Electronic Supplementary Information

A hybrid biocathode: Surface display of O_2-reducing enzymes for microbial fuel cell applications

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A. Materials and Methods:

Materials

o-phenylenediaminedihydrochloride (SIGMA *FAST* OPD), horseradish peroxidase (HRP), glucose oxidase (GOx), 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid (ABTS), laccase, bilirubinoxidase (BOD), methylene blue, potassium chloride phenylmethylsulphonyl fluoride (PMSF), ferric citrate, herring sperm DNA, lithium acetate, goat anti-mouse FITC conjugate, antimycin A (Sigma-Aldrich, St. Luis, MO) ;glucose, (Carlo Erba, Italy); carbenicillin (Bio Pioneer, San Diego, CA); δ-aminolevulinic acid (Merck); bacto-peptone, bacto-casamino acids, Difco yeast nitrogen base without amino acids (BD), galactose (Acros); anti-c-Myc IgG1 (Santa Cruz), goat anti-BOD, donkey anti-goat PE conjugate (Abcam), graphite rods (0.9mm diameter) (Pilot); graphite plates (Fuel Cell Store, San Diego, CA), $CuSO_4$, Nafion® membrane (0.09 mm thick, Alfa Aesar, USA).

Plasmids and strains

Plasmids and yeast that were used or constructed in this study are listed in the following table:

Strain/Plasmid	Characteristics	Source or reference
Yeast strains		
Saccharomyces cerevisiae EBY100 strain	Yeast strain for cell-surface expression	1
Plasmids		
pCTCON	YSD vector	1
pCTCON-PCNA	YSD vector expressing PCNA	Dr. Amir Aharoni, BGU
pC-GOx	YSD vector expressing GOx lacking signal peptide	2
pLCHOOP	YSD vector expressing laccaselacking signal peptide	This study
pC-BOD	YSD vector expressing BOD lacking signal peptide	This study

Strains and media

The *Escherichia coli*, DH5αstrain was used as host strain for recombinant DNA amplification. The *Saccharomyces cerevisiae* EBY100 strain was used for cell-surface expression. *E. coli* were grown in Luria–Bertani medium containing 100 mg/L carbenicillin when necessary, at 37°C with continuous shaking at 200 rpm. EBY100 yeast were grown at 30°C either in YPD media (20g/L glucose, 20g/L peptone, 20g/L yeast extract), or on SC-ura plates. Transformed yeast were grown at 30°C either in SDCAA media (20g/L glucose, 6.7g/L yeast nitrogen base without amino acids, 5g/L Bacto-casamino acids, 6.8g/L$Na_2HPO_4 \cdot 12H_2O$, 9.6g/L$NaH_2PO_4 \cdot H_2O$) or on SC $ura^- trp^-$plates. Induction media for transformed yeast comprised 90% SGCAA media (similar to SDCAA, except that galactose is used instead of glucose), 10% SDCAA media, 3.6 mM δ-aminolevulinic acid, with the addition either of 0.2mM ferric citrate, 0.1mM $CuSO_4$ or 0.22mM $CuSO_4$ for the induction of cells expressing GOx, laccase or BOD, respectively. Induction conditions were incubation for at least 18hr at 20°C, 30°C,or 37°C, with continuous shaking at 200-250rpm.

Construction of plasmids for cell surface display of laccase/BOD

The laccase gene sequence was as follows:

GCTAGCGGTATCGGTCCTGTCGCCGACCTCACCATCACCAACGCAGCGGTCAGCCCCG
ACGGGTTTTCTCGCCAGGCCGTCGTCGTGAACGGTGGCACCCCTGGCCCTCTCATCACC
GGTAACATGGGGGATCGCTTCCAGCTCAATGTCATCGACAACCTCACGGACCACACGA
TGCTGAAGAGCACCAGTATTCACTGGCACGGTTTCTTCCAGAAGGGCACCAACTGGGC
CGACGGTCCCGCCTTCATCAACCAGTGCCCGATCTCATCTGGCCACTCGTTCCTGTATG
ACTTCCAGGTTCCTGACCAGGCTGGCACCTTCTGGTACCACAGTCACTTGTCCACGCAG
TACTGTGATGGTCTGAGGGGTCCGTTCGTTGTTTACGACCCGAATGACCCGGCCGCCG
ACCTGTACGACGTCGACAACGACGACACGGTCATTACCCTTGCGGATTGGTACCACGT
CGCCGCGAAGCTGGGCCCCGCATTCCCTCTCGGCGCCGACGCCACTCTCATCAACGGT
AAGGGACGCTCCCCCAGCACGACCACCGCGGACCTCACTGTTATCAGCGTCACTCCGG
GTAAACGTTACCGTTTCCGCCTGGTGTCCCTGTCGTGCGACCCCAACCACACCTTCAGC
ATCGATGGCCACAACATGACGATCATCGAGACCGACTCGATCAACACGGCGCCCCTCG
TGGTCGACTCCATTCAGATCTTCGCTGCCCAGCGTTACTCCTTCGTGCTCGAGGCCAAC
CAGGCCGTCGACAACTACTGGATTCGCGCCAACCCGAGCTTCGGTAACGTCGGGTTCA
CCGGCGGCATCAACTCGGCTATCCTCCGCTATGATGGCGCCGCTGCCATCGAGCCCAC
CACCACGCAGACCACTTCGACCGAGCCGCTCAACGAGGTCAACCTGCACCCGCTGGTT

GCCACCGCTGTCCCTGGCTCTCCCGCTGCGGGTGGTGTTGACCTGGCCATCAATATGGC
GTTCAACTTCAATGGCACCAACTTCTTCATCAACGGCGCGTCTTTCACGCCCCCGACCG
TGCCTGTCCTCCTCCAGATCATCAGCGGCGCGCAGAACGCGCAGGACCTCCTGCCCTC
CGGCAGCGTATACTCGCTCCCCTCGAACGCCGACATCGAGATCTCCTTCCCCGCCACC
GCCGCTGCCCCCGGTGCGCCCCACCCCTTCCACTTGCACGGGCACGCGTTCGCGGTCG
TCCGCAGCGCCGGCAGCACGGTCTACAACTACGACAACCCCATCTTCCGCGACGTCGT
CAGCACGGGGACGCCTGCGGCCGGTGACAACGTCACCATCCGCTTCCGCACCGACAAC
CCCGGCCCGTGGTTTCTCCACTGCCACATCGACTTCCACCTCGAGGCCGGCTTCGCCGT
CGTGTTTGCGGAGGACATCCCCGATGTCGCATCGGCGAACCCCGTCCCCCAGGCGTGG
TCCGACCTCTGCCCGACCTACGACGCGCGCGACCCGAGCGACCAGGGATCC

The modified laccase *lcc2* gene from *Trametesversicolor* was used for construction of the plasmid after modifications that included the removal of the first 63 nucleotides (encoding the signal peptide as well as the stop codon). A *Nhe*I restriction site was added to the 5' end of the gene and a *Bam*HI site was introduced at the 3' end. The gene was ligated into the *Nhe*I-*Bam*HI sites of the pCTCON vector, yielding the pLCHOOP vector.

The BOD gene sequence was as follows:

GTTGCTCAAATTTCTCCACAATATCCAATGTTCACTGTTCCATTGCCAATTCCACCAGT
TAAACAACCAAGATTGACTGTTACTAATCCAGTTAATGGTCAAGAAATTTGGTATTAT
GAAGTTGAAATTAAACCATTCACTCATCAAGTTTATCCAGATTTGGGTTCTGCTGATTT
GGTTGGTTATGATGGTATGTCTCCAGGTCCAACTTTCCAAGTTCCAAGAGGTGTTGAA
ACTGTTGTTAGATTCATTAATAATGCTGAAGCTCCAAATTCTGTTCATTTGCATGGTTC
TTTCTCTAGAGCTGCTTTCGATGGTTGGGCTGAAGATATTACTGAACCAGGTTCTTTCA
AAGATTATTATTATCCAAATAGACAATCTGCTAGAACTTTGTGGTATCATGATCATGCT
ATGCATATTACTGCTGAAAATGCTTATAGAGGTCAAGCTGGTTTGTATATGTTGACTGA
TCCAGCTGAAGATGCTTTGAATTTGCCATCTGGTTATGGTGAATTCGATATTCCAATGA
TTTTGACTTCTAAACAATATACTGCTAATGGTAATTTGGTTACTACTAATGGTGAATTG
AATTCTTTCTGGGGTGATGTTATTCATGTTAATGGTCAACCATGGCCATTCAAAAATGT
TGAACCAAGAAAATATAGATTCAGATTCTTGGATGCTGCTGTTTCTAGATCTTTCGGTT
TGTATTTCGCTGATACTGATGCTATTGATACTAGATTGCCATTCAAAGTTATTGCTTCT
GATTCTGGTTTGTTGGAACATCCAGCTGATACTTCTTTGTTGTATATTTCTATGGCTGA
AAGATATGAAGTTGTTTTCGATTTCTCTGATTATGCTGGTAAAACTATTGAATTGAGAA
ATTTGGGTGGTTCTATTGGTGGTATTGGTACTGATACTGATTATGATAATACTGATAAA
GTTATGAGATTCGTTGTTGCTGATGATACTACTCAACCAGATACTTCTGTTGTTCCAGC
TAATTTGAGAGATGTTCCATTCCCATCTCCAACTACTAATACTCCAAGACAATTCAGAT
TCGGTAGAACTGGTCCAACTTGGACTATTAATGGTGTTGCTTTCGCTGATGTTCAAAAT

AGATTGTTGGCTAATGTTCCAGTTGGTACTGTTGAAAGATGGGAATTGATTAATGCTG
GTAATGGTTGGACTCATCCAATTCATATTCATTTGGTTGATTTCAAAGTTATTTCTAGA
ACTTCTGGTAATAATGCTAGAACTGTTATGCCATATGAATCTGGTTTGAAAGATGTTGT
TTGGTTGGGTAGAAGAGAAACTGTTGTTGTTGAAGCTCATTATGCTCCATTCCCAGGTG
TTTATATGTTCCATTGTCATAATTTGATTCATGAAGATCATGATATGATGGCTGCTTTC
AATGCTACTGTTTTGCCAGATTATGGTTATAATGCTACTGTTTTCGTTGATCCAATGGA
AGAATTGTGGCAAGCTAGACCATATGAATTGGGTGAATTCCAAGCTCAATCTGGTCAA
TTCTCTGTTCAAGCTGTTACTGAAAGAATTCAAACTATGGCTGAATATAGACCATATGC
TGCTGCTGATGAA

With the same modifications introduced as for the laccase-encoding sequence. The BOD gene sequence was based on the corresponding sequence from *Myrothecium verrucaria*. Nucleotides encoding the first 38 amino acids (corresponding to both the signal peptide and the pro-peptide) were omitted, as was the stop codon. Codon usage was optimized for expression in *Saccharomyces cerevisiae* by the Codon optimization calculator' program (http://www.encorbio.com/protocols/Codon.htm). The BOD gene was ligated into the pCTCON plasmid by the same procedures used for laccase, to yield the pC-BOD vector. Both genes were synthesized and cloned into the pCTCON vector by GenScript (Piscataway, NJ).

Yeast transformation

Yeast transformation was performed using the lithium acetate (LiAc) method: a freshly streaked EBY100 colony was inoculated in 5mL YPD media and grown overnight at 30°C. The overnight culture was used to inoculate100mL YPD media to OD_{600} of 0.1 afterwhichthe cells were grown at 30°C to OD_{600} of1.2. Cells were harvested by centrifugation at 4000rpm for 5 min at 4°C, re-suspended in 50mL H_2O,centrifuged again, re-suspended in 1mL H_2O, harvested (2min, max speed) and re-suspended in 300µL H_2O. Each transformation reaction contained 100µL treated yeast cells, 480µL PEG 50%, 72µl LiAc 1M, 10µl 10mg/mL herring sperm DNA, 30µL H_2O, 30µL plasmid (200-300ng). After a short vortex, the reaction was incubated for 30 min at 30°C. 30µL DMSO was added and the reaction was incubated for 15min at 42°C with continuous shaking. After 30 seconds spin at maximum speed, the pellet was re-suspended in 200µL H_2O and the cells plated onto selective SC-ura-trp plates and grown at 30°C for 48hr.

Fluorescence-Activated cell sorting (FACS)

Transformed yeast were grown overnight in 5 mL SDCAA at 30°C. The culture was refreshed (100 µL starter culture added to 5 mL SDCAA) and grown (4-6hr) to an OD_{600} of 1-1.2. One mL of culture was harvested by centrifugation and re-suspended in 5mL of induction media (with and without $CuSO_4$). Induced culture was grown with shaking (250 rpm) for at least 18 hr at different temperatures (20°C, 30°C, 37°C).

One mL of induced yeast cells, at an OD_{600} of approximately 1.0, was collected by centrifugation. The pellet was washed with 0.5 mL PBSF, rinsed, and pelleted again. BOD- and laccase-modified yeast were labeled with mouse anti-c-Myc IgG1in 50µl of PBSF. BOD-expressing yeast wereadditionally labeled with goat anti-BOD antibodies. Re-suspended cells were incubated for 1hr at 25°C and 400rpm. Yeast cells were collected by centrifugation and washed twice with ice-cold PBSF. After addition of secondary reagents (goat anti-mouse FITC conjugateor donkey anti-goat PE conjugate),cells were incubated on ice and shielded from light for 1hr. After two washes with ice-cold PBSF, the cells were re-suspended in 0.5 mL ice-cold PBSF. A 70µl cell suspension was diluted in 0.5 mL PBS and the mixtureanalyzed by an 'Eclipse' flow cytometer (iCyt, Champaign, IL).

Control cultures: The positive control for myc-labeled surface-expressed proteins was yeast expressing Proliferating Cell Nuclear Antigen (PCNA) on their surface (induced at 30°C). Negative controls were EBY100 yeast (grown in YPD, at 30°C) and induced yeast transformed with either the pLCHOOP or pC-BOD vectors and incubated with secondary antibodies alone.

Laccase and BOD activity assay

Oxidation of the laccase and BOD substrate, o-phenylenediaminedihydrochloride (OPD), changes its absorbance peak at 430nm, allowing the activities of these enzymes to be determined spectrophotometrically.

Yeast cells were washed twice with 0.1M acetate buffer pH, 5 (laccase-expressing yeast) or with phosphate buffer (PB), pH 7 (BOD-expressing yeast), and suspended in 50µL of the same buffer. To each sample, 50µL of SIGMA *FAST*

OPD solution (0.4 mg/mL) was added. The mixture was incubated at 30°C for 2 or 3 hours (BOD) and for 3, 4 or 6 hours (laccase), and the absorbance measured was compared to a standard curve generated with different concentrations of commercial enzyme incubated under the same conditions. From the absorbance of each mixture, a blank value (the same mixture containing neither enzyme nor yeast) was deducted. All measurements were conducted in triplicate and experiments were repeated at least 3 times, always yielding similar results.

Glucose oxidase activity assay

Conducted as described thoroughly in the supplementary data of reference 2.

Cyclic voltammetry (CV)

CV measurements were performed with a VSP potentiostat (BioLogic Science Instruments). The cyclic voltammetry was performed in a three electrode standard cell, using carbon cloth coated with 5% Teflon (Fuel Cell Earth, Stoneham, MA) as the working electrode for the laccase measurements and a graphite rod (0.9mm diameter) for the BOD measurements. A graphite rodwas used asthe counter electrode, and an Ag/AgCl reference electrode was employed (CH instruments, Austin, TX). Measurements were conducted in 0.1 M acetate buffer, pH 5 (laccase) and PB, pH 7 (BOD), with100 µM ABTS added as an electron transfer mediator. The measurement scan rate was 1 mV/s, at a potential range of 0.35–0.7 V. All measurements were carried out at ambient temperature and under aerobic conditions.

Biofuel cell

The biofuel cell was custom-made from two curved glass compartments (cathodic and anodic) with clamping ledges and a Nafion membrane (0.09 mm thick, Alfa Aesar) between them to give an overall 'U'-shape. The voltage and current generated by the biofuel cell were measured by a 2700 multimeter (TES, Taipei, Taiwan) using a resistance decade box (Lutron Electronic Enterprise, Taipei, Taiwan). Measurements were carried out at ambient temperature.Graphite plates (7-11cm^2) were used as the anode. The same graphite plates or carbon cloth with a 5% Nafion coating (18 cm^2) were used as the cathode. The cathode half-cell

contained laccase-expressing yeast (100 mL of culture at OD_{600}=1, washed twice with acetate buffer and suspended in 5 mL of the same buffer) or BOD-expressing yeast (50 mL of culture at OD_{600}=1, washed twice with PB and suspended in 5 mL of the same buffer), 10 μg/mL antimycin A and 5 mM ABTS in an oxygen-saturated environment. The controls used were commercial enzymes, unmodified EBY100 yeast, unmodified yeast with commercial enzymes and buffer containing ABTS with neither yeast nor enzyme. The anode half-cells were kept under a deoxygenated atmosphere and contained induced GOx-expressing yeast (OD_{600}=2.3), 1 mM methylene blue, 0.1 M glucose, 0.1 M acetate buffer, pH 5 (laccase fuel cells) or 0.1 M PB, pH 7 (BOD fuel cells).

Regenerated yeast was prepared as follows: For the cathode half-cell, a yeast sample (100-500 μL) was taken from the working fuel cell and plated on an SC ura^-trp^- plate for 36-48 hours at 30°C. A colony was taken from the plate and incubated overnight at 30°C in SDCAA media (5 mL) as previously described. After another cycle of dilution and overnight incubation in SDCAA, the sample was incubated in induction media and returned to the fuel cell. For the anode half-cell, the process was the same as that of the cathode, except the plating step was omitted.

B. **Results:**

Cloning and transformation

The *Trametes versicolor* laccase gene was cloned into plasmid pCTCON, yielding plasmid pLCHOOP. The *Myrothecium verrucaria* BOD gene was cloned into plasmid pCTCON, yielding the plasmid pC-BOD. The constructed vectors were sequenced and transformed into the *Saccharomyces cerevisiae* EBY100 strain.

FACS analysis

In the yeast surface display system[3], surface expression can be measured through immune fluorescence labeling of either the hemagglutinin or c-Myc epitope tag flanking the expressed protein (Fig S1-A). Surface expression can also be measured through immune fluorescence labeling of the enzyme itself. Laccase

and BOD expression levels were determined by indirect immune fluorescence of a C-terminal myc epitope tag using FACS (Fig.S1–B, C). BOD expression levels were also determined by indirect immune fluorescence of the enzyme (Fig. S1–D). Flow cytometric histograms of cells not expressing enzymes always showed background auto-fluorescence with intensity below 10, which is typical for yeast. Surface expression of laccase and BOD was confirmed by significant labeling of the myc tag attached to each enzyme.

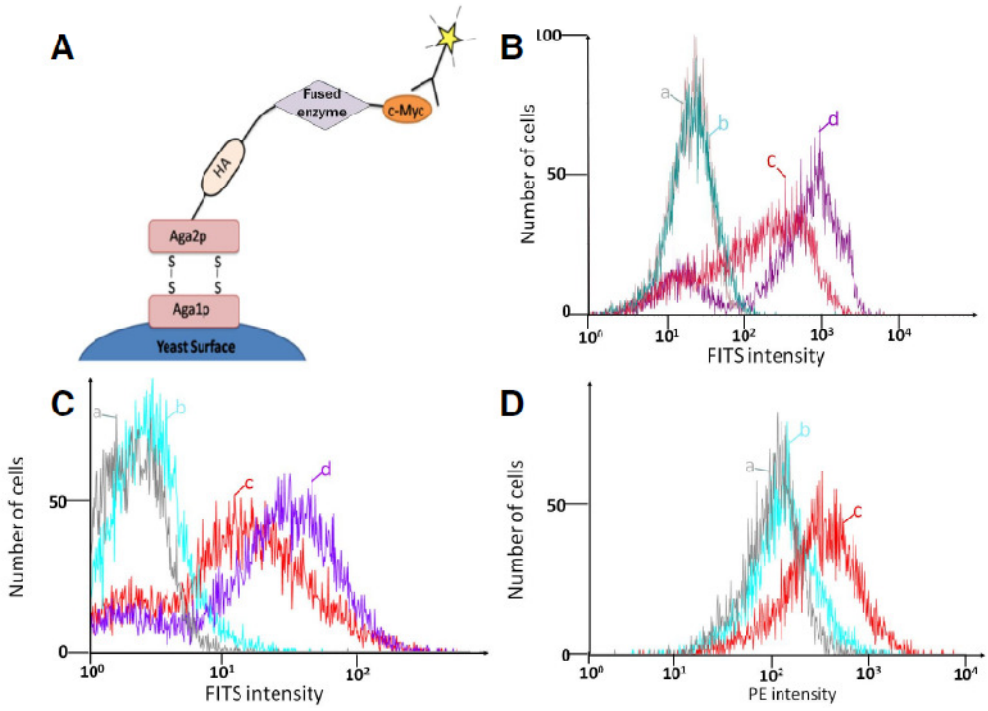

Figure S1: (A) Scheme of yeast surface display. Laccase and BOD are displayed as an Aga2 fusion protein on the surface of yeast. (B, C, D) Histogram analysis of EBY100 cells without added plasmid (negative control, gray), cells displaying PCNA (positive control, purple), cells displayingenzymes (red), and cells displaying enzymes without incubation with primary antibodies (negative control, pale blue). B – Histogram of myc-tagged laccase labeled with anti-myc antibodies.C – Histogram of myc-tagged BOD labeled with anti-myc antibodies.D - Histogram of BOD directly labeled with anti-BOD antibodies.

Biochemical activity of laccase and BOD expressed on yeast

The activities of cell surface displayed laccase/BOD were measured by colorimetric assay. Whole cell activity was measured against standard curves generated using native enzyme. As seen in Fig.S2, transformed yeast induced for expression of surface display products showed significant laccase/BOD activity, while all negative controls showed no activity. BOD expressing yeast grown in induction medium containing 0.22 mM $CuSO_4$ showed a 72% increase in enzyme activity, as compared to the same cells induced in the absence of copper, while laccase-expressing yeast grown in 0.1 mM $CuSO_4$ showed an 85% increase.

Negative controls used were EBY100, uninduced yeast containing the pLCHOOP/pC-BOD plasmids and reaction medium containing neither yeast nor enzymes.

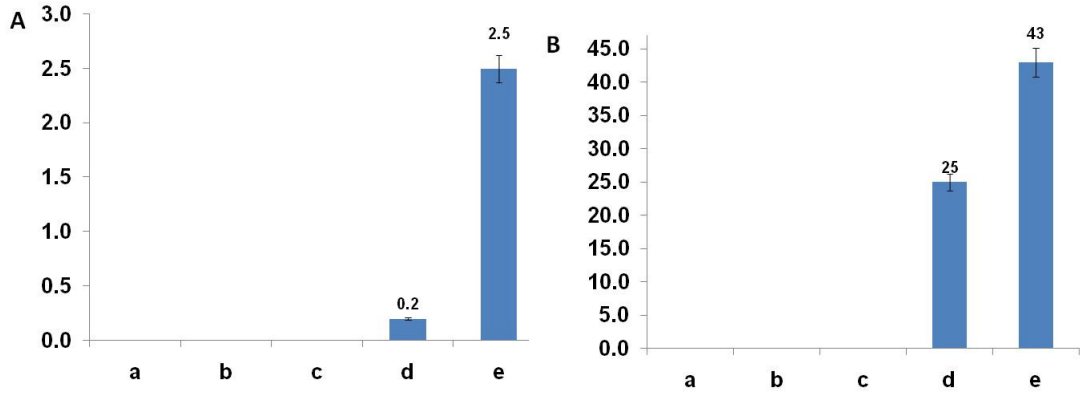

Figure S2: Laccase (A) or BOD (B) activity of different yeast cultures (a) reaction medium with neither yeast nor enzyme; (b) EBY100 yeast; (c) transformed yeast without induction; (d) cells displaying enzyme; (e) cells displaying enzyme, grown in a medium containing 0.1 mM $CuSO_4$.

Biofuel cells

BOD- and laccase-expressing yeast were used as biocatalysts for the reduction of O_2 to water in the cathode chamber. Voltage was measured at different external resistances. Current and power output were calculated, using Ohm's law, and were normalized to the measured electrode surface area. Fig. S6 shows the power output

at different voltages (A) and the polarization curves (B) for BOD. BOD-expressing yeast in the presence of antimycin A showed an open circuit voltage (OCV) of 607mV and a maximum power output of 1.38μW/cm^2, similar to values attained with commercial free BOD biofuel cells (with and without unmodified yeast). BOD-expressing yeast without antimycin A present shown an OCV of only 365mV and power output of 0.053 μW/cm^2, which is closerto the values seen for the fuel cells without enzymes.

Since the addition of antimycin A inhibits oxygen consumption by the yeast, addition of unmodified yeast to the controls did not change their behavior.

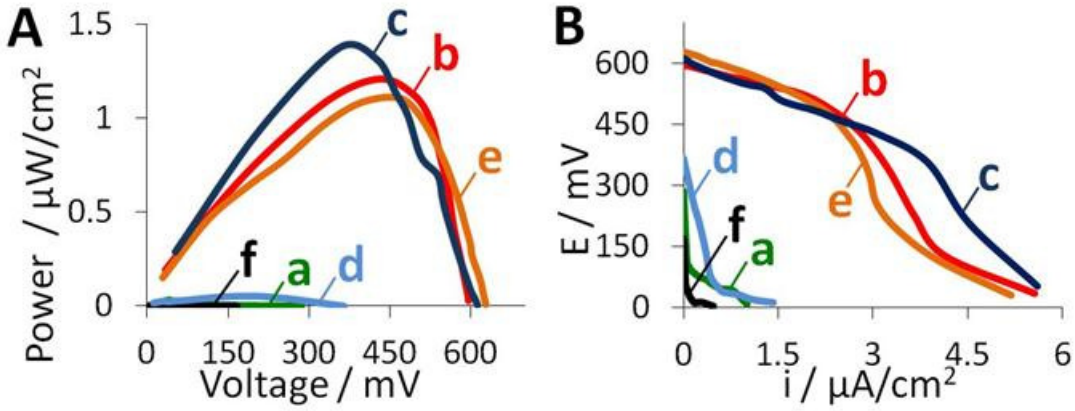

Figure S3: (A) Power output and (B) Polarization curves for biofuel cells containing (a) background solution in the cathode, (b) free BOD, (c) BOD-expressing yeast, (d) BOD-expressing yeast without antimycin A, (e) free BOD and unmodified yeast and (f) unmodified yeast.

The same measurements were conducted for the laccase-expressing yeast. Figure S4 shows the power output at different voltages (A) and the polarization curves (B) for laccase when using graphite rods as electrodes in the cathode, as well as with carbon cloth as electrodes (C,D).Using graphite rods, the laccase-displaying yeast cathode showed an OCV of 215mV, while purified enzyme cathodes showed OCVs of 335mV and 437mV with or without modified yeast, respectively. The maximum power output measured was much lower than that of the purified enzymes. Using carbon cloth as the electrode of the cathode compartments, higher OCV was measured for the laccase-displaying yeast cathode (492 mV), while for the purified enzyme, an OCV of 600mV was measured for cathodes with or without unmodified yeast. Although the OCVs were higher when using carbon cloth electrodes, the fuel cells had a much higher resistance in these instances (up to 200kΩ), resulting in low maximum power outputs, much lower than obtained with purified enzyme, as compared to when carbon rod electrodes were used. These differences can be related to the higher surface area of the carbon cloth, stronger adhesion of the yeast to the electrode (which results in cloth fouling), and to the Nafion coating on the electrode cloth that increases its resistance. Using carbon cloth with no Nafion led to rapid evaporation of the cathode medium.

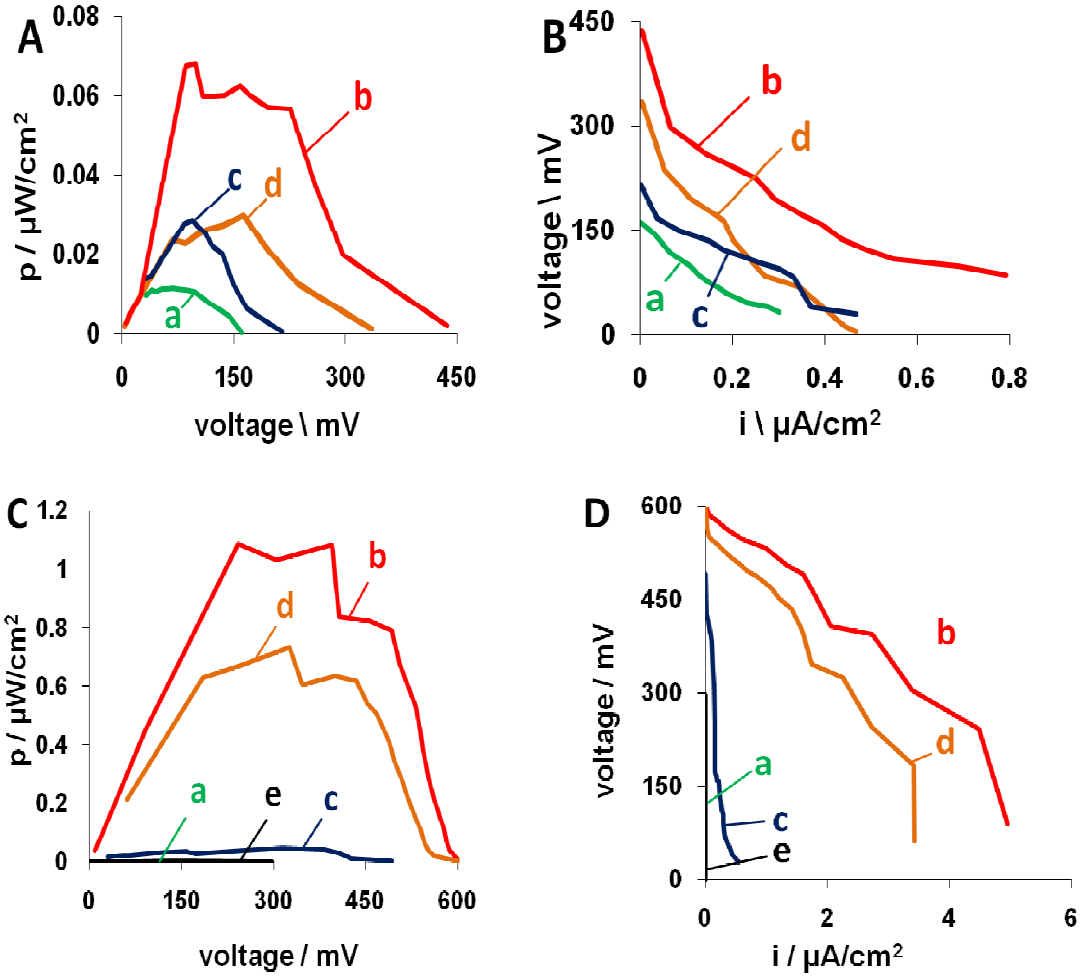

Figure S4:(A)Power output (B) polarization curves for biofuel cells with graphite rod cathodes containing (a) background solution, (b) free laccase, (c) laccase-expressing yeast,(d) free laccase and unmodified yeast, (C) Power output (D) polarization curves for biofuel cells with carbon cloth cathodes containing (a) background solution, (b)commercial free laccase, (c) laccase-expressing yeast,(d) free laccase and unmodified yeast, (e) unmodified yeast.

A long-term study for laccase-displaying yeast was conducted over a period of 30 days. After 21 days, a sample was taken for regeneration of the cells, grown successfully and used to inoculate the cell, after 28 days. These results are presented in Figure S5. It can be seen that although the OCV of the laccase-displaying yeast is lower than of the purified enzyme, the yeast cells are more stable, and the possibility to re-induce these cell enables of the cell for a longer period of time. In contrast, the commercial free enzyme loses its activity and

cannot be regenerated.OCV was measured for biofuel cells containing BOD-expressing yeast and free BOD for 67 days. The fuel cells showed stable OCV for the whole period. Indeed, full activity of the enzymes was measured even after 54 days. After 3 weeks, a sample of solution was taken from each compartment for regeneration of the enzymes. The living yeast in both compartments were able to regenerate the enzymes. Results are shown in figure S6.

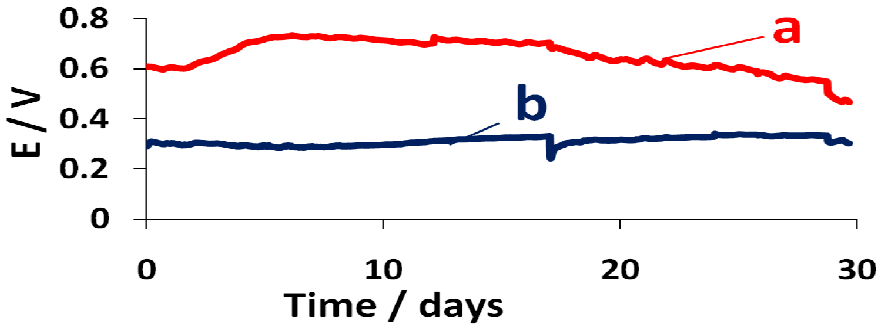

Figure S5: Long-term analysis of laccase biofuel cells containing (a) free laccase and (b) laccase-expressing yeast.

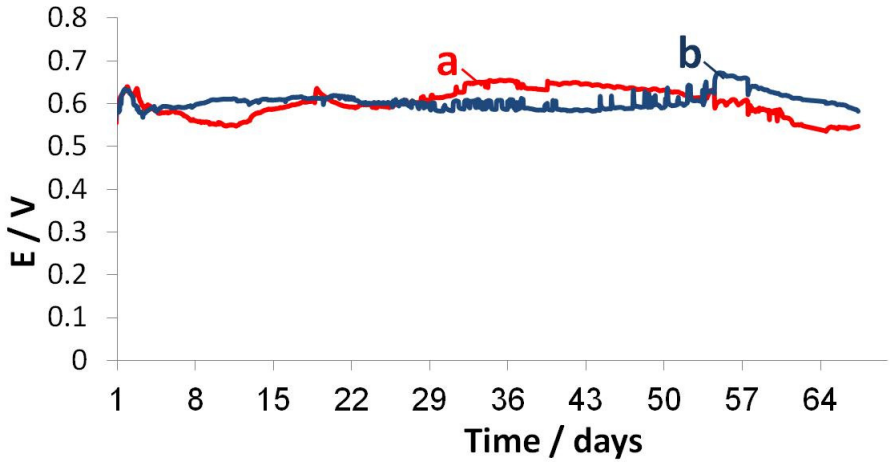

Figure S6: Long term analysis of BOD biofuel cells containing (a) free BOD and (b) BOD-expressing yeast.

References

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3. Jansen, G.; Wu, C.; Schade, B.; Thomas, D. Y.; Whiteway, M. *Gene*, **2005**, 344, 43-51.