Materials and Methods

DNA manipulation and construction of recombinant NDH II strain

The gene expression sequence consists of the isopropyl-beta-D-thiogalactopyranoside (IPTG) responding element (P*lacIq*-*lacI*-P*tac*) and *ndh II* (Gene ID: 4921489, NCBI) which encodes the type II NADH dehydrogenase. The P*lacIq*-*lacI*-P*tac* sequence was designed according to the pMAL-c plasmid (New England Biolabs, USA). The DNA sequence was *in vitro* synthesized (Aitbiotech, Singapore) and amplified by polymerase chain reaction (PCR) using gene specific primers rNDH II-F(5'-CCGGAATTCCCGACACCATCGAATGG-3') / rNDH II-R(5'-CCGCTCGAGTTAGTGCAGCTTGAGGTG AG-3'). The underline was the restriction enzyme sites for *EcoRI* and *XhoI*, respectively. In the thermocycling reactions the high-fidelity hot start enzyme (Kapa, USA) was used and the conditions were as follows: Initial denaturing at 95°C for 5 min; denaturing at 98 °C for 20 sec, annealing at 55 °C for 15 sec, extending at 72 °C for 2 min 6 sec, repeating 2 cycles; denaturing at 98 °C for 20 sec, annealing at 69 °C for 15 sec, extending at 72 °C for 2 min 6 sec, repeating 30 cycles; final extending at 72 °C for 5 min; cooling at 4 °C. The reaction products were purified by PureLink Quick Gel Extraction Kit (Lifetechnologies, USA). Then these products as well as pHG101 empty vector[1] were double digested by *EcoRI* and *XhoI* (NEB, USA) at 37 °C for 12 h, respectively. Both products were mixed by 2 μl Pellet Paint (Merck, USA) followed by 10% (v/v) 3 M NaOAc. After 2 volume of ethanol added and vortexed, they were incubated at -80 °C for 2 h. Then the samples were centrifuged at 13000 rpm for 5 min and the supernatant were removed. The resultant pellets were rinsed with 70% and 100% ethanol respectively and resuspended in sterile deionized (DI) H_2O. 8 μl of DNA fragment dissolved in DI H_2O together with 2 μl of vector in DI H_2O were mixed thoroughly with 10 μl of DNA ligation enzyme solution I (Takara, Japan) and incubated at 16 °C for 16 h. The recombinant products were mixed by 2 μl Pellet Paint followed by 10% (v/v) 3 M NaOAc. After 2 volume of ethanol added and vortexed, they were incubated at -80 °C for 2 h. Then the samples were centrifuged at 13000 rpm for 5 min and the supernatant were removed. The resulting pellets were rinsed with 70% and 100% ethanol respectively, resuspended in 10 μl sterile DI H_2O and then was electroporated into *S. oneidensis* MR-1 electrocompetent cells (700V, 5ms). The cells were immediately transferred into 0.6 ml SOC recovery broth (20% tryptone, 5% yeast extract, 0.5% NaCl, 5% $MgSO_4 7H_2O$ and 20 mM dextrose) and recovered at 30 °C for 1.5 h with shaking at 100 rpm. After selecting with a LB Agar medium containing 50 μg/ml kanamycin, the transformants were screened by PCR and sequenced using specific designed primers V-pHG101-F(5'-GCTAGCCCCGGGTGGTAC-3') / V-pHG101-R(5'-CTTATGTCTATTGCTGGTTTACCG-3'). The recombinant vector was named as pHG-NDH II (Figure S1).

Bacteria strains and cultivation

1 ml *S. oneidensis* MR-1 culture was inoculated in 100 ml LB broth (10g/L NaCl; 10 g/L tryptone; 5 g/L yeast extract) and incubated at 30 °C under shaking at 220 rpm until the optical density of bacterial culture at 600 nm (OD600) reached about 1.0. 1 ml aliquot of the recombinant *S. oneidensis* MR-1 NDH II strain culture was inoculated in 100 ml LB broth containing 50 μg/ml kanamycin and incubated at 30 °C under shaking at 220 rpm. When the OD600 value reached about 0.5, 1 mM IPTG was added to induce overexpression of the type II NADH dehydrogenase for about 6 h. Then both types of bacteria culture were harvested by centrifugation (8000 rpm×8 min). Both bacteria pellets were washed three times with M9 minimal medium (0.5g/L

NaCl; 1g/L NH_4Cl; 3g/L KH_2PO_4; 6.78 g/L Na_2HPO_4; 0.12 g/L $MgSO_4$; 0.011 g/L $CaCl_2$).
The MR-1 strain was suspended in 100 ml anolyte (95% M9 minimal medium plus 5% LB broth containing 18 mM lactate, PH 7.0) and the recombinant strain was suspended in 100 ml anolyte supplemented with 25 μg/ml kanamycin and 0.01 mM IPTG. Both types of bacteria suspension (~10^9 cells / mL) were then transferred into the microbial fuel cell (MFC) anodic chamber respectively and purged with nitrogen gas filtered though 0.2 μm membrane for 30 min to remove oxygen.

MFC construction and operation

Dual-chamber glass MFCs (105 ml volume of each chamber) were constructed with separation by a Nafion 117 membrane (DuPont, USA). Both anode and cathode of MFC were made from carbon cloth (GasHub, Singapore). The geometric surface of cathode was 6 cm^2 while that of anode was 4 cm^2. Before used, carbon cloth was cleaned by acetone and 1 M HCl treatment sequentially and the anode was further treated with oxygen plasma atmosphere by the plasma cleaner system (Harrick, USA) to make it more hydrophilic for better bacteria adhesion.[2] The catholyte was 50 mM $K_3Fe(CN)_6$ plus 50 mM KCl. An external resistor of 2kΩ was used to connect MFC circuits and the voltage across the external resistor was measured by a digital multimeter (ESCORT 3146A).

Colony forming units measurement

Based on the method developed by Merritt *et al,*[3] after the output of MFC turned up stable, the the anodic carbon cloth was aseptically transferred to a 15-ml tube containing 10 ml 4°C pre-cold sterile PBS buffer (8g/L NaCl; 0.2g/L KCl; 1.42g/L Na_2HPO_4; 0.27g/L KH_2PO_4; 1 mM $CaCl_2$; 0.5 mM $MgCl_2$). Then, samples were vortexed thoroughly to make all bacteria detached from the carbon cloth. After the vortexed samples were 10-fold serially diluted, each dilution was plated on a separate LB Agar plate. These plates were incubated at 30 °C for about 18 h and the number of colony forming units (CFU) per unit geometric area of anodic carbon cloth were measured three times independently for both wild-type and recombinant strains employing the cell counter and the results were recorded only when between 50 and 400 colonies per plate were counted.

Membrane protein extraction and quantification

To verify the successful expression of heterogeneous NDH II protein in the membrane, the membrane protein was extracted by using the Membrane I-ReadyPrep protein extraction kit (Bio-Rad, USA) and then quantified by using the BCA protein assay kit-reduing agent compatible (BioVision, USA). Briefly, the samples of NDH II strain induced by IPTG and MR-1 strain were harvested, respectively and the wet cell pellets were resuspended in 0.5 ml M1 buffer and sonicated on ice with an ultrasonic probe to break open the bacterial cells. Then 0.5 ml M2 buffer were added in the cell extracts and mixed well, after which the samples were incubated on ice for 10 min and at 37 °C for 30 min and centrifuged at 16000×g for 5 min. The samples then produced an upper aqueous phase, containing the hydrophilic proteins and a lower detergent-rich phase, containing membrane proteins. Repeated these steps mentioned above once again. Thus, the membrane proteins partitioned into the lower detergent-rich phase while the cytoplasmic and periplasmic proteins were in the aqueous phase. Finally, the concentrations of proteins in the lower phase were measured by BCA method. The bovine serum albumin (BSA)

was used as the protein standard. Protein samples in the 96-well plate were read at 562 nm on a SpectraMax M5 microplate reader system (Molecular Devices, USA) in triplicates.

NAD+ / NADH assay

To detect the intracellular concentrations of NADH and NAD^+ of the bioelectrocatalyst on the anode surface, the NAD^+/NADH quantification colorimetric kit was used (BioVision, USA). Briefly, after the stable output of MFC appeared, the anodic carbon cloth was aseptically transferred into a sterile tube containing pre-cold PBS buffer, which was then vortexed thoroughly to detach all bacteria from the carbon cloth. The samples were centrifuged at 13000 rpm for 6 min and the cell pellets were washed twice with pre-cold PBS and extracted with 400 μl of NAD^+/NADH extraction buffer by subjecting the bacteria cells to three cycles of freeze/thaw followed by homogenization for 5 min[4] and then centrifuged at 13000 rpm for 5 min. The extracted NAD^+/NADH supernatant was filtered through the 10kDa molecular weight cut-off membrane (BioVision, USA). To determine the total concentration of NAD^+/NADH (tNAD), 50 μl of this filtrate from each extracted sample was transferred into a 96-well micro-plate (Thermo scientific, USA) in triplicates. After the NAD cycling mix was added into each well, the plate was incubated at room temperature for 5 min to allow the enzymatic reactions to proceed completely. Subsequently 10 μl of NADH developer was added into each well and incubated at room temperature for 1 h 31 min in the dark. To determine the concentration of NADH, 200 μl of extracted solution from each sample was heated at 60°C in a dry bath for 30 min to decompose NAD^+ and then 50 μl of extracted solution from each sample was transferred into the 96-well plate in triplicates. The rest steps were the same as the procedures of detecting tNAD mentioned above. Eventually, after 1 h 31 min in the dark, the plate was read at 450 nm on a Victor 3 Model Microplate reader (PerkinElmer, USA). The concentration of NAD^+ was calculated as follows: NAD^+= tNAD-NADH.

Characterization of electrochemical behaviors

All electrochemical analyses of bioanode and measurements of MFC performance were performed after a stable output of MFC appeared (~500 h after inoculation during which electrolytes were replenished when required). To reduce the constraint of a whole-cell system on the evaluation of bioelectrocatalyst, the electrochemical analyses of bioanode were conducted in a three-electrode half-cell system (all three electrodes were in the anodic chamber) with an Ag/AgCl (saturated KCl) and a Pt coil as the reference and counter electrodes. All electrochemical experiments were carried out at room temperature (~30 °C) by using VersaSTAT-3F workstation (Princeton Applied Research). The cyclic voltammograms (CVs) were recorded with a scan rate of 1 mV/s from -0.8 V to 0.2 V (versus Ag/AgCl). The chronoamperometry (CA) experiments were performed at 0 V or +0.2 V and the electrochemical impedance spectroscopy (EIS) measurements were carried out over a frequency range of 0.3 Hz to 300 kHz at η =0.2 V with ac amplitude of 10 mV. Tafel plots were recorded for the anode being swept at 1 mV/s from η =0 to 0.25 V, where η =0 is the open circuit potential (OCP) of the anode versus the reference electrode.[5] The measurements of MFC performance were conducted in a whole-cell system. The polarization and power output curves were measured by varying external resistances. Both current density and power density were normalized to the projected surface area of anode. For the discharge experiment, an external load resistor of 2kΩ was connected to MFC and the

voltage across the resistor was recorded. All the experiments were repeated three times and representative results were reported.

High-performance liquid chromatography

Riboflavin in the anodic chamber of MFC was quantitated by the high-performance liquid chromatography (HPLC) analysis employing an Agilent 1260 Infinity HPLC system (Agilent Technologies, USA) with a UV detector. The column was a LiChrospher 100 RP-18 (5 μ m) Hibar RT 250-4 (Merck, USA). Riboflavin was measured (mobile phase: 15% acetonitrile, 50 mM Sodium dihydrogen phosphate, PH 3.0) with a flow of 0.4 ml/min according to the method of Vasilaki *et al*[6] and monitored at 270 nm.

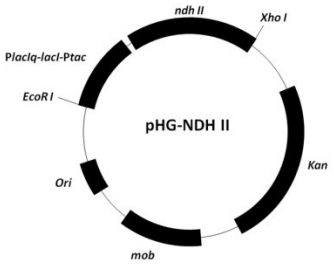

Fig. S1. The schematic of type II NADH dehydrogenase expression vector. The P*laclq*-*lacI*-P*tac*-*ndh II* sequence was inserted into the pHG101 and named as the pHG-NDH II vector.

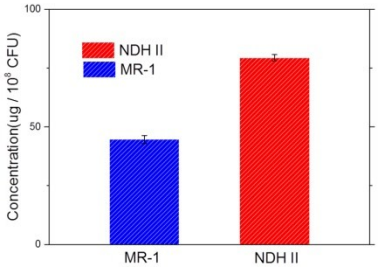

Fig. S2. Membrane protein concentration of MR-1 and NDH II.

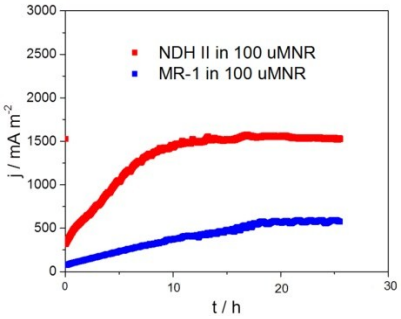

Fig. S3. CA of NDH II and MR-1 bioanodes poised at 0 V with additional 100 μ M NR.

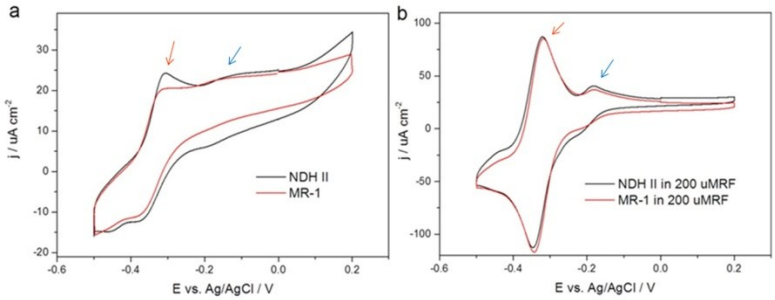

Fig. S4. (a) Nonturnover CV of NDH II and MR-1 bioanodes. (b) nonturnover CV of NDH II and MR-1 bioanodes in 200 uM RF. Orange arrow: peaks from RF; blue arrow: peaks from OM-cytochromes. The potential scan rate is 1 mV/s.

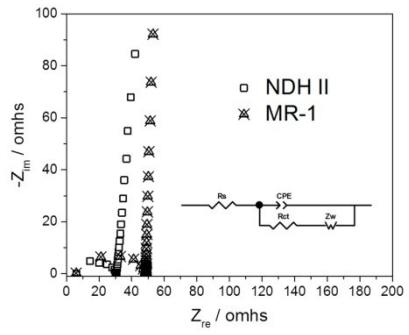

Fig. S5. EIS of the NDH II and MR-1 bioanode in MFCs. The inset is the randle equivalent circuit. The values of CPE of MR-1 and NDH II equal 1.35±0.03 mF (a=0.93±0.02) and 1.36±0.33 mF (a=0.90±0.01), respectively.

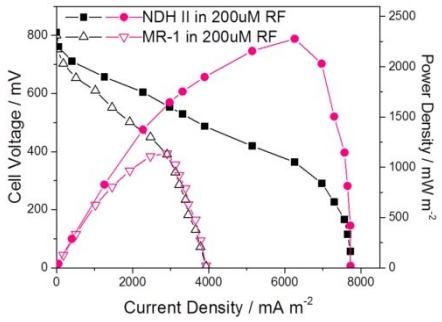

Fig. S6. Power output and polarization curves of NDH II and MR-1 inoculated MFCs with additional 200 μ M RF.

Table S1 Summary of the reported resistances of biofuel cells measured by EIS

Cell type *	**Electrode**	**Bioelectrocatalyst**	**Resistance(Ω)**	**Reference**
D	Graphite felt	Anaerobic sludge	11	*International Journal of Hydrogen Energy*, 2012, **37**, 16935
S with air cathode	Carbon felt	Anaerobic sludge	18.1±2.4	*Electrochimica Acta*, 2011, **58**, 58
SMFC	Carbon paper	Mixed culture in domestic wastewater	2	*Bioresource technology*, 2012, **118**, 412
FMFC	Granular graphite	Mixture of anaerobic and aerobic sludge	20	*Bioresource technology*, 2012, **114**, 308
S with air cathode	Graphite felt	Anaerobic sludge	50	*Journal of Microbial & Biochemical Technology*, 2013, **01**
S	Glassy carbon electrode	Glucose oxidase with OMC; with OMC and PVA**	17.5 for OMC; 5.2 for OMC and PVA	*Journal of Power Sources*, 2010, **195**, 4090
SiMFC	Carbon cloth	River sediment	105.5 for rotating; 78 for non-rotating cathode	*Biosensors & bioelectronics*, 2007, **22**, 3252
D	Carbon cloth	Electrical tension evolved *E.coli* K-12	107.5	*Chemical communications*, 2008, 1290; *Chemical communications*, 2009, 6183
D	Carbon cloth	Effluent from a previous MFC	15.1±0.5	*International Journal of Hydrogen Energy*, 2014, **39**, 19148
D	CHI/VSG***	*Pseudomonas aeruginosa*	150	*Nano letters*, 2012, **12**, 4738

D	CNT/PANI composite	*E.coli* K-12	156	*Journal of Power Sources*, 2007, **170**, 79
D	Graphite felt	*Shewanella oneidensis* DSP10 with additional RF	16	*Biotechnology and bioengineering*, 2009, **104**, 882
D	PANI/TiO_2 composite	*E.coli* K-12	~12.5	*Acs Nano*, 2008, **2**, 113
S	Graphene / nickel foam	*Shewanella putrefaciens*	~6	*RSC Advances*, 2014, **4**, 21788
D	Carbon cloth	GldA overexpressed *E.coli* BL21 (DE3)	125	*Electrochemistry Communications*, 2009, **11**, 1593
D	TiO_2/rGO hybrid on carbon cloth	*Shewanella putrefaciens* CN32	28	*Journal of Power Sources*, 2015, **276**, 208
S	O_2 plasma treated carbon cloth	*Shewanella oneidensis* MR-1; NDH II mutant	42.81±0.02; 24.55±0.15	This work

* Dual chamber: D; single chamber: S; submersible MFC: SMFC; floating MFC: FMFC; sediment MFC: SiMFC;

** Ordered mesoporous carbon: OMC; poly(vinyl alcohol): PVA

*** Chitosan and vacuum-stripped graphene: CHI/VSG

Table S2 Summary of the reported performances of MFCs

Cell type*	**Electrode**	**Bioelectrocatalyst**	**Power density (mW/m^2)**	**Reference**
D	Carbon Cloth	Electrical tension evolved *E.coli* K-12	1300	*Chemical communications*, 2008, 1290
D	NiO/carbon cloth	*Shewanella putrefaciens*	1024±46	*Journal of Power Sources*, 2014, **266**, 226
D	PANI/TiO_2 composite	*E.coli* K-12	1495	*Acs Nano*, 2008, **2**, 113
D	Graphene/ nickel foam	*Shewanella putrefaciens*	3903	*RSC Advances*, 2014, **4**, 21788
D	Carbon fiber brush	*Ochrobactrum* sp. 575	2625**	*RSC Advances*, 2014, **4**, 39839
D	CHI/VSG***	*Pseudomonas aeruginosa*	1530	*Nano letters*, 2012, **12**, 4738

D	Carbon nanocage	GldA overexpressed *E.coli* BL21 (DE3)	1304	*Electrochemistry Communications*, 2009, **11**, 1593
D	rGO-5-Ni composite	*Shewanella oneidensis* MR-1	27000**	*Nanoscale,* 2013, **5**, 10283
D	TiO_2/rGO hybrid on carbon cloth	*Shewanella putrefaciens* CN32	3169	*Journal of Power Sources*, 2015, **276**, 208
D	O_2 plasma treated carbon cloth	*Shewanella oneidensis* MR-1 with biococatalysts	1079.6±5.1	*Chemical communications*, 2015, **51**, 12170
S	Carbon fiber brush	*Shewanella oneidensis* MR-1	332±21	*Biotechnology Bioengeering,* 2010,**105**, 489–498
D	3D graphene/PANI	*Shewanella oneidensis* MR-1	768	*ACS Nano* 2012, **6**, 2394
D	Carbon paper	*Shewanella oneidensis* MR-1	104	*Biosensors and Bioelectronics* 2011, **26**, 3987
S	Graphite felt	SO_3350 mutant MR-1	~110	*Bioscience, Biotechnology, and Biochemistry* 2011, **75**, 2229
D	Carbon cloth	3D rGO hybrid biofilm	843	*Angewandte Chemie International Edition* 2014, **53**, 4480
D	O_2 plasma treated carbon cloth	NDH II mutant	371.5±3.7; 2277.4±11.1 with 200 uM RF	This work

* Dual chamber: D; single chamber: S;

** Unit: mW/m^3;

*** Chitosan and vacuum-stripped graphene: CHI/VSG

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