Enhancement of essential cofactors for *in vivo* biocatalysis

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Supplementary information for this manuscript includes the supplementary text of the full comparison of cofactor enhancement by XR and other methods

Main enzymes for pyridine dinucleotides biosynthesis in bacteria

Pyridine dinucleotides biosynthesis comprises steps of the *de novo* synthesis of oxidized pyridine dinucleotides and the reduction step to generate the reduced form. Generally, NAD^+ synthesis in bacteria requires five enzymes, including aspartate oxidase, quinolinate synthase, quinolinate phosphoribosyltransferase, NaMN adenylyltransferase and NAD^+ synthetase.[1] For $NADP^+$ synthesis, an additional enzyme, NAD^+ kinase, is required to add a phosphate to NAD^+.[1] The first enzyme, aspartate oxidase catalyzes the oxidation of L-aspartate using O_2 (aerobic conditions) or fumarate (anaerobic conditions) to form L-iminosuccinate. The second enzyme, quinolinate synthase catalyzes the condensation of iminosuccinate and dihydroxyacetone phosphate (DHAP) to yield quinolinate. The third enzyme, quinolinic acid phosphoriboyl NaMN transferase converts quinolinate to NaMN. The fourth enzyme, NaMN adenylyltransferase catalyzes adenylation of NaMN to produce nicotinic acid adenine dinucleotide by utilizing ATP as a co-substrate. The last enzyme, NAD synthetase uses ATP and NH_3 to synthesize the NAD^+ product. For $NAD(P)^+$, NAD kinase catalyzes the phosphorylation of NAD^+ to produce $NADP^+$.[1] The enhancement of reduced pyridine dinucleotides is often achieved through addition of a dehydrogenase which can convert a reducing equivalent from an organic substrate to reduce NAD^+ or $NADP^+$ inside cells to generate more NADH or NADPH or reroute the production of NADH to NADPH or *vice versa*.

To synthesize the reduced form of pyridine dinucleotides, three enzymes including glucose 6-phosphate dehydrogenase (G6PD), glyceraldehyde 3-phosphate (GA3P) and formate dehydrogenase (FDH) are commonly applied in the metabolic engineering approach. We only discuss the reactions of GA3P and FDH in this SI while the reaction od G6PD is discussed in the main text. One example of rerouting the production of NADH towards NADPH is the synthesis of lycopene, which requires the condensation of the key glycolytic intermediates, GA3P, pyruvate and NADPH.[2] The overall net reaction of lycopene synthesis is 8GA3P + 8Pyruvate + 16NADPH + 8CTP + 8ATP → 1lycopene + $8CO_2$ + $16NADP^+$ + 8CMP +8 ADP + 12PPi.[2] As the native GA3P dehydrogenase (GA3PD) reaction in *E. coli* is an NAD^+-dependent enzyme which only generates NADH, the endogenous GA3PD in *E. coli* was thus replaced with the *C. acetobutylicum* GA3PD to generate two reducing equivalents of NADPH instead of NADH from the glycolytic pathway of glucose.[3] This strategy increased NADPH from 1.72 mol (in the native cell) to 2.97 mol (in the engineered strain), and yielded 1.95-fold increased lycopene production.[3]

The reaction of FDH has also been used to increase cellular levels of NADH such as in the pathway of alkane production from fatty aldehyde through the reaction of aldehyde deformylating oxygenase (ADO) which requires the redox partners, ferredoxin (Fd) from the reaction of ferredoxin (flavodoxin):$NADP^+$ oxidoreductase (FNR). As the reaction of ADO (Table S1) generates formate as a byproduct, the overexpression of FDH from *Xanthobacter* sp. 91 in the host *E. coli* BL21 (DE3) serves two purposes--to increase the cellular level of NADH and to remove excess formate which can cause a pH drop in the system.[4] The increased ratio of NADH/NAD^+ promotes reduction of Fd by FNR. The reduced Fd can serve as a substrate of ADO, promoting more production of alkane to 50% yield compared to 35% yield of the system without FDH.[4] To summarize the examples of the reduced pyridine dinucleotides synthesis using metabolic engineering approach, the information is shown in Table S1 below.

Table S1. Examples of metabolic engineering approaches to increase levels of intracellular NADH, NADPH and NAD(P)H.

NAD(P)H generating enzymes	Cofactors obtained	NAD(P)H enzymatic reaction	Main enzyme for synthesis of desired products	Main reaction	Products
Glyceraldehyde-3-phosphate dehydrogenase	NADPH	Glyceraldehyde-3-phosphate + $NADP^+$ + Pi → 1,3-bisphophoglycerate + NADPH	Lycopene synthetic operon[3]	Geranyl pyrophosphate (GPP) →→ lycopene (NADPH is required to produce GPP from acetyl-CoA)	Lycopene
Glucose 6-phosphate dehydrogenase	NADPH	Glucose 6-phosphate + NADP → 6-phosphogluconolactone + NADPH	Poly-γ-glutamic acid synthetic operon (PGA synthetase)[5]	L-glutamic acid + ATP → PGA + ADP + Pi	PGA
			Polyketide synthase[6]	Acetyl-CoA + NADPH → polyunsaturated fatty acid (PUFA) + $NADP^+$	PUFA
			Fatty acid synthase[6]	Acetyl-CoA + NADPH → saturated fatty acids (SFA) + $NADP^+$	SFA
			Inositol dehydrogenases[7]	Scyllo-inosose + NADPH → scyllo-inositol + $NADP^+$	Scyllo-inositol
Formate dehydrogenase	NAD(P)H	Formate + $NAD(P)^+$ → CO_2 + NAD(P)H	Aldehyde deformylating oxygenase and its redox partners (Fd/FNR)[4, 8]	Fatty aldehyde + O_2 → alkane + formic acid[4]	Alkane

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