Thermodynamic Constraints on Electromicrobial Protein Production

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Global consumption of protein is projected to double by the middle of the 21st century. However, protein production is one of the most energy intensive and environmentally damaging parts of the food supply system today. Electromicrobial production technologies that combine renewable electricity and CO\textsubscript{2}-fixing microbial metabolism could dramatically increase the energy efficiency of commodity chemical production. Here we present a molecular-scale model that sets an upper limit on the performance of any organism performing electromicrobial protein production. We show that engineered microbes that fix CO\textsubscript{2} and N\textsubscript{2} using reducing equivalents produced by H\textsubscript{2}-oxidation or extracellular electron uptake could produce amino acids with energy inputs as low as 64 MJ kg\textsuperscript{−1}, approximately one order of magnitude higher than any previous estimate of the efficiency of electromicrobial protein production. This work provides a roadmap for development of engineered microbes that could significantly expand access to proteins produced with a low environmental footprint.

Keywords: electromicrobial production, electron uptake, hydrogen oxidation, nitrogen fixation, carbon fixation

INTRODUCTION

Current Methods of Protein Production Are Environmentally Damaging

Current food consumption and farming practices produce a large amount of environmental strain. In particular, the production of livestock for protein leads to significant waste accumulation and energy expenditure (McClements, 2019). The agricultural and food production sectors are responsible for \approx30\% of greenhouse gas emissions, while livestock farming alone accounts for 18\% of emissions (González et al., 2011). Furthermore, the agricultural industry is responsible for 70\% of total freshwater consumption (Heinke et al., 2020). 42\% of freshwater consumption is attributed to livestock production alone (Heinke et al., 2020). But, increased consumption of protein is one of the best ways to improve human, particularly infant, health and productivity in many parts of the world today (Ghosh et al., 2012).

The energy and water consumption of livestock farming will only increase as global appetites increase (Porritt and McCarthy, 2017). First, population will grow to \approx11 billion by 2050 (Proseklov and Ivanova, 2018). Second, the consumption of food, particularly protein, by each individual will also grow thanks to an expected average annual economic growth rate of 3\% from 2014 to 2050 (Tilman et al., 2011; Hawksworth and Chan, 2015). Supplying this increased demand while maintaining the current agricultural areal footprint is expected to require a 75\% increase in agricultural productivity (Proseklov and Ivanova, 2018).
Should agricultural production efficiencies remain stagnant, satisfying the food demands of the world’s growing and increasingly wealthy population with protein will require massive deforestation (Audsley et al., 2010; Tuomisto and Teixeira de Mattos, 2011). Deforestation could eradicate thousands of species and produce large quantities of greenhouse gases, leading to temperature increases exceeding the 2°C warming threshold established by the Paris Climate Agreement, even when ignoring emissions from all other human activity (Voeghele and Nelson, 2019).

Incremental improvements in current food production technologies may not meet future demand and sustainability goals. Current approaches to increasing protein production include advanced livestock breeding, and substitution of livestock protein for insect- and plant-based substitutes. However, all of these approaches depend upon increases in crop yields. But, 78% of the world’s land has natural limitations for agricultural development (Prosokov and Ivanova, 2018), and significant doubts remain about the possibility of increasing crop yields by mid-century (Tilman et al., 2011; Slade et al., 2014; Poore and Nemecek, 2018). Furthermore, increasing water scarcity due to climate change could even depress crop yields in the decades ahead (Slade et al., 2014).

**Autotrophic Metabolism Could Increase the Efficiency of Protein Production**

Autotrophic microbial production of protein is a promising alternative strategy to conventional food production (Ritala et al., 2017; Sillman et al., 2019; Mishra et al., 2020; Leger et al., 2021). In this class of schemes, externally supplied reducing equivalents are used to power microbial N₂ and CO₂-fixing metabolism and synthesis of protein molecules (Gleizer et al., 2020; Hu et al., 2020).

In most systems studied to date, reducing equivalents are supplied by H₂ or methane-oxidation. CO₂ fixation is performed by Calvin-Benson-Bassham cycle, the reverse Krebs cycle or the Wood-Ljungdahl pathway. Autotrophically produced protein has at least two important advantages over traditional protein production methods. Secondly, autotrophic protein production does not depend on the availability of arable land and can be run in a closed system. This greatly reduces water and land consumption and inhibits nitrogen runoff to surrounding environments (Sillman et al., 2019; Nyyssölä et al., 2021). Finally, autotrophic microorganisms can use atmospheric N₂ as a substrate, eliminating the need for thermochemical N₂-fixation (Bothe et al., 2010).

The cost of autotrophic protein production is dropping rapidly. The cost of production of a single protein has reduced from $1 \times 10^6$ kg⁻¹ in 2000 to $\approx 100$ kg⁻¹ in 2019 (Tubb and Seba, 2021). It is projected that the cost of production of a single protein could drop to below $10$ kg⁻¹ by 2025, thereby achieving price parity with animal-based protein products (Tubb and Seba, 2021).

Theoretical analysis suggests that autotrophic protein production could far exceed the efficiency of plant-based protein. Recent analyses of the performance of electromicrobial production of biofuels (Claassens et al., 2019; Salimijazi et al., 2019; Salimijazi et al., 2020), where electrically-supplied reducing equivalents are used to power CO₂ fixation or formic acid assimilation and biofuel, show that these types of schemes could dramatically exceed the efficiency of photosynthetic biofuel production. These results imply that if N₂ fixation were added to these systems, proteins could also be produced at efficiencies exceeding that of photosynthesis. Recent results by Leger et al. (2021) suggest photovoltaic-driven EMP of protein could exceed efficiency of real-world photosynthetic production of protein by at least 2 orders of magnitude.

However, up until now, very few attempts have been made at calculating the upper limit efficiency of EMP amino acid or protein production. This paper presents a model and analyzes the theoretical maximum energetic efficiency for a system of autotrophic microorganisms, fixing CO₂ and N₂ using electrons delivered by either extracellular electron uptake (EEU) (Rowe et al., 2021) or by H₂-oxidation (Liu et al., 2016). These calculations do not predict the performance of any naturally-occurring organism, but do predict an upper limit efficiency for any natural or synthetic organism using these reactions.

**THEORY, RESULTS AND DISCUSSION**

**Theory**

We extended our theoretical framework for calculating the efficiency of electromicrobial production (EMP) of biofuels to calculate the efficiency of amino acid production from electrons, CO₂ and N₂ (Salimijazi et al., 2020). A full set of model parameters and associated values used in this article are shown in Table 1, and a full set of symbols for this article are shown in Supplementary Table S1.

We consider a bio-electrochemical system used to deliver electrons to microbial metabolism (Figures 1A,B). Electrical power is used to generate amino acid (or protein) molecules with an energy per molecule $F_{\text{protein}}$, at a rate $N_{\text{protein}}$. Even though this article strictly considers amino acid synthesis, this can be considered equivalent to protein production from an energetic standpoint as no energy is expended in forming the peptide bond needed to polymerize amino acids. We choose to use the subscript protein rather than AA to avoid confusion with the Avogadro constant, $N_A$. Energy per molecule and molecular weight for each amino acid are shown in Supplementary Table S2. Full derivations of the equations presented here can be found in the supplement to our original electromicrobial production efficiency theory article (Salimijazi et al., 2020), with some changes of symbols used to indicate that we are producing proteins rather than amino acids. If a change of symbol is used, it is indicated in Supplementary Table S1. In our original article (Salimijazi et al., 2020) we focused purely on electrical (or solar) energy to chemical energy (fuel, or on this case protein) conversion.
**TABLE 1** | Electromicrobial protein production model parameters. Model parameters used in this article are based upon model parameters used in a previous analysis of the electromicrobial production of the biofuel butanol (Salimijazi et al., 2020). A sensitivity analysis was performed for all key parameters in this work (Salimijazi et al., 2020). A complete list of symbols used in this work (including symbols for outputs, and intermediate variables) is included in Supplementary Table S1.

| Parameter | Symbol | 1. H<sub>2</sub> with formate | 2. EEU with formate | 3. H<sub>2</sub> with formate | 4. EEU with formate |
|-----------|--------|-----------------------------|---------------------|-----------------------------|---------------------|
| **Electrochemical Cell Parameters** | | | | | |
| Input solar power (W) | P<sub>γ</sub> | 1,000 | 1,000 | 1,000 | 1,000 |
| Total available electrical power (W) | P<sub>e, total</sub> | 330 | 330 | 330 | 330 |
| CO<sub>2</sub>-fixation method | Enzymatic | Electrochemical | | |
| Electrode to microbe mediator | H<sub>2</sub> | EEU | H<sub>2</sub> | EEU |
| Cell 1 anode std. potential (V) | U<sub>cell 1, anode, 0</sub> | N/A | 0.82 | | |
| Cell 1 anode bias voltage (V) | U<sub>cell 1, anode, bias</sub> | N/A | 0.47 | | |
| Cell 1 cathode std. potential (V) | U<sub>cell 1, cathode, 0</sub> | N/A | -0.43 | | |
| Cell 1 cathode bias voltage (V) | U<sub>cell 1, cathode, bias</sub> | N/A | -1.3 | | |
| Cell 1 cathode voltage (V) | U<sub>cell 1, cathode</sub> | N/A | -1.73 | | |
| Cell 1 voltage (V) | U<sub>cell 1</sub> | N/A | 3.02 | | |
| Cell 1 Faradaic efficiency | ξ<sub>I1</sub> | N/A | 0.8 | | |
| Carbons per primary fixation product | Cr | N/A | 1 | | |
| e<sup>-</sup> per primary fixation product | N/A | N/A | 2 | | |
| **Cellular Electron Transport Parameters** | | | | | |
| Membrane potential difference (mV) | ΔU<sub>membrane</sub> | 140 | 140 | | |
| Terminal e<sup>-</sup> acceptor potential (V) | U<sub>Acceptor</sub> | 0.82 | | | |
| Quinone potential (V) | U<sub>Q</sub> | -0.0885 | -0.0885 | | |
| Mtr EET complex potential (V) | U<sub>Mtr</sub> | N/A | -0.1 | | |
| No. protons pumped per e<sup>-</sup> | p<sub>out</sub> | Unlimited | Unlimited | | |
| **Product Synthesis Parameters** | | | | | |
| No. ATPs for product synthesis | | | | See Supplementary Dataset S2 |
| No. NAD(P)H for product synthesis | | | See Supplementary Dataset S2 |
| No. Fd red for product synthesis | | | See Supplementary Dataset S2 |
| Product energy density (J molecule<sup>-1</sup>) | E<sub>protein</sub> | | | See Supplementary Table S2 |

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efficiency, but in this article we expand our theory to calculate the energy (electrical or solar) costs of producing a gram of product ($C_{EP}$ and $C_{SP}$, respectively).

The energy conversion efficiency of the system from electricity to amino acids (or protein) is calculated from the ratio of the amount of chemical energy stored per second ($N_{protein} E_{protein}$), relative to the power input to the system, $P_{e,total}$ (Salimijazi et al., 2020) (basically power out to power in),

$$\eta_{EP} = \frac{N_{protein} E_{protein}}{P_{e,total}}$$

(1)

The total mass of protein produced per second by the system is,
\[
m_{\text{protein}} = N_{\text{protein}} M_{\text{protein}} / N_A.
\]

where \(M_{\text{protein}}\) is the molecular weight of the protein molecule.

The energy cost to produce a unit mass of protein,

\[
C_{EP} = P_e \text{ total} N_A / (m_{\text{protein}}),
\]

Thus, if both the chemical energy per protein molecule and the molecular weight are known (they are for proteins), energy conversion efficiency and energy cost can be easily interconverted,

\[
C_{EP} = N_A E_{\text{protein}} / (\eta_{EP} M_{\text{protein}}),
\]

\[
\text{Thus,}

C_{EP} = P_e \text{ total} N_A / (N_{\text{protein}} M_{\text{protein}}),
\]

For a single bio-electrochemical cell system where CO\textsubscript{2} and N\textsubscript{2} fixation are performed \textit{in vivo} (Figure 1A), the upper limit electrical to chemical conversion efficiency of the system is set by the energy density of an amino acid molecule relative to the amount of charge needed to synthesize it from CO\textsubscript{2} and N\textsubscript{2} (the fundamental charge, \(e\), multiplied by the number of electrons needed for synthesis, \(\nu_{ep}\)) and the potential difference across the bio-electrochemical cell, \(\Delta U_{\text{cell}}\).

\[
\eta_{EP} \leq E_{\text{protein}} / (\nu_{ep} \Delta U_{\text{cell}}).
\]

Thus, the amount of electricity needed to produce a unit-mass of the protein is,

\[
C_{EP} \geq N_A \Delta U_{\text{cell}} \nu_{ep} / M_{\text{protein}}.
\]

A full derivation of Eqs 1, 6 in this article can be found in Section 1 (Eqs 1–9) in the supplement of Salimijazi et al. (2020).

We also consider systems CO\textsubscript{2} reduction is performed electrochemically, and the resulting reduction product (typically a C\textsubscript{1} compound like formic acid) (White et al., 2014; White et al., 2015; Appel et al., 2013) is further reduced enzymatically (Figure 1B). While a C\textsubscript{1} compound like formic acid is lightly reduced (e.g., \(2e^-\) per carbon for formic acid), carbohydrates, biofuels, a protein molecules are more heavily reduced (4–6 \(e^-\) per carbon). Thus, while formic acid can supply all of the carbon and some of the electrons needed to make a protein, it cannot supply all of the electrons, and these need to be supplied by EEU or H\textsubscript{2} oxidation. In these cases, \(\nu_{ep}\) is substituted for the number of additional electrons needed to convert the C\textsubscript{1} product into the final protein product, \(\nu_{e,add}\) (Salimijazi et al., 2020),

\[
\eta_{EP} \leq \frac{E_{\text{protein}} \xi_{12}}{\nu_{e,add} \left( \Delta U_{\text{cell}} \left( \frac{\nu_{e,add} \xi_{12}}{\nu_{e,add} + \Delta U_{\text{cell}}} \right) + \Delta U_{\text{cell}} \right)},
\]

where \(\nu_{ep}\) is the number of primary reduction products (i.e., formic acid molecules) needed to synthesize a molecule of the final product, \(\nu_{e,add}\) is the number of electrons needed to reduce CO\textsubscript{2} to a primary reduction product (i.e., 2 in the case of formic acid), \(\nu_{c,1}\) is the number of carbon atoms per primary fixation product (i.e., 1 in the case of formic acid), \(\xi_{12}\) is the Faradaic efficiency of the bio-electrochemical cell, \(\xi_{11}\) is the Faradaic efficiency of the primary abiotic cell, \(\xi_{c}\) is the carbon transfer efficiency from cell 1 to cell 2. A full derivation of Eq. 8 can be found in Section 10 (Equations 101–118) of the supplement of Salimijazi et al. (2020).

Thus, using Eq. 4, the amount of electricity needed to produce a unit-mass of the protein when using electrochemical CO\textsubscript{2} reduction is,

\[
C_{EP} \geq \frac{\eta_{EP} N_A \left( \Delta U_{\text{cell}} \left( \frac{\nu_{e,add} \xi_{12}}{\nu_{e,add} + \Delta U_{\text{cell}}} \right) + \Delta U_{\text{cell}} \right)}{M_{\text{protein}} \xi_{12}}.
\]

We calculate the electron requirements, \(\nu_{ep}\) or \(\nu_{e,add}\), for amino acid (or protein) synthesis from the number of NAD(P)H (\(\nu_{p,NADH}\)), reduced Ferredoxin (\(\nu_{p,Fd}\)) and ATP (\(\nu_{p,ATP}\)) molecules needed for the synthesis of the molecule, along with a model of the mechanism used for electron delivery to the microbe (Salimijazi et al., 2020).

The key part of our electromicrobial production efficiency theory (Salimijazi et al., 2020) answers the question: how efficiently can energy carried by H\textsubscript{2} or by EEU be transferred into the intracellular reductants needed for metabolism (ATP, NAD(P)H, and Ferredoxin) by use of the inner membrane proton gradient. In the case of both H\textsubscript{2}-oxidation (autotrophic growth of \textit{Ralsstonia eutropha}, the organism used in the Bionic Leaf (Liu et al., 2016), typically uses an atmospheric ratio of 8:1:1 H\textsubscript{2}:O\textsubscript{2}:CO\textsubscript{2} (Brigham et al., 2013)) and EEU (Rowe et al., 2018; Salimijazi et al., 2020; Rowe et al., 2021) mediated electromicrobial production, a micro-aerobic atmosphere needs to be maintained in the cathode chamber. The O\textsubscript{2} concentration in the cathode chamber needs to be just high enough to provide a terminal electron acceptor capable of generating the most proton motive force per electron input into the system, yet low enough to not be reduced by the cathode to H\textsubscript{2}O and short-circuit the electrochemical system. It is notable that both the anode and cathode in the Bionic Leaf exist in the same reaction chamber suggesting that a small amount of O\textsubscript{2} is constantly present. Despite this, the energy efficiency (and by extension Faradaic efficiency) is remarkably high (Liu et al., 2016) (RCv2_1.01).

For systems that rely upon H\textsubscript{2}-oxidation for electron delivery like the Bionic Leaf (Torella et al., 2015; Liu et al., 2016; Salimijazi et al., 2020) (Figure 1C, part 1), the number of electrons needed to synthesize one amino acid molecule is,

\[
\nu_{ep,H_2} = 2\nu_{p,NADH} + 2\nu_{p,Fd} + \nu_{p,ATP} \left( \frac{\Delta G_{\text{ATP}/\text{ADP}} \left( \frac{e \Delta U_{\text{membrane}}}{\text{floor} \left( \frac{U_{H_2} - U_{\text{acceptor}}}{\Delta U_{\text{membrane}}} \right)} \right)}{\xi_{12}} \right),
\]

where \(\Delta G_{\text{ATP}/\text{ADP}}\) is the free energy required for regeneration of ATP, \(\Delta U_{\text{membrane}}\) is the potential difference across the cell’s inner membrane due to the proton gradient, \(U_{H_2}\) is the standard potential of proton reduction to H\textsubscript{2}, \(U_{\text{acceptor}}\) is the standard potential of terminal electron acceptor reduction (typically O\textsubscript{2} + \(2e^-\) to H\textsubscript{2}O), the ceil function rounds up the nearest integer, and the floor function rounds down to the nearest integer. A full derivation of Eq. (10) can be found in Section 2 (Equations 10 to 20) of the supplement for Salimijazi et al. (2020).

The first and second terms in Eq. 10 describe the number of electrons needed to regenerate the NAD(P)H and Ferredoxin.
needed for amino acid synthesis. As the redox potential of H₂ is above those of both NADH and Ferredoxin and both molecules require two electrons to be regenerated, two electrons can be transferred directly from H₂-oxidation. Thus, the number of electrons needed for NAD(P)H regeneration is just double the number of NAD(P)H and Ferredoxin needed for synthesis of the amino acid.

The final term in Eq. 10 calculates the number of electrons needed to regenerate the ATP needed for amino acid synthesis. ATP regeneration involves energy transfer from the incoming electrons to ATP, and charge transfer to O₂. The numerator in the final term of Eq. 10 calculates the number of protons that need to be pumped through the ATP synthase in order to regenerate 1 ATP: the energy needed to regenerate 1 ATP divided by the energy recovered by pumping one proton from the periplasmic side of the inner membrane to the cytoplasmic side. As only integral numbers of protons can be pumped, the ceil function rounds up the result. The denominator in the final term of Eq. 10 calculates how many protons can be pumped from the cytoplasmic side of the membrane to the by sending 1 electron downhill from H₂ to the acceptor (O₂/H₂O). Again, as only integral numbers of protons can be pumped, the floor function rounds down.

The appearance of ΔU_membrane in the numerator and denominator of Eq. 10 is required because the cell and floor functions are numerical (not analytical) and require their arguments to be numerically evaluated before the result can be used in a larger calculation. This is initially counter-intuitive, but captures the core of the unavoidable energy losses imposed by using proton pumping to transduce energy. To illustrate this, consider this example: the result of 7/2 divided by 5/2 is just 7/5 or 1.4 (the twos in the denominators of both terms cancel). However, the result of ceil (7/2) divided by floor (5/2) is different: ceil (7/2) is 4, while floor (5/2) is 2. Thus ceil (7/2) divided by floor (5/2) is 2.0, 43% higher than the result of 7/5.

The inner membrane potential difference, ΔU_membrane, is the largest source of uncertainty in this calculation. Therefore, we present a range of efficiency estimates in Figures 2, 3 and throughout
the text for $\Delta U_{\text{membrane}} = 80 \text{ mV}$ (BioNumber ID (BNID) 10408284 (Milo et al., 2010)) to 270 mV (BNID 107135), with a central value of 140 mV (BNIDs 109774, 103386, and 109775).

For systems that rely upon EEU for electron delivery like *Shewanella oneidensis* (Salimijazi et al., 2020; Rowe et al., 2021) (Figure 1C, part 2),

$$y_{\text{ep,EEU}} = 2y_{p,NADH} + 2y_{p,Fd}$$

$$+ y_{p,\text{ATP}} \frac{\text{cell}(\Delta G_{\text{ATP/ADP}}/c\Delta U_{\text{membrane}})}{\text{floor}((U_Q - U_{\text{acceptor}})/\Delta U_{\text{membrane}})}$$

$$+ y_{p,NADH} \frac{\text{cell}((U_{\text{NADH}} - U_Q)/\Delta U_{\text{membrane}})}{\text{floor}((U_Q - U_{\text{acceptor}})/\Delta U_{\text{membrane}})}$$

$$+ y_{p,Fd} \frac{\text{cell}((U_{\text{Fd}} - U_Q)/\Delta U_{\text{membrane}})}{\text{floor}((U_Q - U_{\text{acceptor}})/\Delta U_{\text{membrane}})}$$  \quad (11)$$

where $U_Q$ is the redox potential of the inner membrane electron carrier, thought to be ubiquinone (Rowe et al., 2018), $U_{\text{NADH}}$ is the standard potential of NAD(P)H, and $U_{\text{Fd}}$ is the standard potential of Ferredoxin. A full derivation of Eq. (11) can be found in Section 7 (Equations 77–91) of the supplement for Salimijazi et al. (2020).

Understanding the division of electron flow between proton motive force generation and electron carrier reduction within the EMP organism will allow us to estimate how low the $O_2$ concentration can be driven.

The overall anode and cathode reactions for $H_2$ evolution,

$$H_2O \rightarrow \frac{1}{2} O_2 + H_2. \quad (12)$$

Thus for every two $H_2$ molecules that are generated, one $O_2$ molecule is also generated.

$$y_{\text{O2, evolved,H}_2} = y_{\text{ep,H}_2}/4. \quad (13)$$

Likewise, for an EEU-mediated system,
\[ \nu_{O_2} = \frac{\nu_{p, EEU}}{4}. \] (14)

How much of this \( O_2 \) is actually needed by the microbe in order to use the \( H_2 \) to generate protein molecules? The redox reaction carried out by complex IV, the terminal oxidase in the aerobic electron transport chain reduces \( O_2 \) to water and \( e^- \) is carried out by complex IV, the terminal oxidase in the cytoplasm (cyt),

\[ \text{O}_2 + 8\text{H}^+ + 4e^- \rightarrow 2\text{H}_2\text{O} + 4\text{H}^+ \] (15)

Thus, one \( O_2 \) molecule is consumed for every 4 electrons sent downhill in energy. Therefore, from Eq. 10, the number of \( O_2 \) molecules needed for \( H_2 \)-mediated EMP is just \( 1/4 \) of the number of electrons used to generate the proton motive force needed to regenerate ATP,

\[ \nu_{O_2, consumed} = \frac{\nu_{p, ATP}}{4} \left( \frac{\text{ceil}(\Delta G_{ATP/ADP}/e\Delta U_{\text{membrane}})}{\text{floor}((\text{U}_{\text{NAD}} - \text{U}_{\text{acceptor}})/\Delta U_{\text{membrane}})} \right) + \frac{\nu_{p, NADH}}{4} \left( \frac{\text{ceil}((\text{U}_{\text{NAD}} - \text{U}_{\text{Q}})/\Delta U_{\text{membrane}})}{\text{floor}((\text{U}_{\text{Q}} - \text{U}_{\text{acceptor}})/\Delta U_{\text{membrane}})} \right) + \frac{\nu_{p, Fd}}{4} \left( \frac{\text{ceil}((\text{U}_{\text{Fd}} - \text{U}_{\text{Q}})/\Delta U_{\text{membrane}})}{\text{floor}((\text{U}_{\text{Q}} - \text{U}_{\text{acceptor}})/\Delta U_{\text{membrane}})} \right). \] (16)

Likewise, for EEU-mediated EMP, the number of \( O_2 \) molecules needed is \( 1/4 \) of the number of electrons used to generate the proton motive force needed to regenerate ATP, \( \text{NAD(P)H} \) and Ferredoxin (but not directly reduce \( \text{NAD(P)} \) or Ferredoxin) (from Eq. 11),

\[ \nu_{O_2, consumed} = \frac{\nu_{p, ATP}}{4} \left( \frac{\text{ceil}(\Delta G_{ATP/ADP}/e\Delta U_{\text{membrane}})}{\text{floor}((\text{U}_{\text{NAD}} - \text{U}_{\text{acceptor}})/\Delta U_{\text{membrane}})} \right) + \frac{\nu_{p, NADH}}{4} \left( \frac{\text{ceil}((\text{U}_{\text{NAD}} - \text{U}_{\text{Q}})/\Delta U_{\text{membrane}})}{\text{floor}((\text{U}_{\text{Q}} - \text{U}_{\text{acceptor}})/\Delta U_{\text{membrane}})} \right) + \frac{\nu_{p, Fd}}{4} \left( \frac{\text{ceil}((\text{U}_{\text{Fd}} - \text{U}_{\text{Q}})/\Delta U_{\text{membrane}})}{\text{floor}((\text{U}_{\text{Q}} - \text{U}_{\text{acceptor}})/\Delta U_{\text{membrane}})} \right). \] (17)

The results of Eqs 13–17 are computed by the CRB_GLYCINE_O2PY code in the ELECTROFOODS package (RCV2_1.0.1).

The \( \text{NAD(P)H} \), \( \text{ATP} \) and \( \text{Fd}_{ed} \) requirements for amino acid synthesis were calculated by balancing reactions for the autotrophic synthesis of the molecule from \( \text{N}_2 \) and \( \text{CO}_2 \) or \( \text{N}_2 \) and formate (\( \text{COOH}^- \)). We enumerated all reaction steps for the production of 19 of the 20 dietary amino acids from acetyl-\text{CoA} and \( \text{NH}_4 \) using data from the KEGG database in Supplementary Dataset S3 (Kanehisa and Goto, 2000; Kanehisa, 2019; Kanehisa et al., 2020). Synthesis of histidine was excluded from these calculations because of technical challenges with stoichiometric balancing due to its inseparable connection with purine synthesis. As a comparison point, and to validate our approach, we also considered the artificial Formolase formate assimilation pathway (Siegel et al., 2015). Finally, in all scenarios, \( \text{N}_2 \) was fixed into metabolism by the iron-molybdenum (FeMo) nitrogenase (Kyoto Encyclopaedia of Genes and Genomes (KEGG) reaction R05185 (Bothe et al., 2010; Hu et al., 2020; Nyysölä et al., 2021).

The overall stoichiometry of autotrophic amino acid synthesis was calculated by a custom flux balance code. Amino acid synthesis reactions (Supplementary Dataset S1) were combined automatically with the \( \text{CO}_2 \)-fixation, \( \text{C}_1 \)-assimilation, and \( \text{N}_2 \) fixation reactions (Supplementary Table S3) by a custom code (Barstow, 2021) into a set of stoichiometric matrices, \( S_p \), for each reaction network.

Each automatically generated stoichiometric matrix was balanced with a custom flux balance program (Barstow, 2021) to find the overall number of NAD(P)H, \( \text{Fd}_{ed} \), and ATP needed for synthesis of each amino acid using each \( \text{CO}_2 \)-fixation or \( \text{C}_1 \)-assimilation pathway.

We consider a species number rate of change vector, \( \dot{n} \), that encodes the rate of change of number of the reactant molecules over a single cycle of the reaction network; a stoichiometric matrix \( S_p \) that encodes the number of reactants made or consumed in every reaction in the network; and a flux vector \( v \) that encodes the number of times each reaction is used in the network. Reactant molecules are denoted as inputs (e.g., \( \text{CO}_2, \text{N}_2, \text{COOH}^- \)), ATP, \( \text{NAD(P)H} \), outputs (e.g., \( \text{H}_2\text{O} \), intermediates, or the target molecule (e.g., the amino acid to be synthesized). For the purposes of this thermodynamic analysis, we consider \( \text{NADH} \) and \( \text{NADPH} \) to be equivalent as they have near identical redox potentials.

The reactant number vector elements for the inputs were calculated by numerically solving the flux balance equation,

\[ n = S_p \nu, \] (18)

under the constraint that number of each intermediate does not change over a reaction cycle, and that number of target molecules increases by 1,

\[ n_i = \begin{cases} 0 & \text{if species } i \text{ is an intermediate} \\ 1 & \text{if species } i \text{ is the target} \end{cases}. \] (19)

The balanced overall stoichiometry for synthesis of each amino acid is shown in Supplementary Dataset S2.

The number of electrons needed to synthesize an average amino acid was found by calculating the average number of \( \text{NAD(P)H}, \text{Fd}_{ed} \), and ATP needed for synthesizing 19 of the 20 amino acids.

**RESULTS AND DISCUSSION**

**Electromicrobial Production of Amino Acids and Protein**

The electrical and solar energy to protein conversion efficiency (\( \eta_{EE} \) and \( \eta_{SP} \)) and the electrical energy consumption per unit mass
(C_{EP}) and cost of solar electricity per unit mass (C_{SP}) for the production of 19 amino acids was calculated for electron uptake by H_{2} transport and oxidation and EEU, and CO_{2} fixation by the Calvin cycle (Figure 2).

Amino acid synthesis has a lower conversion efficiency than purely carbon-containing products due to the high Fd_{red} and ATP requirements of N_{2}-fixation (Supplementary Dataset S1). Despite this, the conversion efficiency either matches, and in most cases exceeds the theoretical maximum conversion efficiency of sunlight to carbohydrate biomass by C_{3} photosynthesis (Figure 2A). However, Arg, Asn, Gly, and Pro synthesis by H_{2} and EEU, and Gln synthesis by EEU have lower conversion efficiencies than C_{4} carbohydrate photosynthesis (Zhu et al., 2008; Zhu et al., 2010). Synthesis of Cys, Ile, Leu, Met, Phe, Tyr and Val exceed the theoretical efficiency of algal photosynthesis (Wijffels and Barbosa, 2010). The average CO_{2}, N_{2}, and electricity conversion efficiency for an average amino acid using the Calvin cycle is 25.2±5.3% when using H_{2}-oxidation, and 23.1±10.3% when using EEU (Figure 2A).

The electrical energy costs (C_{EP}) for individual amino acids using H_{2}-oxidation an the Calvin cycle range from 40.6±8.5 kJ g^{-1} for Asp to 88.2±19.9 kJ g^{-1} for Arg (Figure 2B). Synthesizing the amino acids by EEU rather than H_{2} adds between ~5 and 10 kJ g^{-1}. At projected 2030 prices for solar photovoltaic electricity from the DOE’s SunShot program of 3 ¢ per kWh (United States Department of Energy, 2016), this corresponds to a minimum cost of 0.033 to 0.081 ¢ g^{-1} (Figure 2B). The average amino acid synthesis energy cost using H_{2}-oxidation and the Calvin cycle is 67.9±13.8 kJ g^{-1} (Figure 3B).

As noted before, the energy conversion efficiency of systems using EEU is consistently a few percentage points lower than for systems using H_{2} oxidation (Salimijazi et al., 2020) (Figure 2A). In EEU based systems there is a higher electron requirement, and hence cell current, needed for regeneration of NAD(P)H, Fd_{red} and ATP. Practically, this is almost offset by a lower minimum cell voltage, resulting in a slightly lower conversion efficiency (Salimijazi et al., 2020). Averaged across all amino acids, the efficiency of synthesis for systems using EEU and the Calvin cycle is 23.1±10.3%. This results in an average electrical energy cost that of 74.1±12.1 kJ g^{-1}, about 6 kJ g^{-1} higher than the cost of synthesis using H_{2}-oxidation.

Can we increase the efficiency of electromicrobial production of amino acids? As we have examined before (Salimijazi et al., 2020), we can improve efficiency by swapping the Calvin cycle for the an alternative CO_{2} fixation cycle (Figure 3). As an aside, the only alternative N_{2}-fixation pathway uses the iron-vanadium nitrogenase, that requires 40 ATP and 12 Fd_{red} for each N_{2} fixed (KEGG reaction R12084), compared with 16 ATP and 8 Fd_{red} for the more common iron-molybdenum-cobalt nitrogenase (KEGG reaction R05185).

Not unexpectedly, the order of efficiency of amino acid synthesis efficiency is approximately the same as the order of efficiency of butanol synthesis. As before (Salimijazi et al., 2020), the 4HB cycle, which performed least well for butanol synthesis (Salimijazi et al., 2020), also performed least well for amino acid synthesis. Likewise, the Wood-Ljungdahl pathway performed the best (Figure 3A).

With increasing efficiency comes decreasing electricity cost (Figure 3B). The average cost of producing a gram of amino acid with H_{2}-4HB is 84.3±6.8 kJ g^{-1} and 63.7±6.8 kJ g^{-1} with H_{2}-WL (costs of 0.07 and 0.05 ¢ g^{-1}). Swapping to EEU-4HB increases the costs 97.3±33.1 kJ g^{-1}, and swapping to EEU-WL reduces them to 70.9±23.5 kJ g^{-1} (costs of 0.08 and 0.06 ¢ g^{-1}).

Oxygen Requirements of Electromicrobial Protein Production Are Low

Using Eqs 13–17 and the CB80GLYCINE_O2L2PY code in the ELECTROFOODS package, we find that under nominal conditions (ΔU_{membrane} = 140 mV), for an H_{2}-mediated system using the Calvin cycle, 21.5 e^{-} are needed to synthesize 1 molecule of glycine (supplied by 10.75 H_{2} molecules, or put better, 43 molecules of H_{2} are used to generate 4 glycine molecules). Generating 10.75 molecules of H_{2} by water-splitting co-generates 5.375 molecules of O_{2}. However, only 1.875 molecules of O_{2} are actually needed to generate the ATP needed for glycine synthesis. Thus, almost 2/3rds of the O_{2} generated by water-splitting can be purged from the system to minimize cathode side-reactions.

Likewise, for an EEU-mediated system using the Calvin cycle, 30 e^{-} are needed to generate glycine, releasing 7.5 O_{2} molecules. However, only 4 O_{2} molecules are actually consumed in generating proton motive force. Thus, almost half of the O_{2} generated by water-splitting can be purged from system to minimize cathode side-reactions (RCv2_1.01).}

Electromicrobial Protein Is an Energy-Efficient Alternative to Current Protein Production Technologies

How do the upper-limit efficiencies predicted for EMP protein production compare with real world production efficiencies and energy costs? Most rigorous estimates of the total cradle-to-farm gate energy costs needed to produce a gram of beef, chicken, pork, eggs, and dairy (Williams et al., 2006); soybeans (Pimentel, 2009); insects (Van Huis et al., 2013) and cultured meat (Tuomisto and Teixeira de Mattos, 2011) consider only primary energy inputs. Estimates of primary energy input start at 44 kJ g^{-1} for soybeans (Pimentel, 2009) and go up to 273 kJ g^{-1} for beef (Williams et al., 2006) (Supplementary Table S4).

However, traditional estimates of energy input into protein production are not suitable for an apples-to-apples comparison to the numbers calculated in this article. These estimates consider the energy content of feed stocks such as grain and milk; and infrastructural costs such as transportation to the farm gate and tilling land. In the case of soy bean production, the estimates do not include the energy delivered by sunlight to the system to initially fix CO_{2}, N_{2} and synthesize amino acids. Likewise, for livestock and dairy production, they do not include the energy content of the sunlight needed to produce the feed, only its final energy content.

Traditional energy input estimates of protein production are not wrong. Quite rightly, sunlight has been thought of as free of cost and global warming concerns. Furthermore, traditional
analyses rightly concern themselves with necessary fossil energy inputs. However, as global agricultural production expands, the land for agriculture becomes an increasingly precious commodity. As a result, efficiency of use of sunlight becomes increasingly important.

Likewise, our analysis explicitly ignores infrastructural costs. While we would like to think that bioreactor production of protein could avoid many of these costs, simply thinking this does not make it so. We cannot say so with any certainty if the infrastructure energy costs, such as stirring, heating, gas exchange, are less than the energy inputs associated with agriculture or livestock farming needed to produce a gram of protein.

Estimates of photosynthetic cost of producing protein are the closest comparison point to our work. The closest comparison point to this work is a recent comparison of year round production of protein rich crops, and their protein content with an empirical model of electromicrobial production methods by Leger et al. (2021). The analysis by Leger et al. allows for calculation of the solar energy costs of photosynthetic production (Supplementary Table S5). Energy costs range from 47 MJ g⁻¹ (η_Sp = 0.035%) for soybeans grown in the United States to 408 MJ g⁻¹ (η_Sp = 0.004%) for maize grown in India (Supplementary Table S5).

In contrast, Leger et al. (2021) estimate an averaged sunlight to protein production efficiency of between 0.29% (minimum food production efficiency) and 0.87% (maximum feed production efficiency) using a solar PV driven Methanol-RUMP pathway. These results presented here suggest that these efficiencies, at least instantaneously could be pushed almost an order of magnitude higher.

**CONCLUSION**

In this work, we examined a fundamental, molecular-scale model of electromicrobial production of amino acids. It is important to re-state here that this calculation does not predict the performance of any naturally-occurring organism. It simply considers a set of redox transformations and enzymatic reactions, and predicts an upper limit efficiency for any natural or synthetic organism using these reactions.

Electromicrobial protein production could address many issues surrounding modern protein production including greenhouse gas emissions (Tuomisto and Teixeira de Mattos, 2011; Garnett, 2014; Smetana et al., 2015), nitrogen run-off, and land use (Carpenter, 2005; Audsley et al., 2010; Tuomisto and Teixeira de Mattos, 2011; Guo et al., 2019; Mishra et al., 2020). Recent results by Leger et al. (2021) suggest that the solar to protein conversion efficiency of agriculture could be improved by an order or magnitude by combining PV with electromicrobial production technologies.

We examined electromicrobial protein production systems that assimilate N₂ using a FeMo nitrogenase reaction; assimilate carbon using one of the six known natural CO₂-fixation pathways (3HP/4HB, rTCA, WL, 4HB, CBB, 3HP) pathways or assimilate formic acid with the artificial formolase pathway; and uptake electrons and energy through H₂-oxidation or extracellular electron uptake. The costs of N₂-fixation mean that electromicrobial protein production is likely never to be as efficient as carbohydrate electromicrobial production. But, our results suggest that they could approach it.

The least efficient system (EEU coupled with the 4HB cycle; EEU-4HB) required 97.3 ± 13.2 kJ g⁻¹ of an average amino acid (Figure 3B) (corresponds to an electrical to protein energy conversion efficiency, η_EP = 17.6 ± 2.4%; Figure 3A). The most efficient system (H₂-WL) required only 63.7 ± 5.3 kJ g⁻¹ of amino acids (Figure 3B) (η_EP = 26.9 ± 2.1%; Figure 3A). If supplied with electricity by a perfectly efficient single junction Si PV the EEU-4HB system would produce protein with an efficiency of η_Sp = 5.8%, while the H₂-WL system would produce protein with an efficiency of η_Sp = 8.9%. These results suggest that the process proposed by Leger et al. (2021) could be improved, at least instantaneously, by another order of magnitude.

What’s the best way to achieve the potential of electromicrobial protein production? All of the systems considered in this study rely upon the presence of at least a small amount (≥ a few hundred ppm) O₂ to generate the maximum amount of reducing equivalents from incoming electrons (Torella et al., 2015; Rowe et al., 2018; Rowe et al., 2021).

Natural options exist for carbon assimilation in high efficiency engineered EMP systems. For carbon assimilation, the Calvin cycle, 3HP cycle, and Formolase pathway can all be operated in the presence of O₂. In fact, the H₂-oxidizing microbe *Ralstonia eutropha* (the chassis organism for the Bionic Leaf which uses the Calvin cycle) fixes CO₂ in the presence of at least 1% O₂, while the Fe-oxidizing microbe *Sideroxydans lithothrophicus* ES-1 uses EEU to power CO₂ fixation in a micro-aerobic environment.

However, N₂-fixation poses a uniquely formidable challenge for high efficiency electromicrobial production. Over the past decade, several groups have incorporated genes for N₂-fixation into *E. coli* and demonstrated functional N₂-fixation (Temme et al., 2012; Wang et al., 2013; Li et al., 2016; Yang et al., 2018; Li and Chen, 2020; Ryu et al., 2020). But, despite tantalizing possibilities (MacKellar et al., 2016), all known nitrogenase enzymes are sensitive to O₂. This creates a fundamental incompatibility between EEU and N₂-fixation that needs to be solved.

Creation of an O₂-tolerant nitrogenase may be a tall order for evolution. Unlike other enzymes useful in sustainable energy applications like the hydrogenase (Barstow et al., 2011), there are plenty of evolutionary pressures to drive the creation of an O₂-tolerant nitrogenase. Despite plenty of demand and opportunities for an O₂-tolerant nitrogenase to emerge, nature has not presented one.

To date, nature has solved the problem of operating the nitrogenase in an O₂-rich environment by sequestering it. For example, root nodules in leguminous plants provide an O₂-shielded environment for symbiotic N₂-fixing microbes. Likewise filamentous N₂-fixing cyanobacteria are able to operate the nitrogenase enzyme inside O₂-impermeable
differentiated cells called heterocysts while simultaneously operating oxygenic photosynthesis to generate reducing equivalents in adjacent cells (Bothe et al., 2010). A similar approach, or recent advances in compartmentalization in synthetic biology (Chen and Silver, 2012; Chen et al., 2013; Polka and Silver, 2016; Butterfield et al., 2017; Flamholz et al., 2020), give a menu of options for building a synthetic O2-resistant compartment for the nitrogenase. Achieving this goal is likely to represent a major challenge in synthetic biology.

Development of an O2-resistant compartment will also enable the implementation of highly efficient CO2-fixation pathways like the 3HP/4HB cycle, rTCA cycle and Wood-Ljungdahl pathway in synthetic organisms that simultaneously use O2 as a metabolic terminal electron acceptor.

Failure to operate enzymatic N2-fixation does not spell the end of the road for electromicrobial protein production however. Much as there has been significant development of electrochemical CO2 reduction to C1 compounds, recent developments in electrochemical N2 reduction to ammonia could be a promising complement to biological production of complex amino acids (Guo et al., 2019).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

All code used in calculations in this article is available at https://github.com/barstowlab/electrofoods and is archived on Zenodo at https://doi.org/10.5281/zenodo.5847529.

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AUTHOR CONTRIBUTIONS

Conceptualization, LW and BB; Methodology, BB; Investigation, LW, SM, KR, JS, MH, EN, and BB; Writing - Original Draft, LW, SM, KR, and BB; Writing - Review and Editing, LW and BB; Resources, BB; Supervision, BB.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.820384/full#supplementary-material

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