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Supplementary Information

Functional assembly of a multi-enzyme methanol oxidation cascade on a surface-displayed trifunctional scaffold for enhanced NADH production

Fang Liu[a], Scott Banta[b], and Wilfred Chen[a*]

[a]Department of Chemical and Biomolecular Engineering, University of Delaware, Newark, DE 19716,

[b] Department of Chemical Engineering, Columbia University, New York, NY 10027

1. MATERIALS AND METHODS

1.1 Chemicals

All chemicals were reagent grade or higher and purchased from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). The high fidelity Phusion DNA polymerase (Thermo Scientific, Odessa, TX) and Taq DNA polymerase (Promega, Madison, WI) were used for PCR amplification. The oligonucleotides were synthesized by Integrated DNA technologies (Coraville, IA).

1.2 Strains and media

Escherichia coli strain NEB 5-alpha (*fhuA2* Δ(*argF-lacZ)U169 phoA glnV44* Φ*80*Δ (*lacZ*)M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*) was used as the host for genetic manipulations. *E. coli* SG13009[pREP4] (NaI^S Str^S Rif^S Thi^- Lac^- Ara^+ Gal^+ Mtl^- F^- $RecA^+$ Uvr^+ Lon^+) (QIAGEN) and *E. coli* BL21 (DE3) (F^- *ompT gal dcm lon* $hsdS_B(r_B^- m_B^-)$ λDE3) were used as the hosts for the expression of dehydrogenases. *Saccharomyces cerevisiae* strain EBY100 (*MATα ura3-52 trp1 leu2*Δ*1 his3*Δ*200 pep4::HIS3 prb1*Δ*1.6R can1 GAL* (*pIU211: URA3*)) was

used for the surface display of the trifunctional scaffold. *E. coli* cultures were grown in Luria-Bertani (LB) medium (10.0 g/liter tryptone, 5.0 g/liter yeast extract, 10.0 g/liter NaCl) or Terrific Broth (TB) medium (12.0 g/liter tryptone, 24.0 g/liter yeast extract, 4.0 ml/liter glycerol, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4) with either ampicillin or kanamycin. All yeast cultures were grown in SDC medium (20.0 g/liter dextrose, 6.7 g/liter yeast nitrogen base without amino acids, 5.0 g/liter Casamino Acids).

1.3 Construction of plasmids

To construct the scaffold without the internal cellulose binding domain (CBD), a gene fragment encoding for the cohesin domain from *Clostridium cellulolyticum* was first amplified from plasmid pScaf-ctf[1] with the forward primer 5'-CTA**GCTAGC**GGCGATTCTCTTAAAGTTACAG-3' (the boldface portion is a restriction endonuclease site) and the reverse primer 5'-ACGC**GTCGAC**GCGTGCATG**CCATGG**ATCGATCGTTACACTAC-3' and ligated into the surface display vector pCTCON2 digested with *Nhe*I and *Sal*I to generate plasmid pScaf-c. A gene fragment coding for the cohesin domains from *Clostridium thermocullum* and *Ruminoccocus flavefaciens* was amplified from pScaf-ctf[1] with the forward primer 5'-CATG**CCATGG**ACAACAAAACCACCTGCAAC-3' and the reverse primer 5'-ACGC**GTCGAC**CTTAACAATGATAGCGCCATCAG'-3'. The PCR product was ligated into pScaf-c digested with *Noc*I and *Sal*I to generate pScaf3.

Plasmid pQE9-adh-cc, encoding a His_6-tagged dockerin from *C. Cellulolyticum* fused to the alcohol dehydrogenase (ADH) from *Bacillus stearothermophilus* strain DSM 2334, was obtained by two-step cloning. First, a gene fragment coding for ADH was amplified by PCR from pQE9HSADH[2] with the forward primer 5'-

CCG**GAATTC***ATTAAAGAGGAGAAATTAACT*ATGAAAGCAGCAGTAGTTAACG-3' (the italic portion is part of the ribosome binding site (RBS)) and the reverse primer 5'-CCC**AAGCTT**GGGAGTCGC**GAGCTC**ATCCTCCTTCAATTTTAGTAC-3' and inserted into *EcoR*I/*Hind*III-linearized pQE9 to form pQE9-adh. Similarly, a gene fragment coding for the His_6-tagged dockerin from *C. Cellulolyticum* was amplified from pETEc[1] with primers 5'-CGC **GAGCTC**TGGGTTAAGGGTTCAGGCTGGC-3' and 5'-CCC**AAGCTT**TTAGTGATGGTGATGGTGATGCTGTGTGATTTTTCCTAACAAG-3' and inserted into *Sac*I and *Hind*III linearlized pQE9-adh to form pQE9-adh-cc.

Similarly, to construct the plasmid pQE9-fdh-rf, encoding a His_6-tagged dockerin from *R. flavefaciens* fused to the formate dehydrogenase (FDH) from *Saccharomyces cerevisiae*, a two-step cloning was performed. A gene fragment coding for FDH was amplified by PCR from pQE9HSFDH[2] with the forward primer 5'-CCG**GAATTC***ATTAAAGAGGAGAAATTAACT*ATGTCGAAGGGAAAGGTTTTGC-3' and the reverse primer 5'-GCCGAC**GTCGAC**GCTATGTAGCGC**GAGCTC**TTTCTTCTGTCCATAAGCTCTGG-3' and inserted into *EcoR*I/*Sal*I-digested pQE9 to form pQE9-fdh. A gene fragment coding for the His_6-tagged dockerin from *R. flavefaciens* was amplified from pETGf[1] with primers 5'-CGC**GAGCTC**GATGTTTCAAATAATGTTTACTATG-3' and 5'-GCCGAC**GTCGAC**TTAGTGATGGTGATGGTGATG TTGAGGAAGTGTGATGAG-3', and the PCR product was digested with *Sac*I and *Sal*I and ligated into similarly digested pQE9-FDH to form pQE9-fdh-rf.

To construct pET24a-faldh-ct, the gene encoding for the formaldehyde dehydrogenase (FALDH) from *Pseudomonas putida* was synthesized from GenScript (Piscataway, NJ) and was

amplified by the forward primer 5'- GGAATTC**CATATG**TTGCGCGCTGCCGC-3' and the reverse primer 5'- CGC**GGATCC**CAATGAGTTCTTCTGAGGCAC-3'. The PCR product was inserted into pET24a using NdeI and BamHI to create pET24a-faldh. A gene fragment coding for the His_6-tagged dockerin from *C. thermocellum* was amplified from pETAt[1] using primers 5'-CGC**GGATCC**GGAAACTTCCCGAATCCTTTG-3' and 5'-CCG**CTCGAG**ATAAGGTAGGTGGGGTATGC-3', and the PCR product was digested with *BamH*I and *Xho*I and ligated into pET24a-FALDH to form pET24a-faldh-ct.

1.4 Display of scaffolds on the yeast cell surface

To display the trifunctional scaffolds on the yeast cell surface, yeast cells transformed with pScaf3 were precultured in SDC medium at 30 °C. The overnight culture were inoculated into SGC medium (20.0 g/liter galactose, 6.7 g/liter yeast nitrogen base without amino acids, 5.0 g/liter casamino acids) at an optical density (OD) at 600 nm of 0.1 and induced for 48 h at 20 °C.

1.5 Expression of dockerin-tagged dehydrogenases

The ADH-DocCC fusion protein was expressed in *E. coli* SG13009. Overnight cultures were inoculated into 100 ml LB medium supplemented with 200 μg/ml ampicillin and incubated at 37 ˚C until the OD_{600} reached 0.6-0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 50 μM and cells were grown at 20˚C overnight. *E. coli* SG13009 expressing FDH-DocRF were precultured overnight at 37 ˚C and inoculated into 100 ml of TB medium supplemented with 200 μg/ml ampicillin and incubated at 37˚C until the OD_{600} reached 0.8-1.0. IPTG was added to a final concentration of 50 μM and cells were grown at 20˚C overnight. The FALDH-DocCT fusion protein were expressed in *E. coli* strain BL21(DE3). Overnight cultures were inoculated into 100 ml LB medium supplemented with 1.5% glycerol

and 50 μg/ml kanamycin at an initial OD of 0.05 and incubated at 37 °C until the OD reached 1.5. Then IPTG was added to a final concentration of 400 μM and cells were grown overnight at 20˚C. All cultures were harvested by centrifugation (4000 × g, 10 min) at 4 ˚C, resuspended in binding buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM $CaCl_2$, pH 8.0), and lysed with a sonicator.

1.6 Multi-enzyme cascades assembly on the yeast cell surface

To assembly the individual dehydrogenase on the yeast cell surface, cell lysates containing each dockerin-tagged dehydrogenase were mixed with yeast cells displaying the scaffold for 1 h at room temperature in the binding buffer. To assemble the multi-enzyme cascades, cell lysates mixture containing ADH-DocCC, FALDH-DocCT and FDH-DocRF were incubated with yeast cells displaying the scaffolds. After incubation, cells were washed and harvested by centrifugation (3,000 × g, 10 min) at RT and resuspended in the binding buffer.

1.7 Immunofluorescence microscopy

Yeast cells displaying either the scaffold or the dehydrogenase multi-enzyme cascades on the surface were harvested by centrifugation and washed twice with PBS buffer. Immunofluorescence microscopy was performed using anti-c-Myc or anti-c-His immunoglobulin G as previously described[1].

1.8 Enzymatic activity assay

The alcohol dehydrogenase activity was assayed at 37 °C in a reaction mixture (1 ml) containing 100 mM sodium phosphate buffer, pH 8.5, 5 mM β-NAD and either 100 mM methanol or 10 mM ethanol. The reaction was started by addition of 50 μl ADH-DocCC cell lysates and the production of NADH was measured at 340nm with a spectrophotometer (UV-

1800, Shimadzu, Columbia, MD) at 37 °C. The formaldehyde dehydrogenase activity was performed at 37 °C in 100 mM sodium phosphate, pH 8.5, containing 2 mM β-NAD and 2 mM formaldehyde. Finally, the formate dehydrogenase activity was assayed at 37 °C in 100 mM potassium phosphate, pH 7.0, containing 5 mM β-NAD and 200 mM sodium formate. The enzyme activity was calculated using the following equation and expressed as

$$\text{Units/ml enzyme} = \frac{(\Delta A_{340nm}/\min Test - \Delta A_{340nm}/\min Blank)(1)(df)}{(6.22)(0.05)}$$

, where ΔA_{340nm}/min was obtained using the maximum linear rate of absorption at 340 nm; 1 = total volume (in milliliter) of assay; df = dilution factor; 6.22 = millimolar extinction coefficient of NADH at 340 nm; 0.05 = volume (in milliliter) of enzyme lysates used. The three-enzyme cascade reactions were operated at pH 8.5, which is within the optimum range of all three enzymes.

2. TABLE

Table S1 Specific activities of the three dehydrogenases

	ADH-DocCC	FALDH-DocCT	FDH-DocRF
Enzyme unit (U/mg total protein)	0.028 ± 0.013	0.162 ± 0.012	0.131 ± 0.018

References

1. S.-L. Tsai, J. Oh, S. Singh, R. Chen, W. Chen, *Appl. Environ. Microbiol.* 2009, **75**, 6087.

2. Y. H. Kim, E. Campbell, J. Yu, S. D. Minteer, S. Banta, *Angew. Chem. Int. Ed.* 2013, DOI: 10.1002/anie.201207423.