Thermodynamic Constraints on Electromicrobial Protein Production

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Abstract

Global consumption of protein is projected to double by the middle of the 21st century¹. 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 CO₂-fixing microbial metabolism could dramatically increase the energy efficiency of commodity chemical production²⁻⁵. 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 CO₂ and N₂ using reducing equivalents produced by H₂-oxidation or extracellular electron uptake could produce amino acids with energy inputs as low as 64 MJ kg⁻¹. This work provides a roadmap for development of engineered microbes that could significantly expand access to proteins produced with a low environmental footprint.

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 expenditure⁶. The agricultural and food production sectors are responsible for $\approx 30\%$ of greenhouse gas emissions, while livestock farming alone accounts for 18% of emissions⁷. Furthermore, the agricultural industry is responsible for 70% of total freshwater consumption⁸. 42% of freshwater consumption is attributed to livestock production alone⁸. 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 today⁹.

The energy and water consumption of livestock farming will only increase as global appetites increase. First, population will grow to ≈ 11 billion by 2050¹⁰. 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^{11,12}. Supplying this increased demand while maintaining the current agricultural areal footprint is expected to require a 75% increase in agricultural productivity¹⁰.

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^{13,14}. 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 activity¹⁵.

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 development¹⁰, and significant doubts remain about the possibility of increasing crop yields by mid-century^{11,16,17}. Furthermore, increasing water scarcity due to climate change could even depress crop yields in the decades ahead¹⁶.

Autotrophic metabolism could increase the efficiency of protein production

Autotrophic microbial production of protein is a promising alternative strategy to conventional food production^{5,18-20}. In this class of schemes, externally supplied reducing equivalents are used to power microbial N₂ and CO₂-fixing metabolism and synthesis of protein molecules^{21,22}.

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 three important advantages over traditional protein production methods. First, autotrophic microorganisms can already produce protein at much higher rates than traditional protein sources^{18,19}. 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^{19,23}. Finally, autotrophic microorganisms can use atmospheric N₂ as a substrate, eliminating the need for thermochemical N₂-fixation^{18,19}.

The cost of autotrophic protein production is dropping rapidly. The cost of production of a single protein has reduced from \$1 × 10⁶ kg⁻¹ in 2000 to ≈ \$100 kg⁻¹ in 2019²⁴. 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 products²⁴.

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^{2,4}, 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.*⁵ suggest photovoltaic-driven electromicrobial production (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)²⁵ or by H₂-oxidation²⁶. 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 (ref. ⁴). 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 **Table S1**.

We consider a bio-electrochemical system used to deliver electrons to microbial metabolism (**Figures 1A** and **1B**). Electrical power is used to generate amino acid (or protein) molecules with an energy per molecule E_{protein} at a rate $\dot{N}_{\text{protein}}$. 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 **Table S2**.

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 ($\dot{N}_{\text{protein}} E_{\text{protein}}$), relative to the power input to the system, $P_{e, \text{total}}$ (ref. ⁴),

$$\eta_{EP} = \dot{N}_{\text{protein}} E_{\text{protein}} / P_{e, \text{total}}.$$

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

$$C_{EP} = P_{e, \text{total}} N_A / (\dot{N}_{\text{protein}} M_{\text{protein}})$$

where M_{protein} is the molecular weight of the protein molecule.

For a single bio-electrochemical cell system where CO₂- and N₂- fixation are performed *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 CO₂ and N₂ (the fundamental charge, e , multiplied by the number of electrons needed for synthesis, v_{ep}) and the potential difference across the bio-electrochemical cell, ΔU_{cell} ,

$$\eta_{EP} \leq E_{\text{protein}} / (e v_{ep} \Delta U_{\text{cell}}).$$

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

$$C_{EP} \geq N_A \Delta U_{\text{cell}} e v_{ep} / M_{\text{protein}}.$$

For systems where CO₂ reduction is performed electrochemically, and the resulting reduction product (typically a C₁ compound like formic acid) (refs. ²⁷⁻²⁹) is further reduced enzymatically (**Figure 1B**), v_{ep} is

substituted for number of electrons needed to convert the C_1 product into the final protein product, $v_{e, add}$ (ref. ⁴),

$$\eta_{EP} \leq \frac{E_{protein} \xi_{I2}}{ev_{e, add} \left(\Delta U_{cell1} \left(\frac{v_r v_{er} v_{Cr} \xi_{I2}}{\xi_{I1} \xi_C v_{e, add}} \right) + \Delta U_{cell2} \right)},$$

where v_r is the number of primary reduction products (*i.e.*, formic acid molecules) needed to synthesize a molecule of the final product, v_{er} is the number of electrons needed to reduce CO_2 to a primary reduction product (*i.e.*, 2 in the case of formic acid), v_{Cr} is the number of carbon atoms per primary fixation product (*i.e.*, 1 in the case of formic acid), ξ_{I2} is the Faradaic efficiency of the bio-electrochemical cell, ξ_{I1} is the Faradaic efficiency of the primary abiotic cell 1, ξ_C is the carbon transfer efficiency from cell 1 to cell 2.

Thus, the amount of electricity needed to produce a unit-mass of the protein when using electrochemical CO_2 -reduction is,

$$C_{EP} \geq \frac{ev_{e, add} N_A \left(\Delta U_{cell1} \left(\frac{v_r v_{er} v_{Cr} \xi_{I2}}{\xi_{I1} \xi_C v_{e, add}} \right) + \Delta U_{cell2} \right)}{E_{protein} \xi_{I2}}.$$

We calculate the electron requirements, v_{ep} or $v_{e, add}$, for amino acid (or protein) synthesis from the number of NAD(P)H ($v_{p, NADH}$) reduced Ferredoxin (Fd_{red} ; $v_{p, Fd}$) and ATP ($v_{p, ATP}$) molecules needed for the synthesis of the molecule, along with a model of the mechanism used for electron delivery to the microbe⁴.

For systems that rely upon H_2 -oxidation for electron delivery like the Bionic Leaf^{4,26,30} (**Figure 1C** part 1),

$$v_{ep, H_2} = 2v_{p, NADH} + 2v_{p, Fd} + v_{p, ATP} \frac{\text{ceil}(\Delta G_{ATP/ADP}/e\Delta U_{membrane})}{\text{floor}((U_{H_2} - U_{acceptor})/\Delta U_{membrane})},$$

where $\Delta G_{ATP/ADP}$ is the free energy required for regeneration of ATP, $\Delta U_{membrane}$ 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_2 , $U_{acceptor}$ is the standard potential of terminal electron acceptor reduction (typically $O_2 + 2e^-$ to H_2O), the ceil function rounds up the nearest integer, and the floor function rounds down to the nearest integer.

The inner membrane potential difference, $\Delta U_{membrane}$, is the largest source of uncertainty in this calculation. Therefore, we present a range of efficiency estimates in **Figures 2** and **3** and throughout the text for $\Delta U_{membrane} = 80$ mV (BioNumber ID (BNID) 10408284³¹) 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*^{4,25} (**Figure 1C** part 2),

$$\begin{aligned} v_{ep, EEU} = & 2v_{p, NADH} + 2v_{p, Fd} \\ & + v_{p, ATP} \frac{\text{ceil}(\Delta G_{ATP/ADP}/e\Delta U_{membrane})}{\text{floor}((U_Q - U_{acceptor})/\Delta U_{membrane})} \\ & + v_{p, NADH} \frac{\text{ceil}((U_{NADH} - U_Q)/\Delta U_{membrane})}{\text{floor}((U_Q - U_{acceptor})/\Delta U_{membrane})} \\ & + v_{p, Fd} \frac{\text{ceil}((U_{Fd} - U_Q)/\Delta U_{membrane})}{\text{floor}((U_Q - U_{acceptor})/\Delta U_{membrane})}, \end{aligned}$$

where U_Q is the redox potential of the inner membrane electron carrier, thought to be ubiquinone³², U_{NADH} is the standard potential of NAD(P)H, and U_{Fd} is the standard potential of Ferredoxin.

The NAD(P)H, ATP and Fd_{red} requirements for amino acid synthesis were calculated by balancing networks of reactions for the autotrophic synthesis of the molecule from N_2 and CO_2 or N_2 and formate (COOH^-). We enumerated all reaction steps for the production of 19 of the 20 dietary amino acids from acetyl-CoA and NH_4 using data from the KEGG database in **Dataset S3** (refs. ³³⁻³⁵). 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 consider the synthesis of glucose.

Amino acid synthesis reactions were complemented with reactions for CO_2 -fixation, C_1 -assimilation, and N_2 fixation (**Table S3**). For this article we considered 6 scenarios in which CO_2 was fixed by the well-known Calvin cycle³⁶, the Reductive Tricarboxylic Acid cycle^{37,38}, Wood-Ljungdahl (WL) Pathway³⁶; the 3-hydroxypropionate/4-hydroxybutyrate (3HP-4HB) Pathway^{38,39}; 3-hydroxypropionate (3HP) Cycle⁴⁰; and the Dicarboxylate/4-hydroxybutyrate (4HB) Cycle⁴¹. In addition, we also considered the artificial Formolase formate assimilation pathway⁴². Finally, in all scenarios, N_2 was fixed into metabolism by the iron-molybdenum (FeMo) nitrogenase (Kyoto Encyclopaedia of Genes and Genomes (KEGG) reaction R05185 (refs. 22-24).

The overall stoichiometry of autotrophic amino acid synthesis was calculated by a custom flux balance code. Amino acid synthesis reactions (**Dataset S1**) were combined automatically with the CO_2 -fixation, C_1 -assimilation, and N_2 fixation reactions (**Table S3**) by a custom code⁴³ into a set of stoichiometric matrices, $\mathbf{S}_p$, for each reaction network.

Each automatically generated stoichiometric matrix was balanced with a custom flux balance program⁴³ to find the overall number of NAD(P)H, Fd_{red} , and ATP needed for synthesis of each amino acid using each CO_2 -fixation or C_1 -assimilation pathway.

We consider a species number rate of change vector, $\dot{\mathbf{n}}$, that encodes the rate of change of number of the reactant molecules over a single cycle of the reaction network; a stoichiometric matrix $\mathbf{S}_p$ that encodes the number of reactants made or consumed in every reaction in the network; and a flux vector $\mathbf{v}$ that encodes the number of times each reaction is used in the network. Reactant molecules are denoted as inputs (*e.g.*, CO_2 , N_2 , COOH^- , ATP, NAD(P)H), outputs (*e.g.*, H_2O), intermediates, or the target molecule (*e.g.*, the amino acid to be synthesized). For the purposes of this thermodynamic analysis, we consider NADH and 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,

$$\dot{\mathbf{n}} = \mathbf{S}_p \mathbf{v},$$

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

$$\dot{n}_i = \begin{cases} 0 & \text{if species } i \text{ is an intermediate} \\ 1 & \text{if species } i \text{ is the target} \end{cases}$$

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

The number of electrons needed to synthesize an average amino acid was found by calculating the average number of NAD(P)H, Fd_{red} , 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 (η_{EP} and η_{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 (**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^{44,45}. Synthesis of Cys, Ile, Leu, Met, Phe, Tyr and Val exceed the theoretical efficiency of algal photosynthesis⁴⁶. The average CO_2 , N_2 , and electricity conversion efficiency for an average amino acid using the Calvin cycle is $25.2^{+0.5}_{-3.2}\%$ when using H_2 -oxidation, and $23.1^{+1.0}_{-3.3}\%$ when using EEU (**Figure 3A**).

The electrical energy costs (C_{EP}) for individual amino acids using H_2 -oxidation and the Calvin cycle range from $40.6^{+0.8}_{-5.8} kJ g^{-1}$ for Asp to $88.2^{+1.9}_{-14.1} kJ g^{-1}$ 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 kWh⁴⁷, this corresponds to a minimum cost of 0.033 ¢ g^{-1} to 0.081 ¢ g^{-1} (**Figure 2B**). The average amino acid synthesis energy cost using H_2 -oxidation and the Calvin cycle is $67.9^{+1.3}_{-9.8} kJ g^{-1}$ (**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 oxidation⁴ (**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 efficiency⁴. Averaged across all amino acids, the efficiency of synthesis for systems using EEU and the Calvin cycle is $23.1^{+1.0}_{-3.3}\%$. This results in an average electrical energy cost that of $74.1^{+3.1}_{-12.5} kJ g^{-1}$, about $6 kJ g^{-1}$ higher than the cost of synthesis using H_2 -oxidation.

Can we use biological engineering to increase the energetic efficiency of electromicrobial production of amino acids? As we have examined before⁴, 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 before⁴, the 4HB cycle, which performed least well for butanol synthesis⁴, 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 H₂-4HB is $84.3^{+0.9}_{-6.8} \text{ kJ g}^{-1}$ and $63.7^{+0.7}_{-5.4} \text{ kJ g}^{-1}$ with H₂-WL (costs of 0.07 and 0.05 ¢ g⁻¹). Swapping to EEU-4HB increases the costs $97.3^{+3.3}_{-15.3} \text{ kJ g}^{-1}$, and swapping to EEU-WL reduces them to $70.9^{2.8}_{-9.1} \text{ kJ g}^{-1}$ (costs of 0.08 and 0.06 ¢ g⁻¹).

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 dairy⁴⁸; soybeans⁴⁹; insects⁵⁰ and cultured meat¹⁴ consider only primary energy inputs. Estimates of primary energy input start at 44 kJ g⁻¹ for soybeans⁴⁹ and go up to 273 kJ g⁻¹ for beef⁴⁸ (**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 CO₂, N₂ 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.*⁵. The analysis by Leger *et al.* allows for calculation of the solar energy costs of photosynthetic production (**Table S5**). Energy costs range from 47 MJ g⁻¹ ($\eta_{\text{SP}} = 0.035\%$) for soybeans grown in the US to 408 MJ g⁻¹ ($\eta_{\text{SP}} = 0.004\%$) for maize grown in India (**Table S5**).

In contrast, Leger *et al.*⁵ 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^{14,51,52}, nitrogen run-off, and land use^{13,14,20,53,54}. Recent results by Leger *et al.*⁵ suggest that the solar to protein conversion efficiency of agriculture could be improved by an order of 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 $97.3^{+3.3}_{-15.3} kJ g^{-1}$ of an average amino acid (**Figure 3B**) (corresponds to an electrical to protein energy conversion efficiency, $\eta_{EP} = 17.6^{+0.6}_{-2.4}\%$; **Figure 3A**). The most efficient system (H₂-WL) required only $63.7^{+0.7}_{-5.4} kJ g^{-1}$ of amino acids (**Figure 3B**) ($\eta_{EP} = 26.9^{+0.3}_{-2.1}\%$, **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 $\eta_{SP} = 5.8\%$, while the H₂-WL system would produce protein with an efficiency of $\eta_{SP} = 8.9\%$. These results suggest that the process proposed by Leger *et al.*⁵ 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 ($\geq$ a few hundred ppm) O₂ to generate the maximum amount of reducing equivalents from incoming electrons^{25,30,32}. 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 lithotrophicus* 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-fixation⁵⁵⁻⁶⁰. But, despite tantalizing possibilities⁶¹, 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 hydrogenase⁶², 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 cells⁶³. A similar approach, or recent advances in compartmentalization in synthetic biology⁶⁴⁻⁶⁸, 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 acids⁵⁴.

End Notes

Code Availability

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/doi:10.5281/zenodo.5698500>.

Materials & Correspondence

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Author Contributions

Conceptualization, L.W. and B.B.; Methodology, B.B; Investigation, L.W., S.M., K.R., J.S., M.H., E.N., and B.B; Writing - Original Draft, L.W., S.M., K.R., and B.B.; Writing - Review and Editing, L.W. and B.B.; Resources, B.B.; Supervision, B.B.;

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Competing Interests

The authors declare no competing interests.

Figures

Figure 1. Schematic of amino acid electromicrobial production systems. **(A)** Single bio-electrochemical cell system where electricity is used to power *in vivo* CO₂- and N₂-fixation. **(B)** Dual electrochemical cell system where CO₂ is reduced in the first cell, and then assimilated in the second cell, and combined with enzymatically fixed N₂. **(C)** Long range e⁻ transfer mechanisms considered in this article. In the first, H₂ is electrochemically reduced on a cathode, transferred to the microbe by diffusion or stirring, and is enzymatically oxidized. In the second mechanism, extracellular electron uptake (EEU), e⁻ are transferred along a microbial nanowire (part of a conductive biofilm), or by a reduced medium potential redox shuttle like a quinone or flavin, and are received at the cell surface by the extracellular electron transfer (EET) complex. From the thermodynamic perspective considered in this article, these mechanisms are equivalent.

342 Electrons are then transported to the inner membrane where reverse electron transport is used to regenerate
343 NAD(P)H, reduced Ferredoxin (not shown), and ATP^{25,32}.

Figure 2. Energy conversion efficiency and energy cost of amino acid production. The upper limit energy conversion efficiency and minimum energy cost of amino acid production from CO₂, N₂ and electricity by electromicrobial production systems using the Calvin cycle for CO₂-fixation and either H₂-oxidation or extracellular electron uptake (EEU) were calculated for 19 dietary amino acids (all except histidine) with the ELECTROFOODS package⁴³. NADH, Fd_{red}, and ATP requirements for synthesis of each amino acid are tabulated in **Dataset S2**. This plot can be reproduced using the FIG-CBB_N2_TO_AMINO_ACIDS.PY program in the ELECTROFOODS package⁴³.

(A) Upper limit electrical and solar energy conversion efficiency for amino acids. The left axis shows the electricity to amino acid energy conversion efficiency, while the right axis shows the solar to amino acid conversion efficiency, assuming the system is supplied by a perfectly efficient single-junction Si solar photovoltaic (solar to electrical efficiency of 32.9% (ref. ⁶⁹)). As a first point of comparison, the upper limit solar to biomass energy conversion efficiencies of C₃, C₄ (refs. ^{44,45}), and algal photosynthesis⁴⁶ are marked on the right axis. As a second point of comparison, we have also marked the projected upper limit solar to butanol⁴ and glucose (calculated here) conversion efficiencies by an electromicrobial production system using H₂-oxidation and the Calvin cycle.

(B) Minimum electrical and solar energy costs for the production of a gram of amino acids. The left axis shows the minimum electricity cost, while the right axis shows the minimum cost of that solar electricity, assuming that the US Department of Energy's cost target of 3 ¢ per kWh by 2030 can be achieved⁴⁷.

Figure 3. Changing CO₂-fixation method can improve the performance of amino acid synthesis. The upper limit energy conversion efficiency and minimum energy cost of production of an average amino acid from CO₂ or HCOO⁻, N₂ and electricity by electromicrobial production systems using either H₂-oxidation or extracellular electron uptake (EEU) and one of the 6 naturally-occurring CO₂-fixation pathways or the synthetic Formolase formate assimilation pathway were calculated with the ELECTROFOODS package⁴³. NADH, Fd_{red}, and ATP requirements for synthesis of an average amino acid are tabulated in **Dataset S2**. This plot can be reproduced using the FIG-CBB_N2_TO_AMINO_ACIDS.PY program in the ELECTROFOODS package⁴³. 3HP: 3-hydroxypropionate cycle; 3HP-4HB: 3-hydroxypropionate/4-hydroxybutyrate cycle; 4HB: 4-hydroxybutyrate cycle; CBB: Calvin-Bensson-Bassham cycle; Form: Formolase pathway; rTCA: reductive TCA cycle; WL: Wood-Ljungdahl pathway.

(A) Upper limit electrical and solar energy conversion efficiency for an average amino acid. The left axis shows the electricity to amino acid energy conversion efficiency, while the right axis shows the solar to amino acid conversion efficiency, assuming the system is supplied by a perfectly efficient single-junction Si solar photovoltaic (solar to electrical efficiency of 32.9%⁶⁹).

(B) Minimum electrical and solar energy costs for the production of a gram of an average amino acid. The left axis shows the minimum electricity cost, while the right axis shows the minimum cost of that solar electricity, assuming that the US Department of Energy's cost target of 3 ¢ per kWh by 2030 can be achieved⁴⁷.

Parameter	Symbol	1. H ₂	2. EEU	3. H ₂ with Formate	4. EEU with Formate
Electrochemical Cell Parameters					
Input solar power (W)	P_{γ}	1,000	1,000	1,000	1,000
Total available electrical power (W)	$P_{e, \text{ total}}$	330	330	330	330
CO ₂ -fixation method		Enzymatic		Electrochemical	
Electrode to microbe mediator		H ₂	EEU	H ₂	EEU
Cell 1 cathode std. potential (V)	$U_{\text{cell 1, cathode, 0}}$	N/A		0.82 [Torella2015a]	
Cell 1 cathode bias voltage (V)	$U_{\text{cell 1, cathode, bias}}$	N/A		0.47 [Liu2016a]	
Cell 1 anode std. potential (V)	$U_{\text{cell 1, anode, 0}}$	N/A		-0.43 [Yishai2017a, Zhang2018a]	
Cell 1 anode bias voltage (V)	$U_{\text{cell 1, anode, bias}}$	N/A		1.3 [White2014a]	
Cell 1 voltage (V)	$\Delta U_{\text{cell 1}}$	N/A		3.02	
Cell 1 Faradaic efficiency	ζ_{11}	N/A		0.8 [Rasul2019a]	
Carbons per primary fixation product	v_{Cr}	N/A		1	
e^- per primary fixation product	v_{er}	N/A		2	
Cell 2 (Bio-cell) anode std. potential (V)	$U_{\text{cell 2, anode, 0}}$	-0.41 [Torella2015a]	-0.1 [Bird2011a, Firer-Sherwood2008a]	-0.41	-0.1
Bio-cell anode bias voltage (V)	$U_{\text{cell 2, anode, bias}}$	0.3 [Liu2016a]	0.2 [Ueki2018a]	0.3	0.2
Bio-cell cathode std. potential (V)	$U_{\text{cell 2, cathode, 0}}$	0.82			
Bio-cell cathode bias voltage (V)	$U_{\text{cell 2, cathode, bias}}$	0.47			
Bio-cell voltage (V)	$\Delta U_{\text{cell 2}}$	2 [Liu2016a]	1.59	2	1.59
Bio-cell Faradaic efficiency	ζ_{12}	1.0			
Cellular Electron Transport Parameters					
Membrane potential difference (mV)	$\Delta U_{\text{membrane}}$	140		140	
Terminal e- acceptor potential (V)	U_{Acceptor}	0.82			
Quinone potential (V)	U_{Q}	-0.0885 [Bird2011a]		-0.0885 [Bird2011a]	
Mtr EET complex potential (V)	U_{Mtr}	N/A	-0.1 [Salimijazi2020b]	N/A	-0.1 [Salimijazi2020b]
No. protons pumped per e^-	p_{out}	Unlimited		Unlimited	
Product Synthesis Parameters					
No. ATPs for product synthesis	$v_{\text{p, ATP}}$	See Dataset S2			
No. NAD(P)H for product	$v_{\text{p, NADH}}$	See Dataset S2			
No. Fd _{red} for product	$v_{\text{p, Fd}}$	See Dataset S2			
Product energy density (J molecule ⁻¹)	E_{protein}	See Table S2			

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 butanol⁴. A sensitivity analysis was performed for all key parameters in this work⁴.

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