Electronic Supplementary Material (ESI) for Reaction Chemistry & Engineering.

A One-Pot Biocatalytic and Organocatalytic Cascade Delivers High Titers of 2-Ethyl-2-Hexenal from n-Butanol

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Supplementary materials

S1. General procedures and materials

Unless otherwise noted, all chemicals and solvents were used as received from commercial sources. Water (dd-H2O) used in biological procedures or as a reaction solvent was deionized using a Barnstead E-pure Series 1090 water purification system (Thermo Fisher Scientific, USA). Centrifugations were carried out in a Sorvall Biofuge Stratos Centrifuge (Thermo Electron Corp., USA). Optical density measurements were carried out with Evolution 260 Bio UV-visible spectrometer (Thermo Fisher Scientific, USA) by measuring the optical density at 600 nm. Liquid cultures were shaken in an I24 Incubator (New Brunswick Scientific, USA). Sterilization was carried out in a Sterilmatic Autoclave (Market Forge Industries, USA). Solvent evaporations were carried out with a USA Lab RE-200A rotary evaporator with a recirculating chiller (Glycol Power Pack H35G).

Gas chromatography mass spectrometry analysis was carried out with an Agilent Technologies 7890B GC system paired with a 7693 Autosampler and 5977B MSD. A 30 m Rxi®-5sil MS column with Integra-Guard (Restek #13623-127) was fitted. GC oven temp was initial held at 60 °C for 1 minute, followed by a 15 °C min^{-1} ramp to 120 °C and held for 1 minute. Helium was used as the carrier gas. The injector was maintained at 200 °C, the MSD transfer temperature was 280 °C, and the detector was maintained at 230 °C. 0.5 µL of sample was injected in split injection mode (1:100 split ratio) using 2-methyl-2-pentenal as the internal standard. Selected ion monitoring (SIM) was used.

Calibration curves were generated by diluting 50 mg of the product to 5 mL in a volumetric cylinder. The 10 mg mL^{-1} stock solution was diluted with isooctane to generate calibration levels. 2-methyl-2-pentenal was used as an internal standard. Samples were diluted (1:50) in isooctane (1 mL) before analysis. Spectra were analyzed with Agilent MassHunter Quantitative software.

Proton nuclear magnetic resonance (^{1}H spectra) were acquired with a JEOL ECA-500 spectrometer operating at 500 MHz. Chemical shifts are expressed as parts per million and referenced to residual solvent signal ($CDCl_3$, δ 7.26 ppm; DMSO-d_6, δ 2.50 ppm). Carbon-13 NMR (^{13}C NMR) spectra were acquired with a JEOL-ECA 500 at 125 MHz. Chemical shifts are expressed in parts per million and referenced to residual solvent ($CDCl_3$, δ 77.16 ppm; DMSO-d_6, δ 39.50 ppm). All NMR solvents were acquired from Cambridge Isotope Laboratories

S2. Stains, media, and culture conditions

Komagataella pastoris (Guilliermond) Yamada et al. was purchased from the American Type Culture Collection (ATCC® 28485™). Freeze-dried cells were grown in YPD

media (~125 mL) to mid-exponential phase (~ 48 hours). The cultures were diluted 1:1 with 20 % glucose and allowed to rest for 15 minutes. 1 mL aliquots were portioned into cryogenic tubes, placed in an insulated Styrofoam cooler, and frozen at – 80 ºC. The frozen stock was thawed in a room temperature water bath and resuspended in methanol media (125 mL). Dry cell weight (DCW) was measured by pelleting a volume of liquid culture (6000 × g). The supernatant was discarded and the pellet was transferred to dried aluminum foil boat (100°C, 20 min) with minimal distilled water. The pellet was dried at 100°C until the mass no longer changed (~24 hr). DCW (g/L) was calculated by dividing the mass by the original volume of culture.

YPD media was prepared using the following recipe:

- 10 g/L yeast extract
- 20 g/L peptone
- 20 g/L glucose
- pH adjusted to 5.6 with HCl (10% v/v)

The solution was autoclaved at 121°C for 20 min.

Methanol media was prepared using the following recipe:

- 2.6 g/L potassium phosphate monobasic
- 0.3 g/L potassium phosphate dibasic
- 1.5 g/L ammonium sulfate
- 0.3 g/L magnesium sulfate heptahydrate
- g/L yeast extract
- 1 mg/L iron sulfate heptahydrate
- 5 µg/L copper sulfate monohydrate
- 10 µg/L boric acid
- 10 µg/L manganese sulfate monohydrate
- 70 µg/L zinc sulfate
- 10 µg/L sodium molybdate

The solution was autoclaved at 121°C for 20 min.

After the solution cooled, 10 g/L methanol was added.

S3. Amino acid catalyst screen

The amino acid (100 mM) was dissolved in PBS (~4mL, 50 mM, pH 7.4). The pH was adjusted back to 7.4 with hydrochloric acid (10% v/v) or sodium hydroxide (10% w/v) if necessary. PBS was added to a final volume of 5 mL. The reactions were spiked with butyraldehyde (200 mM). Isooctane (1 mL) was added to each reaction. After 24 hours of reaction at room temperature with rapid stirring, the organic layer was separated and reserved. Product yields were determined by GC-MS.

S4. Catalyst concentration screen

Organocatalyst solutions were prepared in PBS (50 mM, pH 7.4) to deliver final concentrations of 50, 100, and 250 mM. The pH of the PBS/organocatalyst solution was adjusted to 7.4 with hydrochloric acid (10% v/v) or sodium hydroxide (10% w/v), and the solution was autoclaved at 121ºC for 20 minutes.

Whole Cell:
K. pastoris cultures were grown for 72 hours (OD_{600} ~ 2.2) in methanol media (~125 mL). Cultures (10 mL) were pelleted (10 minutes, 5000 x g), and washed with PBS (1 × 10 mL, 50 mM, pH 7.4). The cell pellet was then resuspended in PBS containing the organocatalyst (10 mL) and transferred to a 250 mL Erlenmeyer flask with a screw-cap opening. The reactions were spiked with 1-butanol (200 mM). Isooctane (2 mL) was added to each flask, the flasks were capped, and placed in shaker incubator (30°C, 200 RPM). After 24 hours, the organic layer was separated and reserved. Product yields were determined by GC-MS.

Isolated Enzyme:
In a 250 mL Erlenmeyer flask with a screw-cap opening, catalase (10 mg) was dissolved in PBS containing the organocatalyst (10 mL). The reactions were spiked with 1-butanol (200 mM) and alcohol oxidase (30 units) was added. Isooctane (2 mL) was added to each flask, the flasks were capped, and placed in shaker incubator (30°C, 200 RPM). At 24 hours, the organic layer was separated and reserved. Product yields were determined by GC-MS.

S5. Butanol and enzyme/cell concentration matrix

Whole Cell:
K. pastoris cultures were grown for 72 hours (OD_{600} ~ 2.2) in methanol media (~125 mL). Cell culture suspensions (5, 10, or 20 mL) were pelleted (10 minutes, 5000 × g), and washed with PBS (1 × 10 mL, 50 mM, pH 7.4). All cell pellets were resuspended in PBS (10 mL, 50mM, pH 7.4) containing lysine (250 mM) to create 0.5, 1.0, or 2.0× cell concentrations and transferred to separate 250 mL Erlenmeyer flasks with a screw-cap opening. The reactions were spiked with 1-butanol (100, 200, or 400 mM). Isooctane (2 mL) was added to each flask, the flasks were capped, and placed in shaker incubator (30°C, 200 RPM). At 24 hours, the organic layer was separated and reserved. Product yields were determined by GC-MS.

Isolated Enzyme:
In a 250 mL Erlenmeyer flask with a screw-cap opening, catalase (10 mg) was dissolved in PBS (10 mL, 50 mM, pH 7.4) containing lysine (250 mM). The reactions were spiked with 1-butanol (100, 200, or 400 mM) and alcohol oxidase (15, 30, or 60 units) was added. Isooctane (2 mL) was added to each flask, the flasks were capped, and placed in shaker incubator (30°C, 200 RPM). At 24 hours, the organic layer was separated and reserved. Product yields were determined by GC-MS.

S6. Time course reactions

Whole Cell:
K. pastoris cultures were grown for 72 hours (OD_{600} ~ 2.2) in methanol media (~125 mL). Cell culture suspensions (10 or 20 mL) were pelleted (10 minutes, 5000 × g), and washed with PBS (1 × 10 mL, 50 mM, pH 7.4). All cell pellets were resuspended in PBS (10 mL, 50mM, pH 7.4) containing lysine (250 mM) to create 1.0 or 2.0× cell concentrations and transferred to separate 250 mL Erlenmeyer flasks with a screw-cap opening. The reactions were spiked with 1-butanol (100 or 200 mM). Isooctane (2 mL) was added to each flask, the flasks were capped, and placed in shaker incubator (30 °C, 200 RPM). At 6, 14, 24, 48, and 72 hours a sample (60 µL) of the organic layer was collected and reserved. Product yields were determined by GC-MS.

Isolated Enzyme:
In 250 mL Erlenmeyer flasks with a screw-cap opening, catalase (10 mg) was dissolved in PBS (10 mL, 50 mM, pH 7.4) containing lysine (250 mM). The reactions were spiked with 1-butanol (100, 200, or 400 mM) and alcohol oxidase (15, 30, or 60 units) was added. Isooctane (2 mL) was added to each flask, the flasks were capped, and placed in shaker incubator (30 °C, 200 RPM). At 6, 14, 24, 48, and 72 hours a sample (60 µL) of the organic layer was collected and reserved. Product yields were determined by GC-MS.

S7. pH

Whole Cell:
K. pastoris cultures were grown for 72 hours (OD_{600} ~ 2.2) in methanol media (~125 mL). Cell culture suspensions (20 mL) were pelleted (10 minutes, 5000 × g), and washed with PBS (1 × 10 mL, 50 mM, pH 7.4). All cell pellets were resuspended in PBS (10 mL, 50mM, pH 6, 7.4, or 8) or bicarbonate buffer (10 mL, 50mM, pH 9.2) containing lysine (250 mM) to create 2.0× cell concentrations and transferred to separate 250 mL Erlenmeyer flasks with a screw-cap opening. The reactions were spiked with 1-butanol (100 mM). Isooctane (2 mL) was added to each flask, the flasks were capped, and placed in shaker incubator (30°C, 200 RPM). At 24 hours, the organic layer was separated and reserved. Product yields were determined by GC-MS.

Isolated Enzyme:
In 250 mL Erlenmeyer flasks with a screw-cap opening, catalase (10 mg) was dissolved in PBS (10 mL, 50 mM, pH 6, 7.4, or 8) or bicarbonate/carbonate buffer (10 mL, 50 mM, pH 9.2) containing lysine (250 mM). The reactions were spiked with 1-butanol (100 mM) and alcohol oxidase (60 units) was added. Isooctane (2 mL) was added to each flask, the flasks were capped, and placed in shaker incubator (30°C, 200 RPM). At 24 hours, the organic layer was separated and reserved. Product yields were determined by GC-MS.

S8. Temperature

Whole Cell:
K. pastoris cultures were grown for 72 hours (OD_{600} ~ 2.2) in methanol media (~125 mL). Cell culture suspensions (20 mL) were pelleted (10 minutes, 5000 × g), and washed with PBS (1 × 10 mL, 50 mM, pH 8). All cell pellets were resuspended in PBS (10 mL, 50mM, pH 6, 7.4, or 8) containing lysine (250 mM) to create 2.0× cell concentrations and transferred to separate 250 mL Erlenmeyer flasks with a screw-cap opening. The reactions were spiked with 1-butanol (100 mM). Isooctane (2 mL) was added to each flask, the flasks were capped, and placed in shaker incubator (25, 30, 37, or 45°C, 200 RPM). At 24 hours, the organic layer was separated and reserved. Product yields were determined by GC-MS.

Isolated Enzyme:
In 250 mL Erlenmeyer flasks with a screw-cap opening, catalase (10 mg) was dissolved in PBS (10 mL, 50 mM, pH 8) containing lysine (250 mM). The reactions were spiked with 1-butanol (100 mM) and alcohol oxidase (60 units) was added. Isooctane (2 mL) was added to each flask, the flasks were capped, and placed in shaker incubator (25, 30, 37, or 45°C, 200 RPM). At 24 hours, the organic layer was separated and reserved. Product yields were determined by GC-MS.

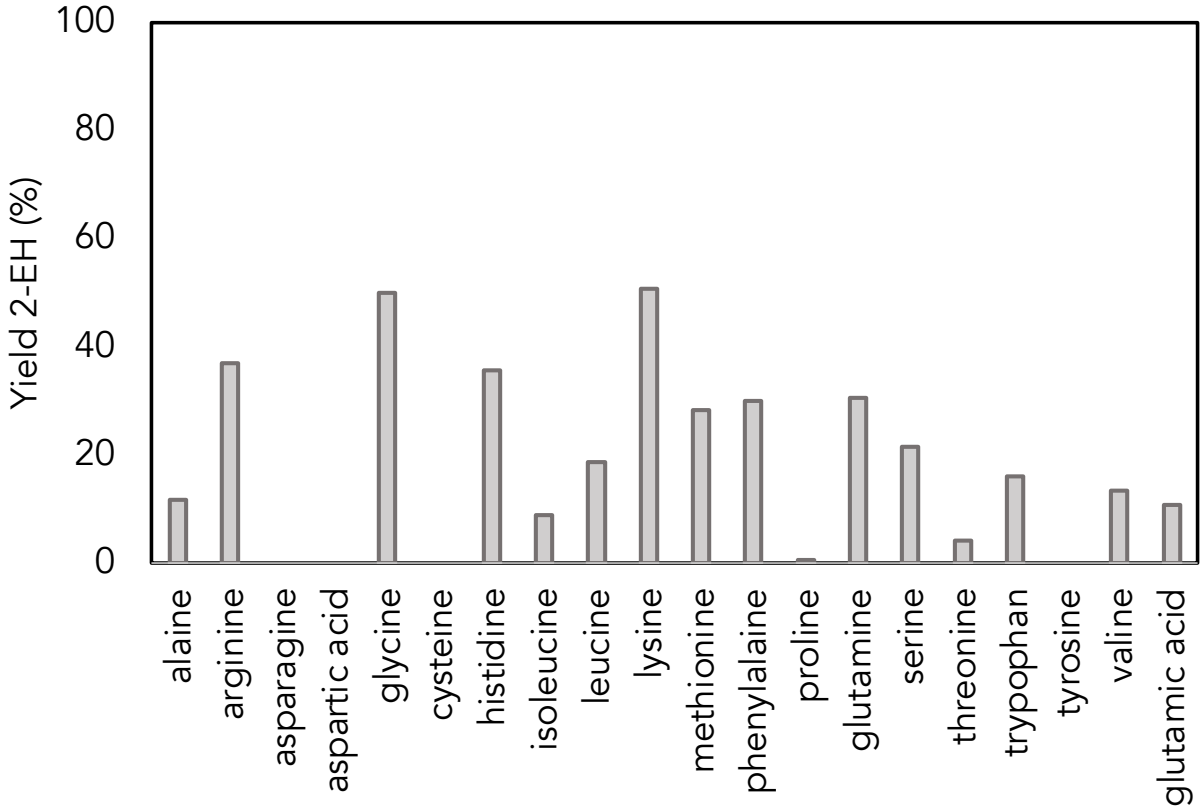

Figure S1. Yield of 2-EH in reactions containing buffer (50 mM PBS), amino acid (100 mM), n-butyraldehyde (200 mM) and isooctane (16.7% v/v). Reactions were run for 24 h at room temperature. Yield of 2-EH was determined by GC-MS.

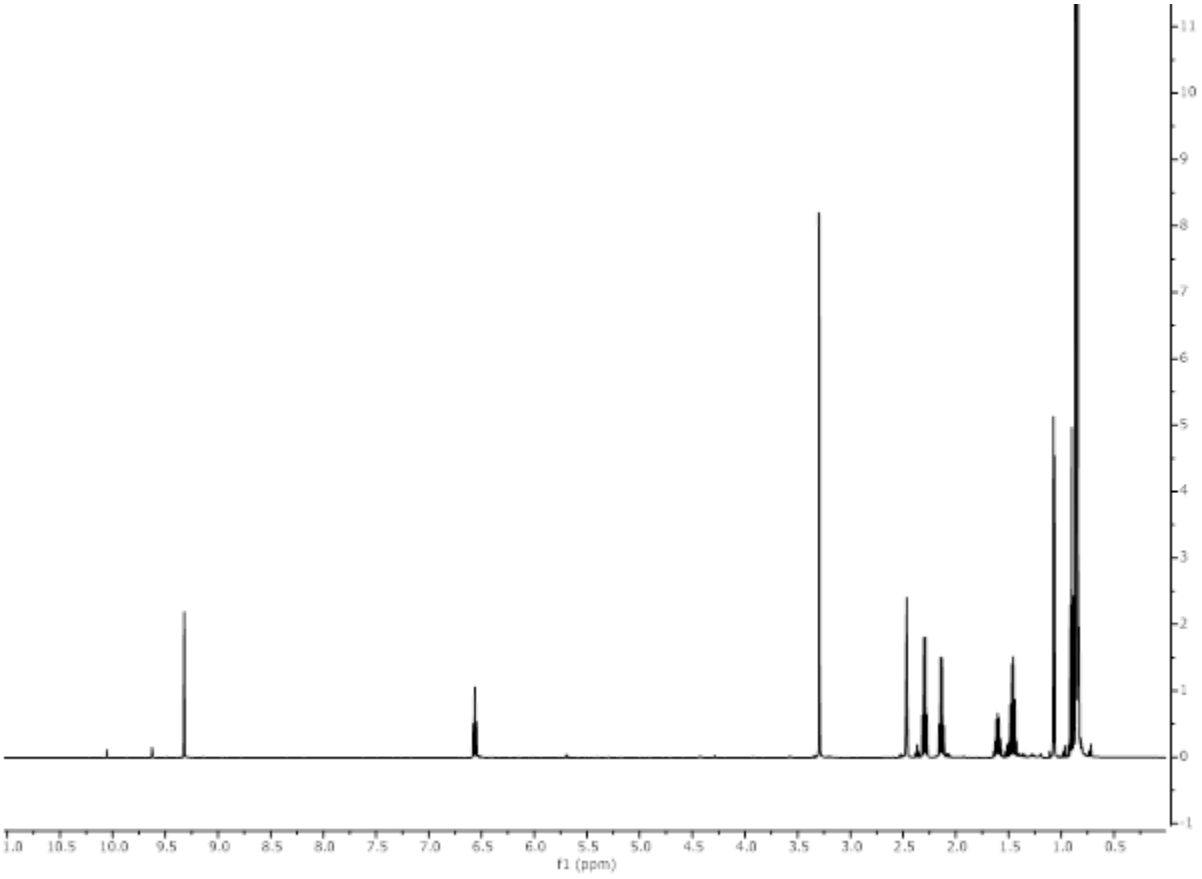

Figure S2. NMR spectra of the isooctane-phase product, 2-ethyl-2-hexenal, after extraction from isooctane with DMSO-d6. Trace isooctane is present at δ ~ 1.6 ppm (m) and 1.1 ppm (d).

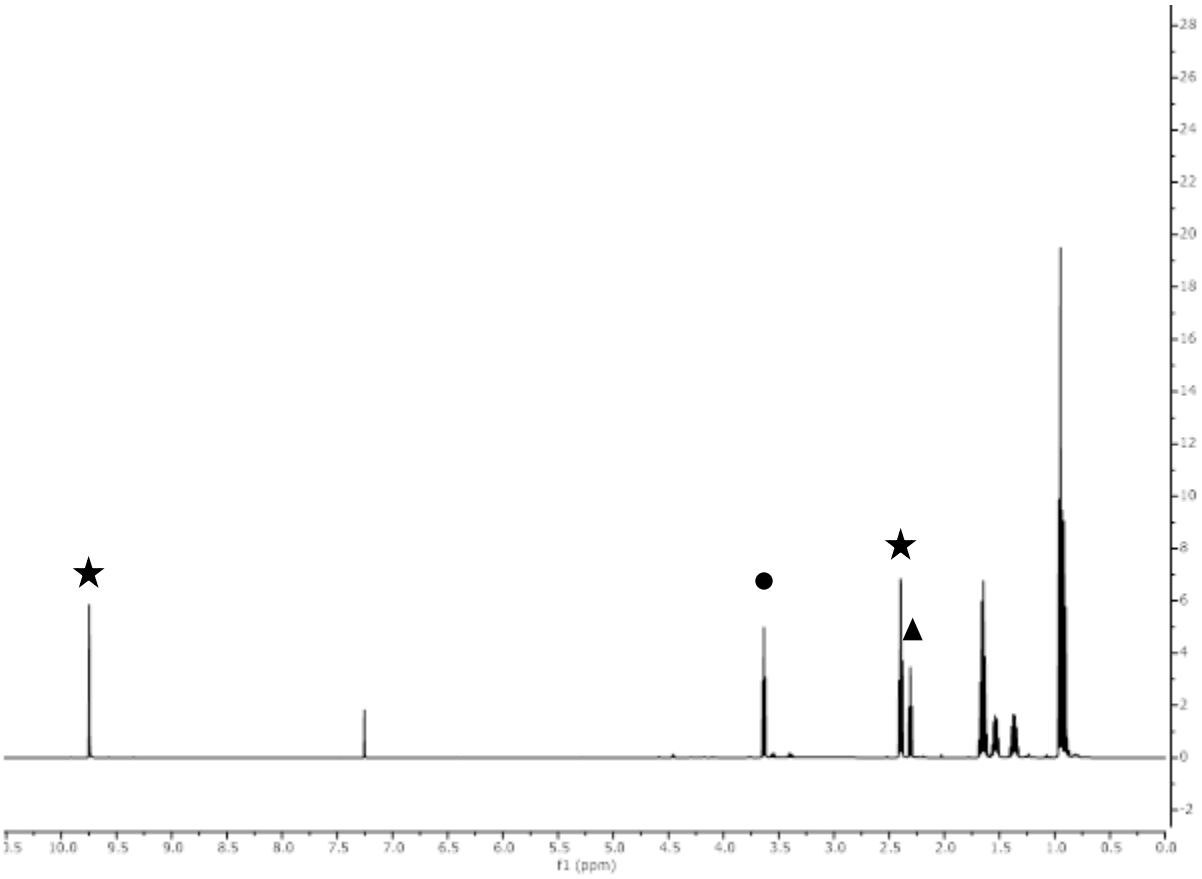

Figure S3. NMR spectra of *K. pastoris* aqueous phase products, butanol (●), butyradehyde (★), and butanoic acid (▲), after acidification (pH<4) and extraction with $CDCl_3$.

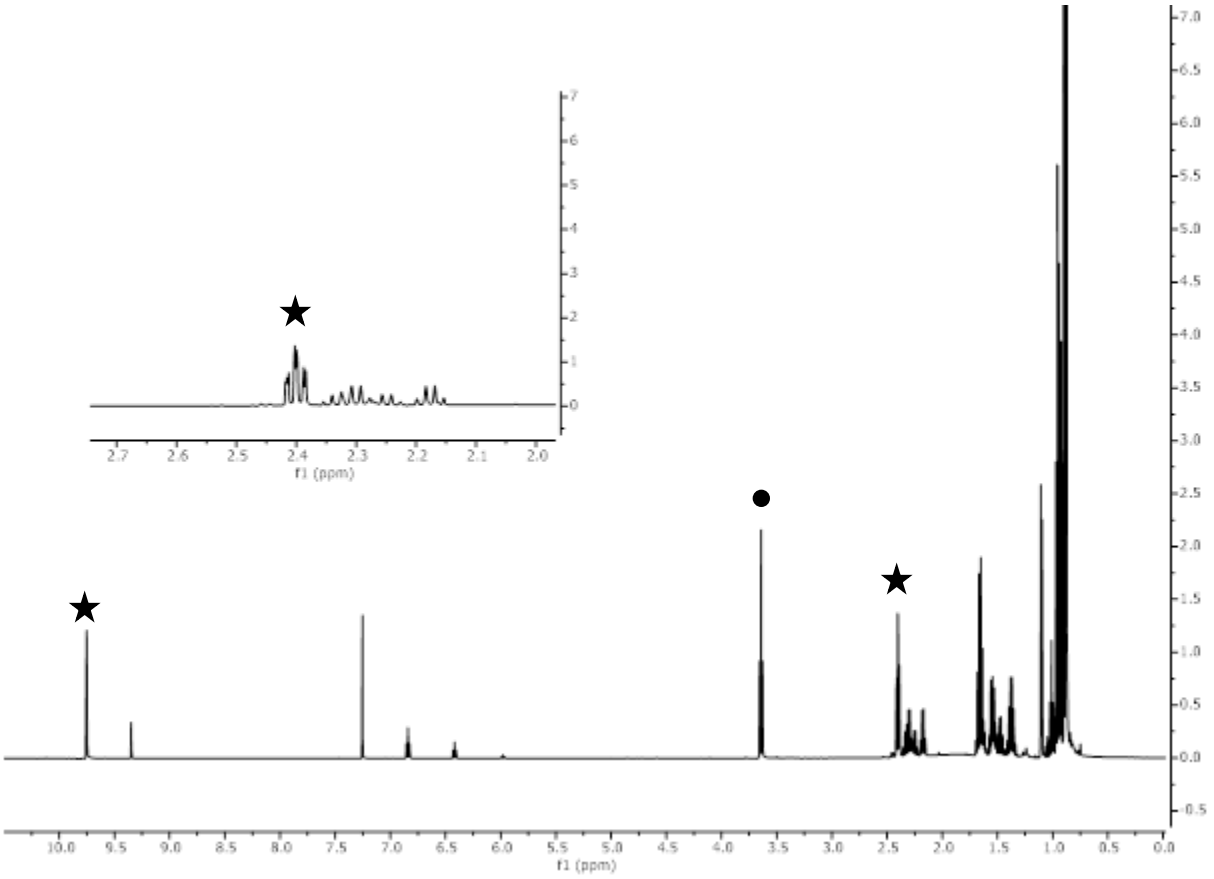

Figure S4. Representative NMR spectra of alcohol oxidase aqueous phase products, butanol (●) and butyraldehyde (★), after acidification (pH<4) and extraction with $CDCl_3$. Trace 2-EH is also present (δ ~ 9.35 ppm).

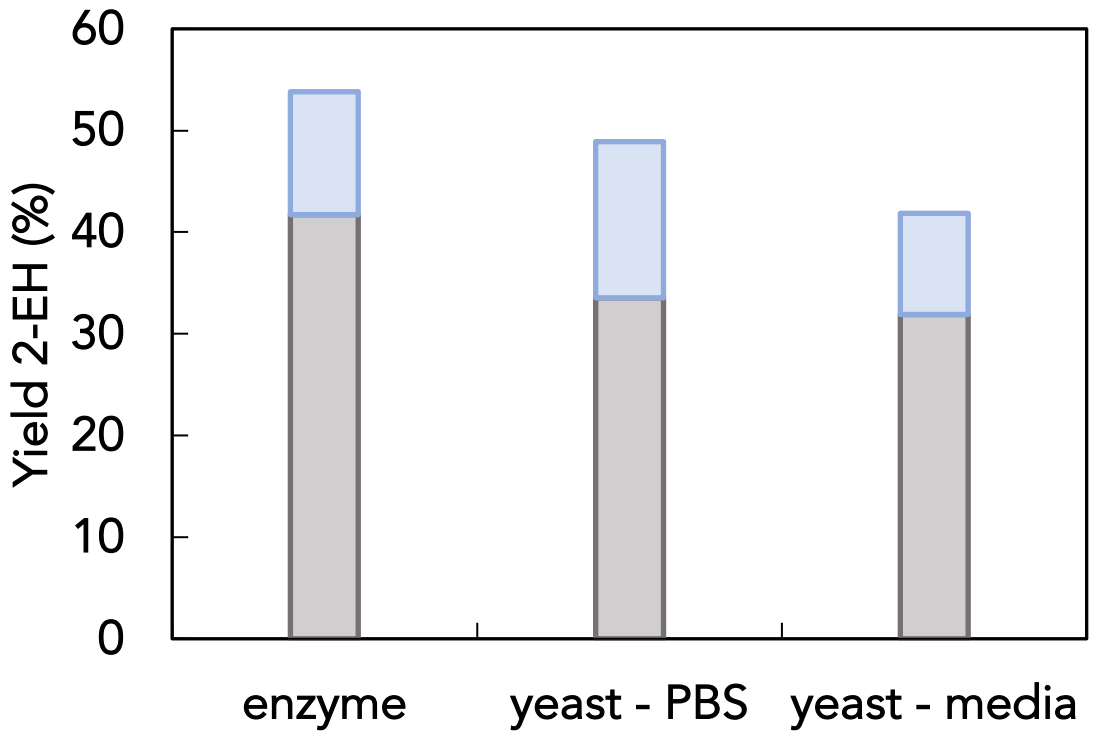

Figure S5. Yields of 2-EH with second addition of n-butanol after 24 h. Reactions were run with alcohol oxidase (60 U) or *K. pastoris* (2×) as indicated, 100 mM n-butanol in PBS, pH 8 containing lysine (250 mM) and an isooctane overlay (16.7%) at 30°C. After 24 h a sample of the isooctane was collected and an additional aliquot of n-butanol was added. Grey bars indicated mg of product (2-EH) produced in the first 24 h; the blue bar indicates additional product produced with second addition of alcohol.