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Supporting Information

3D-Printed Phenacrylate Decarboxylase Flow Reactors for the Chemoenzymatic Synthesis of 4-Hydroxystilbene

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Supporting Information

Contents

Plasmid construction. The *in vitro* recombination method described by Daniel G. Gibson *et al.*[1] was utilized to clone synthetic genes (Geneart) of proteins with a C-terminal His_6-tag and 28-30 bp homologous overlaps into a pET-22b-based vector. After the assembly, DpnI digests were carried out and the reaction mixtures were transformed into chemically competent E.cloni® 5-alpha cells. The GeneJET Plasmid Miniprep Kit (Thermo Scientific) was used for plasmid isolation and purification according to the manufacturer's instructions. The correct assembly and sequence of the gene within the expression plasmids was verified by commercial sequencing (Eurofins Genomics, Germany).

Expression of proteins. For heterologous protein expression, chemically competent *E. coli* BL21(DE3) were transformed with the corresponding expression vector. *E. coli* cells containing the expression vector were selected overnight at 37 °C on LB/agar plates containing 100 µg · mL^{-1} ampicillin. For expression, a single colony was transferred to a suitable volume of LB medium containing 100 µg · mL^{-1} ampicillin and the culture was incubated overnight at 37 °C and 180 rpm in a shaking incubator. The overnight culture was then used to inoculate a 20 times larger volume of 25 °C warm ampicillin containing LB medium. This culture was then incubated for approximately 1.5 h up to an OD_{600} of 0.5 - 1.0 at 37 °C and 180 rpm. After reaching the appropriate cell density, the culture was cooled down to 25 °C for at least 15 min and subsequently induced with IPTG to a final concentration of 0.5 mM. The induced culture was then incubated overnight at 25 °C and then harvested by centrifugation (10,000xg, 10 min, 4 °C). The cells were then resuspended in cold NPI-10 buffer (50 mM NaH_2PO_4, 500 mM NaCl, 10 mM Imidazole, pH 8) and frozen at -80 °C. The volume used for the resuspension was at least one hundredth of the expression volume. For the expression of *Lb*PAD, the culture was cooled down to 20 °C prior to the induction and the expression was carried out overnight at 20 °C.

Protein purification. For the purification, the cell pellets were thawed at 25 °C in a water bath and then incubated with DNaseI and lysozyme for 30 min at 25 °C. After disruption by ultrasonication, the cell lysate was obtained after centrifugation (45,000xg, 1 h, 4 °C), filtered through a 0.45 µm Durapore PVDF membrane (Steriflip, Millipore) and loaded on a 1 mL His60 Ni Superflow Cartridge (Clontech) mounted on an Äkta Pure liquid chromatography system (GE Healthcare, Germany). The purification was carried out with NPI-10 as running buffer and NPI-500 (50 mM NaH_2PO_4, 500 mM NaCl, 500 mM Imidazole, pH 8) as elution buffer. After applying the lysate onto the column, the column was washed with 20 mL 2 % NPI-500 and the protein was eluted with 20 mL using a linear gradient (2 % to 100 % NPI-500). The column outflow was collected in 900 µL fraction and protein containing fractions (detected at 280 nm) were pooled. Subsequently, the buffer was exchanged to PBS buffer using Vivaspin 20, 5000 MWCO (Sartorius).

For small protein amounts (up to 1 mg), the protein was purified using the His-Spin Protein Miniprep (Zymo Research, Germany) according to the manufacturer's instructions subsequent to the ultrasonication step. Subsequently, the buffer was exchanged to 25 mM KP_i buffer (pH 6) using Vivaspin 500, 5000 MWCO (Sartorius).

For characterisation the recombinant and purified proteins were typically analysed by SDS-PAGE using 15 % gels. The bands were stained using Coomassie staining and compared to PageRuler Prestained Protein Ladder (Thermo Scientific). Protein concentrations were determined using UV-Vis spectroscopy and the theoretical molar extinction coefficients at 280 nm (calculated by ProtParam tool, ExPASy).

***p*-Coumaric acid assay.** 50 µL of the enzyme solution (*Es*PAD, *Lp*PAD, *Bm*PAD: 0.2 µM; *Lb*PAD: 0.5 µM) in a 25 mM KP_i reaction buffer (pH 6) was transferred in a UV transparent 96 well microtiter plate and 150 µL of a solution with a concentration of 0.125 mM *p*-coumaric acid in reaction buffer was added. The consumption of *p*-coumaric acid was recorded at 294 nm using a Synergy H1 or MX microplate reader (BioTek) over a period of 10 min at 25 °C. The activity was determined by calculating the slope in the linear range of the decrease of the absorption intensity [OD/min].

Determination of T_{50} values. In order to determine the T_{50} values, 60 µL of the purified enzymes with a concentration of 1 µM in a 25 mM KP_i reaction buffer (pH 6) were incubated for 10 min in temperature gradients ranging from 40 °C to 80 °C for *Es*PAD and 30 °C to 70 °C for *Lp*PAD, *Bm*PAD and *Lb*PAD. Subsequent to this incubation, the samples were cooled on ice for 5 min. The remaining enzyme activity was determined at room temperature using the *p*-coumaric acid assay. The activities were then assigned to the corresponding preincubation temperatures and fitted by sigmoid Boltzmann function using the OriginPro 2019 software (version 9.6.0.172). The inflection point of the Boltzmann function represents the T_{50} value.

RF-HPLC. 4-Vinylphenol and *p*-coumaric acid were detected and quantified by reverse phase HPLC using an Eclipse XDB-C18 column (5 µm, Agilent) with precolumn (Poroshell 120 EC-C18, 2.7 mm, Agilent). The separation was realised at 35 °C with a mobile phase of 35 % acetonitrile and 65 % ddH_2O with 0.1 % trifluoroacetic acid. The flow rate was 1 mL · min^{-1} and the analytes were detected at 210 nm. t_R(*p*-coumaric acid) = 2.2 min, t_R(4-vinylphenol) = 6.2 min (Fig. S8).

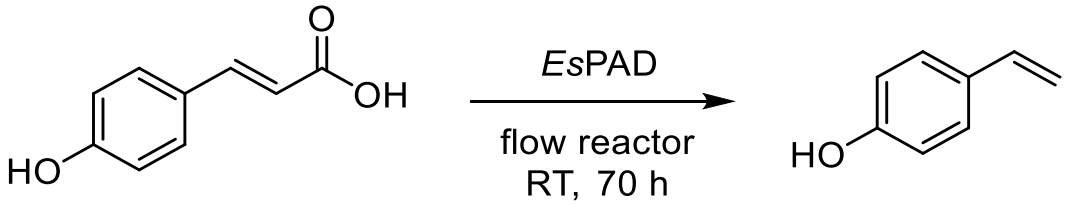
Reactor module fabrication. The reactor module fabrication was realised as described previously[2] with minor changes. Instead of a peltier cooler, the refrigerating circulating thermostat F12-MP (Julabo, Germany) was used to cool the printing surface down to 0 °C and the bioink was prepared with the 25 mM KP_i reaction buffer (Fig. 1a).

Fluidic experiment. All fluidic experiments in this study were carried out in the cylindrical shells of manually shortened 10 mL syringes with one reactor module. The reactor module comprise of an outer circular part containing five parallel, inner strands. This results in six channels with a height of 1 mm, a length of 4.4 mm and widths of 3-12 mm as defined by the inner strands and the outer circle. Spacers were placed in front and behind the reaction modules to widen the inlet and outlet and thus to allow the substrate solution to perfuse all reaction channels equally reducing the risk of dead zones and channelling. The assembly is shown in Figure 3a. For perfusion, a syringe pump Nexus 3000 (Chemyx, USA) with flow rates from 12.5 µL · min^{-1} was employed.

Before assembling the reactor, the reactor modules were brought to room temperature and rehydrated by incubating them in substrate-free reaction buffer for at least 10 min. After assembling the reactor, the reaction chamber was initially quickly filled with approx. 500 µL substrate-containing reaction buffer using the maximal flow rate of the pump to remove all air in the system. After this filling the chosen flow rate was selected and the collection of the outflow was started. 195 µL of the outflow was removed from a collection vessel and mixed with 5 µL of formic acid to stop the enzyme reaction. The decrease in substrate concentration and the increase in product concentration were then quantified using reverse phase HPLC.

Determination of activity after the printing process. To analyse the loss of activity of the enzyme after remelting of the agarose in the printer, samples of freshly prepared bioinks and bioinks after the initial temperature cycle (re-melting at 60 °C and cooling to 35 °C) were generated. Subsequently, the enzyme solution was separated from the agarose matrix using a centrifugal filter unit (Ultrafree-DA, Millipore, Ireland) according to the manufacturer's instructions and the remaining enzyme activities of the samples were determined at room temperature using the *p*-coumaric acid assay.

4-Vinylphenol production. Four flow-reactors each containing one reactor module (*Es*PAD, 100 µM) were perfused with *p*-coumaric acid (1 mM) in KP_i buffer (25 mM, pH 6). The aqueous outflows were combined and extracted with AcOEt three times. The combined organic layers were dried over Na_2SO_4, filtrated, concentrated under vacuum and subjected to column chromatography (AcOEt/n-hexane). The solid product was obtained in a 54.4 % yield.

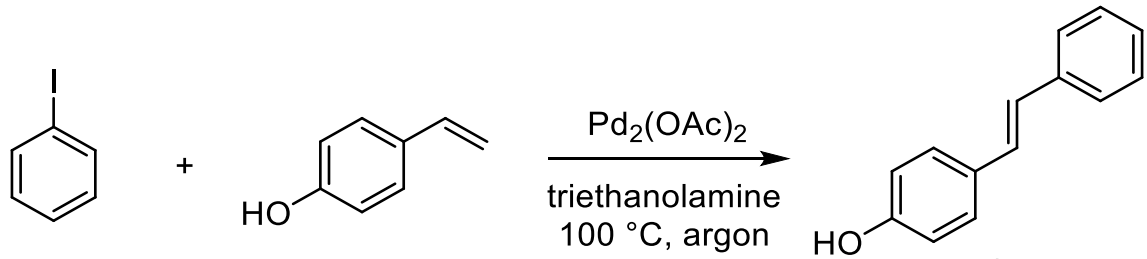


1
$C_9H_8O_3$
M = 164.16 g·mol^{-1}

2
C_8H_8O
M = 120.06 g·mol^{-1}

4-Vinylphenol: ^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm = 5.12 (d, 1H, *J* = 10.8 Hz), 5.19 (s, 1H), 5.60 (dd, 1H, *J* = 17.6, 0.7 Hz), 6.65 (dd, 1H, *J* = 17.6, 10.8 Hz), 6.78-6.82 (m, 2H), 7.28-7.33 (m, 2H). ^{3}C NMR (126 MHz, $CDCl_3$) δ/ppm = 111.6, 115.1, 127.6, 130.6, 136.1, 155.3.

Heck reaction. To a mixture of iodobenzene (1.1 equiv.), triethanolamine (1 eqiv.) and palladium(II)acetate (1 mol%) was added biocatalytically produced 4-vinylphenol (1 equiv.) under argon. After stirring at 100 °C overnight, the reaction was quenched with diluted aqueous hydrochloric acid (1 N) and extracted with AcOEt three times. The combined organic layers were washed with brine, dried over Na_2SO_4, filtrated, concentrated under vacuum and subjected to column chromatography (AcOEt/n-hexane). Product was obtained as white crystals in a 27 % yield.



4
C_6H_5I
M = 203.94 g·mol^{-1}

2
C_8H_8O
M = 120.06 g·mol^{-1}

3
$C_{14}H_{12}O$
M = 196.09 g·mol^{-1}

4-Hydroxystilbene: ^{1}H NMR (500 MHz, DMSO) δ/ppm = 9.59 (s, 1H, Ar-OH), 7.53 (d, *J* = 7.4, 2H, ArH), 7.42 (d, *J* =8.6, 2H), 7.34 (t, *J*=7.7, 2H), 7.22 (t, *J*=7.3, 1H), 7.14 (d, *J*=16.4, 1H), 7.01 (d, *J*=16.4, 1H), 6.77 (d, *J*=8.6, 2H). ^{13}C NMR (126 MHz, DMSO) δ/ppm = 157.34, 137.55, 128.64, 128.45, 128.06, 127.88, 126.97, 126.03, 125.08, 115.55, 39.52.

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Landete2010          1   ----------MTKEFKTLDDFLGTHFIYTYDNGWEYEWYAKNDHTVDYRIHGGMVAGRWV    50
PAD_LB               1   MFAIKKERFMMTKEFKTLDDFLGTHFIYTYDNGWEYEWYAKNDHTVDYRIHGGMVAGRWV    60
                             **************************************************

Landete2010         51   TDQAANIVMLVPGIYKVAWTEPTGTDVALDFVPNEKKLNGTIFFPKWVEEYPEITVTYQN   110
PAD_LB              61   TDQAANIVMLVPGIYKVAWTEPTGTDVALDFVPNEKKLNGTIFFPKWVEEHPEITVTYQN   120
                         **************************************************:*********

Landete2010        111   EHIDLMEESREKYDTYPKLVVPEFANITYMGDAGQDNEDVISEAPYAGMPDDIRAGKYFD   170
PAD_LB             121   EHIDLMEESREKYDTYPKLVVPEFANITYMGDAGQDNEDVISEAPYAGMPDDIRAGKYFD   180
                         ************************************************************

Landete2010        171   SNYKRIKK                                                       178
PAD_LB             181   SNYKRIKK                                                       188
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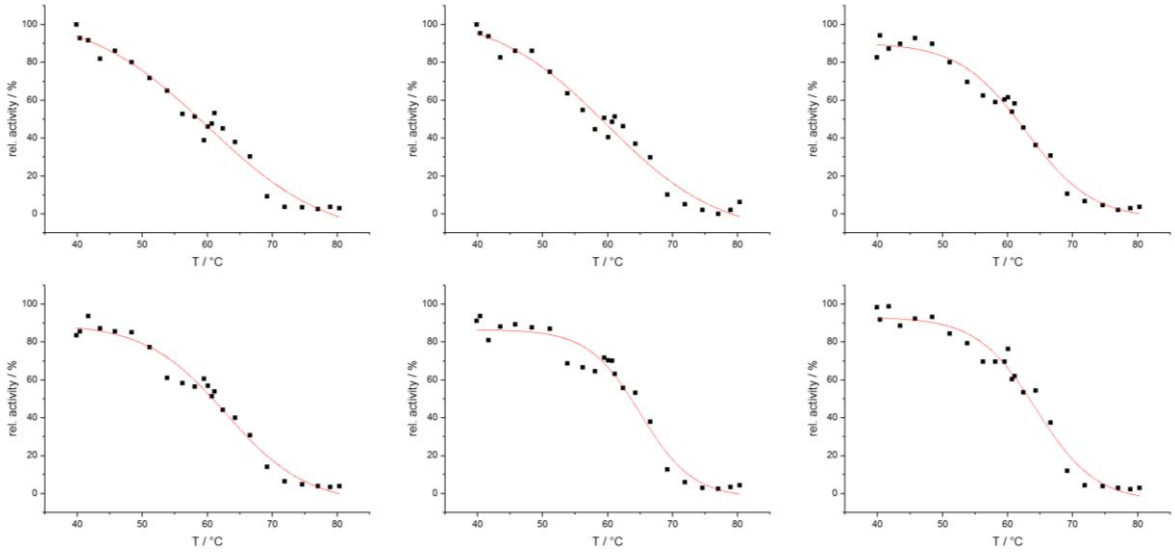
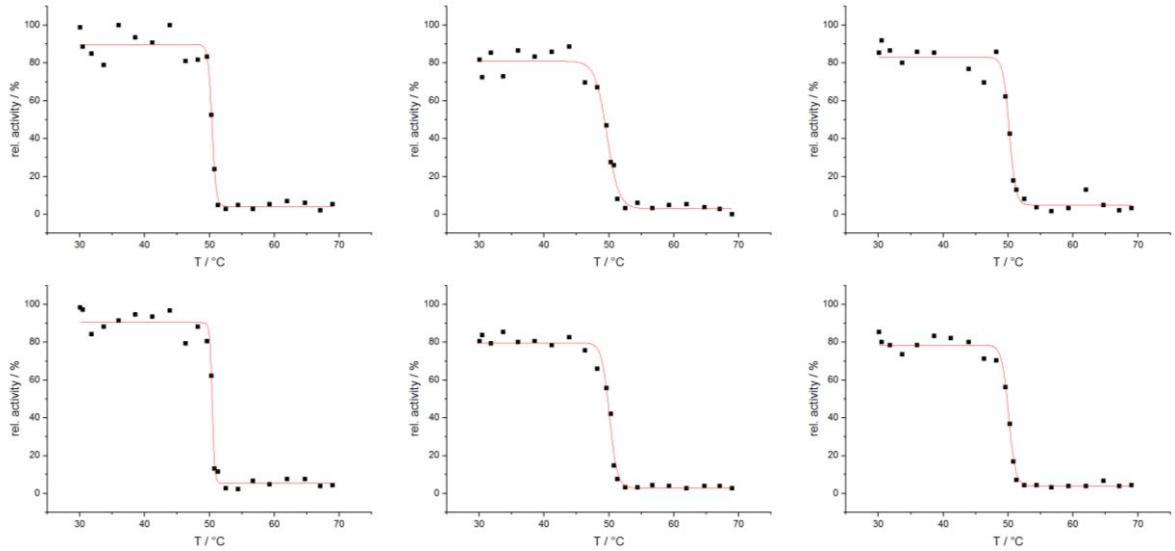
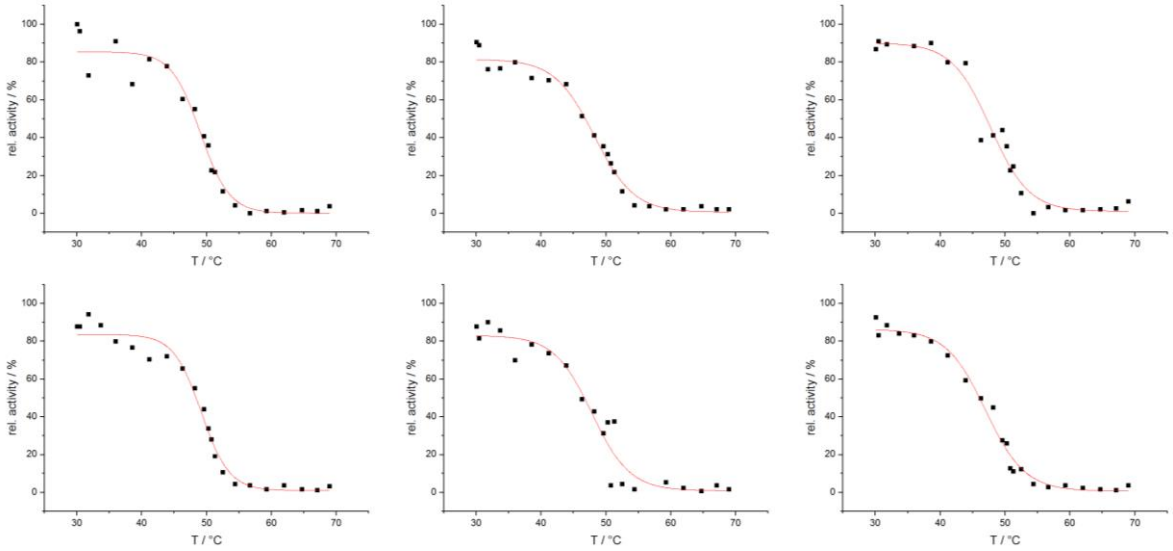
Supplementary Figure 1: Sequential alignment of two PADs from different *L. brevis* stains. Upper sequence: *L. brevis* RM84 published by Landete *et al.*[3] lower sequence: *Lb*PAD from *L. brevis* ATCC 14869 investigated in this study.



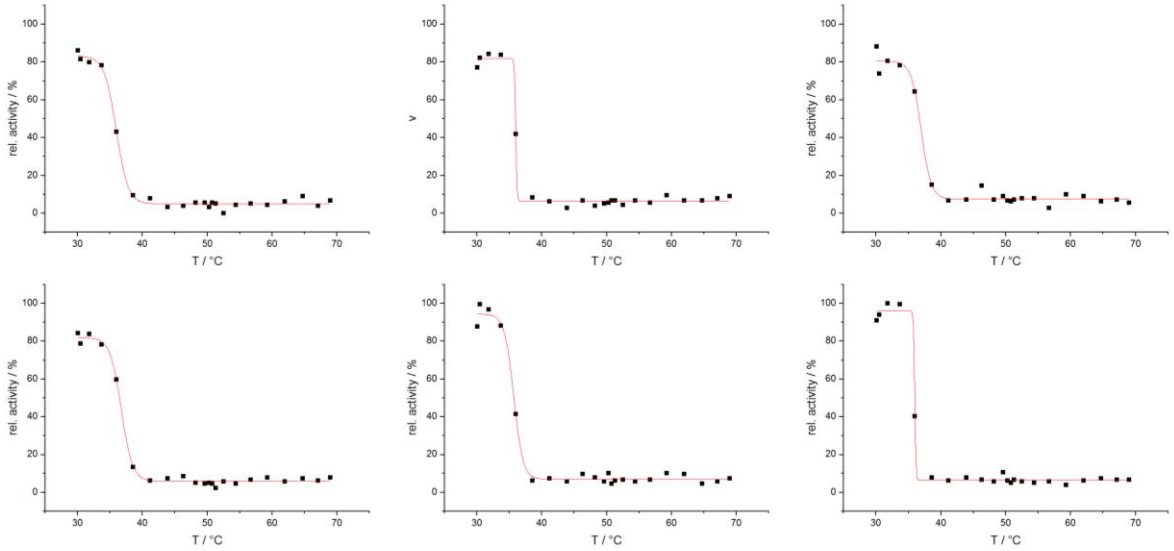
Supplementary Figure 2: Thermal inactivation curves of six independent samples of 1 µM *Es*PAD ranging from 40 °C to 80 °C. Red curves represent the sigmoid Boltzmann fit used for the determination of the T_{50} value.



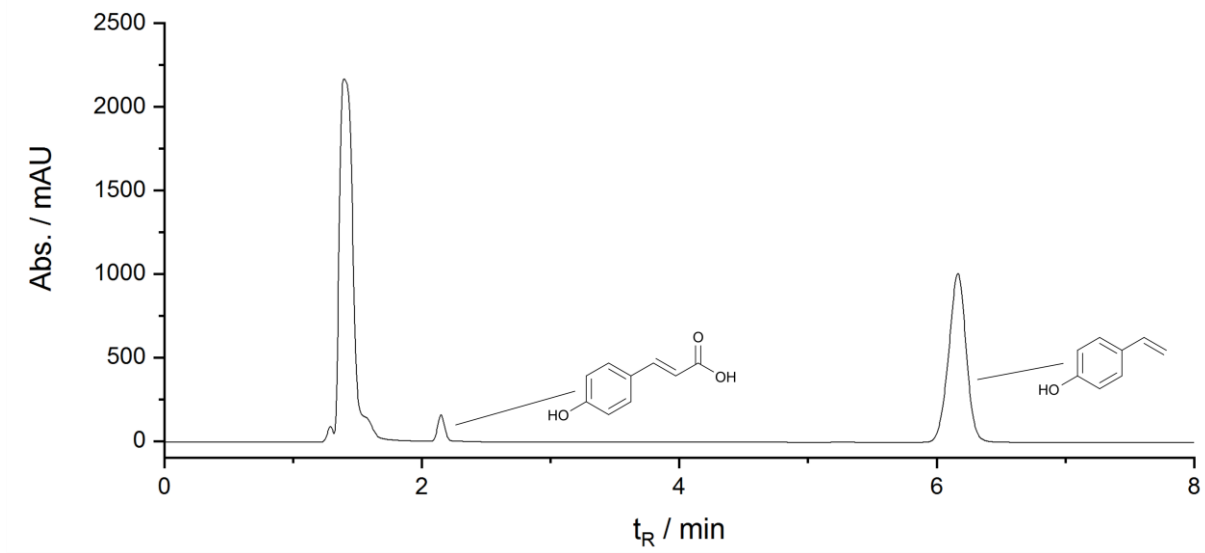
Supplementary Figure 3: Thermal inactivation curves of six independent samples of 1 µM *Lp*PAD ranging from 30 °C to 70 °C. Red curves represent the sigmoid Boltzmann fit used for the determination of the T_{50} value.



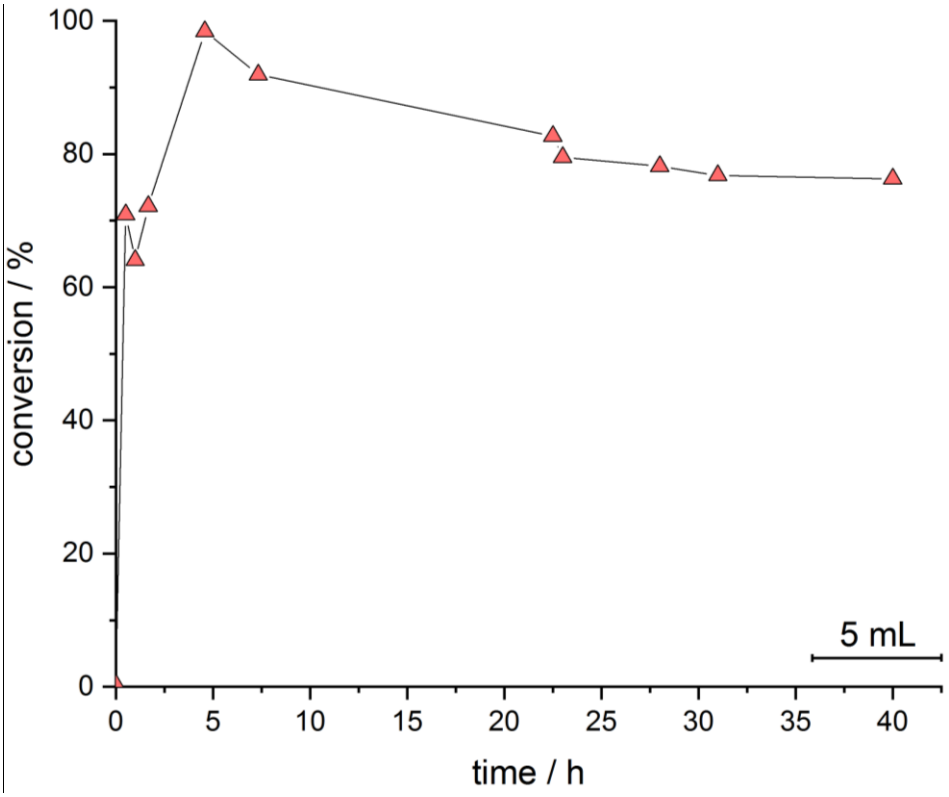
Supplementary Figure 4: Thermal inactivation curves of six independent samples of 1 µM *Bm*PAD ranging from 30 °C to 70 °C. Red curves represent the sigmoid Boltzmann fit used for the determination of the T_{50} value.



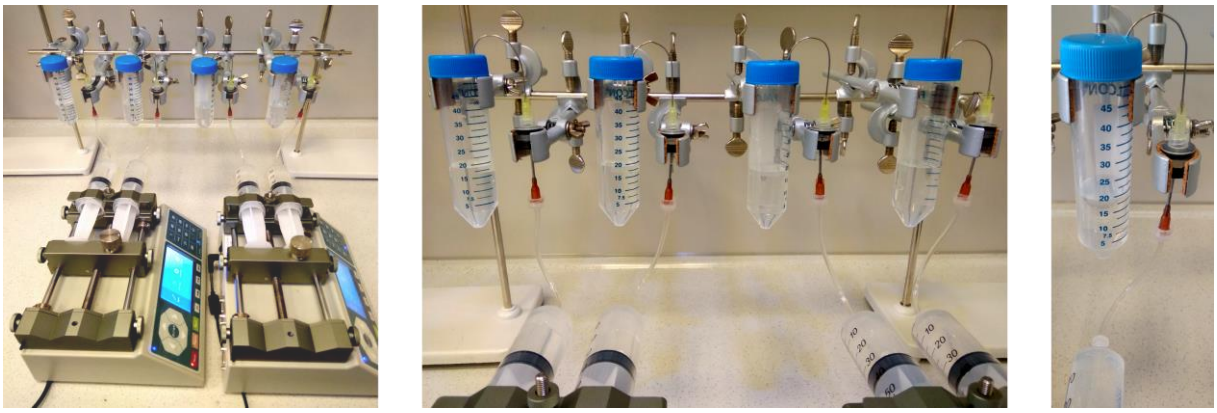
Supplementary Figure 5: Thermal inactivation curves of six independent samples of 1 µM *Lb*PAD ranging from 30 °C to 70 °C. Red curves represent the sigmoid Boltzmann fit used for the determination of the T_{50} value.



Supplementary Figure 6: Exemplary HPLC chromatogram of the conversion of *p*-coumaric acid to 4-vinylphenol employing one reactor module containing 100 µM *Es*PAD. The reaction was stopped *via* addition of formic acid to a final amount of 2.5 % (v/v). The signal was recorded via UV detector at 210 nm. t(Injection Peak) = 1.25 to 1.65 min



Supplementary Figure 7: Conversion of 4-vinylphenol quantified by HPLC in the outflow of the fluidic experiment with one reactor module containing 100 µM *Es*PAD at a flow rate of 12.5 µL · min^{-1}. Full conversion of the substrate (1 mM) is defined as 100 %. The system reaches 4-vinylphenol conversion of up to 98 %. The conversion was determined via HPLC-analysis of the product.



Supplementary Figure 8: Flow reactor set up of four parallel reactors for the production of 4-vinylphenol.

Supplementary Table S1: Amino acid sequences of enzymes used in this study (with GS-linker und His_6-tag in blue)

***Es*PAD-His** (based on UniProt ID: C6F3U5)
MNTFDKHDLSGFVGKHLVYTYDNGWEYEIYVKNENTLDYRIHSGLVGNRWVKDQQAYIVRVGESIY KISWTEPTGTDVSLIVNLGDSLFHGTIFFPRWVMNNPEKTVCFQNDHIPLMNSYRDAGPAYPTEVID EFATITFVRDCGANNESVIACAASELPKNFPDNLKGGGGSGHHHHHH
***Lp*PAD-His** (based on UniProt ID: F9ULL2)
MTKTFKTLDDFLGTHFIYTYDNGWEYEWYAKNDHTVDYRIHGGMVAGRWVTDQKADIVMLTEGIY KISWTEPTGTDVALDFMPNEKKLHGTIFFPKWVEEHPEITVTYQNEHIDLMEQSREKYATYPKLVVP EFANITYMGDAGQNNEDVISEAPYKEMPNDIRNGKYFDQNYHRLNKGGGGSGHHHHHH
***Bm*PAD-His** (based on UniProt ID: A0A109G6Z8)
MEKFIGNHMIYTYENGWEYEIYIKNENTIDYRIHSGMVAGRWVRDQKVDIVKLTEGVYKVSWTEPT GTDVSLNFMPDEKRMHGIIFFPKWVHEHPEITVCYQNDHLNLMHESREKYETYPKYVVPEFADITFI KNVGANNEEVIAQAPYEGMTNDIRAGKLIGGGGSGHHHHHH
***Lb*PAD-His** (based on UniProt ID: U2PH61)
MFAIKKERFMMTKEFKTLDDFLGTHFIYTYDNGWEYEWYAKNDHTVDYRIHGGMVAGRWVTDQAA NIVMLVPGIYKVAWTEPTGTDVALDFVPNEKKLNGTIFFPKWVEEHPEITVTYQNEHIDLMEESREK YDTYPKLVVPEFANITYMGDAGQDNEDVISEAPYAGMPDDIRAGKYFDSNYKRIKKGGGGSGHHH HHH

Supplementary Table S2: Enzymatic activity of all four PAD enzymes with *p*-coumaric acid and caffeic acid as substrates at 25 °C and T_{50} values. All analyses were carried out in technical duplicates and biological triplicates.

Enzyme name	Turnover number with *p*-coumaric acid	Turnover number with caffeic acid	T_{50}
***Es*PAD**	8.9 ± 1.4 s^{-1}	4.5 ± 1.1 s^{-1}	62.5 ± 2.2°C
***Lp*PAD**	5.3 ± 0.7 s^{-1}	6.4 ± 0.7 s^{-1}	50.1 ± 0.3°C
***Bm*PAD**	6.4 ± 0.7 s^{-1}	2.7 ± 0.4 s^{-1}	48.2 ± 0.8°C
***Lb*PAD**	3.2 ± 1.4 s^{-1}	4.2 ± 0.1 s^{-1}	36.2 ± 0.5°C

References

[1] D. G. Gibson, L. Young, R. Y. Chuang, J. C. Venter, C. A. Hutchison, 3rd, H. O. Smith, *Nat. Methods* **2009**, *6*, 343-345.

[2] M. Maier, C. P. Radtke, J. Hubbuch, C. M. Niemeyer, K. S. Rabe, *Angew. Chem., Int. Ed.* **2018**, *57*, 5539–5543.

[3] J. M. Landete, H. Rodríguez, J. A. Curiel, B. d. Las Rivas, J. M. Mancheño, R. Muñoz, *J. Ind. Microbiol. Biotechnol.* **2010**, *37*, 617–624.