

Supporting Information for

Ten-minute protein purification and surface tethering for continuous-flow biocatalysis

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Supplemental Materials and Methods

Unless otherwise indicated, all commercially available reagents and solvents were used directly from the supplier without further purification. The vortex fluid device (VFD) sample tubes were commercial quality borosilicate glass, with an internal diameter of 17.7 mm. Prior to use, each sample tube was cleaned with piranha solution (4:1, conc. sulfuric acid: 30% H₂O₂), rinsed with diH₂O, dried using acetone, and stored in an oven at 160 °C. **Note:** The piranha solution is highly dangerous and corrosive. Therefore, appropriate personal protective equipment should be worn while handling the solution, and only an experienced, well-trained chemist using appropriate safety measures should perform this step. All buffered solutions were prepared with double-deionized water (diH₂O, >18 MV) from a Milli-Q water system (Millipore).

Enzymes, Buffers and Assays

mCherry

Buffer: 50 mM H₂NaPO₄ and 150 mM NaCl was prepared as follows: H₂NaPO₄ (5.999 g) and NaCl (8.766 g) were added to 1.0 L of deionized H₂O and the pH of the solution adjusted to 8.0 at 25 °C with HCl. The volume was adjusted to 1.0 L. The buffer was filter-sterilized through a 0.22 µm filter (Corning), and stored at 25 °C. *Protein solution:* After dialyzing recombinant mCherry into the buffer described above, the protein concentration was determined by measuring its A₂₈₀ using $\epsilon = 34380 \text{ M}^{-1} \text{ cm}^{-1}$ and a MW of 29257 g mol⁻¹. Protein concentration was adjusted either by diluting with buffer or through concentrating with a 10 kDa cutoff concentrator (Sartorius). The purity of the protein was confirmed by 12% SDS-PAGE (Figure S1), and the enzyme was assayed with ≥74% homogeneity.

eGFP

Buffer: 50 mM Na₂PO₄, 300 mM NaCl and 10 mM β-mercaptoethanol was prepared as follows: H₂NaPO₄ (3.000 g), NaCl (8.766 g) and 350 µL of β-mercaptoethanol were added to 500 mL of deionized H₂O and the pH of the solution adjusted to pH 8.0 at 25 °C with HCl. The volume was adjusted to 1.0 L. The buffer was filter-sterilized through a 0.22 µm filter and stored at 25 °C. *Protein solution:* After dialyzing recombinant eGFP into the buffer described above, protein concentration was determined by A₂₈₀ using $\epsilon = 20000 \text{ M}^{-1} \text{ cm}^{-1}$ and a MW of 30152.8 g mol⁻¹. Protein concentration was adjusted either by diluting with buffer or through concentrating with a 10 kDa cutoff concentrator. The purity of the protein was confirmed by 12% SDS-PAGE (Figure S2), and the enzyme was assayed with ≥88% homogeneity.

Alkaline phosphatase

Reaction buffer: 50 mM diethanolamine, 40 mM NaCl, 1 mM MgCl₂•6H₂O, 1 mM NiCl₂•6H₂O, at pH 9.8 buffer was prepared as follows: NaCl (2.324 g), MgCl₂•6H₂O (203 mg) and NiCl₂•6H₂O (237 mg) were dissolved in 800 mL of diH₂O. Diethanolamine (5.257 g) was added to this solution, and the pH adjusted to 9.8 with 1 M NaOH at 25 °C. The volume was adjusted to 1.0

L. The buffer was then filtered-sterilized through a 0.22 μm filter and stored at 4 $^{\circ}\text{C}$. *Dialysis buffer*: 300 mM Tris-HCl, pH 8.0 buffer was prepared as follows: Tris-HCl (47.28 g) was dissolved in 800 mL of dH_2O . The pH of the solution was adjusted to 8.0 with 6 M NaOH at 25 $^{\circ}\text{C}$, and the volume adjusted to 1 L. The buffer was stored at 25 $^{\circ}\text{C}$. *Assay*: 1 mL of activity buffer containing 0.70 mM bis(*p*-nitrophenyl) phosphate sodium salt was added to a glass LCMS vial. The solution was warmed to the required temperature for the specific reaction (25-70 $^{\circ}\text{C}$) in a heating block for two min. After this time, 50 μL of 6.15 μM alkaline phosphatase was added to the solution. The reactions were typically performed for five min unless otherwise indicated. After the indicated reaction time, 200 μL of 4 M NaOH was added to quench the reaction, and 200 μL of this solution was then transferred to a 96-well polystyrene plate (Costar) to measure its absorbance at 405 nm. The molar absorption coefficient of *p*-nitrophenol after the quench described above was 4242 $\text{M}^{-1} \text{cm}^{-1}$. *Enzyme solution*: After dialyzing recombinant alkaline phosphatase into the buffer described above, the protein concentration was determined by A_{280} using $\epsilon = 32890 \text{ M}^{-1} \text{cm}^{-1}$ and a MW of 50212.3 g mol^{-1} . Enzyme concentration was altered either through dilution with buffer or through concentration with a 10 kDa cutoff concentrator. The purity of the protein was confirmed by 12% SDS-PAGE (Figure S3), and the assayed enzyme had $\geq 57\%$ homogeneity, as estimated by ImageJ software.

Phosphodiesterase

Reaction buffer: 50 mM diethanolamine, 40 mM NaCl, 1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1 mM $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, at pH 9.8 buffer was prepared as follows: NaCl (2.324 g), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (203 mg) and $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (237 mg) were dissolved in 800 mL of dH_2O . Diethanolamine (5.257 g) was added to this solution, and the pH adjusted to 9.8 with 1 M NaOH at 25 $^{\circ}\text{C}$. The volume was adjusted to 1.0 L. The buffer was then filtered-sterilized through a 0.22 μm filter and stored at 4 $^{\circ}\text{C}$. *Dialysis buffer*: Phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.2) buffer was prepared as follows: NaCl (8.00 g), KCl (0.20 g), Na_2HPO_4 (1.15 g) and KH_2PO_4 (0.20 g) were dissolved in 800 mL of dH_2O . The pH of the solution was adjusted to 7.2 with 1 M HCl at 25 $^{\circ}\text{C}$. The volume of the buffer was then adjusted to 1.0 L and then filtered-sterilized through a 0.22 μm filter and stored at 25 $^{\circ}\text{C}$. *Assay*: In a 2 mL Eppendorf tube, 1 mL of the buffer containing 0.01 M bis(*p*-nitrophenyl) phosphate sodium salt was warmed to the required temperature for the specific reaction (25-70 $^{\circ}\text{C}$) in a heating block for three min. After this time, 0.1 μL of the dialyzed phosphodiesterase was added to the solution and the reaction assayed for the indicated times. After the reaction time had subsided, 300 μL of 4 M NaOH was added to terminate the reaction. 100 μL of this sample was then transferred to a 96-well micro plate reader, and the solution's absorbance was measured at 405 nm. The molar absorption coefficient of *p*-nitrophenol after the quench described above was 4242 $\text{M}^{-1} \text{cm}^{-1}$. *Enzyme solution*: After dialyzing the recombinant phosphodiesterase into the buffer described above, the protein concentration was determined by Bradford assay. A working solution of 2 mg mL^{-1} enzyme was prepared by diluting the enzyme with buffer or through concentration with a 10 kDa concentrator. The purity of the

protein was confirmed by 12% SDS-PAGE (Figure S4), and the assayed enzyme had $\geq 75\%$ homogeneity.

Esterase

Reaction buffer: 50 mM Na_2HPO_4 at pH 7.0 was prepared as follows: H_2NaPO_4 (1.459 g) and Na_2HPO_4 (3.867 g) were dissolved in 500 mL of dH_2O . The pH of the resulting solution was adjusted to 7.0 at 25 °C with 5.0 M HCl. The volume was adjusted to 1.0 L. The buffer was then filtered-sterilized through a 0.22 μm filter and stored at 4 °C. *Dialysis buffer:* PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.2) buffer was prepared as follows: NaCl (8.00 g), KCl (0.20 g), Na_2HPO_4 (1.15 g) and KH_2PO_4 (0.20 g) were dissolved in 800 mL of dH_2O . The pH of the solution was adjusted to pH 7.2 with 1 M HCl at 25 °C. The volume of the buffer was then adjusted to 1.0 L and then filtered-sterilized through a 0.22 μm filter. Immediately before dialysis, 10% v/v glycerol was added into the solution to maintain protein solubility. *Assay:* In a 15 mL falcon tube, 64 mg of *p*-nitrophenylacetate was re-suspended in 10 mL of ACS Reagent Select™ grade methanol (Sigma) to generate a stable solution that was stored at 4 °C. Then, 3.0 mL of this solution was added to 100 mL of H_2O with rapid mixing before further dilution with 100 mL phosphate buffer (50 nM, pH 7.0) to generate a 0.052 mM stock solution. Esterase (1 μL , 0.038 mM) was diluted into 10 mL of phosphate buffer for testing the enzymatic activity at different temperatures using a heat block. The working enzyme stock (200 μL) was added to the substrate solution (1.10 mL), and the reaction was performed for five min. The reaction was quenched by addition of 1.00 mL of propanol, and 100 μL of the sample was transferred to a 96-well plate before measuring its absorbance at 405 nm. The molar absorption coefficient of *p*-nitrophenol in the solution described above was $6423 \text{ M}^{-1} \text{ cm}^{-1}$. *Enzyme solution:* After dialyzing the recombinant esterase into PBS at pH 8.0, the protein concentration was determined by measuring its A_{280} using $\epsilon = 38390 \text{ M}^{-1} \text{ cm}^{-1}$ and a MW of 38293.2 g mol^{-1} . A working stock of 8.33 μM enzyme solution was prepared by either diluting with buffer or through concentration with a 10 kDa concentrator. The purity of the protein was confirmed by 12% SDS-PAGE (Figure S5), and the assayed enzyme had $\geq 66\%$ homogeneity.

Tobacco epi-aristolochene synthase (TEAS)

Buffer: PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 10 mM β -mercaptoethanol, pH 7.2) buffer was prepared as follows: NaCl (8.00 g), KCl (0.20 g), Na_2HPO_4 (1.15 g) and KH_2PO_4 (0.20 g) were dissolved in 800 mL of dH_2O . The volume was adjusted to 1.0 L. The pH of the solution was adjusted to pH 7.2 with 1 M HCl at 25 °C. The volume of the buffer was then adjusted to 1.0 L and then filtered-sterilized through a 0.22 μm filter. Immediately before dialysis, 7.5% v/v glycerol and β -mercaptoethanol (2.805 mL) were added to the exchange buffer to maintain protein solubility and stability.

Proteins

Production of mCherry

Since mCherry fused to a polyhistidine tag is not commercially available, the protein was prepared using bacterial overexpression as follows:

The gene mCherry from *Discosoma sp.* was purchased from Addgene (Plasmid #27705). The following PCR parameters and oligonucleotides (Integrated DNA Technologies) were used to amplify the mCherry gene. Iproof DNA Polymerase (BioRad) was used for all PCR amplification steps as directed in the manufacturer's instructions. Ten ng of the plasmid was used as the template with the primers below for a PCR as follows: one cycle at 95 °C for five min, 29 cycles at 95 °C for one min, 60 °C for one min, and 72 °C for one min, and one cycle at 72 °C for five min.

mCherryfwd: 5'-GAC GAC GAC AAG GTA GTA GTA GTA GTA ATG GTG AGC AAG GGC GAG GAG GAC AAC ATG GCC ATC-3'

mCherryrev: 5'-GAG GAG AAG CCC GGT TCA CTT GTA CAG CTC GTC CAT GCC GCC GGT GGA GTG GCG GCC CTC-3'

The DNA was then extracted from a 1% agarose gel using a Zymoclean Gel DNA Recovery Kit and sequenced by Genewiz. This DNA was then used with the pET46 Ek/LIC Vector Kit (EMD Millipore Novagen) to generate the mCherry protein expression vector. To isolate the plasmid, *E. coli* NovaBlue cells were used (EMD Millipore, PureLink Quick Plasmid Miniprep Kit) prior to transforming into other heterologous hosts.

Expression and purification of mCherry

The pET46-mCherry construct was transformed *via* heat shock into *E. coli* BL21 Star (DE3) cells. The transformed cells were transferred to an LB agar plate supplemented with 50 µg/mL carbenicillin antibiotic, and incubated at 37 °C for 14 - 16 h. A seed culture was prepared by inoculating a single colony from the plate into 5 mL of LB medium supplemented with 50 µg/mL carbenicillin before shaking at 220 rpm for 6 - 7 h at 37 °C in a 13 mL culture tube. The expression culture was then prepared by inoculating 5 mL of the seed culture per 1.0 L of LB media with 50 µg/mL carbenicillin and then shaking at 220 rpm at 37 °C. When the optical density of the culture reached $A_{600} = 0.6$, overexpression of the mCherry protein with an N-terminal His₁₂ tag was induced through the addition of 0.1 mM IPTG. The induced expression culture was incubated at 20 °C for 18 - 20 h shaking at 220 rpm. The cells were harvested and resuspended in 30 mL buffer (50 mM NaH₂PO₄, 100 mM NaCl pH 8.0, and 100 µL of Halt Protease Inhibitor Cocktail from ThermoFisher). The cell lysate was prepared by sonication (Digital Sonifier 450; std. horn, T <8 °C, 4 × 60 s pulses (1 s on, 2 off), 50% amplitude), and the supernatant purified by VFD-mediated purification as described below. Fractions containing purified protein identified by SDS-PAGE were pooled and concentrated in a 10 kDa microconcentrator. The purity of the protein was confirmed using 12% SDS-PAGE (Figure S1).

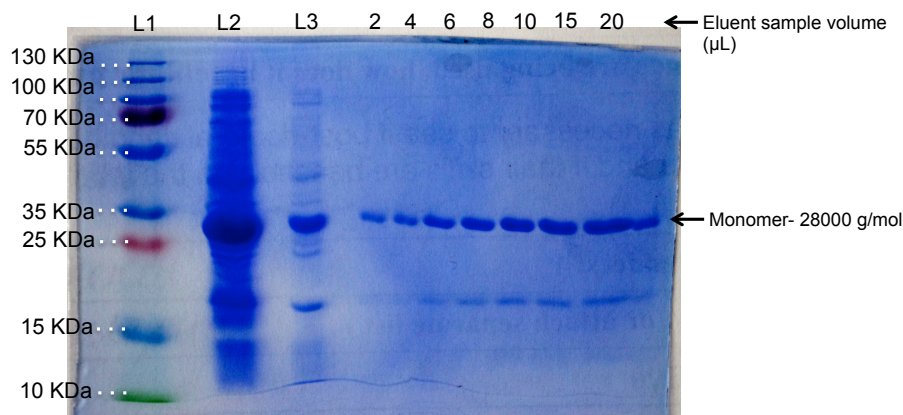


Figure S1 VFD-mediated purification of mCherry. In this 12% tris-glycine SDS-PAGE, each lane was loaded with 10 μ L of sample unless otherwise indicated. **Lane 1.** PageRuler Plus pre-stained protein ladder (ThermoFisher Scientific). **Lane 2.** Cell lysate, **Lane 3.** Flow-through after ten cycles through the VFD. The other lanes shown in this gel show the volume of elution fraction from the VFD after immobilization and purification (*Elution buffer* - 250 mM imidazole, 50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM 2-mercaptoethanol at pH 8.0). Protein homogeneity was estimated to be 74% using ImageJ software.

Production, expression and purification of eGFP

A mammalian expression eGFP vector was a gift from Dr. Gabriel Fenteany and Dr. Alem Kahsai. The eGFP gene was sub-cloned into a pET-28 vector using NdeI and EcoRI restriction enzymes for bacterial protein expression. The vector was first transformed *via* heat shock into *E. coli* BL21 Star (DE3) cells. The transformed cells were transferred to an LB agar plate supplemented with 50 μ g/mL kanamycin antibiotic, and incubated at 37 $^{\circ}$ C for 10 - 12 h. A seed culture was prepared by inoculating a single colony from the transformation plate into 5 mL of LB supplemented with 50 μ g/mL kanamycin antibiotic, then shaking at 220 rpm for 6 - 7 h at 37 $^{\circ}$ C in a 15 mL culture tube. The expression culture was then prepared by inoculating 5 mL of the seed culture per 1.0 L of LB supplemented with 50 μ g/mL kanamycin, then shaking at 220 rpm at 37 $^{\circ}$ C. When the optical density of the culture reached $A_{600} = 0.7$, overexpression of eGFP protein with an N-terminal His₆ tag was induced through the addition of 0.5 mM IPTG. The induced expression culture was incubated at 37 $^{\circ}$ C for 3 h shaking at 170 rpm. The cells were harvested by centrifugation and resuspended and sonicated as described above. The supernatant was purified by VFD-mediated purification as described below. Fractions containing purified protein identified by SDS-PAGE were pooled and concentrated with a 5 kDa concentrator. The purity of the protein was confirmed using 12% SDS-PAGE (Figure S2).

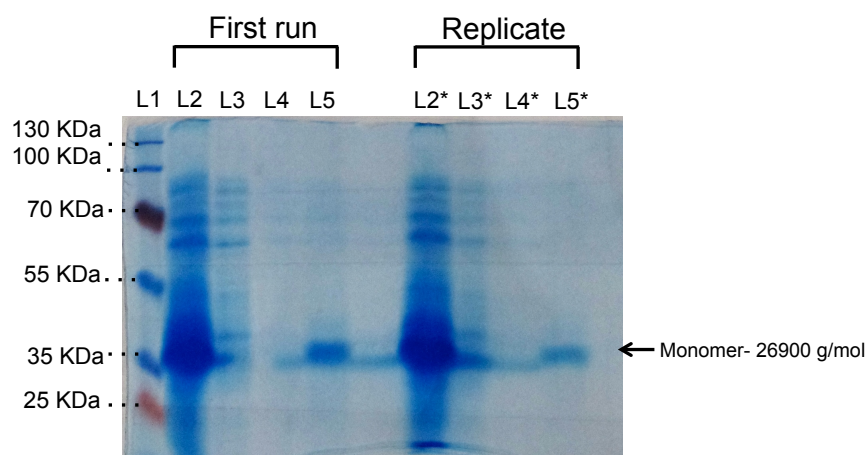


Figure S2 VFD-mediated purification of eGFP. In this 12% tris-glycine SDS-PAGE, each lane was loaded with 10 μ L of sample. **Lane 1.** PageRuler Plus pre-stained protein ladder. **Lane 2.** Cell lysate. **Lane 3.** Flow-through after ten cycles through the VFD. **Lane 4.** The eluent from the wash step (*Wash buffer* - 10 mM imidazole, 50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM 2-mercaptoethanol, pH 8.0). **Lane 5.** The eluent fraction (10 μ L) from the VFD after immobilization and purification (*Elution buffer* - 250 mM imidazole, 50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM β -mercaptoethanol, pH 8.0). Protein homogeneity was estimated to be 88% using the ImageJ software.

Production of alkaline phosphatase

Since alkaline phosphatase containing a polyhistidine tag is not commercially available, the enzyme was prepared using bacterial overexpression as follows.

The alkaline phosphatase gene was extracted from the genomic DNA of BL21 Star (DE3) *E.ia coli* as follows: 3.0 mL of BL21 Star (DE3) cells were cultured overnight in LB at 37 $^{\circ}$ C with shaking at 220 rpm, and were then centrifuged at 14 krpm for two min. The supernatant was discarded and the resulting pellet resuspended in 600 μ L of lysis buffer (4.67 mL TE buffer, 300 μ L 10% SDS, 0.011 g proteinase K, and 10 μ L RNAase), and incubated at 37 $^{\circ}$ C for one h. Then, 300 μ L of phenol and 300 μ L of chloroform were added, and the sample was mixed *via* inversion until homogeneous. The mixture was then centrifuged for five min at 14 krpm, and the upper aqueous phase was extracted. Another 250 μ L portion of both phenol and chloroform were added to the sample before mixing and centrifuging as before. After removing the aqueous phase, three volumes of -20 $^{\circ}$ C molecular biology grade ethanol were added to the sample. The sample was stored at -20 $^{\circ}$ C for 45 min, and then spun down at 14 krpm for ten min. Once the supernatant was discarded, 1 mL of 70% ethanol was added and the sample was spun at 14 krpm for two min. The supernatant was again discarded, and the DNA allowed to air dry before final resuspension in TE buffer (100 μ L).

The following PCR parameters and oligonucleotides were used to extract the alkaline phosphatase gene from the genomic DNA and subsequently clone it into a Novagen pET46 vector.

Herculase ii Fusion DNA polymerase (Agilent Technologies) was used for all PCR amplification steps as directed in the manufacturer's instructions. Touchdown PCR was performed to carry out PCR amplification as follows: one cycle at 98 °C for 30 s, 33 cycles at 98 °C for 10 s, 79.8 °C for 30 s (with a decrease of 0.5 °C every cycle), 72 °C for 30 s, 19 cycles at 98 °C for 10 s, 55.8 °C for 30 s, 72 °C for 3 min 30 s, one cycle at 72 °C for 5.5 min.

EcoliAPForward: 5'-GAC GAC GAC AAG ATG GTG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG-3'

EcoliAP6HReverse: 5'-GAG GAG AAG CCC GGT TCA TCA GTG GTG GTG GTG GTG GTG TTT CAG CCC CAG GGC-3'

The PCR amplification product was extracted from a 1% agarose gel and purified with the Zymoclean Gel DNA Recovery Kit. The gel-extracted DNA was sequenced by Genewiz and then used with the pET46 Ek/LIC Vector Kit to generate the AP protein expression vector. *E. coli* NovaBlue cells were used to isolate the plasmid with the PureLink Quick Plasmid Miniprep Kit prior to transformation into other heterologous hosts.

Expression and purification of alkaline phosphatase

The pET46-AP construct was transformed *via* heat shock into *E. coli* BL21 Star (DE3) cells. The transformed cells were transferred to an LB agar plate supplemented with 50 µg/mL carbenicillin antibiotic, and incubated at 37 °C for 14 - 16 h. A seed culture was prepared by inoculating a single colony from the transformation plate into 5 mL of LB medium with 50 µg/mL carbenicillin antibiotic, then shaking at 220 rpm for 6 - 7 h at 37 °C in a 15 mL culture tube. The expression culture was then prepared by inoculating 5 mL of the seed culture per 1.0 L of LB media with 50 µg/mL carbenicillin, then shaking at 220 rpm at 37 °C. When the optical density of the culture reached A_{600} 0.6 - 0.7, overexpression of AP protein with a C-terminal His₆ tag was induced through the addition of 0.5 mM IPTG. The induced expression culture was incubated at 30 °C for 18 - 20 h shaking at 220 rpm. The cells were harvested and resuspended in 30mL buffer (300 mM Tris-HCl, pH 8.0, 20% w/v sucrose, 0.1 mg/mL lysozyme and 100 µL protease inhibitor cocktail), and allowed to shake at 150 rpm at 4 °C for one h. The cell lysate was prepared by sonication as previously described, and the supernatant then purified by VFD-mediated purification as described below. Fractions containing purified protein identified by SDS-PAGE were pooled and concentrated using a 10 kDa concentrator. The purity of the protein was confirmed using 12% SDS-PAGE (Figure S3). Purification to ≥95% homogeneity was obtained before kinetic assays. For activity assays, the purified recombinant protein was dialyzed into 300 mM Tris, pH 8.0.

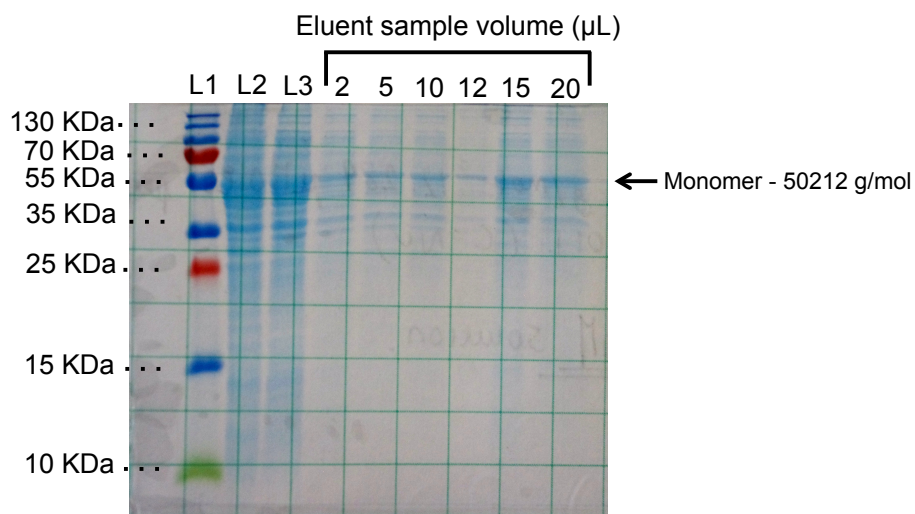


Figure S3 VFD-mediated purification of alkaline phosphatase. In this 12% Tris-glycine SDS-PAGE, each lane was loaded with 10 μL of sample unless otherwise indicated. **Lane 1.** PageRuler Plus pre-stained protein ladder. **Lane 2.** Cell lysate. **Lane 3.** Flow-through after ten cycles through the VFD. Other lanes show the elution fraction from the VFD after immobilization and purification (*Elution buffer* as previously described). The protein fraction visualized in this lane was dialyzed into the assay buffer before further experiments. Protein homogeneity was estimated to be 57% using the ImageJ software.

Production of phosphodiesterase

Since phosphodiesterase fused to a polyhistidine tag is not commercially available, the enzyme was prepared using bacterial overexpression and purification as follows.

The open reading frame encoding phosphodiesterase from *M. jannashchii* was amplified as follows from a plasmid purchased from Addgene in DH5 α cells (Plasmid #11538). The QIAprep Spin Miniprep Kit was used as directed by the manufacturer to isolate the plasmid DNA from an overnight culture of *E. coli* cells. The following PCR parameters and oligonucleotides were used to amplify the gene encoding phosphodiesterase. I-proof DNA polymerase was used for all PCR amplification steps as directed in the manufacturer's instructions. Here, the plasmid (0.25 μL), 25 mM DNTP's (0.25 μL), 10 x PFU buffer (2.50 μL), forward and reverse primers shown below (0.25 μL), iProof enzyme (0.50 μL) and diH $_2$ O (21.0 μL) were subjected to the PCR conditions: one cycle at 98 $^{\circ}\text{C}$ for five min, 25 cycles at 98 $^{\circ}\text{C}$ for one min, 65 $^{\circ}\text{C}$ for one min, and 72 $^{\circ}\text{C}$ for one min, and one cycle at 72 $^{\circ}\text{C}$ for five min.

LIC_Phosphodiesterase_Forward: 5'-GAC GAC GAC AAG ATG AAA ATT GGG ATA ATG AGC GAT ACC CAT GAC-3'

LIC_Phosphodiesterase_Reverse: 5'-GAG GAG AAG CCC GGT TCA TCA TAA CAC TAT CTC CCT ATA CTC CTT-3'.

The resultant DNA fragment (\approx 498 base pairs) was excised from a 1% agarose gel and purified using the QIAquick Gel Extraction Kit. The purified PCR product was then sequenced by

Genewiz, and used with the pET46 Ek/LIC Vector Kit to generate the phosphodiesterase recombinant protein expression vector. *E. coli* Nova blue cells were used to isolate the plasmid prior to transformation into other heterologous hosts.

Expression and purification of phosphodiesterase

The pET46-phosphodiesterase construct was transformed *via* heat shock into *E. coli* BL21 (DE3) cells. The transformed cells were transferred to an LB agar plate supplemented with 50 µg/mL carbenicillin antibiotic, and incubated at 28 °C for nine h. A seed culture was prepared by inoculating a single colony from the transformation plate into 90 mL of LB medium with 50 µg/mL carbenicillin antibiotic and shaking the culture at 225 rpm for 14 - 16 h at 37 °C in a 250 mL baffled flask. The expression culture was then prepared by inoculating 10 mL of the seed culture into 1.0 L of LB media with 50 µg/mL carbenicillin and shaking the culture at 225 rpm in 37 °C (2.0 L baffled flask). When the culture reached an A_{600} of 0.6, overexpression of phosphodiesterase protein with a C-terminal His₆ tag was induced through addition of 0.50 mM IPTG and the mixture was incubated further for three h at 37 °C with shaking at 225 rpm. The cells were harvested and re-suspended in buffer A (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 10 mM β-mercaptoethanol, 100 µL protease inhibitor cocktail). The cell lysate was prepared by sonication as previously described, and the supernatant was purified by VFD-mediated purification as described below. Fractions containing purified protein identified by SDS-PAGE were pooled and concentrated using a 3 kDa microconcentrator. The purity of the protein was confirmed using 12% SDS-PAGE (Figure S4). Purification to ≥85% homogeneity was obtained before subsequent kinetic assays. For activity assays the purified recombinant protein was dialyzed into PBS, pH 7.2.

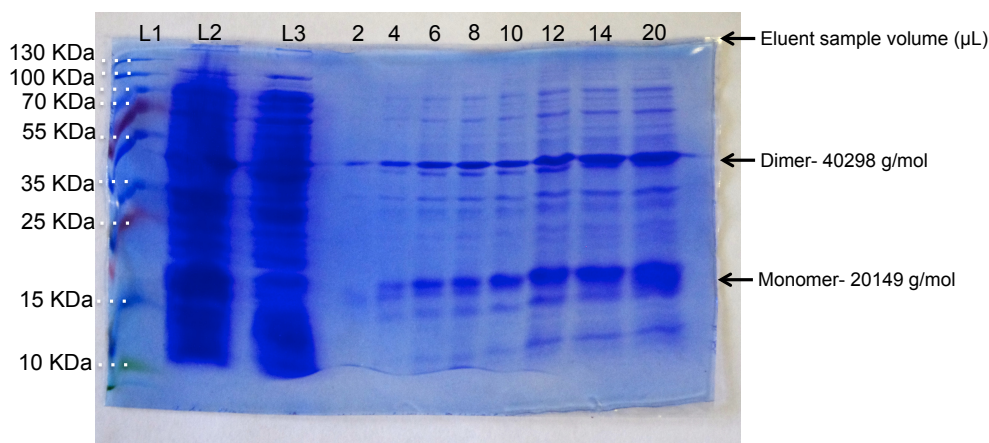


Figure S4 VFD-mediated purification of phosphodiesterase. In this 12% Tris-glycine SDS-PAGE, each lane was loaded with 10 µL of sample unless otherwise indicated. **Lane 1.** PageRuler Plus pre-stained protein ladder. **Lane 2.** Cell lysate, **Lane 3.** Flow-through after ten cycles through the VFD. Other lanes show the volume of elution fraction from the VFD after immobilization and purification (*Elution buffer as previously described*). The purified protein fraction shown here was dialyzed into the assay buffer before further experiments. Protein homogeneity was estimated to be 75% using the ImageJ software.

Production of esterase

Since an esterase containing a polyhistidine tag is not commercially available, the enzyme was prepared using bacterial overexpression as follows.

The genomic DNA of *Lactobacillus plantarum* was purchased from ATCC (#8014D-5). The following PCR parameters and oligonucleotides (IDT) were used to amplify the esterase gene from the genomic DNA by touchdown PCR as follows: one cycle at 98 °C for 30 s, 33 cycles at 98 °C for 10 s, 79.8 °C for 30 s (decreases 0.5 °C every cycle), 72 °C for 30 s, 19 cycles at 98 °C for 10 s, 55.8 °C for 30 s, 72 °C for three min 30 s, one cycle at 72 °C for five min 30 s. Herculase ii Fusion DNA polymerase (Agilent Technologies) was used for the PCR amplification steps.

EstLICNew_Forward: 5'-GAC GAC GAC AAG ATG CCA ACA ATT AAT TCG ATT CAA ACA ACC GTC-3'

EstLICNew_Reverse: 5'-GAG GAG AAG CCC GGT TCA TCA CTA AAT TAA CGC GGC CGC CAT CAC-3'

The PCR product was electrophoresed and excised from a 1% agarose gel before purification using the Zymo Gel DNA Extraction Kit. The purified PCR product was then sequenced by Genewiz, and used with the pET46 Ek/LIC Vector Kit to generate the esterase recombinant protein expression vector. *E. coli* TOP10 cells were used to isolate the plasmid prior to transformation into other heterologous hosts.

Expression and purification of esterase

The pET46-esterase construct was transformed *via* heat shock into *E. coli* BL21 (DE3) cells. The transformed cells were transferred to an LB agar plate supplemented with 50 µg/mL carbenicillin antibiotic, and incubated at 37 °C for 10 - 12 h. A seed culture was prepared by inoculating a single colony from the transformation plate in 25 mL of LB medium with 50 µg/mL carbenicillin antibiotic and shaking the culture at 225 rpm for seven h at 37 °C in a 250 mL baffled flask. The expression culture was then prepared by inoculating 10 mL of the seed culture in 1.0 L of LB media with 50 µg/mL carbenicillin and shaking the culture at 225 rpm in 37 °C (3 L baffled flask). When the optical density of the culture reached $A_{600} = 0.6$, overexpression of esterase protein with a N-terminal His₆ tag was induced through addition of 0.50 mM IPTG, and the culture was incubated further for 16 h at 18 °C with shaking at 170 rpm. The cells were harvested and re-suspended in buffer A. The cell lysate was prepared by sonication as previously described, and the supernatant was purified by VFD-mediated purification as described below. Fractions containing the purified protein identified by SDS-PAGE were pooled and concentrated with a 3 kDa concentrator. The purity of the protein was confirmed using 12% SDS-PAGE (Figure S5). Purification to ≥90% homogeneity was obtained before subsequent kinetic assays. For activity assays, the purified recombinant protein was dialyzed into PBS, pH 7.2.

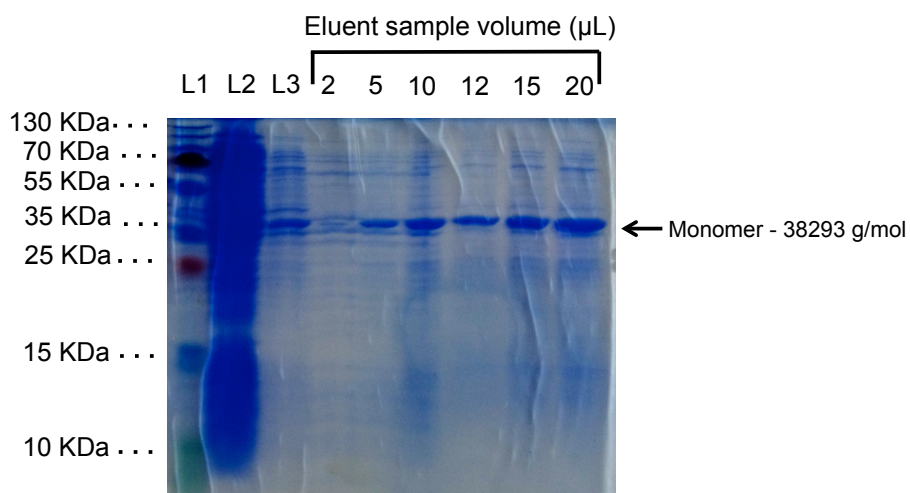


Figure S5 VFD-mediated purification of esterase. In this 12% Tris-glycine SDS-PAGE, each lane was loaded with 10 μL of sample unless otherwise indicated. **Lane 1.** PageRuler Plus pre-stained protein ladder. **Lane 2.** Cell lysate, **Lane 3.** Flow-through after ten cycles through the VFD. Other lanes show the volume of elution fraction from the VFD after immobilization and purification (*Elution buffer as previously described*). The purified protein fraction visualized was dialyzed into the assay buffer before further experiments unless used directly after purification. Protein homogeneity was estimated to be 66% using the ImageJ software.

Expression and purification of TEAS

Our laboratory has previously published the plasmid containing the gene for TEAS.^[1] The pET28-TEAS construct was transformed into *E. coli* BL21 Star (DE3) cells. The transformed cells were transferred to an LB agar plate supplemented with 50 $\mu\text{g}/\text{mL}$ kanamycin antibiotic, and incubated at 37 $^{\circ}\text{C}$ for 14 - 16 h. A seed culture was prepared by inoculating a single colony from the plate into 5 mL of LB medium with 50 $\mu\text{g}/\text{mL}$ carbenicillin antibiotic before shaking at 225 rpm for 6 - 7 h at 37 $^{\circ}\text{C}$ in a 15 mL culture tube. The expression culture was then prepared by inoculating 5 mL of the seed culture per 1.0 L of LB media with 50 $\mu\text{g}/\text{mL}$ kanamycin, then shaking at 220 rpm at 37 $^{\circ}\text{C}$. When the optical density of the culture reached A_{600} - 0.5, the cultures were transferred to an 18 $^{\circ}\text{C}$ incubator with shaking (220 rpm). Once the cultures had reached A_{600} - 0.65, overexpression of TEAS protein with an N-terminal His₆ tag was induced through the addition of 0.5 mM IPTG. The induced expression culture was incubated at 18 $^{\circ}\text{C}$ for 18 - 20 h with shaking at 220 rpm. The cells were harvested and resuspended in buffer (50 mM NaH_2PO_4 , 100 mM NaCl pH 8.0, and 100 μL of Halt Protease Inhibitor Cocktail). The cell lysate was prepared by sonication as previously described, and the supernatant was purified by VFD-mediated purification as described below. Fractions containing purified protein identified by SDS-PAGE were pooled and concentrated with a 3 kDa concentrator. The purity of the protein was confirmed using 12% SDS-PAGE (Figure S6).

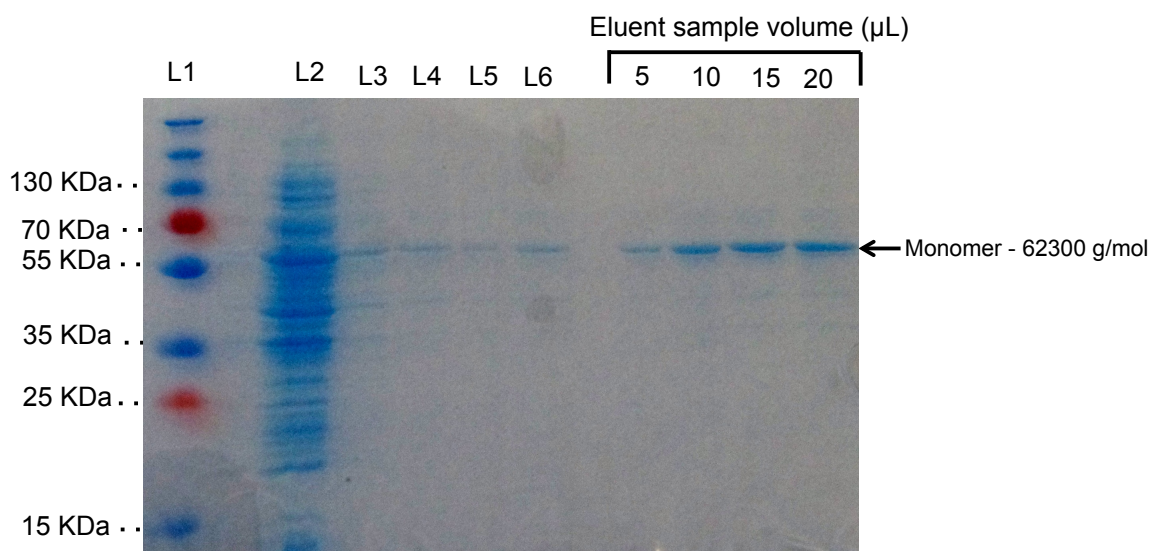


Figure S6 VFD-mediated purification of TEAS. In this 12% tris-glycine SDS-PAGE, each lane was loaded with 10 μ L of sample unless otherwise indicated. **Lane 1.** PageRuler Plus pre-stained protein ladder. **Lane 2.** Cell lysate. **Lane 3.** 10 mM imidazole wash. **Lane 4.** 20 mM imidazole wash. **Lane 5.** 30 mM imidazole wash. **Lane 6.** 40 mM imidazole wash. Other lanes visualized here show the volume of elution fraction from the VFD after immobilization and purification (*Elution buffer as previously described*). Protein homogeneity was estimated to be 93% using the ImageJ software.

VFD Set-up

The vortex fluidic device (VFD) was set to a tilt angle of 45° relative to the horizontal, with the inclined sample tube rotating at 8 krpm. Below are the methods for continuous flow protein purification, immobilization and elution of the purified protein.

Initial optimization studies for protein Immobilization in continuous flow

For the immobilization process, a series of optimization steps were first performed to allow rapid and efficient protein immobilization. The graphs below detail each optimization step and the outcome.

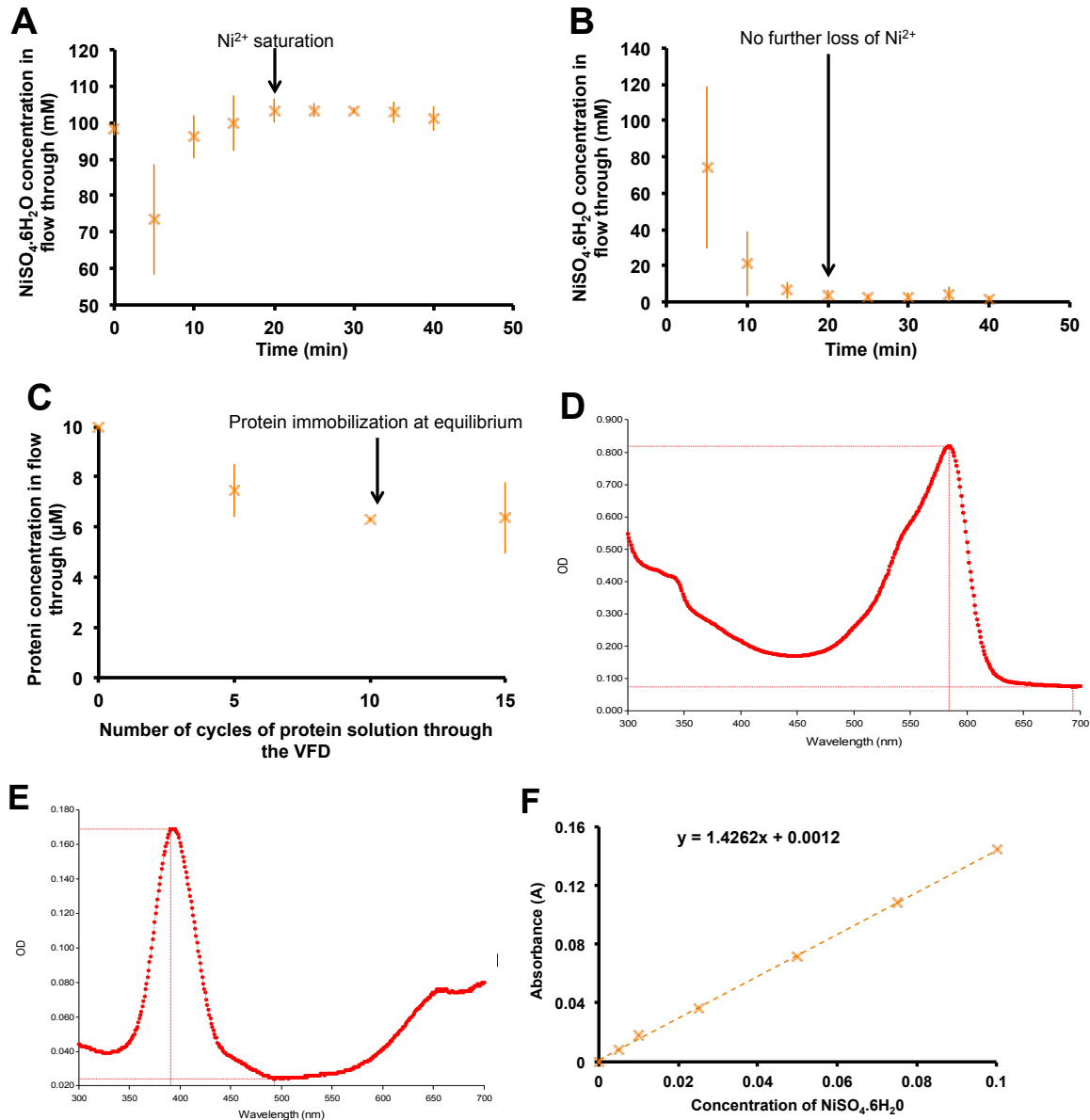


Figure S7 Optimization experiments for protein immobilization onto the reactor surface. For these experiments, 6.00 mL of homogeneous of IMAC resin (4.196 g solid IMAC resin, Bio-Rad, #1560123) was added to the inner surface of the sample tube and the ethanol then removed for 30

min. **A)** After coating the sample tube surface with the IMAC resin, $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ solution (100 mM) was flowed through the reactor at a flow rate of 1.00 mL min^{-1} and a sample collected every five min. For each fraction, 100 μL was added to a 96-well plate and the absorbance measured at 391 nm (λ_{max} for $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ solution, $\epsilon - 3.80 \text{ M cm}^{-1}$). **B)** Following IMAC activation, the residual Ni^{2+} is washed out from the system. PBS buffer was flowed through the reactor at a flow rate of 1.00 mL min^{-1} and a sample collected every five min. Again, for each fraction, 100 μL is added to a 96-well plate and the absorbance measured at 391 nm. **C)** After the reactor has been charged with Ni^{2+} , the residual metal is removed from the reactor; mCherry is then flowed through the reactor and its concentration in the flow-through solution monitored. The starting concentration of mCherry was 10 μM , and optimal immobilization was observed after ten cycles. A 100 μL sample of the flow through was collected every five cycles and the concentration of mCherry determined by its absorbance value at 584 nm (λ_{max} for mCherry, $\epsilon - 72,000 \text{ M}^{-1} \text{ cm}^{-1}$). **D)** The concentration of mCherry was determined by monitoring its λ_{max} of 584 nm. **E)** The concentration of $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ was determined by monitoring its λ_{max} of 391 nm. **F)** A Beer-Lambert plot was constructed to determine the molar extinction co-efficient of the $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ solution in the buffer conditions.

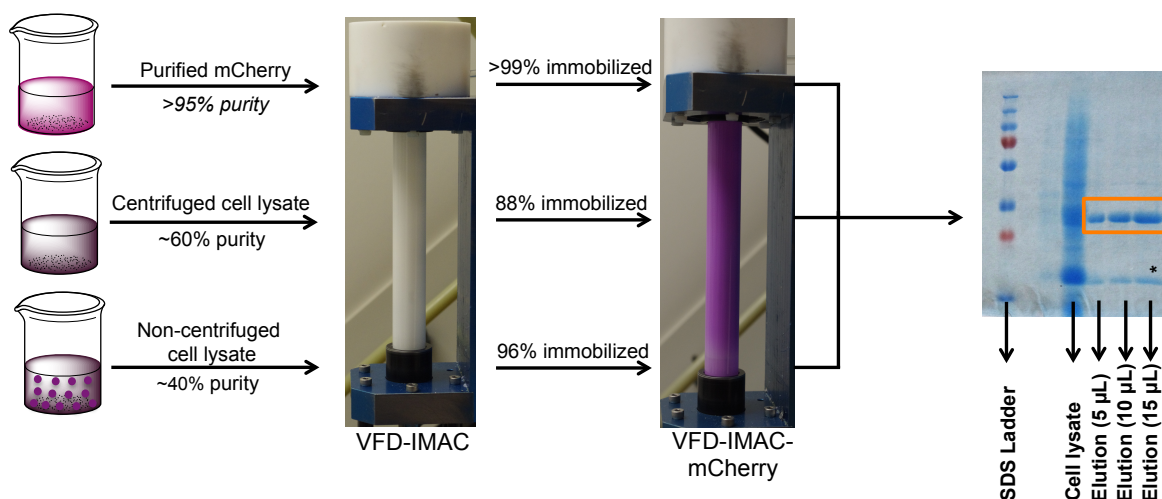


Figure S8 Optimization experiments for protein immobilization from centrifuged and non-centrifuged cell lysate. This figure schematically demonstrates the protein immobilization yields for purified mCherry and then centrifuged and non-centrifuged cell lysate. After coating the VFD reactor with IMAC resin, the protein solution is then flowed through the reactor to extract and purify the protein of interest from solution. An SDS-PAGE of a centrifuged cell lysate after VFD-mediated purification demonstrates the resultant homogeneity. *Indicates protein bound to mCherry, as noted in the main paper.

Initial Studies of Protein Purification in Continuous Flow

Before performing ten-minute protein purification on a VFD, we first suggest testing the system at a lower flow rate as described in these initial studies.

The VFD was fitted with a 17.7 mm internal diameter sample tube and rotated at 8 krpm at a 45° tilt angle. Using a Pasteur pipette, 6.00 mL of homogenous IMAC-resin was added to the rotating to create a thin layer evenly covering the sample tube. The sample tube is then rotated at 25 °C for ten min. At this point, the sample tube rotation is halted, and a jet feed is inserted down the center of the sample tube. The sample tube rotation is then started, and maintained at 8 krpm. The next steps are performed in continuous flow with reagents fed through jet feed driven by a peristaltic or syringe pump. Once transported down the jet feed, the fluid enters the hemisphere of the sample tube, and proceeds up the wall of the sample tube as more fluid is added. As the fluid exits the sample tube, it can be channeled into a collection flask, or recycled back into the reactor tube.

First, a $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ solution is flowed through the VFD (20 mL, 100 mM, 1 mL min⁻¹) to charge the IMAC resin by creation of the nickel complex as depicted in Figure 1A. Following this, PBS (20 mL, pH 7.2, 1 mL min⁻¹) is added to remove any unbound nickel. This fraction is collected and disposed in the correct manner. Next, the protein solution (purified protein, a centrifuged cell lysate, or non-centrifuged cell lysate) is passed through the VFD for ten cycles at a flow rate of 1 mL min⁻¹. At the end of this process, the protein containing the polyhistidine tag is now bound to the reactor, and the collected supernatant contains unwanted protein. Then, imidazole washes are performed to increase the purity of the protein on the reactor. Gradient washes of 10, 20, 30 and 40 mM imidazole are used (20 mL of each wash, 1 mL min⁻¹) and the fractions collected and disposed. The reactor then contains purified protein. At this stage, if the protein will be harvested and used for non-VFD reactions, it can be eluted with imidazole (250 mM, 30 mL, 1 mL min⁻¹). This fraction is then collected and then dialyzed into the reaction buffer of choice.

If the reactor will be used directly for biocatalysis in continuous flow, the reaction buffer is simply flowed through (30 mL, 1 mL min⁻¹) to equilibrate the reactor for the transformation. The substrate can then be flowed through the reactor at the flow rate and concentration of choice. After the experiment, the protein can be eluted and stored as described above. After protein elution from the reactor, the IMAC can be regenerated in continuous flow. For this step, reagents and buffers are flowed through the reactor in the following order.

1. (6 M GnHCl , 0.2 M acetic acid, 20 mL)
2. H_2O (20 mL)
3. SDS (2%, 20 mL)
4. Ethanol (20 mL, 25%)
5. Ethanol (20 mL, 75%)
6. Ethanol (20 mL, 100%)
7. Ethanol (20 mL, 25%)
8. H_2O (20 mL)
9. EDTA (20 mL, 100 mM, pH 8.0)
10. H_2O (20 mL)

11. NiSO₄•6H₂O solution as described above

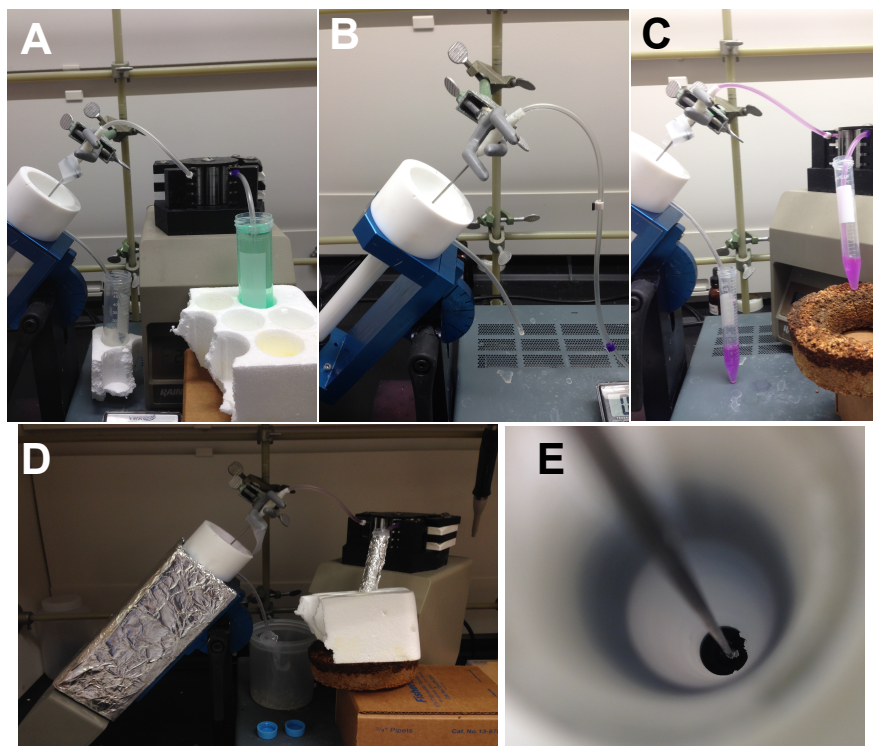


Figure S9 Reactor set up for VFD-mediated protein purification and immobilization. **A)** The IMAC resin is charged with the NiSO₄•6H₂O solution. **B)** The jet feed, a stainless steel injection manifold, is positioned approximately in the center of the rotating tube. **C)** To increase levels of mCherry immobilization, the protein solution is cycled multiple times through the VFD. **D)** As mCherry and eGFP are susceptible to photobleaching, the reactor is protected from light by an aluminum foil covering. **E)** The jet feed releases fluid into the rapidly rotating sample tube, and a drop of fluid is visible here at its tip.

Ten-Minute Protein Purification in Continuous Flow

The flow rate and quantity of reagents needed were optimized for more rapid and efficient protein purification requiring only ten min. The most unstable of the six proteins described above, TEAS, was used for this optimization. First, the flow rate of reagents through the VFD was explored. Using the maximum flow rate of the peristaltic pump (13.30 mL min⁻¹) allowed for rapid purification with no apparent decrease in the purity of the protein. Furthermore, the quantity of NiSO₄•6H₂O flowed through the system was reduced to 10 mL, as previous optimization studies revealed that this quantity saturates Ni²⁺ complexation on the reactor surface. Furthermore, the quantity of PBS flowed through after the NiSO₄•6H₂O solution was also reduced to 10 mL. As demonstrated in Figure S7, after five cycles of protein solution flowing at 13.30 mL min⁻¹ through the reactor, the protein conjugation reaches a state of equilibrium. The last step in the optimized

protocol reduced the volume of imidazole wash solution used. Thus, only 10 mL of 40 mM imidazole was flowed through the reactor tube to remove any non-specific binding. Through this process, the total residence time of the protein through the reactor is only 60 s, and the total purification time was reduced from 320 min to only ten min. Importantly, waste was also reduced from 120 to 33 mL (Figure S10).

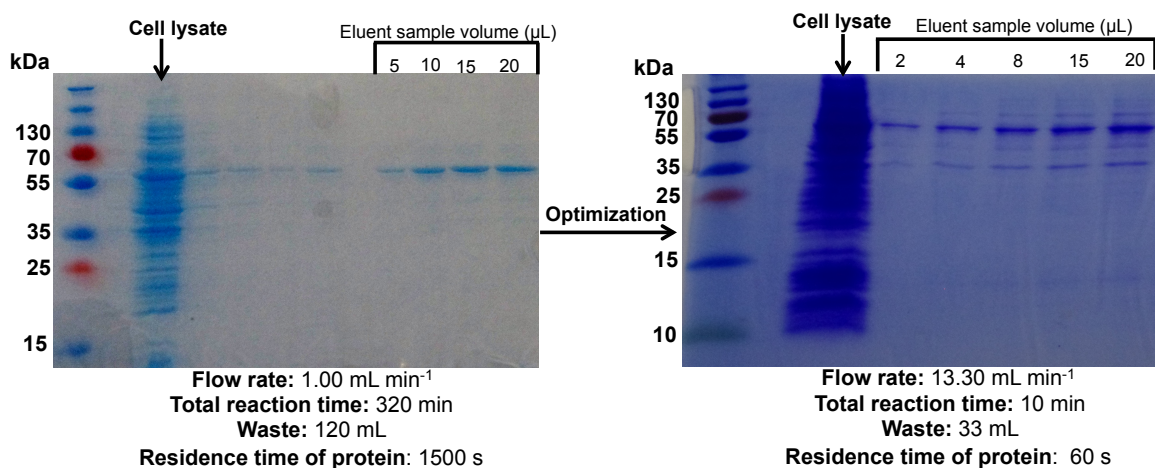


Figure S10 Rapid purification of TEAS. **A)** In this 12% tris-glycine SDS-PAGE a purification time of 320 min yielded homogenous TEAS from cell lysates. **B)** Decreasing this purification time to ten min *via* a series of optimizations afforded protein with adequate homogeneity, but requiring dramatically decreased residence time and producing less waste.

Continuous Flow Reactions for Testing Immobilization Longevity

Next, the robustness of protein attached to the reactor tube using IMAC resin was tested. A non-centrifuged cell lysate of cells overexpressing the mCherry construct was coated on the IMAC-derivatized reactor surface as detailed above. A solution of PBS was then flowed through the reactor tube for five days (1 mL min⁻¹), and the quantity of mCherry in the flow-through solution quantified using UV visible spectroscopy as detailed above. To determine the percentage of mCherry lost from the surface of the reactor, the protein was eluted from the reactor after five days, and its concentration determined. Subtracting the amount of protein removed in the flow through from the total amount of protein in the reactor allowed protein leaching levels to be determined. The experiment was conducted for five days with only 0.34% loss of protein from the surface of the reactor, demonstrating that this approach to protein immobilization could allow significantly longer enzyme reaction times than demonstrated here.

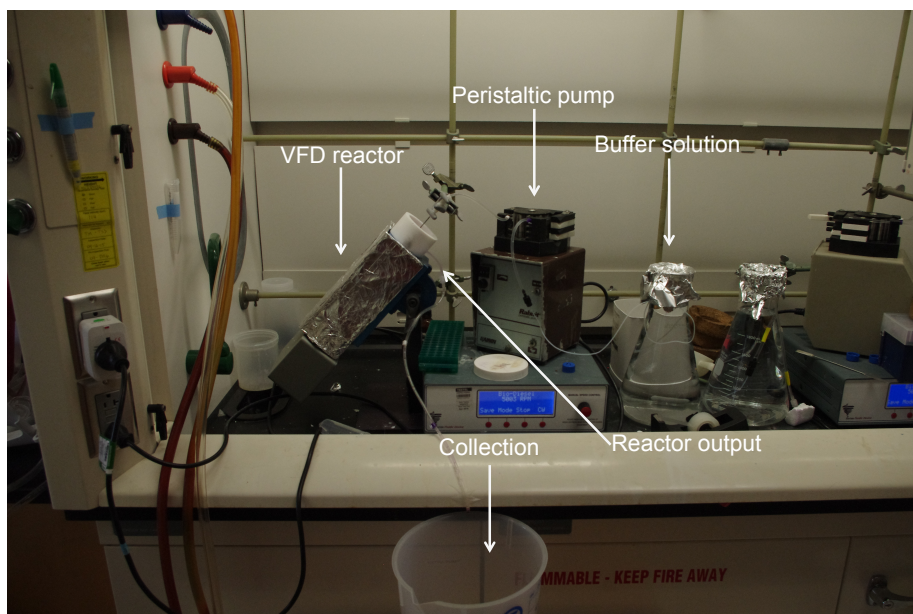


Figure S11 Experimental set up for VFD-mediated continuous flow.

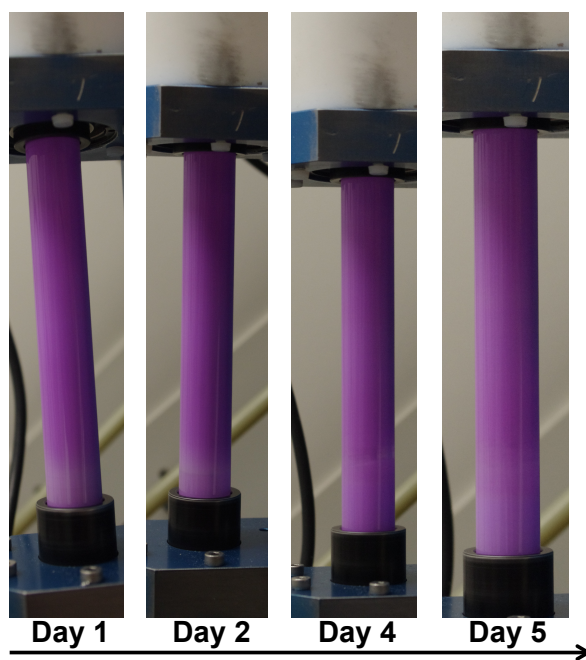


Figure S12 The stability of mCherry on the reactor surface over five days of continuous flow processing. In addition to visual inspection shown here, the concentration of leached and retained mCherry protein was determined as described above.

Creating Enzyme Zones on the Reactor Surface.

Spatially segregated enzyme zones can be created on the reactor surface. IMAC solution (3.00 mL per half a reactor, commercially available suspension in 20% aq. ethanol) and PBS (3.00 mL, pH 8.0) are added to a 15 mL reactor tube. The tube is shaken vigorously for one min, and

then centrifuged in an Eppendorf centrifuge at 1 krpm for five min. The supernatant is discarded, and $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ solution is added (10 mL, 100 mM). The tube was then placed on an orbital shaker, shaking at 225 rpm at 4 °C for two h to charge the IMAC resin. Next, the solution was then centrifuged at 1 krpm for five min. The supernatant was discarded and PBS (3.00 mL, pH 8.0) added. The tube was vigorously shaken for one min and then centrifuged for five min at 8 krpm. 95% of the supernatant was removed from the tube and the protein solution (4.00 mL, pH 8.0) added. For kinetic experiments using stripes, purified protein was used to gain accurate kinetic data. The suspension of IMAC resin is then resuspended by stirring with a spatula, and incubated at 4 °C on an orbital shaker for two h. Next, the tube is centrifuged at 1 krpm for five min before 85% of the supernatant is then discarded.

A sample tube is then inserted into the VFD and rotated at 8 krpm. The protein-bound IMAC was then added to the sample tube by a Pasteur pipette. The IMAC solution is added drop-by-drop to the required zone creating stripes down to a minimum of ~1 cm in width. For every 1 cm zone width, around 250 mg of enzyme-bound IMAC is used, as this creates sharp, distinguishable zones. However, for other patterns, a total of 6.00 g of IMAC coats a single sample tube. After the stripes have been added to the surface of the reactor, the reactor is spun for another ten min. During this time, the IMAC resin becomes a clay-like solid. Next, the jet feed is added down the center of the sample tube as detailed previously, and the reactor is ready for continuous flow biocatalysis.

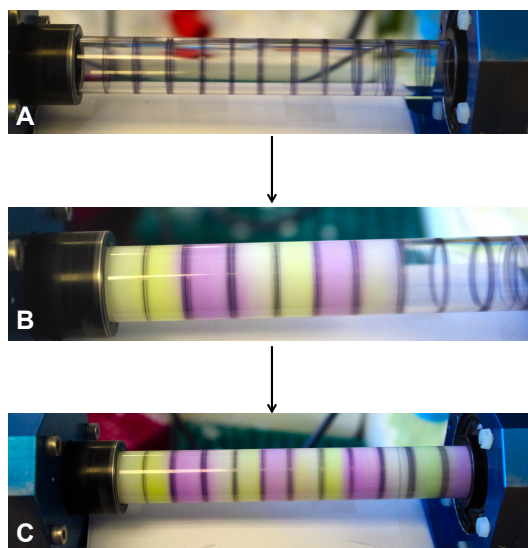


Figure S13 Creating enzymatic zones on the reactor surface. **A)** First, mark the zones with a marker pen onto the surface of the reactor. **B)** Bring the sample tube to 8 krpm, and then add the protein bound IMAC solution to the correct position in the reactor. **C)** Create the stripes by drop-by-drop addition with a Pasteur pipette starting with the bottom of the reactor.

Phosphodiesterase Activity Data

To optimize activity for recombinant expressed phosphodiesterase, enzyme-substrate solutions were subjected to variable reaction conditions including: enzyme concentration, reaction temperature and solvent compatibility. Below each experimental set-up is detailed and the result highlighted.

Phosphodiesterase concentration effects

Recombinantly expressed phosphodiesterase (2.00 mg mL^{-1}) was used to explore the enzyme's activity (Figure S14). For this experiment, the phosphodiesterase buffer was described above, omitting the acetonitrile and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$.

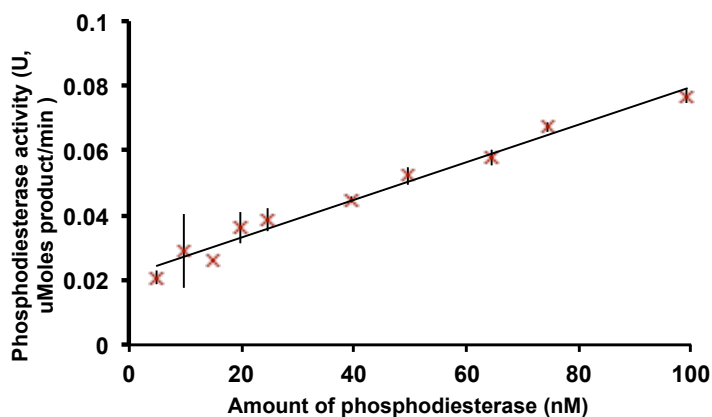


Figure S14 Monitoring the effect of phosphodiesterase quantity on the liberation of *p*-nitrophenol. In this experiment, $1000 \mu\text{L}$ of substrate solution (0.7 mM bis(*p*-nitrophenyl) phosphate sodium salt) was added to a 2.00 mL sample vial, and the solution was heated to $70 \text{ }^\circ\text{C}$ over five min. Next, the required amount of phosphodiesterase was added, and the reaction proceeded for ten min. After ten min, the reaction was quenched with $300 \mu\text{L}$ 4 M NaOH and immediately analyzed as described previously. Error is reported as standard deviation around the mean ($n=3$).

Phosphodiesterase reaction temperature effects

Recombinant expressed phosphodiesterase ($0.1 \mu\text{L}$, 2.00 mg/mL , 4.96 nM) was used to explore the enzyme's activity at various temperatures (Figure S15). For this experiment, the phosphodiesterase buffer was described above, omitting the acetonitrile and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$.

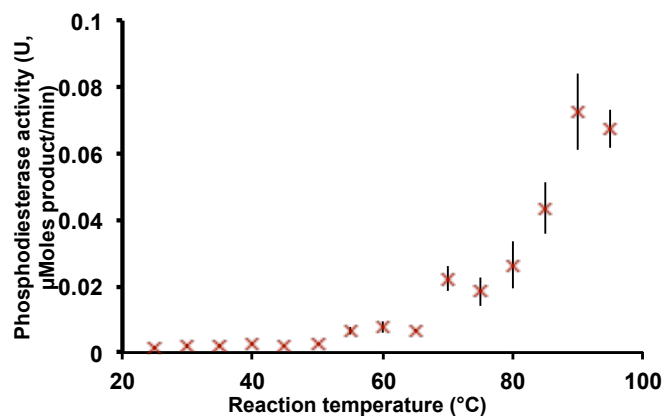


Figure S15 Monitoring the effect of reaction temperature on the liberation of *p*-nitrophenol. For this experiment, 1000 μL of substrate solution (0.7 mM bis(*p*-nitrophenyl) phosphate sodium salt) was added to a 2 mL sample vial, and heated to the indicated temperature over five min. Next, phosphodiesterase (4.96 nM) was added to the solution. After ten min, the reaction was quenched with 300 μL 4 M NaOH, and analyzed as previously described. Error is reported as standard deviation around the mean ($n=3$).

Phosphodiesterase solvent compatibility

To find conditions more appropriate for solubility of the enzyme substrate, recombinant phosphodiesterase (4.96 nM) was exposed to a range of organic solvents, and the enzyme's activity monitored (Figure S16). For this experiment, the buffer was the same as described above, but contained 5% (v:v) of the organic solvent to be tested, and omitted $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$.

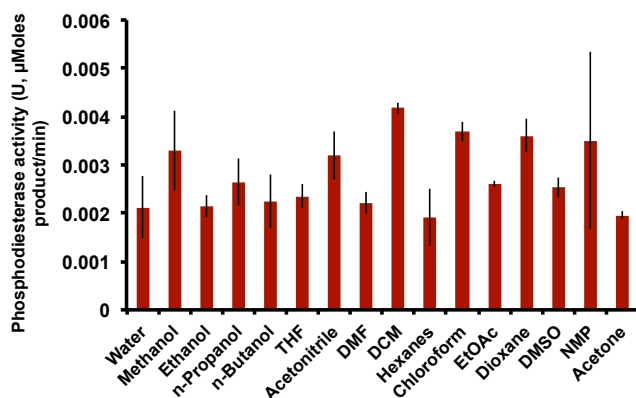


Figure S16 The effect of reaction temperature on the liberation of *p*-nitrophenol. For this experiment, 1000 μL of substrate solution (0.7 mM bis(*p*-nitrophenyl) phosphate sodium salt and 5% of the desired organic solvent) was added to a 2.00 mL sample vial, and was heated to the 70 $^{\circ}\text{C}$ for five min. Once the temperature had been reached, phosphodiesterase (4.96 nM) was added to the solution. After ten min, the reaction was quenched with 300 μL 4 M NaOH and immediately analyzed as described previously. Error is reported as standard deviation around the mean ($n=3$). Hexanes indicates a mixture of hexane isomers.

Phosphodiesterase activity in varying levels of acetonitrile

Recombinant phosphodiesterase (4.96 nM) was used to explore the enzyme's compatibility with a range of acetonitrile concentrations (Figure S17). For this experiment the buffer was as described above, but with varying added quantities of acetonitrile and omitting $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$.

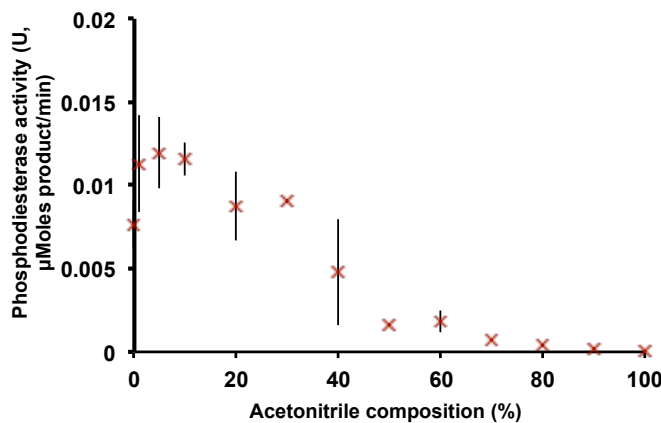


Figure S17 Monitoring the effects acetonitrile composition on product formation during phosphodiesterase catalysis. For this experiment, 1000 μL of substrate solution (0.7 mM bis(p-nitrophenyl) phosphate sodium salt and the indicated levels of acetonitrile) were added to a 2.00 mL sample vial and heated to 70 $^{\circ}\text{C}$ over five min. Once the temperature had been reached, phosphodiesterase (4.96 nM) was added to the solution. After ten min, the reaction was quenched with 300 μL 4 M NaOH, and immediately analyzed as described previously. Error is reported as standard deviation around the mean (n=3).

Phosphodiesterase activity profiles in varying levels of acetonitrile

Recombinant phosphodiesterase (4.96 nM) was used to explore the enzyme's activity profile with higher concentrations of acetonitrile (Figure S18). For this experiment the buffer was as described above, but with various amounts of acetonitrile and omitting $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$. Longer reaction times were needed for effective conversion.

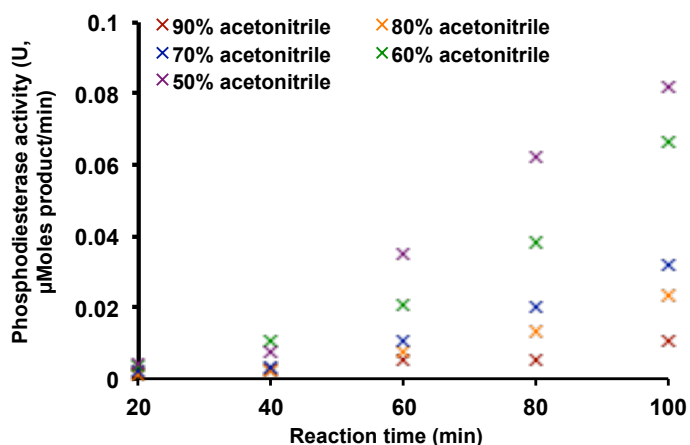


Figure S18 Monitoring the reaction profile for phosphodiesterase in varying higher acetonitrile compositions. For this experiment, 1000 μL of substrate solution (0.7 mM bis(*p*-nitrophenyl) phosphate sodium salt, and the indicated levels of acetonitrile) were added to a 2.00 mL sample vial, and heated to 70 $^{\circ}\text{C}$ over five min. Once the temperature had been reached, phosphodiesterase (4.96 nM) was added to the solution. After ten min, the reaction was quenched with 300 μL 4 M NaOH, and immediately analyzed as described previously. Error is reported as standard deviation around the mean ($n=3$).

Alkaline Phosphatase Activity Data

To optimize activity of recombinant alkaline phosphatase, enzyme-substrate solutions were subjected to various reaction conditions including enzyme concentration, reaction temperature and metal salt preference. Each experimental set-up is detailed below, and the results highlighted.

Alkaline phosphatase activity at various reaction temperatures

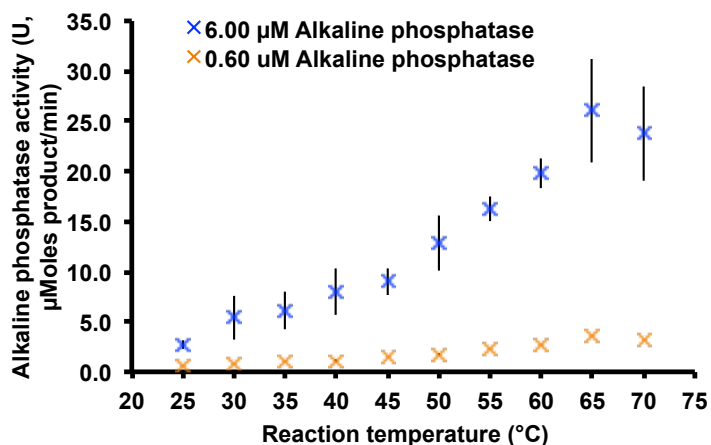


Figure S19 Monitoring the effect of reaction temperature on the activity of alkaline phosphatase. For this experiment, 1000 μL of substrate solution (0.7 mM *p*-nitrophenyl phosphate sodium salt) was added to a 2.00 mL sample vial and allowed to warm to the required temperature over five min. Next, 50 μL of either 6.00 μM or 0.60 μM of alkaline phosphatase was added to the substrate solution and left to react for five min. After the reaction time had ended, the reaction was quenched through addition of 200 μL of 4 M NaOH, and the sample analyzed as described above. Error is reported as standard deviation around the mean ($n=3$).

Alkaline phosphatase activity in various compositions of acetonitrile

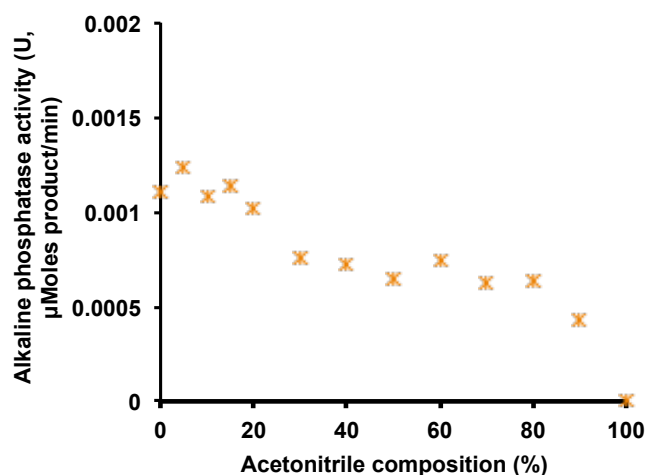


Figure S20 Monitoring the effect of buffer-acetonitrile composition on the activity of alkaline phosphatase. For this experiment, 1000 μL of substrate solution (*p*-nitrophenyl phosphate sodium salt and 0 - 100% acetonitrile) was added to a 2.00 mL sample vial and warmed to 65 $^{\circ}\text{C}$ over three min. Next, 50 μL of 6.0 μM alkaline phosphatase was added, and the reaction proceeded for five min. A control reaction omitting the enzyme was performed under identical conditions to serve as a negative control. The reaction and the negative control were then quenched through addition of 200 μL of 4 M NaOH, and the sample as previously described. Error is reported as standard deviation around the mean ($n=3$).

Alkaline Phosphatase and Phosphodiesterase Comparative Data

To achieve efficient multistep activity, reaction temperature and the metal dependency of the two enzymes were taken into consideration. The data below depict the effects of variable temperature and the concentrations of Ni^{2+} , Mg^{2+} , or a combination of the two on the activity of alkaline phosphatase and phosphodiesterase.

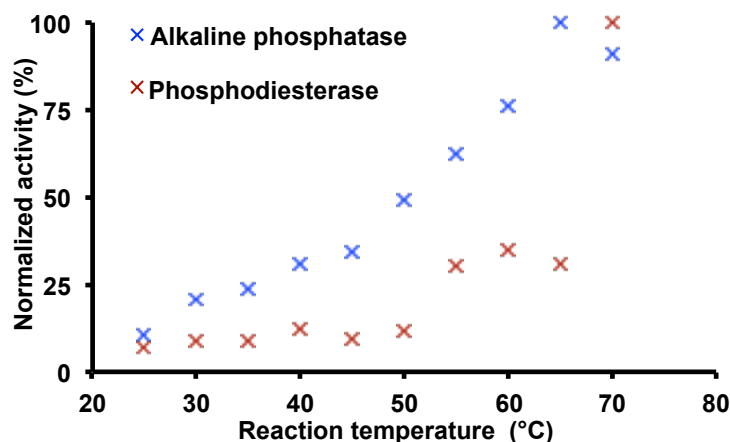


Figure S21 The effect of reaction temperature on phosphodiesterase and alkaline phosphatase. This graph illustrates that both enzymes function more efficiently at higher temperatures. For the experiments discussed in the manuscript, a reaction temperature of 65 °C was used. Though a reaction temperature of 70 °C provides superior activity for phosphodiesterase, the rate limiting enzyme in the dual enzyme process is alkaline phosphatase. Thus, reaction conditions optimal for alkaline phosphatase were selected.

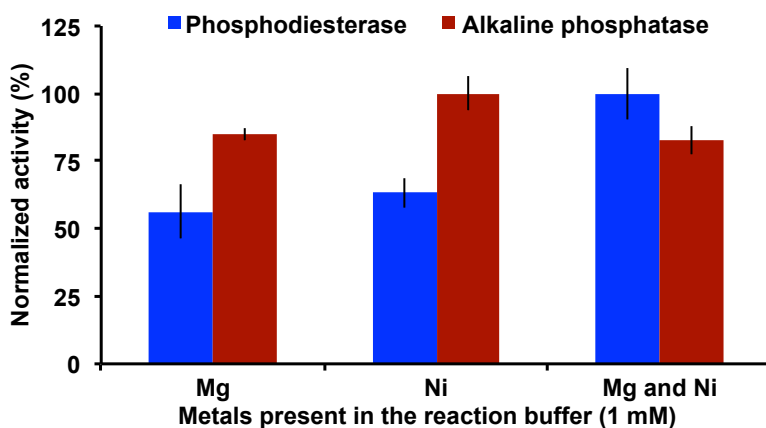


Figure S22 The effect of different metal ions present in the reaction buffer on the activity of phosphodiesterase and alkaline phosphatase. This experiment demonstrates the greater sensitivity of phosphodiesterase on metal ion composition with 1 mM Ni²⁺ and 1 mM Mg²⁺ greatly improving activity. Since alkaline phosphates displays little sensitivity to this variable, the mixture of the two ions was chosen.

Michaelis-Menten Kinetics

To achieve effective multistep activity in continuous flow, both alkaline phosphatase and phosphodiesterase should ideally operate at the same rate. First, the catalytic performance of each protein (k_{cat}/K_m) under theoretical limiting conditions was derived (Figure S23). The alkaline phosphatase reaction mixture contained one equivalent of *p*-nitrophenol, as this is the theoretical maximum of *p*-nitrophenol present in the system during the multistep reaction. After deriving the k_{cat}/K_m values for both proteins, these values are compared, and the concentration of both enzymes adjusted to ensure that zones for the two enzymes can operate at the same catalytic rate. This result was experimentally verified, as a two-fold increase in the amount of *p*-nitrophenol liberated was observed for the reactor with stripes of both enzymes (Figure 2).

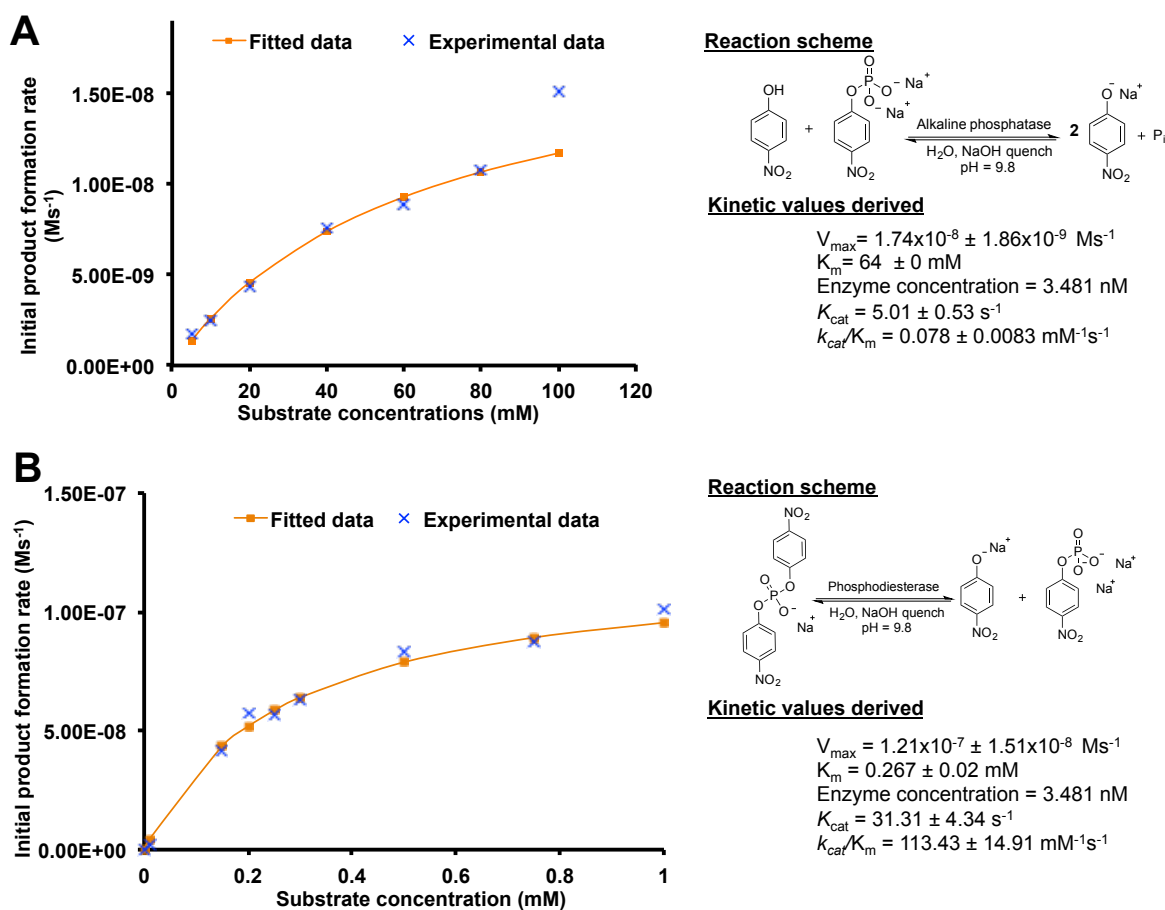


Figure S23 Michaelis-Menten plots for alkaline phosphatase and phosphodiesterase. A)

The Michaelis-Menten values for recombinantly overexpressed alkaline phosphatase identified the dramatic impact of product inhibition by *p*-nitrophenol. **B)** As shown by its k_{cat}/K_m value phosphodiesterase performs 1450-fold faster than alkaline phosphatase under these conditions. Therefore, alkaline phosphatase must be at 1450 greater concentration than phosphodiesterase to achieve similar activity and an efficient multistep substrate conversion. The assay conditions were as follows. A solution of enzyme (100 μL) at the indicated concentrations was heated at 65 $^\circ\text{C}$ for five min, and 900 μL of the substrate solution added. Both reactions were transferred to an ice bath after the reaction to quench the reaction. Then, 100 μL of the reaction solution was added to a 96-well microtiter plate and analyzed as previously described. A non-linear regression analysis fitted the experimental data directly to a Michaelis-Menten curve. Error is reported as the maximum and minimum around the mean ($n=3$).

Multistep Synthesis with Alkaline Phosphatase and Phosphodiesterase

To achieve the multistep activity as demonstrated in Figure 2 of the manuscript, enzymatic-IMAC zones containing phosphodiesterase and alkaline phosphatase were applied to the surface of the reactor as described above. In this experiment, the required concentration of alkaline

phosphatase and phosphodiesterase is first bound to 3.00 mL of homogenous IMAC, and then subsequently added to the reactor surface.

In this procedure, homogenous IMAC resin (3.00 mL) was added to a 15 mL falcon tube. After centrifugation at 1 krpm for five min, the supernatant was removed and $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ solution (3.00 mL, 100 mM) added. This falcon tube was subjected to agitation on an orbital shaker for two h at 4 °C. Next, the falcon tube was centrifuged at 1 krpm for five min. The supernatant was discarded and PBS was added (3.00 mL). The falcon tube was inverted several times, centrifuged at 1 krpm for five min, and then the supernatant removed. This process was repeated three times per falcon tube. After the last volume of PBS had been removed, the IMAC resin was considered charged and ready to bind to the protein of interest.

To a smaller tube (5 mL) was added either phosphodiesterase (5 μL of a 635 $\mu\text{g}/\text{mL}$ protein solution) and enzyme buffer (3.95 mL) to afford a solution with a final concentration of 0.793 $\mu\text{g}/\text{mL}$, or alkaline phosphatase (2.80 mL of a 1638 $\mu\text{g}/\text{mL}$ protein solution) and reaction buffer (1.20 mL) to afford a 4.00 mL solution with a final concentration of 1150 $\mu\text{g}/\text{mL}$. These concentrations were determined by the Michaelis-Menten kinetics described above. The protein solutions (4.00 mL) were added to the falcon tube containing the charged IMAC resin and then subjected to gentle agitation on an orbital shaker for two h at 4 °C. After this time, the falcon tubes were centrifuged at 1 krpm for five min, and 85% of the supernatant was discarded and the remaining slurry stirred until homogenous. Depending upon how many zones were required for each enzyme (2,4,6 or 8 etc.), the reactor was split up into zones of equal length. The protein-IMAC solution was then applied to these zones as described above in Figure S13. In order ensure equal distribution, the 3.00 mL of IMAC-enzyme solution was evenly distributed between each of the zones.

To test the efficiency of the enzyme zones, bis(PNNP) (2.0 mM) was flowed through the reactor at a flow rate of 0.50 mL min^{-1} and at a reactor temperature of 65 °C. The first 6.8 mL of solution exiting the reactor was discarded to allow the system to reach an equilibrium. After this point, small aliquots (1.30 mL) were collected and quenched with NaOH (300 μL) as described above. Ten aliquots are collected and analyzed as described above. Error is reported as standard deviation around the mean (n=10)

References

- [1] J. E. Diaz, C.-S. Lin, K. Kunishiro, B. K. Feld, S. K. Avrantinis, J. Bronson, J. Greaves, J. G. Saven, G. A. Weiss, *Protein Science* **2011**, *20*, 1597-1606.