Productivity enhancement of C=C bioreduction by coupling the in situ substrate feeding product removal technology with isolated enzymes

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ELECTRONIC SUPPLEMENTARY INFORMATION (ESI)

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General methods

Materials. All chemical reagents and solvents were purchased from Sigma-Aldrich and used without further purification. With the only exception of horse liver alcohol dehydrogenase (HLADH, purchased from Sigma-Aldrich), all the enzymes employed were overexpressed in Escherichia coli BL21 (DE3) strains harboring a specific plasmid prepared according to standard molecular biology techniques: pET30a-OYE2 and pET30a-OYE3 from Saccharomyces cerevisiae BY4741 and pKTS-GDH from Bacillus megaterium DSM509.¹

Analytical methods. GC-MS analyses were performed on an Agilent HP 6890 gas-cromatograph equipped with a 5973 mass detector and an Agilent HP-5 (30 m × 0.25 mm × 0.25 μm) column. Method: 60°C (1 min) / 6°C/min / 150°C (1 min) / 12°C/min / 280°C (5 min). Chiral GC analyses of compounds 1b, 3b, 4b were performed on a DANI HT 86.10 gas-chromatograph equipped with a Varian Chirasil-Dex CB (25 m × 0.25 mm) column. Method for compound 1b: 75°C (1 min) / 3°C/min / 119°C (17 min) / 30°C/min / 180°C (5 min). Method for compounds 3b and 4b: 60°C (1 min) / 2°C/min / 150°C (10 min) / 30°C/min / 180°C (5 min). Chiral GC analyses of compound 2b were performed on an Agilent HP 6890 gas-chromatograph equipped with a Mega DACtBSil.BetaCDX (25 m × 0.25 mm × 0.25 μm) column. Method: 60°C (3 min) / 3°C/min / 180°C (2 min) / 30°C/min / 220°C (5 min). Chiral HPLC analyses were performed on a Merck-Hitachi L-4250 chromatograph equipped with a Chiralcel OD column and UV detector (210 nm). For compound 1c: mobile phase n-hexane/i-PrOH 98:2, flow rate 0.6 mL/min. For the methyl ester prepared from 1b: mobile phase n-hexane/i-PrOH 99:1, flow rate 0.6 mL/min. For compound 2c: mobile phase n-hexane/i-PrOH 98:2, flow rate 0.6 mL/min. For compounds 3c and 4c: mobile phase n-hexane/i-PrOH 97:3, flow rate 1.0 mL/min. ¹H and ¹³C NMR spectra were recorded on a Bruker ARX 400 spectrometer (400 MHz ¹H, 100.6 MHz ¹³C) in CDCl₃ solution at r.t., using TMS as internal standard for ¹H and CDCl₃ for ¹³C; chemical shifts δ are expressed in ppm relative to TMS, J values are given in Hz. Optical rotations were determined on a Dr. Kernchen Propol digital automatic polarimeter and are expressed in ° cm³ g⁻¹ dm⁻¹. TLC analyses were performed on Merck Kieselgel 60 F₂₅₄ plates. Protein concentration was determined with the Bio-Rad Protein Assay reagent according to Bradford,² using bovine serum albumine (BSA) as a standard.

Experimental procedures for the biocatalysed reductions

Baker’s yeast-mediated bioreduction

Method A (homogenous phase). To a mechanically stirred mixture of commercial baker’s yeast (250 g) in tap water (1 L) at 30°C, was added a solution of glucose (50 g) in water (100 mL). After 1 hour the substrate 1a (1 g) was added in one portion. The vigorous stirring was continued for 4 days. During that time more baker’s yeast (100 g) and glucose (20 g) were added after 24 and 48 hours. Then, the mixture was filtered on a celite pad and the aqueous phase was extracted with EtOAc (4 × 250 mL). The combined organic phase was concentrated under reduced pressure to afford a brownish oil that was dissolved in CH₂Cl₂ (150 mL). To this solution, after washing with brine (2 × 100 mL) and drying over Na₂SO₄, activated MnO₂ (20 g) was added. After complete conversion of the residual allylic alcohol to the corresponding aldehyde (checked by TLC), the MnO₂ was removed by filtration and the solution was concentrated under reduced pressure. The residue was submitted to column chromatography purification using n-hexane/EtOAc (9:1) as eluent to give, in order of elution, the starting material and the corresponding saturated alcohol.

Method B (SFPR technology). The same procedure of Method A is followed. The substrate 1a-4a adsorbed on XAD 1180 resin (for substrate loading and Xr/s see Table 1) was added in one portion. After 48 h, the mixture was filtered on a sintered glass funnel (porosity 0, >165 μm) and the aqueous phase was extracted again with more resin (10 g). The combined resin crops were washed with acetone (100 mL) and EtOAc (4 × 100 mL). The work-up was carried out as described above.

Enoate reductases-mediated bioreduction

Method A (OYEs). The substrate 1a-4a either dissolved in DMSO or adsorbed on XAD 1180 resin (for substrate loading and Xr/s see Table 1) was added to a solution of glucose (4 eq. with respect to 1a-4a), NADP⁺ (0.1 mM), GDH (4 U mL⁻¹) and OYE (150 μg mL⁻¹) in phosphate buffer (1.0÷10.0 mL, 50 mM, pH 7.0). The mixture was stirred for 12 h in an orbital shaker (160 rpm, 30°C). The solution was decanted and both the resins and the aqueous phase were extracted with EtOAc (2 × 0.5 mL/mL aq), centrifuging after extraction (15000 g, 1.5 min). The combined organic solutions were dried over Na₂SO₄ and concentrated under reduced pressure, yielding the saturated aldehyde or a mixture of saturated aldehyde and starting material. Reactions in optimized conditions were scaled up to preparative scale (50÷150 mg) for product characterization and determination of isolated yields.

Method B (OYEs+HLADH). The same procedure of Method A is followed, adding HLADH (2 U mL⁻¹) and NAD⁺ (0.1 mM) to the reaction mixture.
Experimental procedure for the overexpression of the enzymes in *E. coli* BL21 (DE3)

A 5 mL culture in LB medium containing the appropriate antibiotic (50 μg mL⁻¹ kanamycin for pET-30a, 100 μg mL⁻¹ ampicillin for pKTS) was inoculated with a single colony from a fresh plate and grown overnight at 37°C and 220 rpm. This starter culture was used to inoculate a 200 mL culture, which was in turn grown overnight at the same conditions and used to inoculate a 1.5 L culture. The latter was shaken at 37°C and 220 rpm until OD₆₀₀ reached 0.4-0.5 and then enzyme expression was induced by adding 0.1 mM IPTG (50 ng mL⁻¹ anhydrotetracycline was also added in the case of the pKTS-GDH plasmid). After 5-6 h the cells were harvested by centrifugation (5000 g, 20 min, 4°C), resuspended in 50 mL of lysis buffer (20 mM phosphate buffer pH 7.0, 300 mM NaCl, 10 mM imidazole) and homogenized (Haskel high-pressure homogenizer). The cell-free extract, after centrifugation (20000 g, 20 min, 4°C), was chromatographed on IMAC stationary phase (Ni-Sepharose Fast Flow, GE Healthcare) with a mobile phase composed of 20 mM phosphate buffer, pH 7.0, 300 mM NaCl and a 10-300 mM imidazole gradient. Protein elution was monitored at 280 nm, the fractions were collected according to the chromatogram and dialyzed twice against 1.0 L of 20 mM phosphate buffer pH 7.0 (12 h each, 4°C) to remove imidazole and salts. Purified protein aliquots were stored frozen at –80°C.
(S)-2-methoxy-3-(4-methoxyphenyl)propanal (1b)

$^{[\alpha]}_D^{20} = -21.6$ (c 1.14, CHCl$_3$), ee 94%; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ ppm 9.68 (d, $J = 1.8$ Hz, 1 H), 7.15 (d, $J = 8.5$ Hz, 2 H), 6.85 (d, $J = 8.5$ Hz, 2 H), 3.80 (s, 3 H), 3.76 (ddd, $J = 7.6, 5.2, 2.2$ Hz, 1 H), 3.42 (s, 3 H), 2.79-3.01 (m$_{AB}$, 2 H). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ ppm 203.3, 158.5, 130.3, 128.3, 113.9, 86.6, 58.5, 55.2, 35.5. HRMS (ESI) calcd for C$_{11}$H$_{14}$O$_3$ 194.0943, found 194.0941.
(S)-2-benzylpentanal (2b)

\([\alpha]_D^{20} = -7.0\ (c\ 1.1,\ CHCl_3),\ ee\ 90\%;\)\n
\(^1\mathrm{H}\) NMR (400 MHz, CDCl\(_3\)) \(\delta\) ppm 9.59 (d, \(J = 2.5\) Hz, 1 H), 7.06-7.27 (m, 5 H), 2.78 (m, 2 H), 2.51-2.60 (m, 1 H), 1.17-1.63 (m, 4 H), 0.83 (t, \(J = 7.6\) Hz, 3 H). \(^{13}\mathrm{C}\) NMR (100 MHz, CDCl\(_3\)) \(\delta\) ppm 204.6, 138.9, 128.9, 128.5, 126.3, 53.2, 35.1, 30.8, 20.2, 14.0. HRMS (ESI) calcd for C\(_{12}\)H\(_{16}\)O 176.1201, found 176.1199.
(S)-5-methoxy-1,2,3,4-tetrahydronaphthalene-2-carbaldehyde (3b)

$[\alpha]_D^{20} = -5.0$ (c 1.0, CHCl$_3$), ee 73%; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ ppm 9.79 (d, $J = 1.2$ Hz, 1 H), 7.11 (t, $J = 8.0$ Hz, 1 H), 6.76 (d, $J = 7.4$ Hz, 1 H), 6.68 (d, $J = 7.8$ Hz, 1 H), 3.81 (s, 3 H), 2.87-3.01 (m, 3 H), 2.56-2.70 (m, 2 H), 2.19-2.28 (m, 1 H), 1.68-1.80 (m, 1 H). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ ppm 203.9, 157.2, 135.6, 126.3, 124.9, 121.4, 107.3, 55.2, 46.6, 28.7, 22.7, 22.0. HRMS (ESI) calcd for C$_{12}$H$_{14}$O$_2$ 190.0994, found 190.0991.
(S)-(5-methoxychroman-3-yl)methanol (4c)

\[ \alpha \]D\textsubscript{20} = -6.2 (c 1.17, CHCl\textsubscript{3}), ee 98%; \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) \( \delta \) ppm 7.05 (t, \( J = 8.2 \) Hz, 1 H), 6.48 (d, \( J = 8.2 \) Hz, 1 H), 6.42 (d, \( J = 8.2 \) Hz, 1 H), 4.27 (ddd, \( J = 10.7, 3.1, 1.4 \) Hz, 1 H), 3.95 (dd, \( J = 10.7, 7.7 \) Hz, 1 H), 3.81 (s, 3 H), 3.73 (dd, \( J = 10.8, 5.8 \) Hz, 1 H), 3.64 (dd, \( J = 10.8, 7.8 \) Hz, 1 H), 2.79 (dd, \( J = 17.1, 6.0 \) Hz, 1 H), 2.38 (dd, \( J = 17.1, 7.8 \) Hz, 1 H), 2.29-2.19 (m, 1 H), 1.62 (br s, 1H). \textsuperscript{13}C NMR (101 MHz, CDCl\textsubscript{3}) \( \delta \) ppm 158.2, 155.4, 126.9, 110.2, 109.4, 102.0, 67.3, 63.6, 55.4, 34.4, 21.9. HRMS (ESI) calcd for C\textsubscript{11}H\textsubscript{14}O\textsubscript{3} 194.0943, found 194.0948.
Representative GC/HPLC chromatograms

Methyl (S)-2-methoxy-3-(4-methoxyphenyl)propanoate (prepared from 1b), ee 94% by HPLC

(S)-2-benzylpentanal (2b), ee 90% by GC

(S)-5-methoxy-1,2,3,4-tetrahydronaphthalene-2-carbaldehyde (3b), ee 83% by GC

(S)-(5-methoxochroman-3-yl)methanol (4c), ee 98% by HPLC