Electronic Supplementary Material (ESI) for Catalysis Science & Technology.

Continuous enzymatic stirred tank reactor cascade with unconventional medium yielding high concentrations of (*S*)-2-hydroxyphenyl propanone and its derivatives

Reinhard Oeggl[a,b], Juliane Glaser[a,c], Eric von Lieres[a], and Dörte Rother [a,b*]

Supporting Information

S1. Material and methods

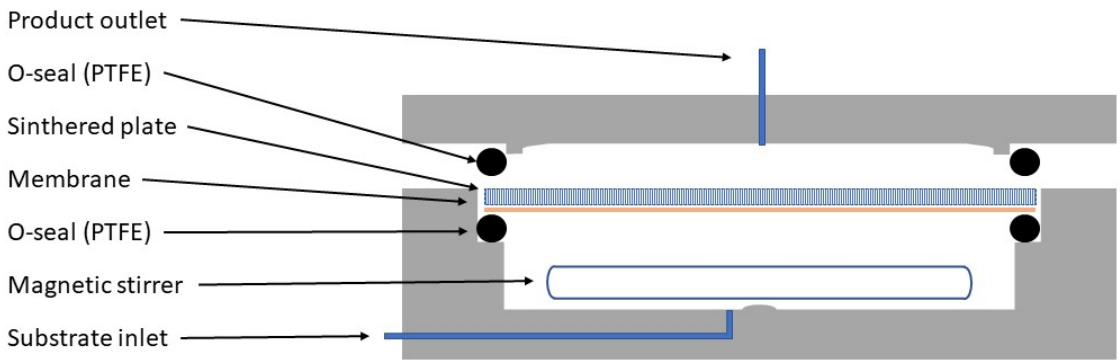

Figure S 1. Scheme of the Jülich enzyme membrane reactor published by Kragl *et al.* The reactor is referred to as membrane reactor because an ultrafiltration membrane is utilized before the outlet to retain the biocatalyst.[1]

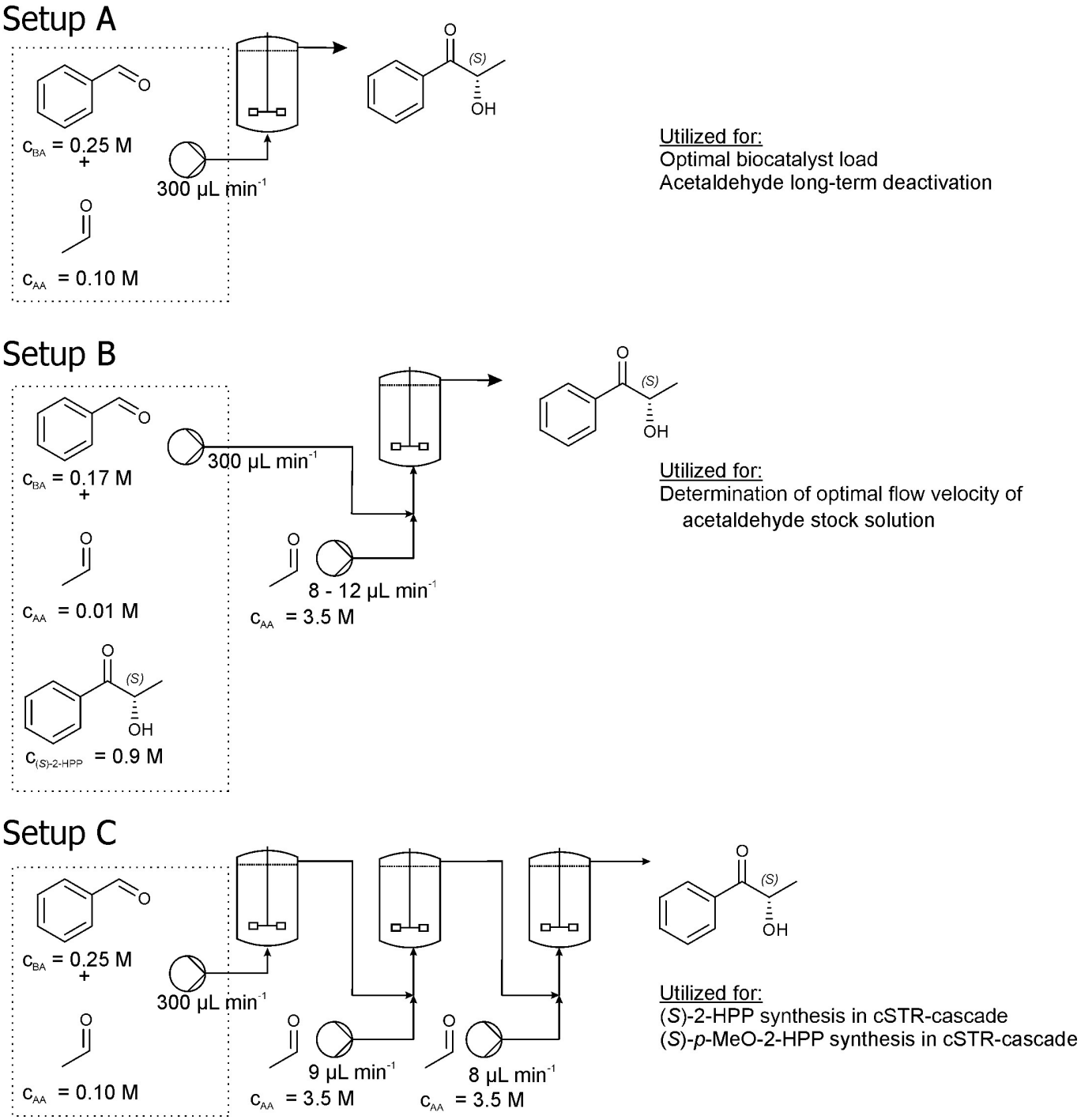

Figure S 2. Detailed scheme of used continuous setups and their application. c(BA) = concentration of benzaldehyde, c(AA) = concentration of acetaldehyde, c((*S*)-2-HPP = concentration of (*S*)-2-HPP.

Table S1. Lower and upper bounds for parameter estimation and initial values handed to optimizer.

Parameter	*Lower bound*	*Upper bound*
v_{MAX}	**10 kat/mol**	**8610 kat/mol**
$K_{M,AA}$	**10 mM**	**500 mM**
$K_{M,BA}$	**10 mM**	**1300 mM**
$K_{inh,AA}$	**1 mM**	**1000 mM**

Table S2. HPLC elution times of benzaldehyde derivatives and the corresponsive products. Benzaldehyde (BA), 2-hydroxy-phenyl-1-propanone (HPP); n.d. = not detected; notably, all fluoro- and chloro- derivatives were detected at 244 nm, while the rest was detected at 210 nm.

	Ortho-substituted			*Meta-substituted*			*Para-substituted*		
	BA	*(S)-HPP*	*(R)-HPP*	*BA*	*(S)-HPP*	*(R)-HPP*	*BA*	*(S)-HPP*	*(R)-HPP*
Fluoro	2.7 min	3.3 min	3.5 min	2.8 min	3.1 min	3.3 min	2.9 min	3.4 min	3.6 min
Chloro	2.6 min	3.4 min	n.d.	2.8 min	3.2 min	3.6 min	3.0 min	3.6 min	n.d.
Bromo	2.7 min	3.4 min	n.d.	2.9 min	3.2 min	n.d.	3.1 min	3.8 min	n.d.
Methoxy	2.8 min	3.5 min	n.d.	2.8 min	3.6 min	n.d.	3.2 min	4.4 min	4.8 min
Nitro	3.8 min	2.6 min	n.d.	3.9 min	n.d.	n.d.	3.9 min	4.2 min	n.d.

Table S3. Physico-chemical parameters of all applied reaction compounds.

	T_{boil} [°C]	T_{melt} [°C]
Methyl-tert-butyl ether	60.4	-102.9
Acetaldehyde	20.8	-119.4
Benzaldehyde	161.8	-36.5
(S)-HPP	304.6	39.7

S2. Results and discussion

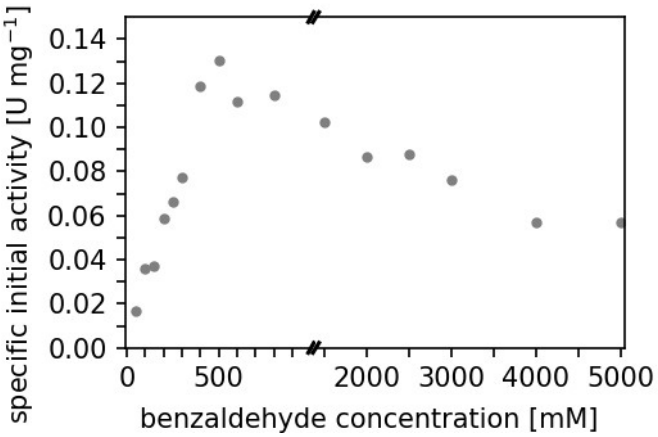

Figure S 3. Benzaldehyde optimum of the reaction to (*S*)-2-HPP by *PpB*FD varL461A. Reaction conditions: 20 mg mL^{-1} *Pp*BFD varL461A LWC were suspended in 100 mM acetaldehyde and the indicated concentration of benzaldehyde in MTBE. The reaction was started by adding 20 µL mL^{-1} 1 M TEA buffer pH 10 with 5 mM $MgSO_4$ and 1 mM ThDP; 30 °C, 1000 rpm, n=1

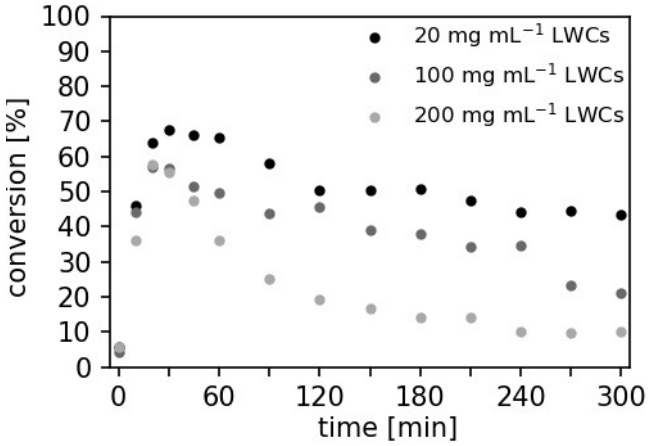

Figure S 4. Optimal cell load in one cSTR unit. Depicted is the conversion of acetaldehyde and benzaldehyde to (*S*)-2-HPP in MARS**.** Reaction conditions: the indicated amount of *Pp*BFD varL461A LWC was placed inside of an EMR chamber. A substrate solution of 250 mM benzaldehyde and 100 mM acetaldehyde in MTBE was pumped at 0.3 mL min^{-1}; 300 rpm, 30 °C, n=1

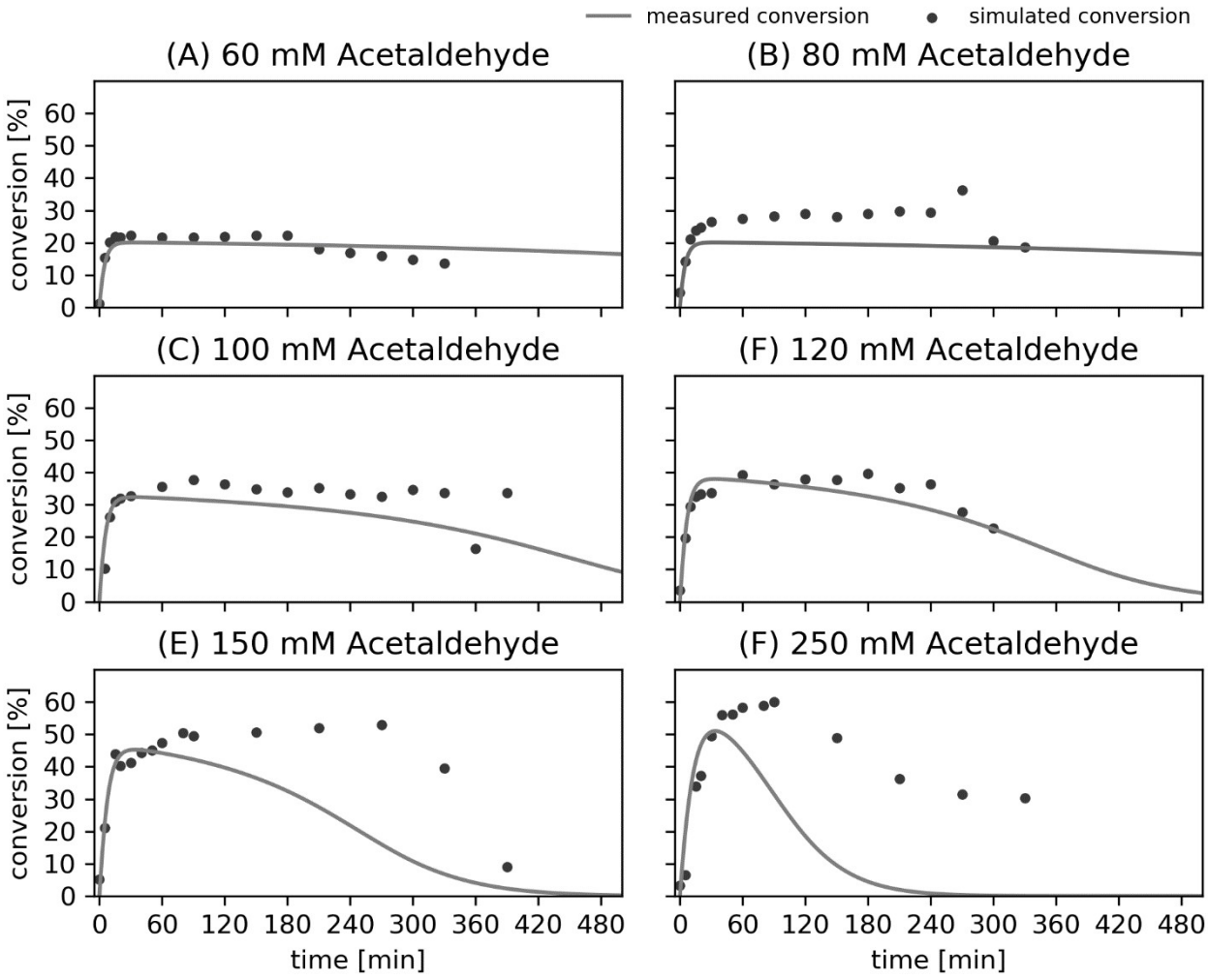

Figure S 5. Fitting results of acetaldehyde impact on the ligation of acetaldehyde and benzaldehyde to (*S*)-2-HPP in a single cSTR unit at varying acetaldehyde concentrations and 250 mM benzaldehyde in the feed. The Michaelis-Menten parameters are estimated together with the constant of the staged deactivation model. Depicted is the conversion of acetaldehyde and benzaldehyde to (*S*)-2-HPP in micro-aqueous reaction system

(MARS). A: Fit of S-HPP conversion at 60 mM acetaldehyde. B: Fit of (*S*)-2-HPP conversion at 80 mM acetaldehyde. C: Fit of (*S*)-HPP conversion at 100 mM acetaldehyde. D: Fit of (*S*)-2-HPP conversion at 120 mM acetaldehyde. E: Fit of (*S*)-2-HPP conversion at 150 mM acetaldehyde. F: Fit (*S*)-2-HPP conversion at 250 mM acetaldehyde; dashed line = simulated values; black points = measured data

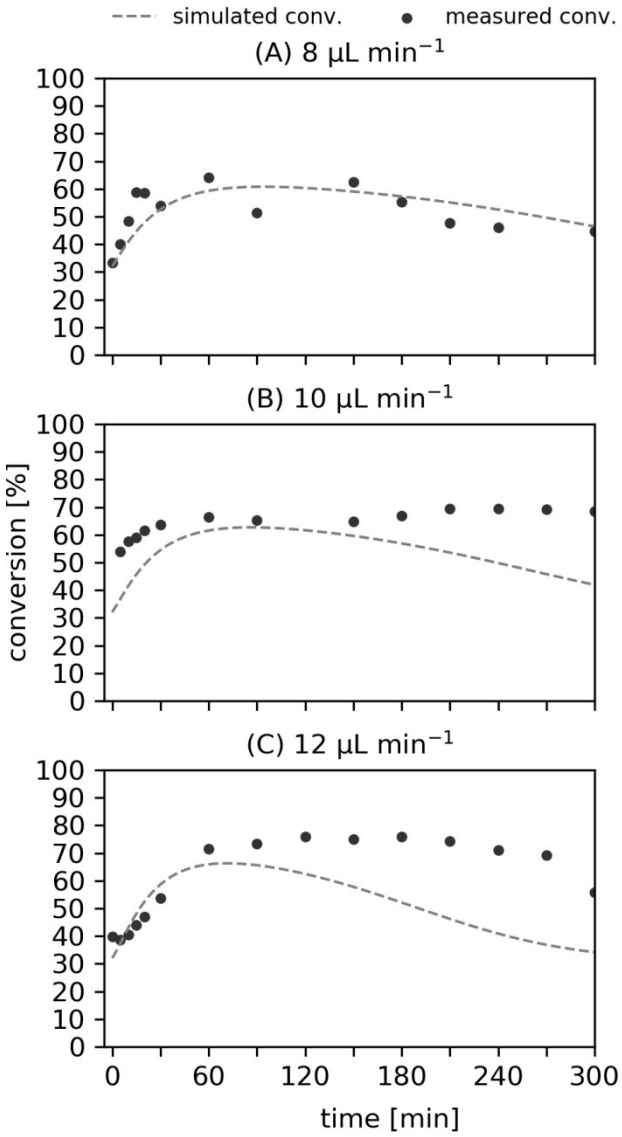

Figure S 6. Measured and predicted effect of different acetaldehyde stock pumping velocities. The staged deactivation model was used, and the Michaelis-Menten parameters were estimated from process data of a single cSTR unit. The model predicted the effect of different pumping velocities for an acetaldehyde stock solution for pumping velocities of (A) 8 µL min^{-1}, (B) 10 µL min^{-1}, and (C) 12 µL min^{-1}. Reaction conditions: 2 mg *Pp*BFD varL461A LWC; the substrate solution of 250 mM benzaldehyde and 100 mM acetaldehyde was pumped at 0.3 mL min^{-1}. The acetaldehyde stock solution of the indicated concentration was pumped at the depicted velocity. Both solutions met at a T-mixing unit prior to entering the reaction chamber. 30 °C, 300 rpm, $n_{technical}$=1; see Figure S2 setup B.

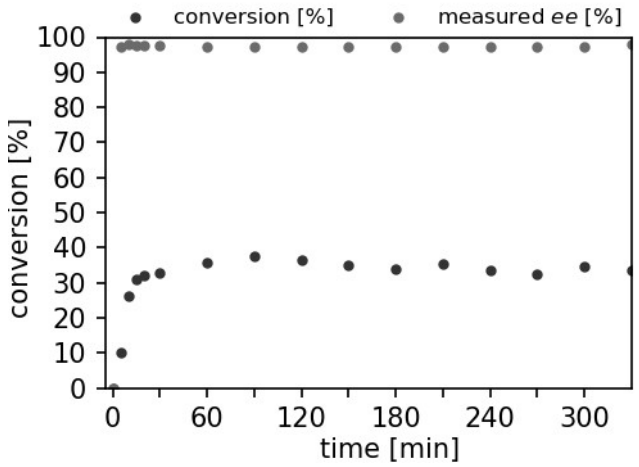

Figure S 7. Continuous operation of one single cSTR unit. Displayed is the conversion of benzaldehyde. Reaction conditions: 2 mg *Pp*BFD varL461A LWC; substrate solution of 250 mM benzaldehyde and 100 mM acetaldehyde pumped at 0.3 mL min^{-1}; 30 °C, 300 rpm, n=1; scheme see S2

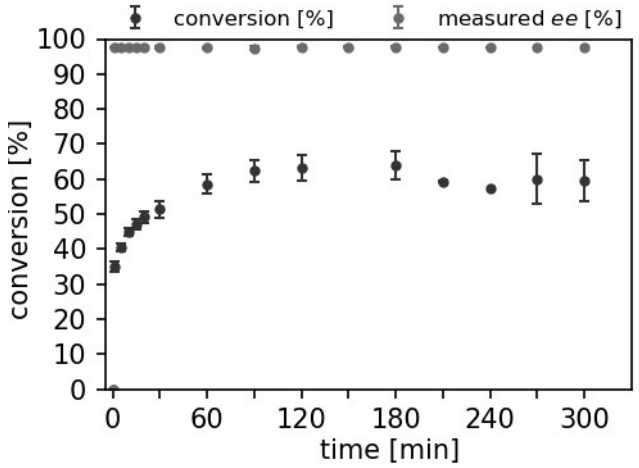

Figure S 8. Continuous operation of two cSTR-cascade units in line. Reaction conditions: 2 mg *Pp*BFD varL461A LWC; The substrate solution of 250 mM benzaldehyde and 100 mM acetaldehyde pumped at 0.3 mL min^{-1}. A 3.5 M acetaldehyde stock solution was pumped at 0.01 mL min^{-1}. Both solutions met at a T-mixing unit prior to entering the reaction chamber. 30 °C, 300 rpm, n=3; scheme see S2

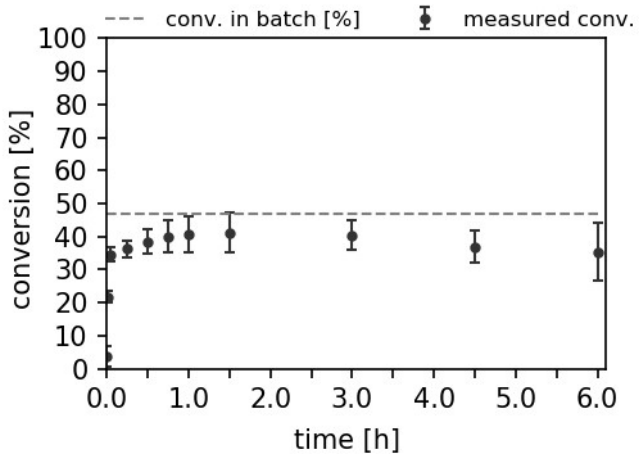

Figure S9. Continuous operation for the synthesis of *para*-methoxy-(*S*)-2-HPP in a cSTR-c consisting of three units. Reaction conditions: 2 mg *Pp*BFD varL461A LWC; The substrate solution of 250 mM *para*-methoxy-benzaldehyde and 100 mM acetaldehyde pumped at 0.1 mL min^{-1}. A 3.5 M acetaldehyde stock solution was pumped at 0.0035 mL min^{-1}. Both solutions met at a T-mixing unit prior to entering the reaction chamber. 30 °C, 300 rpm, n=3; scheme see S2

S7 Estimation of intracellular ThDP concentration and its demand for (*S*)-HPP conversion in the presented MARS

First, the endogenous concentration of ThDP in the reaction setup was estimated based on a literature concentration of intracellular metabolites in the cytoplasm and literature that relates dry cell weight of *E. coli* with wet cell weight of *E. coli* cells.

The reaction setup contained 20 mg whole cells in 1 mL. The concentration of ThDP per g wet weight was defined to be 143 nM [2]. 1 g wet weight was defined to equal 0.3 g dry weight [3].

Hence 1 g dry cells contain a concentration of 476 nM, which means that the application of 20 mg *Pp*BFD varL461A provided an intrinsic concentration of 9.5 nM ThDP.

Second, the concentration of *Pp*BFD varL461A was estimated assuming that the weight of a regular *E. coli* cell constitutes by 55% of protein [3]. It was assumed that the amount of heterologous protein in the setup is approximately 58%, which means that approximately 1.1 mg *Pp*BFD varL461A were present in the setup. Assuming a molecular weight of 56 kDa native *Pp*BFD varL461A, the concentration of *Pp*BFD varL461A can be calculated in a volume of 1 mL. According to equation S1.

Equation S1.

$$c = \frac{m}{V * MW}$$

C = concentration [mol L^{-1}]
m = weight [g]
V = volume [L]
MW = molecular weight [g mol^{-1}]

One tetramer of native *Pp*BFD varL461A has four active sides, which each bind one molecule of ThDP. Hence about 19 μM *Pp*BFD varL461A would be present in the setup, which is by a factor of 8000 more enzyme to ThDP.

S8 Catalyst cost-effectiveness

Outlined here is the estimation for the catalyst cost-efficiency, which is calculated as defined in equation S2.

Equation S2.

$$catalyst\ cost - efficiency\ [\%] = \frac{biocatalyst\ costs\ [€\ kg^{-1}]}{value\ of\ the\ generated\ product\ [€\ kg^{-1}]} * 100$$

According to Tufvesson *et al.* the value for a fine chemical product can be generalized in a potential calculation to 15 € kg^{-1} and the costs for the production of a whole cell catalyst to 35 € kg^{-1}. The acceptable catalyst cost-efficiency is defined as 10 %.[4]

Based on these assumptions and the known reaction conditions as defined in the material and methods section, the catalyst cost-efficiency can be estimated.

First, the costs for biocatalyst production are calculated. In the reaction setup three cSTR units are connected in series to a cSTR-c. Each unit contains 1 g whole cell catalyst, which is a total of 3 g whole cell catalyst. The production of this amount of catalyst is based on the defined assumptions 0.105 €.

Thus 1 g whole cell catalyst synthesizes 190 mmol L^{-1} (*S*)-2-HPP in 8 h, which is equal to 16 retention times.

Second, the amount of synthesized product by the cSTR-c is calculated. Here, 190 mmol L^{-1} are generated in 8 h until the catalyst dies off. This duration accounts to 16 retention times. The retention volume in one cSTR unit is 9 mL. With three cSTR-c units in series, the total retention volume is 27 mL. The molecular weight of (*S*)-2-HPP is 150.18 g mol^{-1}. Based on the equation S1 the whole cSTR-s generates 12.3 g (*S*)-2-HPP in one retention time. This accounts to 0.431 €.

Hence, the catalyst cost-efficiency is approximately 24 % and thus by a factor of 2.5 higher than would be acceptable based on the assumed prices.

For comparison, also the efficiency of a comparable *fed*-batch reaction to the above calculated cSTR-s efficiency is presented. In a *fed*-batch synthesis in MARS by Wachtmeister *et al.* respectable product concentrations of 340 mM (*S*)-2-HPP were synthesized in 6 h with 100 mg L^{-1} whole cell catalyst in a reaction volume of 1 mL. Following the same calculation route the catalyst costs account to 0.0035 €. The output of the reaction is 0.051 g (*S*)-2-HPP, which has an estimated value of 0.0007 €. In this case the catalyst cost efficiency is 4500 % and thereby exceeds the acceptable catalyst costs by 450-fold.

S9: Modeling of acetaldehyde provoked irreversible enzyme deactivation

The proposed mechanism of acetaldehyde enzyme deactivation is based on binding of acetaldehyde to one (arbitrary) subunit of the enzyme tetramer. The tetramer consists of a dimer of dimers with two active sides within each dimer. These active sides are located between the monomers. Due to conformational changes of this subunit, the catalytic active sides that are located between two neighboring subunits are impaired. Hence, by the deactivation of one subunit the active enzyme tetramer can lose up to half of its catalytic active sides (less if they are already deactivated trough another monomer). As four subunits can be subsequentially impaired in any order, there are 16 of enzyme isomers in different stages of deactivation that can be categorized into 4 groups as shown in table S4).

Table S4. List of different *Pp*BFD varL461A isoenzymes. The roman index refers to the number of stereoconformer active subunits. The Arabic number refers to the specific order of active and deactivated subunits. The total activity refers to the fraction with respect to the fully active complex..

Enzyme species	*Number of active subunit*				*Total Activity*
	IV	*III*	*II*	*I*	
E_{IV}	1	1	1	1	1.00
$E_{II,1}$	0	1	1	1	0.50
$E_{II,2}$	1	0	1	1	0.50
$E_{II,3}$	1	1	0	1	0.50
$E_{II,4}$	1	1	1	0	0.50
$E_{I,1}$	0	0	1	1	0.25
$E_{I,2}$	0	1	1	0	0.25
$E_{I,3}$	1	0	0	1	0.25
$E_{I,4}$	1	1	0	0	0.25
$E_{0,1}$	0	1	0	1	0.00
$E_{0,2}$	1	0	1	0	0.00
$E_{0,3}$	0	0	0	1	0.00
$E_{0,4}$	0	0	1	0	0.00
$E_{0,5}$	0	1	0	0	0.00
$E_{0,6}$	1	0	0	0	0.00
$E_{0,7}$	0	0	0	0	0.00

Fully active enzyme E_{IV} can be deactivated to half of its activity by impairment of the first, second, third, or fourth subunit by irreversible binding of acetaldehyde. Those states are denoted by $E_{II,1}$, $E_{II,2}$, $E_{II,3}$, and $E_{II,4}$, where the roman index denotes the number of remaining active catalytic centers. Those enzymes can be further impaired in their activity at any further subunit, to either have only a quarter of original activity or none, depending on which subunit is deactivated. Those enzymes are described by the species $E_{I,1} - E_{0,2}$. The enzymes who are still active by a quarter, species $E_{I,1} - E_{I,4}$, can be further deactivated to zero activity, species $E_{0,3} - E_{I,6}$. Binding of acetaldehyde to complexes that are already fully deactivated, species $E_{0,1} - E_{0,6}$, is not relevant for the purpose of this model. As the deactivation by acetaldehyde is irreversible, no enzyme can change from inactive to active states. Enzyme pools with half of their original activity are denoted by E_{II}, those pools with a quarter of their original activity are denoted by E_{I}, and those with no activity by E_0. Reaction rate equations according to this mechanism are presented in eq. (S1).

$$\frac{dc_{E_{IV}}}{dt} = -4\,k_D c_{AA} c_{E_{IV}} \qquad \text{(S1a)}$$

$$\frac{dc_{E_{II,1}}}{dt} = k_D c_{AA}\left(c_{E_{IV}} - 3\,c_{E_{II,1}}\right) \qquad \text{(S1b)}$$

$$\frac{dc_{E_{II,2}}}{dt} = k_D c_{AA}\left(c_{E_{IV}} - 3\,c_{E_{II,2}}\right) \quad \text{(S1c)}$$

$$\frac{dc_{E_{II,3}}}{dt} = k_D c_{AA}\left(c_{E_{IV}} - 3\,c_{E_{II,3}}\right) \quad \text{(S1d)}$$

$$\frac{dc_{E_{II,4}}}{dt} = k_D c_{AA}\left(c_{E_{IV}} - 3\,c_{E_{II,4}}\right) \quad \text{(S1e)}$$

$$\frac{dc_{E_{I,1}}}{dt} = k_D c_{AA}\left(c_{E_{II,1}} + c_{E_{II,2}} - 2c_{E_{I,1}}\right) \quad \text{(S1f)}$$

$$\frac{dc_{E_{I,2}}}{dt} = k_D c_{AA}\left(c_{E_{II,1}} + c_{E_{II,4}} - 2c_{E_{I,2}}\right) \quad \text{(S1g)}$$

$$\frac{dc_{E_{I,3}}}{dt} = k_D c_{AA}\left(c_{E_{II,2}} + c_{E_{II,3}} - 2c_{E_{I,3}}\right) \quad \text{(S1h)}$$

$$\frac{dc_{E_{I,4}}}{dt} = k_D c_{AA}\left(c_{E_{II,3}} + c_{E_{II,4}} - 2c_{E_{I,4}}\right) \quad \text{(S1i)}$$

$$\frac{dc_{E_{0,1}}}{dt} = k_D c_{AA}\left(c_{E_{II},1} + c_{E_{II},3} - 2c_{E_{0,1}}\right) \quad \text{(S1j)}$$

$$\frac{dc_{E_{0,2}}}{dt} = k_D c_{AA}\left(c_{E_{II},2} + c_{E_{II},4} - 2c_{E_{0,2}}\right) \quad \text{(S1k)}$$

$$\frac{dc_{E_{0,3}}}{dt} = k_D c_{AA}\left(c_{E_{I,1}} + c_{E_{I,3}} + c_{E_{0,1}} - c_{E_{0,3}}\right) \quad \text{(S1l)}$$

$$\frac{dc_{E_{0,4}}}{dt} = k_D c_{AA}\left(c_{E_{I,1}} + c_{E_{I,2}} + c_{E_{0,2}} - c_{E_{0,4}}\right) \quad \text{(S1m)}$$

$$\frac{dc_{E_{0,5}}}{dt} = k_D c_{AA}\left(c_{E_{I,2}} + c_{E_{I,4}} + c_{E_{0,1}} - c_{E_{0,5}}\right) \quad \text{(S1n)}$$

$$\frac{dc_{E_{0,6}}}{dt} = k_D c_{AA}\left(c_{E_{I,3}} + c_{E_{I,4}} + c_{E_{0,2}} - c_{E_{0,6}}\right) \quad \text{(S1o)}$$

$$\frac{dc_{E_{0,7}}}{dt} = k_D c_{AA}\left(c_{E_{0,3}} + c_{E_{0,4}} + c_{E_{0,5}} + c_{E_{0,6}}\right) \quad \text{(S1p)}$$

$$c_{E_{II}} = c_{E_{II,1}} + c_{E_{II,2}} + c_{E_{II,3}} + c_{E_{II,4}} \quad \text{(S1q)}$$

$$c_{E_I} = c_{E_{I,1}} + c_{E_{I,2}} + c_{E_{I,3}} + c_{E_{I,4}} \quad \text{(S1r)}$$

$$c_{E_0} = c_{E_{0,1}} + c_{E_{0,2}} + c_{E_{0,3}} + c_{E_{0,4}} \quad \text{(S1s)}$$

$$c_{BFD} = c_{E_{IV}} + \frac{c_{E_{II}}}{2} + \frac{c_{E_I}}{4} \quad \text{(S1t)}$$

$$v_D = k_D \left(4c_{E_{IV}} + 3\, c_{E_{II}} + 2c_{E_I} + 2c_{E_{0,1}} + 2c_{E_{0,2}} + c_{E_{0,3}} + c_{E_{0,4}} + c_{E_{0,5}} + c_{E_{0,6}} \right) \quad \text{(S1u)}$$

Here, v_D denotes the consumption velocity of acetaldehyde due to deactivation of the enzymes. This consumption could be neglected but is considered for completeness of the mass balance of acetaldehyde in eq. (2b).

Alternatively, a simple generic approach for modeling the enzyme deactivation is tested for comparison:

$$\frac{dc_{BFD}}{dt} = -\, k_D c_{AA} c_{BFD} \quad \text{(S1v)}$$

$$v_D = k_D\, c_{BFD} \quad \text{(S1w)}$$

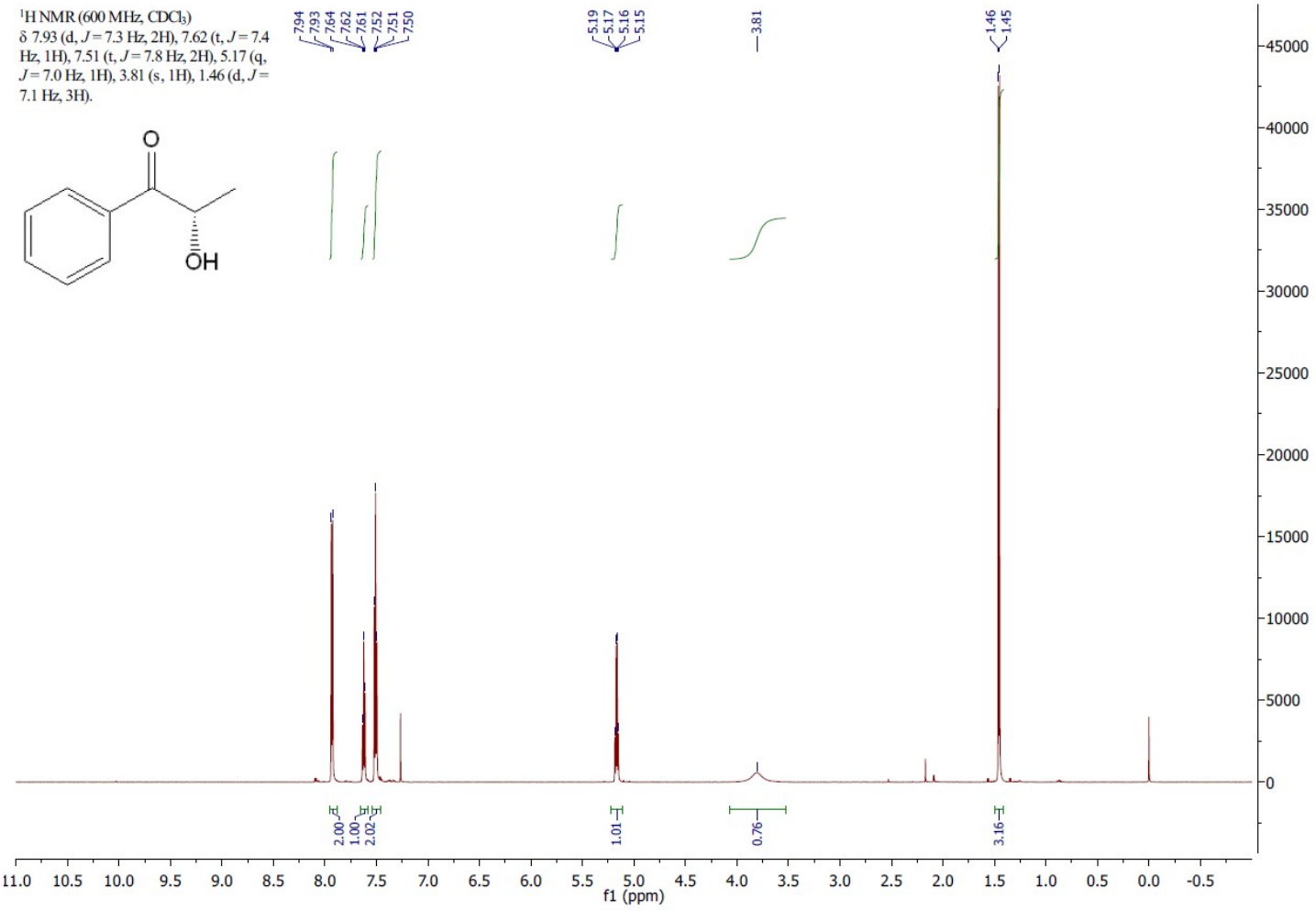

Figure S10. ^{1}H NMR of (*S*)-2-hydroxy-phenyl-1-propanone.

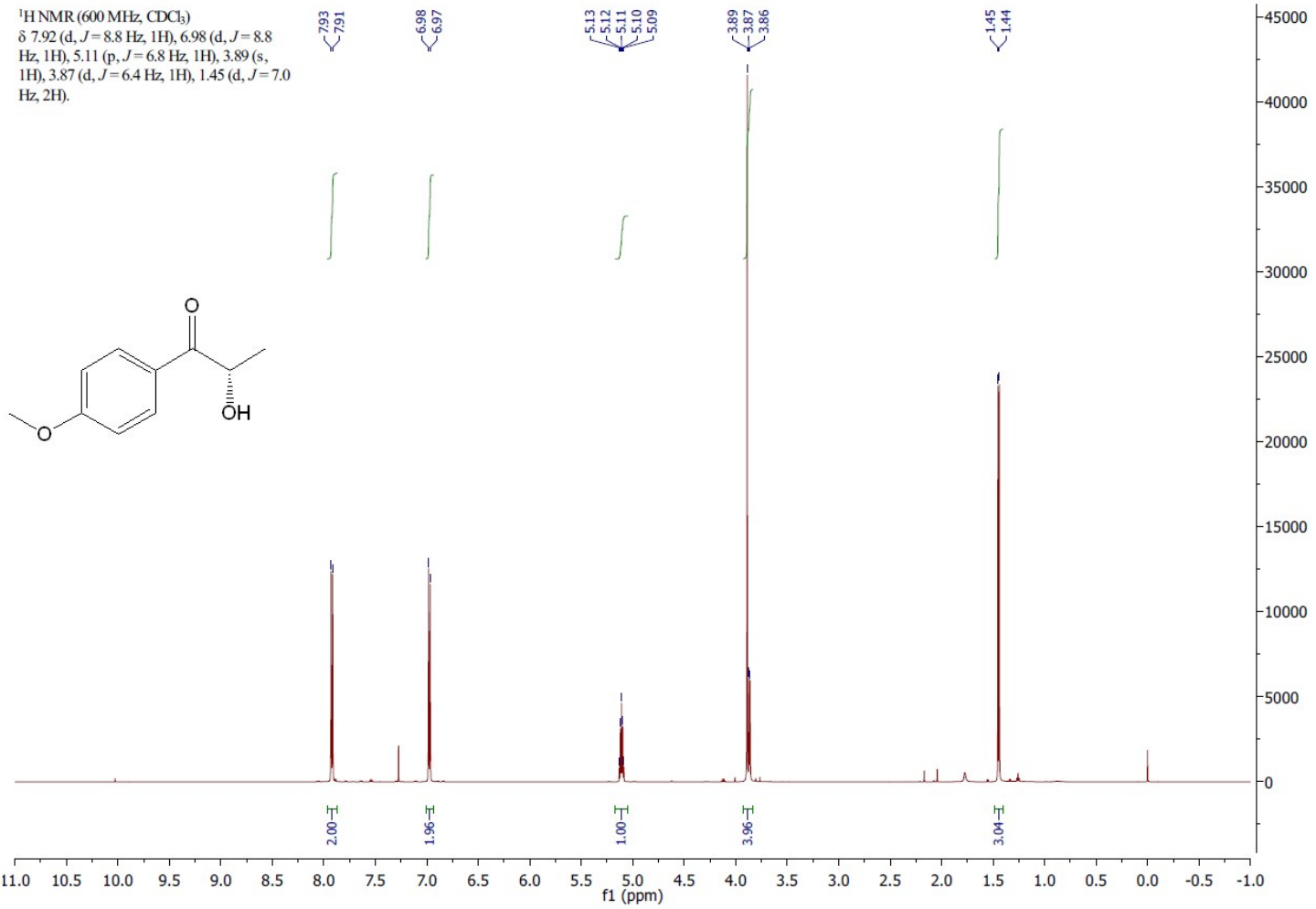

Figure S11. ^{1}H NMR of (*S*)-2-hydroxy-1-(*para*-methoxy-phenyl)propanone.

References

1 B. U. Kragl, D. Gygax, O. Ghisalba and C. Wandrey, 1991, **101**, 827–828.

2 A. F. Makarchikov, B. Lakaye, I. E. Gulyai, J. Czerniecki, B. Coumans, P. Wins, T. Grisar and L. Bettendorff, *Cell. Mol. Life Sci.*, 2003, **60**, 1477–1488.

3 R. Milo, Overall macromolecular composition of *E. coli* cell, http://bionumbers.hms.harvard.edu/bionumber.aspx?id=111490&ver=7&trm=E. coli weight&org=.

4 P. Tufvesson, J. Lima-Ramos, M. Nordblad and J. M. Woodley, *Org. Process Res. Dev.*, 2011, **15**, 266–274.

5 J. Wachtmeister, A. Jakoblinnert and D. Rother, *Org. Process Res. Dev.*, 2016, **20**, 1744–1753.