Supplementary Information

Experimental section

acid dissolved in (a) 7% isopropanol and methanol (50:50 v/v) and (b) 8% DMSO

Experimental section

Materials

2-Hexenedioic acid (98%, a mixture of *cis*- and *trans*-isomers) was synthesized by BOC Sciences (Shirley, NY, USA). *trans*-Cinnamic acid, 3-methyl-2-cyclohexenone (98%), 2-cyclohexen-1-one (≥95%), acrolein, *trans*-2-butenoic acid (98%), *cis,cis*-muconic acid (≥97%), *trans,trans*-muconic acid (98%), and adipic acid (≥99.5%) were purchased from Sigma (St. Louis, MO, USA). Phusion® High-Fidelity DNA Polymerase was purchased from New England Biolabs (Whitby, Ontario, Canada). In-Fusion HD plus EcoDry for ligation-independent cloning was obtained from Clontech (Mountain View, CA, USA). All other chemicals were purchased from Sigma.

Bioinformatic analysis

Multiple sequence alignment was prepared using Clustal Omega[1] and ESPript 3.0.[2] The protein domains of ERs were analyzed using InterPro server.[3] The structure of ER-BC was modeled using I-TASSER server[4] and a structure modeled with *E. coli* DCR (PDB code 1PS9) as a template was used for structure analysis.

Cloning, expression, and purification of proteins

Genes encoding the selected OYE and ER proteins (Tables S1 and S2) were PCR-amplified from genomic DNA and cloned into a p15Tv-Lic plasmid via a ligation-independent method as previously described.[5] Recombinant ER plasmids were transformed into the *E. coli* BL21 (DE3) *ΔiscR* strain for over-expression of iron-sulfur containing proteins.[6] *E. coli* transformants were grown aerobically at 37°C in Terrific Broth (TB) medium (1 L) supplemented with 100 μg/mL ampicillin until the culture optical density (OD 600 nm) reached 0.6-0.8. At this point, cultures were transferred to tightly closed flasks with a magnetic stir bar, and protein expression was induced with 0.4 mM IPTG after 30 min of anaerobic pre-cultivation. Cultures were incubated with DMSO (50 mM) as the final electron acceptor anaerobically for 15-19 h at room temperature on a magnetic stir plate. E. coli cells were harvested by centrifugation (9,000 *g*), and cell pellets were stored in liquid N_2 for whole-cell biotransformation or protein purification. Protein purification was performed in an anaerobic glove box (Coy Laboratory Products, Grass Lake, MI, USA) under an atmosphere of 80% N_2, 10% H_2, and 10% CO_2. All buffers were degassed and sparged with argon before use. Cell pellets were resuspended in lysis buffer (400 mM NaCl, 50 mM HEPES, pH 7.5, 5% glycerol, 5 mM imidazole, 0.05% Tween-20, 1 mg/mL lysozyme, 3 U/mL benzonase, 0.5 mM EDTA, and 1 mM DTT) and incubated at room temperature for 30 min. Cell lysates were cleared by centrifugation (60,000 *g*) and the supernatant was incubated with a Ni-affinity resin (Qiagen, Valencia, CA, USA) at 4°C for 1 h. The resin was then washed with 100 mL of washing buffer (400 mM NaCl, 50 mM HEPES, pH 7.5, 5% glycerol, 30 mM imidazole, 0.5 mM EDTA, and 1 mM DTT) and eluted with elution buffer (400 mM NaCl, 50 mM HEPES, pH 7.5, 5% glycerol, 250 mM imidazole, 0.5 mM EDTA, and 1 mM DTT).

Purified ERs were frozen and stored in liquid N_2. Protein concentration was determined using Bradford assay and protein purity was evaluated using 10% SDS-PAGE gels.
Recombinant OYE plasmids were transformed into the E. coli BL21-Gold (DE3) strain (Stratagene, La Jolla, CA, USA). *E. coli* transformants were grown at 37°C in TB medium (1 L) supplemented with 100 μg/mL of ampicillin until the OD at 600 nm reached 0.6-0.8. Protein expression was induced with 0.4 mM IPTG, and *E. coli* cells were grown overnight at 16°C. *E. coli* cells were harvested by centrifugation (9,000 *g*), and cell pellets were resuspended in lysis buffer (400 mM NaCl, 50 mM HEPES, pH 7.5, 5% glycerol, and 5 mM imidazole) followed by sonication to break cells. Lysates were cleared by centrifugation (60,000 *g*), and the supernatant was incubated with Ni-affinity resin at 4°C for 30 min. The resin was then washed with washing buffer (400 mM NaCl, 50 mM HEPES, pH 7.5, 5% glycerol, and 30 mM imidazole) and eluted with elution buffer (400 mM NaCl, 50 mM HEPES, pH 7.5, 5% glycerol, and 250 mM imidazole). Purified OYEs were frozen in liquid N_2 and stored at −80°C.

UV-visible absorption spectra

UV-visible absorption spectra were obtained using a rubber-capped quartz cuvette and Cary 50 spectrophotometer (Varian Medical Systems, Palo Alto, CA, USA). For complete oxidation of ERs, purified proteins (6.5 mg/mL for ER-CA and 4.6 mg/mL for ER-BC) were kept overnight on ice in the presence of atmospheric oxygen, and the absorption spectra of oxidized proteins were recorded. For complete reduction and subsequent re-oxidation of ERs, purified proteins were degased and sparged with argon, then NADH in excess (~ 3 mM) was added to protein solutions and the absorption spectra of reduced proteins were recorded. After that, 6 mM *trans*-cinnamic acid was added to protein solutions, and the absorption spectra of re-oxidized proteins were recorded.

Protein identification by LC-MS

Peptide identification from SDS-PAGE was conducted following a protocol modified from Shevchenko et al.[7] Following coomassie staining, gels were washed in ultrapure water for several hours and protein bands were excised and transferred into microcentrifuge tubes. Gel fragments were destained for 30 min at room temperature in 100 μL of ammonium bicarbonate/acetonitrile (1:1, vol/vol). 500 μL of acetonitrile was added, and samples were incubated until gel pieces became white. Solution was removed, gel pieces covered with 50 μL of trypsin buffer (13 ng/μL trypsin in 10mM ammonium bicarbonate, pH 8.5, and 10% acetonitrile) and left on ice for 30 min. An additional 50 μL of trypsin buffer was added and left for 90 min on ice followed by addition of 20 μL of 10 mM ammonium bicarbonate, pH 8.5, and incubation at 37°C overnight. Cleanup of samples was done using Omix C18 pipette tips (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer instructions, vacuum-dried and re-suspended in 100 μL of 0.1% formic acid.
Samples were analyzed by MS/MS on a Thermo Q-Exactive mass spectrometer equipped with a Nanospray Flex Ion Source, Thermo Scientific Acclaim PepMap RSLC 50 μm x 15 cm, nanoViper C18, 2 μm column and an Easy-nLC 1000 HPLC. The mobile phase consisted of two eluants: 0.1% formic acid (Buffer A) and acetonitrile (Buffer B). The conditions used were; 5 min at up to 10% B, 101 min at up to 40% B, 103 min at up to 95% B, and 120 min

with up to 95% B, with a flow rate of 250 nL/min. Raw data files were converted with MSconvert and analyzed with X!Tandem using a custom peptide database in GPM Manager.

Enzyme assays

Enzyme activity of purified ERs was measured spectrophotometrically in 96-well plates after incubation for 3-5 min at 30°C in an anaerobic glove box as previously described.[8] Buffers and reagents were degassed and sparged with argon. Reaction mixtures contained 100 mM potassium phosphate buffer (pH 7.0), NADH (0.5 mM), indicated substrates, and proteins (0.1-50 μg) in a final volume of 200 μL. Enzyme reactions were monitored by following the decrease in absorbance at 340 nm due to oxidation of NADH to NAD^+ or NADPH to $NADP^+$. Kinetic parameters (k_{cat}, K_m, and K_i) of ER-CA and ER-BC were determined by the substrate inhibition equation ($v = V_{max} / (1 + K_m [S] + [S] / K_i)$) or the Hill equation ($v = V_{max}$ [illegible] $[S]^n / (K_m^n + [S]^n)$. Prism software (version 5.02; GraphPad Software, San Diego, CA, USA) was used for nonlinear regression as previously described.[9]

To measure the conversion yield of ER-BC *in vitro*, enzyme reactions were performed overnight at 37°C in 1.5 ml HPLC vials closed with rubber stoppers. Reaction mixture degassed and sparged with argon contained 100 mM HEPES (pH 7.5), NADH (1 mM), indicated substrates, 40 mM sodium formate, 20 ug formate dehydrogenase (UniProt ID P33160), and 40 ug ER-BC in a final volume of 200 uL. Tubes were incubated at 37°C on a shaker (200 rpm). After 12h of incubation, reaction mixture was filtered with 10 kDa spin filters (PES membrane, VWR) and analysed with HPLC or LC-MS.

To measure the conversion yield of ER-BC *in vivo*, *E. coli* cells over-expressing recombinant ER-BC were grown under anaerobic conditions, harvested by centrifugation (9,000 *g*), and washed with 10 mM sodium phosphate (pH 7.0) containing 1% NaCl. An appropriate amount of *E. coli* cells (54-154 mg dry cells/mL) was resuspended in the same buffer. Reaction mixtures including *E. coli* cells, substrates, and cofactor were transferred to 5 mL glass tubes tightly capped with air-tight rubber stoppers. Reaction mixtures contained 54 mM sodium phosphate (pH 7.0), 3% glucose, 0.4% NaCl, one of substrates (0.7 mM of *cis,cis*- or *trans,trans*-isomer of muconic acid, 3 mM *trans*-cinnamic acid, 5 mM 2-cyclohexene-1-one, 20 mM 3-methyl-2-cyclohexenone, 20 mM *trans*-2-butenoic acid, and 20 mM 2-hexenedioic acid), and kanamycin (100 μg/mL) in a final volume of 2 mL. Tubes were incubated at 37°C on a shaker (200 rpm). After 24h of incubation liquid samples were centrifuged (18,000 *g*), supernatant was analysed for substrates and products concentrations with HPLC or LC-MS.

Purified OYEs were screened for hydrogenation activity against acrolein, 2-cyclohexen-1-one, *cis,cis*-muconic acid, *trans,trans*-muconic acid, and 2-hexenedioic acid. Enzymatic activity was measured spectrophotometrically in 96-well plates after incubation for 20 min at room temperature in an anaerobic chamber. All buffers and reagents were degassed and sparged with argon to minimize the substrate-independent oxidation of NADPH. Reaction mixtures contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM NaCl, 0.25 mM NADPH, indicated substrates (1 mM) and protein (2.5 μg) in a final volume of 200 μL. Enzyme activity was monitored by following the decrease in absorbance at 340 nm due to oxidation of NADPH to NADP+.

All results of *in vitro* experiments are means from at least three independent determinations. Control experiments were performed in parallel to correct substrate-independent oxidation of cofactors. All buffers and reagents used in anaerobic reactions were rigorously sparged with argon to remove traces of oxygen before use.

Protein thermostability (T_{agg}) and oxygen tolerance

Temperature-dependent aggregation of purified ER-CA and ER-BC (0.4 mg/mL) in 100 mM HEPES (pH 7.0), 100 mM NaCl, 0 or 1 mM *trans*-cinnamic acid was monitored using differential static light scattering (Stargazer; Harbinger Biotechnology and Bioengineering Corporation, Toronto, ON, Canada) as previously described.[10] Protein thermostability can be assessed by monitoring protein aggregation during thermal denaturation. 50 µL of protein solutions was placed in a 384-wll plate, and covered with 50 µL of mineral oil to prevent evaporation of water. Protein samples were heated from 27 to 85°C at a rate of 1 °C/min and transition temperature (T_{agg}) was determined using the BioAactive software.

Purified ER-CA (100 µL, 2.9 mg/mL) and ER-BC (100 µL, 4.6 mg/mL) were placed in 5 mL glass tubes tightly capped with air-tight rubber stoppers. Anaerobic tubes were filled with an anaerobic gas mixture (80% N_2, 10% H_2, and 10% CO_2), and aerobic tubes were filled with atmospheric air. The tubes were kept on ice for one week, and small aliquots of ERs (5 µL) were withdrawn by a syringe every day to measure the residual activity of ERs in a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0), 0.5 mM NADH, 0.5, indicated substrates (0.75mM *trans*-cinnamic acid for ER-CA and 0.25 mM 2-hexenedioic acid for ER-BC), and protein (1 µg) in a final volume of 200 µL.

Whole-cell biotransformation

E. coli cells over-expressing recombinant ERs were grown under anaerobic conditions (as indicated above), harvested by centrifugation (9,000 *g*), and washed with 500 mL of 10 mM sodium phosphate (pH 7.0) containing 1% NaCl. An appropriate amount of *E. coli* cells (54-154 mg dry cells/mL) was resuspended in 2 mL of the same buffer. Reaction mixtures including *E. coli* cells, substrates, and cofactor were transferred to 10 mL glass tubes tightly capped with air-tight rubber stoppers. Reaction mixtures contained 54 mM sodium phosphate (pH 7.0), 3% glucose, 0.4% NaCl, substrates (0.7 mM of *cis,cis*- or *trans,trans*-isomer of muconic acid, or 20 mM of 2-hexenedioic acid), and kanamycin (100 µg/mL) in a final volume of 4 mL. Due to low solubility of muconic acid in aqueous buffer solutions (~200 mg/L = ~1.4 mM), it was used at 0.7 mM final concentration for whole-cell biotransformations. Tubes were incubated at 37°C on a shaker (200 rpm). Liquid samples (500 µL) were withdrawn with gas-tight and argon-sparged syringes. Enzyme reactions were stopped by the addition of 2 volumes of cold (−20°C) acetonitrile/methanol/100 mM formic acid mixture (40:40:20 vol/vol). Samples were cooled at −20°C for 1h and centrifuged at 4°C (18,000 *g*). Supernatants were dried in a vacuum centrifuge, dissolved in 5mM H_2SO_4 or 0.1% formic acid, filtered through 10 kDa centrifugal filters, and stored at −20°C for HPLC or LC-MS analysis respectively. For the whole-cell biotransformation of 2-hexenedioic acid by ER-BC, ER-CA, ER-MT, ER-CK, ER-CL, and ER-CT cells, final cell concentrations were 103, 86, 90, 87, 103 and 95 mg dry cells/mL, respectively, whereas 87, 90, 86, 88, and 94 mg dry cells/mL were used for whole-cell biotransformations of *cis,cis*-muconic acid by

ER-BC, ER-CA, ER-MT, ER-CK, and ER-CL, respectively. For the whole-cell biotransformation of *trans,trans*-muconic acid by ER-BC, ER-CA, ER-MT, ER-CK, and ER-CL, we used 84, 154, 89, 88, and 86 mg dry cells/mL, respectively. Negative control experiments were performed using *E. coli* cells harboring empty plasmids. All results of *in vivo* experiments are means from at least two independent determinations.

Product analysis by HPLC, LC-MS, and NMR

Standards and reaction samples were analyzed by HPLC using a Varian ProStar HPLC system (Varian Medical Systems, Palo Alto, CA, USA) equipped with an Aminex HPX-87H column (300 ⌀ 7.8 mm) (Bio-Rad Laboratories Inc., Hercules, CA, USA) and Pursuit 5 C18 (150 x 4.0 mm) (Varian Medical Systems, Palo Alto, CA, USA). Samples were eluted with 5 mM H_2SO_4 at a flow rate of 0.3 ml/min (Aminex HPX-87H), or with methanol (B) - 0.1% formic acid (A) gradient (Pursuit 5 C18). The solvent gradient was 0 min, 10% B; 10 min, 10% B; 25 min, 44% B; 35 min, 10% B; followed by equilibration for 5 min with 10% B. The concentration of substrates and products was determined by linear regression based on the peak areas at 210 nm (adipic acid and 2-hexenedioic acid), 254 nm (*trans*-cinnamic acid, 3-phenylpropanoic acid, 2-cyclohexen-1-one, and 3-methyl-2-cyclohexenone), 270 nm (3-methylcyclohexanone and cyclohexanone) and RI detection (crotonic acid and butyric acid).

The LC-MS platform consists of a Dionex Ultimate 3000 UHPLC system and a Q-Exactive mass spectrometer equipped with a HESI source (Thermo Scientific, Waltham, MA, USA). Control of the system and data handling was performed using Thermo Xcalibur 2.2 software and Chromeleon 7.2 software. Liquid chromatography was conducted on a Hypersil Gold C18 column (50 mm ⌀ 2.1 mm, 1.9 µm particle size, Thermo Scientific) equipped with a guard column. The pump was run at a flow rate of 200 µL/min. Water containing 0.1% formic acid was used as solvent A, whereas solvent B was acetonitrile. The solvent gradient was 0 min, 0% B; 4 min, 0% B; 15 min, 100% B; 24 min, 100% B; 25 min, 0% B; 30 min, 0% B; followed by equilibration for 5 min with 0% B. Autosampler temperature was maintained at 5°C, and injection volume was 10 µL. Data collection was done in negative ionization mode with a scan range *m/z* 120-800, resolution 70,000 at 1 Hz, AGC target of 3e6 and a maximum injection time of 200 ms. Concentrations of the reaction substrates and products in the samples were determined by linear regression using standard solutions of *cis,cis*-muconic acid (*m/z* 141.0162), *trans,trans*-muconic acid (*m/z* 141.0163), cyclohexanone (*m/z* 96.05751), cyclohexanone (*m/z* 98.07316), 2-hexenedioic acid (*m/z* 143.0319), and adipic acid (*m/z* 145.0475). Linear detection ranges for adipic acid, muconic acid, and 2-hexenedioic acid was 0.25-2.00 mM, 0.25-1.00 mM, and 0.25-2.00 mM, respectively.

Isolated adipic acid was dried to completeness in a SpeedVac, and 1 mg of sample was dissolved in DMSO-d6 to prepare the sample for 1H NMR. 1H NMR spectrum for the adipic acid sample was collected on an Agilent DD2 700 MHz spectrometer at the Nuclear Magnetic Resonance facility in the Department of Chemistry at the University of Toronto.

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Table S1. OYE proteins used in this work.

UniProt ID	GenBank ID	Microorganism	Activity [U/mg protein][a]	
			Acrolein	2-Cyclohexen-1-one
1. Q9KCT8	BAB05200.1	*Bacillus halodurans* C-125	ND	0.13
2. P54550	CAB14314.1	*Bacillus subtilis* subsp. subtilis str. 168	ND	0.21
3. Q7NSC5	AAQ61162.1	*Chromobacterium violaceum* ATCC 12472	ND	0.07
4. P77258	AAC74722.1	*Escherichia coli* K12	ND	0.18
5. Q8Y4H1	CAD00549.1	*Listeria monocytogenes* EGD-e	ND	0.08
6. Q82S49	CAD86422.1	*Nitrosomonas europaea* ATCC 19718	ND	0.19
7. Q9HZR5	AAG06320.1	*Pseudomonas aeruginosa* PAO1	ND	0.13
8. Q9HW45	AAG07744.1	*Pseudomonas aeruginosa* PAO1	ND	0.15
9. Q88PD0	AAN66545.1	*Pseudomonas putida* KT2440	ND	0.15
10. Q88NF7	AAN66878.1	*Pseudomonas putida* KT2440	0.06	0.25
11. Q88MU0	AAN67100.1	*Pseudomonas putida* KT2440	ND	0.14
12. Q887W0	AAO54700.1	*Pseudomonas syringae* pv. tomato str. DC3000	ND	0.18
13. Q883F8	AAO55915.1	*Pseudomonas syringae* pv. tomato str. DC3000	ND	0.13
14. Q87X32	AAO57808.1	*Pseudomonas syringae* pv. tomato str. DC3000	ND	0.09
15. Q48MP2	AAZ36642.1	*Pseudomonas syringae* pv. phaseolicola 1448A	ND	0.14
16. Q48LU5	AAZ33328.1	*Pseudomonas syringae* pv. phaseolicola 1448A	ND	0.18
17. Q48EK4	AAZ35385.1	*Pseudomonas syringae* phaseolicola 1448A	ND	0.22
18. Q0SBS7	ABG95009.1	*Rhodococcus* sp. RHA1	0.08	ND
19. Q0RXM6	ABG99960.1	*Rhodococcus* sp. RHA1	ND	ND
20. Q99VD2	BAB57118.1	*Staphylococcus aureus* subsp. aureus Mu50	ND	ND
21. Q82MZ3	BAC69220.1	*Streptomyces avermitilis* MA-4680	0.05	0.23
22. Q92YK2	AAK65532.1	*Sinorhizobium meliloti* 1021	ND	ND
23. Q8E9V9	AAN57126.1	*Shewanella oneidensis* MR-1	ND	ND
24. Q8PDM8	AAM39626.1	*Xanthomonas campestris* pv. campestris str. ATCC 33913	ND	0.15
25. Q03558	AAA83386.1	*Saccharomyces cerevisiae*	0.17	0.23

[a] The cut-off value for enzyme activity of OYE is 0.05 U/mg protein. ND = not detected. No OYEs showed enzymatic activity against 2-hexenedioic acid or *cis,cis*- and *trans,trans*-isomers of muconic acid.

Table S2. ER proteins used in this work.

UniProt ID	GenBank ID	Microorganism
1. F7ZQU1 (**ER-CA**)	AEI32805.1	*Clostridium acetobutylicum* DSM 1731
2. A5N665 (**ER-CK**)	EDK32796.1	*Clostridium kluyveri* DSM 555
3. D8GRM6 (**ER-CL**)	ADK16394.1	*Clostridium ljungdahlii* DSM 13528
4. O52933 (**ER-CT**)	CAA71086.1	*Clostridium tyrobutyricum* DSM 1460
5. O52935 (**ER-MT**)	CAA76082.1	*Moorella thermoacetica* ATCC 39073
6. G2TQU6 (**ER-BC**)	AEO99944.1	*Bacillus coagulans* 36D1

Table S3. Identification of ER-CT in SDS-PAGE gels using LC-MS.

Proteins	Peptides[a]		log(e)[b]	log(I)[c]	Protein Coverage [%][d]		Molecular Weight [kDa]	GenBank ID
	Total	Unique			Measured	Corrected		
ArnA	467	39	-411.6	10.01	44	57	74.2	ACT44004.1
HtpG	90	23	-219.6	8.47	37	46	71.4	ACT42323.1
DnaK	18	11	-80	6.89	21	24	69.1	ACT41916.1
ER-CT	18	7	-57.4	7.27	11	13	72.9	CAA71086.1
GFAT	15	6	-49.4	7.11	9.7	12	66.9	ACT45408.1

[a] Total peptides: total number of peptides used for protein assignment, unique peptides: number of unique peptide sequences associated with protein assignment. Proteins with more than 10 total peptides were only shown in this table.

[b] log(e): base-10 log of the expectation that any particular protein assignment was made at random (E-value).

[c] log(I): base-10 log of the sum of fragment ion intensities used for protein assignment.

[d] % measured: amino acid coverage of assigned protein, % corrected: amino acid coverage of assigned protein, corrected for peptides unlikely to be observed.

Table S4. Kinetic parameters of purified ER-CA and ER-BC.

	Substrates[a]					
Enzymes	*trans*-Cinnamic acid			2-Hexenedioic acid		
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
	[s^{-1}]	[mM]	[s^{-1} mM^{-1}]	[s^{-1}]	[mM]	[s^{-1} mM^{-1}]
ER-CA	9.7 ± 1.4	0.40 ± 0.10	24.0 ± 2.1	0.214 ± 0.044	20.5 ± 0.60	0.0104 ± 0.001
ER-BC	1.1 ± 0.35	0.68 ± 0.30	1.6 ± 0.27	2.86 ± 0.114	18.9 ± 1.37	0.151 ± 0.005

[a] *trans*-cinnamic acid was titrated with 1N NaOH, and 2-hexendioic acid was dissolved in 14% isopropanol.

Table S5. *In vivo* substrate conversion by *E. coli* cells expressing ER-CK, ER-CL, or ER-MT.

Substrates	Substrate concentration, mM	*in vivo* substrate conversion, % (24 h incubation)		
		ER-CK	ER-CL	ER-MT
1. 2-Cyclohexen-1-one [a]	5 mM	19.9±3.7	18.4±2.6	11.9±1.7
2. 3-Methyl-2 Cyclohexenone	20 mM	BDL	BDL	BDL
3. *trans*-2-Butenoic acid	20 mM	MC	MC	MC
4. *trans*-Cinnamic acid	3 mM	MC	MC	MC
5. 2-Hexenedioic acid	20 mM	102.1±5.6	87.8±3.0	45.3±1.8
6. c*is,cis*-Muconic acid	0.7 mM	BDL	BDL	70.0±6.5
7. *trans,trans*-Muconic acid	0.7 mM	75.7±2.18	80.5±0.48	77.6±3.1

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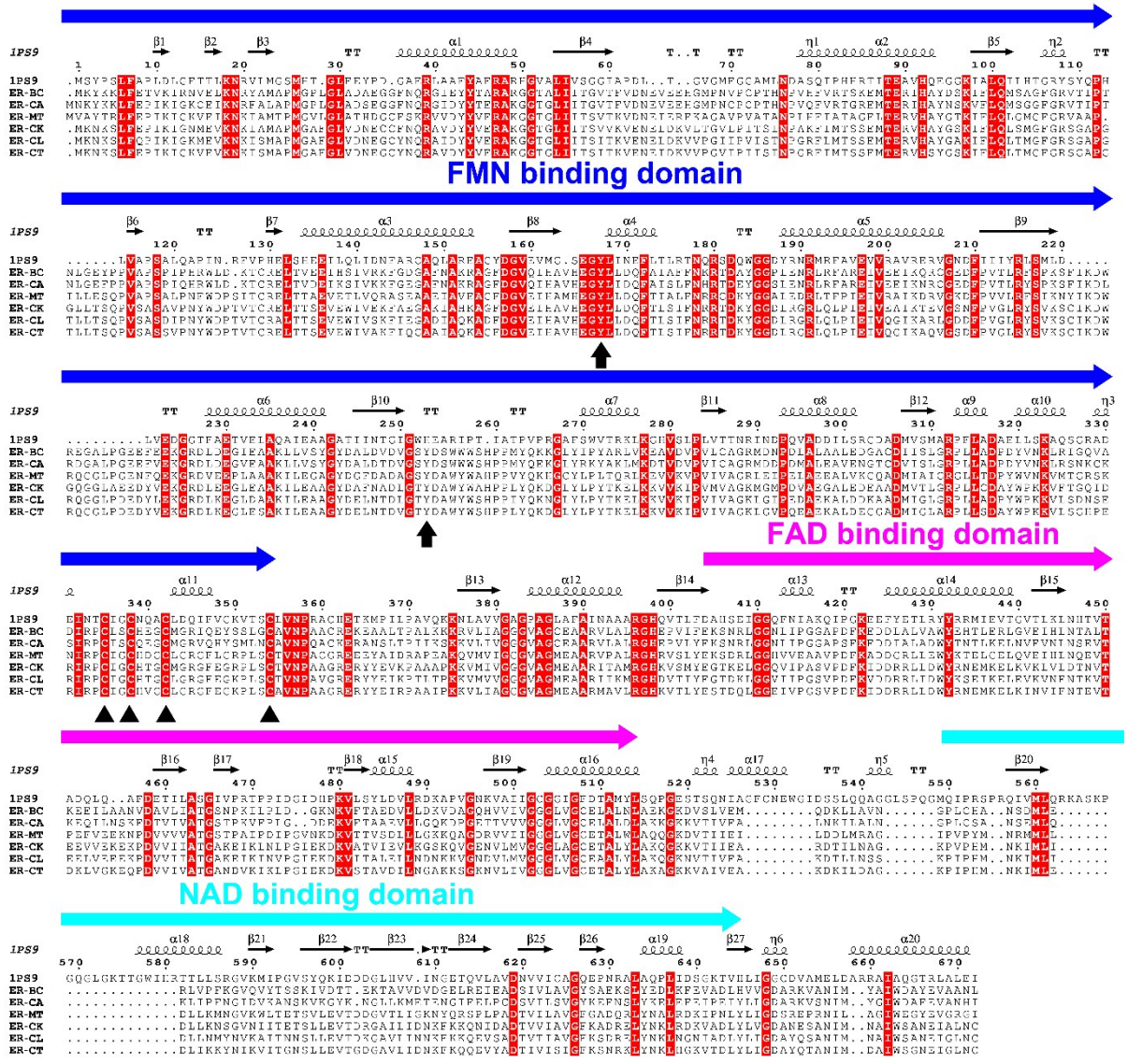
[a] *E. coli* cells without ERs can hydrogenate 2-cyclohexen-1-one due to the presence of the endogenous N-ethylmaleimide reductase NemA (Uniprot ID P7725) (Table S1). This background activity was subtracted from experimental data.
BDL-below detection limit, MC –metabolized by cell

Figure S1. Sequence analysis of ERs characterized in this work. Multiple sequence alignment of six ERs and *E. coli* DCR. ER-BC (*Bacillus coagulans*, AEO99944.1) exhibits 30, 72, 51, 51, 49, and 50% sequence identity with *E. coli* DCR (*E. coli*, CDJ73774.1), ER-CA (*C. acetobutylicum*, AEI32805.1), ER-MT (*Moorella thermoacetica*, CAA76082.1), ER-CK (*C. kluyveri*, EDK32796.1), ER-CL (*C. ljungdahlii*, ADK16394.1), and ER-CT (*C. tyrobutyricum*, CAA71086.1), respectively. The residues conserved in six ERs and *E. coli* DCR are highlighted in red. The conserved cysteine residues coordinating an [4Fe-4S] cluster are indicated by black triangles. The secondary structure elements derived from the *E. coli* DCR structure (PDB code 1PS9) are shown, and the DCR catalytic residues (Tyr166 and His252) are indicated by black arrows.



The protein domains are indicated by the colored arrows.

Figure S2. Structural analysis of *E. coli* DCR and ER-BC. (a) The ribbon diagram of the *E. coli* DCR protomer (PDB code 1PS9) showing the four cofactors bound (shown as sticks): $NADP^+$ (cyan), FAD (purple), [4Fe-4S] cluster (yellow and orange), and FMN (dark blue). The six ERs also have the same cofactors but use NADH instead of NADPH. (b) Structural model of ERBC constructed using the DCR structure as a template. In the ER-BC model, the positions of protein domains are indicated by the models of bound cofactors (shown as sticks): FMN (dark blue; aa 5-356), FAD (purple, aa 404-514), NAD^+ (or $NADP^+$) (cyan, aa 525-646), and [4Fe-4S] cluster (yellow and orange), which were superimposed into the ER-BC model using the DCR structure.

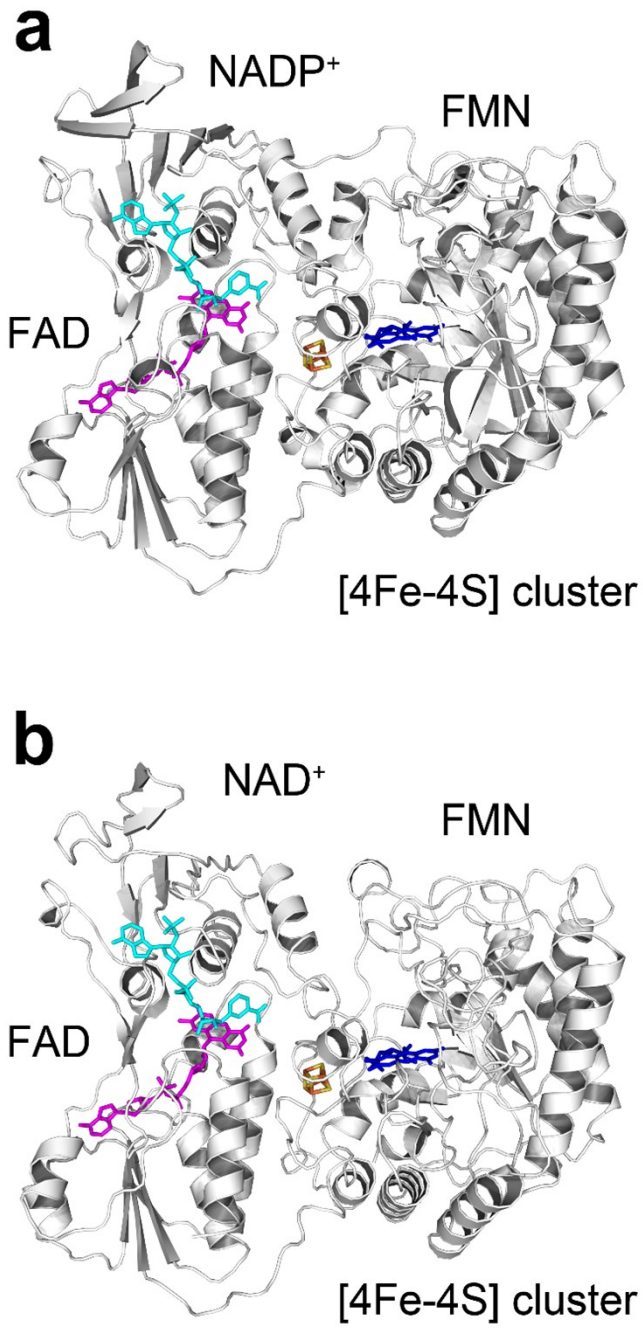


Figure S3. MS and NMR analyses of adipic acid purified from whole cell biotransformation of (1) 2-hexenedioic acid (20 mM) and (2) *cis,cis*-muconic acid sodium salt (40 mM). (a) MS analysis: total ion chromatogram from C18 purification of adipic acid isolated using Aminex column under negative ionization. (b) MS spectrum of the C18 adipic acid peak. M/Z value for adipic acid under negative ionization shown. (c) 1H NMR spectrum of purified adipic acid. Hydrogen atoms are labelled numerically with respect to corresponding to carbon centers from inset structure with coupling indicated in parenthesis. 80mM *cis,cis*-muconic acid sodium salt was prepared by titrating with 1N NaOH at room temperature.

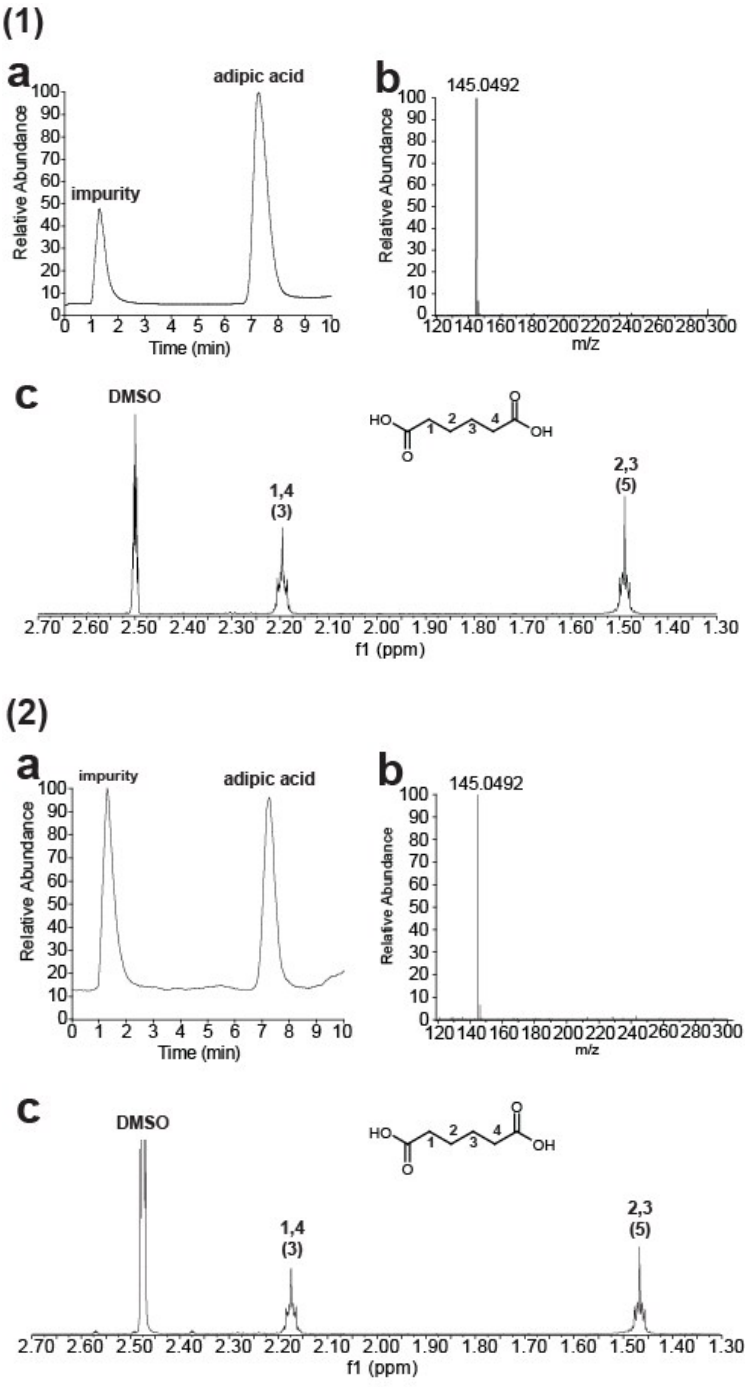


Figure S4. Whole-cell biotransformation of 2-hexenedioic acid (20 mM) to adipic acid by *E. coli* cells expressing recombinant (a) ER-CL or (b) ER-MT. Adipic acid in culture supernatants was identified and quantified using HPLC.

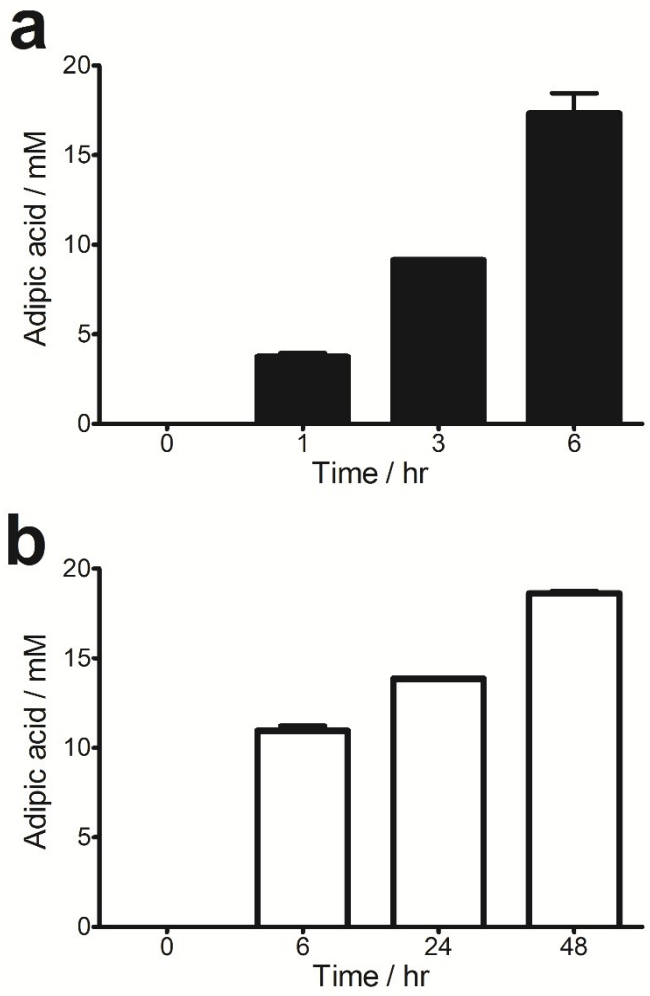


Figure S5. SDS-PAGE analysis of the expression and purification of ERs from *E. coli* cells. (a) ER-CA, ER-CK, and ER-BC. (b) ER-MT, ER-CL, and ER-CT. The lane labels indicate: P, cell lysate pellet; S, cell lysate supernatant; E, eluate from a Ni^{2+} affinity column (purified ER); M, protein markers. LC-MS analysis of the 67-75 kDa gel bands in the purified ER-CT sample (lane E) identified five soluble proteins including ER-CT (see Table S3).

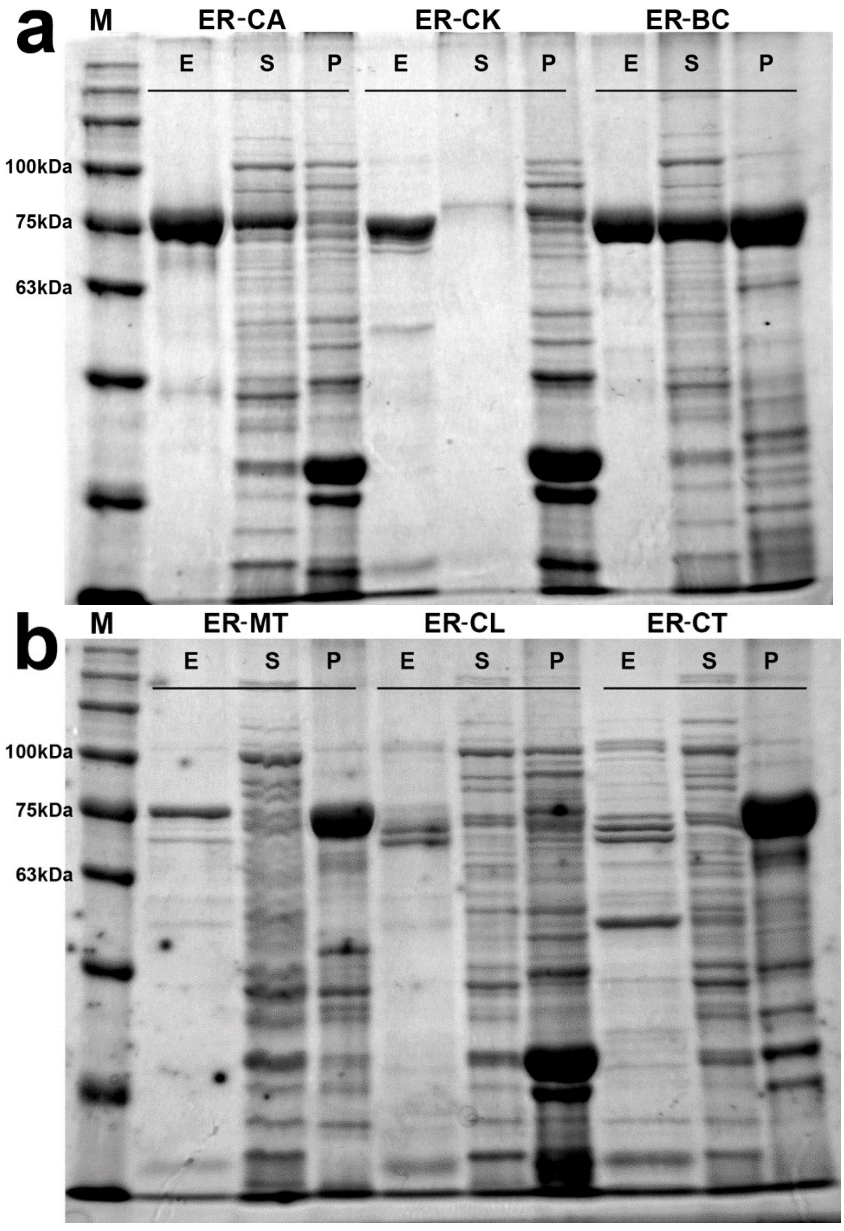


Figure S6. *In vitro* hydrogenation activity of purified ER-BC with (a) NADPH and (b) NADH with 20 mM 2-hexenedioic acid.

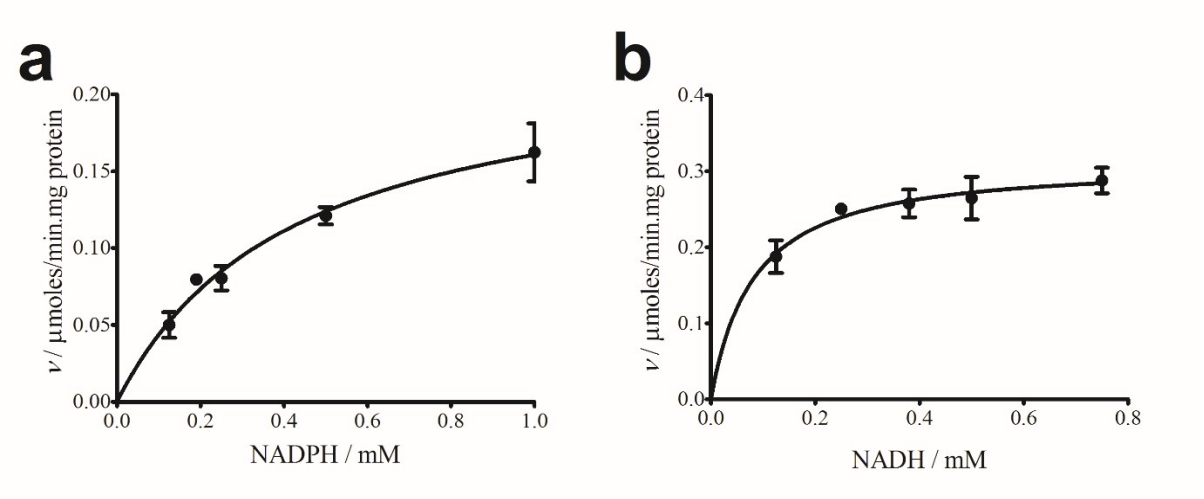


Figure S7. *In vitro* hydrogenation activity of purified ER-CA and ER-BC against 2-hexenedioic acid dissolved in aqueous buffer.

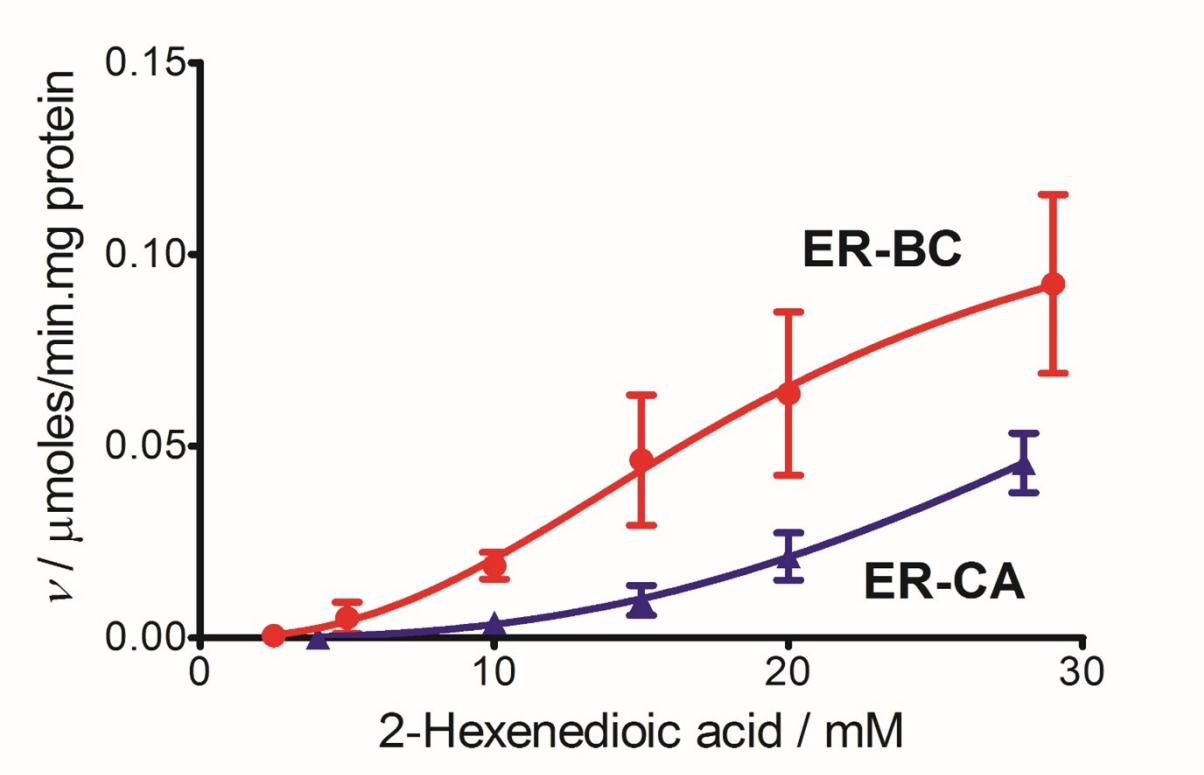


Figure S8. *In vitro* hydrogenation activity of purified ER-BC against 2-hexenedioic acid dissolved in (a) 7% isopropanol and methanol (50:50 v/v) and (b) 8% DMSO.