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SUPPORTING INFORMATION

Bio-derived Production of Cinnamyl Alcohol via a Three Step Biocatalytic Cascade and Metabolic Engineering

Evaldas Klumbys, Ziga Zebec, Nicholas J. Weise, Nicholas J. Turner, and Nigel S. Scrutton

BBSRC/EPSRC Centre for Synthetic Biology of Fine and Speciality Chemicals, Manchester Institute of Biotechnology & School of Chemistry, University of Manchester, 131 Princess Street, M1 7DN, Manchester, UK

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General methods

Analytical grade reagents and solvents were obtained from Sigma-Aldrich, AlfaAesar or Fisher Scientific and used without further purification, unless stated otherwise. NADH and NADPH were acquired from Melford. ATP, L-Phenylalanine, *trans*-Cinnamic and *trans*-Cinnamaldehyde were purchased from Sigma-Aldrich. Restriction enzyme kits, expression vectors and laboratory strain *E. coli* were purchased from New England Biolabs (NEB). The L-phenylalanine over production strain, *E. coli* NST (ATCC 31882) was purchased from ATCC®. Microbiological media ingredients were obtained from ForMedium™ (LB, TB and M9) and prepared according to the recommended protocols provided. GDH (CDX-901) was kindly supplied by Codexis alcohol dehydrogenase from *Saccharomyces cerevisiae* (ScADH) (≥300 units/mg protein) was obtained from Sigma. *Mycobacterium marinum* carboxylic acid reductase (MCAR) and *Bacillus subtilis* phosphopantetheine transferase (Sfp) were provided in pET-21a and pCDF-1b expression vectors respectively. *Anabaena variabilis* phenylalanine ammonia lyase (AvPAL) was provided in a pET-16b plasmid.

Experimental Procedures

Preparation of the lyophilised biocatalyst

A pET-16b expression plasmid containing the His_6-tagged open reading frame for AvPAL was used as for previous studies[1] and transformed into *E. coli* BL21(DE3) protein production strain (New England Biolabs) according to the supplier's protocol. Expression of the gene encoding AvPAL was conducted according to previously reported methods.[1] LB medium (5 mL, supplemented with kanamycin or ampicillin) was inoculated with a single colony of *E. coli* BL21(DE3) containing the suitable plasmid and grown for 16 h at 37°C and 250 rpm. This starter culture was then used to inoculate LB-based auto-induction medium[2] (800 mL, supplemented with 50 μg mL^{-1} kanamycin), which was incubated at 18 °C and 250 rpm for 4 days. The cells were pelleted by centrifugation (4000 rpm, 12 min) and separated from the supernatant for storage of the wet cell mass at -20°C until further use. In the case of AvPAL a lyophilised dry cell powder formulation was used as reported previously.[3] The isolated cell mass was flash frozen in liquid nitrogen and freeze dried using a Heto Power Dry LL1500 Freeze Dryer for 16-24 h. The dry cell mass was then ground into a fine powder and stored at –20°C until required.

MCAR Whole Cell Biocatalyst Preparation

For pre-culture a single colony was grown in 100 ml either Terrific Broth (TB) or Lysogeny Broth (LB) containing 100 μg/ml ampicillin and 50 μg/ml streptomycin over night at 37 °C and 190 rpm. MCAR expression was followed as mentioned next. 5 ml of pre-culture was transferred to 500 ml TB containing the same concentration of antibiotics and grown at 37 °C and 180 rpm shaking. At exponential growth phase (OD_{600}=0.6-0.8) cells were induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside and incubated overnight (20h) at 20 °C and 180 rpm. Cells were pelleted (6,000 rpm, 8 min, 4°C) and washed with 0.5 % sodium chloride solution. The cells were then pelleted again (4,000 rpm, 8 min, 4 °C) and lyophilized using a Heto Power Dry LL1500 Freeze Dryer for 16-24 h. The dry cell mass was then ground into a fine powder and stored at –20°C until required.

Purification of MCAR

E. coli cells with overexpressed MCAR PPant were lysed in 100 ml 50 mM potassium phosphate buffer pH 8.0, 500 mM NaCl and 10 mM imidazole buffer. Cells were disrupted by the French Press at 1500 psi and cell debris removed by centrifugation at ~48,000 x g, 4 °C for 1 h. Cell lysate was filtrated using 0.45 μm Minisart NML syringe filters (surfactant free cellulose acetate membrane) and loaded on Ni-IDA resin (Generon). Stepwise elution performed with 50 ml buffer at 4°C. The sample after Ni-IDA was concentrated by the combined use of Amicon stirred cell concentrator (76 mm, 100,000 MWCO polyethersulfone membrane Discs from generon) and Vivaspin

20 (100,000 MWCO PES membrane GE Healthcare) at 2,800 x g up to 6 ml at 4 °C. 3 ml was loaded onto the HiLoad 16/60 Superdex 200 with 5 ml loop and isocratically eluted in 50 mM potassium phosphate buffer pH 8.0, 150 mM NaCl at 1 ml/min flow rate at 4 °C. Eluent with high A_{280} absorbance was collected and appropriate fractions selected according to SDS-PAGE analyses. These were then concentrated using a Vivaspin 20 centrifugation tube (100,000 MWCO PES membranes from GE Healthcare) at 2,800 x g (4°C). The retained enzyme isolate was then frozen as several protein ball stocks by dropping protein solution slowly in liquid nitrogen for storage at -80 °C.

Reaction with AvPAL

AvPAL reactions were performed in 1 ml volume containing 10 mM L-Phe, 100 mM potassium phosphate buffer (pH 7.5), 1 – 5 mg of AvPAL dried cells at 30 °C, 250 rpm vertical shaking and 24 h. 400 µl of sample was spun down and filtered using standard filter vial 0.45 µm PVDF (Thomson instrument company) for HPLC analysis.

CAR and CAR-ADH Biotransformations

Reactions were performed in 100 mM potassium phosphate buffer (pH 7.5) containing 10 mM $MgCl_2$, 1 mg/ml MCAR dried cells, 15 mM D-glucose, 10 mM ATP, 10 U GDH, 500 µM $NADP^+$ and 5 mM *trans*-cinnamic acid in 1ml volume. In addition, 0.05 mg/ml ADH, 500 µM NAD^+ and 15 mM D-glucose were supplied to test the double reduction cascade. Biotransformations were incubated at 30 °C, 250 rpm vertical shaking. Following an appropriate reaction time, the mixture was acidified with 100 µl concentrated HCl and extracted using 2 x 400 µl ethyl acetate before drying with anhydrous magnesium sulphate. The sample was then derivatised using 200 µl methanol and 10 µl 2.0 M (Trimethylsilyl) diazomethane solution in hexane (28 °C, 60 min, 250rpm). Derivatization was terminated by addition of 2 µl glacial acetic acid before incubation for a further 20 min. Product and starting material were identified using GC and GC-MS to assign peaks, with conversions determined via comparison of integrated peak areas.

Preparative Scale Synthesis of Cinnamyl Alcohol

The reaction was performed in 100 mM potassium phosphate buffer (pH 7.5) with 3 mg/ml of AvPAL dried cells and 10 mM L-Phe (107 mg, 65 ml final volume) in a round bottom flask (30 °C, 250 rpm). After sufficient conversion to cinnamic acid the reaction mixture was centrifuged to remove lyophilized AvPAL-containing cells and the supernatant separated and supplemented with 10 mM $MgCl_2$, 1 mg/ml MCAR dried cells, 30 mM D-glucose, 10 mM ATP, 10 U GDH, 0.05 mg/ml ADH, 500 µM $NADP^+$, 500 µM NAD^+ (130 ml final volume). The new reaction mixture was then incubated at 30 °C with 250 rpm agitation until full conversion was reached. Monitoring of the AvPAL and CAR-ADH reactions was performed via sampling and HPLC / GC analyses as previously described. Following the complete biotransformation, the reaction mixture was centrifuged down at 4,000 rpm for 20 min and products extracted 3 times with ethyl acetate (1:1/v:v). The organic phase was dried with anhydrous magnesium sulphate, filtered and concentrated using rotary evaporator to obtain crude yellowish oil which solidified upon cooling to room temperature. The cinnamyl alcohol product was then purified by flash chromatography using a silica gel column (pore size 60 Å, 220-440 mesh particle size, 35-75 µm particle size) with isocratic elution (dichlomethane:methanol - 98:2). This yielded colourless oil which solidified upon cooling. A small sample of the isolated product was dissolved in 800 µl $CDCl_3$ for ^1H- and ^{13}C-NMR analyses. These were recorded using Bruker Biospin instrument operating at 400 MHz.

In-vivo production of cinnamyl-alcohol in *E. coli* NST

Molecular cloning

In order to convert L-phenylalanine to cinnamyl alcohol, we constructed the plasmid pZZ-Eva2 (Figure S6) that contains the most efficient genes/parts (srPAL, ADH/KRED, CAR11, Sfp and P_{Rham}) determined by biocatalysis. First, each part was separately amplified from the corresponding expression vector by polymerase chain reaction (PCR) using the Phusion-polymerase (NEB). The parts were gel purified (Macherey-nagel) and *DpnI* digested (NEB), always following the manufacturers protocol. Next, we fused srPAL-ADH/KRED and P_{Rham}-Sfp using overlap extension PCR (OE-PCR) to form a single part,[4] resulting in a total of three parts (srPAL-ADH/KRED =2704bp, CAR= 3636bp and P_{Rham}-Sfp =881bp). Finally, inverse PCR was employed to linearize the plasmid backbone pBbE8kRFP.[5] All three parts and the backbone were fused together to form a single vector (Figure S6) in a molar ratio 2:2:2:1 using the In-Fusion®-Kit (Takara). The plasmid's insert region, which represents the bioengineered pathway, was fully sequenced by Sanger sequencing. All details regarding oligonucleotides, templates and PCR product-sizes can be found in Table S1&S2. The srPAL-ADH/KRED-CAR11 operon is under the control of an L-arabinose inducible promoter, and Sfp is under the control of an L-rhamnose inducible promoter. The pZZ-Eva2 encodes kanamycin resistance gene and a colE1 origin of replication with 20-30 copies per cell.

Culturing conditions, cinnamyl alcohol extraction and quantification

We obtained the L-phenylalanine overproduction strain *E. coli* NST (ATCC 31882)[6] in order to have a stable intracellular supply of substrate for *in-vivo* production of cinnamyl alcohol. The *E. coli* NST strain can produce up to 1mg L^{-1} of L-phenylalanine in 24h,[7] using glycerol (1.5% *v/v*) and glucose (0.25%) as carbon sources that enter the cell glycolysis pathway for overproduction of L-phenylalanine via the shikimate pathway. Our production media was TB or M9 with glycerol (1.5% *v/v*) and glucose (0.25%) as carbon sources, while M9-Gly had only glycerol (1.75% *v/v*) and M9-Glu had only glucose (1.75%) (Figure 5 and S5). Since *E. coli* NST is auxotrophic for tyrosine and tryptophan, the production media were supplemented with 30 mg L^{-1} of each of the two amino acids.

E. coli NST was grown in LB medium until optical density (OD) of 0.2, and then made electro-competent for transformation with pZZ-Eva2. In order to prepare the inoculum for the *in-vivo* production experiment, three single colonies of *E. coli* NST transformed with pZZ-Eva2 were picked, transferred into 1.5ml tubes and grown in 300 µl of LB media supplemented with 50 mg L^{-1} kanamycin for 3h at 30°C while shaking at 1000rpm (Eppendorf, Thermo-shaker). 5ml of each production media variant (TB, M9, M9-Gly, M9-Glu) was inoculated with 50µl of the starting inoculum of each starting colony and incubated at 30°C for 3h at 200rpm (New Brunswick Scientific). All cultures were induced with 50mM arabinose and 0.01% L-rhamnose and harvested 24h, 48h and 72h after induction to test cinnamyl alcohol production and measure OD. To extract cinnamyl alcohol from the culture, 1ml of culture was transferred to a 2ml tube, and 100 µl 1M HCl was added with 500µl ethyl acetate and vortexed for 10 s. The phases were separated by centrifugation for 5 min at 13000 rpm, and the organic phase was collected in a fresh 1.5ml tube containing anhydrous $MgSO_4$ to remove any residual aqueous phase. This process of organic solvent extraction was repeated twice (2 x 500µl), while collected in the same tube filed with anhydrous $MgSO_4$ for drying, briefly vortexed and centrifuged for 3min at 13000rpm. Derivatization was performed in a 2ml tube mixing 600µl of the dried ethyl acetate, 200 µl methanol and 20 µl 2.0 M trimethylsilyldiazomethane, incubated for 1h at 28 °C, 600 rpm (thermo-block) in a fume hood. To stop the reaction 4 µl glacial acetic acid (2 µl/10 µl derivatization agent) was added to the mixture, followed by 20 min incubation at 28 °C, 600 rpm. Finally, 600µl of ethyl acetate containing the internal standard secondary benzene (0.1%) was added to the mix in the 2ml tube, mixing the ethyl acetate extract from the culture and the ethyl acetate with the internal standard in a 1:1 ratio. This final solution was transferred to a GC vial, processed on GC (see below) and quantified by GC area using standards (Sigma).

Analytical Methods

HPLC analysis

Reverse phase HPLC was performed on an Agilent 1200 Series LC system equipped with a G1379A degasser, a G1312A binary pump, a G1329 autosampler unit, a G1316A temperature controlled column compartment and a G1315B diode array detector. Conversion for the PAL-catalysed reaction was calculated from reverse phase liquid chromatography performed using a ZORBAX Extend-C18 column (50 mm × 4.6 mm × 3.5 µm, Agilent). Mobile phase: NH_4OH buffer (0.35% w/v, pH 10.0) / MeOH (in a ratio of 90:10). Flow rate: 1 mL min^{-1}. Temperature: 40°C. Detection wavelength: 210 nm. Peaks were assigned via comparison with commercially available standards. Starting material and product distributions were derived from integrations of peak areas with using a response factor of 2.3 to account for the higher UV absorbance of **2**. Retention times for compounds **1** and **2** were 2.3 and 5.4 minutes respectively.

GC Analysis

Volatile extracts (1 µL) from the CAR, CAR-ADH and *in vivo* cultures were analysed by gas chromatography on an Agilent Technologies 7890A GC system equipped with an FID detector and a 7693 autosampler. A DB-WAX column (30 m; 0.32 mm; 0.25 µm film thickness; JW Scientific) was used to separate the compounds. The injector temperature was set at 220°C with a split ratio of 10:1 (1 µL injection). The carrier gas was helium with a flow rate of 1.5 mL/min and a pressure of 9.2 psi. The following oven program was used: 100°C (0 min hold), ramp to 200°C at 4°C/min (0 min hold), and ramp to 240°C at 20 °C/min (1 min hold). The FID detector was maintained at a temperature of 250°C with a flow of hydrogen at 30mL/min.

GC-MS Analysis

Reaction products were primarily analysed by GCMS using an Agilent Technologies 7890B GC equipped with an Agilent Technologies 5977A MSD. The products were separated on a DB-WAX column (30 m x 0.32 mm i.d., 0.25 µM film thickness, Agilent Technologies). The injector temperature was set at 240 °C with a split ratio of 20:1 (1 µL injection). The carrier gas was helium with a flow rate of 2 mL/min and a pressure of 4.6 psi. The following oven program was used: 100°C (0 min hold), ramp to 20°C at 4 °C/min (0 min hold), and ramp to 240 °C at 20 °C/min (1 min hold). The ion source temperature of the mass spectrometer (MS) was set to 230 °C and spectra were recorded from m/z 50 to m/z 250. Compound identification was carried out using authentic standards and comparison to reference spectra in the NIST library of MS spectra and fragmentation patterns.

HRMS of compound 4

HRMS analyses were performed using an Agilent 6510 Q-TOF mass spectrometer connected to an Agilent 1200 Series LC system.

Steady-state kinetic parameters for MCAR

The substrate was dissolved in DMSO. Data were recorded by monitoring the rate of NADPH oxidation at 340 nm over a maximum period of 60 s (fitted over 30 s) under the following reaction conditions: 100 mM KH_2PO_4/K_2HPO_4 pH 7.5, 10 mM $MgCl_2$, 1 mM ATP, 200 µM NADPH, 0.3 µM MCAR enzyme at 30 °C. Analysis was performed on a Varian Cary 300 Bio UV-visible spectrophotometer using 1.5 mL UV-Cuvette semi-micro Brand® plastic cuvettes suitable for UV measurements. The data was fitted with modified Michaelis-Menten steady-state kinetic equations with substrate inhibition.

Supplementary Data Figures

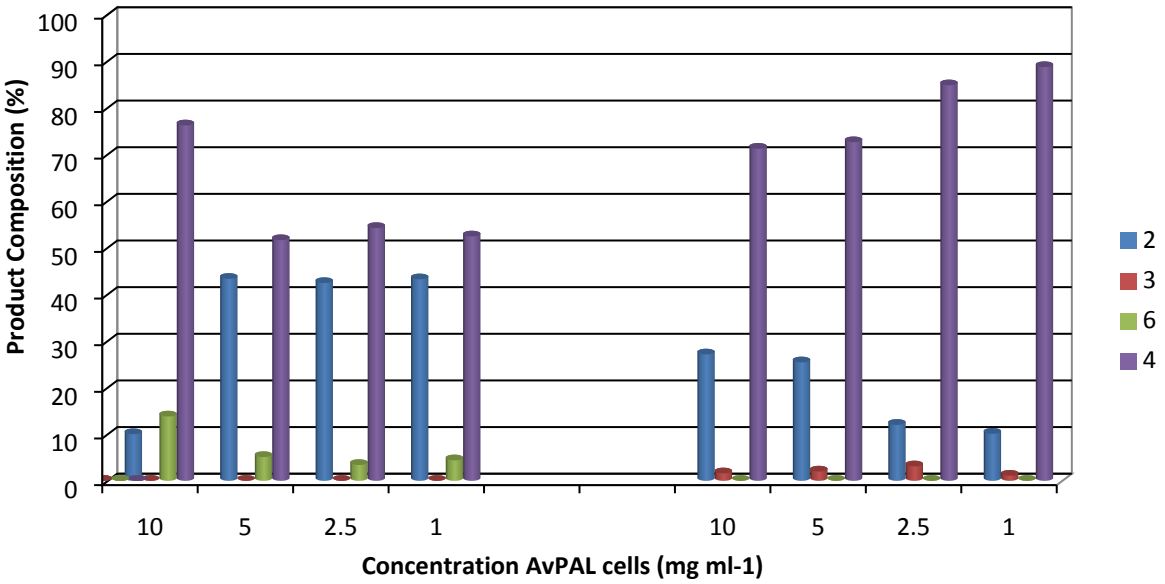


Figure S1. Comparison of one-pot triple enzyme cascade (left) and partition process (right) involving addition of CAR and ADH after completion of the PAL reaction. Partition reaction was performed in 100 mM potassium phosphate buffer (pH 7.5), AvPAL dried cells and 10 mM L-Phe at 30 °C, 250 rpm 1 ml final volume. After 20 h the reaction mixture was spun down and 500 µl supernatant supplemented with 10 mM $MgCl_2$, 1 mg/ml MCAR dried cells, 30 mM D-glucose, 10 mM ATP, 10 U GDH, 0.05 mg/ml ADH, 500 µM $NADP^+$, 500 µM NAD^+ up to final volume of 1 ml. Incubated at 30 °C, 250 rpm 20h. One-pot reaction had the same conditions but starting concentration of substrate was 5 mM instead of 10 mM and reaction was stopped after 20 h.

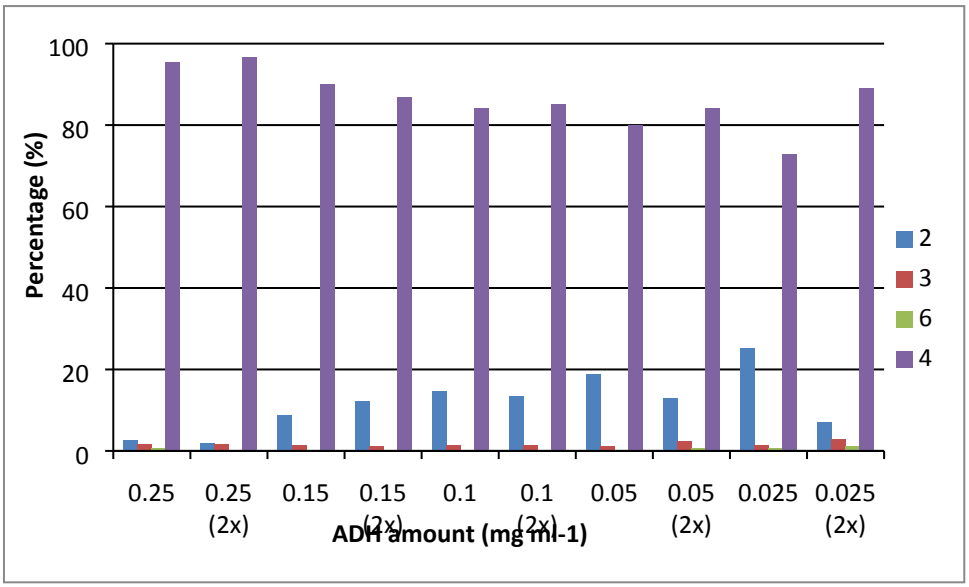


Figure S2. Effect of ScADH loading and batch addition on conversion of **2** in combination with MCAR, following initial production by AvPAL. The reaction was performed in 100 mM potassium phosphate buffer (pH 7.5), 5 mM *trans*-cinnamic acid, 10 mM MgCl2, 1 mg mL-1 MCAR lyophilised cells, 0.25 – 0.025 mg mL-1 ScADH, 30 mM D-glucose, 10 mM ATP, 10 U GDH, 500 µM $NADP^+$, 500 µM NAD^+ final volume 1 mL at 30 °C, 250 rpm, 22 h. 2x indicates the second addition of ADH after 4h.

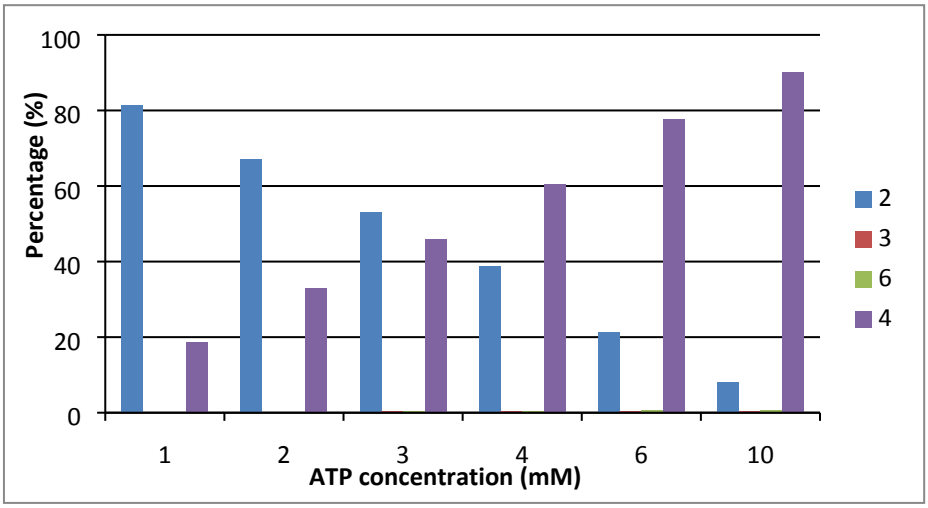
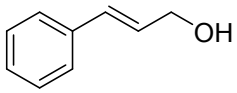


Figure S3. The impact of ATP concentration on the CAR / ADH cascade. The reaction was performed in 100 mM potassium phosphate buffer (pH 7.5), 5 mM *trans*-cinnamic acid, 10 mM $MgCl_2$, 1 mg mL^{-1} MCAR lyophilised cells, 0.25 mg mL^{-1} ScADH, 30 mM D-glucose, 1 - 10 mM ATP, 10 U GDH, 500 µM $NADP^+$, 500 µM NAD^+ final volume 1 mL at 30 °C, 250 rpm, 22 h.

Characterisation data of 4 from preparative scale biotransformation

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer (400.1 MHz) without additional internal standard. Chemical shifts are reported as δ in parts per million (ppm), calibrated against residual solvent signal.



(*R*)-3-amino-3-(3-fluorophenyl)propanoic acid (1c)

^{1}H NMR ($CDCl_3$): δ 7.16-7.31 (m, 5H, ArH), 6.50-6.54 (d, 1H, J = 16 Hz, C=CH), 6.23-6.30 (dt, 1H, J = 16, 8 Hz, $C=CHCH_2$), 4.21-4.23 (dd, 2H, J = 8, 4 Hz, $C=CHCH_2$); ^{13}C NMR ($CDCl_3$): 136.70, 131.09, 128.60, 128.54, 127.69, 126.48, 63.65.

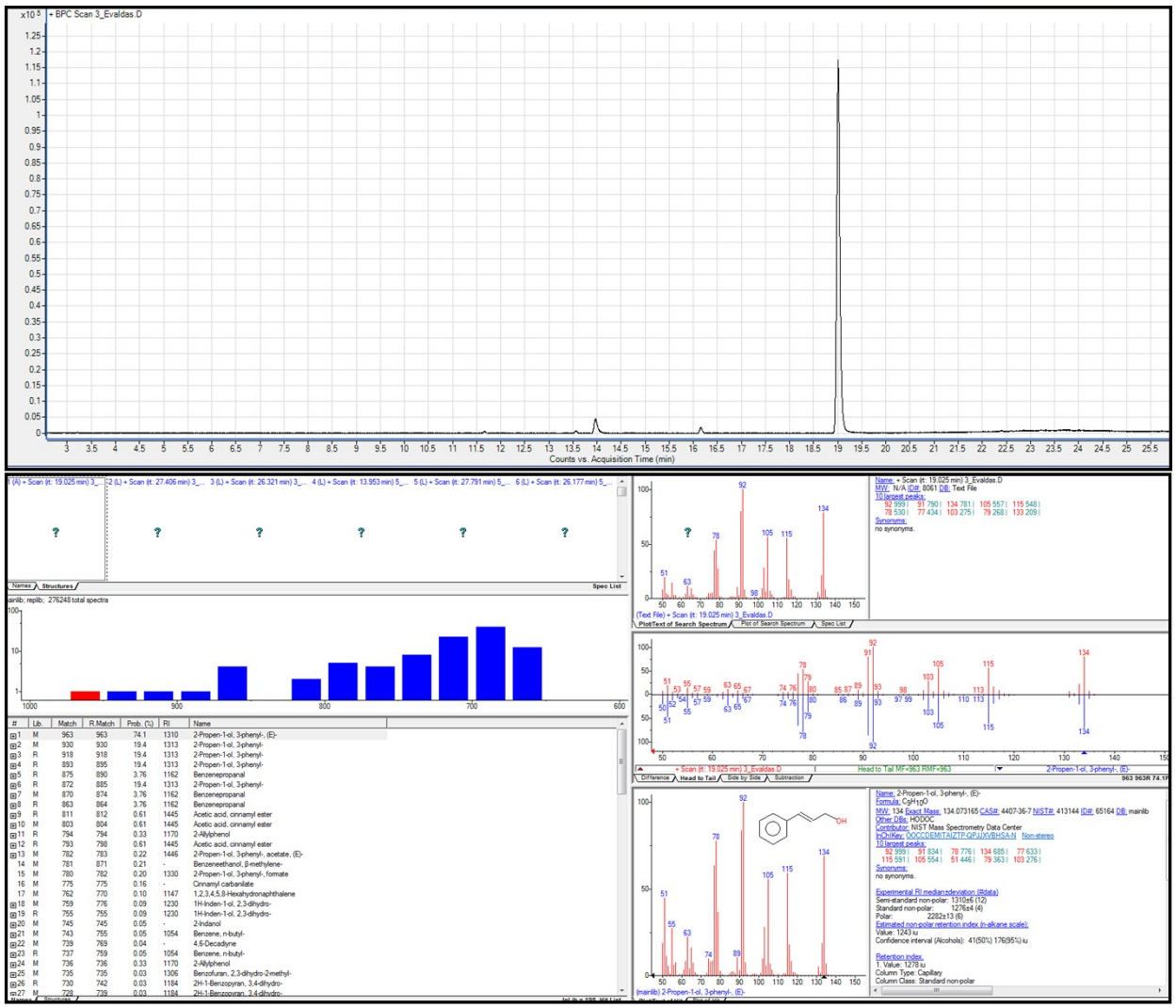


Figure S4. Top graph shows chromatogram from GC-MS after flash purification. Bottom graph shows the ionization spectrum and prediction patterns of the main peak from GC-MS chromatogram.

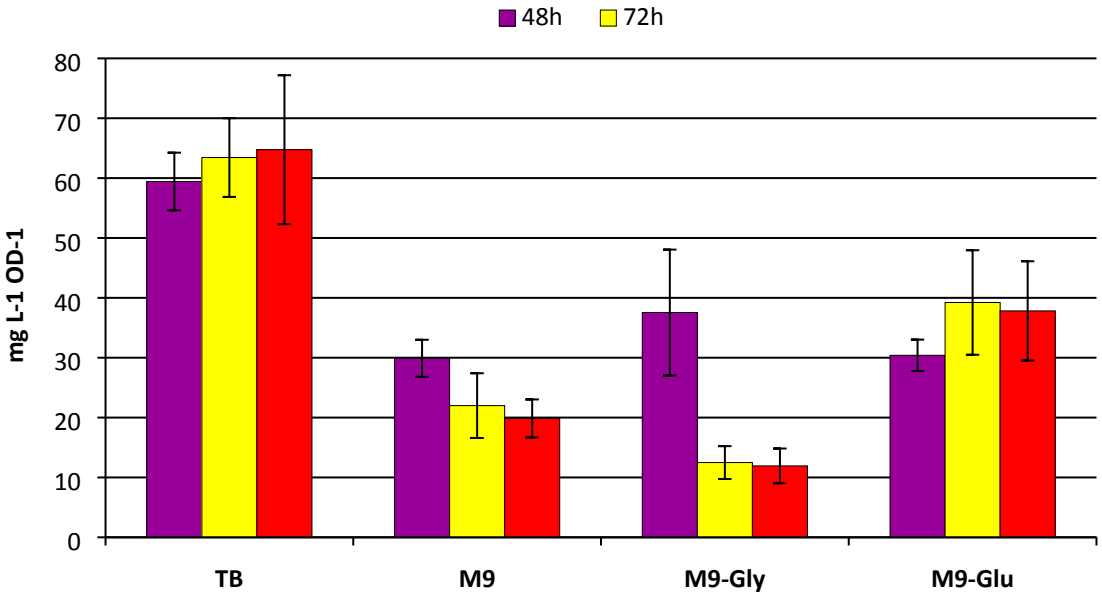


Figure S5. Quantification of *in-vivo* Cinnamyl alcohol produced 24h, 48 and 72h after induction, normalized by cell density (OD). Error bars present the standard deviation (SD) of three biological replicates.

Figure S6. Schematic representation of the vector pZZ-Eva2 used for *in-vivo* cinnamyl-alcohol production.

>pZZ-Eva2 (recombinant)

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Figure S7. The FASTA format, representing the full size nucleic acid sequences (10446bp) of recombinant plasmid pZZ-Eva2, as it was used in the *in-vivo* experiment.

Table S1. Basic information on oligonucleotides used for polymerase chain reactions (PCR) in this study.

Primer name	**Application**	**Product Size (bp)**	**Template**
srPAL+O_Fw	PCR & OE-PCR	1638bp & 2704bp	pET-16b
srPAL_Rv	PCR & Sequencing	1638bp	pET-16b
KRED+O_Fw	PCR	1081bp	pET-28a
KRED+O_Rv	PCR & OE-PCR	1081bp & 2704bp	pET-28a
CAR11_Fw	PCR & Sequencing	3636bp	pET-21a
CAR11_Rv	PCR & Sequencing	3636bp	pET-21a
Rha+CAR_Fw	PCR & OE-PCR	210bp & 881bp	Synthetic
Rha+Sfp_Rv	PCR	210bp	Synthetic
Sfp_Fw	PCR	692bp	pCDF-1b
Sfp+O_Rv	PCR	692bp	pCDF-1b
Full-INs-Eva2_Rv	OE-PCR & Sequencing	881bp	Synthetic
Full-Ins_Fw	Sequencing	ND	pZZ-Eva2
KRED-seq_Fw	Sequencing	ND	pZZ-Eva2
KRED-seq_Rv	Sequencing	ND	pZZ-Eva2
CAR-seq_2Fw	Sequencing	ND	pZZ-Eva2
CAR-seq_3Fw	Sequencing	ND	pZZ-Eva2
CAR-seq_4Fw	Sequencing	ND	pZZ-Eva2
CAR-seq_5Fw	Sequencing	ND	pZZ-Eva2

ND = not defined.

Table S2. Oligonucleotides used in this study.

Primer name	Sequence 5'-3'
srPAL+O_Fw	ttgggaattcaaaaGATCTaggaggATAAAGAAATGCACACCATGGACACTGC
srPAL_Rv	ttaGTCCCGCCCGGCGT
KRED+O_Fw	GCCGGGCGGGACtaaaggaggATAAAGAAATGCGGACGATGAAGGCA
KRED+O_Rv	atgaTGaCTGCTGCCCATTTCTTTATcctcctTTATTGGCCCGTGTTCAGC
CAR11_Fw	ATGGGCAGCAGtCATCATCAT
CAR11_Rv	ttAGAGCAGGCCGAGCTG
Rha+CAR_Fw	CAGCTCGGCCTGCTCTaaaaagtcaaaagcctccgaccg
Rha+Sfp_Rv	AATTCCGTAAATCTTGCCCATTTCTTTATcctcctttcattacgaccagtctaaaaagcgc
Sfp_Fw	ATGGGCAAGATTTACGGAATT
Sfp+O_Rv	tcgaGtttGGATCCTTATAAAAGCTCTTCGTACGAGACCA
Full-INs-Eva2_Rv	tcgaGtttGGATCCTTATAAAAGCT
Full-Ins_Fw	ttgggaattcaaaaGATCTagg
KRED-seq_Fw	AATTCATGGTGGCCCCG
KRED-seq_Rv	AACTGCACGCCGAGGTG
CAR-seq_2Fw	GCTCGTGTTCGACTACCGC
CAR-seq_3Fw	ACTCCCCGTATCCCCGA
CAR-seq_4Fw	ATCTTCCAGGTCGAGGTGC
CAR-seq_5Fw	TGGAGCCCTCGGCCTT

>AvPAL(*Anabaena variabilis*)

MKTLSQAQSKTSSQQFSFTGNSSANVIIGNQKLTINDVARVARNGTLVSLTNNTDILQGIQASCDYINNAVESGEPIYGV
TSGFGGMANVAISREQASELQTNLVWFLKTGAGNKLPLADVRAAMLLRANSHMRGASGIRLELIKRMEIFLNAGVTPY
VYEFGSIGASGDLVPLSYITGSLIGLDPSFKVDFNGKEMDAPTALRQLNLSPLTLLPKEGLAMMNGTSVMTGIAANCVY
DTQILTAIAMGVHALDIQALNGTNQSFHPFIHNSKPHPGQLWAADQMISLLANSQLVRDELDGKHDYRDHELIQDRYS
LRCLPQYLGPIVDGISQIAKQIEIEINSVTDNPLIDVDNQASYHGGNFLGQYVGMGMDHLRYYIGLLAKHLDVQIALLASP

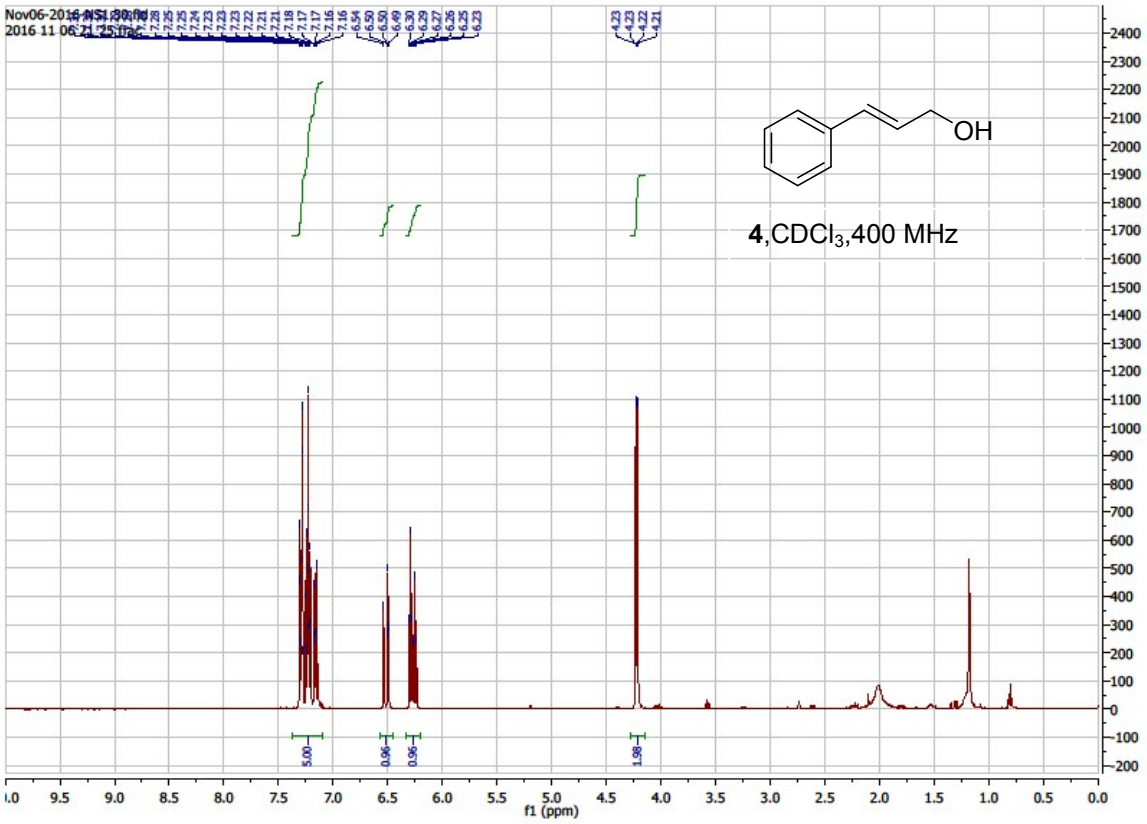
EFSNGLPPSLLGNRERKVNMGLKGLQICGNSIMPLLTFYGNSIADRFPTHAEQFNQNINSQGYTSATLARRSVDIFQNY
VAIALMFGVQAVDLRTYKKTGHYDARACLSPATERLYSAVRHVVGQKPTSDRPYIWNDNEQGLDEHIARISADIAAGG
VIVQAVQDILPCLH

>MCAR(*Mycobacterium marinum*)
MSPITREERLERRIQDLYANDPQFAAAKPATAITAAIERPGLPLPQIIETVMTGYADRPALAQRSVEFVTDAGTGHTTLRL
LPHFETISYGELWDRISALADVLSTEQTVKPGDRVCLLGFNSVDYATIDMTLARLGAVAVPLQTSAAITQLQPIVAETQPT
MIAASVDALADATELALSGQTATRVLVFDHHRQVDAHRAAVESARERLAGSAVVETLAEAIARGDVPRGASAGSAPGT
DVSDDSLALLIYTSGSTGAPKGAMYPRRNVATFWRKRTWFEGGYEPSITLNFMPMSHVMGRQILYGTLCNGGTAYFV
AKSDLSTLFEDLALVRPTELTFVPRVWDMVFDEFQSEVDRRLVDGADRVALEAQVKAEIRNDVLGGRYTSALTGSAPIS
DEMKAWVEELLDMHLVEGYGSTEAGMILIDGAIRRPAVLDYKLVDVPDLGYFLTDRPHPRGELLVKTDSLFPGYYQRAE
VTADVFDADGFYRTGDIMAEVGPEQFVYLDRRNNVLKLSQGEFVTVSKLEAVFGDSPLVRQIYIYGNSARAYLLAVIVPT
QEALDAVPVEELKARLGDSLQEVAKAAGLQSYEIPRDFIIETTPWTLENGLLTGIRKLARPQLKKHYGELLEQIYTDLAHG
QADELRSLRQSGADAPVLVTVCRAAAALLGGSASDVQPDAHFTDLGGDSLSALSFTNLLHEIFDIEVPVGVIVSPANDLQ
ALADYVEAARKPGSSRPTFASVHGASNGQVTEVHAGDLSLDKFIDAATLAEAPRLPAANTQVRTVLLTGATGFLGRYLA
LEWLERMDLVDGKLICLVRAKSDTEARARLDKTFDSGDPELLAHYRALAGDHLEVLAGDKGEADLGLDRQTWQRLADT
VDLIVDPAALVNHVLPYSQLFGPNALGTAELLRLALTSKIKPYSYTSTIGVADQIPPSAFTEDADIRVISATRAVDDSYANG
YSNSKWAGEVLLREAHDLCGLPVAVFRCDMILADTTWAGQLNVPDMFTRMILSLAATGIAPGSFYELAADGARQRAH
YDGLPVEFIAEAISTLGAQSQDGFHTYHVMNPYDDGIGLDEFVDWLNESGCPIQRIADYGDWLQRFETALRALPDRQR
HSSLLPLLHNYRQPERPVRGSIAPTDRFRAAVQEAKIGPDKDIPHVGAPIIVKYVSDLRLLGLL

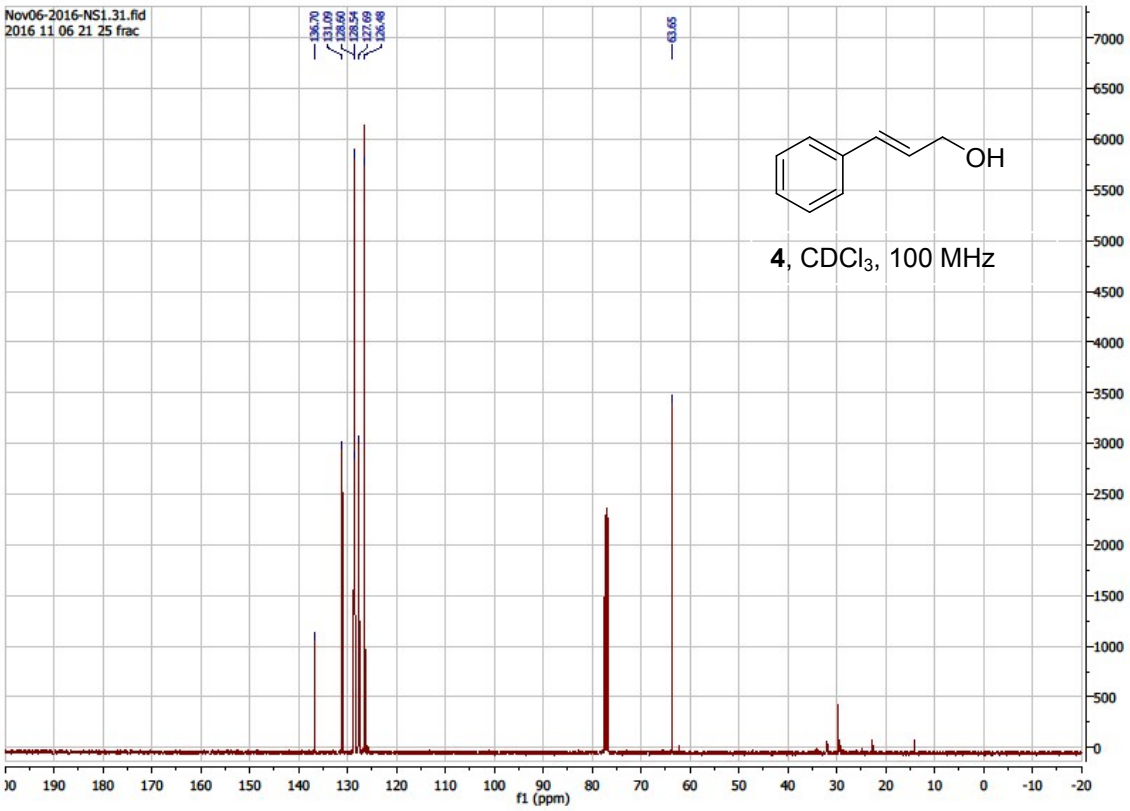
>BsSfp(*Bacillus subtilis*)
MGKIYGIYMDRPLSQEENERFMTFISPEKREKCRRFYHKEDAHRTLLGDVLVRSVISRQYQLDKSDIRFSTQEYGKPCIPD
LPDAHFNISHSGRWVIGAFDSQPIGIDIEKTKPISLEIAKRFFSKTEYSDLLAKDKDEQTDYFYHLWSMKESFIKQEGKGLSL
PLDSFSVRLHQDGQVSIELPDSHSPCYIKTYEVDPGYKMAVCAAHPDFPEDITMVSYEELL

Figure S8. The FASTA format amino acid sequences of recombinant AvPAL, MCAR and BsSfp enzymes as used in biotransformation and biocatalyst preparation procedures.

^{1}H NMR of compound 4 from biotransformation



^{13}C NMR of compound 4 from biotransformation



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