A high-energy-density sugar biobattery based on a synthetic enzymatic pathway

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High-energy-density, green, safe batteries are highly desirable for meeting the rapidly growing needs of portable electronics. The incomplete oxidation of sugars mediated by one or a few enzymes in enzymatic fuel cells suffers from low energy densities and slow reaction rates. Here we show that nearly 24 electrons per glucose unit of maltodextrin can be produced through a synthetic catabolic pathway that comprises 13 enzymes in an air-breathing enzymatic fuel cell. This enzymatic fuel cell is based on non-immobilized enzymes that exhibit a maximum power output of 0.8 mW cm\(^{-2}\) and a maximum current density of 6 mA cm\(^{-2}\), which are far higher than the values for systems based on immobilized enzymes. Enzymatic fuel cells containing a 15% (wt/v) maltodextrin solution have an energy-storage density of 596 Ah kg\(^{-1}\), which is one order of magnitude higher than that of lithium-ion batteries. Sugar-powered biobatteries could serve as next-generation green power sources, particularly for portable electronics.
The rapidly growing demand for powering portable electronic devices is driving the development of better batteries with features such as enhanced energy-storage densities, high levels of safety, fast rechargeability, biodegradability and small environmental footprints. The rechargeable lithium-ion battery is often the system of choice because it offers a high energy density, has a flexible and light-weight design and has a longer lifespan than comparable battery technologies. The energy-storage density of a typical lithium-ion battery is approximately 0.54 MJ kg\(^{-1}\) (that is, 150 Wh kg\(^{-1}\)). The widespread use of metal-catalysed batteries also raises many concerns, primarily related to safety, toxic metal pollution and the availability of costly, limited, irreplaceable or rare metal resources.

Enzymatic fuel cells (EFCs) are emerging electrobiochemical devices that directly convert chemical energy from a variety of fuels into electricity using low-cost biocatalyst enzymes. Inspired by living cells that can utilize complex organic compounds (for example, starch and glycerogen) as stored energy sources, sugar-powered EFCs represent the next generation of biodegradable, highly safe biobatteries. Compared with microbial fuel cells, EFCs usually generate much higher power densities in terms of mW cm\(^{-2}\). This feature highlights their great potential for powering a variety of portable electronic devices in the near future.

In principle, fuels used in EFCs can have high energy-storage densities if they are completely oxidized. For example, the combustion energy of glucose is 15.5 MJ kg\(^{-1}\). Glucose can release up to 3.574 Ah kg\(^{-1}\), which is 85-fold greater than the energy released by lithium-ion batteries (42 Ah kg\(^{-1}\)). Most EFCs run on complex organic compounds (for example, glucose, methanol, glycerol and so on). Therefore, high energy-storage density potentials can only be achieved if these fuels are completely oxidized to CO\(_2\), as occurs in living cells through carbohydrate metabolic pathways. However, natural catabolic pathways may not be feasible for practical use in EFCs, because nearly all of these pathways require costly and labile reagents, such as ATP, coenzyme A and complex cellular membranes. The incomplete oxidation of organic fuels mediated by one or a few enzymes in EFCs results in low energy densities, waste of fuel and inhibition of enzymes by the metabolic products. In vitro synthetic enzymatic pathways have been constructed on the anode compartments in EFCs for deep or complete oxidation of methanol, ethanol, glycerol and glucose. However, previous studies did not provide quantitative evidence (for example, Faraday efficiency) for the complete oxidation of organic fuels in microbial fuel cells.

 Sugars are appealing fuels for EFCs because they are abundant, renewable, inexpensive in terms of $ GJ \(^{-1}\), non-toxic, safe for storage and distribution, and carbon neutral over the entire life cycle. Starch is the most widely used energy-storage compound in nature. The catabolism of starch allows for a slow and nearly constant release of chemical energy in living cells that is different from its monomer glucose. Maltodextrin, a partially hydrolysed starch fragment, is a superior fuel to glucose in EFCs, because maltodextrin has 11% higher energy density than glucose. Maltodextrin is also less costly because glucose is the main product of its enzymatic hydrolysis, and low-cost linear maltodextrin can be made from cellulose. An equivalent weight of maltodextrin has a much lower osmotic pressure than glucose. Moreover, it can provide slowly metabolized glucose-1-phosphate for more stable electricity generation in closed EFCs. Maltodextrin has been used as a fuel for EFCs, but only two electrons could be generated per glucose unit.

In this study, a synthetic ATP- and COA-free catabolic pathway that comprises 13 enzymes in an air-breathing EFC is constructed to completely oxidize the glucose units of maltodextrin, yielding nearly 24 electrons per glucose. We find that the EFC based on non-immobilized enzymes exhibits a maximum power output far higher than those based on the immobilized enzymes. These sugar-powered biobatteries feature high energy-storage densities and high safety. Thus, these batteries represent next-generation micropower sources that could be especially useful for portable electronics.

Results

Comparison of non-immobilized and immobilized enzymes. Enzyme immobilization on the surface of conductive electrodes is widely used in nearly all EFCs. Enzymes are immobilized using a variety of methods, including gel entrapment, physical adsorption, chemical covalent linking and immobilization with nanoparticles and nanotubes. These methods originated from biosensors that focus on achieving reproducible signals by immobilizing commercially available mesophilic enzymes to enhance their stability without concern for slow reaction rates. However, enzymes immobilized on the surface of solid electrodes generally exhibit much lower activities (for example, 1%) due to enzyme deactivation and poor fuel transfer from the bulk solutions to the immobilized enzymes.

To achieve constant high-power EFCs, we considered an alternative strategy for mediating electron transfer in the EFCs without immobilizing the enzymes. Our strategy retains the enzymatic activity and facilitates mass transfer by immobilizing the electron mediator (that is, vitamin K\(_3\) (VK\(_3\))) on the surface of the electrode. The stability of enzymes can be addressed by the use of thermoenzymes. In this study, the thermoenzymes were produced in E. coli and purified by three methods: heat precipitation, His-tag/nickel charged resin and adsorption of cellulose-binding-module-tagged proteins on a cellulose adsorbent (Supplementary Fig. S1 and Supplementary Table S1). Two typical enzyme immobilization approaches for EFCs were used as controls: polymer matrix entrapment in a quaternary tetrabutylammonium bromide (TBAB)-modified Nafion (Fig. 1a(1)) and covalent binding on carbon nanotubes (CNTs) (Fig. 1a(2)). To compare the EFCs equipped with non-immobilized enzymes with the two EFCs equipped with immobilized enzymes, equivalent amounts of glucose-6-phosphate dehydrogenase (G6PDH) and diaphorase (DI) were used to test the polarization and the power outputs of the EFCs for g6p fuel (Fig. 1b,c). The mass transport region for the non-immobilized EFCs occurred at higher current densities compared with the covalent binding-based EFCs (Fig. 1b), suggesting the influence of enhanced mass transport for the non-immobilized enzymes. The EFC based on non-immobilized G6PDH exhibited the highest power density of 0.13 mW cm\(^{-2}\), three times higher than that of the covalent binding method. The EFC based on the TBAB-modified Nafion polymer entrapment method had the lowest maximum power density of 0.0013 mW cm\(^{-2}\), which was only 4% of the density for the covalent binding method. The G6PDH immobilized by Nafion polymer entrapment and the covalent binding retained 0.2 and 6% of its non-immobilized activity, respectively. The DI immobilized by Nafion polymer entrapment and the covalent binding retained 0.4% and 7.5% of its non-immobilized activity, respectively (Supplementary Table S2). These data for enzyme activity clearly suggest that a dramatic activity loss occurs due to enzyme immobilization. The power density data validate the feasibility of using non-immobilized enzyme(s) to achieve high-power output in EFCs.

Complete oxidation of maltodextrin. To release the maximum electron potential from each glucose unit (that is, 24 per glucose),...
we designed a non-natural enzymatic pathway containing 13 enzymes (Fig. 2a). This synthetic pathway consists of four functional modules: g6p generation from maltodextrin mediated by α-glucan phosphorylase (α-GP) and phosphoglucomutase (PGM) (equation 1); two reduced NADH generated from g6p mediated by two NAD-dependent G6PDH and 6-phosphogluconate dehydrogenase (6PGDH) (equation 2); NADH electro-oxidation through non-immobilized DI to immobilized VK₃ that generates 2 electrons per NADH (equation 3); and 5/6 moles of g6p regeneration from 1 mole of ribulose-5-phosphate via a hybrid pathway that comprises enzymes in the pentose phosphate, glycolysis and gluconeogenesis pathways (equation 4). The overall anode reaction for the combination of equations 1–4 approximately results in equation 5. Clearly, each glucose unit from maltodextrin can generate 24 electrons on the anode via this de novo pathway (equation 5).

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(C₆H₁₀O₅)_n + P₁ \rightarrow g6p + (C₆H₁₀O₅)_{n-1} \quad (1)
\]

\[
g6p + H₂O + 2NAD⁺ → ribulose-5-phosphate + CO₂ + 2NADH + 2H⁺ \quad (2)
\]

\[
NADH + H⁺ → 2H⁺ + 2e⁻ \quad (3)
\]

\[
6\text{Ribulose-5-phosphate} + H₂O \rightarrow 5\ g6p + \text{phosphate} \quad (4)
\]

\[
(C₆H₁₀O₅)₇ + 7H₂O → 24e⁻ + 6CO₂ + 24H⁺ \quad \text{(anode compartment)} \quad (5)
\]

The pathway utilizes two NAD-dependent G6PDH and 6PGDH to generate NADH differently from natural NADP-dependent enzymes in the pentose phosphate pathway used for anabolism. The above pathway does not require either ATP or CoA, which are very costly and unstable in EFCs. Moreover, phosphate ions can be recycled to maintain constant pH and ion concentrations. This cyclic pathway design is different from the linear pathways typically used in EFCs.

The power densities from maltodextrin fuel (that is, 2 mM glucose units) were compared for three EFCs that used one dehydrogenase (that is, G6PDH), two dehydrogenases (that is, G6PDH and 6PGDH) or the entire pathway (Fig. 2b). The open circuit potentials were similar for the three EFCs (~ 0.7 V). When only G6PDH was used, the EFC exhibited a maximum power density of 0.011 mW cm⁻². When a second dehydrogenase (6PGDH) was added, the maximum power density increased by a factor of 2, to 0.024 mW cm⁻². When eight additional enzymes were added to reconstitute the entire pathway (Fig. 2a), the maximum power density increased slightly to 0.026 mW cm⁻². The corresponding maximum current density was 35% higher than the current density of the system based on two dehydrogenases (Fig. 2b).

To quantitatively validate the complete oxidation of the glucose units of maltodextrin, we measured the Faraday efficiency from NADH to electrons through the DI and VK₃ in the air-breathing EFC (Supplementary Fig. S2). Under oxygen-free conditions for the anode compartment, the Faraday efficiency of the EFC was 97.6 ± 3.0% (Supplementary Fig. S3), suggesting that the electro-enzymatic oxidation of NADH is highly efficient. Moreover, the removal of oxygen from the anode compartment was essential for obtaining a high Faraday efficiency and preventing the non-selective oxidation of NADH. In a 15-mL EFC containing 13 enzymes and a low concentration of maltodextrin at room temperature (Fig. 2c), the current density increased to a peak value of 0.12 mA cm⁻² at 24 h and then decreased slowly due to substrate consumption. After > 150 h, the current output decreased to nearly zero. The cumulative electric charge generated was 48.9 C relative to the theoretical electric charge generated based on the consumption of the glucose units (that is, 53.0 C, 1 mole of glucose unit can generate 24 × 96,485 C in principle). This result suggests a cumulative
Faraday efficiency of 92.3% with 1 mole of glucose generating 22.2 moles of electrons. It was noted that the negative control (that is, the same EFC without the substrate) did not generate significant current outputs (Supplementary Fig. S4). The Faraday efficiency was higher than that of the microbial fuel cell based on glucose (83%) because cell-free biosystems do not waste organic fuels on cell growth and byproduct formation, as demonstrated previously. Our system provides the first quantitative evidence for nearly 24 electrons produced per glucose unit in an EFC. Moreover, our data suggest that we can convert all of the chemical energy from the sugar into electrical energy and increase the energy density of the EFC by one order of magnitude.

**High-energy-density high-power EFCs.** Power density is another important consideration in EFCs. To increase the power density, we optimized a number of factors, including the EFC configuration, the enzyme loading and the experimental conditions under which the non-immobilized G6PDH acts on g6p. The optimal CNT loading was 3 mg cm$^{-2}$ of carbon paper (Supplementary Fig. S5a). The six electrodes stacked together as a...
three-dimensional anode increased the maximum power density by 50% and the maximum current density by fourfold (Supplementary Fig. S5a,b). Increasing the enzyme loading from 1 to 10 U per cell drastically increased the maximum power density and maximum current density to 0.35 mW cm\(^{-2}\) and 4.1 mA cm\(^{-2}\), respectively, at room temperature (23\(\degree\)C; Supplementary Fig. S5c). Elevating the temperature to 50\(\degree\)C doubled the maximum power density to 0.8 mW cm\(^{-2}\) (Supplementary Fig. S5d).

The EFC that comprises 13 non-immobilized enzymes based on 15% (wt/v) maltodextrin generated the maximum power density of 0.4 mW cm\(^{-2}\) at a scanning rate of 1 mV s\(^{-1}\) at room temperature. The EFC generated a nearly constant power output of \(\sim 0.32\) mW cm\(^{-2}\) for 60 h in a closed system (Fig. 3). In addition, a stack of two cuvette-based EFCs can power a digital clock and a light-emitting diode (LED) (Supplementary Fig. S6), suggesting that these EFCs could be used to power a number of electronic devices in the near future.

**Discussion**

The complete oxidation of the glucose units of the 15% maltodextrin solution means that the energy-storage density of this sugar-powered EFC can be as high as 596 Ah kg\(^{-1}\), which is more than one order of magnitude higher than the energy-storage densities for lithium-ion batteries and primary batteries (Fig. 4 and Supplementary Table S3). Although the voltages of the EFCs (for example, 0.5 V) are much lower than the voltages of lithium-ion batteries (3.6 V), the energy density of the 15% sugar-powered EFC can reach up to 298 Wh kg\(^{-1}\), several times that of common rechargeable batteries (for example, Pd acid, NiMH and lithium-ion batteries) and higher than that of common primary batteries (for example, zinc-carbon, alkaline and Li-MnO\(_2\) batteries; Fig. 4). The cuvette-based EFC (Supplementary Fig. S6) has an energy-storage density of \(\sim 238\) Wh kg\(^{-1}\) for the entire system, because the weight of the combined electrode materials, the plastic cuvette and the membrane electrode assembly accounts for \(\sim 20\%\) of the entire device weight. Such biobatteries may be regarded as environmentally friendly, disposable primary batteries, because they have better energy densities and less environmental impact.

In addition to the one order-of-magnitude improvement in the energy density of the sugar biobatteries via this synthetic pathway, relative to the system with one redox enzyme (Fig. 4), the biobatteries equipped with non-immobilized enzyme cascades might be refilled by the addition of the sugar solution, because the sole gaseous product (CO\(_2\)) can be easily released from the anode compartment and the non-immobilized enzymes are not washed out of the EFCs. The non-immobilized enzyme EFC was tested by adding the sugar solution twice (Supplementary Fig. S7). However, the decreased performance of the EFCs suggested more research and development needed for extending the lifetime of EFCs.

These sugar biobatteries represent a new type of rechargeable battery. One of the greatest advantages of fuel cells compared with closed primary and secondary batteries is that they are open systems that use high-energy-density fuels (for example, H\(_2\), methanol, glucose and maltodextrin) that can be fed into the fuel cell device continuously (for example, proton exchange membrane fuel cells) or sporadically (for example, direct methanol fuel cells and sugar batteries\(^{4,27}\)). When the weight ratio of the fuel to the fuel cell system is large enough (that is, 5–10) or if the fuel cell is refilled a number of times, the energy density of the entire system, including the fuel, a fuel tank and a fuel cell system, can be close to the theoretical energy density of the fuel that is used. Clearly, the use of water-free chemicals as fuels is more attractive in terms of energy-storage density (Fig. 4). However, a separate fuel tank and a complicated fuel feeding system is required in such a system.

Maltodextrin is a better EFC fuel than alcohols (for example, methanol) or glucose. Maltodextrin is slowly utilized via the synthetic pathway to generate a nearly constant power output (Fig. 3) rather than a peak power over a short time\(^{10}\). In addition, most enzymes cannot work well in high concentrations of alcohol or glucose due to inhibition or low water activity. For example, the highest methanol concentration that can be used in EFCs is \(\sim 0.5\) M, resulting in a lower energy-storage density of 40.2 Wh kg\(^{-1}\) (Fig. 4)\(^{28}\). Similarly, high concentrations of glucose (for example, 0.4 M)\(^{7}\) lead to high osmotic pressures (\(\sim 9.85\) atm) that can impair enzyme activity\(^{29}\). Compared with the six-enzyme EFC that oxidizes glucose to CO\(_2\) (ref. 13), the inherently low, but

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**Figure 3 | Continuous power and current outputs for the 13-enzyme fuel cell.** The EFC has an external load of 150 \(\Omega\) and is run at room temperature. The experimental conditions are 100 mM HEPES, pH 7.5, buffer containing 15% wt/v maltodextrin, 10 mM MgCl\(_2\), 0.5 mM MnCl\(_2\), 4 mM NAD\(^+\), 0.5 mM thiamine pyrophosphate, 40 mM sodium phosphate, 5 mM DTT and non-immobilized enzyme loading conditions of 30 units of 1–4 enzymes, 10 units of 5–12 enzymes, 80 units of DI, 50 mg l\(^{-1}\) kanamycin, 40 mg l\(^{-1}\) tetracycline, 40 mg l\(^{-1}\) cycloheximide, 0.5 g l\(^{-1}\) sodium azide, 1 g l\(^{-1}\) BSA and 0.1% Triton X-100 in a 15-ml closed anode compartment.

**Figure 4 | Comparison of energy densities among batteries and EFCs.** EFCs are powered by 500 mM methanol, 7.2% wt/v glucose or 15% wt/v maltodextrin or dehydrated fuels at a voltage of 0.5 V. More information is available in Supplementary Table S3.
promiscuous, activities of one enzyme that catalyses several substrates results in very low power densities. The use of less than ten enzymes for implementing complex reactions for the production of biocommodities, fine chemicals and pharmaceuticals seems to be not economically prohibitive26,30-33. One of the most important issues for sugar biobatteries is extending their lifetime. This involves improving the stability of enzymes, cofactors and mediators3. A preliminary diagnostic experiment was conducted to study the decreased performance of the non-immobilized EFCs (Supplementary Fig. S8a). The addition of the new substrate and enzyme mixture to the EFC resulted in a quarter of the maximum power output, suggesting that enzyme deactivation is one of the causes of the decreased power output after more than 1 week of operation at room temperature. Instead of using immobilized enzymes similar to that in most EFCs, we prolonged the lifetime of enzymes using non-immobilized thermoenzymes isolated from (hyper)thermophilic microorganisms34. Clearly, relatively non-stable thermoenzymes, such as phosphoglucone isomerase, z-GP and PGM, isolated from thermophiles can be replaced with enzymes from hyperthermophiles or engineered mutant enzymes generated by protein engineering (that is, rational design, directed evolution or a combination of methods). In addition, the half-life time of the non-immobilized enzymes increased from 5.0 days to 7.7 days through the addition of 1 g L
-1 BSA and 0.1% Triton X-100 (Supplementary Fig. S8b), suggesting that the formulation of enzyme mixture can also be adjusted to prolong the lifetime of non-immobilized enzyme mixtures. Furthermore, replacement of old anodes with new anodes doubles the power output to nearly half of the maximum power output (Supplementary Fig. S8a), indicating that leaching of adsorbed VK3 from the anode results in lower power outputs. Therefore, it will be important to adopt a better method to immobilize VK3-like mediators on the surface of anodes35.

In addition to the enhanced stability of the enzymes, mediator, future research and development on high-power, sugar-powered EFCs should focus on increasing the power density, prolonging the lifetime of the EFCs and replacing the platinum in the cathodes with laccase2 or bilirubin oxidase4. With respect to the first goal, the power density of the EFCs could be increased to 10 mW cm
-2 or higher, using a combination of newly developed high-electrical conductivity and high-surface-area nanomaterials as electrodes, such as graphene foams34 or carbonized nanofibre aerogels37,38. The performance can also be improved using better mediators39, more active enzymes or enzymatic complexes40, and optimized enzyme ratios predicted by in silicon models41. With respect to the second goal, the use of more stable thermoenzymes, better mediators and cofactors, and better formulation of enzymes can prolong the lifetime of EFCs to months or longer at room temperature, similar to the proteases used in liquid detergents. Moreover, costly and labile NAD can be replaced with low-cost and stable biocatalysts42,43.

To summarize, high-energy-density sugar biobatteries that use this synthetic catabolic pathway could represent the next generation of environmentally friendly power sources, because of their many appealing features, such as high energy density, safety, biodegradability and low cost catalysts without the need for costly metals and rare earth elements. However, the degradation of EFCs due to the relatively short lifetimes of the enzymes, cofactors and mediators must be resolved before EFCs can be used on a large scale.

Methods

Chemicals. All chemicals, including maltodextrin (dextrose equivalent of 4.0–7.0, that is, a measured degree of polymerization of 19), VK3, nicotinamide adenine dinucleotide (NAD, including both the oxidized form (NAD+)) and the reduced form (NADH)), poly-l-lysine (PLL, molecular weight, ~70–150 kDa), dithiothreitol (DTT), 1,3-dimethyl-2-imidazolidinone (DMI), hydroxide (EDC) and N-hydroxy succinimide were reagent grade or higher and purchased from Sigma-Aldrich (St Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA), unless otherwise noted. Restriction enzymes, T4 ligase and Phusion DNA polymerase were purchased from New England Biolabs (Ipswich, MA, USA). Oligonucleotides were synthesized either by Integrated DNA Technologies (Corvallis, IA, USA) or Fisher Scientific. The carbon paper (AvCarb MGL200) used in the anodes was purchased from Fuel Cell Earth (Stoneham, MA, USA). Membrane electrode assemblies consisting of Nafion 212 membranes and a carbon cloth cathode modified with Pt and Rh were purchased from Fuel Cell Store (San Diego, CA, USA). COOH-functionalized multiwall CNTs with an outer diameter of <8 nm and a length of 10–30 μm were purchased from Cheap-Tubes (Brattleboro, VA, USA). Regenerated amorphous cellulose used in enzyme purification was purchased from Avicel PH105 (FMC, Philadelphia, PA, USA) through its dissolution and regeneration, as described elsewhere42. Escherichia coli Top10 was used as a host cell for DNA manipulation and E. coli BL21 Star (DE3) (Invitrogen, Carlsbad, CA, USA) was used as a host cell for recombinant protein expression. Luria–Bertani medium including either 100 mM L-ampicillin or 50 mM L-kanamycin was used for E. coli cell growth and recombinant protein expression36.

Production and purification of recombinant enzymes. The E. coli BL21 Star (DE3) strain harbouring plasmid pMD18 was grown to an OD
-600 of 0.6–0.8. Protein expression was induced by adding 100 mM of isopropyl-β-D-1-thiogalactopyranoside during 30 min of overnight incubation. The cells were harvested by centrifugation at 4 °C and washed once with 20 mM HEPES (pH 7.5) containing 0.3 M NaCl. The cell pellets were resuspended in the same buffer and lysed by ultrasonication (Ultrasonic Scientific Sonic Dismembrator Model 500; 5-s pulse on and off, total 300 s at 50% amplitude). After centrifugation, the target proteins the supernatants were purified. Three approaches were used to purify the various recombinant proteins (Supplementary Table S1). His-tagged proteins were purified by the Profinity IMAC Ni-Charged Resin (Bio-Rad, Hercules, CA, USA). Fusion proteins containing a cellulose-binding module and self-cleavage intein were purified through high-affinity adsorption on a large-surface-area resin, and amorphous cellulose44,45. Heat precipitation at 80 °C was used to purify ribose-5-phosphate isomerase, ribulose-5-phosphate epimerase, triosephosphate isomerase (TIM) and aldolase46,47. The purity of the recombinant proteins was examined by SDS–PAGE (Supplementary Fig. S1).

Enzyme activity assays. Cladstridium thermocellum z-GP activity was assayed in 100 mM HEPES buffer (pH 7.5) containing 1 mM MgCl
2, 5 mM DTT, 30 mM maltodextrin and 10 mM sodium phosphate at 23 °C for 5 min. The reaction was stopped with the addition of 10 mM EDTA, and neutralized with KOH. The z-GP activity was measured using a glucose hexokinase/G6PDH assay kit (Pointe Scientific, Canton, MI, USA) supplemented with PGM. C. thermocellum PGM activity was measured in 100 mM HEPES buffer (pH 7.5) containing 5 mM MgCl
2, 0.5 mM MnCl
2 and 5 mM glucose-1-phosphate at 23 °C for 5 min. The G6P product was determined using a hexokinase/G6PDH assay kit44,48. Geobacillus stearothermophilus G6PDH activity was assayed in 100 mM HEPES buffer (pH 7.5) containing 100 mM NaCl, 2 mM G6P, 2 mM NAD+, 5 mM MgCl
2 and 0.5 mM MnCl
2 at 23 °C. An increase in the absorbance due to the formation of NADH was measured at 340 nm44. Morella thermostatica G6PDH activity was measured in a 100 mM HEPES buffer (pH 7.5) containing 2 mM 6-phosphogluconate, 2 mM NAD+, 5 mM MgCl
2 and 0.5 mM MnCl
2 at 23 °C for 5 min39.

Thermotoga maritima ribose-5-phosphate isomerase activity was assayed using a modified Dische’s cysteine–carbazole method. (D)-Ribulose-5-phosphate epimerase activity was determined on a substrate of (D)-ribose-5-phosphate as described previously41.

Thermus thermophilus transketolase activity was measured in a 50 mM Tris/HCl (pH 7.5) buffer containing 0.8 mM D-xylulose-5-phosphate, 0.8 mM D-ribose-5-phosphate, 5 mM MgCl
2, 0.5 mM thiamine pyrophosphate, 0.15 mM NADH, 60 U mL
-1 of TIM and 20 U mL
-1 of glyceraldehyde 3-phosphate dehydrogenase. The reaction was started with the addition of transketolase at 23 °C. The Δ-α-glycerophosphate 3-phosphate product was quantified by measuring the consumption of NADH measured at 340 nm for 5 min. T. maritima transaldolase activity was assayed as reported previously49. T. thermophilus TIM activity was determined in 50 mM Tris/HCl (pH 7.5) containing 5 mM MgCl
2, 0.5 mM MnCl
2, 0.5 mg mL
-1 BSA, 20 U mL
-1 of glyceraldehyde 3-phosphate dehydrogenase and 0.25 mM NADH50. T. thermophilus fructose-1,6-bisphosphate aldolase was assayed in a 50 mM Tris/HCl buffer (pH 7.5) at 23 °C with 1.9 mM fructose-1,6-bisphosphate as a substrate and glyceraldehyde 3-phosphate as the product. The aldolase activity was determined by measuring the consumption of 0.15 mM NADH, 60 U mL
-1 of TIM and 20 U mL
-1 of glyceraldehyde 3-phosphate dehydrogenase at 340 nm59.
T. maritima fructose-1,6-bisphosphatase activity was determined based on the release of phosphate.

C. thermophilum phosphoglucosamine isomerase activity was assayed at 23 °C in 100 mM HEPES (pH 7.5) containing 10 mM MgCl₂, 0.5 mM MnCl₂ and 5 mM fructose 6-phosphate. After 3 min, the reaction was stopped with the addition of HClO₄ and neutralized with KOH. The g6p product was analysed at 37 °C with a hexokinase/G6PDH assay kit.

G. stearothermophilus DI activity was assayed in 10 mM PBS solution containing 0.16 mM NADH and 0.1 mM dichlorophenolindophenol at 23 °C. A decrease in the absorbance at 600 nm due to the consumption of dichlorophenolindophenol was measured using a spectrometer.

Enzyme immobilization. Two enzyme immobilization methods were used to prepare the anodes equipped with the immobilized enzymes. Method 1 was based on the entrapment of enzymes into a quaternary ammonium-bromide-salt-modified Nafion. The casting solution was 10 mM NaOH, 0.16 mM NADH and 0.1 mM dichlorophenolindophenol at 23 °C, and power outputs were normalized to 1 cm² of anode area because the reaction rate depends on the enzyme loading conditions of 30 units of 1–4 enzymes, 10 units of 5–12 enzymes, 80 units of DI, 0.1 M NAD⁺ and 100 mM NaNO₂ at 4 °C before use.

For preparation of the non-immobilized enzyme anodes, 1 or 3 mg of CNTs were added to the surface of a 1 cm² carbon paper (AvCarb MGL200) from Fuel Cell Earth using PLL (molecular weight, ~70–150 kDa) as described previously. A 10-μl solution of 0.29 M VK₃ dissolved in acetone was deposited on the dry CNT-containing anode under a hood. After 2 h of acetone evaporation, the water-insoluble VK₃ was deposited onto the anode through physical adsorption.

Electrochemical characterization of EFCs. All electrochemical tests were performed using a 1000B Multi-Potentiostat (CH Instruments Inc., Austin, TX, USA) interfaced to a personal computer (PC). Experimental data pertaining to current and power outputs were normalized to 1 cm² of anode area because the reaction occurring at the anode was the rate-limiting step and the oxidation of protions mediated by Pt in the membrane electrode assemblies was not rate limiting. The measurements of open circuit potential and linear sweep voltammetry were performed at a scan rate of 1 mV s⁻¹.

For the comparison of the power generation from the immobilized and non-immobilized enzyme EFCs (Fig. 1), the electrolytes contained 10 mM g6p, 100 mM HEPES buffer (pH 7.5), 0.5 mM NAD⁺, 0.5 mM MnCl₂, and 10 μM G6PDH. Each unit of G6PDH and 40 units of DI were either immobilized on the electrodes or attached to electrodes.

When maltodextrin was used as a substrate (Fig. 2), the electrolytes contained 100 mM HEPES buffer (pH 7.5), non-immobilized enzymes, 0.1 mM maltodextrin, 4 mM NAD⁺, 4 mM sodium phosphate, 10 mM G6PDH, and 0.5 mM MnCl₂. One unit of G6PDH and 40 units of DI were either immobilized on the electrodes or dissolved in the electrolyte.

Supplementary Table S1. Amperometry was conducted at 0 V to achieve the maximal current density. The EFC with 0.2 mM g6p was run for 2 days until nearly zero current was obtained, before a solution of 0.1 mM maltodextrin (that is, ~1.9 mM glucose) was added. The complete oxidation of maltodextrin took ~1 week at room temperature and the remaining maltodextrin was quantified using a 5A-20 starch assay kit (Sigma-Aldrich). The Faraday efficiency was calculated according to

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\text{Faraday efficiency (FE)} = \frac{\text{current (A)}}{\text{Faraday constant (A·mol⁻¹)}} \times \text{weight of substrate (g)}
\]

where \(\text{Faraday constant (A·mol⁻¹)}\) is 96485. The EFCs were operated at 23 °C for 60 h. The power density was measured for 60 h at 23 °C (Fig. 3).

References


