Characterizing Efficiency of Multi-Enzyme Cascade-Based Biofuel Cells by Product Analysis

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The performance of biofuel cells with enzyme cascades have normally been characterized with open circuit potential, power density, and current density measurements. In this work, we demonstrate that with the method of quantitative product analysis by mass spectrometry, we can obtain other valuable information about the biofuel cell efficiency. Faradaic efficiency, coulombic efficiency and product efficiency were calculated for a six-enzyme glucose biofuel cell system. Oxidation pathway bottlenecks were determined with quantitative mass spectrometry measurements via direct infusion. These measurements and calculations give an in-depth understanding of the bioelectrocatalytic bottlenecks in the enzyme cascade for the target fuel (glucose).

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Utilizing enzymes as biocatalysts in biofuel cells has expanded the diversity of potential fuels for fuel cells[1–5] and offered the opportunity to utilize more complex molecules (such as glucose,[6] fructose,[7] glycerol,[8] pyruvate,[9] lactate[10] and many others) as energy sources. For more efficient use of fuels, multiple enzyme systems (enzyme cascades) were designed to perform sequential oxidations of fuels to form small molecule final product, such as carbon dioxide, and produce maximum electrical energy per fuel molecule. Palmore et al. introduced the use of enzyme cascades in 1998, when they utilized a three-enzyme cascade to completely oxidize methanol.[11] In 2008, Sokic-Lazic et al. reported a bioanode that used an enzyme cascade to mimic the Krebs cycle and achieved complete oxidation of ethanol at a bioanode.[12] By mimicking the complete Krebs cycle on a carbon electrode, the power density was increased by 8.71-fold compared to a single enzyme-based ethanol biofuel cell. In 2009 and 2011, complete oxidations of other complex fuels (pyruvate[9] and lactate[10]) with Krebs cycle enzymes were reported, which resulted in 26-fold enhancements in power density performance compared to a single enzyme-based biofuel cell. In 2011, Xu et al. demonstrated the complete oxidation of glucose with a non-natural oxidation pathway.[6] A six-enzyme cascade was utilized to perform a 12-step, 24-electron oxidation of glucose. The power density showed an almost 50-fold increase with the cascade. Enhancements have also been observed for glucose in a bi-enzyme cascade[13] and for the deep and complete oxidation of maltodextrin.[14,15]

Studies of the utilization of enzyme cascades in biofuel cell systems have demonstrated that deeper or complete oxidation of fuels can increase the power density and current density of biofuel cells. However, in order to have a better understanding on the performance of an enzyme cascade system, there are more questions to be answered: (1) How much of the electricity produced is from the designed oxidation pathway (faradaic efficiency)? (2) What ratio of the theoretical maximum amount of electrical energy in the fuel is produced during the biofuel cell operation process (coulombic efficiency)? (3) What percentage of the fuel is completely oxidized to the final product(s) (product efficiency)? To answer these questions, a careful analysis on the intermediates and products of the biofuel cell systems needs to be carried out. Product analysis not only gives the quantitative analysis needed to calculate the different types of efficiencies mentioned above, but also provides evidence to investigate the bottleneck step(s) in the oxidation pathway which is crucial information for bioanode cascade optimization and, therefore, fuel cell performance optimization.

Faradaic efficiency, coulombic efficiency, and product efficiency describe different aspects of biofuel cell performance. Faradaic efficiency is defined as the fraction of charge passed to form intermediates and product(s) in an electrochemical process divided by the total charge passed in the electrochemical cell.[16] It is an important criterion to determine whether the oxidation is strictly following a desired pathway or if there are other (in this case non-enzymatic) processes occurring that are utilizing electrons. Coulombic efficiency describes the percentage of the theoretical maximum charge that is passed during the biofuel cell operation if all reactant were to form the final oxidized product (carbon dioxide).[16] Higher coulombic efficiency represents a higher degree of oxidation of the fuel. Product efficiency reflects the percentage of fuel molecules that have been completely oxidized to final product(s) molecules. High product efficiency means low intermediate buildup in the oxidation process and is representative of no large catalytic bottlenecks in the enzyme cascade.

The three different types of efficiencies provide more detailed information and another method to evaluate the enzyme cascades utilized in biofuel cell systems aside from open circuit potential, current and power densities. In this work, we will demonstrate the detailed, mass spectrometric product analysis on a previously reported glucose oxidation system that utilizes a six-enzyme cascade to oxidize glucose to the final product, carbon dioxide.[6] PQQ-dependent glucose, gluconate, alcohol and aldehyde dehydrogenases, oxalate oxidase and aldolase were immobilized on carbon fiber electrodes to perform the complete oxidation of glucose (as shown in Figure 1). The biofuel cell operation products were quantitatively analyzed and the biofuel cell efficiencies were calculated, compared, and discussed.

Experimental

Enzyme extractions and bioanode fabrication.— PQQ-dependent enzymes were extracted from *Gluconobacter sp.* (DSM 3504) and aldolase was extracted from *Sulfolobus Solfataricus* (DSM 1616) as described previously.[6] Oxalate oxidase was provided as a gift from Amano. The 5 wt.% tetrabutylammonium bromide (TBAB)-modified Nafion membrane suspension was prepared as discussed in reference.[17] Enzyme/TBAB-modified Nafion casting solutions were made with 1:1 ratio of TBAB-modified Nafion and enzyme cascade solution. Enzyme ratios were calculated based on specific activities. Casting solutions of total concentration of 20 mg protein/mL were vortexed in preparation for coating on electrodes. A 100 μL aliquot of casting mixture was pipetted onto the 1 cm^2 electrode, allowed to soak into the Toray carbon fiber paper electrode, and dried in the hood for 12 hours. Prepared electrodes were stored at 4°C until use.

Physical cell apparatus.— The biofuel cell anode compartment contained 2 mL of pH 6.5 phosphate buffer solution with 1 mM glucose and 1 M KNO_3 electrolyte. The air-breathing cathode

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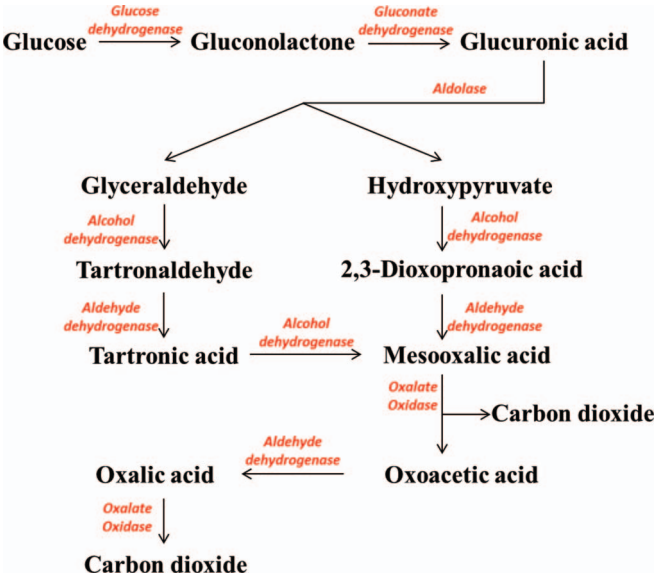

Figure 1. Designed non-natural oxidation pathway of glucose with a six-enzyme cascade.

compartment consisted of a 2.5 × 2.5 cm piece of an ELAT electrode with 20% platinum on Vulcan XC-72 (E-Tek) hot-pressed to a 3 × 3 cm Nafion NRE212 membrane.

Electrochemical measurements.— All electrochemical data were collected and analyzed with a CH Instruments 650 potentiostat interfaced to a PC. Battery discharge was performed for 120 hours at 5 mV cell potential. The experiment was terminated when the current density decreased to 2% of the maximum.

Quantitative mass spectrometry measurements.— Quantitative mass spectrometry was performed with a WATERS LCT Premier XE (TOF). All samples and standards were measured with the same operating conditions. Negative ESI ionization mode was applied with capillary voltage of 1200.0 V and sample cone of 15.0 V. Desolvation temperature was 145°C and source temperature was 75°C. Samples were infused at a rate of 10 μL/minute. A 1 mM aliquot of intermediate product standards in the same buffer solutions were used as standards. Products concentrations were calculated by comparing the peak heights of products in the sample and 1 mM standards.[18,19]

Results and Discussion

The theoretical maximum charge that could pass during the oxidation of the 2 mL 1 mM glucose solution was 4.63 C (1 mole of glucose can generate 24 electron × 96,485 C/mole of electrons). During the 120 hours of biofuel cell operation time, an accumulative of 3.07 C of charge had passed. A small amount of product solution was sampled immediately after biobattery discharge for quantitative mass spectrometry measurements. The results of the mass spectrometry analysis show that the product solution has no detectable glucose. It contains 0.15 mM gluconic acid (in equilibrium with the cyclic ester form gluconolactone in aqueous solution), 0.16 mM glucuronic acid, 0.35 mM mesooxalic acid and 0.2 mM oxalic acid as shown in Table I and Figure 2. With this information, product efficiency, Coulombic efficiency and faradaic efficiency were calculated as shown below.

Product efficiency is the amount of glucose that had been completely converted to carbon dioxide (mM) divided by the total amount of glucose in the fuel solution. The amount of carbon dioxide formed in the process can be calculated by using the total amount of carbon in glucose fuel and subtracting the amount of carbon left in the final solution. The calculated result (Eq. 1) shows that 44.8% of the glucose fuel was completely converted to carbon dioxide. 55.2% of the glucose was partially oxidized and existed in the solution in the form of intermediates (gluconic acid, glucuronic acid, mesooxalic acid, and oxalic acid).

Table I. Quantitative mass spectrometry measurement results.

Product	Sample peak height	1 mM standard peak height	Concentration (mM)
Gluconic acid	934	6223	0.15
Glucuronic acid	498	3112	0.16
Mesooxalic acid	16558	47308	0.35
Oxalic acid	5657	28286	0.2

$$[6 \times 1mM - (6 \times 0.15mM + 6 \times 0.16mM + 3 \times 0.35mM + 2 \times 0.2mM)]/(6 \times 1mM) = 44.8\% \quad [1]$$

Coulombic efficiency is the fraction of charge that passed to form products along the pathway divided by the theoretical maximum amount of charge in this process. The charge passed in the biofuel cell operation can be calculated from the measured intermediate concentrations. Calculation result shows that 63% of the theoretical maximum amount of charge was transferred during the 120 hours operation. The charge passed to form carbon dioxide is:

$$0.002L \times 96485\,C/mole\ e \times [1mmole/L \times 0.448 \times 0.024\,mole\ e/mmole] = 2.07\,C \quad [2]$$

The charge passed to form 0.15 mM gluconolactone is:

$$0.002L \times 96485\,C/mole\ e \times [0.15\,mmole/L \times 0.002\,mole\ e/mmole] = 0.06\,C \quad [3]$$

The charge to form 0.16 mM glucuronic acid is:

$$0.002L \times 96485\,C/mole\ e \times [0.16\,mmole/L \times 0.004\,mole\ e/mmole] = 0.12\,C \quad [4]$$

The charge passed to form 0.35 mM mesooxalic acid is:

$$0.002L \times 96485\,C/mole\ e \times [0.35\,mmole/L \times 0.012\,mole\ e/mmole \times 0.5] = 0.41\,C \quad [5]$$

The charge passed to form 0.2 mM oxalic acid is:

$$0.002L \times 96485\,C/mole\ e \times [0.20\,mmole/L \times 0.02\,mole\ e/mmole \times 0.33] = 0.25\,C \quad [6]$$

Therefore, the Coulombic efficiency is

$$(2.07 + 0.06 + 0.12 + 0.41 + 0.25)\,C/4.63\,C = 63.0\% \quad [7]$$

Note: The fractions 0.5 and 0.33 used in Eq. 5 and Eq. 6 represent that to form 1 molecule of mesooxalic acid and oxalic acid, 0.5 and 0.33 molecules of glucose are converted, respectively.

Faradaic efficiency is the fraction of the charge that passed in the biofuel cell operation to form products along the pathway divided by the total amount of charge transferred in this process. Since the products above produce 2.91C of charge and the total charge passed is 3.07C, then the faradaic efficiency is 95.0%, indicating that the oxidation of glucose is following the desired non-natural pathway and very little, non-enzymatic byproducts were formed during the biofuel cell operation.

The product analysis results also show that there is only gluconolactone (in the form of gluconic acid), glucuronic acid, mesooxilic acid and oxalic acid in the product solution, which indicates that the PQQ-dependent glucose, alcohol and aldehyde dehydrogenase have higher catalyzing rate than PQQ-dependent gluconate dehydrogenase, aldolase and oxalate oxidase, and the latter three enzymes are the bottlenecks of the oxidation pathway. The results are within

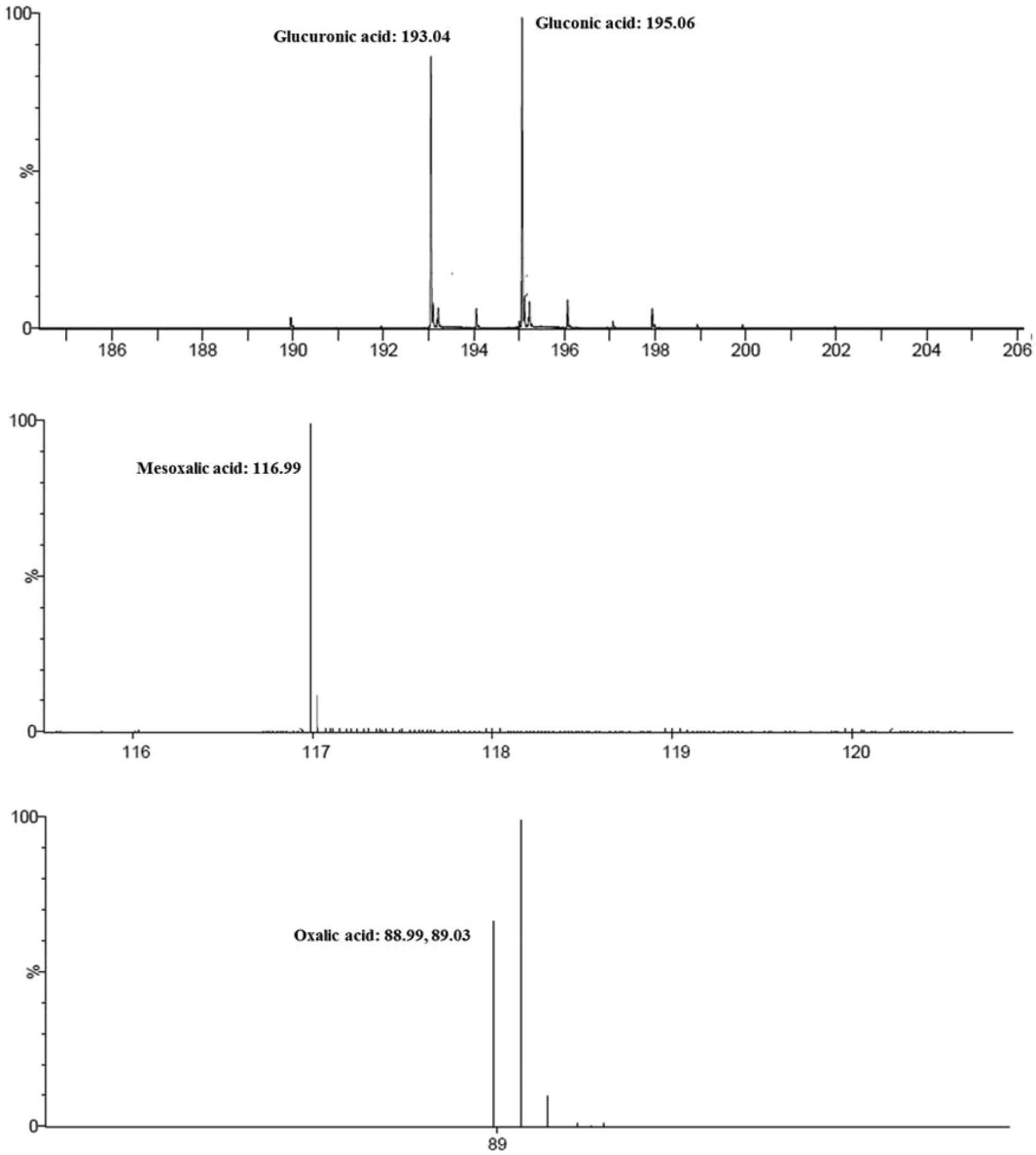

Figure 2. Mass spectrometry analysis of bulk electrolysis product solution. Gluconic acid, glucuronic acid, mesooxalic acid and oxalic acid were detected. Sample was run in negative ionization mode, which is forming [M-H+]; peaks are molecular weight −1.

our expectation based on the enzyme activity assays results. For instance, this data shows that oxalate oxidase catalyzing the reaction of mesooxalic acid and oxalic acid is a bottleneck in the enzyme cascade pathway. This is expected, since the specific activity of oxalate oxidase to mesooxalate is 0.75 U/mg and the specific activity of oxalate oxidase to oxalate substrate is 3.2 U/mg, which are both low specific activities for enzyme cascades. This indicates that optimization of this glucose oxidation cascade could be achieved by increasing the amount of those rate limiting enzyme ratios in the multiple enzyme system or improving the specific activity of those rate limiting enzymes.

Conclusions

In this work, we demonstrated the use of mass spectrometric product analysis to evaluate a multi-enzyme biofuel cell system. A previously reported six-enzyme cascade for glucose oxidation was used as an example to calculate faradaic efficiency, Coulombic efficiency and product efficiency which give valuable information on the performance of the biofuel cell. Faradaic efficiency of 95% shows that little non-enzymatic electrochemical products are being formed at the electrode. A Coulombic efficiency of 63% and product efficiency of 45% was calculated with the example system. The product analysis results also gave in-depth information on the bottlenecks of the designed oxidation pathway. These measurements and calculations are very important for multiple enzyme biofuel cell systems and should be given more attention when designing an enzyme cascade for biofuel cells.

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