Supplementary Information

Synthetic engineering of a new biocatalyst encapsulating [NiFe]-hydrogenases for enhanced hydrogen production

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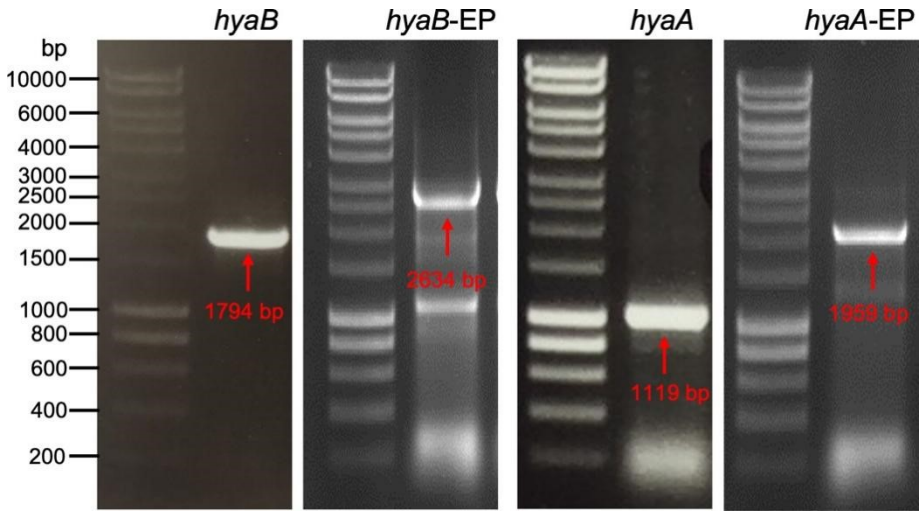
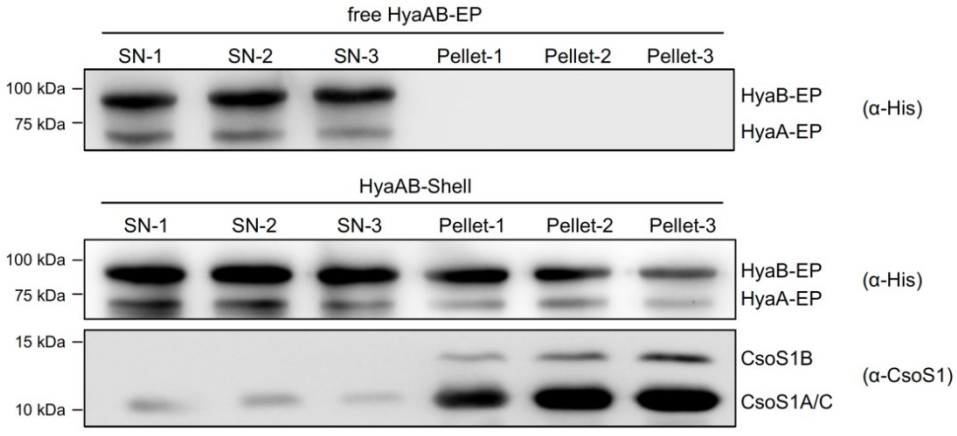
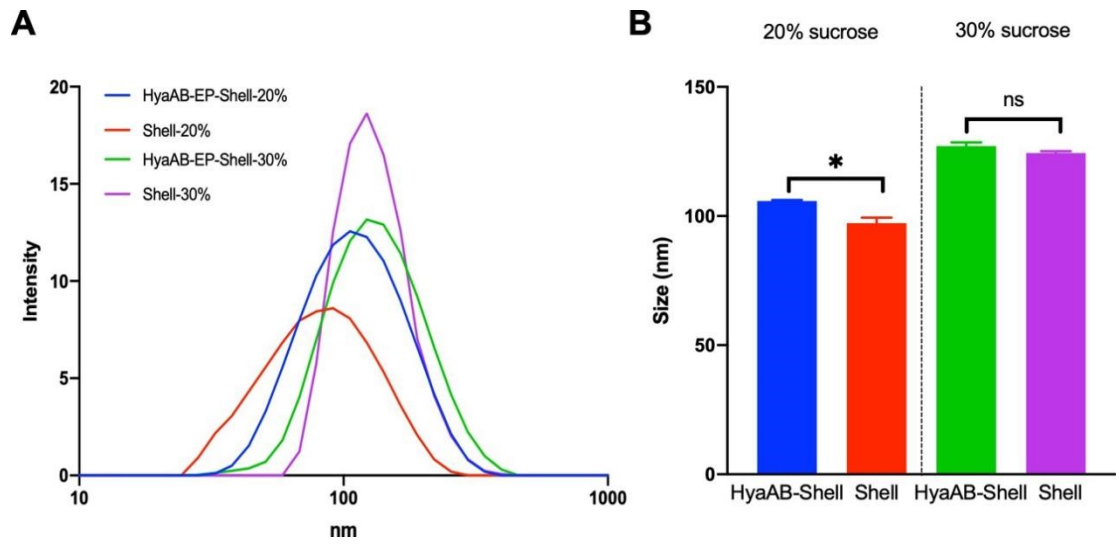


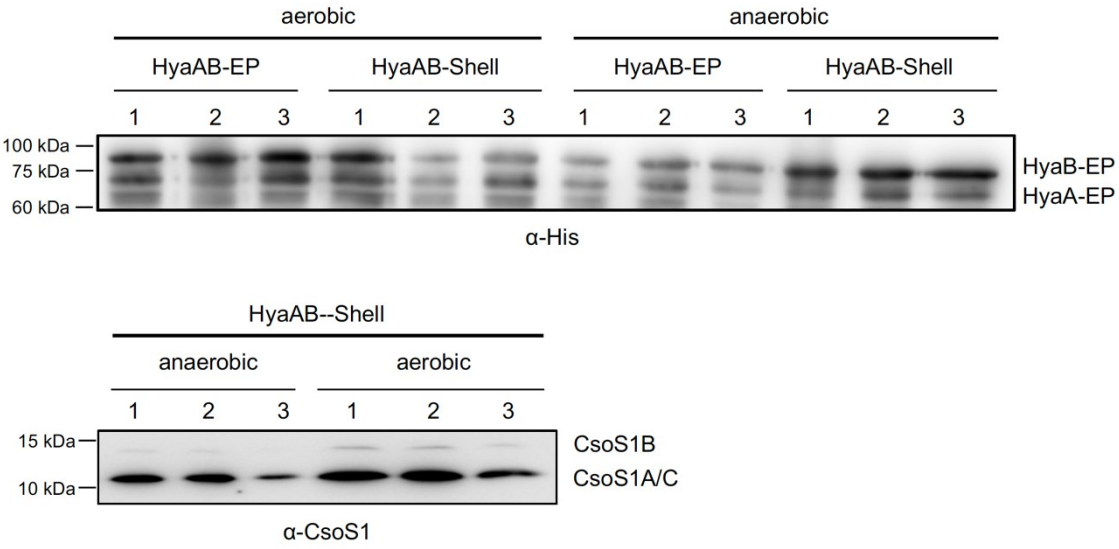
Supplementary Figure 1. Construction of the vectors expressing EcHyd-1, verified by PCR (*hyaA, hyaB, hyaA*-EP, *hyaB*-EP).



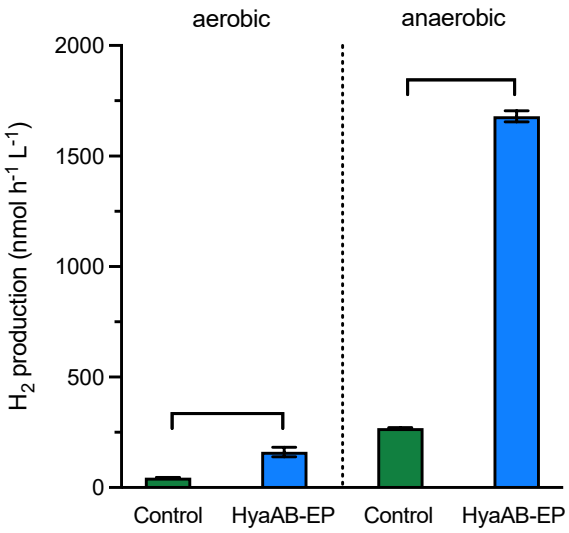
Supplementary Figure 2. Encapsulation of HyaAB with recombinant α-carboxysome shells. Immunoblot analysis of the supernatants and pellets of cell extracts after 50,000 x *g* centrifugation from *E. coli* strains producing HyaAB-EP alone or co-expressing HyaAB-EP with α-carboxysome shells (with three biological repeats), using anti-His and anti-CsoS1 antibodies, respectively.



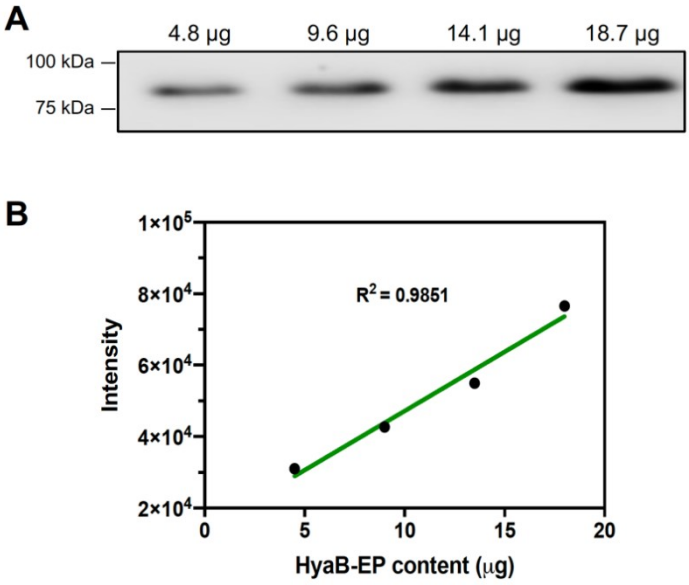
Supplementary Figure 3. Comparison of the sizes of HyaAB-Shell assemblies and empty shells. (**A**) Diameters of α-carboxysome shells and HyaAB-Shell assemblies in the 20% and 30% sucrose fractions after sucrose gradient centrifugation, measured by Dynamic Light Scattering (DLS). (**B**) Average diameters of HyaAB-Shell catalysts and empty shells in the 20% and 30% sucrose fractions measured by DLS. Values represent mean ± standard deviation, *n* = 3 biologically independent experiments. *ns*, non-significant difference. * *p* = 0.02, two-tailed unpaired t-test.



Supplementary Figure 4. Immunoblot analysis of HyaAB-Shell and unencapsulated HyaAB. Heterologously expressed *Ec*Hyd-1 and α-carboxysome shell proteins were determined using anti-6xHis-tag and anti-CsoS1A/C/B antibodies.



Supplementary Figure 5. *In vivo* hydrogen production of wild-type *E. coli* BL21(DE3) and *E. coli* cells expressing free HyaAB-EP after 16-hour induction under aerobic (left) and anaerobic (right) conditions. ***, p = 0.001, ****, p < 0.0001, two-tailed unpaired t-test. Error bars represent the standard deviation of the mean of three biological replicates.



Supplementary Figure 6. A linear correlation was observed between the quantitation of HyaB-EP by BCA protein assay kit and densitometric quantitation from immunoblot analysis. (**A**) Immunoblot analysis of free HyaB-EP using an anti-His antibody. Isolated free HyaB-EP at various protein concentrations were loaded onto SDS-PAGE gels for quantification by immunoblot analysis. (**B**) The linear relationship between HyaB-EP content quantified by BCA protein assay and immunoblot analysis based on three biologically independent experiments. Densitometric quantitation of HyaB-EP levels was determined by using ImageJ.

Supplementary Table 1. Primers used in this study. Overlap sequences for Gibson Assembly are underlined.

Primers	Nucleotide sequences	Note
pCDF-HyaB-FW	CCATCACCATCATCACCACAGCCAGAGCACTCAGTACGAAACT	amplification of *hyaB* (plasmid pCDF-HyaB)
pCDF-HyaB-RV	TCGACTTAAGCATTATGCGGCCGCATTAACGCACCTGCACGGAGATCAGC	
pCDF-HyaA-FW	GTTAAGTATAAGAAGGAGATATACACATCACCATCATCACCACATGAATAACGAGGAAACA	amplification of *hyaA* (plasmid pCDF-HyaAB)
pCDF-HyaA-RV	CGCAGCAGCGGTTTCTTTACCAGACTCATGCCTGTTTATCCTC	
pCDF-HyaB-EP-FW	CCATCACCATCATCACCACAGCCAGAGCACTCAGTACGAAACT	amplification of *hyaB* (plasmid pCDF-HyaB-EP)
pCDF-HyaB-EP-RV	ACGCACCTGCACGGAGATCAGCTCG	
pCDF-HyaB-EP-FW	CGAGCTGATCTCCGTGCAGGTGCGTACGAGCACCCCAGAGCCC	amplification of *csoS2*-Cterm (plasmid pCDF-HyaB-EP)
pCDF-HyaB-EP-RV	AGCATTATGCGGCCGCAAGCTTCAACCGCGCGCGCCGCC	
pCDF-HyaA-FW	GTTAAGTATAAGAAGGAGATATACACATCACCATCATCACCACATGAATAACGAGGAAACA	amplification of *hyaA* (plasmid pCDF-HyaAB-EP)
pCDF-HyaA-RV	ACCCATTTGCGGAATATCGACCACG	
pCDF-HyaA-EP-FW	CGTGGTCGATATTCCGCAAATGGGTACGAGCACCCCAGAGCCC	amplification of *csoS2*-Cterm (plasmid pCDF-HyaB-EP)
pCDF-HyaA-EP-RV	CGCAGCAGCGGTTTCTTTACCAGACTCGATCAACCGCGCGCGCCGCC	
HyaB-seq-FW	GGATCTCGACGCTCTCCCT	Sequencing primers for pCDF-HyaB/pCDF-HyaB-EP
HyaB-seq-RV	CGATTATGCGGCCGTGTACAA	
HyaA-seq-FW	TTGTACACGGCCGCATAATC	Sequencing primers for pCDF-HyaAB/pCDF-HyaAB-EP
HyaA-seq-RV	GCTAGTTATTGCTCAGCGG	