S. cerevisiae possesses numerous advantageous biological features, such as being robust, easily handled, mostly non-pathogenic and having high catalytic rates, etc., which can be considered as merits for being used as a promising biocatalyst in microbial fuel cells (MFCs) for electricity generation. Previous studies have developed efficient MFC configurations to convert metabolic electron shuttles, such as cytoplasmic NADH, into usable electric current. However, no studies have elucidated the maximum potential of S. cerevisiae for current output and the underlying metabolic pathways, resulting from the interaction of thousands of reactions inside the cell during MFC operation. To address these two key issues, this study used in silico metabolic engineering techniques, flux balance analysis (FBA), and flux variability analysis with target flux minimization (FATMIN), to model the metabolic perturbation of S. cerevisiae under the MFC energy extraction. The FBA results showed that, in the cytoplasmic NADH-dependent mediated electron transfer (MET) mode, S. cerevisiae had a potential to produce currents at up to 5.781 A/gDW for the anaerobic and 6.193 A/gDW for the aerobic environments. The FATMIN results showed that the aerobic and anaerobic metabolisms are resilient, relying on six and five contributing reactions respectively for high current production. Two reactions, catalyzed by glutamate dehydrogenase (NADH) (EC 1.4.1.3) and methylene tetrahydrofolate dehydrogenase (NADH) (EC 1.5.1.5), were shared in both current-production modes and contributed to over 80% of the identified maximum current outputs. It is also shown that the NADH regeneration was much less energy costly than biomass production rate. Taken together, our finding suggests that S. cerevisiae should receive more research effort for MFC electricity production.

**Keywords:** MFC, microbial fuel cell, *Saccharomyces cerevisiae*, bioelectricity, flux balance analysis, flux variability analysis, flux minimization, FATMIN

**Introduction**

*Saccharomyces cerevisiae* is associated with anthropic environments and is well known for food or beverage (alcoholic) fermentation.\(^1\) Besides these applications, this yeast species has been employed as an eukaryotic model organism in molecular and cell biology; for example, the characteristics of many proteins can be discovered by studying their homologs in *S. cerevisiae*.\(^1-4\) Recently, it has been shown that *S. cerevisiae* can be processed to produce potential advanced biofuels such as long chain alcohols and isoprenoid- and fatty acid-based biofuels, which have physical properties that more closely resemble petroleum-derived fuels.\(^5\) Nevertheless, biofuels need to be further combusted to produce usable energy in the form of electricity. To circumvent the disadvantages of biofuel combustion and directly convert the metabolic reducing potential inside the cell into electricity, another device, named a microbial fuel cell (MFC), has also been proposed. In a typical MFC configuration, microorganisms are grown in the anodic compartment and obtain electrons from breaking-down substrate during growth.\(^6\) The gained electrons can be harnessed and transported out of cells to an electrode via two main operational modes, mediated electron transfer (MET), and direct electron transfer (DET) modes.\(^6\) MET mode involves soluble redox mediators (such as bromocresol green [BG] and neutral red [NR])\(^6\) that act as electron relays repeatedly cycling between the metabolism of the cell and the electrode,\(^6\) whereas DET mode is the case where electrons are directly expelled by some microorganisms via membrane-associated proteins (e.g., cyt-type cytochrome).\(^6\) In either of these modes, the physiological effect of the immersion of a microbe in the anodic compartment is that an electron drain is introduced by the external environment. Because of the key role of redox carriers in most aspects of metabolism, operation of the metabolic network is fundamentally affected by this. The focus of this article is to model the resulting rebalancing of metabolic fluxes by using flux balance...
analyses (FBA). Further exposition of the working principle of an MFC can be found in several review articles.\textsuperscript{2,5,11} Both MET and DET modes can be used in MFCs based on \textit{S. cerevisiae}.\textsuperscript{11} In the DET mode, the yeast-based MFC could so far only produce an extremely low current output, at a scale of microamperes.\textsuperscript{12} In addition, it is unclear what mechanisms on the cell wall are responsible for DET current production.\textsuperscript{14} In the MET mode, mediators are required to facilitate the transfer of electrons to the anode, because this yeast is thought incapable of extracellular mediator synthesis.\textsuperscript{15} Because glycolysis occurs in the cytosol of the yeast cell rather than in the mitochondria, the electron shuttle, NADH, is easily accessible to a mediator molecule present in the cell membrane of the yeast and has been proposed as the main electron supplier in a yeast-based MFC.\textsuperscript{15,20} Based on these characteristics, MFCs using yeast can be directly applied in fermenters for in situ power generation.\textsuperscript{15} Other electron carriers (such as FADH\textsubscript{2}) may also contribute to current production, but these reduced molecules are regenerated by much fewer reactions than NADH, which is widely used in many reactions of the energy metabolism (e.g., glycolysis and Krebs cycles).

Since the biological entity, \textit{S. cerevisiae}, in the MFCs are considered as the fundamental part that determines the current and power output of MFCs and draws a distinction between MFC and other chemical fuel cells, it would be desirable to know the inherent restrictions and mechanisms of the yeast for current generation and to understand how the energy extraction perturbs the metabolic mechanisms of microorganisms in MFCs. Although some studies have discussed the metabolisms for supplying electrons in MFCs based on empirical knowledge of the textbook biochemical pathways or limited results arising from reductionist investigative methods, there is still an urgent demand for knowledge about the metabolic pathways resulting from the interaction of thousands of reactions inside the yeast cell during MFC operations. This task can be solved by in silico modeling of genome-scale metabolic network reconstructions (GENRE), using the recently developed constraint-based modeling approaches.\textsuperscript{17,18} However, these novel methods had not been used in MFC research area until recently.\textsuperscript{19,20}

In this study, to identify the maximum potential of \textit{S. cerevisiae} to sustain the energy extraction process in yeast-based MFCs, FBA, and multi-objective optimization\textsuperscript{21-24} was employed to investigate the metabolic capability of the yeast to supply an excess flux of cytosolic NADH to reduce the oxidized media tors (or anode), under two broad metabolic types, anaerobic and aerobic growth. A genome-scale metabolic model of \textit{S. cerevisiae} (yeast 5) was chosen as the backbone for the present flux balance modeling, since it has been tested extensively against experimental results for prediction of several growth conditions, including byproduct synthesis, in a previous study.\textsuperscript{25} Only minimal changes were made to the model for our study, to represent the diversion of redox carriers in a MFC. In addition, flux variability analysis with target flux minimization (FATMIN)\textsuperscript{26} was implemented to elucidate pertinent reactions underlying the maximum current output. Finally, robustness analysis was conducted to establish the feedstock costs for the cytosolic NADH-linked electricity generation in \textit{S. cerevisiae}.

Results and Discussion

Impact of the redox perturbation on the biomass production. Figure 1 shows how production of mediated electron transfer (MET) aimed at cytosolic NADH competes with biomass production for metabolic resources. MET influenced the production of the NADH and biomass in the same way for both anaerobic and aerobic conditions, that is, the increase in MET drove the NADH regeneration rates in the two growth modes toward their maximum attainable values, and the corresponding NADH flux consumed for cell maintenance and biomass formation rates toward zero. This indicates most of the energy originally for growth is converted to electricity.

At the base state optimized for growth, the total turnover rate of NADH was 19.74 mmol/gDW/h and 67.34 mmol/gDW/h for the anaerobic and aerobic condition respectively. Compared with the base states, the anaerobic and aerobic metabolisms had a potential to increase their NADH regeneration rates by about 5.469 (447%) and 1.736 (71.6%) folds respectively under the highly NADH-perturbed metabolic states, achieving the highest NADH_mfc flux values of about 108 mmol gDW\textsuperscript{−1} h\textsuperscript{−1} for the anaerobic growth and 115.6 mmol gDW\textsuperscript{−1} h\textsuperscript{−1} for the aerobic growth, which were achievable when biomass production rate was suppressed to 0.012 and 0.01284 h\textsuperscript{−1} in the anaerobic and aerobic modes respectively. This indicates that aerobic respiration can increase the maximum biomass production rate, but does not substantially influence the metabolic capability for NADH regeneration. At the same substrate consumption rate, the fermentation can regenerate NADH to a level that is sufficient to sustain similar electric current output as aerobic growth.

The MET production rates shown in the top parts of Figure 1 for the two growth cases, only show a linear dependence on biomass growth for small growth rates. Starting from the unperturbed state of zero MET production at the right hand side of the plots and based on the slope changes in the lines connecting two neighboring data points, the relationships between varying biomass production rate and the MET (NADH_mfc) rate can be divided into three stages (see Fig. 1). These are: an increase in MET without changing biomass growth (Stage I), a downward curving nonlinear rise of MET vs. growth rates (Stage II), and a linear dependence of the rise in MET on drop in biomass formation (Stage III). Stage III only appears in the left corner of the figures, where the line connecting the points clustered indicates there is a linear relationship between the biomass production and the current output.

The slope of the NADH production vs. biomass growth curve as in Figure 1 gives a direct measure of the efficiency of the underlying pathways. A large slope means that NADH production increases by a large amount for a given cost in terms of biomass production and is therefore highly efficient and vice versa. In the discussions below, we base interpretations of reallocations
reallocation of energy resources inside cells can elevate the slight increase in MET rate, which obviate the degradation of the intrinsic biological objective, to grow. However, rerouting the energy flow through different metabolic pathways could quell the net NADH production (MET) curve, the Stage I relationship indicates that solutions that are based on

Figure 1. The relationships of the biomass production rate and cytosolic NADH flux diverted toward anode (NADH_mfc). The NADH supplying rate in the MET mode and the reducing equivalent consumption rate for cellular use, as functions of biomass production rate. The red line represents the maximal NADH_mfc flux for a feasible biomass production rate, while any point within the pink area represents all allowable NADH_mfc and biomass production rates. The blue area represents the total cytosolic NADH consuming flux for normal cellular function. The distance between the two lines across the pink and blue areas represents the total available reducing equivalent flux in the cell at a metabolic state related to a specific biomass production rate; inset, enlargement of boxed area. Dashed line with arrowheads indicates which two data points are paired up. The points are simulated by bi-objective optimization involving varying coefficients (λ) assigned for the growth and MET maximizations. A more detailed discussion of the significance of the λ is given in the Methods section. Briefly, it is a parameter that continuously adjusts the metabolic state of the cell through a range stretching from pure growth without the extraction of current (at one extreme) to at state in which all metabolic resources are taken up by supplying electric current and no growth, at the other extreme.

of metabolic resources to pathways with different efficiencies on this evidence directly derived from the modeling results.

Looking at the net NADH production (MET) curve, the Stage I relationship indicates that solutions that are based on
some cellular functions, which, for example, are unnecessary in optimal growth conditions, but may be important to survival in adverse conditions. This also implies that maximization of growth rate is not the sole goal for eukaryote.

The Stage II indicates that the metabolic pathways have different efficiencies and capability in NADH regeneration. Some pathways possess higher efficiencies in converting the metabolic resource into NADH but lower maximum capability in turnover rate and some others vice versa. When the current output was reduced, the metabolic pathways with higher efficiencies were selected to minimize the metabolic resource cost in order to maximize growth rate. When the current output rose to a higher level, those pathways with lower efficiencies but higher upper bounds of the NADH flux had to be used instead.

The Stage III behavior begins at very low growth rate values (less than about 10% of the highest growth rates) in each of the two modes. The Stage III results from the fact that, only a certain set of pathways are able to sustain the very high current output and the flux through these pathways are linearly proportional to current output and biomass production rate.

Figure 1 also shows that a rising MET production rate reduce the cytosolic NADH consumption rate until it finally reaches zero, which indicates that no cytosolic NADH fluxes were consumed for cell maintenance. The curve plotted for the NADH consumption rate vs. the biomass production can be classified into three types: Type I is a reduction in the NADH consumption rate without changing the biomass production rate; Type II represents a decrease in the NADH consumed for maintenance, associated with a drop in biomass production rate; Type III is the phenomenon that (nearly) no cytosolic NADH flux was consumed for cell maintenance accompanied by a decline in the biomass production.

A correspondence can be found between the types of consumption behaviors and the stages displayed by the MET production curves. Stage I coincides with Type I, which is reflected by the observation that the increase in the MET is accompanied by a drop in the NADH consumption for cellular maintenance. This indicates that increasing NADH flux for internal use would be the primary mechanism implemented by the yeast to relieve the redox perturbation. The similar mechanism has also been seen for the NADH-targeted MET mode of C. reinhardtii. This suggests that excess cytosolic NADH may be a common occurrence in eukaryotic microorganisms.

The Stage II occurs with both Type II and III. This indicates that in Stage II, there is a continuous adjustment in the metabolic strategies underlying the MET current production. The reduction in NADH consumption for maintenance may contribute to the major portion of the elevated NADH flux diverted to the mediator in MFCs in the beginning (i.e., the metabolic status undergoing a relatively lower degree of the current-producing perturbation). As the NADH flux shunt further augments, the intracellular pathways that consume cytosolic NADH are disfavored by the metabolism. The cell opts for metabolic pathways that can excessively produce NADH flux for oxidized mediators, while trying to maximize biomass production rate. When entering Stage III of the MET, the cell redirects nearly all of the metabolic resource to NADH regeneration pathways that can supply a high flux of NADH.

Both Stage I and Type I behaviors occurred in the cases of C. reinhardtii and S. cerevisiae. The coincidence indicates that the multiplicity of eukaryotic functions make these two microorganisms tolerable to hostile situations, since it is originally reflected that there are more routes of energy flow underlying more physiological conditions. In addition, different from C. reinhardtii, where the shape of cytosolic NADH consumption rate changed in a complex manner, that of S. cerevisiae is much simpler. This discrepancy may be a result of the photosynthesis which contains a number of energy pathways providing metabolic fuel to the cell growth.

To further elucidate which one of the two growth conditions can better cope with the NADH-related perturbation, we performed fractional benefit analysis, which calculated a measure called the fractional benefit B as discussed in the methods section. Briefly, B is the sum of the fractions of maximal growth and NADH production respectively, achieved in a particular metabolic state. Figure 2 shows the result of this measure applied to the reported simulations. Starting from wild type growth rates (right hand side of the figures), an increase in B values can be seen for the Stage I and Type I behavior. The apex points of the B value curves correspond to the highest achievable combined benefit. The maximum B value for the anaerobic growth is a little bit higher than that for the aerobic growth. This suggests that, even though the aerobic growth has a potential to funnel more excess NADH for current production than the anaerobic condition, but the loss of NADH can cause more adverse effects on the aerobic than the anaerobic growth. Both growth conditions can yield a maximum B value of above 75%, which is much higher than those of all other three microorganisms (G. sulfurreducens, C. reinhardtii, and Synechocystis sp. PCC 6803) that we studied previously. This indicates that additional NADH perturbations would result in less hostile effects on S. cerevisiae than the other three microorganisms and thus S. cerevisiae has the greatest suitability for current production among the four.

The metabolic strategies supporting the high NADH regeneration rate in the anaerobic and aerobic modes are elucidated in the next section.

**Metabolic strategies for increasing flux of reducing equivalents.** A list of identified reactions (enzymes) that were responsible for the promoted diversion of the cytosolic NADH flux toward current production is summarized in Table 1. In the anaerobic fermentation, S. cerevisiae relied on six reactions to regenerate NADH from NAD⁺ at the high rate, subject to the stoichiometry and biological constraints applied. Two of six NADH production routes, namely glutamate dehydrogenase (NAD) (EC: 1.4.1.3) and methylene-tetrahydrofolate dehydrogenase (NAD) (EC: 1.5.1.5), were the main NADH suppliers. The two enzyme catalyzed reactions were capable of producing up to 77.48% (85.3 mmol/gDW/h) and 81.23% (95.37 mmol/gDW/h) respectively of the maximum net NADH supplying rate (105.1 mmol/gDW/h). Nevertheless, only the combined fluxes of these two reactions that made up any
percentages within a range of 99.4 to 103.8% of the maximum net NADH flux were viable. This indicates there are unlimited metabolic states that can achieve the FBA identified maximum net NADH shunt. The overflow (the percentage above 100%) can be off-set by the consuming NADH fluxes, for instance, the glutamate dehydrogenase (NAD) catalyzed reaction can be reversible and consume up to 3.948 (3.757%) of the maximum net NADH flux.

Another significant portion (22.47%) of the maximum net NADH flux was supplied by four reactions, catalyzed by alcohol dehydrogenase (ethanol to acetaldehyde) (EC: 1.1.1.1), formate dehydrogenase (EC: 1.2.1.2), glyceraldehyde-3-phosphate dehydrogenase (EC: 1.2.1.12), and phosphoglycerate dehydrogenase (EC: 1.2.1.2), respectively. These four reactions had rigid flux values, which accounted for 5.76%, 7.20%, 5.89%, and 3.62% of the maximum net NADH flux respectively. This indicates that they are essential to growth.

All other NADH producing reactions jointly contributed up to only 0.06% of the maximum net NADH flux. This suggests that, in order to achieve a surplus NADH flux of 105.1 mmol/gDW/h, nearly all of the metabolic resources need to be reallocated toward the four aforementioned reactions. In addition, the rigid variability (vi_min/vi_max > 0.99) of these reactions indicates that these reactions were much preferred for the biomass growth, rather than NADH-targeted current production.

Furthermore, it was found that the flux through the reaction catalyzed by alcohol dehydrogenase (EC: 1.1.1.1) merely had a flux of 6.049 mmol/gDW/h under the high degree of NADH perturbation. This is contrary to a previous reporting where alcohol dehydrogenase (EC: 1.1.1.90), and malate dehydrogenase (EC: 1.2.1.12), and phosphoglycerate dehydrogenase (EC: 1.2.1.2), glyceraldehyde-3-phosphate dehydrogenase (EC: 1.2.1.1), and methylene tetrahydrofolate dehydrogenase (NAD) (EC: 1.5.1.5), had a potential to solely supply up to 81.71% of the maximum net NADH flux. Each of the two reactions, identified to have a capability to replenish the loss of cytosolic NADH under high current output. Each of the two reactions, catalyzed by glutamate dehydrogenase (NAD) (EC: 1.5.1.5), were used by the two metabolic states that can achieve the FBA identified maximum net NADH flux. However, the presently computed metabolic flux model elucidate that the three pathways for NADH regeneration are not the metabolic pathways responsible for the excess NADH regeneration, subject to the stoichiometry and substrate uptake constraints. Instead, two reactions (EC: 1.4.1.3) in nitrogen metabolism and (EC: 1.5.1.5) in folate biosynthesis are the only two routes allowing a high NADH regeneration rate.

Aerobic growth. Under aerobic respiration, five reactions were identified to have a capability to replenish the loss of cytosolic NADH under high current output. Each of the two reactions, catalyzed by glutamate dehydrogenase (NAD) (EC: 1.4.1.3) and methylene tetrahydrofolate dehydrogenase (NAD) (EC: 1.5.1.5), had a potential to solely supply up to 81.71% of the maximum net NADH flux (113.31 mmol/gDW/h); and any combinations of the reaction flux amount to 81.71–81.75% of the maximum net NADH flux were attainable. About 18.06% of the maximum net NADH flux was supplied by alcohol dehydrogenase (EC: 1.1.1.90), glyceraldehyde-3-phosphate dehydrogenase (EC: 1.2.1.12), and malate dehydrogenase (EC: 1.1.1.85). These three reactions had fixed fluxes, implying their enzymatic activities were critical to cell growth.

Comparison of the identified reactions chosen by anaerobic and aerobic growths shows that three reactions, glutamate dehydrogenase (NAD) (EC: 1.4.1.3), glyceraldehyde-3-phosphate dehydrogenase (EC: 1.2.1.12), and methylene tetrahydrofolate dehydrogenase (NAD) (EC: 1.5.1.5), were used by the two modes to achieve a high NADH regeneration rate. This indicates that 87.70% of the maximum net NADH flux in the anaerobic mode and 85.50% of the maximum flux in the aerobic mode were generated through the same pathways.

To demonstrate how nutrient uptake is channeled to biomass growth and current yield respectively, Table 2 compares the corresponding fluxes at different glucose uptake rates.

Figure 2. The effect of varying biomass production on the fractional benefit. The fractional benefit B plotted on the vertical axis, is a measure of success in achieving the combined goals of maximal growth rate and MET flux. Maximizing one of these at a time, as at the endpoints, gives only B = 50%. The graphs show that relative to this, gains in MET flux can more than offset losses of growth rate in S. cerevisiae metabolism.

### Table 2

| Glucose Uptake Rate (g DW/h) | Fractional Benefit for Biomass Production | Fractional Benefit for Current Production |
|----------------------------|------------------------------------------|------------------------------------------|
| 0.01                       | 60%                                      | 40%                                      |
| 0.1                        | 50%                                      | 50%                                      |
| 0.2                        | 40%                                      | 60%                                      |
| 0.3                        | 30%                                      | 70%                                      |

Effect of varying glucose uptake rate on predicted biomass and NADH production rates. Table 2 summarizes the linear functions obtained for biomass and NADH_mfc production rates vs. glucose uptake rates. Column 3 shows that the optimal glucose-to-biomass efficiency was heavily suppressed under the high current output condition in both anaerobic and aerobic growth.
Comparison of columns 3 and 4 indicates that NADH regeneration is much less substrate-costly than the biomass production. Compared with anaerobic growth, the efficiency of conversion of glucose to biomass was 66.67% higher, but the efficiency for glucose to NADH was just 7.83% higher in the aerobic growth. This implies that the intake of oxygen can promote the biomass production but has fewer effects on NADH regeneration. Finally, the two operation modes are compared for their theoretically maximum current output in Figure 3 and Table 3. Comparison of amperage outputs: Overall, the maximum current output achieved for aerobic growth (6.19 A/gDW) was about 714% higher than for its anaerobic counterpart (5.781 A/ gDW). Since aerobic metabolism can more efficiently use substrate than the anaerobic, aerobic MFCs were expected to be capable of generating more current than the anaerobic one. This anticipation is in keeping with the present study’s prediction, which implies that the intake of oxygen can promote the biomass production but has fewer effects on NADH regeneration.

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Comparison of amperage outputs: Overall, the maximum current output achieved for aerobic growth (6.19 A/gDW) was about 714% higher than for its anaerobic counterpart (5.781 A/gDW). Since aerobic metabolism can more efficiently use substrate than the anaerobic, aerobic MFCs were expected to be capable of generating more current than the anaerobic one. This anticipation is in keeping with the present study’s prediction, which implies that the intake of oxygen can promote the biomass production but has fewer effects on NADH regeneration.
in the same study, a 0.006A/g can be calculated, which is still much lower than the presently computed value (5.714 A/g). The large discrepancy between the previously reported current per cell gram and the presently computed one may be attributed to the fact that (1) the cells were not in optimal metabolic states, (2) not every single cell participated in current output, (3) the mediator, i.e., methylene blue (MB), could not penetrate the cell wall and/or cytoplasmic membrane, and thus could not interact with the cytoplasmic metabolic activity efficiently, (4) and the engineering design might not efficiently collect all the electrons funneled away from the microorganism.

The eukaryotic respiratory chain has a complicated architecture which is advantageous to the cell energy balance. The cell membrane contains higher protein content, which corresponds to the higher rate of redox reactions between cytoplasm and inter-membranous space. This feature has been proposed to facilitate S. cerevisiae attached to the anode to convey electrons to the electric circuit without aids of artificial mediators in an MFC. The intracellular redox mediators (i.e., NAD+/NADH and FAD+/FADH) were suggested to sustain a current output of up to 282.85 mA/m². However, without knowledge of the cell density on the anode of the MFC, it is impossible convert the unit (mA/m²) of the reported current density into a unit of cell density on the anode of the MFC, it is impossible convert the unit (mA/m²) of the reported current density into a unit of amperage per gram dry weight cell, and therefore we are unable to compare the current output of the yeast cell in this particular MFC to the theoretical maximum value computed here.

Another more recent study investigated the possibility of S. cerevisiae to transfer electrons to an extracellular electron acceptor through DET mode and found that the cells adhered to the anode were able to sustain power generation in a media-tor-less MFC configuration. It is proposed that the surface con-fined species are responsible for the DET, however, the power performance of this MFC was extremely low (0.003 W m⁻² and 0.03 A/m²). With a reported cell sample weight of 0.13 g and the anode surface area of 4 cm², the current density can be trans-lated into 0.000009231A/g. Since the DET mode is based on surface confined species and it is unclear what those species are, identification of the protein involved in such a DET mode is required, before the in silico method presented in this study can be employed to reveal the innate capability of the DET mode based on S. cerevisiae.

Conclusions

In this study, the anaerobic and aerobic growth modes of S. cerevisiae were modeled to study its maximum potential for MFC current output and underlying metabolic metabolisms. It was shown that a similar level of maximum current outputs can be achieved for the two growth modes (i.e., 5.781 A/gDW for the anaerobic growth and 6.193 A/gDW for the aerobic growth), whereas under aerobic conditions the yeast achieved nearly twice the anaerobic growth rate. To achieve the highest efficiency of NADH regeneration and maximize the current output, the aerobic and anaerobic metabolisms relied on two reactions, catalyzed by glutamate dehydrogenase (NADH) (EC 1.4.1.3) and methylene tetrahydrofolate dehydrogenase (NADH) (EC 1.5.1.5), for NADH production. Besides, the robustness analysis indicated that the NADH regeneration was much less energy costly than the biomass production rate. Taken together, our findings suggest that S. cerevisiae should be re-evaluated and receive more research effort for MFC electricity generation.

Materials and Methods

General modeling assumptions. The modeling was conducted based on the three assumed conditions: (1) The MFC reactor is a chemostat that can provide all the necessary nutrients to maintain the optimal microbial growth; (2) Only a pure S. cerevisiae culture is used for electricity generation; (3) A putative ideal mediator (e.g., Neutral red) can be found to enter the cytoplasm, intercept the electrons from NADH-involved metabolic activity and convey the electrons to the anode; and (4) The uptake rate of the organic substrate (i.e., glucose) is allowed to vary but is constrained to a realistic range to reflect the observed uptake rates for optimal growth.

Modeling electrode interactions. The interactions with an electrode were captured by introducing two reactions into the model reconstruction (Table 4). These reactions represent the net reaction between the reducing equivalents and the electrodes in the MFC.

The two added reactions reflect a well-established understanding that mediators in an MFC enter the cell and interact with cytosolic NADH in the metabolism to extract energy (in the form of electrons) from these electron shuttles, as schematically shown in Figure 4. Because NADH is a major electron carrier in microbial metabolism connecting many different cellular processes, known as a "hub" in terms of network theory, the effects of the NADH drain that the added reactions creates, propagates throughout the metabolic network. In this way, the modeling can estimate the maximum power achievable for a microorganism in
maximization of the production of an overflow flux of desirable reducing equivalent, cytoplasmic NADH, so as to restore the redox balance perturbed by the energy extraction process during MFC operation. The bi-level objective optimization can result in a set of Pareto-optimal points, forming a Pareto front, which describes the trade-off relationship between the growth rate of the organism and the regeneration rate of a plethora NADH flux. Through varying the relative weights associated with each objective, the impact of the enhanced energy extraction on the growth of the cell can be evaluated. The bi-objective equation in this study is defined as follows in terms of the biomass growth flux \( F_B \) and the reducing equivalent diversion flux \( F_N \):

\[
O = (1 - \lambda) F_B + \lambda F_N
\]

A unit conversion factor \( g \) is explicitly provided because in the model, \( F_N \) and \( F_B \) are measured respectively in units (mmol/gDW/h or g/gDW/h or [h⁻¹]). This ensures that \( \lambda \) is a dimensionless fraction that can be directly interpreted as the relative contribution of the NADH flux to the combined objective.

Fractional benefit analysis. The bi-objective optimization conducted in this study can assess how metabolic energy loss during current production influences the biomass growth. However, the mathematical optimization method based on such a bi-objective function cannot clarify the combined benefit resulting from an optimized metabolic state. Therefore, this requires implementation of fractional benefit analysis to mathematically quantify either extreme on the Pareto front; even though only one of the

![Figure 4. A schematic of the modeled anodic mechanisms where the cytoplasmic NAD+/NADH cycle as the electron supplier. Microbes take up substrates (glucose) generating carbon dioxide and proton. This process yield electrons for metabolic benefit, i.e., growth, and reduces Med_\( \text{oxy} \) in the cytosol into Med_\( \text{red} \), which diffuses into contact with the electrode, where Med_\( \text{red} \) reduces the electrode generating electrical current. The oxidized form, Med_\( \text{oxy} \), diffuses back through the anolyte for reuse by the microbes. The radial red circle highlights the redox cycle supplying the electron to the anode, which is investigated in the present modeling.](image)

Table 2. The summary of linear functions of the biomass production and MET rates in the heterotrophic, photoautotrophic, and mixotrophic modes

| Metabolic type | Substrate | Growth \( |\text{gDW/gDW/h}| \) | MET \( |\text{gDW/gDW/h}| \) (mmol/gDW/h) | \( y_c \) | \( y_m \) |
|---------------|-----------|----------------|-------------------------------|------|------|
| Anaerobic     | glucose   | \( y = 0.0261x \) | \( y_m = 6.971x \) | 0.0261 | 6.971 |
| Aerobic       | glucose   | \( y = 0.0035x \) | \( y_m = 7.518x \) | 0.0035 | 7.518 |

Note: The FBA simulations were performed by changing the photon uptake rate and the glucose uptake rate with the maximization of the objectives. \( y \) denotes the MET rate (mmol/gDW/h or gDW/gDW/h) or growth rate (gDW/gDW/h), whereas \( x \) represents the substrate (i.e., glucose) uptake rates (mmol/gDW/h). \( y_c \) represents the optimal growth, whereas \( y_m \) denotes the perturbed growth under cytosolic NADH deprivation.

Table 3. Comparison of predicted amperage output under theoretical maximum current output condition and 5% of optimal growth rate condition

| Mode          | Biomass production rate (gDW/gDW/h) | Electron (mmol gDW⁻¹ h⁻¹) | Amperage (A gDW⁻¹) | Coulombic efficiency (%) | W gDW⁻¹ |
|---------------|----------------------------------|--------------------------|------------------|--------------------------|---------|
| Anaerobic     | 0.01303                          | 215.7                    | 5.781            | 89.87%                   | 4.798   |
| Aerobic       | 0.01303                          | 231.1                    | 6.193            | 96.29%                   | 5.141   |

MFC, as well as elucidate the response of energy metabolism to the perturbation induced by electricity generation.

Objective equation. The objective equation was formulated as described in our recent studies. Briefly, we constructed a bi-objective equation that comprises two objectives: one is the maximization of the growth rate, which reflects the belief that microbes evolve to optimize their metabolisms for maximizing biomass production (growth rate) and has been experimentally validated by numerous studies; the other is the maximization of the production of an overflow flux of desirable reducing equivalent, cytoplasmic NADH, so as to restore the redox balance perturbed by the energy extraction process during MFC operation. The bi-level objective optimization can result in a set of Pareto-optimal points, forming a Pareto front, which describes the trade-off relationship between the growth rate of the organism and the regeneration rate of a plethora NADH flux. Through varying the relative weights associated with each objective, the impact of the enhanced energy extraction on the growth of the cell can be evaluated. The bi-objective equation in this study is defined as follows in terms of the biomass growth flux \( F_B \) and the reducing equivalent diversion flux \( F_N \):

\[
O = (1 - \lambda) F_B + \lambda F_N
\]

A unit conversion factor \( g \) is explicitly provided because in the model, \( F_N \) and \( F_B \) are measured respectively in units (mmol/gDW/h or g/gDW/h or [h⁻¹]). This ensures that \( \lambda \) is a dimensionless fraction that can be directly interpreted as the relative contribution of the NADH flux to the combined objective.
objective terms is maximized, the other term also has a value and will contribute (positively or negatively) to the overall benefit. In the fractional benefit analysis, each of $F_a$ and $F_e$ is scaled as a fraction of the maximal values $F_a^*$ and $F_e^*$ they can ever achieve, i.e., the values when each is independently maximized. As the maximum of each of these fractions is 1, a plausible measure of the combined benefit achieved in any particular metabolic state is the value of the quantity we denote as the fractional benefit $B$:

$$B = \frac{1}{2} \left( \frac{F_a}{F_a^*} + \frac{F_e}{F_e^*} \right)$$

A value of $B$ in excess of 50%, indicates that gains in one objective more than compensates for losses in the competing objective.

Conversion of units of flux and current. Current (in amperes) was integrated over time and converted to electrons recovered by using the following conversions: 1 C = 1 A × 1 s, 1 C = 6.24 × 10^18 electrons, and 1 mol = 6.02 × 10^23 electrons (the Faraday constant converted into A/g as follows:

$$96485 \text{ C/mol}$$

was integrated over time and converted to electrons recovered by objective.

Due to various potential losses associated with MFC operation, such as ohmic resistances, concentration polarization and kinetic constraints. These are not taken into account in the calculations reported here.

The MFC standard cell potential calculated as above and shown in (Table 6) shows that as long as the same electron donors are used, the choice of biocatalyst will have little effect on the cell potential.

Calculation of the viable growth rate. To obtain a realistic upper value for the current that can be extracted from a microbe, we chose 5% of the maximum theoretical biomass production rate as the minimum viable growth rate. This percentage value is more likely to be achievable in practice, when compared with 1% assumed as the viability threshold for computational identification of the lowest growth rate in several previous studies.

**Table 4.** The added reactions for modeling the interaction of the microorganism and the electrode in MFCs

| Operation mode of the MFC | Reaction ID | Reaction |
|---------------------------|------------|---------|
| MET                       | 1nadh_mfc  | nadh → nadh_mfc |
|                           | 2nadh_mfc  | nadh_mfc → nadh + h_amm |
| h_amm: the H ions as the by-product released from the reaction 2nadhmfc |

**Table 5.** The standard potential of the redox reactions involving the electron donor and acceptor for the MFC (measured at pH 7).

| Redox couple | $E^\text{cell}$ (V) |
|--------------|---------------------|
| Anode        | MET NAD$^+$ + H$^+$ → NADH | −0.32 |
| Cathode      | O$_2$ + 4H$^+$ → 2H$_2$O | +0.83 |

**Table 6.** The theoretical limit of standard anode potentials of MFC based on S. cerevisiae

| Electron transfer mode | $E^\text{cathode}$ | $E^\text{anode}$ | $\Delta E^\text{cell}$ |
|------------------------|---------------------|-------------------|------------------------|
| MET                    | −0.32               | 0.51              | 0.83                   |

**Table 7.** The added reactions for modeling the interaction of the microorganism and the electrode in MFCs.

| Reaction ID | Reaction |
|------------|---------|
| 1nadh_mfc  | nadh → nadh_mfc |
| 2nadh_mfc  | nadh_mfc → nadh + h_amm |
| h_amm: the H ions as the by-product released from the reaction 2nadhmfc |

**Table 8.** The added reactions for modeling the interaction of the microorganism and the electrode in MFCs.

| Reaction ID | Reaction |
|------------|---------|
| 1nadh_mfc  | nadh → nadh_mfc |
| 2nadh_mfc  | nadh_mfc → nadh + h_amm |
| h_amm: the H ions as the by-product released from the reaction 2nadhmfc |

**Table 9.** The standard potential of the redox reactions involving the electron donor and acceptor for the MFC (measured at pH 7).

| Redox couple | $E^\text{cell}$ (V) |
|--------------|---------------------|
| Anode        | MET NAD$^+$ + H$^+$ → NADH | −0.32 |
| Cathode      | O$_2$ + 4H$^+$ → 2H$_2$O | +0.83 |

**Table 10.** The theoretical limit of standard anode potentials of MFC based on S. cerevisiae

| Electron transfer mode | $E^\text{cathode}$ | $E^\text{anode}$ | $\Delta E^\text{cell}$ |
|------------------------|---------------------|-------------------|------------------------|
| MET                    | −0.32               | 0.51              | 0.83                   |

All simulations were performed on glucose as the sole carbon source. The exchange of ammonium, protons, iron(2$^+$), phosphate, potassium, sodium, sulfate, and water were all unconstrained. The maximum rate of the glucose uptake is set to 0.0268 A/g. Based on stoichiometric information, 1 mol of glucose supplies 24 mol of electrons. The theoretical power outputs of the three tested modes. An upper limit for the cell voltage is calculated in this work based on formal potentials of the biological and electro-chemical redox processes, as given by:

$$\Delta E^\text{cell} = E^\text{cathode} - E^\text{anode}$$

Here, $\Delta E^\text{cell}$ is the standard cell potential (aka., electromotive force); $E^\text{cathode}$ is the standard potential of cathode oxidation, $E^\text{anode}$ is the standard potential of anode reduction. The formal potentials of the anode and cathode used for calculation of power density in the three operation modes are summarized in Table 5.

The real potential derived from the MFC will be reduced due to various potential losses associated with MFC operation, such as ohmic resistances, concentration polarization and kinetic effects.
that consume it) and minimize this collective flux while leaving the value of the objective unchanged. By this way, the flux in reactions that contribute both productive and futile flux components is limited to just the productive part. Because of the flux balance conditions, futile fluxes are eliminated also from other reactions that form part of the pathway.

**FATMIN** is a pipeline algorithm comprising FBA and flux variability analysis (FVA) and therefore can be performed with either COBRA or OptFlux according to users’ preference. The maximum reducing equivalent production rate for auxiliary growth rates between the data points were calculated using the interpolation function in Mathematica 8.0 (Wolfram Research, Inc.). All the FBA and FATMIN results are detailed in the Supplemental Tables.

**Disclosure of Potential Conflicts of Interest**
No potential conflict of interest was disclosed.

**Supplemental Materials**
Supplemental materials may be found here:
www.landesbioscience.com/journals/bioe/articles/26222

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