Electronic Supplementary Material (ESI) for Green Chemistry.

Asymmetric synthesis of (*S*)-phenylacetylcarbinol – closing a gap in C-C bond formation

--- Electronic Supplementary Information ---

S5. Reaction optimisation of (*S*)-PAC synthesis with *Ap*PDC-E469G-I468A-W543F in batch

S6. Access to (*S*)-PAC concentrations >300 mM (45 g/L) combining optimal reaction conditions in fed-batch

S7. Enlargement of the product platform of functionalized α-hydroxy ketones

S8. References

S1. *Acetobacter pasteurianus* pyruvate decarboxylase (*Ap*PDC)

The enzyme *Ap*PDC is a pyruvate decarboxylase (PDC) from the organism *Acetobacter pasteurianus* (E.C 4.1.1.1). Its natural function is in the oxidative metabolism of the bacterium.[1] The enzyme is a homo-tetramer comprising of four subunits, each with an α/β-mixed fold and one active site (Figure S1, PDB-entry: 2VBI). The cofactor, thiamine diphosphate (ThDP), is coordinated in the active side by a magnesium-ion in a V-shaped conformation between two *Ap*PDC subunits.[2, 3]

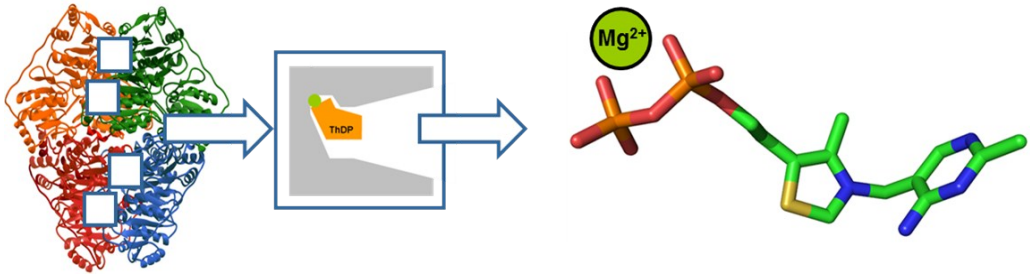

Figure S1: protein crystal structure (left), active site scheme (middle) and cofactor (right) of *Ap*PDC Crystal structure of *Ap*PDC (homotetramer, PDB-entry: 2VBI) with active sites schematically presented as blue squares □ (left). Scheme of one active site with the cofactor thiamine diphosphate represented in orange, magnesium ion in green and surrounding amino acids in grey (middle). Stick model of ThDP with bound magnesium ion (right).

The carboligation reaction is catalysed in two reaction steps. Initially, a so-called donor molecule binds to the ThDP-ylide. In case of an α-keto acid, the molecule is decarboxylated and an enamine-carbanion intermediate is formed; in case of an aldehyde deprotonation instead of decarboxylation occurs. In the second step, the C-C-bond is formed by a nucleophilic attack of the carbanion to an aldehyde (proton acceptor). Subsequently, the product is released and the cofactor regenerated.[4]

S1.1 Rational enzyme design of *Ap*PDC for (*S*)-PAC synthesis: identification of hot spots

With variant *Ap*PDC-E469G, published in 2011,[3] (*S*)-PAC could be synthesised with an *ee* of ~87 % when isolated enzymes[5] and 43-60 % (depending on the reaction conditions) when whole cells[6] were used.

As stereoselectivity was only moderate, not meeting industrial requirements, further enzyme engineering was done. On basis of the structure-function investigations for the variant *Ap*PDC-E469G two amino acids were identified as targets for further improving the (*S*)-selectivity: isoleucine in position 468 (I468) and tryptophan in position 543 (W543).

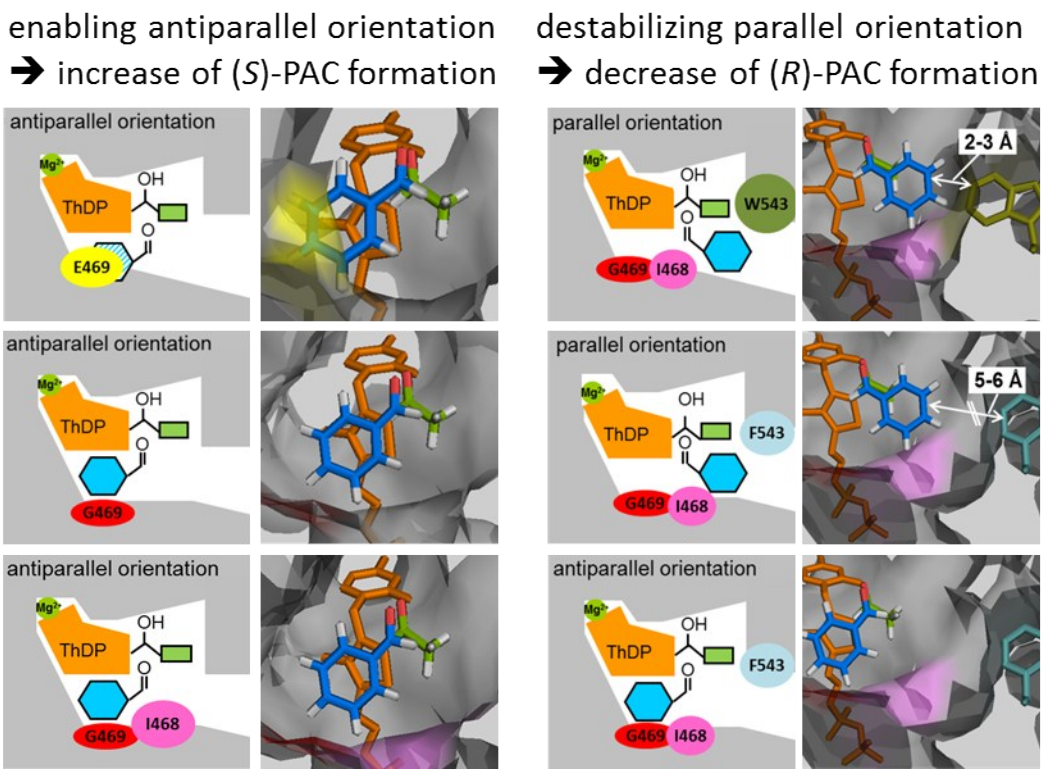

Figure S2: Selected target positions for further enzyme engineering of *Ap*PDC-E469G for stereoselective (*S*)-PAC synthesis

Left ("enabling anti-parallel orientation"): isoleucine in position 468 might destabilize the anti-parallel orientation of donor and acceptor molecule. An exchange to a smaller amino acid might further open the (*S*)-pocket such as described for the amino acid exchange E349G (see chapter 1.2).

Right ("destabilizing parallel orientation"): tryptophan in position W543 might be involved in a stabilization of the benzaldehyde in the anti-parallel orientation since π−π-stacking might occur in the distance of 2-3 Å of the aromatic rings. In theory, an exchange e.g. to phenylalanine (W543F) might enhances the distance and thus could lead to an destabilization of the unwanted (*R*)-PAC synthesis

S2. Enzyme engineering of *Ap*PDC-E469G

S2.1 Site-saturated mutagenesis

Site saturated mutagenesis using "NDT" codon degeneracy (with: "N" being able to pair with all DNA-bases, "D" pairing as adenine, guanine or thymine and "T" representing thymine) allows an exchange of the target amino acid by 12 of the 20 natural amino acids. These 12 amino acids (glycine, valine, leucine, isoleucine, phenylalanine, tyrosine, asparagine, serine, cysteine, aspartic acid, histidine, arginine) cover a variety of chemical properties of side chains: aliphatic, aromatic, polar or non-polar.[7]

S2.1.1 Primer for site-saturation mutagenesis

Primers of approximately 30 bases were designed incorporating the NDT or the complementary AHN codon to mutate position 468 and 543 in *Ap*PDC-E469G.

Primer for Site Saturated Mutagenesis generating *Ap*PDC-E469G-I468X
5´ CCGTGGCTATGTCNDTGGCATCGCCATTC 3´
5´ GAATGGCGATGCCAHNGACATAGCCACGG 3´

Primer for Site Saturated Mutagenesis generating *Ap*PDC-E469G-W543X
5´GGATATGCTGGTTCAANDTGGCCGCAAGGTTGC 3´

5´GGCAACCTTGCGGCCAHNTTGAACCAGCATATC 3´

S2.1.2 Polymerase chain reaction

Polymerase chain reaction (PCR) was performed using the Novagen® KOD Polymerase PCR Kit in accordance with the manual protocol with following setup:

PCR-solution:

1x PCR-buffer
5 % (v/v) DMSO
2 mM $MgSO_4$
0.2 mM nucleotide
0.25 pmol primer forward
0.25 pmol primer reverse
0.1 ng/µL DNA template

PCR temperature profile:

	duration (min)	temperature (°C)	repetitions
initializing	2:00	95	
denaturation	0:20	95	20x
annealing	1:00	58-80	
elongation	6:00	70	
termination	10:00	70	

As a template *Ap*PDC-E469G incorporated in pET-21a(+) plasmid was used:

TGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCC
CTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGG
GTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTT
TTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTT
GATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGT
TTACAATTTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGA
GACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGG
CATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAA
CTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGC
GGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACA
GAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGAC
AACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAAT
GAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGCAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTC
TAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTAT
TGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTAT
CTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCA
GACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGAC
CAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGT
AATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTG
GCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATAC
CTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATA
AGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGT
GAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGA
GGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTC
AGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCT
GCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAG
TCAGTGAGCGAGGAAGCGGAAGAGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATATGGTGCACTCT
CAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTATACACTCCGCTATCGCTACGTGACTGGGTCATGGCTGCGCCCCGACACCCGC
CAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTC
AGAGGTTTTCACCGTCATCACCGAAACGCGCGAGGCAGCTGCGGTAAAGCTCATCAGCGTGGTCGTGAAGCGATTCACAGATGTCTGCCT
GTTCATCCGCGTCCAGCTCGTTGAGTTTCTCCAGAAGCGTTAATGTCTGGCTTCTGATAAAGCGGGCCATGTTAAGGGCGGTTTTTTCCTGT
TTGGTCACTGATGCCTCCGTGTAAGGGGGATTTCTGTTCATGGGGGTAATGATACCGATGAAACGAGAGAGGATGCTCACGATACGGGTTA
CTGATGATGAACATGCCCGGTTACTGGAACGTTGTGAGGGTAAACAACTGGCGGTATGGATGCGGCGGGACCAGAGAAAAATCACTCAGG
GTCAATGCCAGCGCTTCGTTAATACAGATGTAGGTGTTCCACAGGGTAGCCAGCAGCATCCTGCGATGCAGATCCGGAACATAATGGTGCA
GGGCGCTGACTTCCGCGTTTCCAGACTTTACGAAACACGGAAACCGAAGACCATTCATGTTGTTGCTCAGGTCGCAGACGTTTTGCAGCAG
CAGTCGCTTCACGTTCGCTCGCGTATCGGTGATTCATTCTGCTAACCAGTAAGGCAACCCCGCCAGCCTAGCCGGGTCCTCAACGACAGG
AGCACGATCATGCGCACCCGTGGGGCCGCCATGCCGGCGATAATGGCCTGCTTCTCGCCGAAACGTTTGGTGGCGGGACCAGTGACGAA
GGCTTGAGCGAGGGCGTGCAAGATTCCGAATACCGCAAGCGACAGGCCGATCATCGTCGCGCTCCAGCGAAAGCGGTCCTCGCCGAAAA
TGACCCAGAGCGCTGCCGGCACCTGTCCTACGAGTTGCATGATAAAGAAGACAGTCATAAGTGCGGCGACGATAGTCATGCCCCGCGCCC
ACCGGAAGGAGCTGACTGGGTTGAAGGCTCTCAAGGGCATCGGTCGAGATCCCGGTGCCTAATGAGTGAGCTAACTTACATTAATTGCGTT
GCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTAT
TGGGCGCCAGGGTGGTTTTTCTTTTCACCAGTGAGACGGGCAACAGCTGATTGCCCTTCACCGCCTGGCCCTGAGAGAGTTGCAGCAAGC
GGTCCACGCTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGATGGTGGTTAACGGCGGGATATAACATGAGCTGTCTTCGGTATCGTCGTA
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CATCGCAGTGGGAACGATGCCCTCATTCAGCATTTGCATGGTTTGTTGAAAACCGGACATGGCACTCCAGTCGCCTTCCCGTTCCGCTATC
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ATTTGCTGGTGACCCAATGCGACCAGATGCTCCACGCCCAGTCGCGTACCGTCTTCATGGGAGAAAATAATACTGTTGATGGGTGTCTGGT
CAGAGACATCAAGAAATAACGCCGGAACATTAGTGCAGGCAGCTTCCACAGCAATGGCATCCTGGTCATCCAGCGGATAGTTAATGATCAG
CCCACTGACGCGTTGCGCGAGAAGATTGTGCACCGCCGCTTTACAGGCTTCGACGCCGCTTCGTTCTACCATCGACACCACCACGCTGGC

ACCCAGTTGATCGGCGCGAGATTTAATCGCCGCGACAATTTGCGACGGCGCGTGCAGGGCCAGACTGGAGGTGGCAACGCCAATCAGCA
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GGCTTCAGCGCGGAAGGCTACGCCCGTTCTAACGGGGCTGCGGCAGCGGTTGTCACCTTCAGCGTTGGCGCCATTTCCGCCATGAACGC
CCTCGGCGGCGCCTATGCCGAAAACCTGCCGGTTATCCTGATTTCCGGCGCGCCCAACAGCAATGATCAGGGCACAGGTCATATCCTGCA
TCACACAATCGGCAAGACGGATTACAGCTACCAGCTTGAAATGGCCCGTCAGGTCACCTGTGCCGCCGAAAGCATTACCGACGCTCACTCC
GCCCCGGCCAAGATTGACCACGTCATTCGCACGGCGCTGCGCGAGCGTAAGCCGGCCTATCTGGACATCGCGTGCAACATTGCCTCCGA
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GGGCGAAGTCTCGAACCCCGGCGTGCAGGAACTGGTGGAGACCTCCGACGCACTGCTGTGCATCGCCCCCGTATTCAACGACTATTCAAC
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TTACCCTGCGCGCCTTCCTGCAGGCTCTGGCGGAAAAAGCCCCCGCGCGCCCGGCCTCCGCACAGAAAAGCAGCGTCCCGACGTGCTCG
CTCACCGCGACATCCGATGAAGCCGGTCTGACGAATGACGAAATCGTCCGTCATATCAACGCCCTGCTGACATCAAACACGACGCTGGTG
GCAGAAACCGGCGATTCATGGTTCAATGCCATGCGCATGACCCTGCCGCGCGGTGCGCGCGTGGAACTGGAAATGCAGTGGGGCCATAT
CGGCTGGTCCGTGCCCTCCGCCTTCGGCAATGCCATGGGCTCGCAGGACCGCCAGCATGTGGTGATGGTAGGCGATGGCTCCTTCCAGC
TTACCGCGCAGGAAGTGGCTCAGATGGTGCGCTACGAACTGCCCGTCATTATCTTTCTGATCAACAACCGTGGCTATGTCATTGGCATCGC
CATTCATGACGGCCCGTACAACTATATCAAGAACTGGGATTACGCCGGCCTGATGGAAGTCTTCAACGCCGGAGAAGGCCATGGACTTGG
CCTGAAAGCCACCACCCCGAAGGAACTGACAGAAGCCATCGCCAGGGCAAAAGCCAATACCCGCGGCCCGACGCTGATCGAATGCCAGA
TCGACCGCACGGACTGCACGGATATGCTGGTTCAATGGGGCCGCAAGGTTGCCTCAACCAACGCGCGCAAGACCACTCTGGCCCTCGAG
CACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCA
TAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGAT

After the PCR, 5 µL of the PCR-product was loaded on a 1 % (w/v)-agarose “test”-gel. After 20 minutes at 120 V in TRIS-acetate-EDTA-buffer (50 mM TRIS-acetate; 1 mM EDTA, pH 8) the gel was stained with ethidium bromide. The PCR-preparation with the highest DNA concentration was purified with a PCR purification kit (QIAquick PCR Purification Kit, QIAGEN AG) according to the companies´ protocol. The DNA concentration was determined with a Nano-Drop device and used for transformation of *E. coli* BL21(DE3) cells.

S2.1.3 Transformation of *E. coli* BL21(DE3) and *E. coli* DH5α cells

E. coli BL21(DE3) strains were transformed by heat shock. 50 µL competent cells were thawed on ice and 100 ng of plasmid DNA was pipetted to each aliquot. After incubation on ice for 30 min, a heat shock at 42 °C was performed for 90 sec. The aliquots were then incubated (3 min, on ice) before 500 µL SOC-Medium were added. Afterwards, the cell suspension was incubated at 37 °C and 350 rpm for 45 min. Finally, the aliquots were centrifuged at 13.000 rpm for 30 sec to pellet the cells. The pellet was resuspended in 100 µL SOC-Medium and plated on LB-agar-plates containing ampicillin (100 µg/mL) as selection marker.

As a positive control for the PCR-products, the template *Ap*PDC-E469G was used and treated in all further steps always analogously.

SOC-medium
"Super Optimal Catabolite-repression broth"

20 g/L	tryptone
5 g/L	yeast extract
10 mM	NaCl
2.5 mM	KCl
10 mM	$MgCl_2$
20 mM	glucose

LB-medium
"Lysogeny Broth"

10 g/L	NaCl
10 g/L	peptone
5 g/L	yeast extract

S2.1.4 Cultivation and expression in flower plates

46 Colonies of the incubated (15-18 hours at 37°C) agar plates were picked and cultivated for 24 h at 20 °C and 850 rpm in a 1 mL Nerbe-Plate® in LB-medium (chapter 2.1.3). One additional well served as negative control and was not inoculated. Another additional well served as positive control and was inoculated with *E. coli* BL21(DE3) *Ap*PDC-E469G. This complete plate was used on the one hand as a preculture for overexpression (expression plate) in FlowerPlates® (m2p-labs, Germany) and on the other as a master plate. Every well in the FlowerPlate® contained 1.5 mL autoinduction medium and was inoculated with 10 µL preculture. The plate was then cultivated for 48 h at 20 °C and 850 rpm. For storage of the master plate, glycerol (300 µL) was added as cryo-protectant and then frozen at -80 °C. During preparation of AI-medium, peptone and yeast extract were dissolved in 795 mL H_2O and autoclaved. All other components were prepared in separate stock solutions, sterile filtrated and added directly before use.

AI-medium
"autoinduction medium"

12 g/L	peptone
24 g/L	yeast extract
90 mM	KPi-buffer (pH=7.5)
0.5 g/L	glucose
2 g/L	lactose
10 mg/L	ampicillin
6.3 g/L	glycerin

S2.1.5 Cell disruption, carboligation reactions and TTC-assay for determination of active enzyme

In order to allow the investigation of the carboligation reaction in duplicate, two aliquots of each 500 µL cell suspension of the 'expression plate' were transferred to 96-well 1 mL Nerbe-Plate®. The plate was centrifuged at

4.000 rpm for 3 min to pellet the cells. The supernatant was discarded and the pellet resuspended in 420 µL KPi-buffer (50 mM potassium phosphate, pH 6.5) containing 2.5 mM $MgSO_4$, 0.1 mM ThDP and 1 mg/mL lysozyme (lysozyme from hen egg; Fluka BioChemika). The plate was incubated for 1 h at 20 °C and 400 rpm. To pellet the cell debris the plate was centrifuged for 10 min at 4.000 rpm. 250 µL of supernatant were transferred to a 2 mL Nerbe-Plate®. The crude cell extract was mixed with 250 µL of a solution containing 40 mM benzaldehyde and 400 mM of pyruvate in 50 mM KPi-buffer (pH 6.5) containing 2.5 mM $MgSO_4$, 0.1 mM ThDP. Subsequently, the plate was incubated for 24 h at 20-23 °C to let the carboligation reaction take place. As a rapid test, a triphenyl tetrazolium chloride (TTC)-assay[8] was conducted to determine whether any 2-hydroxy ketone was formed. Therefore 100 µL sample were transferred to a 96-well plate and 40 µL of TTC-reagent (1 g/L TTC in 17.5 (v/v %) ethanol and 0.75 M NaOH) was added. Red coloration indicates the presence of 2-hydroxy ketones. All steps described in this chapter were carried out semi-automatically by a TECAN® robotic system.

S2.1.6 Screening for increased stereoselectivity of (*S*)-PAC by chiral HPLC-Analysis

Positive hits identified in the TTC assay were analyzed by HPLC analytics in more detail. Therefore 200 µL of sample were extracted with 200 µL heptane. 150 µL of the organic phase were transferred to a HPLC vial and analyzed using a Chiralpak IC-3 (150 mm x 2.1 mm x 3 µm, Chiral Technologies) column with an IC-pre-column on an Agilent 1100 Series HPLC. Program and typical retention times:

HPLC program

length	24 min
flow	0.5 mL/min
mobile phase	25 % (v/v) isopropanol
	75 % (v/v) heptane

Retention times and wavelength

chemical	retention time [min]	wavelength [nm]
(*R*)-PAC	12.3	210
(*S*)-PAC	12.9	210
benzaldehyde	11.4	254

S2.1.7 Identification and DNA-sequencing of positive screening hit

For identification of positive screening hits, defined as the once showing higher stereoselectivity for (*S*)-PAC synthesis in carboligation reaction (chapter S2.1.6) as gained with *Ap*PDCE469G, the respective cells of the respective 'master plate' vial (see chapter S2.1.4) were used for inoculation and cultivation. For sequencing, the plasmid DNA was isolated using the Miniprep® Kit (QIAGEN AG) according to the manual and subsequently the DNA was sequenced by LGC Genomics sequencing services (https://shop.lgcgenomics.com).

S2.2 Site-directed mutagenesis via QuikChange™ PCR

In order to create the selected triple variant *Ap*PDC-E469G-I468A-W543F, QuikChange™ (Agilent Technologies) a PCR was performed using the coding gene of *Ap*PDC-E469G-T384G-I468A-W543F as a template.[9]

Primer for QuikChange™ generating the gene encoding *Ap*PDC-E469G-I468A-W543F
5´CTGGTGGCAGAAACCGGCGATTCATG 3´
5´CATGAATCGCCGGTTTCTGCCACCAG 3´

The PCR (chapter S2.1.2) was performed with a temperature gradient of 58.6 °C - 75.6 °C. For transformation (chapter S2.1.3) *E. coli* DH5α cells were used. Colonies were picked and cultivated (37 °C for 15-18 h at 150 rpm in 10 mL LB-medium in a 100 mL shake flask). DNA was isolated and sequenced as described (chapter S2.1.7).

S2.3 Summarized results of the *Ap*PDC-mutagenesis for (*S*)-PAC synthesis

For site-saturation mutagenesis (chapter S2.1), 48 colonies of each selected position (*Ap*PDC-E469G-I469X and *Ap*PDC-E469G-W543X) had been picked (after transformation of the PCR products in *E. coli* BL21(DE3) cells; chapter S2.1.1-2.1.3), cultivated (chapter S2.1.4), the cells disrupted (chapter S2.1.5) and stereoselectivity of active variants (analyzed by TTC-assay; chapter S2.1.5) analyzed in more detail by chiral HPLC-analytics (chapter S2.1.6). Variants, which showed higher (or almost as high) stereoselectivities for (*S*)-PAC synthesis as the starting variant *Ap*PDC-E469G, were identified by sequencing of the respective isolated DNA from the master plate (chapter S2.1.7). In parallel, *Ap*PDC-E469G-I468A-W543F was generated via site-directed mutagenesis (see chapter S2.2) and (*S*)-PAC synthesis performed analogously to the site-saturation mutagenesis experiments performed. Positive screening hits are summarized in Figure S3.

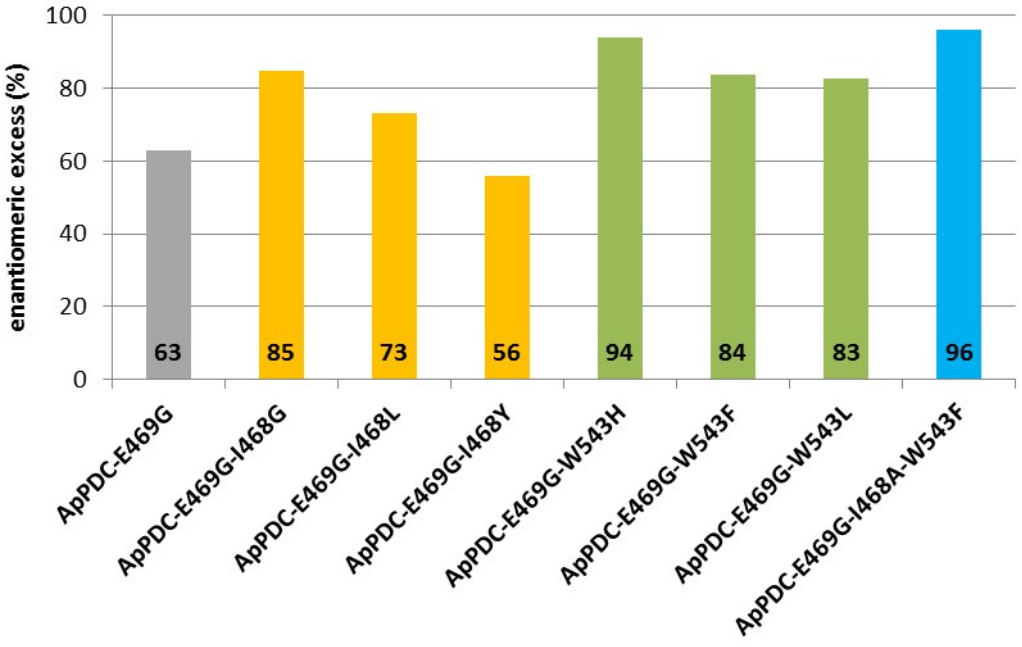


Figure S3: Screening results of variants with improved stereoselectivity for (*S*)-PAC synthesis compared to the starting variant *Ap*PDC-E469G generated by site-saturation (chapter S2.1) and site-directed (chapter 2.2) mutagenesis.

Reaction conditions: 40 mM benzaldehyde; 400 mM pyruvate; 50 mM potassium phosphate (Kpi) buffer (pH 6.5), 2.5 mM $MgSO_4$; 0.1 mM ThDP; T=20 °C; 800 rpm, 800 µL reaction volume in 1.5 mL closed glass vials; crude cell extract preparation as described in chapter S2.1

S3. Production and purification of *Ap*PDC-variants (0.2 – 1 L)

S3.1 Production of *Ap*PDC-variants in 200 mL to 1 L scale

Protein production of *Ap*PDC-variants in *E. coli* BL21(DE3) cells for biotransformation besides the initial screening (chapter 2) were performed in 200 mL to 1 L autoinduction medium. Competent *E. coli* BL21(DE3) cells were transformed (chapter S2.1.3) with the respective plasmid DNA and plated on agar plates containing 100 µg/mL ampicillin. Pre-cultures were inoculated in LB-medium from single colonies in a volume of 10 mL (in 50 mL shaking flaks). After incubation for 15-18 h at 37 °C, the main culture (AI-medium (chapter 2.1.4); 20 % (v/v) filling volume in a baffled shaking flask) was inoculated with 1 % (v/v) pre-culture and cultivated at 20 °C for 48 h. Afterwards, cells were harvested (6.000 rpm; 30-45 min.) and stored at -20 °C.

S3.2 Crude cell extract preparation

10 g frozen cells (chapter S3.1) were re-suspended to 30 % (w/v) in 50 mM KPi-buffer, pH 6.5 containing 2.5 mM $MgSO_4$ and 0.1 mM ThDP. If crude cell extract was used for subsequent enzyme purification, 10 mg/mL lysozyme (lysozyme from hen egg; Fluka BioChemika) was added and stirred on ice for 20 min. The cell suspension was disrupted by ultrasonication (4x2 min with 1 min breaks) using a S14D-sonotrode (Hielscher Ultrasonics GmbH) with an amplitude of 0.5. Cell debris was removed by centrifugation (18,000 rpm; 45 min; 4 °C; Beckman Coulter). The supernatant (“crude cell extract”, CCE) was stored on ice and was either used for immediate protein purification (chapter S3.3) or was lyophilized (chapter S3.4).

S3.3 Protein purification

S3.3.1 Immobilized metal-ion affinity chromatography (IMAC)

Purification has been limited to the most promising variants (as was deduced from their performance in the crude cell extract experiments). An Äkta purifier (Amersham Bioscience; units: pH/C-900; UV-900; P-900) was used. A Ni-NTA-column (60 mL “Ni-NTA superflow”; QIAGEN AG) was washed with 3 column volumes equilibration buffer (50 mM KPi-buffer, pH 6.5, containing 2.5 mM $MgSO_4$ and 0.2 mM ThDP). The cell-free CCE (chapter S3.2) was loaded and the column flushed with equilibration buffer. Afterwards, washing buffer (equilibration buffer pH 6.5+ 50 mM imidazole) was used to remove weakly bound proteins. When a stable baseline was reached again, the elution buffer (equilibration buffer pH 7.5 + 250 mM imidazole) was used to elute the target protein. The elution fractions were pooled and desalted by size exclusion chromatography (chapter S3.3.2).

S3.3.2 Size exclusion chromatography (SEC)

SEC was used for desalting of the IMAC-elution fractions (chapter S3.3.1). A 1 L SEC-column (material: Sephadex G25; Pharmacia Biotech) was equilibrated with 2 column volumes 10 mM KPi-buffer, pH 6.5, containing 2.5 mM $MgSO_4$ and 0.2 mM ThDP. The samples were loaded and the column flushed with the same buffer until a stable baseline was reached on an Äkta purifier (Amersham Bioscience; units: pH/C-900; UV-900; P-900). The elution fractions with the target protein were identified by UV absorption at 280 nm, pooled and stored at -20 °C.

S3.4 Lyophilization of crude cell extracts (CCE) and purified enzymes

The frozen SEC fractions (chapter S3.3.2) or crude cell extracts (chapter S3.2) were lyophilized at reduced pressure of 0.22 mbar and -80 °C (LOC-1M; Martin Christ Gefriertrocknungsanlagen GmbH). The freeze-dried enzyme was stored at -20 °C.

S4. Detailed reaction analysis of positive screening hits and selected *Ap*PDC-variants

S4.1 Analysis of benzaldehyde and phenylacetylcarbinol (PAC)

For chiral analytics of (*S*)- and (*R*)-PAC and simultaneous determination of benzaldehyde and PAC concentrations, a reaction sample were diluted 1-10 in acetonitrile. In order to remove precipitated enzyme the sample was centrifuged for 1 min at 13,000 rpm and the supernatant analyzed using a Chiralpak IC column (150 mm x 2.1 mm x 3 µm, Chiral Technologies) equipped with a DAD detector, using the program reported below. Typical retention times are also listed:

HPLC program (flow: 1 mL/min)

min	acetonitrile (%)
0	35
7	35
8	60
14	60
15	35
18	35

Retention times and wavelength

	retention time (min)	wavelength (nm)
benzaldehyde	8.0	254
(*S*)-PAC	5.9	210
(*R*)-PAC	6.3	210

S4.2 Carboligation reactions with *Ap*PDC-variants used as whole cell catalysts or purified enzymes

Positive screening hits, defined as those variants showing higher stereoselectivity for (*S*)-PAC synthesis compared to the starting variant *Ap*PDC-E469G (see chapter S2) in crude cell extracts, were further investigated with respect to their implementation as purified enzyme or whole cells catalyst. Experiments at different reaction conditions were performed in closed glass vials at 20 °C and 850 rpm.

S4.2.1 Carboligation reactions with whole cell biocatalysts

Whole cell biocatalysts containing the respective variants (chapter S3.2) were used in a concentration of 50 mg/mL wet cell weight. As a control, the starting variant *Ap*PDC-E469G was used under identical reaction conditions. Reaction conditions were the following: 20 mM benzaldehyde; 200 mM pyruvate; 50 mM KPi-buffer (pH 6.5), 2.5 mM MgSO4; 0.1 mM ThDP; 20 °C; 800 rpm, 800 µL reaction volume in 1.5 mL closed glass vials. At selected time points samples were taken (chapter S4.1) over a period of 21 h. Stereoselectivities, determined after 5.5 h, are shown in the main manuscript in Figure 3. Progress curves of the reactions are shown in Figure S4.

In these initial screenings the enantiomeric purity (see Fig. 3 – main article, standard deviation <5 %) was of higher interest than the accurate conversion values. The typical error of this or other reaction setups in such low substrate

concentrations in screening setups is <20 %. As a yes/no answer this was appropriate in order to select variants with high *ee* values and moderate to high conversion (>50 %).

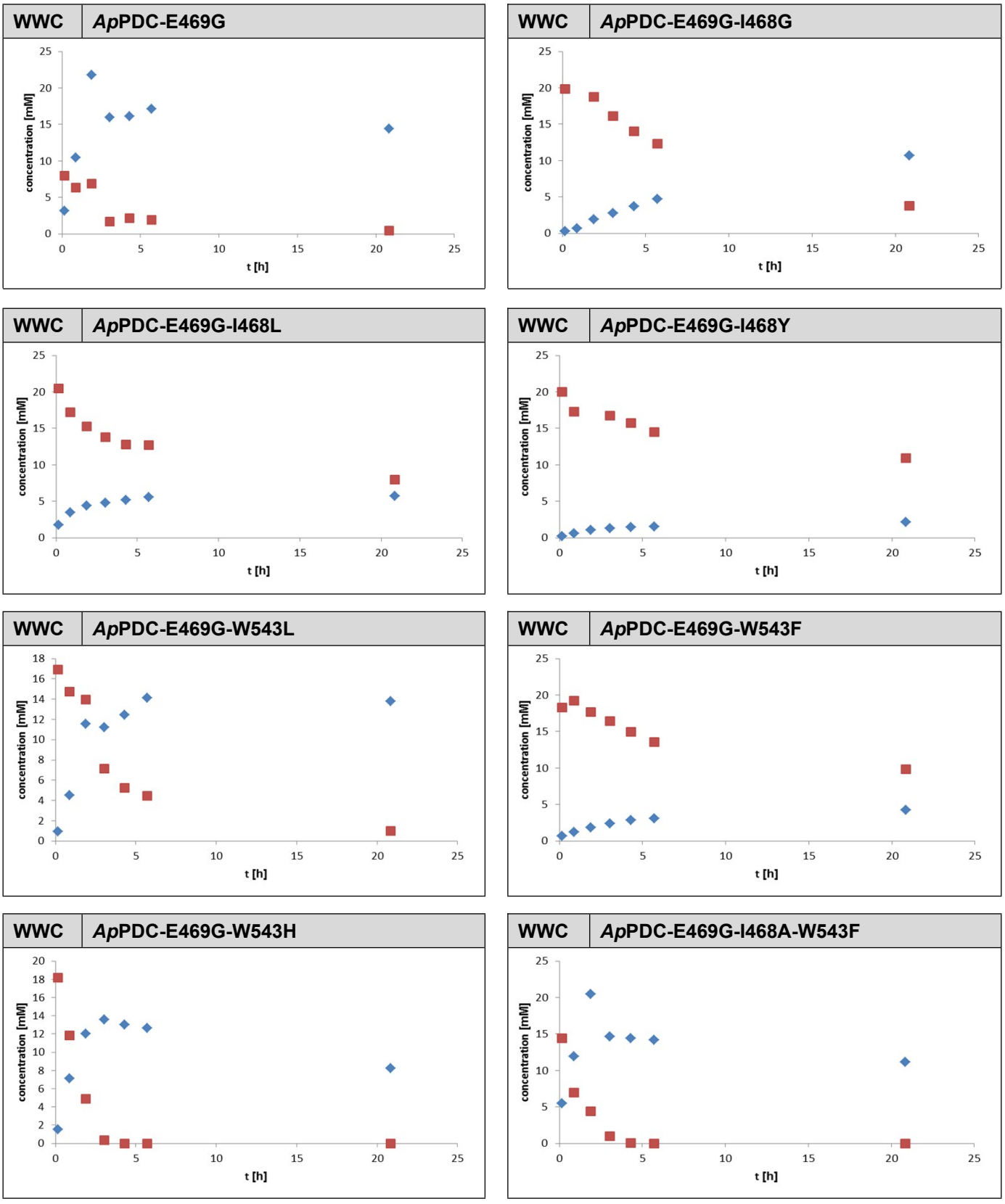


Figure S4: Progress curves of (*S*)-PAC syntheses catalysed by different *Ap*PDC-variants used as whole cell biocatalyst (WWC).

Reaction conditions: 20 mM benzaldehyde; 200 mM pyruvate; 50 mM KPi-buffer (pH 6.5), 2.5 mM $MgSO_4$; 0.1 mM ThDP; 20 °C; 800 rpm, 800 µL reaction volume in 1.5 mL closed (by plastic sheet) glass vials; whole cell biocatalyst concentration: 50 mg/mL. Blue diamonds: PAC [mM], red squares: benzaldehyde [mM]

A. Baraibar demonstrated in his doctoral thesis that 0.1 mM of ThDP has to be present in the environment of whole cell catalyst in order to maintain maximal reactivity.[6] As very high concentrations of ThDP-depending enzymes are overexpressed in the applied whole cells, questioning if sufficient cofactors can be supplied by the cell, 0.1 mM ThDP as well as 2.5 mM $MgSO_4$ have been added to the buffer to avoid possible cofactor limitations and therewith activity decreases.

S4.2.2 Carboligation reactions with purified enzymes

Purified, lyophilized enzyme variants (chapter S3.3-3.4) were investigated with respect to productivity and selectivity in 50 mM KPi-buffer containing 2.5 mM $MgSO_4$ and 0.1 mM ThDP at different pH values. 40 mM benzaldehyde and 200 mM pyruvate were incubated with 1 mg/mL purified protein (chapter S2.1) at 850 rpm and 22 °C, 800 µL reaction volume in 1.5 mL closed glass vials. Samples were taken (chapter S4.1) at selected time points over a period of 30 h. Stereoselectivities are shown in the main manuscript in Figure 3. Conversions are shown in Figure S5.

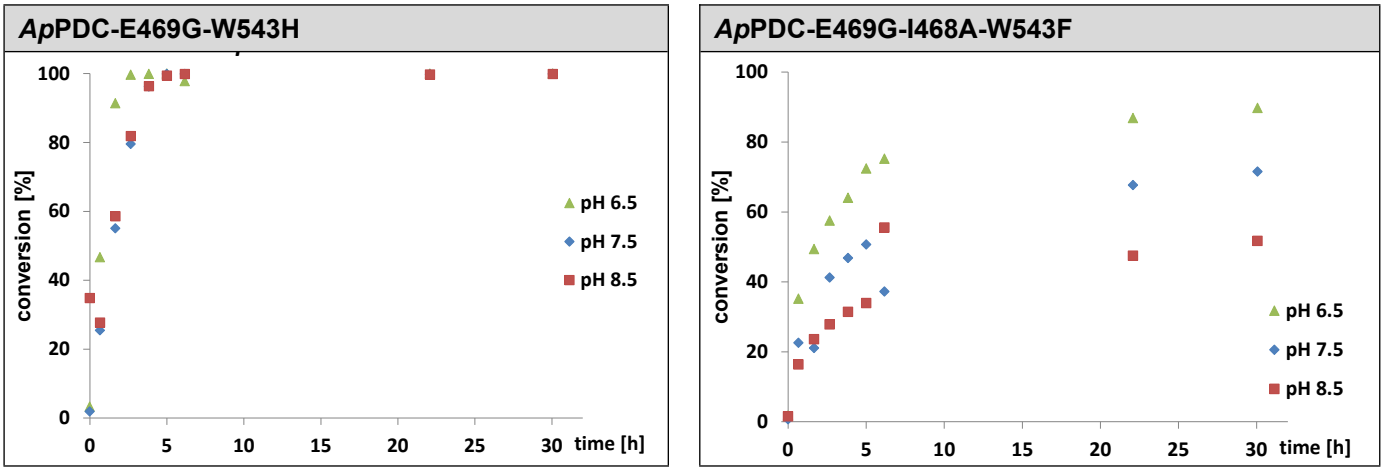


Figure S5: Carboligation reactions with *Ap*PDC-E469G-W543H (left) and *Ap*PDC-E469G-I468A-W543F (right) used as purified enzymes.

Conversion is given in % relative to the initial benzaldehyde concentration.

Reaction conditions: 40 mM benzaldehyde; 200 mM pyruvate; 50 mM KPi-buffer with three different pH-values, 2.5 mM $MgSO_4$; 0.1 mM ThDP; 22 °C; 800 rpm, 800 µL reaction volume in 1.5 mL closed glass vials; protein concentration: 1 mg/mL.

S4.2.3 Carboligation reactions with wet whole cells overexpressing *Ap*PDC-E469G-I468A-W543F in batch - preparative scale

Exemplarily, the batch scale with variant *Ap*PDC-E469G-I468A-W543F was scaled up to 250 mL. Wet whole cells containing the enzyme variant *Ap*PDC-E469G-I468A-W543F were investigated with respect to product concentration and selectivity in 100 mM KPi-buffer pH 6.5 containing 2.5 mM $MgSO_4$ and 0.5 mM ThDP. 40 mM benzaldehyde and 120 mM pyruvate were incubated with 50 mg/mL wet whole cells at 850 rpm and 22°C. Samples were taken at selected time points over a period of 8 h (Figure S6). After 8 h work-up was done as following. Cells were first removed by centrifugation (8.500 rpm; 45 min, 4°C). The aqueous phase was extracted three times with

a i-prop:EtOAC 1:1 mixture (3 x 250 mL). The combined organic phases were washed with brine (3 x 200 mL), dried over $MgS0_4$, and the solvent removed under reduced pressure. The product (*S*)-PAC was characterized by ^{1}H-NMR and ^{13}C-NMR (600 MHz, $CDCl_3$). Spectral data were in accordance with previously reported.[10] (*S*)-PAC was obtained with a purity of 74 %, determined by quantitative NMR using maleic acid as internal standard. Predominant impurities were 2-propanol, remaining benzaldehyde and HPP. The *ee* was 93.7 % (determined *via* HPLC, see S4.1).

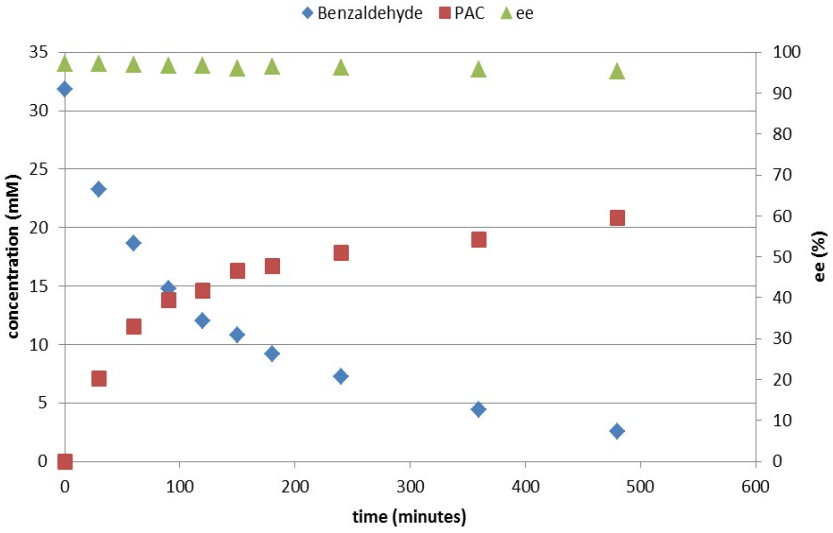


Figure S6: Carboligation reaction in 250 mL preparative scale with *Ap*PDC-E469G-I468A-W543F used as wet whole cell catalyst.

Reaction conditions: 100 mM KPi-buffer pH 6.5 containing 2.5 mM $MgSO_4$ and 0.5 mM ThDP. 40 mM benzaldehyde and 120 mM pyruvate were incubated with 50 mg/mL wet whole cells at 850 rpm and 22°C. Samples were taken at selected time points over a period of 8 h.

S5. Reaction optimisation of (*S*)-PAC synthesis with *Ap*PDC-E469G-I468A-W543F in batch

S5.1 Selection of co-solvents for higher solubility of substrates and products

In buffer (50 mM potassium phosphate + 0.2 mM ThDP + 2.5 mM $MgSO_4$, 25 °C) benzaldehyde has a solubility of ~60 mM and PAC ~150 mM (determination: 300 mM of the respective compound was rapidly stirred at 1000 rpm in this buffer for 60 min, centrifuged for 10 min at 13.000 rpm and concentration determined in the supernatant).

Since emulsions and phase interfaces often cause enzyme denaturation, different water-miscible organic co-solvents were tested to increase the substrate and product concentrations. Benzaldehyde solutions with a concentration of 150 mM were prepared using the minimal amount of the respective co-solvent to completely dissolve the benzaldehyde.

A solution containing 200 mg/mL *Ap*PDC-E469G-I468A-W543F wet cells was prepared in double concentrated pyruvate/buffer-solution (600 mM pyruvate in 100 mM potassium phosphate pH 6.5 + 0.4 mM ThDP + 5 mM $MgSO_4$). Benzaldehyde was dissolved in the respective organic co-solvent and deionized water was added to give the final 2-fold concentrations of solvent and benzaldehyde (e.g. final concentration: 150 mM benzaldehyde, 30 % DMSO => 2-fold stock: 300 mM benzaldehyde, 60 % DMSO). Both stock solutions were mixed 1:1 to give the respective final reaction solutions. Reactions (1 mL solution in 2 mL closed glass vials) were shaken in an

Eppendorf Thermo Shaker at 600 rpm and 22 °C for 16 h, before PAC concentrations and ee-values were determined (see chapter S4.1, data reported in Figure S7).

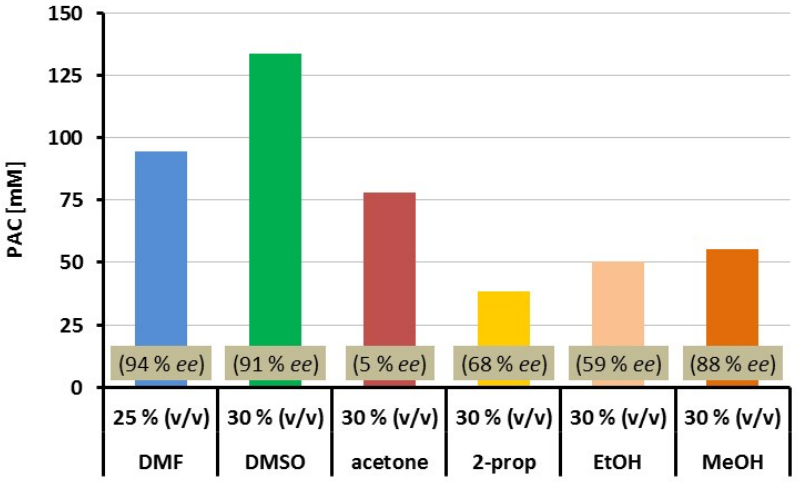


Figure S7: Carboligation reactions towards (*S*)-PAC starting from 150 mM benzaldehyde in presence of the minimal required amount of co-solvent for solubilisation and using 100 mg/mL *Ap*PDC-E469G-I468A-W543F wet cells as biocatalyst.

Reaction conditions: X % (v/v) co-solvent, 150 mM benzaldehyde, 300 mM pyruvate, 50 mM potassium phosphate, 0.2 mM ThDP, 2.5 mM $MgSO_4$, pH 6.5, 100 mg/mL wet cells. 1 mL reaction volume in 2 mL closed glass vials, 22 °C, 600 rpm shaking, 16 h reaction time.

DMF: dimethylformamide, DMSO: dimethylsulfoxide, 2-prop: 2-propanol, EtOH: ethanol, MeOH: methanol

While high conversions and still good *ee*-values could be achieved with DMSO and DMF, the final product concentration and the stereoselectivity were significantly reduced in reactions with acetone, isopropanol, and ethanol. Similar results have been found for *Ap*PDC-E469G in reactions with 20 mM benzaldehyde.[5] More detailed investigations on co-solvent effects lead to the hypothesis that compounds with appropriate electrochemical and steric properties can compete with the benzaldehyde for binding in the (*S*)-pocket, which impairs the stereoselectivity of the (*S*)-PAC formation (see Chapter S1) at higher co-solvent concentrations.[5]

S5.2 Influence of co-solvents and benzaldehyde on the half-life of the enzyme

In order to analyse the influence of different co-solvents as well as benzaldehyde on the enzyme stability, several investigations were performed. Cell suspensions with/without benzaldehyde, with/without co-solvents were prepared analogously as described in chapter S5.1 (50 mM potassium phosphate (pH 6.5), 0.2 mM ThDP, 2.5 mM $MgSO_4$, 0-30 % (v/v) co-solvent, 0-100 mM benzaldehyde and 100 mg/mL wet cells of *Ap*PDC-E469G-I468A-W543F). 5 mL of each suspension was stirred at 22 °C. At different time points, 100 µL of sample were withdrawn and added to 400 µL of an assay solution (150 mM benzaldehyde, 300 mM pyruvate, 50 mM potassium phosphate, 30 % (v/v) DMSO, 0.2 mM ThDP, 2.5 mM $MgSO_4$, pH 6.5) to measure the residual activity of the biocatalyst. Activity was measured as a discontinuous HPLC-based activity assay (sample preparation and HPLC-measurement - see chapter S4.1).

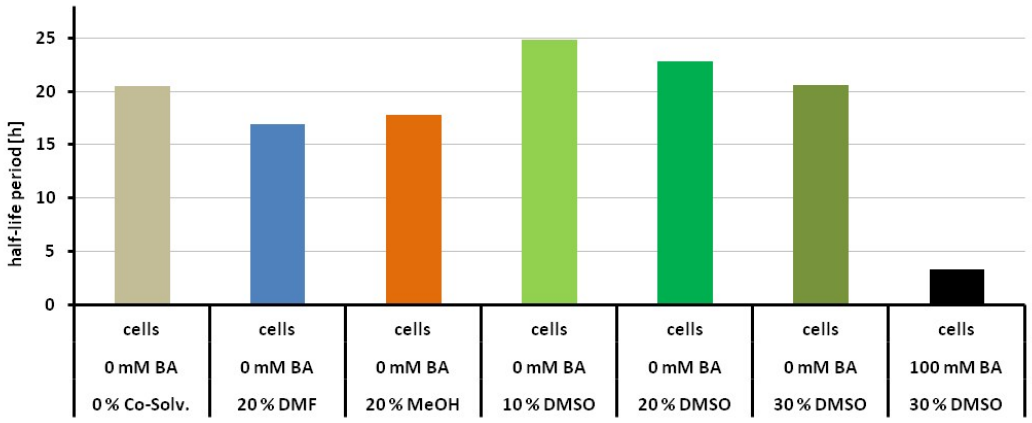


Figure S8: Influence of different co-solvents, co-solvents concentrations, benzaldehyde on half-life of *Ap*PDC-E469G-I468A-W543F as wet cell catalysts

Reaction conditions of incubation solution: 50 mM potassium phosphate (pH 6.5), 0.2 mM ThDP, 2.5 mM $MgSO_4$, 0-30 % (v/v) co-solvent, 0 mM or 100 mM benzaldehyde and 100 mg/mL wet cells of *Ap*PDC-E469G-I468A-W543F. 5 mL closed glass vial, 100 rpm, 22 °C.

DMF: dimethylformamide, DMSO: dimethylsulofxide, MeOH: methanol, Co-Sol.: co-solvent

Assay solution: 150 mM benzaldehyde, 300 mM pyruvate, 50 mM potassium phosphate, 30 % (v/v) DMSO, 0.2 mM ThDP, 2.5 mM $MgSO_4$, pH 6.5. 100 µL "incubation solution" + 400 µL 'assay solution' were incubated in 2 mL glass closed vials at 600 rpm, 22 °C.

The half-life of *Ap*PDC-E469G-I468A-W543F in wet *E. coli* cells incubated in buffer (50 mM potassium phosphate, 0.2 mM ThDP, 2.5 mM $MgSO_4$) without benzaldehyde or co-solvents is ~20 h (Figure S8). DMSO at concentration sbetween 10-20 % even has a positive effect which is in good agreements with previously published data for the wild-type *Ap*PDC.[11] The half-life of *Ap*PDC-E469G-I468A-W543F in wet *E. coli* cells is reduced in presence of DMF and ethanol. Clearly significantly negative influence on the half-life was detected in the presence of benzaldehyde.

With benzaldehyde concentrations of 150 mM a high PAC-concentration of ~130-140 mM was reached (Figure S5). Different effects might hamper higher product concentrations such as: pyruvate concentration, DMSO amount, PAC-inhibition or pH changing due to pyruvate consumption. The influence of all these effects is described in the next chapter (S5.3).

S5.3 Influence of different reaction parameters on the (*S*)-PAC synthesis

S5.3.1 Effect of pyruvate concentration on reactions with 150 mM benzaldehyde

To analyse if higher pyruvate concentrations influence the reaction performance, biotransformations with 150 mM benzaldehyde and 150-1000 mM pyruvate, were set-up in 50 mM potassium phosphate (pH 6.5), 0.2 mM ThDP, 2.5 mM $MgSO_4$, 30 % (v/v) DMSO, with 100 mg/mL *Ap*PDC-E469G-I468A-W543F wet cells analogously to the preparation described in chapter S5.1. After 16 h incubation (22 °C, 600 rpm, 1 mL reaction volume in 2 mL closed glass vials), the PAC-concentration was determined by HPLC (chapter S4.1).

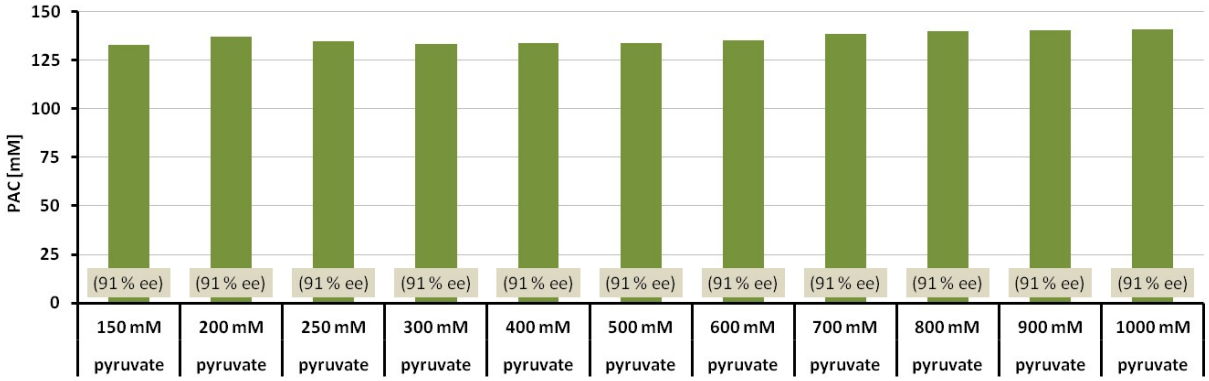


Figure S9: **Reactions with 150-1000 mM pyruvate and 100 mg/mL *Ap*PDC-E469G-I468A-W543F wet cells**
Reaction conditions: 150 mM benzaldehyde, 150-1000 mM pyruvate, 30 % DMSO, 50 mM potassium phosphate (pH 6.5), 0.2 mM ThDP, 2.5 mM $MgSO_4$, 100 mg/mL *Ap*PDC-E469G-I468A-W543F as wet cells. 1 mL reaction volume incubated in 2 mL closed glass vials, 22 °C, 600 rpm shaking, 16 h reaction time.

Pyruvate concentrations up to 1 M clearly do not negatively influence the absolute PAC-concentration (Figure S9). The stereoselectivity as well as the activity of the catalysts remains the same, independent on the pyruvates concentration. However, in terms of reaction costs, it is important to mention that equimolar concentrations of PAC and benzaldehyde are most useful as the absolute concentrations do not significantly different from each other.

S5.3.2 Effect of the DMSO concentration on reactions with 150 mM benzaldehyde

In order to investigate if PAC concentrations >150 mM can be reached with higher initial benzaldehyde concentrations (and respectively higher DMSO concentrations to dissolve the benzaldehyde), reactions with 22.5-40 % (v/v) DMSO, 150 mM benzaldehyde, 300 mM pyruvate, 50 mM potassium phosphate (pH 6.5), 0.2 mM ThDP, 2.5 mM $MgSO_4$ and 100 mg/mL *Ap*PDC-E469G-I468A-W543F wet cells were set up analogously to reactions described before (see chapter S5.1). Similar results were obtained with the different amounts of DMSO (Figure S10) indicating no DMSO inhibition for concentration at least up to 40 mM.

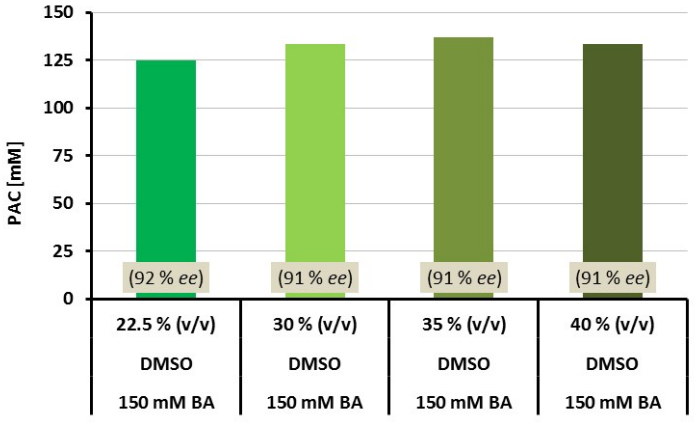


Figure S10: **Summary of reactions with 22.5-40 % (v/v) DMSO, 150 mM benzaldehyde, 300 mM pyruvate 100 mg/mL *Ap*PDC-E469G-I468A-W543F as wet cells**
Reaction conditions: X % (v/v) co-solvent, 150 mM benzaldehyde, 300 mM pyruvate, 50 mM potassium phosphate, 0.2 mM ThDP, 2.5 mM $MgSO_4$, pH 6.5, 100 mg/mL wet cells. 1 mL reaction volume in 2 mL closed glass vials, 600 rpm shaking, 22 °C, 16 h reaction time.
DMSO: dimethylsulfoxide, BA: benzaldehyde.

S5.3.3 Effects of different initial PAC-concentrations (quick test for product inhibition)

Typically, Michaelis-Menten kinetics would give clear inhibition constants. However, since only a discontinuous HPLC-assay for activity measurements is available for these enzymes, and time-consuming two-substrate kinetics would be required to gain detailed information, a simpler setup was chosen. To estimate a potential inhibition of the enzyme at higher product concentrations, biotransformations were performed in presence of increasing concentrations of (*S*)-PAC concentration.

For these experiments, a stock solution containing 150 mM benzaldehyde, 400 mM pyruvate, 50 mM potassium phosphate (pH 6.5), 37.5 % DMSO, 0.2 mM ThDP, 2.5 mM $MgSO_4$ was prepared. To four aliquots of this solution different concentrations of (*S*)-PAC were added. A cell suspension of 500 mg/mL was prepared in 50 mM potassium phosphate buffer, pH 6.5, 0.2 mM ThDP, 2.5 mM $MgSO_4$. To 800 µL of the stock solution 200 µL cell suspension was added (yielding into dilution of 20 %). The reaction solutions were incubated in 2 mL closed glass vials at 600 rpm, 22 °C and the product concentration analysed at different time points by HPLC measurements (chapter S4.1).

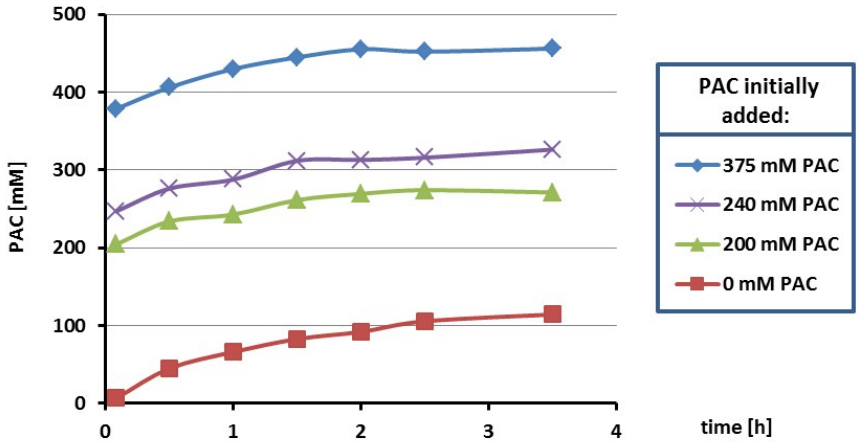


Figure S11: Influence of different initial (*S*)-PAC-concentrations on reactions with *Ap*PDC-E469G-I468A-W543F used as wet-cell-catalysts (=> test for product inhibition)

Reaction condition: 0-375 mM PAC, 120 mM benzaldehyde, 300 mM pyruvate, 50 mM potassium phosphate buffer, pH 6.5, 30 % (v/v) DMSO, 0.2 mM ThDP, 2.5 mM $MgSO_4$, 100 mg/mL *Ap*PDC-E469G-I468A-W543F as wet-cells, incubated in 2 mL glass closed vials at 600 rpm, 22 °C.

The results show that even in the presence of initial PAC-concentrations of 375 mM, further product formation was clearly detectable (Figure S11). Therefore, a PAC-related product inhibition could not be observed at least up to ~375 mM.

S5.3.4 Effects of different initial pH-values

During decarboxylation of pyruvate and subsequent carboligation with benzaldehyde to (*S*)-PAC, pyruvic acid is consumed. Thus, the pH could increase even in buffered systems, if it is not controlled which would impair the activity of the enzyme. To test the possible pH-effects reactions in a 5 mL scale were performed and the final pH of the reaction solution was checked (Figure S12).

Reactions with 150 mM benzaldehyde, 300 mM pyruvate, 30 % (v/v) DMSO, 100 mM potassium phosphate (pH 6.5), 0.2 mM ThDP, 2.5 mM $MgSO_4$, 100 mg/mL *Ap*PDC-E469G-I468A-W543F wet cells were set up analogously

to the experiments described previously (chapter 5.1). Here two different initial pH values were chosen: pH 6.5 and pH 5.0.

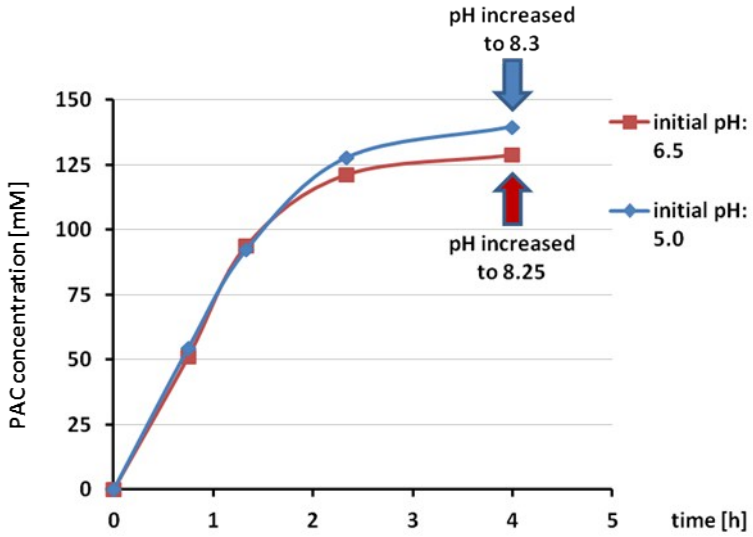


Figure S12: Reactions with 30 % (v/v) DMSO, 150 mM benzaldehyde, 300 mM pyruvate and 100 mg/mL *Ap*PDC-E469G-I468A-W543F as wet cells

Reaction conditions: 150 mM benzaldehyde, 300 mM pyruvate, 30 % DMSO, 100 mM potassium phosphate (pH 6.5: red and pH 5.0: blue), 0.2 mM ThDP, 2.5 mM $MgSO_4$, 100 mg/mL *Ap*PDC-E469G-I468A-W543F as wet cells. 5 mL reaction volume incubated in 10 mL closed glass vials, 200 rpm stirring. On the ordinate axis concentration of PAC is shown.

Due to the consumption of pyruvic acid the pH increased from initially pH 5.0 (Figure S11, blue) and pH 6.5 (Figure S12, red) to pH >8. The *Ap*PDC, an enzyme from *Acetobacter pasteurianus*, has a pH-optimum in the acidic range (pH 5-6.5). At pH values above pH 8 the enzyme is almost completely inactivated.[11] As a consequence, the pH must be controlled by continuous acid addition.

S6 Access to (*S*)-PAC concentrations >300 mM (45 g/L) combining optimal reaction conditions in fed-batch

S6.1 Reaction setup with benzaldehyde feed and pH-control

Since (*S*)-PAC is a valuable intermediate for the synthesis of chiral drugs such as nor(pseudo)ephedrine, ephedrine, metaraminol, levonordefrin and others, reactions with high product concentrations are required to proof the industrial relevance of (*S*)-PAC synthesis with this new catalyst.

As described before, product concentrations up to 120 mM (*S*)-PAC were reached in simple batch reactions modes when co-solvents like DMF or DMSO were added to increase benzaldehyde and PAC solubility (see chapter S5.1). While the toxicity of DMF is much higher than the one of DMSO and in addition DMSO has stabilisation effect in terms of biocatalyst half-life (see chapter S5.2), DMSO was selected as a co-solvent of choice. However, an upper limit of ~120 mM PAC could not be exceeded (chapter S5.1), although neither a PAC-induced product inhibition (chapter S5.3.3) nor pyruvate (chapter S5.3.1) and DMSO (chapter S5.3.2) significantly hampered the reaction. However, an increase of pH up to pH >8 during the course of the reaction due to the consumption of pyruvic acid (chapter S5.3.4) was detected, which inactivates the enyzme.[11] Therefore, a pH-control ('718 STAT Titrino'; Metrohm AG) was applied for concentrations >120 mM. Since pyruvic acid is consumed during the reaction, a 3 M pyruvic acid feed solution (in water; pH ~1.05) was used to keep the pH constant at 6.5. Moreover, benzaldehyde (and even more not dissolved benzaldehyde) caused significant deactivation of the biocatalyst (chapter S5.2). Therefore and because even with e.g. 30 % (v/v) DMSO only 150 mM benzaldehyde is soluble, benzaldehyde needs to be fed during the reaction. Conclusively, in a fed-batch reaction the feed-rate (LA-160; Landgraf Laborsysteme GmbH) was adjusted in a way that the benzaldehyde concentration was kept below the solubility limit, but high enough not to limit the reaction (➔ between 20-120 mM). Setup of the reaction is depicted in Figure S13.

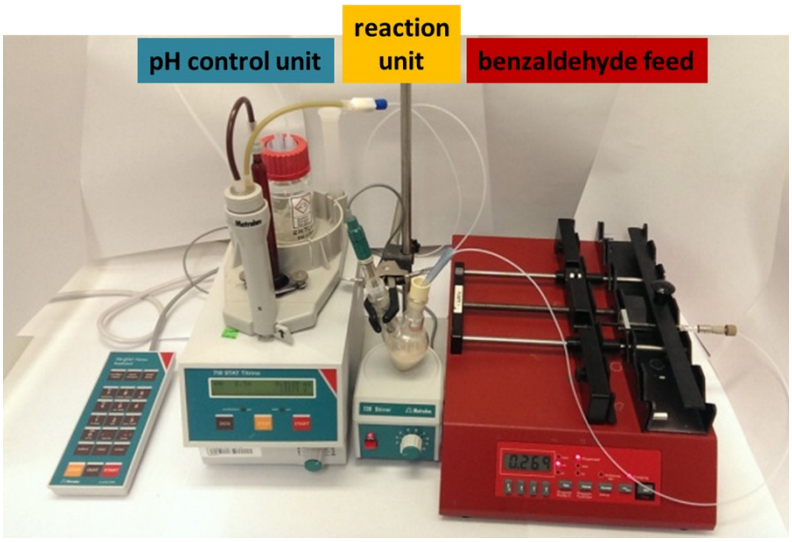


Figure S13: Setup for the production of high (*S*)-PAC concentrations in reactions with co-solvents, pH-control (left, blue) and benzaldehyde-feed (right, red)

S6.2 *Ap*PDC-E469G-I468A-W543F wet cells; 30 % (v/v) DMSO; benzaldehyde-feed and pH-control

Similar to the experiments described before (chapter S5.3), a 25 mL reaction solution with the following initial starting parameters was set-up: 100 mM benzaldehyde, 300 mM pyruvate, 30 % DMSO, 50 mM potassium phosphate buffer, pH 6.5, 0.5 mM ThDP, 2.5 mM $MgSO_4$, 200 mg/mL *Ap*PDC-E469G-I468A-W543F wet cells. The

25 mL reaction volume was incubated at 22 °C at 400 rpm (stirring with magnetic bar). A 3 M pyruvic acid solution was used to keep the pH constant at 6.5. The benzaldehyde feed rate was adjusted upon determination of the benzaldehyde concentration at different time points of the reaction by HPLC (chapter S4.1)

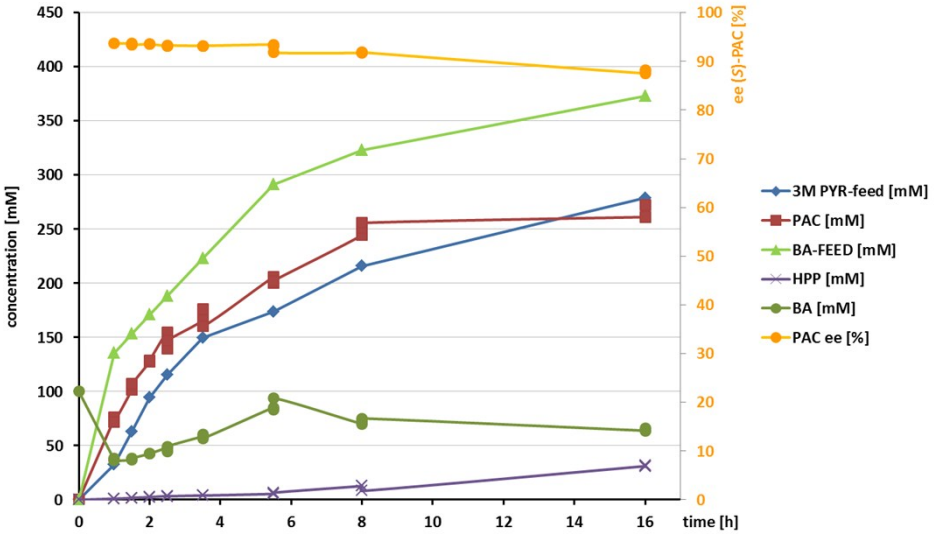


Figure S14: (*S*)-PAC synthesis with 30 % (v/v) DMSO, 100 mM benzaldehyde, 300 mM pyruvate and 200 mg/mL *Ap*PDC-E469G-I468A-W543F wet cells, benzaldehyde feed and pH-control (3 M pyruvic acid)

Initial reaction conditions: 100 mM benzaldehyde, 300 mM pyruvate, 30 % DMSO, 50 mM potassium phosphate buffer, pH 6.5, 0.5 mM ThDP, 2.5 mM $MgSO_4$, 200 mg/mL *Ap*PDC-E469G-I468A-W543F wet cells. 25 mL reaction volume incubated at 22 °C; 400 rpm stirring.

pH kept constant at 6.5 by online pH-measuring and pH-stat coupled with 3 M pyruvic acid feed (blue).

Benzaldehyde was fed (light green) with an external syringe pump such that the concentration was kept >50 mM, but below the solubility limit <<150 mM; ideal benzaldehyde concentration <100 mM.

Using this set-up, the product concentration increased to values higher than 150 mM (Figure S14, red). It can be clearly seen that the amount of pyruvic acid fed is almost equal to the amount of PAC produced, which is in line with the stoichiometry of the reaction. After 8 h reaction time, ~250 mM PAC (=37.5 g/L) with an *ee* for (*S*)-PAC of 91 % was formed. The amount of the regioisomer HPP (2-hydroxy-1-phenylpropan-1-one) is with ~4 % relatively low. After 8 h the product concentration did not increase further. Since strong PAC-induced product inhibition does not occur up to values of at least 375 mM (see chapter S5.3.3), biocatalyst inactivation might be caused by the substrate benzaldehyde (half-life in presence of benzaldehyde: <4 h; see chapter S5.2).

S6.3 Influence of whole cell background on the isomerisation of (*S*)-PAC

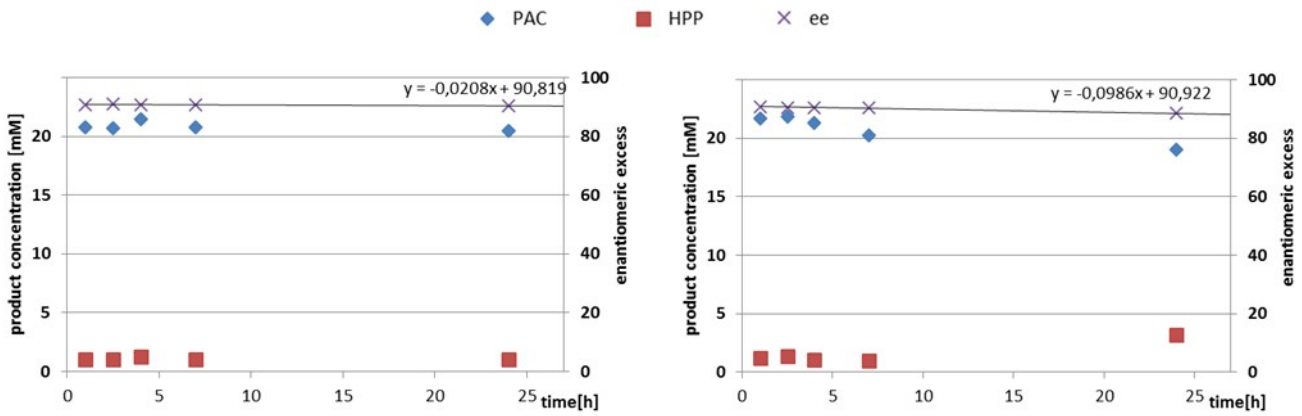


Figure S15: Stereoselectivity changes of (*S*)-PAC in the presence of purified enzyme (A) and whole *E. coli* cells with empty plasmid (B). Reaction conditions: A- 0.5 mg/ml purified ApPDC-E469G-I468A-W543H, 20 mM PAC, 50 mM KPi pH 6.5; B- 50 mg/ml *E. coli* BL21(DE3) cells with empty-plasmid , 20 mM PAC, 50 mM KPi pH 6.5.

When PAC is incubated with whole cells (50 mg/ml BL21(DE3)-empty-plasmid without any carboligase expressed, Figure S15, B) the PAC concentration decreases significantly over time while the regioisomer HPP is formed. This is not the case if PAC is incubated under the same conditions with purified enzyme (Figure S15, A). Moreover, the *ee* decreases by a factor of 5 faster in the presence of cells compared to isolated enzymes. This suggests that a background isomerisation is catalysed by *E.coli* cells, which can be e.g. catalysed by keto-enol-isomerases of the glycolysis.

S7 Enlargement of the product platform of functionalized α-hydroxy ketones

To enlarge the product platform of functionalized α-hydroxy ketones 21 benzaldehyde-derivatives were tested. The substrate scope contained *ortho*-, *meta*- and *para*-substituted benzaldehydes with various functional groups (see Figure S16), which differ in size and chemical properties.

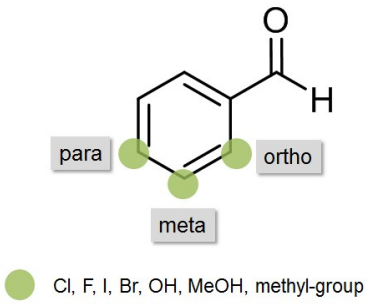


Figure S16: Tested substrates for *Ap*PDC-wt and variants. Benzaldehyde derivatives with functional groups in *ortho-, meta-.* and *para*- position.

S7.1 General methods

All reactions were performed with purified and lyophilized enzymes (procedure described in chapter 3). Protein concentrations were determined with the NanoDrop® spectrophotometer at 280 nm. Molecular weight and

extinction coefficient at 280 nm were determined for each protein by the ExPASy ProtParam tool.[12] The calculated molecular weight was 61.13 kDa and the extinction coefficient was calculated as 69955 $M^{-1}cm^{-1}$ measured in water. Reactions were performed in analytical scale (1 mL total volume) with pyruvate decarboxylase from *Acetobacter pasteurianus* (*Ap*PDC) wild type and 3 variants:

1 – *Ap*PDC-wildtype (wt),

2 – *Ap*PDC-E469G,

3 – *Ap*PDC-E469G/W543H,

4 – *Ap*PDC-E469G/I468A/W543F

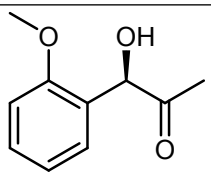
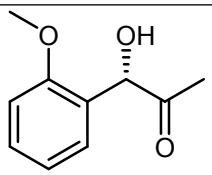
Reaction conditions: *Ap*PDC (1.5 mg/mL), buffer (50 mM potassium phosphate buffer pH 6.5 containing 0.1 mM ThDP, 2.5 mM $MgSO_4$, 10 % DMSO), benzaldehyde and derivatives (10 mM), pyruvate (50 mM), 20 °C, 600 rpm, 16 h.

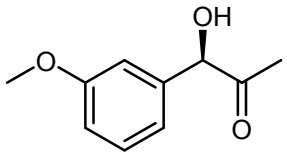
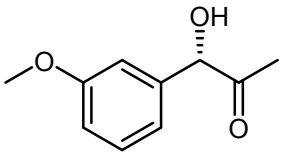
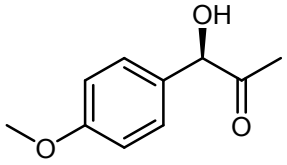
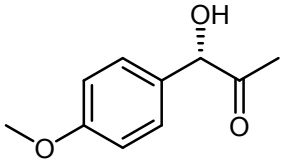
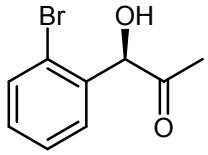
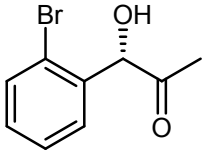
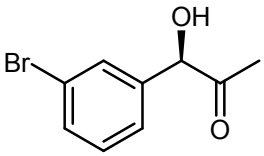
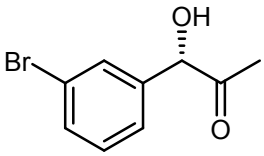
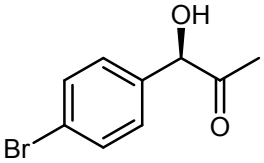
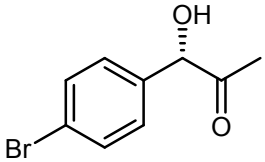
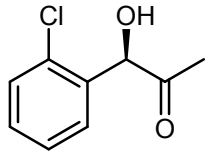
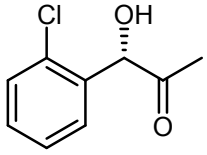
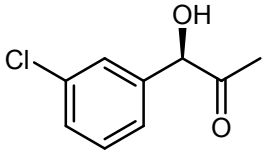
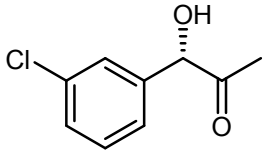
Conversions were determined based on substrate decrease by GC/MS after extraction with ethyl acetate (150 µL sample and 200 µL ethyl acetate). GC MS analyses were obtained on a HP 6890 N Series GC system (EI 70 eV) with a HP 5973 Network Mass Selective Detector (Agilent), provided with a DB-5MS column (Agilent, 30 m × 0.25 mm, 0.25 µm thick stationary phase). Following conditions were used: injector temperature: 250 °C, detector temperature: 300 °C, temperature program: 60 °C for 3 min, then temperature raise with 20 °C·min^{-1} to 280 °C, then hold for 4 min, flow rate 25 mL·min^{-1}. CD spectra were recorded on a Jasco J-810 spectrometer (Jasco International Co.) as a solution in acetonitrile.

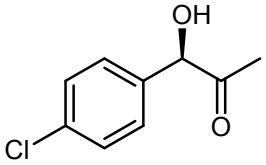
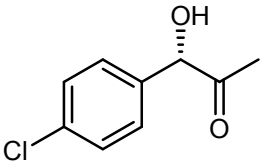
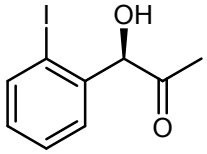
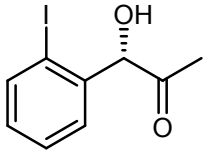
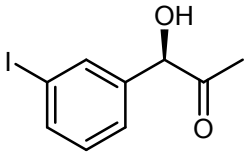
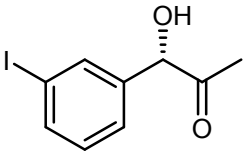
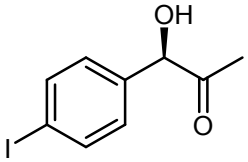
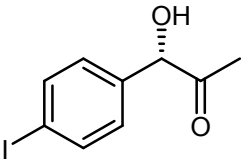
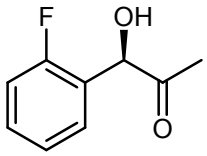
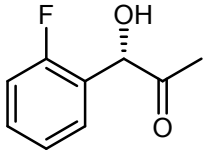
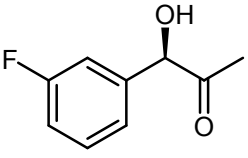
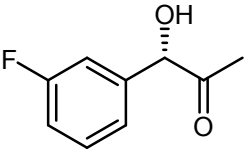
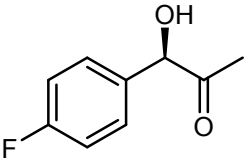
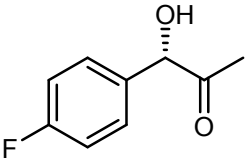
Enantiomeric excess was determined by chiral-phase High-Performance Liquid Chromatography (HPLC-DAD) after extraction with ethyl acetate (300 µL sample and 200 µL ethyl acetate). HPLC analysis was carried out on a HP 1100 chromatography system (Agilent). The following columns were used: Daicel Chiralcel OB 10 µm (250 mm × 4.6 mm), Daicel Chiralcel OD-H 5 µm (250 mm × 4.6 mm), CS Chromatographie chiral-OM 5 µm (250 mm × 4.6 mm), Reposil Chiral-OM 10 µm (250 mm × 10 mm), Daicel Chiralpak AD 10 µm (250 mm × 4.6 mm), Phenomenex Lux Cellulose-1 5 µm (250 mm × 4.6 mm), Daicel Chiralpak AS-H 5 µm (250 mm × 4.6 mm), and CS Chromatographie MultoHigh Chiral-AM-HR 5 µm (250 mm × 4.6 mm).

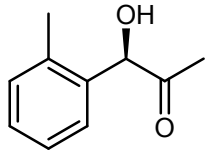
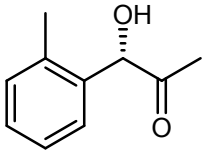
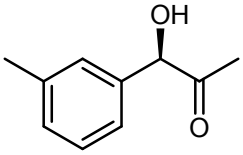
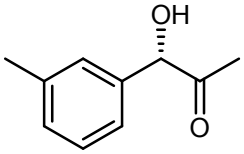
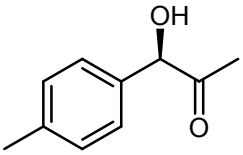
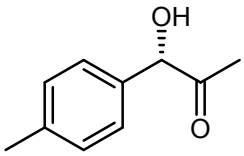
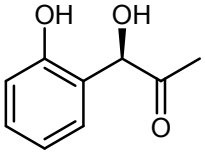
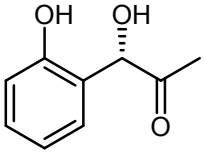
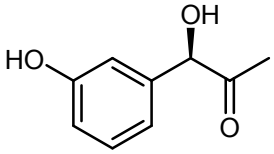
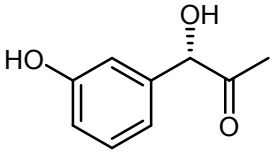
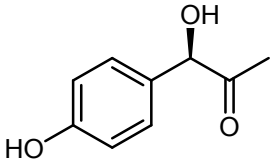
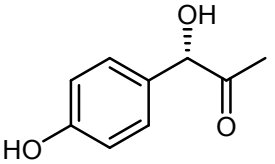
S7.2 Analytical data for conversion and *ee*-determination

The reactions were performed as described above in chapter S7.1.

compound		**detection method**
Methoxy-PAC-derivatives		
 (*R*)-1-hydroxy-1-(2-methoxyphenyl)propan-2-one	 (*S*)-1-hydroxy-1-(2-methoxyphenyl)propan-2-one	Conversion and (*R*)- and (*S*)-enantiomer determined according to Loschonsky *et al.* 2014 [13] respective compound 20a

 (*R*)-1-hydroxy-1-(3-methoxyphenyl)propan-2-one	 (*S*)-1-hydroxy-1-(3-methoxyphenyl)propan-2-one	Conversion and (*R*)- and (*S*)-enantiomer determined according to Loschonsky *et al.* [13] respective compound 20b
 (*R*)-1-hydroxy-1-(4-methoxyphenyl)propan-2-one	 (*S*)-1-hydroxy-1-(4-methoxyphenyl)propan-2-one	Conversion and (*R*)- and (*S*)-enantiomer determined according to Loschonsky *et al.* [13] respective compound 20c
Bromo-PAC-derivatives		
 (*R*)-1-(2-bromophenyl)-1-hydroxypropan-2-one	 (*S*)-1-(2-bromophenyl)-1-hydroxypropan-2-one	Conversion and (*R*)- and (*S*)-enantiomer determined according to Loschonsky *et al.* [13] respective compound 17a
 (*R*)-1-(3-bromophenyl)-1-hydroxypropan-2-one	 (*S*)-1-(3-bromophenyl)-1-hydroxypropan-2-one	Conversion determined according to Loschonsky *et al.* [13] respective compound 17b
 (*R*)-1-(4-bromophenyl)-1-hydroxypropan-2-one	 (*S*)-1-(4-bromophenyl)-1-hydroxypropan-2-one	Conversion and (*R*)- and (*S*)-enantiomer determined according to Loschonsky *et al.* [13] respective compound 17c
Chloro-PAC-derivatives		
 (*R*)-1-(2-chlorophenyl)-1-hydroxypropan-2-one	 (*S*)-1-(2-chlorophenyl)-1-hydroxypropan-2-one	GC/MS: t_R = 7.07 min HPLC DAD: Daicel Chiralpak OD-H 5 µm, 20 ºC, 0.75 mL min−1, *n*-hexane/propan-2-ol = 95:5; t_R(*R*) = 16.6 min, t_R(*S*) = 13.6 min. (*R*)-enantiomer was assigned according to the reaction mechanism of the wildtype enzyme, which was (*R*)-selective with all tested substrates so far (*S*)-enantiomer showed identical UV-spectrum
 (*R*)-1-(3-chlorophenyl)-1-hydroxypropan-2-one	 (*S*)-1-(3-chlorophenyl)-1-hydroxypropan-2-one	Conversion and (*R*)- and (*S*)-enantiomer determined according to Loschonsky *et al.* [13] respective compound 16b

 (*R*)-1-(4-chlorophenyl)-1-hydroxypropan-2-one	 (*S*)-1-(4-chlorophenyl)-1-hydroxypropan-2-one	Conversion and (*R*)- and (*S*)-enantiomer determined according to Loschonsky *et al.* [13] respective compound 16c
Iodo-PAC-derivatives		
 (*R*)-1-hydroxy-1-(2-iodophenyl)propan-2-one	 (*S*)-1-hydroxy-1-(2-iodophenyl)propan-2-one	Conversion and (*R*)- and (*S*)-enantiomer determined according to Loschonsky *et al.* [13] respective compound 18a
 (*R*)-1-hydroxy-1-(3-iodophenyl)propan-2-one	 (*S*)-1-hydroxy-1-(3-iodophenyl)propan-2-one	Conversion and (*R*)- and (*S*)-enantiomer determined according to Loschonsky *et al.* [13] respective compound 18b
 (*R*)-1-hydroxy-1-(4-iodophenyl)propan-2-one	 (*S*)-1-hydroxy-1-(4-iodophenyl)propan-2-one	Conversion and (*R*)- and (*S*)-enantiomer determined according to Loschonsky *et al.* [13] respective compound 18c
Fluoro-PAC-derivatives		
 (*R*)-1-(2-fluorophenyl)-1-hydroxypropan-2-one	 (*S*)-1-(2-fluorophenyl)-1-hydroxypropan-2-one	GC/MS: t_R = 7.79 min HPLC DAD: Daicel Chiralpak AS-H 5 µm, 20 ºC, 0.75 mL min−1, *n*-hexane/propan-2-ol = 90:10; t_R(*R*) = 9.6 min, t_R(*S*) = 10.6 min. (*R*)-enantiomer was assigned according to the reaction mechanism of the wildtype enzyme, which was (*R*)-selective with all tested substrates so far (*S*)-enantiomer showed identical UV-spectrum
 (*R*)-1-(3-fluorophenyl)-1-hydroxypropan-2-one	 (*S*)-1-(3-fluorophenyl)-1-hydroxypropan-2-one	Conversion and (*R*)- and (*S*)-enantiomer determined according to Loschonsky *et al.* [13] respective compound 15b
 (*R*)-1-(4-fluorophenyl)-1-	 (*S*)-1-(4-fluorophenyl)-1-	Conversion determined according to Loschonsky *et al.* [13] respective compound 15c

hydroxypropan-2-one	hydroxypropan-2-one	
Methyl-PAC-derivatives		
 (*R*)-1-hydroxy-1-(2-methylphenyl)propan-2-one	 (*S*)-1-hydroxy-1-(2-methylphenyl)propan-2-one	Conversion and (*R*)- and (*S*)-enantiomer determined according to Loschonsky *et al.* [13] respective compound 21a
 (*R*)-1-hydroxy-1-(3-methylphenyl)propan-2-one	 (*S*)-1-hydroxy-1-(3-methylphenyl)propan-2-one	Conversion and (*R*)- and (*S*)-enantiomer determined according to Loschonsky *et al.* [13] respective compound 21b
 (*R*)-1-hydroxy-1-(4-methylphenyl)propan-2-one	 (*S*)-1-hydroxy-1-(4-methylphenyl)propan-2-one	Conversion and (*R*)- and (*S*)-enantiomer determined according to Loschonsky *et al.* [13] respective compound 21c
Hydroxy-PAC-derivatives		
		GC/MS: t_R = 9.0 min
 (*R*)-1-hydroxy-1-(3-hydroxyphenyl)propan-2-one	 (*S*)-1-hydroxy-1-(3-hydroxyphenyl)propan-2-one	Conversion determined according to Loschonsky *et al.* [13] respective compound 19b
 (*R*)-1-hydroxy-1-(4-hydroxyphenyl)propan-2-one	 (*S*)-1-hydroxy-1-(4-hydroxyphenyl)propan-2-one	Conversion determined according to Loschonsky *et al.* [13] respective compound 19c

S7.3 Comparison of conversion and *ee* sorted by the functional group position (*ortho, meta, para*)

To be able to state a general behavior or reaction mechanism the results were sorted by the position of the functional group.

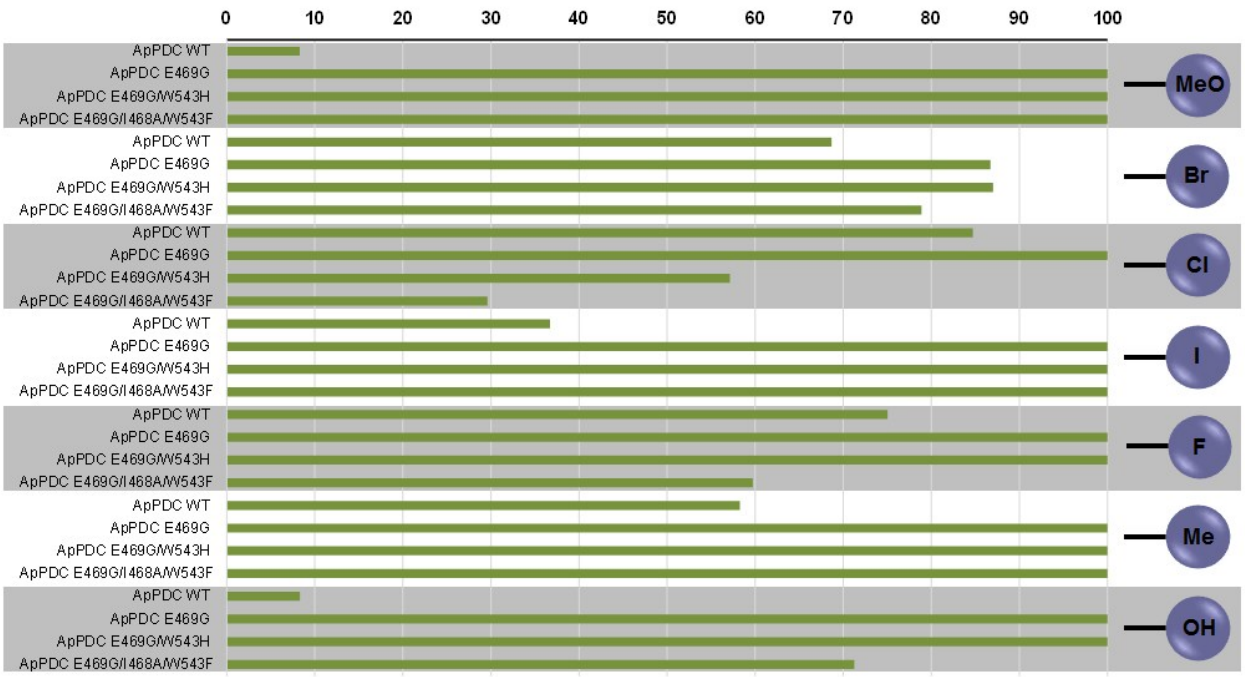


Figure S17: Conversion of all *meta*-substituted benzaldehyde derivatives. Substrate scope of the pyruvate decarboxylase from *Acetobacter pasteurianus* (*Ap*PDC), wild type and variants. 1 – *Ap*PDC-wt, 2 – *Ap*PDC-E469G, 3 – *Ap*PDC-E469G/W543H, 4 – *Ap*PDC-E469G/I468A/W543F. Reaction conditions: *Ap*PDC (1.5 mg/mL), buffer (50 mM potassium phosphate buffer pH 6.5, 0.1 mM ThDP, 2.5 mM $MgSO_4$, 10 % DMSO), benzaldehyde and derivatives (10 mM), pyruvate (50 mM), 20 °C, 600 rpm, 16 h. % conversions determined by GC/MS.

Derivatives with functional groups in ***meta*-position** are the best accepted substrates in the tested setup, as conversions are the highest and every combination showed at least a conversion around 8 %, most much higher conversions (see Figure S17). In 14 of the 28 tested combinations full conversion was achieved. The single and the double mutant showed highest conversions.

Table S1: Influence of the functional group in *meta*-position: **conversion**

enzyme	**low conversion high conversion**
*Ap*PDC-E469G	Br < F/Cl/I/Me/MeO/OH
*Ap*PDC-E469G/W543H	Cl < Br < F/I/Me/MeO/OH
*Ap*PDC-E469G/I468A/W543F	Cl < F < OH < Br < I/Me/MeO

Iodine, methyl- and methoxy-group in meta-position showed no influence on the conversion of the benzaldehyde derivatives for all tested variants. Higher effect on the conversion was observed with bromide for the single variant and chloride for the double and triple variant.

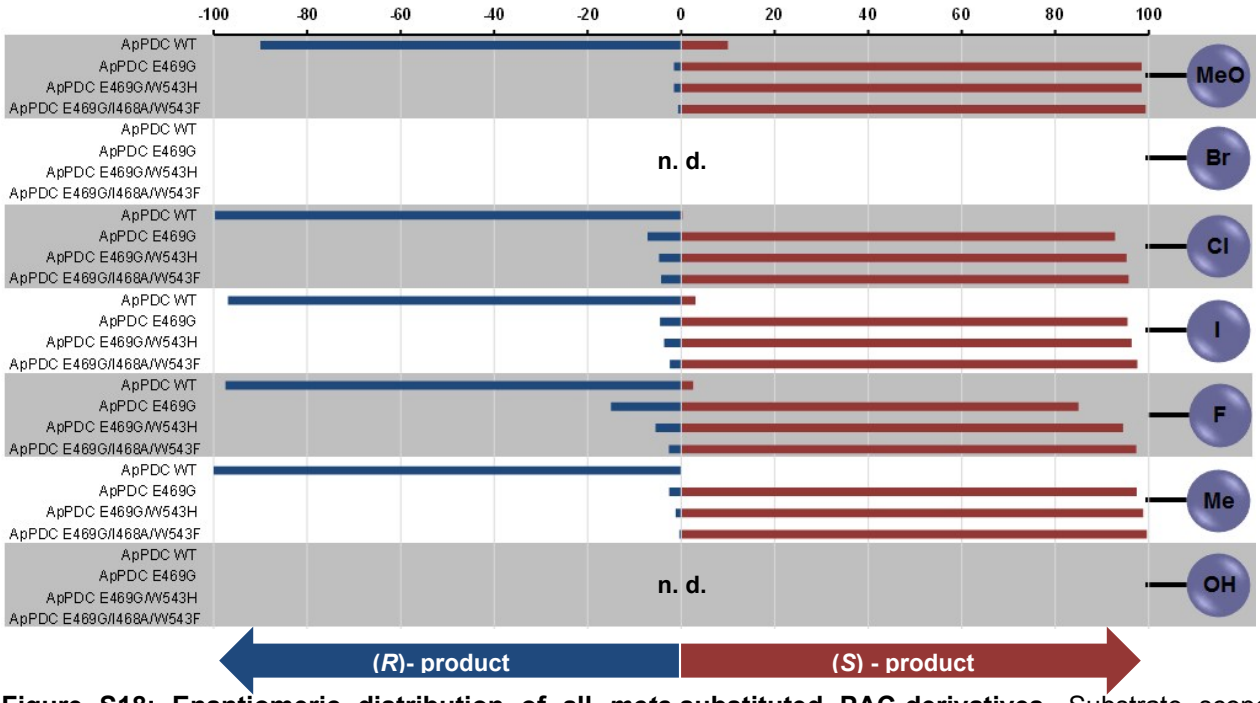


Figure S18: Enantiomeric distribution of all *meta*-substituted PAC-derivatives. Substrate scope of the pyruvate decarboxylase from *Acetobacter pasteurianus* (*Ap*PDC), wild type and variants. 1 – *Ap*PDC-wt, 2 – *Ap*PDC-E469G, 3 – *Ap*PDC-E469G/W543H, 4 – *Ap*PDC-E469G/I468A/W543F. Reaction conditions: *Ap*PDC (1.5 mg/mL), buffer (50 mM potassium phosphate buffer pH 6.5, 0.1 mM ThDP, 2.5 mM $MgSO_4$, 10 % DMSO), benzaldehyde and derivatives (10 mM), pyruvate (50 mM), 20 °C, 600 rpm, 16 h. Enantiomeric excess was determined by chiral phase HPLC. N. d.: not determined.

All meta-substituted substrates were converted (*R*)-selectively with the WT and highly (*S*)-selectively with all three variants (see Figure S18, Table S2).

Table S2: Influence of the functional group in *meta*-position: **enantiomeric excess**

enzyme	***ee*-shifting from (*S*) → (*R*)** **high** **low**	**not determined**
*Ap*PDC-E469G	F > Cl > I > Me/MeO	OH (n.d.), Br (n.d.)
*Ap*PDC-E469G/W543H	F > Cl > I > Me/MeO	OH (n.d.), Br (n.d.)
*Ap*PDC-E469G/I468A/W543F	Cl > F > I > Me/MeO	OH (n.d.), Br (n.d.)

In general, the influence of a functional group in *meta*-position on the *ee* is marginal. Fluoride and chloride seem to have the highest influence.

***Ortho*-substituted** benzaldehydes are in general well accepted by the variants. 7 of the 28 testes combinations showed full conversion and 4 others more than 80 %. In contrast, except of fluoro- and chloro-benzaldehyde the wildtype enzyme does not accept *ortho*-substituted benzaldehydes (Figure S19).

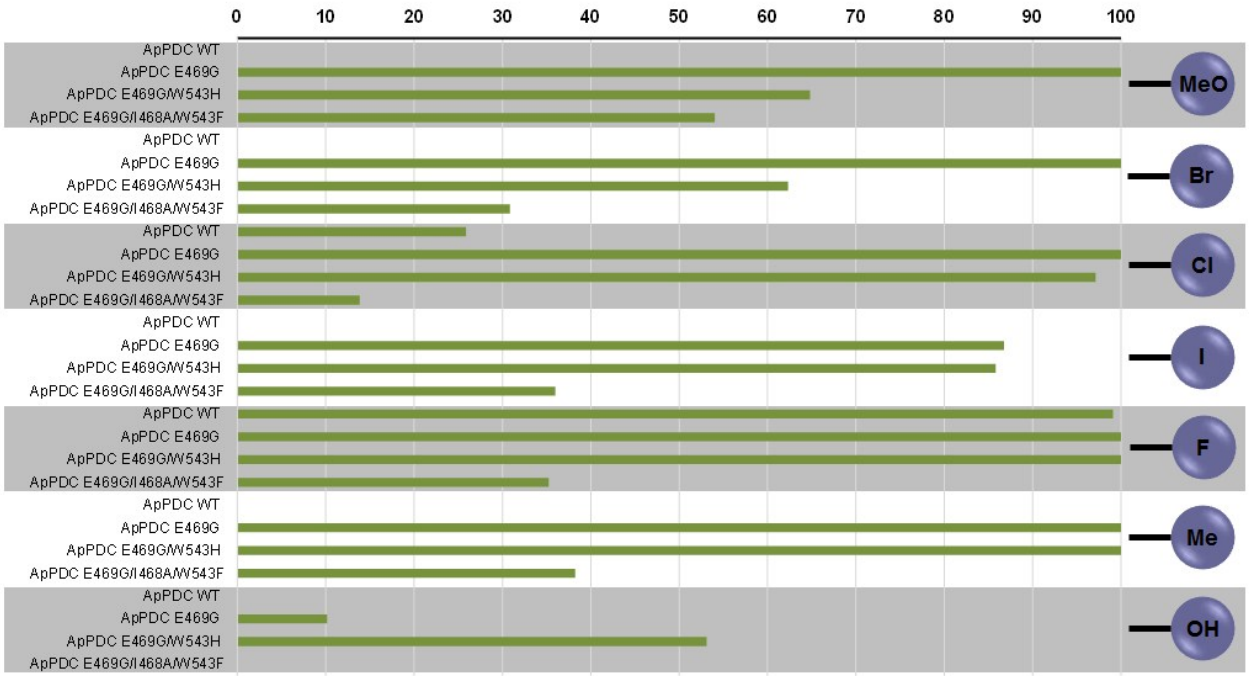


Figure S19: Conversion of all *ortho*-substituted benzaldehyde derivatives. Substrate scope of the pyruvate decarboxylase from *Acetobacter pasteurianus* (*Ap*PDC), wild type and variants. 1 – *Ap*PDC-wt, 2 – *Ap*PDC-E469G, 3 – *Ap*PDC-E469G/W543H, 4 – *Ap*PDC-E469G/I468A/W543F. Reaction conditions: *Ap*PDC (1.5 mg/mL), buffer (50 mM potassium phosphate buffer pH 6.5, 0.1 mM ThDP, 2.5 mM $MgSO_4$, 10 % DMSO), benzaldehyde and derivatives (10 mM), pyruvate (50 mM), 20 °C, 600 rpm, 16 h. % conversions determined by GC/MS.

For the three tested *Ap*PDC variants the influence of the various functional groups differed a lot (Table S3). For the single mutant, only the hydroxy-group and iodide had a negative influence on the conversion. For the double variant the hydroxy-group and bromide had the highest negative influence on the conversion but conversion was still over 50 %. For all other *ortho*-substituted benzaldehydes conversion was between 80 and 100 %. In case of the triple mutant the conversions were distinctly decreased. Just conversion with a methoxy-group substituted benzaldehyde resulted in conversion higher than 50 %. For the residual *ortho*-substituted substrates the conversion dropped below 40 % and less.

Table S3: Influence of the functional group in *ortho*-position: **conversion**

enzyme	**low conversion** **high conversion**	**no conversion (n.c.)**
*Ap*PDC-E469G	OH < I < MeO/Br/Cl/F/Me	
*Ap*PDC-E469G/W543H	OH < Br < MeO < I < Cl < Me/F	
*Ap*PDC-E469G/I468A/W543F	Cl < Br < F < I < Me < MeO	OH (n.c.)

In contrast to the *meta*-substituted benzaldehyde derivatives the *ee* is shifted towards the *R*-products for *ortho*-substituted benzaldehyde (Figure S20). The three variants are still (*S*)-selective for numerous *ortho*-substituted benzaldehydes, but the overall picture showed more (*R*)-product formation. Therefore, a functional group in *ortho* position seems to have more influence on the *ee* than in meta-position.

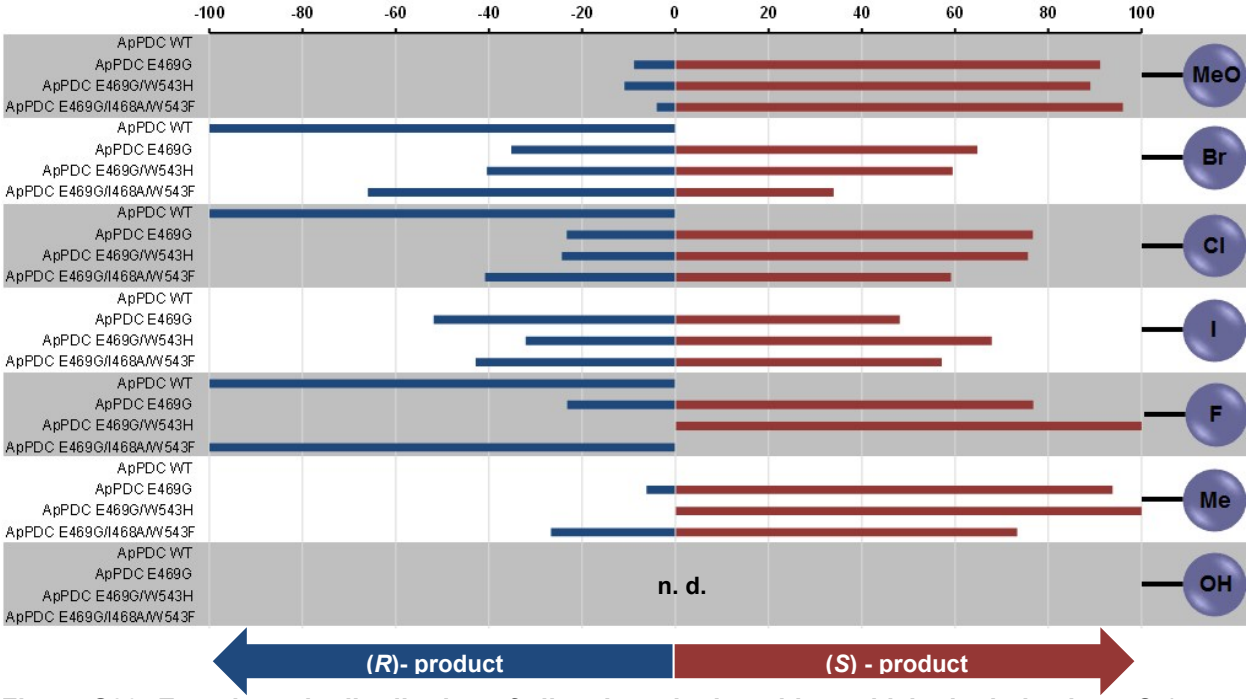


Figure S20: Enantiomeric distribution of all *ortho*-substituted benzaldehyde derivatives. Substrate scope of the pyruvate decarboxylase from *Acetobacter pasteurianus* (*Ap*PDC), wild type and variants. 1 – *Ap*PDC-wt, 2 – *Ap*PDC-E469G, 3 – *Ap*PDC-E469G/W543H, 4 – *Ap*PDC-E469g/I468A/W543F. Reaction conditions: *Ap*PDC (1.5 mg/mL), buffer (50 mM potassium phosphate buffer pH 6.5, 0.1 mM ThDP, 2.5 mM $MgSO_4$, 10 % DMSO), benzaldehyde and derivatives (10 mM), pyruvate (50 mM), 20 °C, 600 rpm, 16 h. Enantiomeric excess was determined by chiral phase HPLC. N. d.: not determined.

Table S4: Influence of the functional group in *ortho*-position: **enantiomeric excess**

enzyme	***ee*-shifting from (*S*) → (*R*)** **high** **low**	**not determined (n.d.)**
*Ap*PDC-E469G	I > Br > Cl/F > MeO > Me	OH (n.d.)
*Ap*PDC-E469G/W543H	Br/I > Cl > MeO > Me/F	OH (n.d.)
*Ap*PDC-E469G/I468A/W543F	F > I > Br > Cl > MeO > Me	OH (n.c.)

Halogen groups have the highest influence on the *ee* (Table S4). This effect might be attributed to the electronegativity of these substituents.

The conversions of ***para*-substituted** substrates are generally comparable to the conversions of *ortho*-substituted substrates. With 8 out of 28 tested combinations full conversion could be reached, with 3 others more than 80 % conversion. Only with a hydroxy-group in *para*-position no conversion was gained in general. Further, a methoxy-group in *para*-position in combination with the triple mutant led to no product formation (Figure S21).

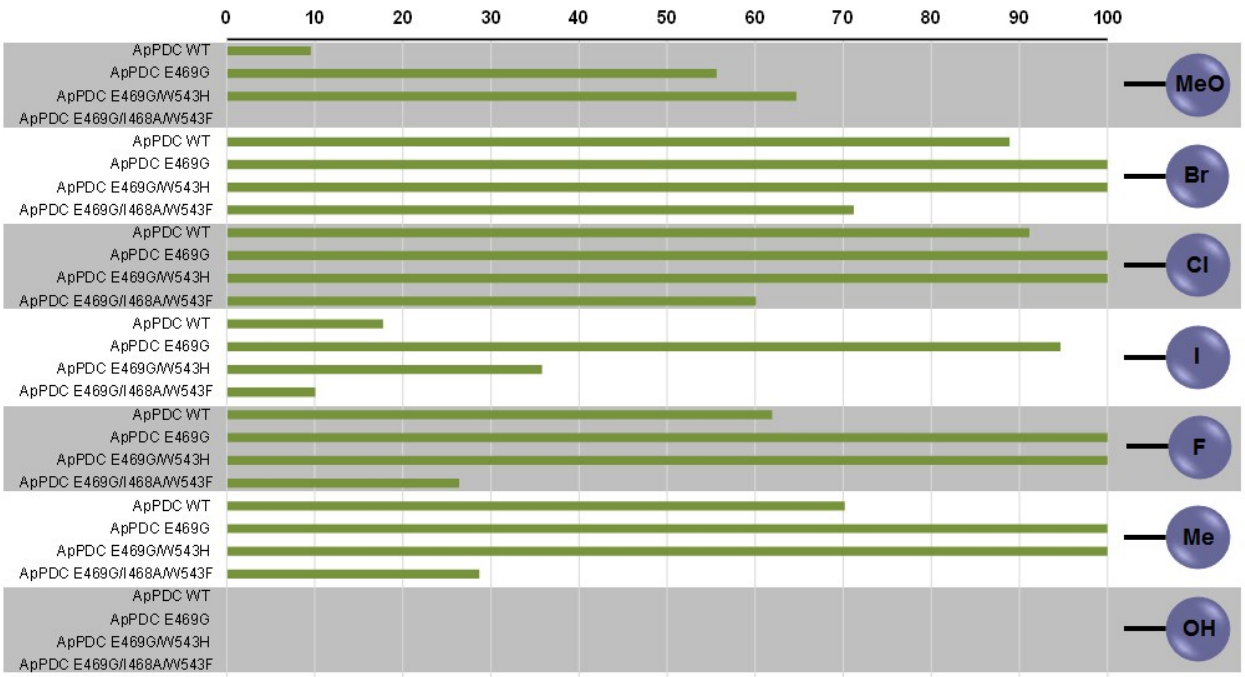


Figure S21: Conversion of all *para*-substituted benzaldehyde derivatives. Substrate scope of the pyruvate decarboxylase from *Acetobacter pasteurianus* (*Ap*PDC), wild type and variants. 1 – *Ap*PDC-wt, 2 – *Ap*PDC-E469G, 3 – *Ap*PDC-E469G/W543H, 4 – *Ap*PDC-E469G/I468A/W543F. Reaction conditions: *Ap*PDC (1.5 mg/mL), buffer (50 mM potassium phosphate buffer pH 6.5, 0.1 mM ThDP, 2.5 mM $MgSO_4$, 10 % DMSO), benzaldehyde and derivatives (10 mM), pyruvate (50 mM), 20 °C, 600 rpm, 16 h. % conversions determined by GC/MS.

Table S5: Influence of the functional group in para-position: **conversion**

enzyme	**low conversion high conversion**	**no conversion (n.c.)**
*Ap*PDC-E469G	MeO < I < Br/Cl/F/Me	OH (n.c.)
*Ap*PDC-E469G/W543H	I < MeO < Br/Cl/F/Me	OH (n.c.)
*Ap*PDC-E469G/I468A/W543F	I < F < Me < Cl < Br	OH (n.c.), MeO (n.c.)

For the single and double mutant bromide, chloride, fluoride and a methyl-group seem to have no influence on the conversion of the substrates with *para*-substitutions (Table S5). The ranking above reveals that the iodide and the methoxy-group had opposing effects for the single and the double mutant. With iodide in *para*-position the single mutant showed much higher conversion, whereas the double mutant showed a significantly lower conversion with this substrate. With the methoxy-group in *para*-position it behaves the other way around.

The influence of a substituted functional group in *para*-position seems to be much higher for the triple mutant. Conversions were in general much lower for this variant with *para*-substituted benzaldehydes. Especially with iodide conversion dropped below 10 %. It is hard to distinguish whether the influence caused by steric hindrance or if it is due to chemical properties, as there is no direct observable trend.

With functional groups in *para*-position the *ee* is shifted towards the (*R*)-product (Figure S22). Most of the variants which are (*S*)-selective benzaldehyde derivatives show surplus of the (*R*)-stereoisomer when using *para*-substitutes. Only for the methyl- and the methoxy-group the double and the triple mutant remain (*S*)-selective.

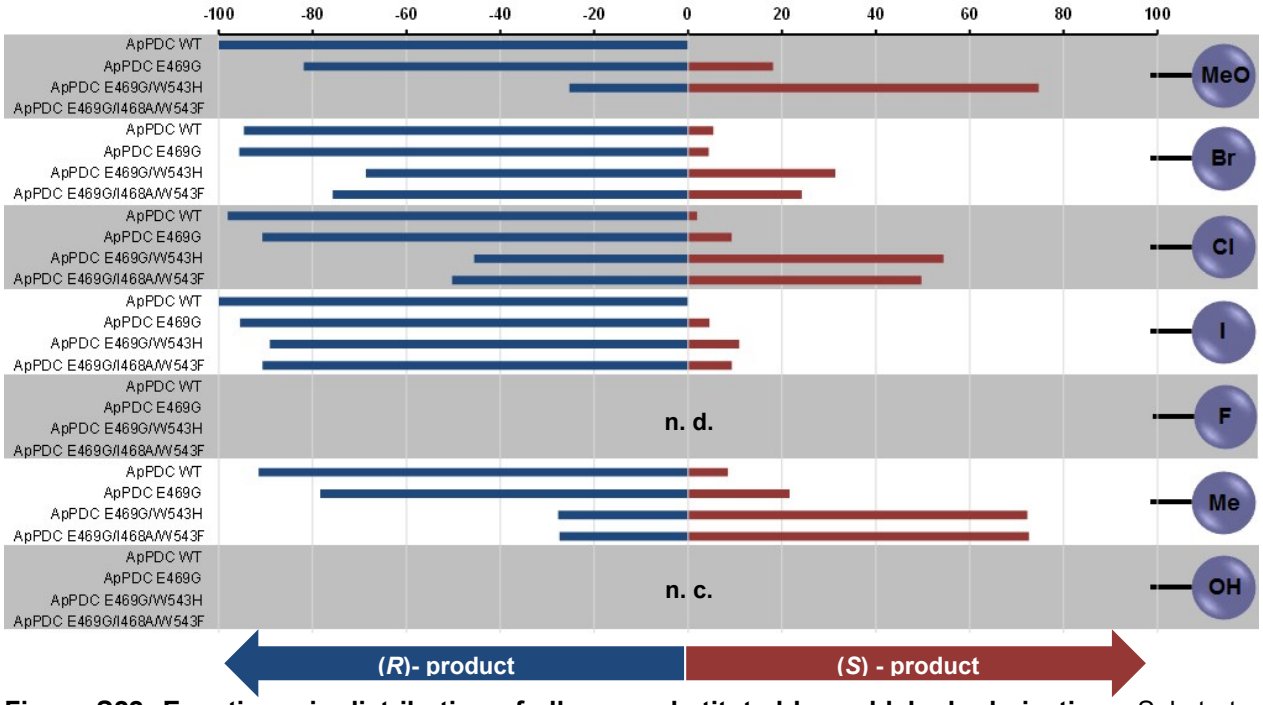


Figure S22: Enantiomeric distribution of all *para*-substituted benzaldehyde derivatives. Substrate scope of the pyruvate decarboxylase from *Acetobacter pasteurianus* (*Ap*PDC), wild type and variants. 1 – *Ap*PDC-wt, 2 – *Ap*PDC-E469G, 3 – *Ap*PDC-E469G/W543H, 4 – *Ap*PDC-E469G/I468A/W543F. Reaction conditions: *Ap*PDC (1.5 mg/mL), buffer (50 mM potassium phosphate buffer pH 6.5, 0.1 mM ThDP, 2.5 mM $MgSO_4$, 10 % DMSO), benzaldehyde and derivatives (10 mM), pyruvate (50 mM), 20 °C, 600 rpm, 16 h. Enantiomeric excess was determined by chiral phase HPLC. N. d.: not determined. N. c.: no conversion.

Table S5: Influence of the functional group in *para*-position: **enantiomeric excess**

enzyme	***ee*-shifting from (*S*) → (*R*)** **high** **low**	**no conversion (n.c.)/ not determined (n.d.)**
*Ap*PDC-E469G	I > Br > Cl > MeO > Me	OH (n.c.), F(n.d.)
*Ap*PDC-E469G/W543H	I > Br > Cl > Me/MeO	OH (n.c.), F(n.d.)
*Ap*PDC-E469G/I468A/W543F	I > Br > Cl > Me	OH (n.c.), F(n.d.), MeO (n.c)

Halogen groups have the highest influence on the *ee* (Table S6). This effect might be attributed to the electronegativity of these substituents.

S8. References

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