Supporting Information

Aldehyde Production in Crude Lysate-Based and Whole-Cell Biotransformation Using a Noncanonical Redox Cofactor System

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A. Methods

Plasmids and Strains

All plasmids and strain MX102 were taken from previous work in our lab [1] (Table S1). All plasmids were transformed by electroporation. When multiple plasmids were used in a strain, they were transformed simultaneously. When applicable, the following antibiotic concentrations were used, unless stated otherwise: 100 mg/L ampicillin, 50 mg/L kanamycin, 50 mg spectinomycin, or 20 mg/L chloramphenicol.

Crude Lysate Biotransformations

Cell-free reactions were performed with crude *E. coli* lysates in a modular system. In this system, each protein was overexpressed and processed separately. Then, different lysates were mixed at controlled ratios to complete the full system. *E. coli* strain MX102 was transformed with a plasmid expressing XenA (pEK102), GDH WT (pEK101), or GDH Ortho (pLZ216). 4 mL seed cultures containing 2xYT medium supplemented with 1% w/v mannitol and appropriate antibiotics were incubated at 30 ℃ while shaking at 250 r. p. m. for 16 hours. Next, 0.5% v/v seed cultures were used to inoculate 250 mL of 2xYT medium supplemented with 1% w/v mannitol and 200 mg/L ampicillin in a 500 mL baffled shake flask. Cultures were incubated at 30 ℃ while shaking at 250 r. p. m. for 4 hours. Protein expression was induced with 0.5 mM IPTG and 0.1% w/v arabinose. Cultures shook for an additional 10 hours under the same conditions. To harvest and process the cells, cultures were centrifuged at 4000 RCF for 20 minutes at 4 ℃. The resulting cell pellets were washed 3 times in 250 mL of ice-cold wash buffer containing 120 mM potassium acetate, 28 mM magnesium acetate, and 20 mM tris base pH 8.2. After washing, cells were resuspended in 0.7 mL of wash buffer per 1 g wet-cell-weight of pelleted cells. The resuspended cells were then lysed by French press. The cell lysate was clarified by centrifugation twice at 20,000 RCF for 20 minutes at 4 ℃. When stated in the text, an additional ultracentrifugation step was applied by centrifuging the supernatant from the preceding centrifugation step at 30,000 RCF for 30 minutes at 4 ℃. The clarified lysate was aliquoted into 1.5 mL microcentrifuge tubes and stored at -80 ℃ until use. The total protein concentration in the lysate was quantified by Bradford Assay.

Cell-free reactions were performed at a 1 mL working volume in 2 mL glass vials sealed with a PTFE-lined cap at 37 ℃ without shaking. Clarified cell lysate was thawed on ice. The XenA-enriched lysate was mixed with the GDH WT- or GDH Ortho-enriched lysate at various ratios to create a complete cycling system. The total lysate protein concentration was maintained constant at 4.5 mg/mL in the final reaction mixture. Lysate mixture was spiked into pre-warmed, concentrated assay buffer to start the reaction. Two different reaction mixtures were used depending on whether Buffer A or phosphate buffer was being investigated. The working concentration and composition of the Buffer A reaction mixture was 12 mM magnesium acetate, 10 mM ammonium acetate, 130 mM potassium acetate, 10 mM potassium phosphate, 200 mM D-glucose, 1 mM of oxidized cofactor (when optimizing, cofactor concentration was varied at 0, 0.1, 0.2, 0.5, 1, 2, 5, or 10 mM), and 500 mg/L citral. The working concentration and composition of the phosphate buffer reaction mixture was 200 mM potassium phosphate at pH 7.0, pH 7.5, or pH 8.0, 200 mM NaCl, 200 mM D-glucose, 0.1, 0.2, or 0.5 mM NMN^+, and 500 mg/L citral. Samples were taken intermittently for analysis. Samples were extracted with an equal volume of chloroform containing 200 mg/L octanol as an internal standard. The organic fraction was used for GC-FID analysis, as detailed below.

Determination of Molar Concentrations of Recombinant Protein in Crude Lysates

The molar concentration of XenA and GDH Ortho in crude lysates was determined by comparing the level of specific protein activity to a standard curve derived from purified proteins. To generate the purified proteins, *E. coli* strain BL21 (DE3) was transformed with a plasmid expressing XenA (pEK102) or GDH Ortho (pLZ216) with a 6x His-tag fusion at the N terminus. 4 mL seed cultures containing LB medium and appropriate antibiotics were incubated at 37 ℃ for ~16 hours while shaking at 250 r.p.m.. The seed cultures were used to inoculate 10 mL of LB medium with appropriate antibiotics in a 50 mL conical tube. Protein expression was induced with 0.5 mM IPTG. The tube cap was loosely affixed with tape. The cultures were then incubated at 30 ℃ for 24 hours while shaking at 250 r.p.m.. Proteins were purified using His-Spin Protein Purification Miniprep kit (Zymo Research Corporation). The purified protein concentrations were quantified by Bradford assay.

To generate the standard curves, purified protein was serially diluted with His-Elution Buffer (Zymo Research Corporation). The dilution series was spiked into assay buffer to start the reactions. For XenA, the assay buffer contained 200 mM potassium phosphate at pH 7.5, 0.2 mM NADPH, and 5 mM ketoisophorone at 37 °C. The initial reaction rate was determined by observing the decrease in light absorption at 340 nm, corresponding to the rate of NADPH consumption. Reactions containing no substrate were run to determine the non-specific XenA activity rate. Reaction rate was determined by subtracting the no substrate reaction rate from the reaction rate with substrate. For GDH Ortho, the assay buffer contained 50 mM Tris-Cl at pH 8, 140 mM D-glucose, 3 mM NMN^+ at 25 °C. The initial reaction rate was determined by observing the increase in light absorption at 340 nm, corresponding to NMNH production. The rate of cofactor consumption or formation was plotted against enzyme concentration, and a best-fit line was fit to the data. The equation of the best-fit line was used to determine molar concentrations of recombinant protein in crude lysate. To test XenA and GDH Ortho concentration in crude lysates, crude lysates were diluted with His-Elution Buffer (Zymo Research Corporation), and the diluted lysates were spiked into identical assay buffer as their respective purified proteins. Reaction rate was measured in the same way. The reaction rate in the crude lysates was compared to the standard curve to obtain the concentration of the respective recombinant protein in the lysates.

Resting Whole-Cell Biotransformation

Three plasmids, one expressing XenA (selected from pEK102 or pLZ217), one expressing one of the GDH variants (selected from pLZ216, pSM106, or pSM107), and pSM109 expressing a glucose transport facilitator were transformed into MX102. 4 mL seed cultures containing 2xYT medium supplemented with 1% w/v mannitol and appropriate antibiotics were incubated at 30 ℃ while shaking at 250 r.p.m. for 16 hours. Next, 0.5% v/v seed cultures were used to inoculate 100 mL of 2xYT medium supplemented with 1% w/v mannitol in a 250 mL baffled shake flask. Cultures were incubated at 30 ℃ while shaking at 250 r. p. m. for 4 hours. Protein expression was induced with 0.5 mM IPTG and 0.1% w/v arabinose. Cultures were shaken for an additional 10 hours under the same conditions. Cells were collected by centrifugation in 50 mL conical tubes for 15 minutes at 20 ℃ at 3,750 r.p.m.. The supernatant was discarded, and the cells were washed three times with 50 mL of room temperature 100 mM potassium phosphate (pH 7.4), followed by resuspension in room temperature 100 mM potassium phosphate at pH 7.4 to a standardized OD_{600} = 50. 1 mL of washed cells were spiked into 4 mL of concentrated assay buffer in a 15 mL conical tube. The working concentration and composition of buffer contained 100 mM potassium phosphate (pH 7.4), 200 mM D-glucose, 2 mM NMN^+, and 0.5 g/L citral. The tubes were sealed tight and mounted horizontally in an incubating shaker. The tubes were incubated at 30 ℃ while shaking at 250 r.p.m. for 3 hours. After incubation, 0.5 mL of cell suspension was pelleted,

and 200 µL of supernatant was extracted with an equal volume of chloroform containing 200 mg/L octanol as an internal standard. The organic fraction was analyzed by GC-FID, as detailed below.

GC-FID Analysis

All GC analysis was performed on an Agilent 6850 (Agilent Technologies) equipped with an FID. An Agilent DB-WAXetr capillary column (30 m x 0.56 mm x 1 µm) was used for separation. The inlet and detector were held at 250 and 260 ℃, respectively. The GC was operated in constant flow mode with a flowrate of 3 mL/min. Helium was used as the carrier gas. Air and hydrogen were supplied to the FID at 350 and 40 mL/min, respectively. All gasses were purchased from Airgas. 5 µL of sample was injected with a 2:1 split ratio. The oven was held at 150 ℃ for 15 minutes, then ramped to 240 ℃ at a rate of 20 ℃/min before equilibration back to 150 ℃. Elution times are as follows: citral, 7.26 and 8.42 minutes; citronellal, 4.08 minutes; citronellol, 8.72 minutes; nerol, 10.04 minutes; geraniol, 11.55 minutes; octanol, 4.59 minutes.

Determination of Relative Plasmid Expression Levels in Whole Cells

Strain MX102 was transformed with two sets of plasmids. The first set expressed XenA on a P_{LlacO1} ColE1 *ori* vector (pEK102) and GDH Ortho on a P_{BAD} RSF *ori* (pSM106). The second set expressed the same genes, but on the opposite vector (pLZ217 and pEK101 for XenA and GDH Ortho, respectively). Cells were cultured identically as resting whole-cell biotransformations detailed in the previous Methods section. 10 mL of culture was incubated on ice for 15 minutes, and then pelleted by centrifugation at 4 ℃. The supernatant was discarded. The cell pellet was resuspended in 1 mL of ice-cold 35 mM Tris-Cl at pH 8.0. The resuspended cells were lysed by bead beading with glass beads. Lysates were quickly cooled in an ice water bath to remove any heat accumulated from bead beating. The lysate was clarified by centrifugation at 20,000 RCF for 20 minutes at 4 ℃. The clarified lysate was transferred to a fresh 1.5 mL microcentrifuge tube on ice for immediate analysis. The protein concentration in the lysate was determined by Bradford Assay.

To measure the relative expression levels of the vector, GDH Ortho activity was measured by the change in light absorption at 340 nm, due to NMNH formation using a SpectraMax M3 at room temperature. No substrate was supplied to XenA, so change in light absorption is a measure of only GDH Ortho activity, not cycling activity. Lysate was spiked into concentrated assay buffer to start the reaction. The working concentration and composition of assay buffer was 35 mM Tris-Cl at pH 8.0, 140 mM glucose, 2 mM NMN^+.

B. Current Methods for Aldehyde Production in Microbes

In *Saccharomyces cerevisiae*, pathway modules have been compartmentalized in organelles to physically isolate aldehydes from competing enzymes [2,3]. In *Escherichia coli*, Kunjapur and coworkers developed an aromatic aldehyde accumulating *E. coli* strain, termed reduced aromatic aldehyde reduction (RARE) strain, by disrupting six aldehyde reductases [4]. They then used the RARE strain in the production of benzaldehyde and vanillin [4]. Similarly, Rodriguez and coworkers generated an aliphatic aldehyde accumulating strain by screening 44 candidate aldehyde reductases and ultimately identifying and disrupting 13 genes [5].

C. Supporting Figures and Tables

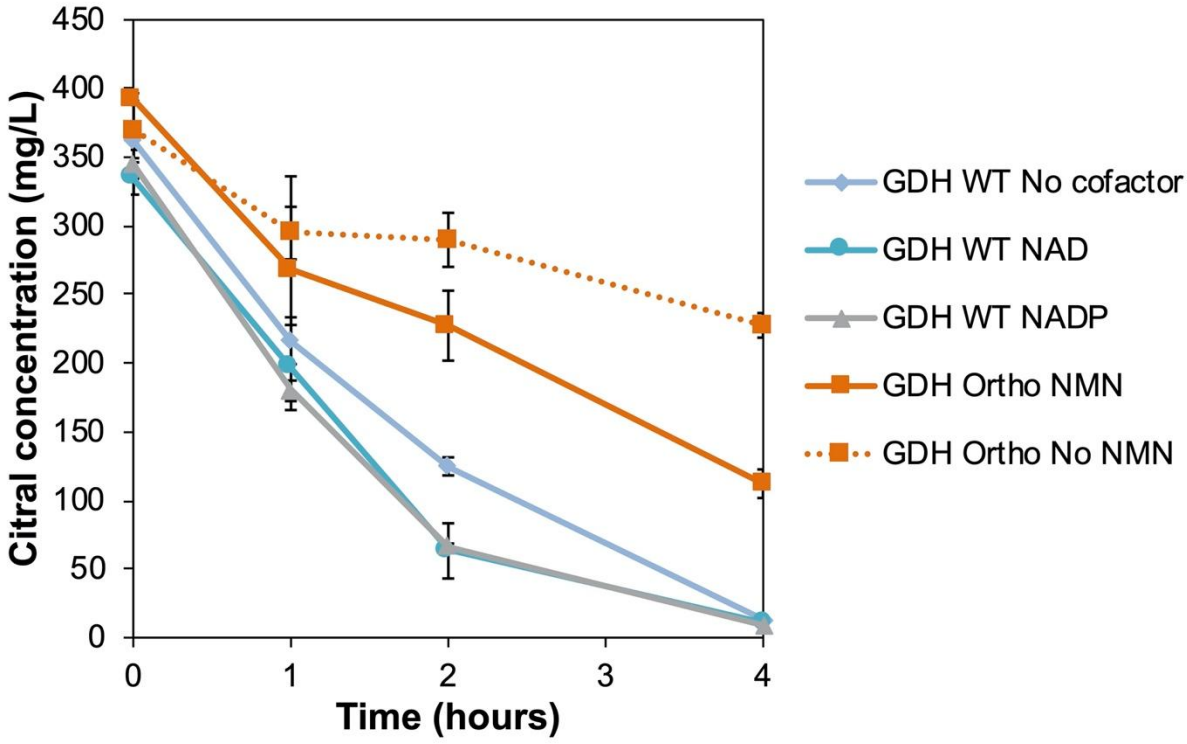


Figure S1: Citral consumption in crude lysate-based biotransformation

Crude *Escherichia coli* lysates were used to pair *Pseudomonas putida* enoate reductase XenA with wild type or engineered *Bacillus subtilis* glucose dehydrogenase (GDH WT or GDH Ortho, respectively) to convert citral to citronellal. When GDH WT was supplied, citral was readily consumed by XenA and endogenous aldehyde dehydrogenases. When GDH Ortho was supplied, citral was still present after four hours. However, when GDH Ortho was supplied, citral consumption was not proportional to aldehyde or alcohol product formation (Figure 2B). This indicates aldehyde oxidation and conversion to sulcatone are likely responsible for citral and citronellal degradation, as discussed previously [6]. Confirming this, preliminary results showed the presence of neric acid, geranic acid, citronellic acid, and sulcatone by gas chromatography in the crude lysate reactions (data not shown). Reactions were performed in assay buffer containing 12 mM magnesium acetate, 10 mM ammonium acetate, 130 mM potassium acetate, 10 mM potassium phosphate, 200 mM D-glucose, 1 mM of oxidized cofactor, and 500 mg/L citral. Lysates were mixed at a 1:1 protein ratio. Lysate was spiked into concentrated assay buffer to a final concentration of 4.5 mg/mL of protein in solution. Reactions were incubated at 37 °C without shaking. A detailed method can be found in the supplemental information.

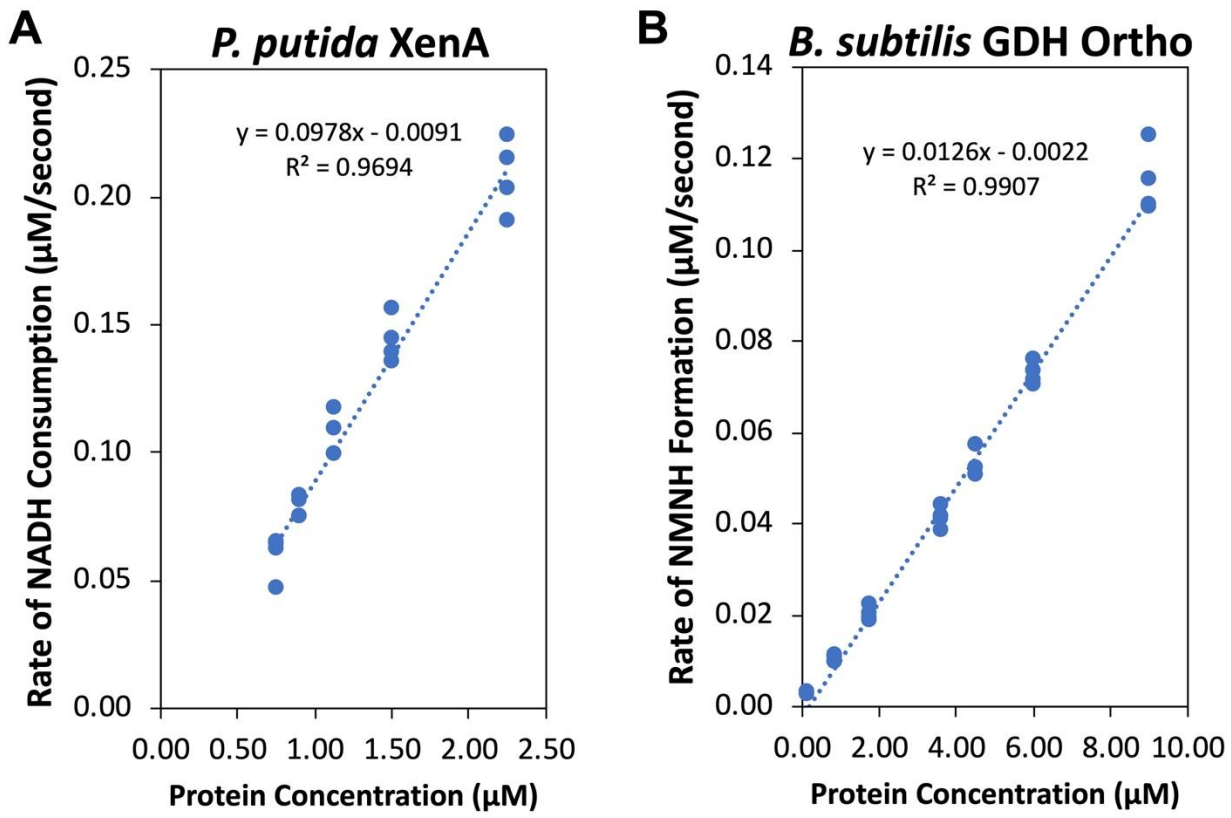


Figure S2: Standard curves used to determine recombinant protein concentration in crude lysates

To determine the molar concentration of recombinant protein in the crude *E. coli* lysates, specific protein activity standard curves were generated using purified protein. XenA (pEK102) or GDH Ortho (pLZ216) were expressed in *E. coli* strain BL21 (DE3) and purified using Zymo Research His-Spin Protein Miniprep. A dilution series of purified protein was spiked in assay buffer, and the initial reaction rate was monitored by measuring the change in light absorption at 340 nm using a spectrophotometer. (A) Purified *P. putida* XenA was spiked into assay buffer containing 200 mM potassium phosphate at pH 7.5, 0.2 mM NADPH, and 5 mM ketoisophorone at 37 °C. The initial reaction rate was determined by observing the decrease in light absorption at 340 nm, corresponding to the rate of NADPH consumption. No substrate reactions were run to determine the background. Reaction rate was determined by subtracting the no substrate reaction rate from the reaction rate with substrate. (B) Purified *B. subtilis* GDH Ortho was spiked into assay buffer containing 50 mM Tris-Cl at pH 8.0, 140 mM D-glucose, 3 mM NMN^+ at 25 °C. The initial reaction rate was determined by observing the increase in light absorption at 340 nm, corresponding to NMNH production. 4 replicates were run at each protein concentration. Reaction rate was plotted against enzyme concentration, and a best-fit line was fit to the data. The equation of the best-fit line was used to determine molar concentrations of recombinant enzymes in crude lysate.

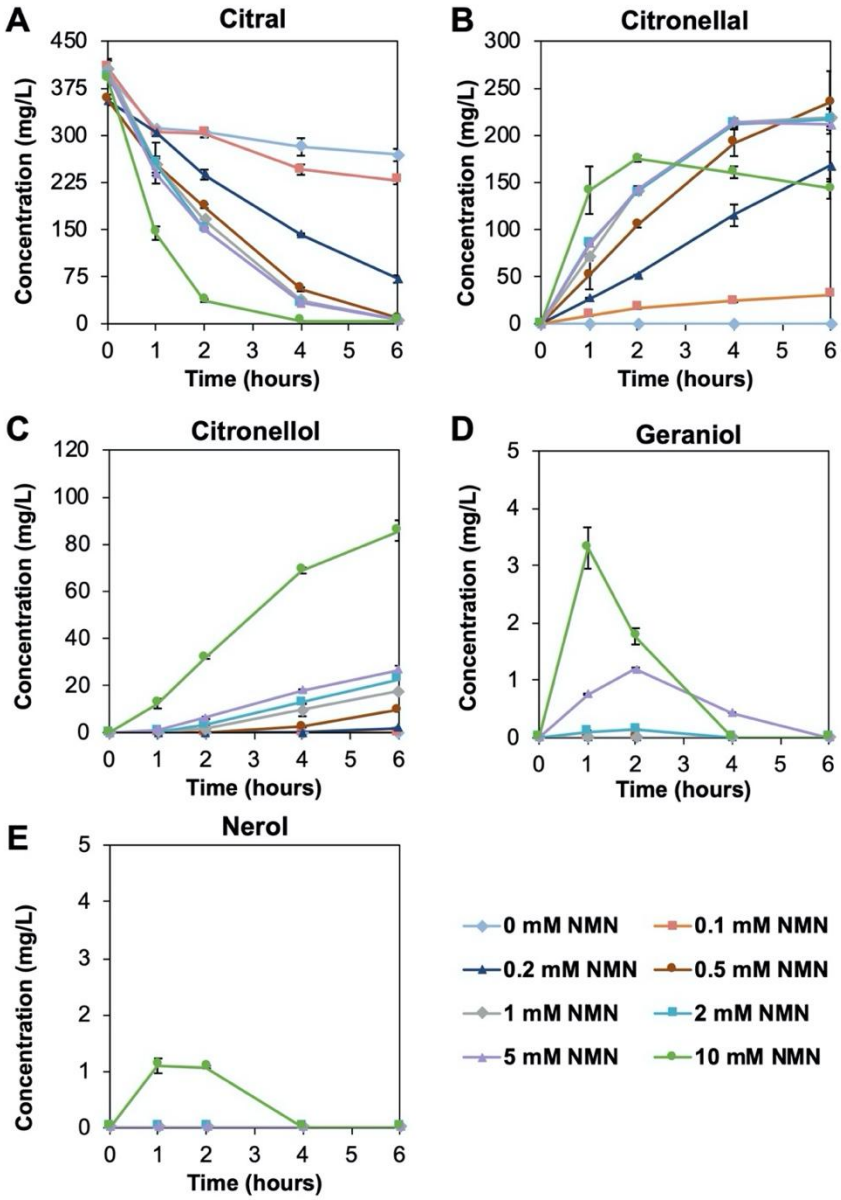


Figure S3: Time-course of citral biotransformation and optimization in Buffer A

(A-E) Citral consumption and product formation with varying NMN^+ supplementation. An increase of NMN^+ facilitated an increase in the rate of substrate conversion, but also a decrease in product purity. Low concentrations of NMN^+ (0.1, 0.2 and 0.5 mM) facilitated significant improvement of product purity and elimination of alcohol byproducts, geraniol and nerol, at the cost of low levels of substrate conversion. Reactions were performed in assay buffer containing 12 mM magnesium acetate, 10 mM ammonium acetate, 130 mM potassium acetate, 10 mM potassium phosphate, 200 mM D-glucose, 0-10 mM oxidized cofactor, and 500 mg/L citral. Lysates were mixed at a 7:1 XenA:GDH Ortho mass ratio. Lysate was spiked into concentrated assay buffer to a final concentration of 4.5 mg/mL of protein in solution. Reactions were incubated at 37 °C without shaking. A detailed method can be found in the Methods section. Values are an average of at least three replicates, and error bars represent one standard deviation.

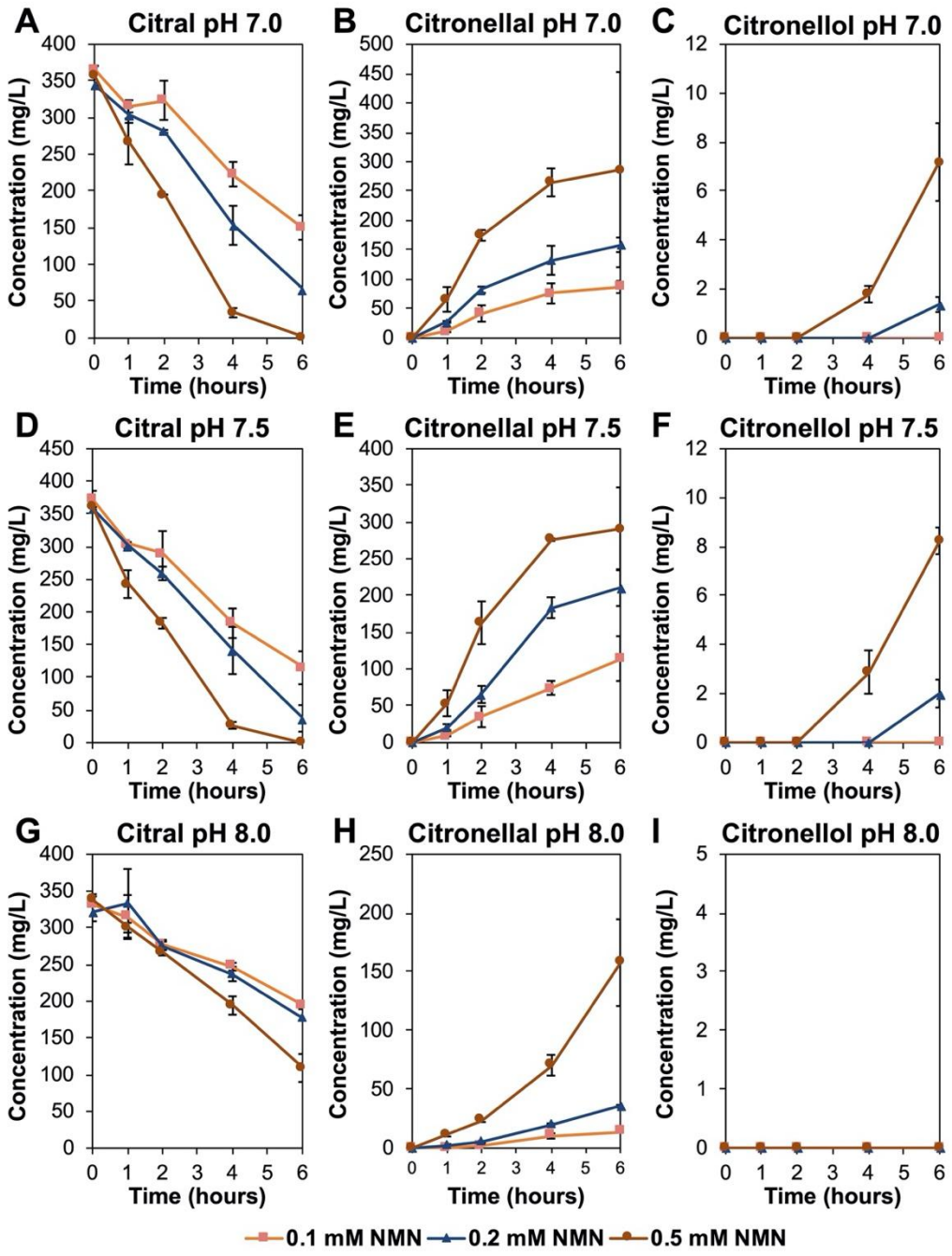
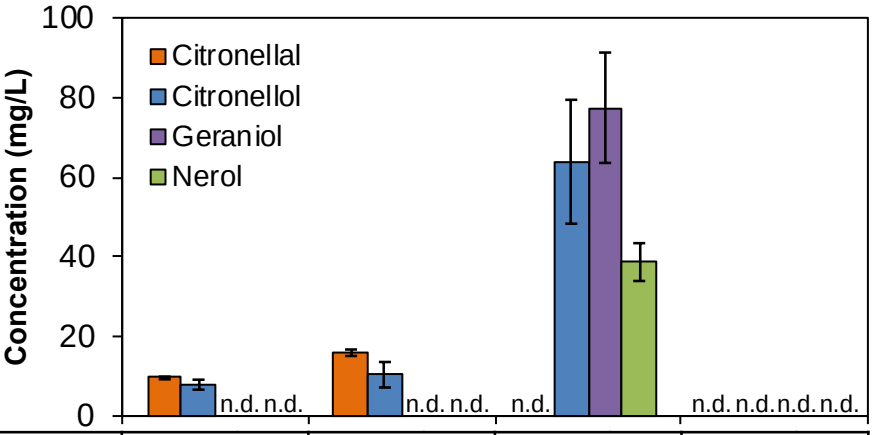


Figure S4: Time-course of citral biotransformation and optimization in Phosphate Buffer

(A-I) Citral consumption and product formation with varying NMN^+ supplementation and buffer pH. Nerol and geraniol were not detected at any condition tested. Reactions were performed in assay buffer containing 200 mM potassium phosphate at pH 7.0, 7.5, or 8.0, 200 mM NaCl, 200 mM D-glucose, 0.1-0.5 mM of oxidized cofactor, and 500 mg/L citral. Lysates were mixed at a 7:1 XenA:GDH Ortho mass ratio. Lysate was spiked into concentrated assay buffer to a final concentration of 4.5 mg/mL of protein in solution. Reactions were incubated at 37 °C without shaking. A detailed method can be found in the Methods section. Values are an average of at least three replicates, and error bars represent one standard deviation.



XenA	Low	High	High	High
GDH	High	Low	Low	Low
GDH Variant	Ortho	Ortho	WT	Ortho
NMN^+	2 mM	2 mM	2 mM	0 mM

Figure S5: Resting *E. coli* whole-cell biotransformation 24-hour timepoint

The 24-hour timepoint of resting whole-cell biotransformation from Figure 5B. Resting *E. coli* whole cells expressing *P. putida* XenA, *B. subtilis* GDH Ortho, and a glucose transporter, Glf, from *Z. mobilis*. The relative expression level between XenA and GDH Ortho was modulated by expressing them individually on two, multicopy plasmids with different replication origins and promoters. When High XenA expression was paired with low GDH Ortho expression, 16 mg/L of citronellal is produced with a product purity of 62%. When the expression levels between XenA and GDH Ortho are inverted, citronellal is only produced at 10 mg/L with a product purity of 55%. With GDH WT, only alcohol byproducts derived from citral and citronellal were produced. With no cofactor, no substrate conversion was observed. When compared with the 3-hour timepoint (Figure 5B), low XenA expression with high GDH Ortho expression increased the level of citronellal produced (from 6 to 10 mg/L) with improved product purity (from 42 to 55%). However, high XenA expression with low GDH Ortho expression resulted in decreased citronellal production (from 33 to 16 mg/L) and decreased product purity (from 83% to 62%). Whole-cell biotransformation was performed with resting *E. coli* cells at an OD600 of 10 with 200 mM D-glucose, 2 mM NMN^+, and 500 mg/L citral, at 30 ℃ for 24 hours while shaking at 250 r.p.m. Values are an average of at least three replicates, and the error bars represent one standard deviation. n.d., not detected.

Table S1: Strains and plasmids used in this study

Strains	**Description**	**Reference**
BL21 (DE3)	*E.* coli, protein expression strain for protein purification	Invitrogen
MX102	*E. coli* Δ(*araD-araB*)*567*, Δ*lacZ4787*(::rrnB-3), *λ-*, *rph-1*, Δ(*rhaD-rhaB*)*568*, *hsdR514*, Δ*pncC*, Δ*pgi*, Δ*zwf*, Δ*gntK::kan*	[1]
Plasmids	**Description**	**Reference**
pEK101	P_{LlacO1}*::Bs gdh*, ColE1 *ori*, Amp^R	[1]
pEK102	P_{LlacO1}*::Pp xenA*, ColE1 *ori*, Amp^R	[1]
pLZ216	P_{LlacO1}*::Bs gdh* I195R-A93K-Y39Q-S17E, ColE1 *ori*, Amp^R	[1]
pLZ217	P_{BAD}*::Pp xenA*, RSF *ori*, $Spec^R$	[1]
pSM106	P_{BAD}*::Bs gdh* I195R-A93K-Y39Q-S17E, RSF *ori*, $Spec^R$	[1]
pSM107	P_{BAD}*::Bs gdh*, RSF *ori*, $Spec^R$	[1]
pSM109	P_{LlacO1}*::Zm glf*, p15A *ori*, Cm^R	[1]

Abbreviations indicate source of genes: *Bs*, *Bacillus subtilis*; *Pp*, *Pseudomonas putida*; *Zm*, *Zymomonas mobilis*

Table S2: Accession numbers for proteins used in this study

Protein Name	Full Name	Protein ID
Pp XenA	NADH: flavin oxidoreductase/NADH oxidase	Q9R9V9
Bs Gdh	Glucose 1-dehydrogenase	P12310
Zm Glf	Glucose facilitated diffusion porin	P21906

D. References

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