Electronic Supplementary Material (ESI) for Green Chemistry.

SUPLEMENTARY INFORMATION

Cell-free biocatalytic syntheses of L-pipecolic acid: a dual strategy approach and process intensification in flow

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Content:

1. Supplementary materials and methods:

- *Cloning and characterisation of He-P5C:*

A gene coding for a pyrroline-5-carboxylate dehydrogenase was identified in the genome of *Halomonas* elongata DSM 2581. The gene, HELO_4115, was amplified from the genome by PCR following the manufacturers instructions using the pair of primers 5' AAAAAGGATCCCCCCATGGCAAGCCAAGTCACC 3' and 5' AAAAAGAATTCTCATCAGCGCTTGCCGAGTTCG 3', which were designed to incorporate a restriction site at each end of the gene. The gene was subsequentially cloned into a pRSET B vector digested with the same restriction enzymes. The new plasmid was called pRSET-B-HeP5C.

- *Kinetic characterisation of He-P5C:*

He-P5C kinetic behaviour was characterised concerning both its cofactor affinity and preference and L-thiazolidine-4-carboxylic acid (T4C). As for the cofactor, a fixed amount of pyrrolidine-6-carboxylate was used with various concentration of either NADH or NADPH in phosphate buffer pH 8. The consumption of the reduced cofactor was measured at 340 nm for 2 minutes measuring every 10 seconds. One unit of enzymatic activity was defined as the consumption of 1 nmol of cofactor per minute and mg of enzyme.

For the cyclic amino acid analogue T4C, a fixed amount of 1 mM of NAD^+ and various concentrations up to 20 mM of T4C. One unit of enzymatic activity was defined as the reduction of 1 nmol of cofactor per minute and mg of enzyme.

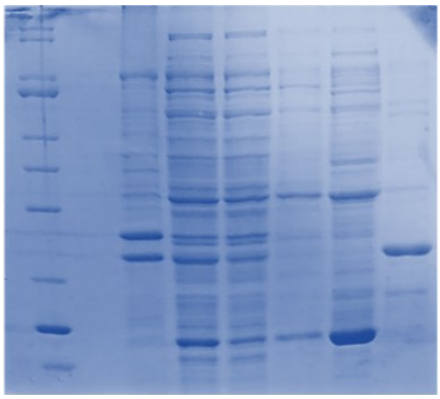


Figure S1: SDS-page analysis of He-P5C expression. 1: Cell extract after expression; 2: Soluble fraction; 3: Insoluble fraction; 4: Flow through in IMAC; 5: Washing step; 6. Purified protein fraction.

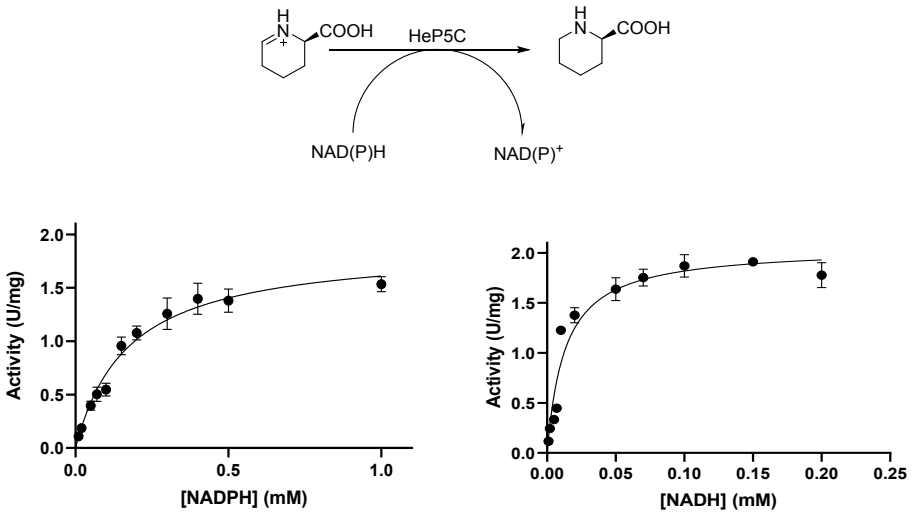


Figure S2: Kinetics of He-P5C for NAD^+ and NADH. Activities were measured with a fixed concentration of P6C and variable concentration of the cofactor in 50 mM phosphate buffer pH 8 at 25ºC.

3. Multi-enzymatic system with HeWT and He-P5C:

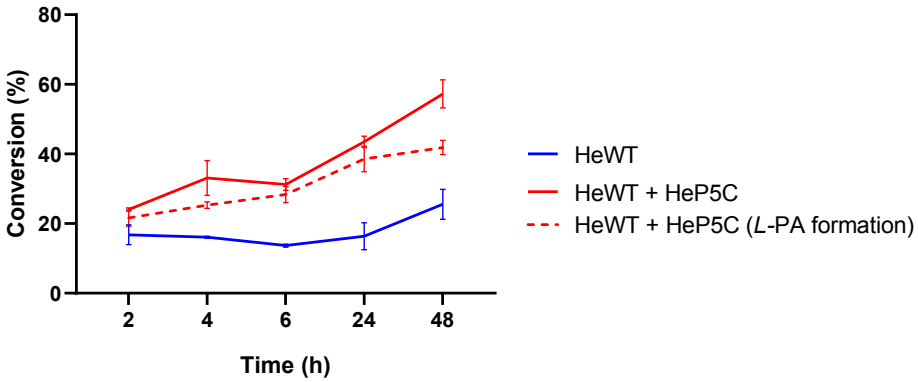


Figure S3: Conversion, calculated as the depletion of L-lysine, for the biotransformation using the transaminase alone (blue) and the whole cascade (red). The dotted red line represents the conversion by measuring the L-PA formation. Reaction conditions are 2 mg/mL HeWT, 1 mg/mL He-P5C, 50 mM of lysine, 2 equivalent pyruvates as amino acceptor, 10 mM NADH, 0.1 mM PLP in 100 mM phosphate buffer pH 10. Values correspond to the mean between two independent measures with the SD.

A. **B.**

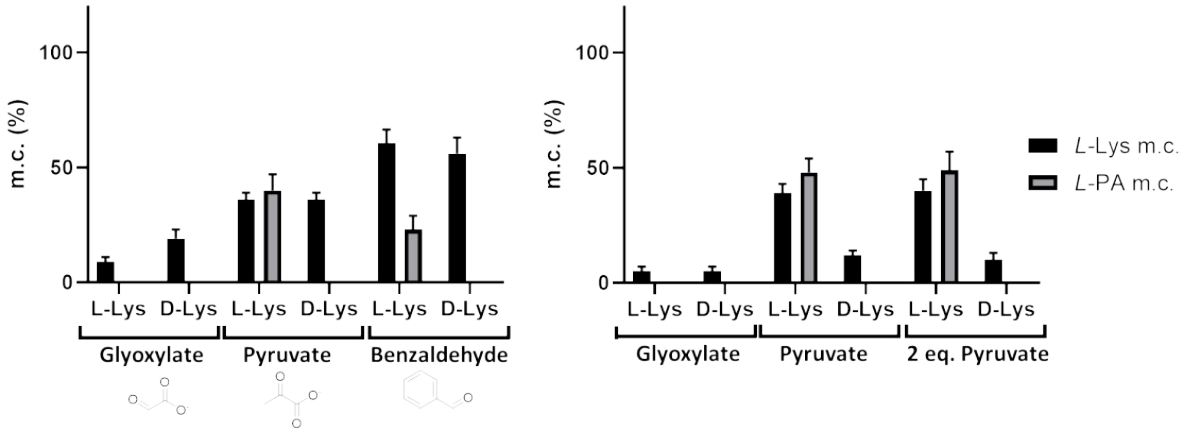


Figure S4: Conversion of L-lysine (black) and total conversion to L-PA (grey). A. Reaction conditions are 2 mg/mL HeWT, 1 mg/mL He-P5C, 10 mM of lysine (A) or 50 mM of lysine (B), 1 equivalent of amino acceptor, if not stated otherwise, 0.1 mM PLP in 100 mM phosphate buffer pH 8 with equimolar concentration of the NADH. Conversions at 48h are shown. Lysine conversion was measured by substrate depletion by HPLC. Values correspond to the mean between two to three independent measures with the SD.

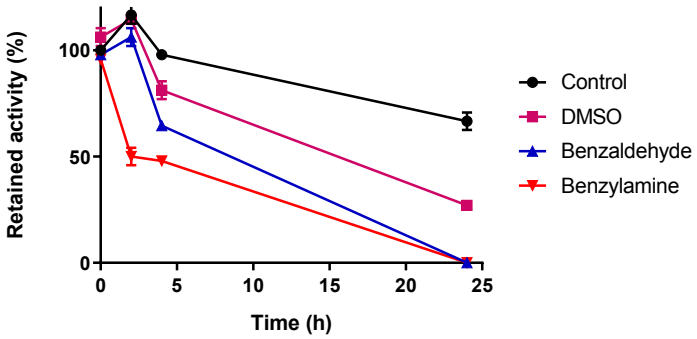


Figure S5: Stability of HeP5C in the reaction conditions with benzaldehyde as the amino acceptor. A solution of 0.25 mg/mL of He-P5C was incubated with 10% DMSO or 10 mM benzaldehyde or benzylamine in 50 mM phosphate buffer pH8 at 37 ºC for 24 h. The stability was measured by using the standard activity assay for He-P5C after incubation in the different conditions for the time specified. The control was HeP5C in 50 mM phosphate buffer pH8 with no additional cosolvent.

Table S1: Effect of the recycling system in L-PA formation. The reactions were performed with 50 mM of each substrate with 2 mg/mL HeWT, 2 mg/mL of He-P5C and 2 mg/mL Cb-FDH or Bm-GDH with 1 mM NAD^+ in 50 mM carbonate buffer pH 10 at 37ºC.

		L-lysine depletion (%)	L-PA (%)
Cb-FDH	**NAD^+**	59 ± 1	58 ± 2
Bm-GDH	**NAD^+**	27 ± 4	6 ± 1
	$NADP^+$	57 ± 4	32 ± 2
No recycling		26 ± 4	14 ± 2

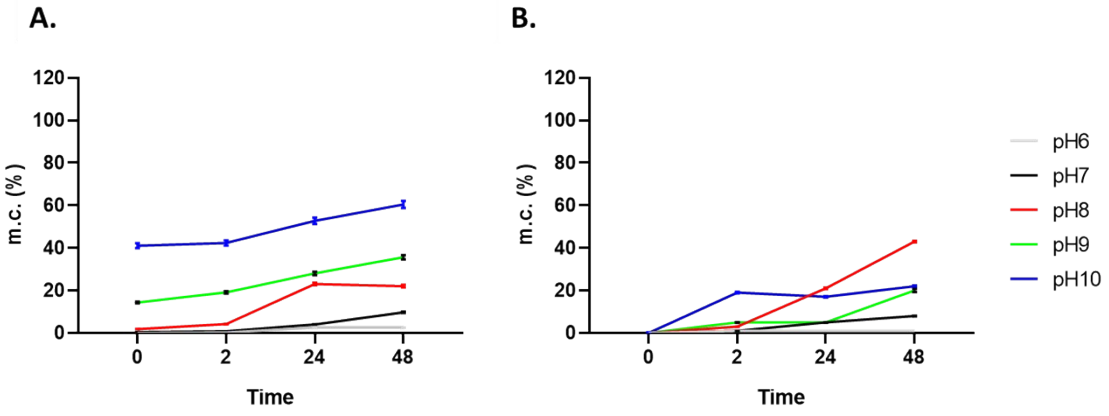


Figure S6: pH dependence of PA formation. Reaction conditions were 2 mg/mL HeWT, 1 mg/mL He-P5C, 1 mg/mL Bm-GDH, 50 mM lysine, 50 mM pyruvate in the adequate buffer with 1 mM $NADP^+$ in 100 mM phosphate buffer or carbonate buffer. Values correspond to the average of two independent measures with the SD. **A.** Lysine conversion into P6C catalysed by HeWT at different pH. **B.** PA formation at different pH.

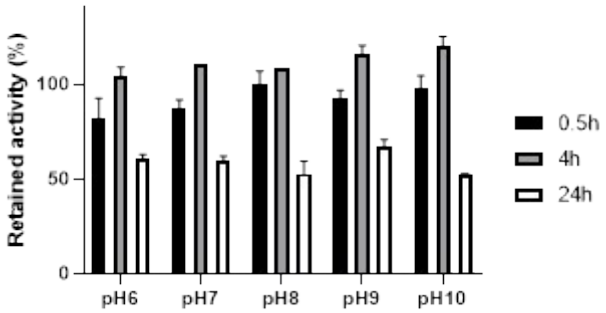


Figure S7: Stability of He-P5C at different pH values. The activity is shown as percentage of the initial activity of the protein using the standard activity assay.

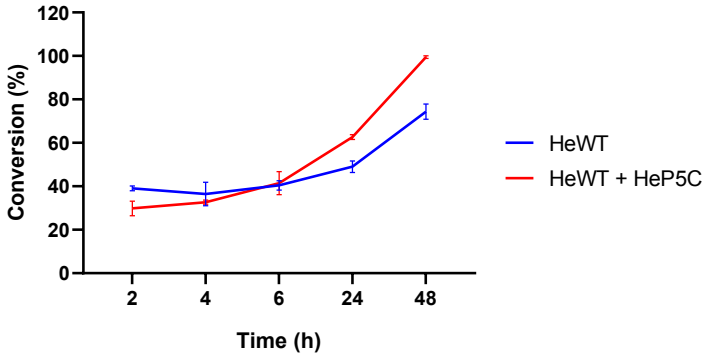


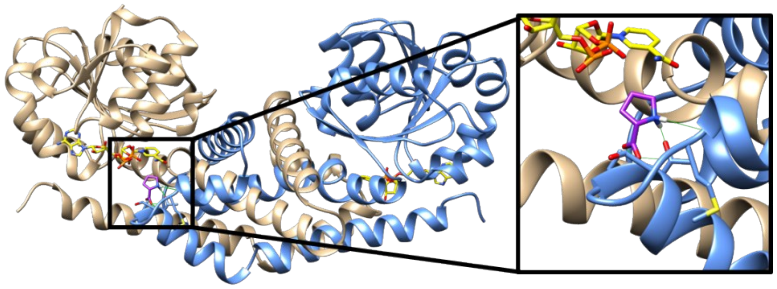
Figure S8: Conversion, calculated as the depletion of ornithine, for the biotransformation using the transaminase alone (blue) and the whole cascade (red). Reaction conditions are 2 mg/mL HeWT, 1 mg/mL He-P5C, 50 mM of L-ornithine, 2 equivalent pyruvates as amino acceptor, 10 mM NADH, 0.1 mM PLP in 100 mM phosphate buffer pH 8. Values correspond to the average of two independent measures.

4. Redox neutral cascade:

Table S2. Influence of physico-chemical parameters of supports on the recovered activities of immobilised enzymes.

Code	Matrix	Reactive group	Rec. activity Gs-Lys6DH (%)	Rec. activity He-P5C (%)
EC-EP/S	Methacrylate	Epoxy	63	<5
EP403/S		Epoxy	61	<5
EP113/S		Epoxy	53	<5
EC-HFA/S		Amino-epoxy	47	<5
HFA403/S		Amino-epoxy	36	<5
AG-Ep	Agarose 6BCL	Epoxy	91	10

A.



B.

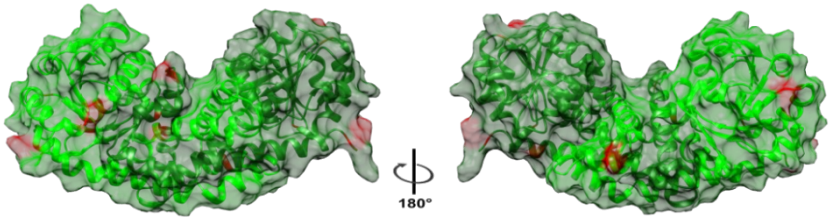
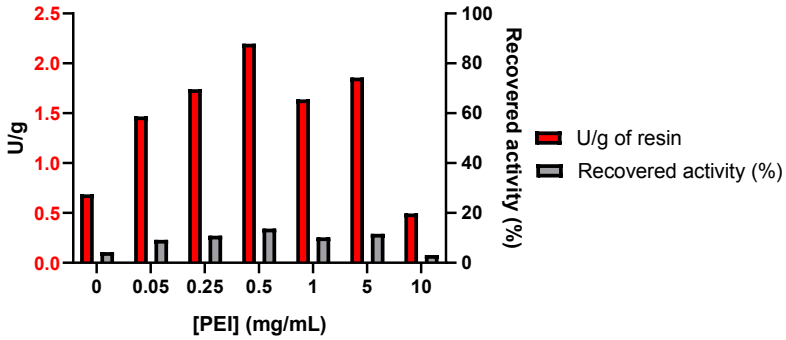


Figure S9. Modelling analysis of He-P5C structure. **A.** Image of the He-P5C model represented as ribbons. The two chains are shown in pink and blue. NAD^+ is shown in yellow and the proline substrate, docked into the active site, in purple. Hydrogen bonds between proline and the substrate binding residues are shown in green. **B.** Image of the homology modelled He-P5C. The two different chains are shown in two different shades of green with their corresponding surface representation. The lysine residues in red.

A.



B.

EP403/S	EP403/S + PEI	EP113	EP113 + PEI	Ag-Ep + PEI
2 ± 0.5	8.4 ± 1	1.7 ± 0.5	8.6 ± 1	21 ± 2

Figure S10: Optimization of He-P5C immobilisation. **A.** Effect of PEI addition during the immobilisation of He-P5C. EP403/S was used as a support. Values correspond to one measurement. **B.** Recovered activity (%) of immobilised He-P5C on different supports in presence of PEI. The average of three measures and its SD are shown.

5. Cofactor co-immobilisation:

For this purpose, two PEI with different molecular weight (60 kDa and 270 kDa) were tested for the reversible co-immobilisation of the cofactor, following the immobilisation strategy depicted in Figure S9. PEI 270 kDa showed a 1.5-fold better immobilisation yield for the cofactor that could be explained by the higher number of amine groups on the surface of the support. Moreover, the support coated with PEI 270 kDa retains the cofactor more efficiently since a lower K_d value was obtained (Table S3).

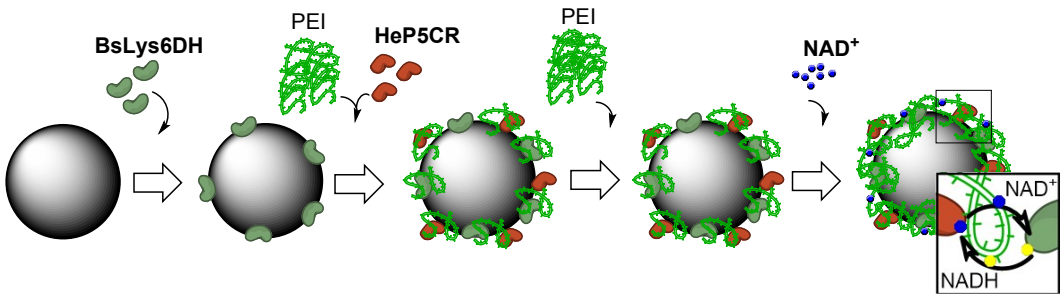


Figure S11: Scheme of the co-immobilisation of both enzymes and the cofactor.

Table S3: Optimization of PEI-coating for cofactor co-immobilisation. K_d was calculated following the equation: $Q = Q_{max} \times X / (K_d + X)$; where [$Q$] means the concentration of immobilised cofactor (mM) and [X] is the concentration of cofactor (mM) in the bulk after plotting the abs_{340nm} obtained when 0.2-50 mM of cofactor were offered to the resin. Q_{max} corresponds to the maximal possible absorption.

PEI (kDa)	Immobilisation yield (%)	K_d (µmol/g)
60	19.3 ± 1.2	24.1 ± 1.2
270	28.9 ± 4.1	11.2 ± 3.4

The biocatalysts with co-immobilised cofactor were trialled in batch reactions (50 mM phosphate buffer pH 8) obtaining the similar conversion (~90%) to the biotransformation with free cofactor. However, the reuse of the self-sufficient biocatalyst could not be performed due to the elution of the co-immobilised cofactor. Then, the self-sufficient biocatalysts were tested in batch reactions but using 10 mM phosphate buffer, showing a lower stability compared to the biocatalyst with free cofactor (Figure S10). Since the association/dissociation equilibrium between the cofactor and the positively charged support requires a low ionic strength (10 mM), the activity of both enzymes was assayed in the different buffered solutions (10 mM and 50 mM). No significant differences were observed for Gs-Lys6DH. However, the He-P5C activity either free or immobilised on a support happened to be lower in buffers with low ionic strength (Figure S11), maintaining half of the activity in 10 mM phosphate buffer in the best scenario. This fact could be explained by the halophile origin of the enzyme.

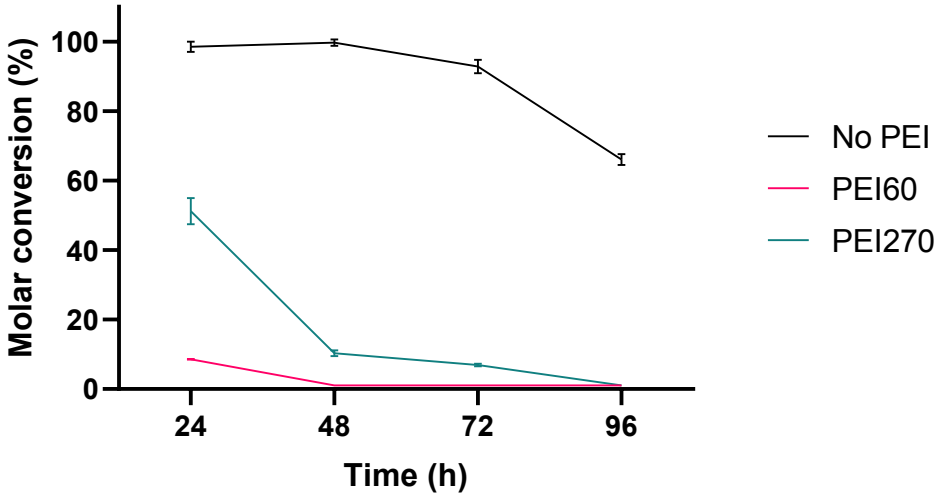


Figure S12. Batch biotransformations by using self-sufficient biocatalysts. All the biocatalysts contained 2 mg/g He-P5C and 2 mg/g Gs-Lys6DH. 8 µmol/g NAD^+ were co-immobilised on AG-Ep/PEI60 while 23 µmol/g NAD^+ were co-immobilised on AG-Ep/PEI270. The reactions were performed with 10 mM L-Lysine (and 0.1 mM NAD^+ in case of free cofactor) in 10 mM phosphate buffer pH 8 at 37 ºC.

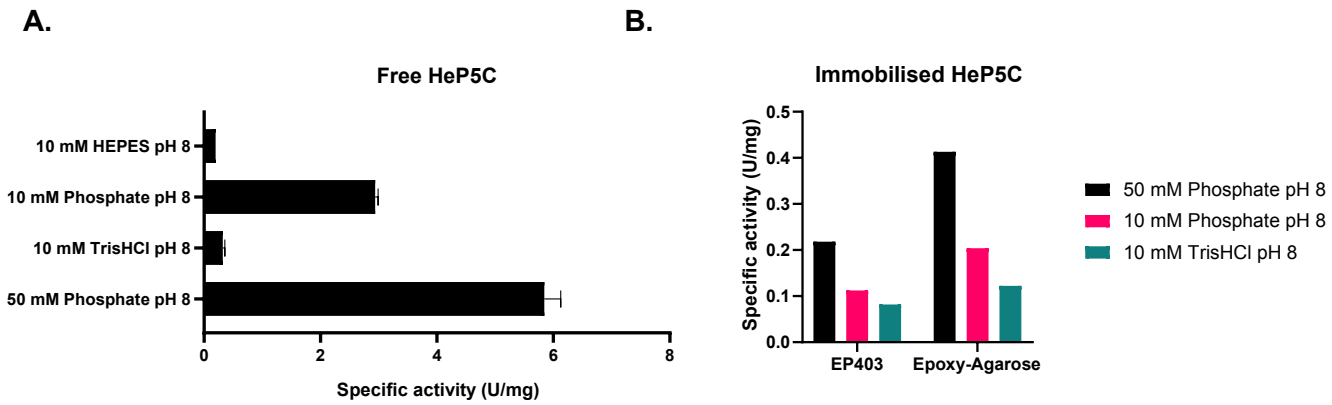


Figure S13: Effect of ionic strength on the specific activity of free He-P5C (**A**) and immobilised He-P5C (**B**) in different buffered solutions.

6. Process intensification:

Table S4: Optimization of flow conditions to produce L-pipecolic acid. The reactions were performed with 10 mM L-Lysine in 50 mM phosphate buffer pH 8 in a 2 mL packed-bed reactor. The flow rates were 200 μL/min (10 min R.T.), 100 μL/min (20 min R.T.) and 67 μL/min (30 min R.T.).

NAD^+ (mM)	Temperature (ºC)	R.T. (min)	L-PA formation (%)
1	37	10	10 ± 2
1	37	20	58 ± 1
1	37	30	99 ± 4
0.5	37		70 ± 3
0.5	40		46 ± 1
0.1	37		28 ± 1

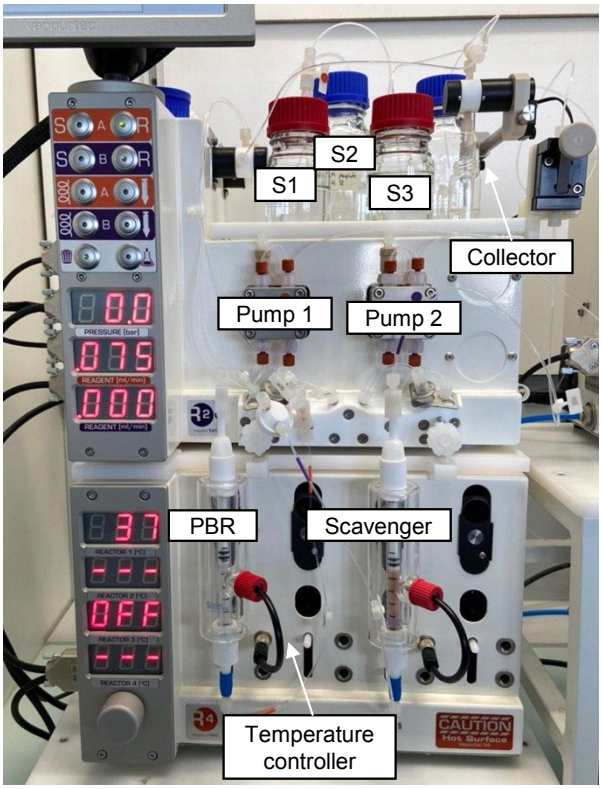


Figure S14. Scheme of the continuous flow packed-bed reactor (PBR) with in-line purification system. Firstly, the substrate solution (S1) containing 10 mM L-lysine and 1 mM of cofactor in 50 mM phosphate buffer at pH 8 was pumped (Pump 1) towards the PBR connected to the scavenger column (Amberlyst® A26). In a second step, a solution (S2) of 50 mM phosphate buffer at pH 8 was flushed (Pump 1) through the scavenger column. Finally, the desorption solution (S3) which contains 100 mM phosphate buffer at pH 6 was used (Pump 2) to elute the NAD^+ trapped into the column allowing its easy reuse for another flow reaction.

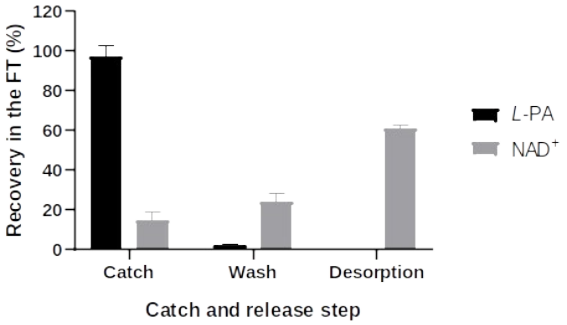


Figure S15. In-line recovery of pipecolic acid and NAD^+ in flow using a packed bed column of Amberlyst® A26. Values are the average of three measures and the SD is shown.