Supporting Information:

Controllable Display of Sequential Enzymes on Yeast Surface with Enhanced Biocatalytic Activity towards Efficient Enzymatic Biofuel Cells

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1. Supplementary Experimental Methods

1.1 Strains, media and chemicals

The recombinant strains and plasmids in the present study were listed in **Table S1**. *Escherichia coli* DH5α (F-φ80 lacZΔM15Δ(lacZYA-argF) U169 endA1 recA1 hsdR17 (rk-,mk+)supE44λ-thi-1 gyrA96 relA1 phoA) was used for recombinant DNA manipulation. *S. cerevisiae* EBY100 and *Pichia pastoris* X-33 (Invitrogen, Carlsbad, CA) were used for yeast cell surface display of the scaffoldins and secreted expression of recombinant proteins. *E. coli* was cultured in Luria-Bertani (LB) medium supplemented with ampicillin (100 μg mL^{-1}) or zeocin (25 μg mL^{-1}). Yeasts were grown in YPD media. *S. cerevisiae* EBY100 transformants were selected on SD-trp plates, and galactose was used to replace glucose in SD-trp media for protein expression induction. *P. pastoris* X-33 transformants were selected on YPDS plates supplemented with zeocin at 100 μg mL^{-1}. The recombinant cells were percultured in BMGY medium, and then induced in BMMY with 1% methanol and 10 mM $CaCl_2$. All restriction enzymes were obtained from Fermentas (MBI Fermentas, Canada). Graphene was kindly given by Dr. Liwei Liu in Suzhou Institute of Nano-tech and Nano-bionics, Chinese Academy of Sciences. Poly(acrylic acid) (PAA) and 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were obtained from Aladdin Reagent Company (Shanghai, China). D-glucose, starch and methylene blue (MB) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Cation exchange membrane was purchased from the Green Environmental Protection Technology Co., Ltd (Hangzhou, China). Laccase (Lac) from *Trametes versicolor* and Nafion® 117 solution containing 5% Nafion were purchased from Sigma-Aldrich (St. Louis, USA). Lac was purified using a dialysis membrane (cutoff molecular weight: 10 kD) before use. The specific activity of Lac is 13.6 and 21.2 U mg^{-1} for before and after dialysis, respectively. All other chemicals were used as received without further purification. Ultrapure water was prepared using a Milli-Q system (Millipore, Billerica, MA, USA).

1.2 Construction, assay and evaluation of the displayed sequential enzyme systems

1.2.1 Plasmid construction

Primers used for plasmid construction are summarized in **Table S2**. Plasmid pYD1 and pPICZαA were used as the parent vector for construction and expression of fusion protein. Plasmids bearing the genes of Coh-Doc pair proteins were presented by Dr. Yingang Feng in QIBEBT, Qingdao. A gene encoding scaffoldin with CohC-CohT was obtained by performing PCR using primer pair cohC-cohT For/Rev using pET28NS-C1-T3 as the template. The

amplified fragment was cloned into Nhe I/Bam HI-digested plasmid pYD1 to form pYD1-cohC-cohT. CohC encoding gene was amplified using primer pair cohC-F1 For/ cohC-R1 Rev. The product was digested by Xho I and Apa I and ligated into pYD1-cohC- cohT to form pYD1-cohC-cohT-cohC. To obtain plasmid pYD1-cohC-cohC-cohT, the fragment of cohC gene was amplified by PCR with primers cohC-F2 For and cohC-R2 Rev, which was cut with Nhe I and Bam HI and ligated into the corresponding sites of pYD1, and then the gene fragment of cohC-cohT was amplified with primers cohC-cohT2 For/Rev, which was cut with Bam HI and Xho I and ligated into the corresponding sites of above vector. In order to construct plasmid pYD1-cohC-cohC-cohT-cohC, CohC encoding gene was amplified with primers cohC-F1 For and cohC-R1 Rev, which was cut with Xho I and Apa I and ligated into the corresponding sites of pYD1-cohC-cohC-cohT. In order to construct plasmid pYD1-cohC-cohC-cohT-cohC, the fragment of cohc gene was amplified by PCR with primers cohC-F2 For and cohC-R2 Rev, which was cut with Nhe I and Bam HI and ligated into the corresponding sites of pYD1-cohC-cohT-cohC. These plasmids were transformed in S. cerevisiae EBY100 using the standard lithium acetate procedure. Corresponding DocC and DocT encoding genes were amplified by PCR using docC For/Rev, docT For/Rev as primers from pET28NS-docC and pET28NS-docT, respectively. *S. fibuligera* genomic DNA was obtained from Prof. Zhenming Chi (Ocean University of China, Qingdao) as a gift. Gene fragment coding for GA were amplified with *S. fibuligera* genomic DNA as the template using primer pair ga For/ Rev, and GOx encoding gene was amplified by PCR from genomic DNA of *A. niger* using primers gox For and gox Rev. GA gene and DocC gene were fused by Overlap Extension PCR to obtain GA-DocC fusion gene, which was inserted into pPICZαA vector to form pPICZαA-ga-docC. To generate pPICZαA-gox-docT, the same method was used. Both of two secreted expression vectors were separately transformed in P. pastoris X-33 by electroporation method.

1.2.2 Enzyme assays

$$\text{starch} \xrightarrow{\text{GA}} \text{glucose} \qquad (1)$$

$$\text{glucose} + O_2 \xrightarrow{\text{GOx}} \text{gluconolactone} + H_2O_2 \qquad (2)$$

GA activity was measured at room temperature for 15 min in 50 mM HAc–NaAc buffer (pH 5.0) containing 0.1% soluble starch. Glucose was detected using glucose detection kit bought from Jiancheng, China. GA enzyme activity (one unit) is defined as the enzyme amount that generates 1 μmol glucose per min under the given assay conditions. GOx activity assay was used as described before [1]. Briefly, enzyme samples were added into 100 mM phosphate buffer (pH 7.4) containing 10 mM glucose, 0.17 mM *o*-dianisidine dihydrochloride and 2 U

mL^{-1} horseradish peroxidase (HRP). The enzymatic reaction was carried out at room temperature. Then the absorbance at 500 nm was recorded. One unit of GOx activity is defined as the enzyme amount that 1 μmol H_2O_2 per minute under the above assay condition.

1.2.3 The overall reaction rate of GA&GOx-yeast catalyzing starch

Starch was used as substrate, which could be finally transformed into D-gluconolactone and hydrogen peroxide by GA&GOx-yeast (eqs.1, 2). The overall reaction was conducted for 15 min in 50 mM HAc–NaAc buffer (1 mL, pH 5.0) containing reaction cocktail (0.1% soluble starch, 0.17 mM *o*-dianisidine dihydrochloride, 2 U mL^{-1} HRP) and GA&GOx-yeast cells (OD_{600nm}=1). The overall reaction rate (nmol H_2O_2 min^{-1} mL^{-1}) is defined as the amount of generated H_2O_2 (nmol mL^{-1}) under the above assay condition. All the experiments were repeated at least three times.

1.2.4 Estimation of expressed GA and GOx numbers on the yeast cell surface

The calculation of the apparent numbers of enzymes displayed per cell was carried out based on the the previous reports [2, 3]. The activity of the immobilized enzymes on the cell surfaces is assumed to be the same as that of the free enzymes. To determine the copy number of GA and GOx enzymes on the yeast cells surface, enzymatic activity assays were conducted using various amounts of purified dockerin-fused GA and dockerin-fused-GOx, respectively. The calibration curves were obtained by plotting the enzyme activities varying with the amounts of purified dockerin-fused enzymes ranging from 0 to 150 μg. The amounts of expressed enzymes on the surface of the strains were estimated on basis of the calibration curves. Finally, the numbers of displayed enzyme molecules on the cell surface were calculated on the basis of the molecular weights of dockerin-GA and dockerin-GOx along with the numbers of yeast cells.

1.2.5 Testing the stability of engineered yeast

Yeast cells co-displayed GA and GOx were incubated at 4 ℃ and room temperature, respectively. To evaluate the activity at 4 °C, it is necessary to bring the sample back to the room temperature, to measure and then to return the samples to 4 °C. Meanwhile, the mixture of the same amount of free enzymes was treated under the same conditions. Over a period of 30 days, equal volume of each sample solution was taken to check the overall reaction rate as described above every 5 days.

1.3 Construction of EBFCs and their performance testing

1.3.1 Preparation of PAA dispersed graphene

PAA-graphene complex was prepared by dispersing 2 mg graphene into 1 mg mL^{-1} PAA in water under vigorous sonication for 30 min until a homogeneous dispersion resulted.

1.3.2 Apparatus and electrochemical measurements

Electrochemical measurements were carried out with a CHI660E potentiostat (CH Instruments, Chenhua, Shanghai, China). The half-cell measurement was performed in a conventional three-electrode system using the as-prepared electrode as working electrode, a saturated calomel electrode (SCE) as reference electrode, and a Pt wire electrode as auxiliary electrode. All potentials were reported in this paper were recorded versus this reference.

2. Supplementary Tables:

Table S1. Strains and plasmids used in this study

Strain	Plasmid	Description
EBY	pYD1	No surface display (negative control)
EBY-C_1T_1	pYD1-cohC-cohT	Surface display of scaffoldin with CohC-CohT on *S. cerevisiae* EBY100
EBY-$C_1T_1C_1$	pYD1-cohC-cohT-cohC	Surface display of scaffoldin with CohC-CohT-CohC on *S. cerevisiae* EBY100
EBY-C_2T_1	pYD1-cohC-cohC-cohT	Surface display of scaffoldin with CohC-CohC-CohT on *S. cerevisiae* EBY100
EBY-$C_2T_1C_1$	pYD1-cohC-cohC-cohT-cohC	Surface display of scaffoldin with CohC-CohC-CohT-CohC on *S. cerevisiae* EBY100
X33-GA	pPICZαA-ga-docC	Secreted expression of GA-DocC fusion protein in *P. pastoris*
X33- GOx	pPICZαA-gox-docT	Secreted expression of GOx-DocT fusion protein in *P. pastoris*

Table S2. PCR primers used in this study

Primer	Sequence (5'→3')
cohC-cohT For	CTAGCTAGCATCCCTGGCGATTCTCTTAAAG
cohC-cohT Rev	CGGGATCCTCACAGATCCTCTTCTGAGATGAGTTTTTGTTCAGGTGTTGT AGGTGTTGTAGG
cohC-F1 For	CCGCTCGAGATCCCTGGCGATTCTCTTAAAG
cohC-R1 Rev	TCCGGGCCCTTGAGTACCAGGATCTATAGTTACAC
cohC-F2 For	CTAGCTAGCATCCCTGGCGATTCTCTTAAAG
cohC-R2 Rev	CGGGATCCTTGAGTACCAGGATCTATAGTTACAC
ga For	GGAATTCATGAACACTGGACATTTCC
ga Rev	CAATTACTGGGTCAGGATCTGTAAGAAGTTCAATCAATTT
gox For	CCGCTCGAGAAAAGAGAGGCTGAAGCTAGGAGCAATGGCATTGAAG
gox Rev	GACGTCGCCGTATAATTTAGTCTGCATGGAAGCATAATCTTCCAAG
docC For	AAATTGATTGAACTTCTTACAGATCCTGACCCAGTAATTG
docC Rev	GGGGTACCGTGTTGCTTGGAAGCTTACTTACC
docT For	CTTGGAAGATTATGCTTCCATGCAGACTAAATTATACGGCGACGTC
docT Rev	AAGGAAAAAAGCGGCCGCGTTCTTGTACGGCAATGTATC

Table S3. Amino acid sequences of dockerin-fused proteins

dockerin-fused protein	Amino acid sequence
GA-DocC	MNTGHFQAYSGYTVARSNFTQWIHEQPAVSWYYLLQNIDYPEG QFKSAKPGVVVASPSTSEPDYFYQWTRDTAITFLSLIAEVEDHSF SNTTLAKVVEYYISNTYTLQRVSNPSGNFDSPNHDGLGEPKFNV DDTAYTASWGRPQNDGPALRAYAISRYLNAVAKHNNGKLLLAG QNGIPYSSASDIYWKIIKPDLQHVSTHWSTSGFDLWEENQGTHF FTALVQLKALSYGIPLSKTYNDPGFTSWLEKQKDALNSYINSSG FVNSGKKHIVESPQLSSRGGLDSATYIAALITHDIGDDDTYTPFN VDNSYVLNSLYYLLVDNKNRYKINGNYKAGAAVGRYPEDVYN GVGTSEGNPWQLATAYAGQTFYTLAYNSLKNKKNLVIEKLNYD LYNSFIADLSKIDSSYASKDSLTLTYGSDNYKNVIKSLLQFGDSF LKVLLDHIDDNGQLTEEINRYTGFQAGAVSLTWSSGSLLSANRA RNKLIELLTDPDPVIVYGDYNNDGNVDALDFAGLKKYIMAADH AYVKNLDVNLDNEVNAFDLAILKKYLLGMVSKLPSN
GOx-DocT	MRSNGIEASLLTDPKDVSGRTVDYIIAGGGLTGLTTAARLTENPN ISVLVIESGSYESDRGPIIEDLNAYGDIFGSSVDHAYETVELATNN QTALIRSGNGLGGSTLVNGGTWTRPHKAQVDSWETVFGNEGW NWDNVAAYSLQAERARAPNAKQIAAGHYFNASCHGTNGTVH AGPRDTGDDYSPIVKALMSAVEDRGVPTKKDFGCGDPHGVSM FPNTLHEDQVRSDAAREWLLPNYQRPNLQVLTGQYVGKVLLS QNGTTPRAVGVEFGTHKGNTHNVYAEHEVLLAAGSAVSPTILE YSGIGMKSILEPLGIDTVVDLPVGLNLQDQTTATVRSRITSAGAG QGQAAWFATFNETFGDYSEKAHELLNTKLEQWAEEAVARGGFH NTTALLIQYENYRDWIVNHNVAYSELFLDTAGVASFDVWDLLPF TRGYVHILDKDPYLHHFAYDPQYFLNELDLLGQAAATQLARNI SNSGAMQTYFAGETIPGDNLAYDADLSAWTEYIPYHFRPNYHG VGTCSMMPKEMGGVVDNAARVYGVQGLRVIDGSIPPTQMSSH VMTVFYAMALKISDAILEDYASMQSGSGSGSGSGTKLYGDVND DGKVNSTDAVALKRYVLRSGISINTDNADLNEDGRVNSTDLGIL KRYILKEIDTLPYKN

Table S4. The numbers of expressed enzyme units on the yeast cell surface

Strain	GA-DocC	GOx-DocT
EBY-C_1T_1-GA-GOx	38000	37000
EBY-$C_1T_1C_1$-GA-GOx	72000	37000
EBY-$C_2T_1C_1$-GA-GOx	64000	21000

Note: In order to investigate the molecular ratio of GA to GOx on the cell surface, the numbers of expressed enzyme units on the bifunctional yeast cell surface were determined. It was estimated to be about 38000 GA-DocC and 37000 GOx-DocT enzymes per cell (calculated ratio, 1.03:1) when the scaffoldin was CohC-CohT. The correct 1:1 binding ratio demonstrated the proper folding of fusion proteins. As expected, the number of GA-DocC doubled, but that of GOx-DocT retained the same level as CohC domain doubled. However, a downward trend was observed when the scaffoldin was CohC-CohC-CohT-CohC. Specifically, there were about 64000 GA-DocC enzymes and 21000 GOx-DocT enzymes per cell. The calculated ratio (3.05:1) was very close to our designed value (GA:GOx=3:1).

3. Supplementary Figures:

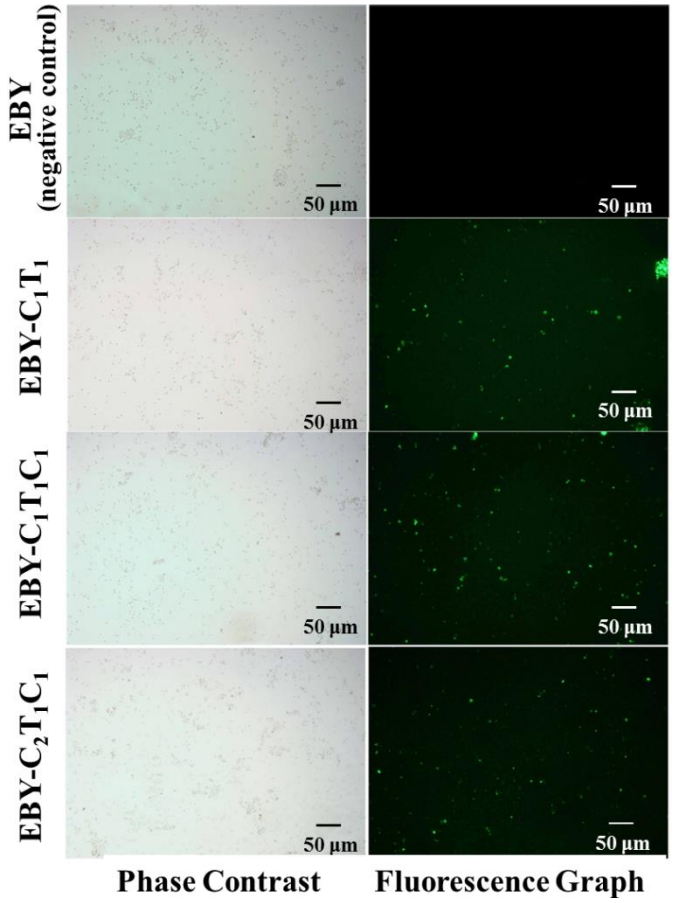

Figure S1. Phase-contrast and immunofluorescence micrographs of yeast cells displaying synthetic scaffoldins

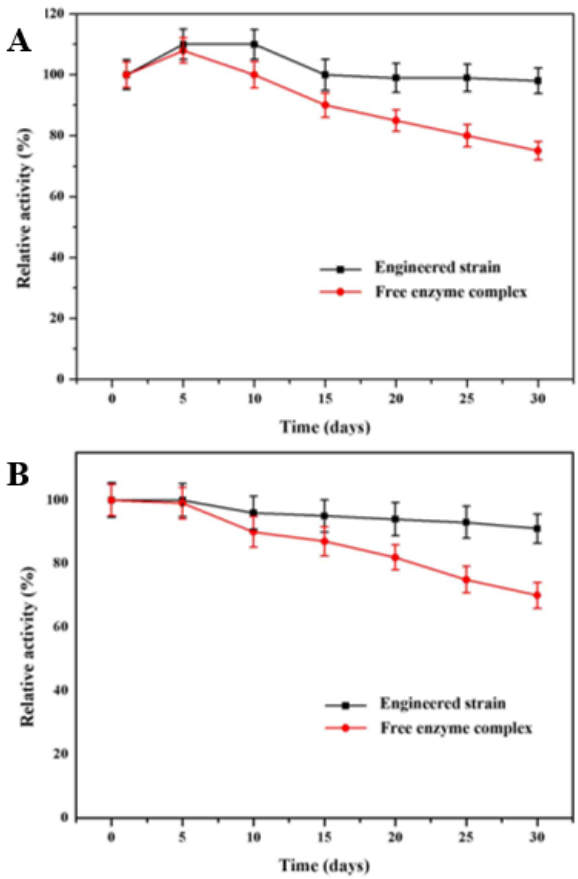

Figure S2. Long-term stability of sequential enzymes co-displayed on cell surface (EBY-$C_1T_1C_1$-GA-GOx) and the same amount of free enzyme complex at 4 ℃ (A) and room temperature (B). Error bars represent the standard error of three replicates.

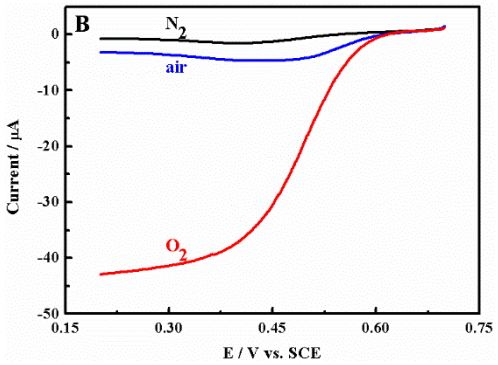

Figure S3. (A) CVs of the Lac/graphene/GCE biocathode in 0.2 M pH 5.0 McIlvaine buffer containing 0.5 mM ABTS under (a) N_2-saturated atmosphere, (b) ambient air, and (c) O_2-saturated atmosphere. Scan rate: 20 mV s^{-1}. (B) Polarization curves of the Lac/graphene/GCE biocathode in 0.2 M pH 5.0 McIlvaine buffer containing 0.5 mM ABTS under (a) N_2-saturated atmosphere, (b) ambient air, and (c) O_2-saturated atmosphere. Scan rate: 1 mV s^{-1}.

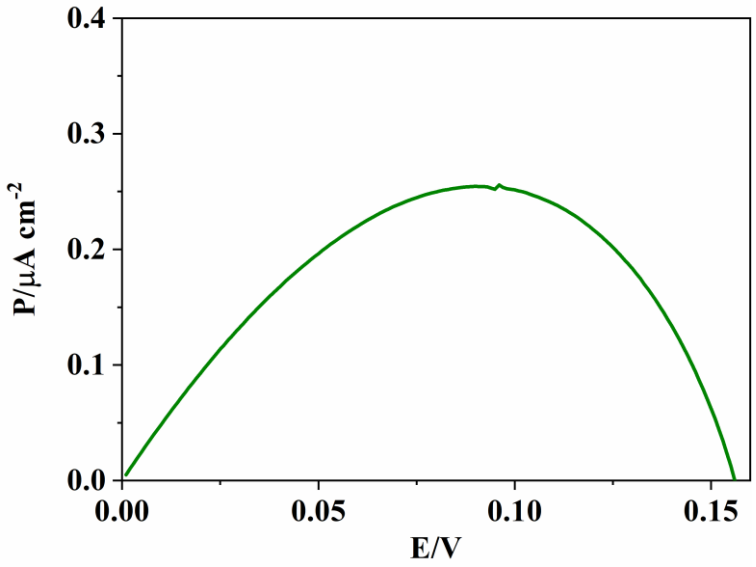

Figure S4. Power density-voltage profiles of the blank control cell consisting of a yeast/graphene/GCE based anode and a Lac/graphene/GCE based cathode. 0.2 M McIlvaine buffer (pH 5.0) containing 0.5 mM MB and 1.0% (w/w) starch was used for the anode compartment, while O_2-saturated 0.2 M McIlvaine buffer (pH 5.0) with 0.5 mM ABTS was used for the cathode compartment.

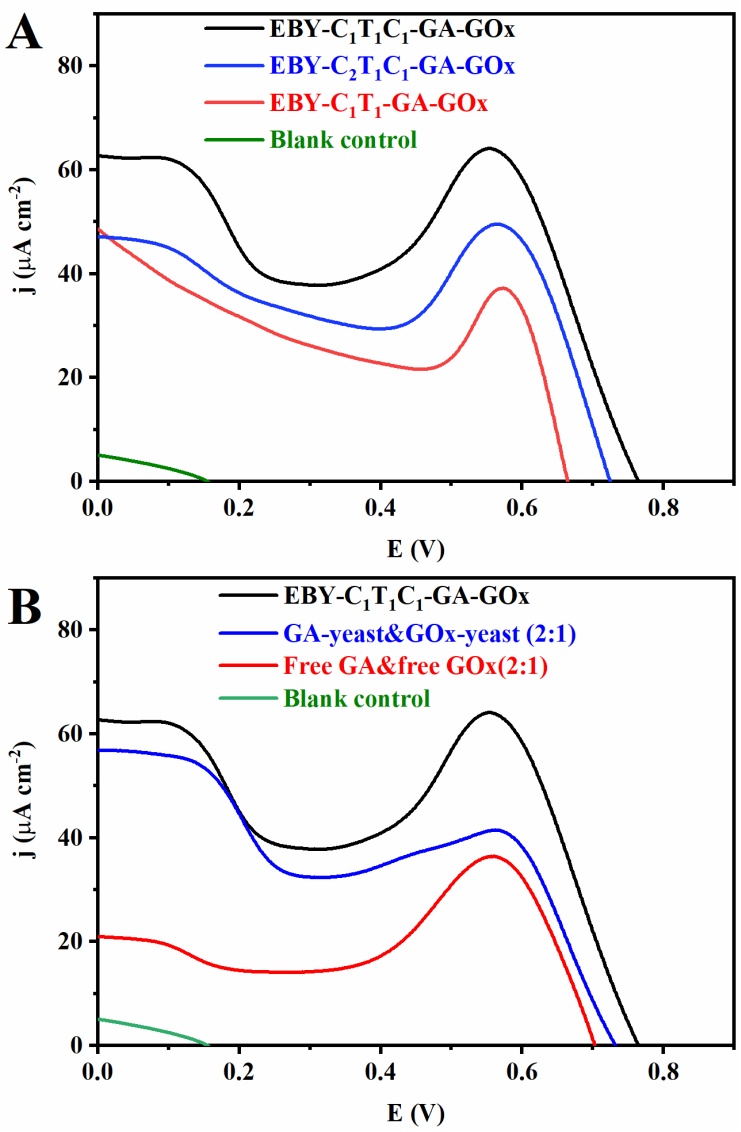

Figure S5. Polarization curves of two-compartment starch/O_2 EBFCs on different bioanodes, which were fabricated by depositing biocatalysts onto graphene/GCE. 0.2 M McIlvaine buffer (pH 5.0) containing 0.5 mM MB and 1.0% (w/w) starch was used for the bioanode compartment, while the Lac/graphene/GCE was used throughout as the biocathode, in which O_2-saturated 0.2 M McIlvaine buffer (pH 5.0) with 0.5 mM ABTS was used for the biocathode compartment. Blank control indicates a cell consisting of a yeast/graphene/GCE based anode and a Lac/graphene/GCE based cathode.

References:

[1] H.W. Wang, Q.L. Lang, L. Li, B. Liang, X.J. Tang, L.R. Kong, M. Mascini, A.H. Liu, Yeast surface displaying glucose oxidase as whole-cell biocatalyst: construction, characterization, and its electrochemical glucose sensing application, Anal. Chem., 85 (2013) 6107-6112.

[2] A.S. Wieczorek, V.J.J. Martin, Effects of synthetic cohesin-containing scaffold protein architecture on binding dockerin-enzyme fusions on the surface of Lactococcus lactis, Microb. Cell Fact., 11 (2012) 160.

[3] S.H. Lee, J.H. Choi, S.H. Park, J.-i. Choi, S.Y. Lee, Enantioselective resolution of racemic compounds by cell surface displayed lipase, Enzyme Microb. Technol., 35 (2004) 429-436.