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Supplementary Information

Sustainable chemo-enzymatic NADP(H) synthesis from biomass-derived xylose, polyphosphate, and nicotinamide

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Supplementary Materials and Methods

Heterologous protein expression and purification

For standard expression and purification of recombinant enzymes, recombinant probiotic *Escherichia coli* Nissle cultures were cultivated under aerobic conditions at 37℃ in a shaker containing Terrific Broth at 200 rpm until a specific optical density was reached ($OD_{600\ nm}$ 0.6). Protein overexpression was induced with isopropyl β-D-1-thiogalactopyranoside (0.1 mM), and the culture was then incubated overnight at 16℃ while agitating at 200 rpm. Recombinant proteins were purified to at least 95% homogeneity using immobilized metal affinity chromatography as previously described [1]. In brief, cells were collected by centrifugation at 8,000 × g for 10 min at 4℃ and resuspended in binding buffer ((4-(2-hydroxyethyl)-L-piperazineethanesulfonic acid) (HEPES)-Na (pH 7.5; 25 mM), NaCl (0.3 M), $MgCl_2$ (1 mM), glycerol (5%, v/v), and imidazole (5 mM)). The cells were then homogenized *via* sonication in an ice bath for 10 min (1 s on and 2 s off). The lysate was centrifuged at 20,000 × g for 20 min at 4℃, and the supernatant was passed through a glass gravity column containing 0.5 mL Ni Sepharose™ High-Performance resin (GE Healthcare; Chicago, IL, USA). After washing with wash buffer (HEPES-Na (pH 7.5; 25 mM), NaCl (0.3 M), $MgCl_2$ (1 mM), glycerol (5%, v/v), and imidazole (25 mM)), proteins were eluted with 0.4 mL of elution buffer (HEPES-Na (pH 7.5; 25 mM), NaCl (0.3 M), $MgCl_2$ (1 mM), glycerol (5%, v/v), and imidazole (300 mM)). Protein purity was then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (**Figure S6**). The protein stock solutions were flash-frozen in liquid nitrogen, stored at −80℃, and thawed immediately before use. The N-His_6 tag was retained for all experiments.

Preparation of sugarcane bagasse-derived crude xylose

Sugarcane bagasse-derived xylose was prepared by following the previous report[2]. Briefly, sugarcane bagasse was collected from a local vendor (Zhongli Tourist Night Market, Taoyuan, Taiwan). The bagasse ash was first heat-dried at 75℃ for five days. The dried bagasse ash was then ground into small fragments, and then 25 g of the ash was hydrolyzed within reaction mixtures (500 mL) containing HCl (3%, v/v). The reaction mixtures were incubated at 121℃ for 42 min, followed by titration with NaOH (1 N) to pH 7.0. The bagasse hydrolysate was then centrifuged at 4,000 × g for 20 min at 25℃, and the supernatant was concentrated by a rotary evaporator under 190 hPa atmosphere and in a constant temperature oil bath (Tokyo Rikakikai Co., Ltd.; Tokyo,

Japan) at 65°C. The xylose-rich supernatant was filter-sterilized using a 0.2 μm Supor® membrane syringe filter (Pall Life Sciences; Port Washington, NY, USA) and was stored at 4°C until use. The concentrations of xylose contained in the hydrolysates were then estimated by measuring $\lambda_{553\ nm}$ *via* UV-Vis scanning on a SpectraMax® iD3 microplate reader (Molecular Devices; San Jose, CA, USA)[3]. Subsequently, the acquired $\lambda_{553\ nm}$ values were then converted to the xylose titers based on a standard curve derived from the varying concentrations of the corresponding xylose (ranging from 0.3–5.0 mM; **Figure S7**). The produced crude xylose was then used as a substrate for subsequent experiments.

Microalgal polyP synthesis and purification

Microalgae *Chlorella vulgaris* (*C. vulgaris*) was purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and used for polyP synthesis as previously described[4]. Briefly, *C. vulgaris* was cultivated in 2 L Erlenmeyer flasks containing sterilized wastewater (1 L; pH adjusted to neutral) at room temperature with continuous shaking (200 rpm). The *C. vulgaris* biomass was collected by centrifugation at 4430 × g for 10 min at room temperature and then resuspended in a buffer containing HEPES-KOH (pH 7.0; 20 mM), KCl (0.15 M), and ethylenediaminetetraacetic acid (EDTA) (5 mM) at a pellet to buffer ratio of 1 : 3. The cells were lysed *via* ultrasonication for 20 min (3 s on and 3 s off) and the cell-lysate containing polyP was subsequently incubated at 100 °C for 10 min, followed by centrifugation at 8000 × g for 3 min at room temperature to separate the cell debris from the supernatant containing the polyP. The polyP concentration within the supernatant was then quantified by the TBO method (see below). The supernatant containing polyP was stored at −80 °C for further use in subsequent experiments.

Quantification of polyphosphate (polyP) using the toluidine blue O (TBO) assay

ATP regeneration was verified by monitoring polyP consumption as described previously[5]. PolyP was quantified through a metachromatic assay, which is based on the concentration-dependent decrease in λ_{630nm} due to the metachromatic reaction between TBO and polyP. Briefly, a sample (2.5 μL) was added to the TBO solution (250 μL; 15 μg/mL) containing acetic acid (0.1 N) in a 96-well microplate, and the mixture was allowed to react at room temperature. The λ_{630nm} was then measured by a SpectraMax® iD3 microplate reader (Molecular Devices; San Jose, CA, USA). The standard curve was established using varying concentrations of commercial polyP (sodium polyP (25-mer)) (ranging from 8.0–20 mM or 0.5–8.0 mM; **Figure S8**).

High-performance liquid chromatography (HPLC) analysis

(i) D-xylulose-5-phosphate (Xu5P) analysis

The completed reactions of enzymatic synthesis of Xu5P were collected and filtered through the Amicon® Ultra-0.5 centrifugal filter (cut-off: 3 kDa) (Merck KGaA; Darmstadt, Germany), and the flowthrough fractions were used for HPLC analysis by following a protocol described previously[6]. The Xu5P was separated on a 7.8 × 300 mm Aminex HPX-87H Column (Shimadzu; Nakagyo-ku, Kyoto, Japan) with the HPLC system (HITACHI Ltd.; Chiyoda-ku, Tokyo, Japan), and an autosampler all controlled by a ChromNAV Ver.2.04.05 software system (JASCO; Hachioji, Tokyo, Japan) for chromatographic analysis (**Figure S8A**). The mobile phase consisted of H_2SO_4 (5 mM). The samples were diluted with H_2SO_4 (5 mM) using a 0.22-μm hydrophilic polytetrafluoroethylene (PTFE) syringe filter (ANPEL, Shanghai, China). The total regeneration time for the HPLC column is 30 min. Aliquots of 10 μL were injected into the instrument by autosampler, and the subsequent elution was performed at a flow rate of 0.6 mL/min. Subsequently, the acquired peak areas were converted to the respective Xu5P concentrations based on a standard curve derived from the varying concentrations of the corresponding Xu5P (**Figure S9**).

(ii) NAD(H) and NADP(H) analysis

The completed reactions of the one-pot NAD(P)H synthesis were collected and filtered through the Amicon® Ultra-0.5 centrifugal filter (cut-off: 3 kDa) (Merck KGaA; Darmstadt, Hesse, Germany), and the flowthrough fractions were used for HPLC analysis by following a protocol described previously[7]. The NAD(P)(H) were separated on a 150 mm × 4.60 mm Luna® C18 column packed with 5-μm particles (Phenomenex; Torrance, CA, USA) with the HPLC system (HITACHI Ltd.; Chiyoda-ku, Tokyo, Japan), an MD4010 photodiode array detector (JASCO; Hachioji, Tokyo, Japan) and an autosampler all controlled by a ChromNAV Ver.2.04.05 software system (JASCO; Hachioji, Tokyo, Japan) for chromatographic analysis (**Figure S8B**). The $\lambda_{340\,nm}$, indicative of NAD(P)H, was simultaneously recorded during the separation while the $\lambda_{254\,nm}$ was recorded for $NAD(P)^+$ detection. The mobile phase consisted of buffer A (potassium dihydrogen phosphate (pH 5.0; 20 mM)) and buffer B (acetonitrile (10%, v/v), potassium dihydrogen phosphate (pH 5.0; 200 mM)). The pH of buffer A was adjusted with phosphoric acid (10 M), and both buffers were filtered through a 0.22-μm membrane filter. The total regeneration time for the HPLC column is 50 min. Aliquots of 10 μL were injected into the instrument by autosampler, and

the subsequent elution was performed at a flow rate of 0.4 mL/min. All gradient changes were linear. The gradient conditions were as follows: initial conditions are 100% buffer A; from 25.5–30 min, the gradient changes to 50% buffer A; from 30 to 33 min, the gradient changes to 0% buffer A; from 33 to 50 min, the gradient changes to 100% buffer A and remains at this condition until the next injection. The column is equilibrated in 100% buffer A for 50 min before the next sample injection. Subsequently, the acquired peak areas were converted to the respective NAD(P)(H) concentrations based on a standard curve derived from the varying concentrations of the corresponding NAD(P)H (**Figure S9**).

(iii) Tetrahydrofolate (THF) analysis

The completed reactions of the one-pot THF synthesis were collected and filtered through the Amicon® Ultra-0.5 centrifugal filter (cut-off: 3 kDa) (Merck KGaA; Darmstadt, Hesse, Germany), and the flowthrough fractions were used for HPLC analysis by following a protocol described previously[8]. The THF was separated on a 150 mm × 4.60 mm Luna® C18 column packed with 5-µm particles (Phenomenex; Torrance, CA, USA) with the HPLC system (HITACHI Ltd.; Chiyoda-ku, Tokyo, Japan), an MD4010 photodiode array detector (JASCO; Hachioji, Tokyo, Japan) and an autosampler all controlled by a ChromNAV Ver.2.04.05 software system (JASCO; Hachioji, Tokyo, Japan) for chromatographic analysis (**Figure S8C**). The $\lambda_{290\ nm}$, indicative of folate compounds, was simultaneously recorded during the separation. The mobile phase consisted of buffer A (acetonitrile) and buffer B (PBS (pH 2.2; 30 mM)). Both buffers were filtered through a 0.22-µm membrane filter. The total regeneration time for the HPLC column is 36 min. Aliquots of 10 µL were injected into the instrument by autosampler, and the subsequent elution was performed at a flow rate of 0.8 mL/min. All gradient changes were linear unless stated. The gradient conditions were as follows: initial conditions are 5% buffer A (isocractic flow); from 8.0 to 23 min, the gradient changes to 24% buffer A; from 23 to 28 min, the gradient changes to 5% buffer A; from 33 to 50 min, the gradient changes to 5% buffer A and remains at this condition until the next injection. The column is equilibrated in 5% buffer A for 50 min before the next sample injection. Subsequently, the acquired peak areas were converted to the respective THF and folic acid (FA) concentrations based on a standard curve derived from the varying concentrations of the corresponding standards (**Figure S10**).

Supplementary Figures

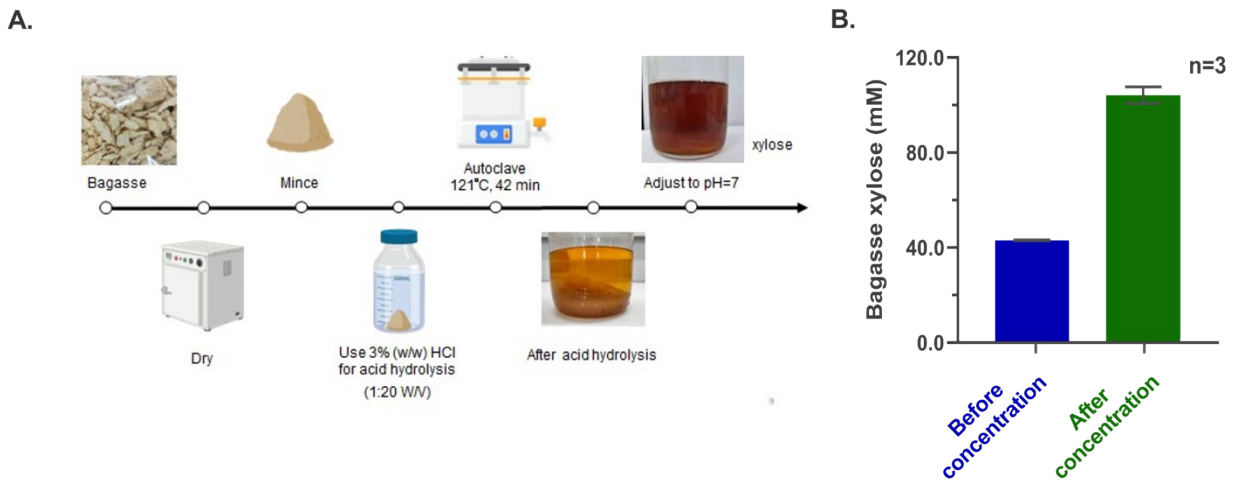

Figure S1. Sugarcane bagasse-derived crude xylose production *via* thermal acidic hydrolysis. **(A)** Schematic diagram of crude xylose production. **(B)** The xylose titers in the sugarcane bagasse hydrolysates before and after concentration were estimated from the standard curves established by measuring the $\lambda_{553\ nm}$ of varying concentrations of xylose standards (ranging from 0.3–5.0 mM; **Figure S10**). The bars and columns represent the standard deviations and means of three independent experimental replicates, respectively.

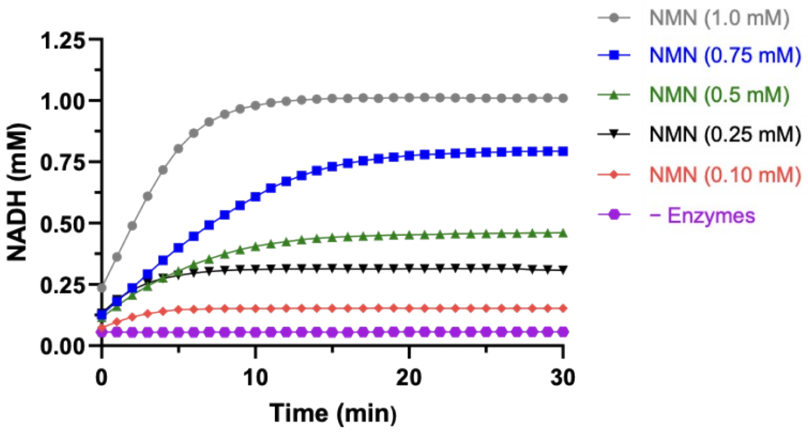

Figure S2. Enzymatic synthesis of reduced nicotinamide adenine dinucleotide (NADH) from nicotinamide mononucleotide (NMN). (A) Time-dependent analysis of NADH synthesis *via* an optical assay ($\lambda_{340\ nm}$). The reactions were conducted with varying concentrations of NMN (ranging

from 0.1–1.0 mM). The NADH concentrations were estimated from the standard curves established by measuring the $\lambda_{340\,nm}$ of varying NADH concentrations (ranging from 0.10–2.0 mM; **Figure S9**).

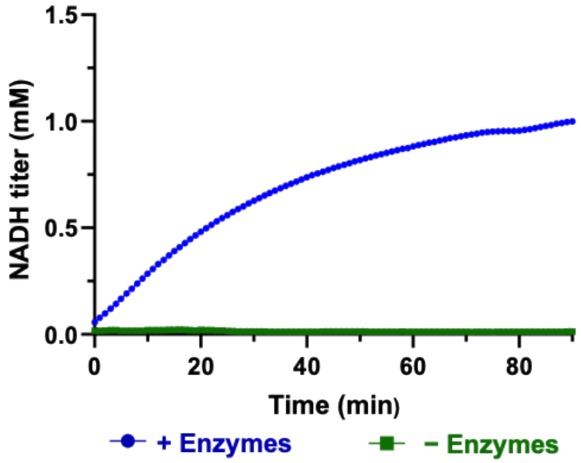

Figure S3. One-pot enzymatic synthesis of reduced nicotinamide adenine dinucleotide (NADH) from ribose-5-phosphate (R5P). Time-dependent analysis of NADH synthesis *via* an optical assay ($\lambda_{340\,nm}$). The reactions were conducted with or without all required enzymes. The NADH concentrations were estimated from the standard curves established by measuring the $\lambda_{340\,nm}$ of varying concentrations of NADH standards (ranging from 0.12–2.0 mM; **Figure S9**).

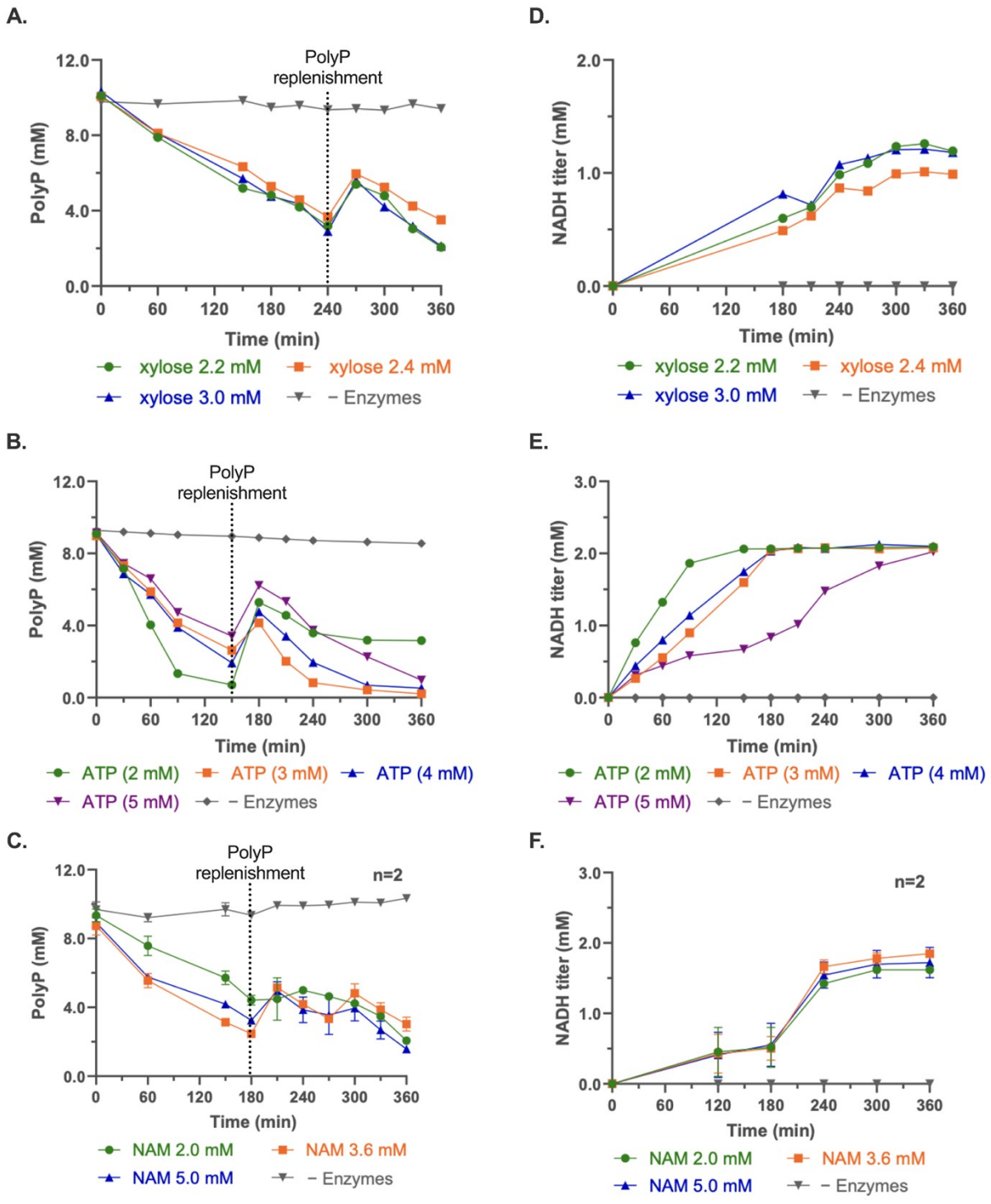

Figure S4. Time-dependent analysis of polyphosphate (polyP) consumption and nicotinamide adenine dinucleotide (NADH) synthesis in the one-pot, one-step enzymatic synthesis of NADH from commercial xylose with varying concentrations of xylose, ATP, or nicotinamide (NAM). (A–C) Time-dependent analysis of polyP consumption *via* the toluidine

blue O (TBO) assay during the one-pot, one-step enzymatic NADH synthesis reaction. The concentrations of polyP were estimated from the standard curves established *via* TBO assay with varying concentrations of polyP standards (ranging from 0.5–20.0 mM; **Figure S7**). Dashed lines represent the time when polyP was replenished. **(D–F)** Time-dependent analysis of NADH synthesis *via* an optical assay ($\lambda_{340\ nm}$). The NADH titers were estimated from the standard curves established by measuring the $\lambda_{340\ nm}$ of varying concentrations of NADH standards (ranging from 0.12–2.0 mM; **Figure S7**). The reactions were conducted (A, D) with varying xylose concentrations, (B, E) with varying ATP concentrations (ranging from 2.0–5.0 mM), or (C, F) with varying nicotinamide concentrations (ranging from 2.0–5.0 mM) as well as without all required enzymes.

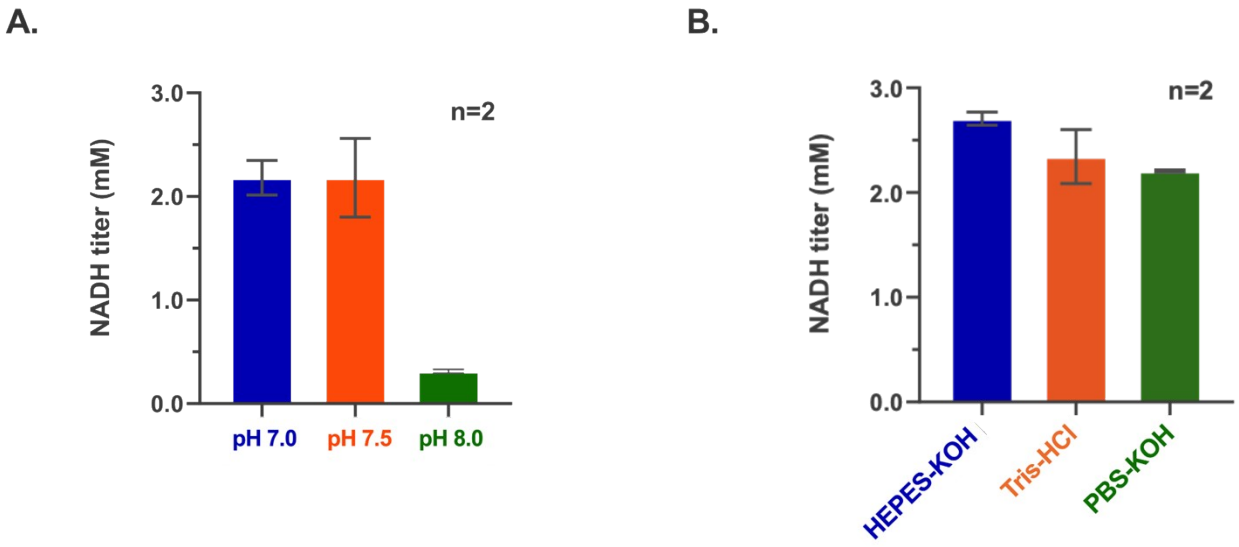

Figure S5. Optimization of one-pot, one-step enzymatic synthesis of nicotinamide adenine dinucleotide (NADH) from commercial xylose. (A, B) The NADH titers in the enzymatic synthesis reactions were estimated from the standard curves established *via* high-performance liquid chromatography (HPLC) analysis by measuring the peak areas of varying concentrations of NADH standards (ranging from 0.12–2.0 mM; **Figure S9**). The reactions were conducted in (A) varying values of pH or (B) different buffer compositions (HEPES-KOH, PBS-KOH, or Tris-HCl; pH 7.5). The bars and columns represent the ranges and averages of two independent experimental replicates, respectively.

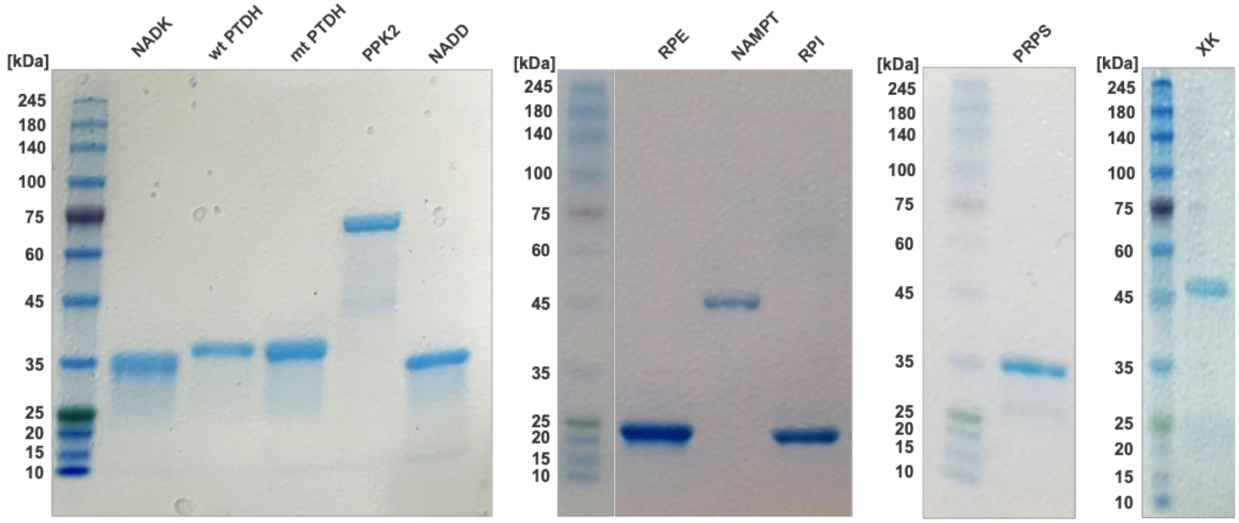

Figure S6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel images of purified recombinant proteins. Nicotinamide adenine dinucleotide kinase (NADK; 34 kDa), nicotinamide phosphoribosyltransferase (NAMPT; 54 kDa), nicotinamide mononucleotide adenylyltransferase (NMNAT; 33 kDa), polyphosphate kinase 2 (PPK2; 72 kDa), phosphoribosyl pyrophosphate synthetase (PRPS; 36 kDa), phosphite dehydrogenase (wild-type (wt PTDH) and mutant-type (mt PTDH); both 38 kDa), ribulose-phosphate 3-epimerase (RPE; 24 kDa), ribose-5-phosphate isomerase (RPI; 24 kDa), and xylulokinase (XK; 55 kDa)

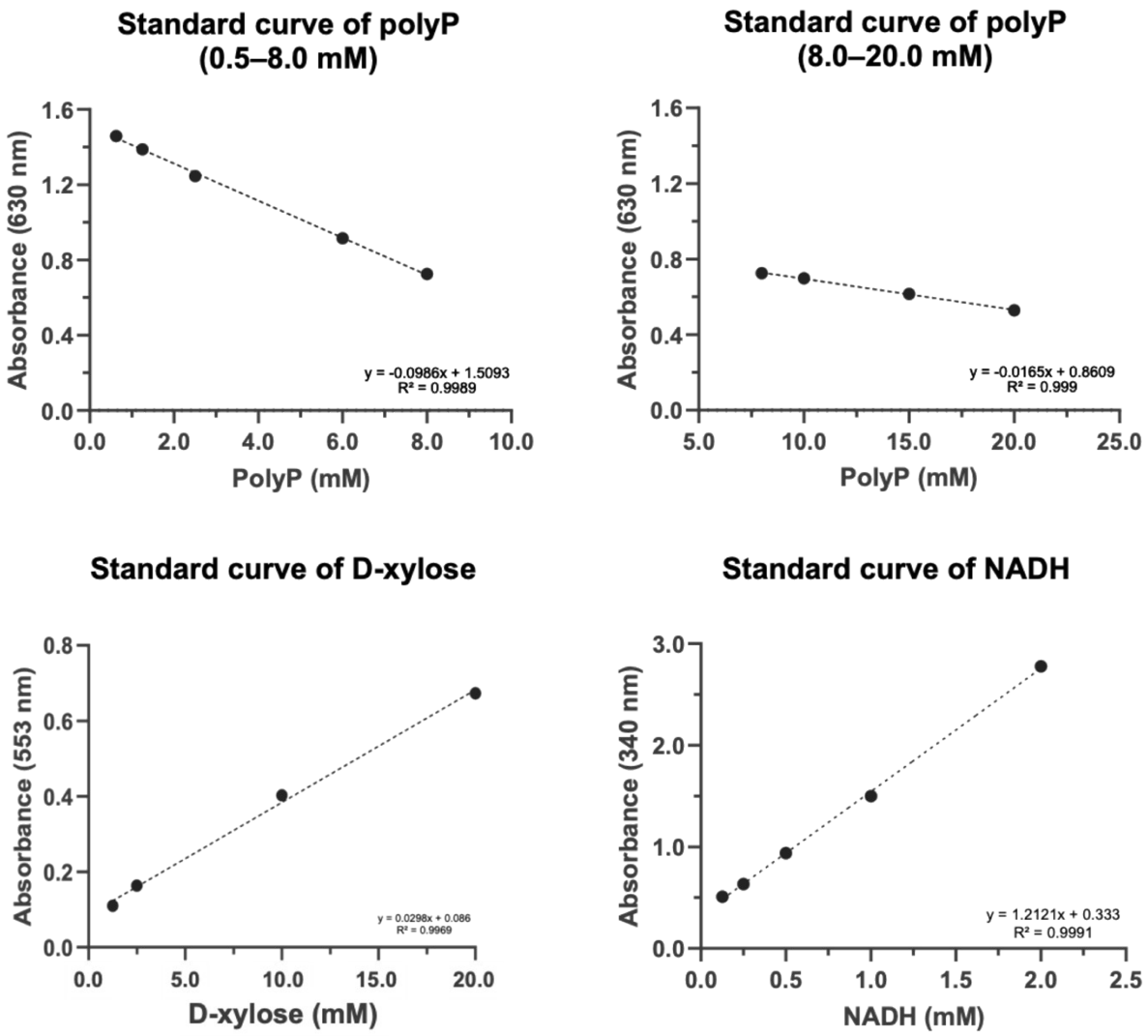

Figure S7. Standard curves of polyphosphate (polyP), xylose, and reduced nicotinamide adenine dinucleotide phosphate (NADH) determined by UV-Vis analysis.

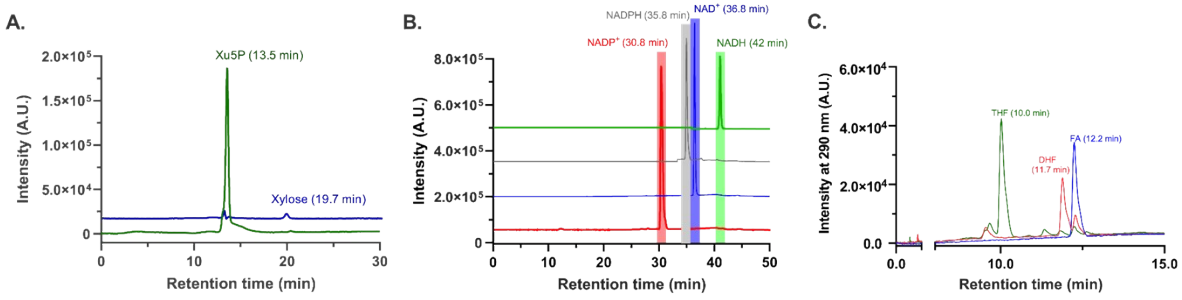

Figure S8. High-performance liquid chromatography (HPLC) chromatograms of dihydrofolate (DHF), folic acid (FA), nicotinamide adenine dinucleotide (NAD(H)), nicotinamide adenine dinucleotide phosphate (NADP(H)), tetrahydrofolate (THF), xylose, and D-xylulose-5-phosphate (Xu5P). (A) HPLC chromatograms of xylose (19.7 min) and Xu5P (13.5 min). **(B)** HPLC chromatograms of NAD^+ (36.8 min), $NADP^+$ (30.8 min), NADH (42 min) and NADPH (35.8 min). **(C)** HPLC chromatograms of DHF (11.7 min), FA (12.2 min), and THF (10.0 min).

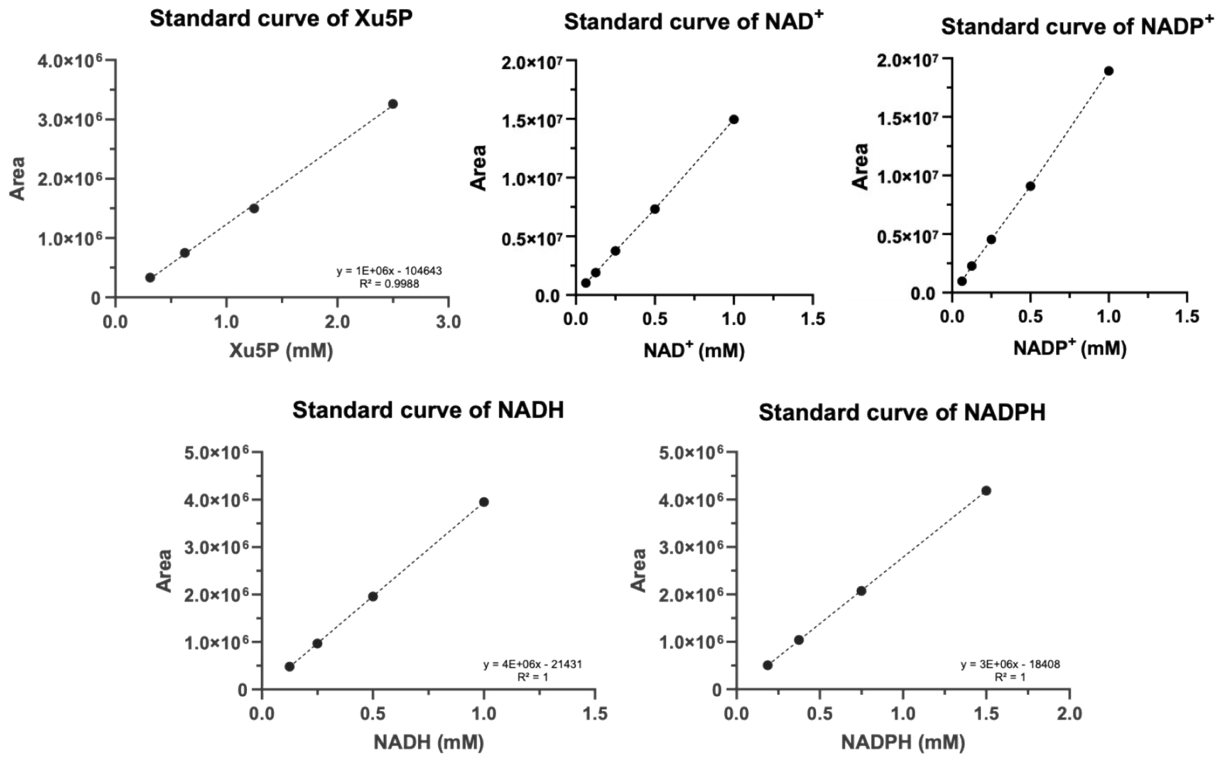

Figure S9. Standard curves of D-xylulose-5-phosphate (Xu5P; 13.5 min), nicotinamide adenine dinucleotide (NAD^+; 36.8 min), nicotinamide adenine dinucleotide phosphate ($NADP^+$; 30.8 min), reduced NAD^+ (NADH; 42 min), and reduced $NADP^+$ (NADPH; 35.8 min) determined by high-performance liquid chromatography (HPLC) analysis.

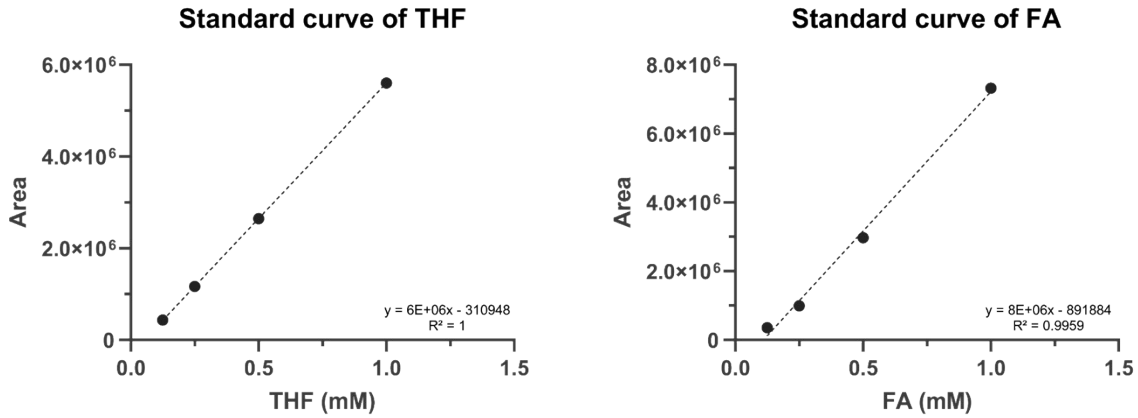

Figure S10. Standard curves of tetrahydrofolate (THF; 10.0 min) and folic acid (FA; 12.2 min) determined by high-performance liquid chromatography (HPLC) analysis.

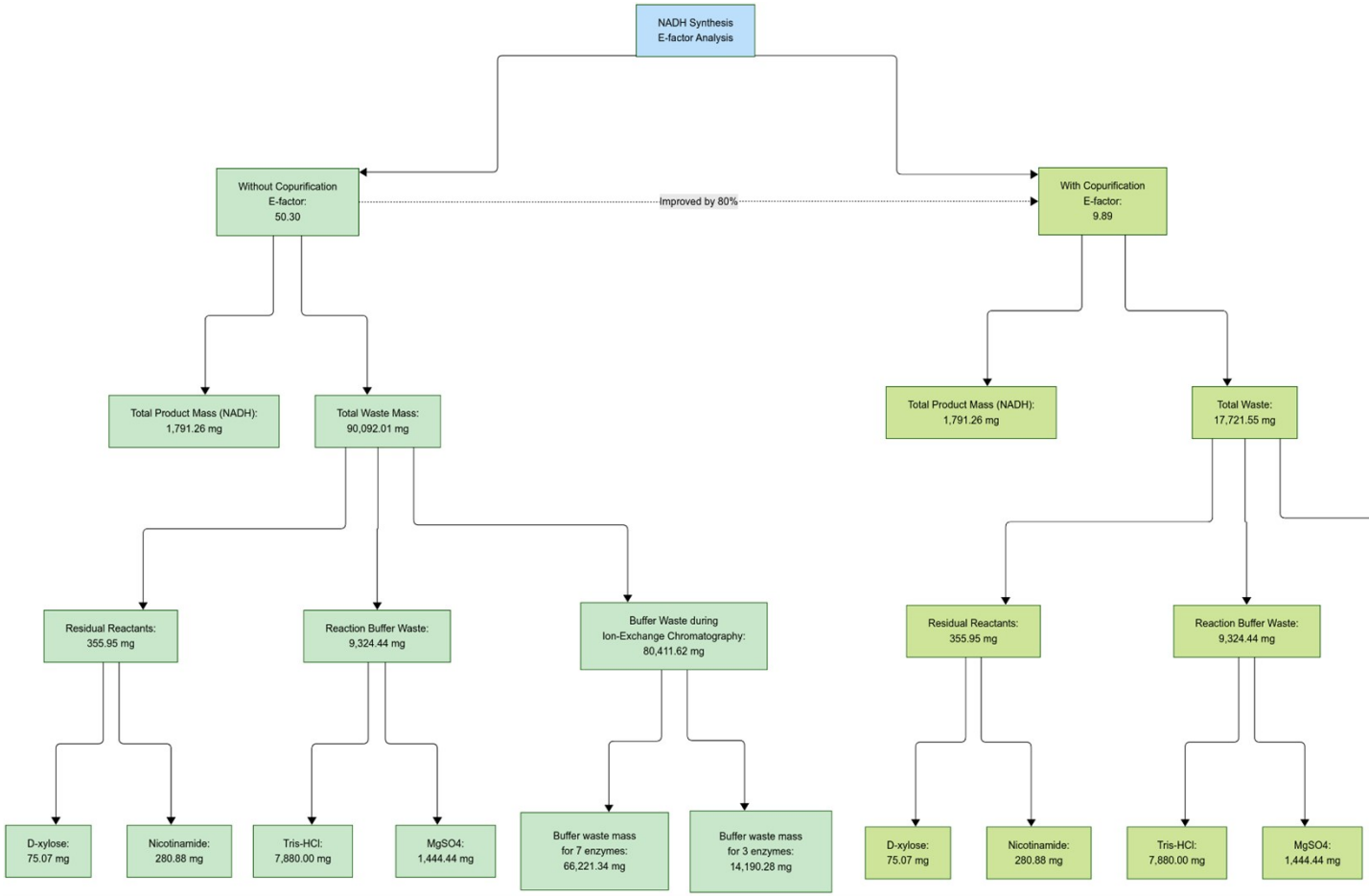
NADH Synthesis
E-factor Analysis
Without Copurification
E-factor:
50.30
Improved by 80%
With Copurification
E-factor:
9.89
Total Product Mass (NADH):
1,791.26 mg
Total Waste Mass:
90,092.01 mg
Total Product Mass (NADH):
1,791.26 mg
Total Waste:
17,721.55 mg
Residual Reactants:
355.95 mg
Reaction Buffer Waste:
9,324.44 mg
Buffer Waste during
Ion-Exchange Chromatography:
80,411.62 mg
Residual Reactants:
355.95 mg
Reaction Buffer Waste:
9,324.44 mg
D-xylose:
75.07 mg
Nicotinamide:
280.88 mg
Tris-HCl:
7,880.00 mg
MgSO4:
1,444.44 mg
Buffer waste mass
for 7 enzymes:
66,221.34 mg
Buffer waste mass
for 3 enzymes:
14,190.28 mg
D-xylose:
75.07 mg
Nicotinamide:
280.88 mg
Tris-HCl:
7,880.00 mg
MgSO4:
1,444.44 mg

Figure S11. Synthesis tree of E-factor analysis for NADH synthesis

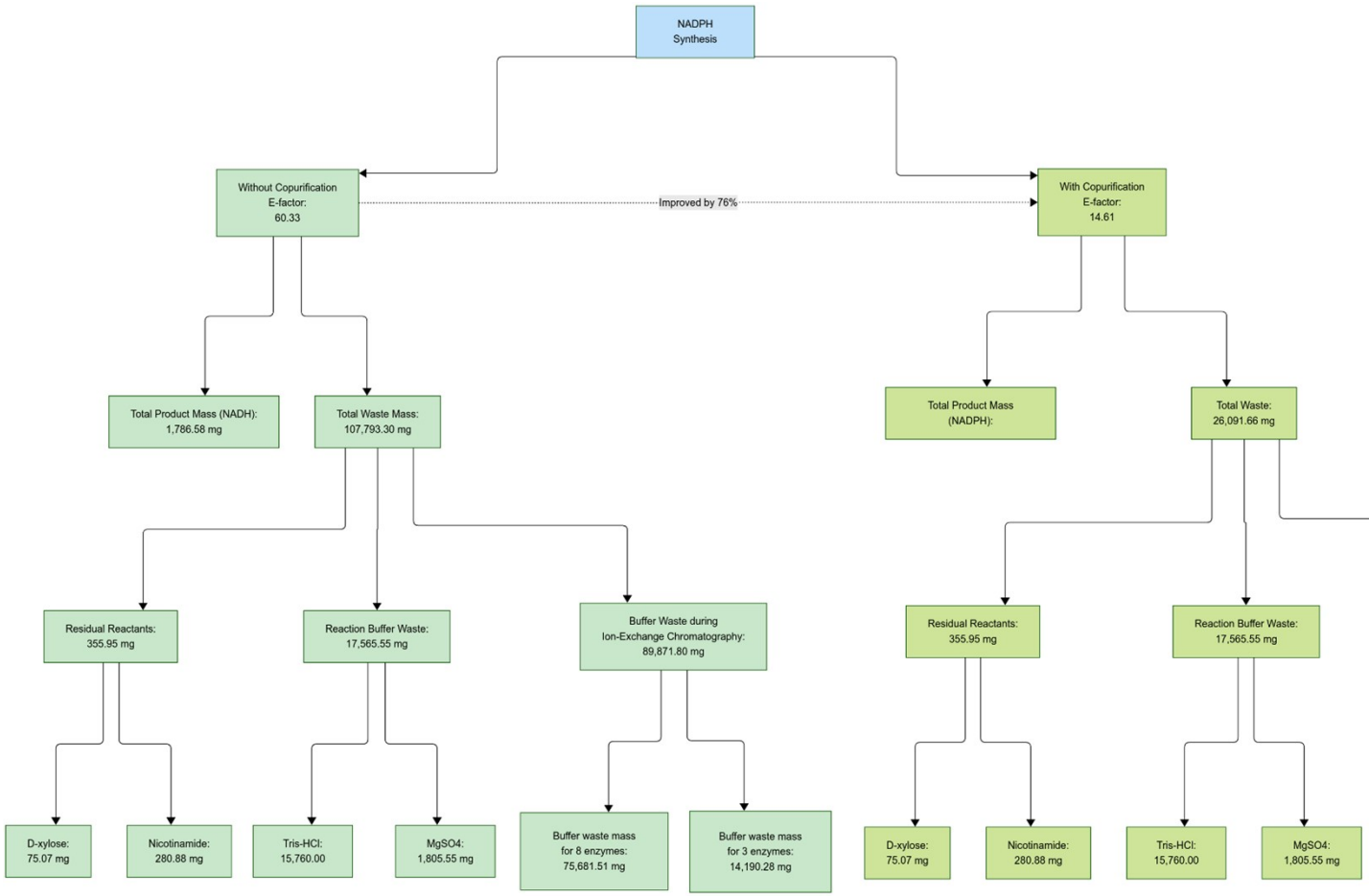
NADPH Synthesis
Without Copurification E-factor: 60.33
Improved by 76%
With Copurification E-factor: 14.61
Total Product Mass (NADH): 1,786.58 mg
Total Waste Mass: 107,793.30 mg
Total Product Mass (NADPH):
Total Waste: 26,091.66 mg
Residual Reactants: 355.95 mg
Reaction Buffer Waste: 17,565.55 mg
Buffer Waste during Ion-Exchange Chromatography: 89,871.80 mg
Residual Reactants: 355.95 mg
Reaction Buffer Waste: 17,565.55 mg
D-xylose: 75.07 mg
Nicotinamide: 280.88 mg
Tris-HCl: 15,760.00
MgSO4: 1,805.55 mg
Buffer waste mass for 8 enzymes: 75,681.51 mg
Buffer waste mass for 3 enzymes: 14,190.28 mg
D-xylose: 75.07 mg
Nicotinamide: 280.88 mg
Tris-HCl: 15,760.00
MgSO4: 1,805.55 mg

Figure S12. Synthesis tree of E-factor analysis for NADPH synthesis

Supplementary Tables

Table S1. The list of recombinant proteins and commercial enzymes used in this work.

Enzyme	Abbreviation	Molecular weight (kDa)	UniProt accession	Host organism	Expression vector
Dihydrofolate reductase	DHFR	18	Q1GAP1	*Homo sapiens*	pET28a (+)
Nicotinamide adenine dinucleotide kinase	NADK	34	P0A7B3	*Escherichia coli*	pET28a (+)
Nicotinamide phosphoribosyltransferase	NAMPT	54	A0A979FZM9	*Chitinophaga pinensis*	pET28a (+)
Nicotinamide mononucleotide adenylyltransferase	NMNAT	33	Q9HAN9	*Homo sapiens*	pET28a (+)

Inorganic pyrophosphatase	PPase	32	P00817	*Saccharomyces cerevisiae*	a
Polyphosphate kinase 2	PPK2	72	A0A6N4SMB5	*Cytophaga hutchinsonii*	pMCSG19
Phosphoribosyl pyrophosphate synthetase	PRPS	36	P9WKE3	*Mycobacterium tuberculosis*	pET28a (+)
Phosphite dehydrogenase	Wild-type (wt) PTDH	38	O69054	*Pseudomonas stutzeri*	pET28a(+)
	Mutant-type (mt) PTDH (E175A, A176R)	38	–	–	pET28a(+)
Ribulose-phosphate 3-epimerase	RPE	24	I3DTP3	*Bacillus methanolicus*	pET28a (+)
Ribose-5-phosphate isomerase	RPI	24	P0A7Z0	*Escherichia coli*	pET28a (+)
Xylose isomerase	XI	N.A.	N.A.	*Streptomyces murinus*	a
Xylulokinase	XK	55	Q9WXX1	*Thermotoga maritima*	pET28a(+)

a: PPase and XI were purchased from Roche (10108987001) and Sigma-Aldrich (G4166), respectively.

Table S2. Comparison of the NMN/NAD(P)H synthesis strategies.

Strategy	Substrates	Product	Time	Titer	Percentage yield	Note	Reference
Chemical synthesis	Tetraacetyl ribose, NAM	NMN, NAD	27 h (NMN), 43 h (NAD^+)	N.A.	80% (NMN), 58% (NAD^+)	Undesired isomer	Lee et al.[9]
Fermentative synthesis	Glucose, NAM	NMN	24 h	0.5 g/L	2.3%	Low titer	Liu et al.[10]
Fermentative synthesis	Glucose, NAM	NMN	37 h	6.8 g/L	86%	Time-consuming	Shoji et al.[11]
Fermentative synthesis	Glucose, NAM	NMN	18 h	17.2 g/L	53%	Low percentage yield	Maharjan et al.[12]
Fermentative synthesis	Glucose, NAM	NMN	25 h	16.2 g/L	97%	Time-consuming	Huang et al.[13]
Fermentative synthesis	Glucose, xylose, NAM	NMN	96 h	46.7 g/L	35%	Time-consuming, low percentage yield	Gan et al[14]
Enzymatic synthesis	NAM, starch	NMN	24 h	0.9 g/L	87%	Require NADH	Li et al.[15]
Enzymatic synthesis	NAM, D-ribose	NMN	3 h	8.1 g/L	81%	Competing reaction due to the use of cell-lysates	Peng et al.[16]
Chemo-enzymatic synthesis	Sugarcane bagasse, NAM	NADH, NADPH	6 h (NADH), 4 h (NADPH)	2.0 g/L	85%	High titer, high percentage yield, quick	This study

Table S3. Materials prices

Material/Component	Price	Link (obtained on April 3rd, 2025)
Phosphite	0.55 USD/g	https://www.sigmaaldrich.com/US/en/product/sigald/215112
D-xylose	Free	–
NAM	0.29 USD/g	https://www.sigmaaldrich.com/US/en/product/sigma/72340
ATP	85.31 USD/g	https://www.sigmaaldrich.com/US/en/product/aldrich/a26209?utm_source=google&utm_medium=cpc&utm_campaign=19476915493&utm_content=147935828074&gad_source=1&gclid=CjwKCAjwwLO_BhB2EiwAx2e-30eKhq9_mpF4p40EDXNfJjSg4qL7MYLM2AjHDnnuaf_YLY5vZKvmhxoCJ64QAvD_BwE
PolyP	Free	–
Tris-HCl	0.378 USD/g	https://www.sigmaaldrich.com/US/en/product/roche/10812846001
Glycerol (100%)	161 USD/L	https://www.sigmaaldrich.com/US/en/product/sigma/g5516?srsltid=AfmBOoooSVD0q30qj6nS0_yDrZxNT4W9r-nJrhIrosLOEMNkMTTXDPoq
KCl	0.1 USD/g	https://www.sigmaaldrich.com/US/en/product/sigald/s9888
HEPES (1 M)	904 USD/L	https://www.sigmaaldrich.com/US/en/product/sigma/h0887

Supplementary Data

Data S1. Thermodynamic calculation of the *in vitro* one-pot enzymatic NAD(P)H synthesis process

$\Delta_rG'^m$ values were calculated under the following experimental conditions: 1 M reactant concentration, pH 8.0, 25°C, pMg 2.0, and 0.10 M ionic strength. $\Delta_rG'^m$ of each reaction was estimated using the eQuilibrator tool (https://equilibrator.weizmann.ac.il/).

For NAD^+ synthesis

D-xylose + ATP → ~~Ribose 5-phosphate~~ + ADP ($\Delta_rG'^m = -20.5 \pm 3.9$ kJ/mol)

+) ~~Ribose 5-phosphate~~ + ATP → ~~Phosphoribosyl pyrophosphate~~ + AMP ($\Delta_rG'^m = -4.5 \pm 2.8$ kJ/mol)

+) ~~Phosphoribosyl pyrophosphate~~ + Nicotinamide → ~~NMN~~ + ~~PP_i~~ ($\Delta_rG'^m = -14.8 \pm 10.3$ kJ/mol)

+) ~~NMN~~ + ATP → NAD^+ + ~~PP_i~~ ($\Delta_rG'^m = -5.0 \pm 5.7$ kJ/mol)

+) ~~$2PP_i$~~ + $2H_2O$ → $4P_i$ ($\Delta_rG'^m = 2 \times (-30.9 \pm 0.4)$ kJ/mol)

Overall reaction:

D-xylose + Nicotinamide + 3ATP + $2H_2O$ → NAD^+ + ADP + AMP + $4P_i$ ($\Delta_rG'^m = -106.6 \pm 9.9$ kJ/mol) (1)

Moreover, ATP can be regenerated from AM(D)P using polyP by PPK2.

$$ADP + PolyP_{(n)} \rightarrow ATP + PolyP_{(n-1)} \ (\Delta_rG'^m = \sim 0 \text{ kJ/mol}) \quad (3)$$

$$AMP + PolyP_{(n-1)} \rightarrow ATP + PolyP_{(n-3)} \ (\Delta_rG'^m = \sim 0 \text{ kJ/mol}) \quad (2)$$

Overall reaction = (1) + (2) + (3)

+) D-xylose + Nicotinamide + ~~3ATP~~ + $2H_2O$ → NAD^+ + ~~ADP~~ + ~~AMP~~ + $4P_i$ ($\Delta_rG'^m = -106.6 \pm 9.9$ kJ/mol) (1)

+) ~~ADP~~ + $PolyP_{(n)}$ → ~~ATP~~ + ~~$PolyP_{(n-1)}$~~ ($\Delta_rG'^m = \sim 0$ kJ/mol) (2)

+) ~~AMP~~ + ~~$PolyP_{(n-1)}$~~ → ~~ATP~~ + $PolyP_{(n-3)}$ ($\Delta_rG'^m = \sim 0$ kJ/mol) (3)

$$D\text{-xylose} + Nicotinamide + ATP + PolyP_{(n)} + 2H_2O \rightarrow NAD^+ + 4P_i + PolyP_{(n-3)}$$

($\Delta_rG'^m = -106.6 \pm 9.9$ kJ/mol) (4)

For NADH synthesis

NAD^+ is reduced to synthesize NADH by phosphite dehydrogenase (PTDH).

$$Phosphite + NAD^+ + H_2O \rightarrow P_i + NADH \ (\Delta_rG'^m = -63.6 \text{ kJ/mol}) \quad (5)^*$$

*Assuming that the standard reduction potentials of phosphite and NAD(P)H are −0.65 and −0.32 V, respectively, the Gibbs free energy of the (6) was estimated by the Nernst equation.$\Delta_rG'^m = -nF\Delta E = -2 \times 96.485 \times (-0.32-(-0.65)) = -63.6$ kJ/mol (n, number of electrons transferred; F, Faraday constant (96.485 kJ/mol·V); ΔE, standard reduction potential difference ($Eelectron_{acceptor} - Eelectron_{donor}$))

Overall reaction = (4) + (5)

+) D-xylose + Nicotinamide + ATP + $PolyP_{(n)}$ + $2H_2O$ → ~~NAD^+~~ + $4P_i$ + $PolyP_{(n-3)}$

($\Delta_rG'^m = -106.6 \pm 9.9$ kJ/mol) (4)

+) Phosphite + ~~NAD~~$^{\pm}$ + H_2O → P_i + NADH ($\Delta_rG'^m$ = −63.6 kJ/mol) (5)*

D-xylose + Nicotinamide + ATP + Phosphite + $PolyP_{(n)}$ + $3H_2O$ → NADH + $5P_i$ + $PolyP_{(n-3)}$

($\Delta_rG'^m$ = −170.2 ± 9.9 kJ/mol)

For NADPH synthesis

NAD^+ is sequentially phosphorylated by NAD^+ kinase (NADK).

ATP + NAD^+ → ADP + $NADP^+$ ($\Delta_rG'^m$ = −13.3 ± 5.7 kJ/mol) (6)

ATP can be regenerated from AM(D)P using polyP by PPK2.

ADP + $PolyP_{(n-3)}$ → ATP + $PolyP_{(n-4)}$ ($\Delta_rG'^m$ = ~0 kJ/mol) (3)

Finally, $NADP^+$ is reduced to synthesize NADPH by PTDH.

Phosphite + $NADP^+$ + H_2O → P_i + NADPH ($\Delta_rG'^m$ = −63.6 kJ/mol) (7)*

Overall reaction = (4) + (6) + (3) + (7)

+) D-xylose + Nicotinamide + ATP + $PolyP_{(n)}$ + $2H_2O$ → ~~NAD~~$^{\pm}$ + $4P_i$ + ~~$PolyP_{(n-3)}$~~

($\Delta_rG'^m$ = −106.6 ± 9.9 kJ/mol) (4)

+) ~~ATP~~ + ~~NAD~~$^{\pm}$ → ~~ADP~~ + ~~NADP~~$^{\pm}$ ($\Delta_rG'^m$ = −13.3 ± 5.7 kJ/mol) (6)

+) ~~ADP~~ + ~~$PolyP_{(n-3)}$~~ → ~~ATP~~ + $PolyP_{(n-4)}$ ($\Delta_rG'^m$ = ~0 kJ/mol) (3)

+) Phosphite + ~~NADP~~$^{\pm}$ + H_2O → P_i + NADPH ($\Delta_rG'^m$ = −63.6 kJ/mol) (7)

D-xylose + Nicotinamide + ATP + Phosphite + $PolyP_{(n)}$ + $3H_2O$ → NADPH + $5P_i$ + $PolyP_{(n-4)}$

($\Delta_rG'^m$ = −183.5 ± 15.6 kJ/mol)

Data S2. E-factor calculation of the *in vitro* one-pot enzymatic NAD(P)H synthesis process. (water is not considered waste in our calculation).

E-factor for NADH synthesis (without a copurification system):

Overall reaction equation for NADH synthesis:

D-xylose + Nicotinamide + ATP + Phosphite + $PolyP_{(n)}$ + $3H_2O$ → NADH + $5P_i$ + $PolyP_{(n-3)}$

Total enzyme concentration: 0.985 mg/mL

Reaction volume: 1 L

Final NADH concentration: 2.7 mM

Molar mass of NADH: 663.43 mg/mmol

1. Total product mass of NADH

Mass of NADH (mg) = Concentration (mM) × Volume (L) × Molar mass (mg/mmol)

= 2.7 mM × 1 L × 663.43 mg/mmol

= 1,791.26 mg

2. Waste mass calculation

The waste includes the residual reactants and the discarded buffer during the cascade reaction and ion-exchange chromatography for enzyme purification:

(A) Waste mass for the residual reactants (D-xylose and nicotinamide):

Waste mass (mg) = Concentration (mM) × Volume (L) × Molar mass (mg/mmol)

Molar mass of D-xylose: 150.13 mg/mmol

Molar mass of nicotinamide: 122.12 mg/mmol

Residual D-xylose: 0.5 mM × 1 L × 150.13 mg/mmol = 75.07 mg

Residual nicotinamide: 2.3 mM × 1 L × 122.12 mg/mmol = 280.88 mg

Total waste mass:

75.07 mg (D-xylose) + 280.88 mg (nicotinamide) = 355.95 mg

(B) Discarded buffer waste

(i) The reaction buffer waste (Tris-HCl (50 mM) and $MgSO_4$ (12 mM))

Molar mass of Tris: 157.6 mg/mmol

Molar mass of $MgSO_4$: 120.37 mg/mmol

Tris-HCl waste: 50 mM × 1 L × 157.6 mg/mmol = 7,880.00 mg

Mg^{2+} ($MgSO_4$) waste: 12 mM × 1 L × 120.37 mg/mmol = 1,444.44 mg

Total buffer waste mass during the cascade reaction: 7,880.00 mg (Tris) + 1,444.44 mg ($MgSO_4$) = 9,324.44 mg

(ii) The buffer waste during ion-exchange chromatography

Molar mass of HEPES: 238.3 mg/mmol

Molar mass of glycerol: 92.09 mg/mmol

Molar mass of NaCl: 58.44 mg/mmol

Molar mass of $MgCl_2$: 95.21 mg/mmol

Molar mass of imidazole: 68.08 mg/mmol

- Binding buffer (HEPES-Na (25 mM), glycerol (5%, v/v; 5 mM), NaCl (300 mM), $MgCl_2$ (1 mM), and imidazole (5 mM)):
  (25 mM × 238.30 mg/mmol + 5 mM × 92.09 mg/mmol + 300 mM × 58.44 mg/mmol + 1 mM × 95.21 mg/mmol + 5 mM × 68.08 mg/mmol) × 1L = 24,385.56 mg

- Wash buffer (HEPES-Na (25 mM), glycerol (5%, v/v; 5 mM), NaCl (300 mM), $MgCl_2$ (1 mM), and imidazole (25 mM)):
  (25 mM × 238.30 mg/mmol + 5 mM × 92.09 mg/mmol + 300 mM × 58.44 mg/mmol + 1 mM × 95.21 mg/mmol + 25 mM × 68.08 mg/mmol) × 1L = 25,747.16 mg

- Elution buffer (HEPES-Na (25 mM), glycerol (5%, v/v; 5 mM), NaCl (300 mM), $MgCl_2$ (1 mM), and imidazole (300 mM)):
  (25 mM × 238.30 mg/mmol + 5 mM × 92.09 mg/mmol + 300 mM × 58.44 mg/mmol + 1 mM × 95.21 mg/mmol + 300 mM × 68.08 mg/mmol) × 1L = 44,469.16 mg

Total buffer waste mass for one enzyme purification: 24,385.56 mg (Binding buffer) + 25,747.16 mg (Wash buffer) + 44,469.16 mg (Elution buffer) = 94,601.88 mg

Assuming that the enzymes are purified with a yield of 5 g (7 enzymes) and 10 g (3 enzymes), 0.5 g is used in each experiment (PPase is provided from a commercial source).

Buffer waste mass for 7 enzymes with a yield of 5 g:
94,601.88 mg / (5 g / 0.5 g) × 7 = 66,221.34 mg

Buffer waste mass for 3 enzymes with a yield of 10 g:
94,601.88 mg / (10 g / 0.5 g) × 3 = 14,190.28 mg

Total buffer waste mass for the 10 enzyme purification:
66,221.34 mg + 14,190.28 mg = 80,411.62 mg

Total waste mass (residual reactants + reaction buffer waste + buffer waste during ion-exchange chromatography):
355.95 mg (residual reactants) + 9,324.44 mg (reaction buffer waste) + 80,411.62 mg (buffer waste during ion-exchange chromatography) = 90,092.01 mg

Altogether, the E-factor for NADH synthesis (without a copurification system) is: 90,092.01 mg (total waste mass) / 1,791.26 mg (total product mass) = **50.30**

E-factor for NADH synthesis (with a copurification system)

By copurifying 10 enzymes, total buffer waste mass during ion-exchange chromatography is reduced by a factor of 1/10. While the NADH product mass remains the same, the total waste mass decreases significantly.

Total buffer waste mass during ion-exchange chromatography with a copurification system:
80,411.62 mg / 10 = 8,041.16 mg

Total waste mass (residual reactants + reaction buffer waste + buffer waste during ion-exchange chromatography):
355.95 mg (residual reactants) + 9,324.44 mg (reaction buffer waste) + 8,041.16 mg (buffer waste during ion-exchange chromatography) = 17,721.55 mg

Therefore, the E-factor for NADH synthesis (with a copurification system) is: 17,563.84 mg (total waste mass) / 1,791.26 mg (total product mass) = **9.89**

E-factor for NADPH synthesis (without a copurification system)

Overall reaction equation for NADPH synthesis

D-xylose + Nicotinamide + ATP + Phosphite + $PolyP_{(n)}$ + $3H_2O$ → NADPH + $5P_i$ + $PolyP_{(n-4)}$

Total enzyme concentration: 1.185 mg/mL

Reaction volume: 1 L

Final NADPH concentration: 2.4 mM

The molar mass of NADPH: 744.4 mg/mmol

1. **Total product mass of NADPH**

Mass of NADPH (mg) = Titer (mM) × Volume (L) × Molar mass (mg/mmol)

= 2.4 mM × 1 L × 744.41 mg/mmol

= 1,786.58 mg

2. Waste mass calculation

The waste mass calculation is the same as the one for the NADH synthesis process except for the buffer waste.

(i) The reaction buffer waste

Tris-HCl Buffer Waste (NADPH synthesis process is two-stage):

50 mM × 1 L × 157.6 mg/mmol × 2 = 15,760.00 mg

Mg^{2+} ($MgSO_4$) waste: 15 mM × 1 L × 120.37 mg/mmol = 1,805.55 mg

Total buffer waste mass during the cascade reaction: 15,760.00 mg (Tris) + 1,805.55 mg ($MgSO_4$) = 17,565.55 mg

(ii) The buffer waste during ion-exchange chromatography

Assuming that the enzymes are purified with a yield of 5 g (8 enzymes) and 10 g (3 enzymes), 0.5 g is used in each experiment.

Buffer waste mass for 8 enzymes with a yield of 5 g:

94,601.88 mg / (5 g / 0.5 g) × 8 = 75,681.51 mg

Buffer waste mass for 3 enzymes with a yield of 10 g:

94,601.88 mg / (10 g / 0.5 g) × 3 = 14,190.28 mg

Total buffer waste mass for the 10 enzyme purification:

75,681.51 mg + 14,190.28 mg = 89,871.80 mg

Total waste mass (residual reactants + reaction buffer waste + buffer waste during ion-exchange chromatography):

355.95 mg (residual reactants) + 17,565.55 mg (reaction buffer waste) + 89871.80 mg (buffer waste during ion-exchange chromatography) = 107,793.30 mg

Therefore, the E-factor for NADPH synthesis (without a copurification system) is: 107,793.30 mg (total waste mass) / 1786.58 mg (total product mass) = **60.33**

E-factor for NADPH synthesis (with a copurification system)

The total buffer waste mass during ion-exchange chromatography is reduced by a factor of 1/11 with a copurification system.

Total buffer waste mass during ion-exchange chromatography with a copurification system: 89,871.80 mg / 11 = 8,170.16 mg

Total waste mass (residual reactants + reaction buffer waste + buffer waste during ion-exchange chromatography):
355.95 mg (residual reactants) + 17,565.55 mg (reaction buffer waste) + 8,170.16 mg (buffer waste during ion-exchange chromatography) = 26,091.66 mg

Therefore, the E-factor for NADPH synthesis (with a copurification system) is: 26,091.66 mg (total waste mass) / 1786.58 mg (total product mass) = **14.61**

Data S3. Cost calculation for chemo-enzymatic NADPH synthesis

Cost for chemo-enzymatic NADPH synthesis was calculated by using the material prices listed in Table S3.

1. Cost per protein purification

Given that HEPES buffer and glycerol account for most of the buffer preparation costs, the cost for buffer preparation can be calculated as described below.

Binding buffer/Wash buffer/Elution buffer (USD/L)

- HEPES-Na (25 mM): 25 mL of 1 M stock = 904 USD/L × 0.025 L = 22.60 USD
- Glycerol (5%, v/v): 50 mL of 100% glycerol = 161 USD/L × 0.05 L = 8.05 USD

Total cost for buffers = 30.65 USD/L

Given that 40mL, 20 mL, and 1 mL of binding buffer, wash buffer, elution buffer were used for every protein purification, respectively, total buffer cost per protein purification can be calculated as below.

- Binding buffer: 40 mL × 30.65 USD/L = 1.22 USD

- Wash buffer: 20 mL × 30.65 USD/L = 0.61 USD
- Elution buffer: 1 mL × 30.65 USD/L = 0.03 USD

Total buffer cost per protein purification = 1.86 USD

2. Cost for NADPH synthesis

(i) Mass of NADPH:

- NADPH titer: 2.0 g/L
- Reaction volume: 300 μL = 0.0003 L
- Mass of NADPH = 2.0 g/L × 0.0003 L = 0.0006 g

(ii) Enzyme costs (0.05 mg/mL was used for every NADPH synthesis reaction):

- 8 enzymes at 5 g/L yield: Each enzyme uses 0.05/5 = 0.01 (1%) of a purification batch
- 3 enzymes at 10 g/L yield: Each enzyme uses 0.05/10 = 0.005 (0.5%) of a purification batch

  Altogether, total enzyme fraction = (8 × 0.01) + (3 × 0.005) = 0.08 + 0.015 = 0.095

  Given that cost per enzyme purification is 1.86 USD,

  total enzyme cost = 0.095 × 1.86 USD = 0.177 USD

(iii) Reaction components:

- Phosphite: 10 mM × 0.0003 L = 0.003 mmol = 0.000249 g (MW = 83 g/mol)
  Cost = 0.000249 g × 0.55 USD/g = 0.000137 USD
- Nicotinamide: 5.0 mM × 0.0003 L = 0.0015 mmol = 0.000183 g (MW = 122 g/mol)
  Cost = 0.000183 g × 0.29 USD/g = 0.000053 USD
- ATP: 3.2 mM × 0.0003 L = 0.00096 mmol = 0.000472 g (MW = 491.2 g/mol)
  Cost = 0.000472 g × 85.31 USD/g = 0.0402 USD
- Tris-HCl: 50 mM × 0.0003 L = 0.015 mmol = 0.00237 g (MW = 157.6 g/mol)
  Cost = 0.00237 g × 0.378 USD/g = 0.000896 USD
- KCl: 20 mM × 0.0003 L = 0.006 mmol = 0.000447 g (MW = 74.55 g/mol)
  Cost is negligible
- $MgCl_2$: 8.0 mM × 0.0003 L = 0.0024 mmol = 0.000228 g (MW = 95.2 g/mol)
  Cost is negligible
- $FeSO_4$: 0.01 mM × 0.0003 L = 0.000003 mmol = 0.00000046 g (MW = 151.9 g/mol)
  Cost is negligible

- Glycerol (for enzyme storage): 5% solution, 0.0003 L × 0.05 = 0.000015 L
  Cost = 0.000015 L × 161 USD/L = 0.00241 USD

Total material cost = 0.0437 USD (sum of all reagent costs)

Total cost = enzyme cost + material cost = 0.177 USD + 0.0437 USD = 0.221 USD

Therefore, NADPH (USD/g) = 0.221 USD / 0.0006 g = 368.33 USD/g

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