media (Carl Roth, Germany) was used. The solutions were autoclaved at 121 °C for 20 min. Agar plates were additionally in the liquid media with kanamycin (50 µg mL^{-1}), streptomycin (100 µg mL^{-1}), and gentamycin (50 µg mL^{-1}). The antibiotic stock-solutions were sterile filtered (Filtropur S, PES, 0.2 µm, Sarstedt, Germany) before adding to the media. One litre M9* medium contained 9.0 g KH_2PO_4, 20.34 g Na_2HPO_4, 1.0 g NH_4Cl, and 0.5 g NaCl. pH was adjusted to pH 7.1 using NaOH (10 M). After sterilization, the medium was supplemented with 2 ml of a 1 M $MgSO_4$ solution and 1 mL trace element solution US* (Bühler et al., 2002)[†]. Cells were grown in M9* medium supplemented with 1 % (w/v) glucose as the sole carbon source. Kanamycin (50 µg mL^{-1}), streptomycin (100 µg mL^{-1}), and gentamycin (50 µg mL^{-1}) were added when appropriate.

Electrochemical hydrogenation of aromatics

All experiments were carried out under potentiostatic control by a potentiostat/galvanostat (VSP 150 BioLogic Science Instruments, France) using a three-electrode setup consisting of a working electrode (WE), a Ag/AgCl sat. KCl reference electrode (RE, SE 11, 0.197 V vs. SHE, Xylem Analytics Germany Sales GmbH & Co. KG/Sensortechnik Meinsberg, Germany) and a platinum counter electrode (for details on electrode size see below). The electrochemical system was operated as two chamber electrochemical cell with the cathode and anode chambers interfaced via an ion exchange membrane (fumasep FKS-PET-130, FUMATECH BWT GmbH, Bietigheim-Bissingen, Germany). All experiments were conducted at room temperature (~24 °C), using a phosphate buffer solution (1M KH_2PO_4/K_2HPO_4) as supporting electrolyte (SE). Previous to every experiment, the electrodes were subjected to mechanical and electrochemical pre-treatment, as follows:

Mechanical pretreatment: An emulsion of water (200 µL) with Al oxide and Ti oxide (50 mg) was prepared. The suspension was transferred to a nylon/diamond pad (polishing kit PK-4, Bioanalytical, IN, USA), and electrodes were cleaned using rotating movements for 2 min. After washing the paste off the electrodes, they were covered with water and ultrasonicated at 35 kHz for 30 s (Super RK103H, Sonorex, Germany). After washing three times with water, electrodes were drained and subjected to electrochemical pretreatment.

Electrochemical pretreatment: After the mechanical pretreatment, electropolishing of the WE in the SE (60 mL) was performed using cyclic voltammetry (CV) from E_1 = −0.6 V to E_2 = 2.0 V at a scan rate of v = 100 mV s^{-1} for 20 cycles while stirring continuous. After CV, the electrodes were washed with water.

Electrochemical reduction of aromatic compounds

The electrochemical reduction of aromatics was performed in a divided system. For the cathode material screening were used Pb (1.0 x 1.0 cm, 2.0 cm^2 geometric area of both faces), Ag (1.0 x 1.0 cm, 2.0 cm^2), Pt-Ti (1.0 x 1.0 cm, 2.0 cm^2), and Pt (1.0 x 1.0 cm, 2.0 cm^2), as monolithic electrodes. The suspended heterogeneous catalysts were used in combination with Pt (1.0 x 1.0 cm, 2.0 cm^2), 0.66 mol% of catalyst (according catalyst loading) for phenol and 1.0 mol% of catalyst for the electrochemical hydrogenation of the artificial aromatic mixture (ArMix).

The cathode chamber was separated via a custom-made glass tube interfaced by an ion exchange membrane. The working volume of the cathode chamber was 60 mL of a mixture of aromatic compound(s)/SE, at a final concentration of 0.1 M for phenol hydrogenation, and 0.16 M for the hydrogenation of the ArMix (constituted of Syringol, Guaiacol, Phenol and Catechol, in a molar ratio of 1/1.6/4/4)[‡]. The anode chamber consisted of a platinum electrode (Pt, 2 x (1.0 x 1.0 cm), 4.0 cm^2 geometric area of both faces) immersed in 10 mL of the corresponding SE. The distance between both electrodes was kept at 1.0 cm. The electrochemical hydrogenation was carried out under potentiostatic control at a fixed potential of -1.6 V. Reactions were performed for 22 h, and samples were taken from the working chamber every hour for the first 5 hours. The pH and conductivity of the cathode chamber were measured at the beginning and at the end of the experiments using a

[†] Bühler, B., Witholt, B., Hauer, B., Schmid, A. "Characterization and application of xylene monooxygenase for multistep biocatalysis"*Appl. Environ. Microbiol.*, **2002**, *68*, 560-568 (https://doi.org/10.1128/AEM.68.2.560-568.2002).

[‡] As shown in Katahira, R. *et. al. ACS Sustain. Chem. Eng.*, **2016**, *4*, 1474–1486 (https://doi.org/10.1021/acssuschemeng.5b01451), different experimental conditions for lignin depolymerization yield a different composition and molar ratio of the monomers in the mixture. Herein, we adjusted the molar ration of the artificial mixture according the solubility of the different monomers in the reaction medium.

SevenExcellence S470 (Mettler-Toledo, Greifensee, Switzerland) with an InLab Micro Pro pH electrode and an InLab 710 conductivity electrode (both Mettler-Toledo, Greifensee, Switzerland). Both electrodes were calibrated with commercial buffer solutions (Mettler-Toledo, Greifensee, Switzerland).

Organism and preculture

Pseudomonas cultivations were carried out in baffled Erlenmeyer shaking flasks with a liquid volume of maximally 20 % of the total volume in a Celltron shaker (Infors HT, Bottmingen, Schweiz) at 30 °C and 150 rpm. *Pseudomonas* was cultivated in an LB preculture for ~24 h, from which M9* media was inoculated (100µL) and incubated for another 24 h. This pre-culture was used to inoculate the M9* media at a starting concentration of 0.01 g L^{-1}.

Bioreactor system

Bioreactor experiments were conducted in bubble column reactors (Fig. S1). The reactors consist of a 775 mL glass column as the main vessel (Fig S1; 3), with a height of 50 cm, a diameter of 5 cm, and a working volume of 500 mL. These columns were placed in a heated water bath (Fig S1; 4), and the temperature was regulated by a water heater (Fedour, China) and distributed by an aquarium pump (Cokdez, China). Sterile filtered (Midisart™ 2000 PTFE Air Filter, 0.2 µm, Satorius, Germany) air was controlled at 1 L min^{-1} by gas flowmeters (Shiwaki, China) (Fig S1; 5) and supplied to the bioreactors. Heterologous gene expression was induced with 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) (Glentham Life Sciences, England) and m-Toluic acid (Sigma Aldrich, United States). The substrate (Fig S1; 1) was continuously pumped at 41.3 µL min^{-1} by a multichannel cassette pump (Watson-Marlow pumps group, United Kingdom) (Fig S1; 2).

Figure S1. Experimental setup of bubble column-bioreactors. 1: Substrate feedstock; 2: Pump with feeding line; 3: Bubble columns with 500 mL working volume; 4: Water bath with temperature regulation at 30 °C; 5: Gas supply line for 1 L min^{-1} sterile filtered air.

Bioreactor experiments

Bioreactor experiments in the bubble columns consist of two process steps. In the initial growth phase, the bioreactors were inoculated with the cell suspension from the M9* preculture at 0.01 g L^{-1}. This growth phase was performed overnight until the biomass reached ca. 0.2 g L^{-1}. Then, induction was performed by adding both inducers (IPTG and m-Toluic acid) to initiate the heterologous gene expression. In the second phase, the biotransformation was started after 4 h of induction by continuously feeding the electrochemical solution at a 41.3 µL min^{-1}. Two different solutions from the electrochemical conversion were used as feedstock-solution. The first electrochemical solution mainly consists of cyclohexanol (28 mM). In contrast, the composition of the second electrochemical solution consists of syringol (3.6 mM), cyclohexanol (28 mM), 2-Methoxycyclohexanol (isom.) (10.8 mM), (1S,2S)-2-Methoxycyclohexanol (2.8 mM), 2,6-dimethoxycyclohexan-1-ol (isom.) (1.4 mM), cis-1,3-Cyclohexanediol (13.6 mM), trans-1,1-Cyclohexanediol (11.6 mM). After 5 hours, the substrate feeding was stopped, and the reactors were kept running overnight. The final liquid sample was taken after 20 h of biotransformation. In case of intense foaming during reactor cultivations, 50 to 100 µL of antifoam A (Sigma-Aldrich, United States) was added.

GC–MS for quantification of electrochemical hydrogenation of aromatic compounds

Samples from the electrochemical reactor were taken before, during (one sample every hour for the first 5 hours) and after the electrolysis of the aromatic compound(s). Samples were prepared for analysis by diluting aliquots of acidified water (1mL H_2SO_4 at 1000 mL milliQ water; pH=2) with a dilution factor 1:50. Samples taken during and after the electrolysis were filtered with 0.2 µm PTFE filter previous dilution for analysis.

Diluted samples were analyzed via gas chromatography–mass spectrometry (GC–MS) (GC 7890A and MSD 5975C InertXL, Agilent, Santa Clara, USA), using a DB-FATWAX capillary column (30 m × 250 µm × 0.25 µm, Agilent, Santa Clara, USA) with Helium as carrier gas and 1-Butanol as internal standard. The initial temperature was 50°C (held for 2 min) and it was increased to 250°C with a temperature ramp of 15 K min^{-1}. Phenol, 2-methoxyphenol (guaiacol), 2,6-dimethoxyphenol (syringol), cyclohexanone, 2-methoxycyclohexanone, 2-methoxycyclohexanol, cyclohexanol and methoxycyclohexane were identified using retention times and mass spectra of pure reference compounds, and quantified using external standards (5

levels). The concentration of 2-methoxycyclohexanol (isomer), 2-hydroxy-cyclohexanone, 2,6-dimethoxycyclohexan-1-ol (isomer 1), 2,6-dimethoxycyclohexan-1-ol (isomer 2), *cis*-1,2-cyclohexanediol and *trans*-1,2-cyclohexanediol that were not calibrated, was estimated using an average response factor of all calibrated cyclohexanols and cyclohexanons.

HPLC for quantification of catechol and adipic acid

Catechol present in the ArMix (diluted in water to 1:500), as well as the produced adipic acid (diluted in acidic water to 1:1) were quantified by HPLC. Samples from the anodic chamber after the electrolysis (diluted in water to 1:20) were subjected to qualitative HPLC analysis.

For samples from the ArMix and from the anodic chamber, HPLC analysis was performed at 208 nm with the gradient shown in **Table S1** with a constant flow of 0.8 mL min^{-1}. Measurements were carried out at room temperature for 30 min. Between each sample the column was re-equilibrated for 5 min to the starting measurement conditions. Phenol, catechol, syringol and guaiacol were identified using retention times of pure reference compounds and quantified with external standards. Thus, a 7-point calibration in the range 0.2–12 mg L^{-1} was performed based on a mixture of the mentioned compounds.

Table S1. HPLC gradient flow with acetonitrile + 0.01% H_2SO_4 (solvent A) and water + 0.01% H_2SO_4 (solvent B).

time [min]	solvent A [%]	solvent B [%]
0.00	2.0	98.0
3.00	2.0	98.0
25.00	95.0	5.0
29.00	95.0	5.0
30.00	2.0	98.0

HPLC analysis for adipic acid quantification was performed at 208 nm with the gradient shown in **Table S2** with a constant flow of 0.8 mL min^{-1}. Measurements were carried out at room temperature for 20 min. Between each sample the column was re-equilibrated for 5 min to the starting measurement conditions. Adipic acid was identified using retention time of pure reference compound and quantified with external standards. Thus, a 7-point calibration in the range 0.1–5 mg mL^{-1} was performed based on a pure adipic acid standard.

Table S2. HPLC gradient flow with acetonitrile + 0.01% H_2SO_4 (solvent A) and water + 0.01% H_2SO_4 (solvent B).

time [min]	solvent A [%]	solvent B [%]
0.00	10.0	90.0
7.00	10.0	90.0
15.00	95.0	5.0
19.00	95.0	5.0
20.00	10.0	90.0

Biomass analysis

From the liquid samples, biomass concentration was calculated using $OD_{450\,nm}$measurement. The correlation factor of biomass dry weight to $OD_{450\,nm}$ was determined during one experiment. Therefore, the cell suspension was centrifuged at 4500 rpm for 3 minutes. The supernatant was disposed, and the cell pellet was washed twice with deionized water by resuspension and centrifugation. The pellets were dried for 24 h at 105 °C and the weight was measured. In comparison with the $OD_{450\,nm}$ values of the samples, a mean correlation factor of 0.188 was calculated.

Coulombic efficiency (CE)

The coulombic efficiency (CE) was calculated by relating the charge needed to synthesize the desired product(s) (Q), which is derived from the produced amount of substance n within the electrolyzed solution (the volume was assumed to be constant) determined by GC-MS or HPLC analysis, in relation to the total charge (Q_{total}):

$$CE = \frac{Q}{Q_{total}} \times 100\ \%$$

with

$$Q = n \times z \times F$$

where n is the amount of substance in mol, z is the number of transferred electrons per molecule, and F = 96485 C mol^{-1} is the Faraday constant. Q_{total} can be derived from the integrated current (i) of the electrochemical hydrogenation:

$$Q_{total}(t) = \int_0^t i(t)dt$$

Statistical analysis

All experiments were done in at least three independent replicates ($n \geq 3$). In this regard independent replicates means that the phenol/ArMix solution, the following electrolysis of the solution, the biosynthesis of adipic acid, and finally the sample preparation was performed fully independent for each single replicate. All values are given as the mean ± standard deviation if not stated otherwise.

2. Overview of the performance for the electrochemical hydrogenation of aromatics

Figure S2 gives an overview about the performance of the hydrogenation of phenol for the three most promising catalyst of the tested one, as well as the hydrogenation of an artificial aromatic mixture (ArMix) using Rh/C (5wt%) as catalyst. In the case of phenol reduction performed with Pt/C (20wt%) and Rh/C (5wt%), the mass balance is consistently reaching about 70%, and the electrochemical hydrogenation of the ArMix with Rh/C (5wt%) yielded a mass balance of about 90%. HPLC analysis showed that over the time, a small portion of phenol and constituents of the ArMix migrated to the anode chamber and consequently were oxidized. The lack of HPLC calibration for the oxidation products, did not allow to quantify them and include the results in the mass balance calculations.

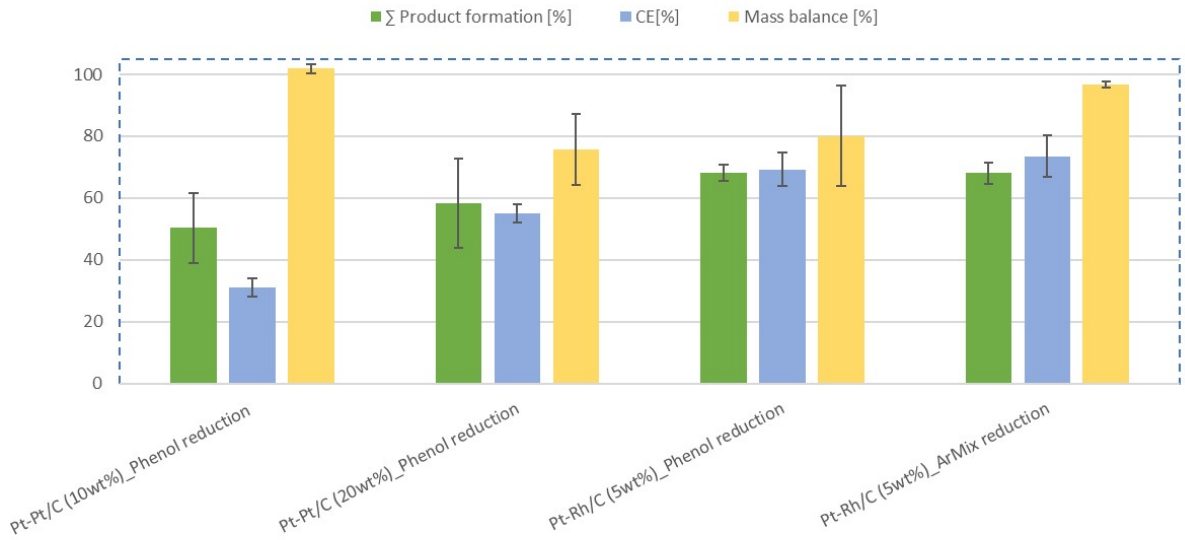

Figure S2. Overview of the performance for the electrochemical reduction of phenol (using three promising catalysts) and an artificial aromatic mixture (ArMix, with Rh/C (5wt%)). Reactions were performed using a two-chamber electrochemical cell in a three-electrode setup consisting of a working electrode (cathode) set at -1.6 V vs the Ag/AgCl sat. KCl reference electrode and a counter electrode (Pt). In all cases a phosphate buffer solution (1M KH_2PO_4/K_2HPO_4) was used as supporting electrolyte. Results correspond to 5h reaction time and standard deviation is indicated (n=3).

The potential of -1.6 V was chosen to ensure the formation of enough of H_2 (gas or in ststu nascendi) to be available at ambient pressure for the suspended catalysts to perform the hydrogenation of the aromatic substrates. As shown in **Figure S3**, for those systems which indeed allowed for the formation of cyclohexanol, i.e., suspended catalysts on activated carbon (e.g. Rh/C (5wt%)), cyclic voltammetry did not provide substantial valuable information regarding onset potential of the hydrogenation reaction, because the hydrogenation (reduction) of the aromatic substrates is not taking place on the surface of the monolithic electrode.

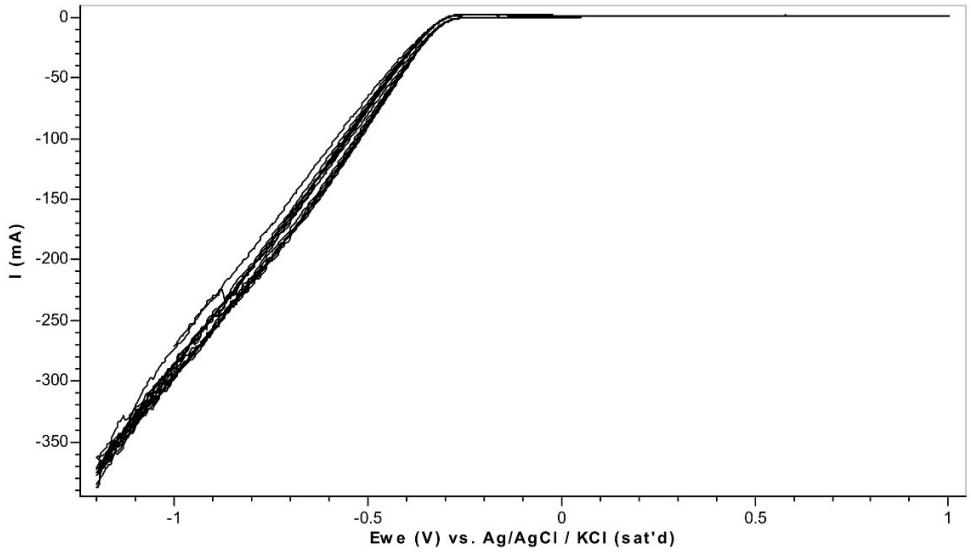

Figure S3. Collection of cyclic voltammetry scans for a solution of 100 mM phenol in 1M KH_2PO_4/K_2HPO_4 as supporting electrolyte. Monolithic Pt(s) (1.0 × 1.0 cm, 2.0 cm^2) was used as working electrode. Cyclic voltammetry (CV) from E_1 = −1.2 V to E_2 = 1.0 V at a scan rate of v = 100 mV s^{-1} for 15 cycles while stirring continuous.

3. E-factor and benchmarking analysis

E-factor calculations: The E-factor (or environmental factor) determines the amount of waste generated per product equivalent and has been evaluated as[§, **]:

$$E = \frac{m_{waste}}{m_{product}}$$

Table S3: E-factor calculations for single and mixed substrates for this study

Components	Single substrate [g]	Mixture [g]
Na_2HPO_4	10.17	10.17
KH_2PO_4	4.50	4.50
NaCl	0.25	0.25
NH_4Cl	0.50	0.50
$MgSO_4$	1.00	1.00
Streptomycin	0.05	0.05
Kanamycin	0.03	0.03
Gentamycin	0.03	0.03
Glucose	5.00	5.00
Substrate	0.06	0.10
Water	510.85	510.85
Sum	532.43	532.48
Sum w/o water	21.58	21.62
Adipic acid	0.05	0.095
E-factor	10821.75	5553.56
E-factor w/o water	438.56	225.52

w/o water: without water, E-factor: kg waste kg product^{-1}

[§] Sheldon, R. A. "The E Factor: fifteen years on" *Green Chemistry*, **2007**, *9*, 1273-1283 (https://doi.org/10.1039/B713736M).
[**] Sheldon, R. A. "Fundamentals of green chemistry: efficiency in reaction design" *Chemical Society Reviews*, **2012**, *41*, 1437-1451 (https://doi.org/10.1039/C1CS15219J).

Table S4: Comparison of adipic acid process performance parameters

Process	Starting compound	Product	Catalyst	Space-time- yield ($g\ L^{-1}\ h^{-1}$)	Molar yield (%)	Product ($g\ L^{-1}$)	References
Bioprocess	Catechol	Adipic acid	Biocatalyst (*E. coli*.)	6.6 × 10-5	0.241	0.0016	Kruger 2020[§§]
Bioprocess	Guaiacol	Adipic acid	Biocatalyst (*E. coli*.)	0.27	77	0.55	Suitor 2020[***]
Bioprocess	4-hydroxybenzoic acid	Adipic acid	Biocatalyst (*P. putida* KT2440)	0.02	17	2.5	Niu 2020[†††]
Bioprocess	Cyclohexane	Adipic acid	Biocatalyst (*P. taiwanensis*)	1.28	9	10.2	Bretschneider 2022[‡‡‡]
Bioprocess	Glycerol	Adipic acid	Biocatalyst (*E. coli*.)	0.8	72	68	Zhao et al., 2018[§§§]
Electroprocess*	Phenol	Cyclohexanol	Electrocatalyst	n.a.	64.9 (± 8.6)	8.16 (± 0.13)	This study
Bioprocess*	Cyclohexanol	Adipic acid	Biocatalyst (*P. taiwanensis*)	0.02	60.8 (± 2.7)	0.10 (± 0.0045)	This study
Combined (Electroprocess+Biological) process*	Phenol	Adipic acid	Electrocatalyst + Biocatalyst (*P. taiwanensis*)		39.5	0.10 (± 0.0045)	This study
Electroprocess*	Aromatic mixture**	Mixture of functionalized cyclohexanes***	Electrocatalyst	n.a.	83.4 (± 4.2)	15.27 (± 0.91)	This study
Bioprocess*	Mixture of functionalized cyclohexanes***	Adipic acid and other compounds	Biocatalyst (*P. taiwanensis*)	0.04	68.3 (± 1.7)	0.19 (± 0.0049)	This study
Combined (Electroprocess+Biological) process*	Aromatic mixture**	Adipic acid and other compounds	Electrocatalyst + Biocatalyst (*P. taiwanensis*)		57.0	0.19 (± 0.0049)	This study

* Calculated for endpoint of electrosynthesis (22h) and5 h for biotransformation

** As described in section 1, page S2

*** As described in Figure 4b (manuscript)

[§§] Kruyer, N.S., Wauldron, N., Bommarius, A.S. *et al.* "Fully biological production of adipic acid analogs from branched catechols" Sci Rep, **2020**, *10*, 13367 (https://doi.org/10.1038/s41598-020-70158-z).

[***] Suitor, J.T, Varzandeh, S., Wallace, S. "One-Pot Synthesis of Adipic Acid from Guaiacol in Escherichia coli" *ACS Synth. Biol.*, **2020**, *9*, 2472–2476 (https://doi.org/10.1021/acssynbio.0c00254).

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[‡‡‡] Bretschneider, L., Heuschkel, I., Bühler, K., Karande, R., Bühler, B. "Rational orthologous pathway and biochemical process engineering for adipic acid production using *Pseudomonas taiwanensis* VLB120" *Met. Eng.*, **2022**, *70*, 206–217 (https://doi.org/10.1016/j.ymben.2022.01.014).

[§§§] Zhao, M., Huang, D., Zhang, X., Koffas, M.A.G., Zhou, J., Deng, Y. "Metabolic engineering of *Escherichia coli* for producing adipic acid through the reverse adipate-degradation pathway" *Met. Eng.*, **2018**, *47*, 254–262 (https://doi.org/10.1016/j.ymben.2018.04.002).