Renewable electrons-driven bioinorganic nitrogen fixation

a superior route toward green ammonia?

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Broader context

More than 90% of the ammonia production worldwide is still dependent on the century-old, fossil fuel-driven, energy-intensive, and high-impact Haber-Bosch process. In the past years, several alternative approaches, including biological, electrochemical, plasma chemical, thermochemical, and photochemical processes, have been intensively studied for ammonia synthesis. Among these scenarios, biological nitrogen fixation (BNF), using living microorganisms termed diazotrophs to reduce nitrogen to ammonia, has attracted increasing attention. Noteworthy, BNF in nature is limited by the availability of electrons and energy mainly from inorganic reduced substances (e.g., H_2, H_2S, Fe^{2+}) in soil or organics from plant roots, and thus, cannot meet the current and future industrial needs. More recently, green bioelectrocatalytic nitrogen-fixation systems (e-BNF) use enzymes or bacteria as catalysts to produce ammonia using unlimited electrons (or H_2) directly from the electrode has sparked increasing research interest because of sustainability, mild operation, high selectivity, and high energy efficiency. This perspective outlines the history and present state of e-BNF for ammonia synthesis and fundamentals and potential challenges, emphasizing the bioinorganic aspects. The synergistic effort that merges synthetic biology, material science, and biochemistry to advance the e-BNF process toward field application is also proposed.

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Ammonia is crucial for the fertilizer industry and the global chemical economy. However, the conventional Haber-Bosch process for NH_3 synthesis is energy and capital-intensive and associated with high greenhouse gas emissions (1.44% of global CO_2 emissions). Thus, green ammonia synthesis that supports the green energy transition and sustainable development has become a research hotspot. Among others, nature-inspired bioelectrocatalytic nitrogen fixation (e-BNF), which combines the advantages of electrocatalysis, enzymes/microbes, and renewable energy, is emerging as one of the cutting-edge carbon-neutral, energy-efficient, and potentially sustainable strategies for ammonia synthesis. Nevertheless, the development of e-BNF is still in its infancy. Herein, we present a systematic assessment of the historical development and current state of e-BNF for ammonia synthesis. First, we revisit the conventional Haber-Bosch process and abiotic electrocatalysis approaches and access the fundamentals, merits, and challenges of bioinorganic e-BNF in the context of electrochemistry and bioelectrochemistry. Second, the electron transfer mechanisms, and enzyme- and microbial cells-based e-BNF are thoroughly discussed. In the end, we discuss future developments and perspectives on bioelectrocatalytic ammonia synthesis.

Ammonia synthesis: 100-year-old Haber-Bosch process and recent abiotic electrocatalysis

Ammonia, one of the largest-volume commercial compounds on the planet, plays a crucial role in nitrogen fertilization, hydrogen storage, clean fuels, and other chemical manufacturing [1]. In the past century, NH_3 is mainly produced by the Haber-Bosch process, known from the pioneering work of German physical chemist Fritz Haber and industrial chemist Carl Bosch [2]. From a mechanistic point of view, the Haber-Bosch method comprises several primary steps, including dissociative coactivation of N_2 adsorption and reduction of surface nitride via dihydrogen (H_2) passing through surface NH_x intermediates, and the formation and desorption of NH_3 [3] (Fig. 1). Unfortunately, there is a contradiction between the unfavorable thermodynamical barrier and the kinetic properties of the catalysts. The former requires temperatures below 200 °C, while the latter demands a temperature over 400 °C to sufficiently maintain catalyzing activity (e.g., Fe-based catalyst) [4]. The ammonia synthesis reaction in this process is given as:

$$N_{2(g)} + 3H_{2(g)} \rightarrow 2NH_{3(g)},\ \Delta H^0 = -92.2\ kJ{\cdot}mol^{-1}$$

Therefore, the high temperature (350–550 °C) and high pressure (150–350 atm) required by the current Haber-Bosch process are the results of both thermodynamic and kinetic considerations [5]. Meanwhile, multiple passes and cooling between phases to upgrade transformation are indispensable. Moreover, NH_3, N_2, and H_2 are the cardinal composition streams that depend on the equilibrium thermodynamics caused by temperature, pressure, flow rate, the stoichiometric ratio of N_2 to H_2, catalysts, and reactor architecture. As a result, this industrial process is energy- and capital-intensive and accompanied by a vast investment in fossil fuels. It also brings a range of environmental issues such as CO_2 emissions and water pollution [6].

Hence, exploring a novel eco-friendly ammonia synthesis roadway accompanied by reasonable energy investment and minimal environmental disturbance, particularly under moderate reaction conditions, is fundamental to sustainable development and green inhabitation for human beings. In addition, some companies have been working on alternative ammonia production approaches for a couple of years. Table 1 summarizes some technologies for ammonia production that have been piloted in real commercial uses. These methods mainly aimed to employ a more renewable feedstock to produce ammonia, however, the overall processes still cannot overcome the inherent limitations.

In light of the challenges in the Haber-Bosch process, a majority of efforts have been directed toward developing alternative methods capable of converting N_2 to ammonia under ambient circumstances. Heterogeneous electrocatalytic ammonia production, which can produce ammonia from N_2 and water utilizing clean and renewable energy resources, has received attention because of its remarkable selectivity and energy conversion efficiency [7, 8]. Electrocatalytic ammonia production uses substantially less energy and has a lower carbon footprint than conventional technologies like the Haber-Bosch process while still attaining NH_3 at an impressive production rate and yield [9]. In the electrocatalytic ammonia production process, H^+ generated in the anode diffuses into the cathode and combines with N_2 to catalyze into NH_3 driven by external voltages. The typical electrocatalysts can be classified into three groups: (i) metal-based materials, which can be further subdivided into noble metal (e.g., Ru, Rh, Pt, and Au), transition metal (e.g., Fe and Ni), and metal oxide (e.g., Fe_2O_3); (ii) metal-free materials (e.g., N-dopped porous carbon); and (iii) their hybrids. Liu et al. synthesized a nanocomposite of PC/Sb/$SbPO_4$ (a metal-based electrocatalyst and PC refers to phosphorus-doped carbon) that could effectively electrocatalyze N_2 reduction with high activity and superior selectivity. The NH_3 production rate reached 16.70 $\mu g{\cdot}h^{-1}{\cdot}mg^{-1}$ with 31% Faradaic efficiency at −0.15 V vs. reversible hydrogen electrode (RHE) in the acid electrolyte (0.1 M HCl), and 13.40 $\mu g{\cdot}h^{-1}{\cdot}mg^{-1}$ with 34% Faradaic efficiency at -0.10 V vs. RHE in the neutral solution (0.1 M Na_2SO_4) [10]. Under a neutral pH, the nanocomposite catalyst achieved

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outstanding performances of NH_3 synthesis among all non-noble metal-based electrocatalysts studies [10]. Similarly, Liu et al. developed an electrochemical excitation of boron-rich covalent organic frameworks bypassing the restricted boron-doping amount in the carbon. The density functional theory calculations further demonstrated that the boron atoms endowed a unique electronic structure for N_2 absorption and thus substantially lowered the energy barrier of N_2 dissociation [11]. Most importantly, this is to date the first observation to achieve the highest Faradaic efficiency of 45.43% (with 12.53 μg $NH_3 \cdot h^{-1} \cdot mg^{-1}$ production rate) among the reported metal-free catalysts for nitrogen reduction [11].

The ins and outs of electrocatalytic ammonia synthesis have been well addressed in several excellent reviews. Deng et al. summarized the recent efforts in the electrocatalysis of ammonia under mild conditions and discussed potential strategies to overcome the competition between the N_2 reduction reaction and the H_2 evolution reaction [12]. Hu et al. deeply overviewed ammonia production by electrochemical approaches from principles, practices, and challenges, the role of catalysts and electrolytes, and an initial techno-economic assessment to estimate global nitrogen resources [13]. While the electrocatalytic ammonia synthesis processes show advantages over the conventional Haber-Bosch process, the low turnover, poor Faradaic efficiency, and exorbitant expenses of the sacrificial reductants are still the key challenges that limit the wider application.

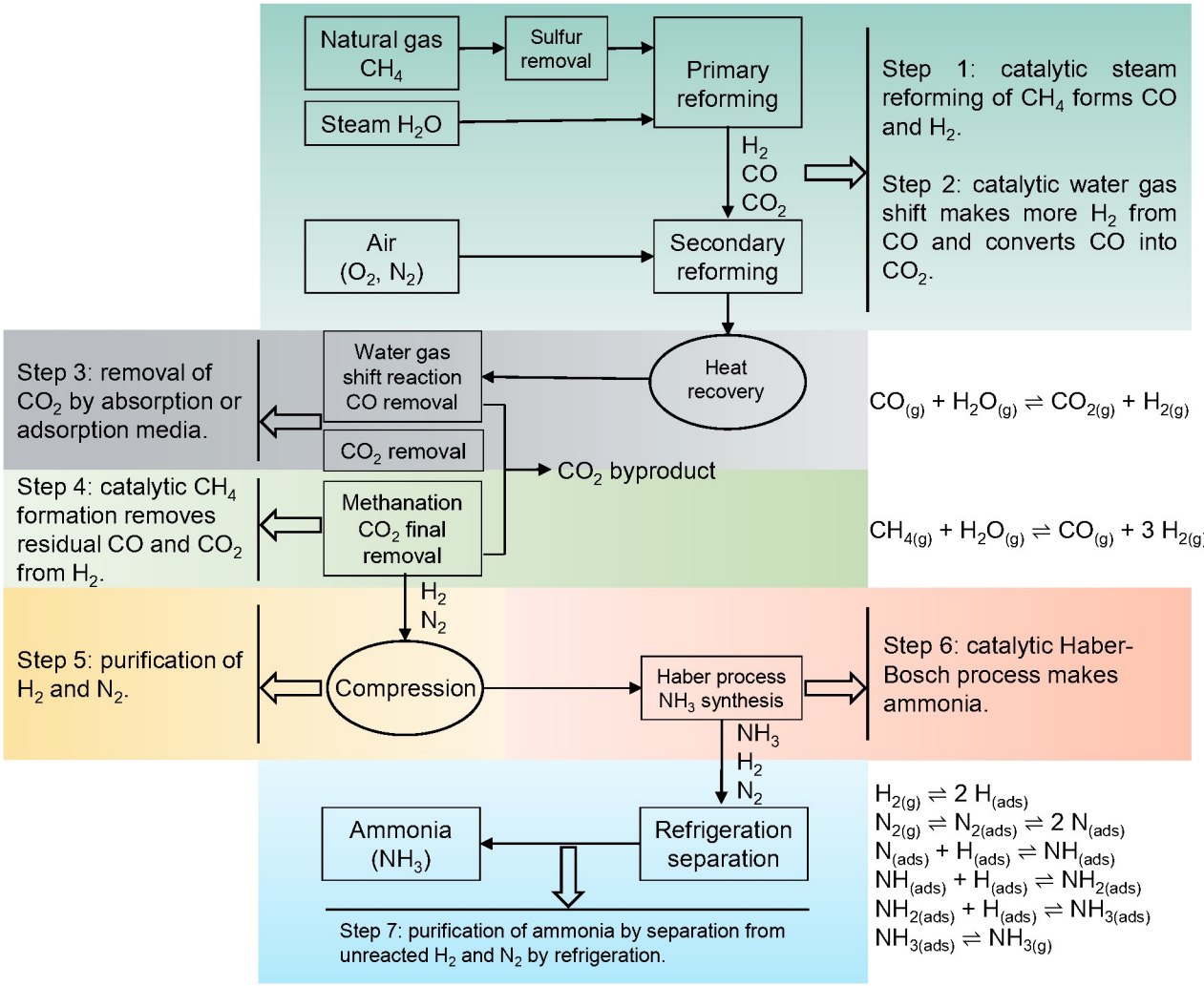

Fig. 1 The Haber-Bosch process for ammonia production.

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Table 1. Pilot technologies by different companies for ammonia production.

Technologies	Raw materials	Production capacity (ton·d^{-1})	Advantage	Company
The NH_3 500– Standalone Fuel Synthesizer	Air, water, and renewable energy from solar, wind, or tidal power	500 L·d^{-1}	On-site production Portable design	NH_3 Canada Inc.
Gasification & catalytic conversion	Woody biomass	360 (urea)	Biomass and CO_2 as feedstock	BioNitrogen
On-site biogas-to-nitrogen technology	Biogas	20 (NH_3) & 35 (urea)	On-site production Cost competitive	Agrebon Inc.
Water electrolysis-based processes	Natural gas	151	Green and renewable	Fortescue Future Industries & Incitec Pivot Limited

Hybrid biology and electrocatalysis: opening a new era for green ammonia

Biological nitrogen fixation (BNF) driven by nitrogen-fixing bacteria in natural environments is essential for sustaining the global biogeochemical nitrogen cycle. Nitrogen-fixing bacteria are also called diazotrophs, which mainly constitute free-living, symbiotic, and associated bacteria capable of reducing N_2 into NH_3 or its aqueous ion form, i.e., ammonium (NH_4^+). BNF is a self-regulating process that can easily occur at ambient pressure and temperature without greenhouse gas emission. Nevertheless, N_2 is fixed into intracellular biomass, which is more suitable for biofertilizer applications [14, 15]. Therefore, downstream processing is required to extract pure ammonia from the nitrogen-rich biomass. To produce extracellular ammonia, ammonia-assimilation inhibitors, e.g., methionine sulfoximine and phosphinothricin, are required to restrain the incorporation of ammonia into amino acids in the pathway of glutamine synthetase and glutamate synthase. Knocking out glutamine synthetase and glutamate synthase genes or shackling their expression is another imperative to intensify extracellular ammonia excretion [16]. Still, the BNF process relies on reducing powers or energy sources that are pretty constrained in natural environments. As a result, the natural BNF process cannot fulfill present and future NH_3 demands.

More recently, bioelectrocatalytic nitrogen fixation (e-BNF), which uses solid electrodes as electron donors to provide diazotrophic bacteria with substantial and accessible reducing power and ATP for their metabolism, has sparked research interests [17]. Besides living cells, the e-BNF process could also be catalyzed by enzymes such as diverse oxidoreductases (mainly divided into metalloenzymes and non-metalloenzymes), called enzymatic electrocatalysis [18]. As the predominant enzyme-electrocatalysts, oxidoreductase, the large molecule (constitutes insulating protein shell and small redox cofactor), could catalyze redox reactions and transfer electrons between the electrode and the enzyme-substrate with various enzyme cofactors [19]. The primary metalloenzyme cofactors can be classified into heme [20], Fe-S cluster [21], copper [22], molybdenum [23], and tungsten centers [24], which play a vital role in the catalytic function of oxidoreductases. Since the whole microbes function as tiny and self-replicating bioreactors and can perform complex metabolic networks inside cells, they can catalyze a greater spectrum of redox reactions, which are less substrate-specific than those catalyzed by isolated oxidoreductases, and the reduced equivalents can be generated by the cells' metabolic activity [25, 26].

Electrons flowing from the electrode (e.g., anode, oxidation of organic waste stream, or water splitting) to the other electrode (cathode) can be further utilized to foster biosynthetic machinery of a microbial cell for commodities synthesis. Electroactive nitrogen-fixing bacteria could conduct electrochemical interactions or reactions with electrodes for ammonia synthesis, mainly through direct and indirect extracellular electron transfer pathways. Direct electron transfer is often done by membrane-bound redox/electron transport proteins (mainly multi-heme c-type cytochromes [27, 28]), or electrically conductive protein nanowires (controversially composed of PilA-based nanowires, also named e-pili [29, 30], or cytochrome-based nanowires [31, 32], that have been widely investigated in the model electroactive bacteria, *Geobacter sulfurreducens* [33, 34] and *Shewanella* oneidensis [35, 36]. Indirect (i.e., mediated) electron transfer requires redox mediators such as (i) self-secreted electron shuttles like flavins [37] (e.g., riboflavin, flavin adenine dinucleotide, and flavin mononucleotide [38]); (ii) reducing equivalents (i.e., cofactors) nicotinamide adenine dinucleotide NAD^+ and its reduced form NADH, and its phosphorylated form NADPH, which can act as intracellular [39, 40] or extracellular electron mediator [41]; (iii) small molecular electron carriers, e.g., H_2 [42], formate [43] and methanol [44]; (iv) complex organic molecules, e.g., neutral red [45], humic substances [46] and humic acid analog (e.g., anthraquinone-2,6-disulfonate [47]); or (v) conductive minerals, e.g., manganese [48] and iron (magnetite nanoparticles [49]). The ecological landscape often contains many of the previously listed electron mediators [50] that can facilitate extracellular electron transfer for microorganisms free of the charge, i.e., no synthesis of mediators is required. With access to these free mediators, diverse Gram-positive bacteria can perform extracellular electron transfer using fewer electron transfer steps than mineral-respiring Gram-negative bacteria [51, 52]. The following criteria are typically taken into consideration for the determination of exogenous mediators: (i) widely accessible or simply producible via electrochemistry, (ii) electropositive enough to reduce NAD(P)H

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directly for effective energy transfer to cellular metabolism, and (iii) highly soluble in aquatic solution and non-highly toxic [53].

As increasingly sophisticated extracellular electron transfer mechanisms are investigated in the biotechnology application of Electromicrobiology [54, 55], the e-BNF routes provide an alternative avenue to synthesize ammonia. Compared to other approaches, e-BNF empowers unique merits of potentially sustainable, widespread scopes of the feed substrates, ambient reaction conditions, high selectivity, and high energy efficiency [56]. Despite this route being still restricted to small lab-scale development, it may potentially circumvent the economic, climate, and environmental challenges encountered by conventional methods. While e-BNF intrigues scientists' interests and research publications, a crucial review of the fundamentals and recent advances is still missing. Milton et al. and Rapson et al. recently outlined the key advances in nitrogenase bioelectrocatalysis ammonia under ambient conditions, respectively [57, 58]. They both started from the structure and biochemistry of nitrogenase mainly focusing on Mo nitrogenase and then listed a variety of recent studies on improving ammonia production rate in this field as well as the optimization strategies that were outlined. Thus, the scientific advancement and potential application of enzymatic and microbiological electrocatalysis for ammonia synthesis were systematically reviewed in this work. Meanwhile, we further posed the promising directions that could lead to accomplishing a simple self-sustained system for ammonia synthesis. We anticipated that the interdisciplinary integrations of synthetic biology, material science, and microbiology with an emphasis on electromicrobiology would open up a new horizon in bioelectrocatalytic green ammonia synthesis.

Enzymatic electrocatalysis

Nitrogenases

Nitrogenases, as an enzyme cascade, composed of a homodimeric reducing component protein (Fe protein) and a catalytic protein (generally molybdenum-dependent, i.e., Mo-Fe protein, and partially vanadium-dependent, i.e., V-Fe protein, and iron-dependent, i.e., Fe-Fe protein), play a decisive role in catalyzing the reaction of the biological N cycle. The Mo nitrogenase exists in all diazotrophs, the sole one known in diazotrophs having symbiotic interactions with higher plants. The V nitrogenase and Fe nitrogenase are two more Mo-free alternatives that exist in free-living bacteria and cyanobacteria. They all require Fe and essentially distinguish from corresponding metal cofactors at crucial active sites, such as Fe-Mo cofactor, Fe-V cofactor, and Fe-Fe cofactor (Fig. 2A). Mo nitrogenase consisting of Fe protein and Mo-Fe protein is perceived as the chief enzyme in BNF, which possibly has been most studied in *Azotobacter vinelandii* [57, 59]. *nifH* gene-encoded reductase Fe protein in *A. vinelandii* is a ca. 66 kDa homodimer with a single [4Fe-4S] cluster that connects both monomers (ligated by two cysteine residues on each monomer) and may attach one ATP or similar nucleoside phosphates on each monomer. *nifD* and *nifK* genes encoded catalytic Mo-Fe protein in *A. vinelandii* is a ca. 240 kDa $\alpha_2\beta_2$ tetramer, with each αβ unit forming a transitory connection with the Fe protein (Fig. 2B) [57]. Each αβ half comprehends a P cluster, i.e., [8Fe-7S] cluster that connects the αβ half and is mainly ligated by cysteine residues in both α subunit of NifD and β subunit of NifK, though additional serine and tyrosine residues located around the P cluster are regarded to play a crucial role in transient states of the P cluster which is formed in the catalytic process [60]. Also, the Mo-Fe protein's α subunit includes a Fe-Mo cofactor, i.e., [7Fe-Mo-9S-C-homocitrate], which is ligated by a histidine and a cysteine residue, and where N_2 is reduced to NH_3 (Fig. 2A-B).

However, several recent investigations have uncovered that V nitrogenase and Fe nitrogenase may also contribute to 70% of total nitrogen fixation in certain diazotrophs (e.g., cyanolichens and bryophytes) [61, 62]. Thus, in the BNF process for terrestrial ecosystems, these two viable surrogate nitrogenases can also delineate a plethora of ecological properties.

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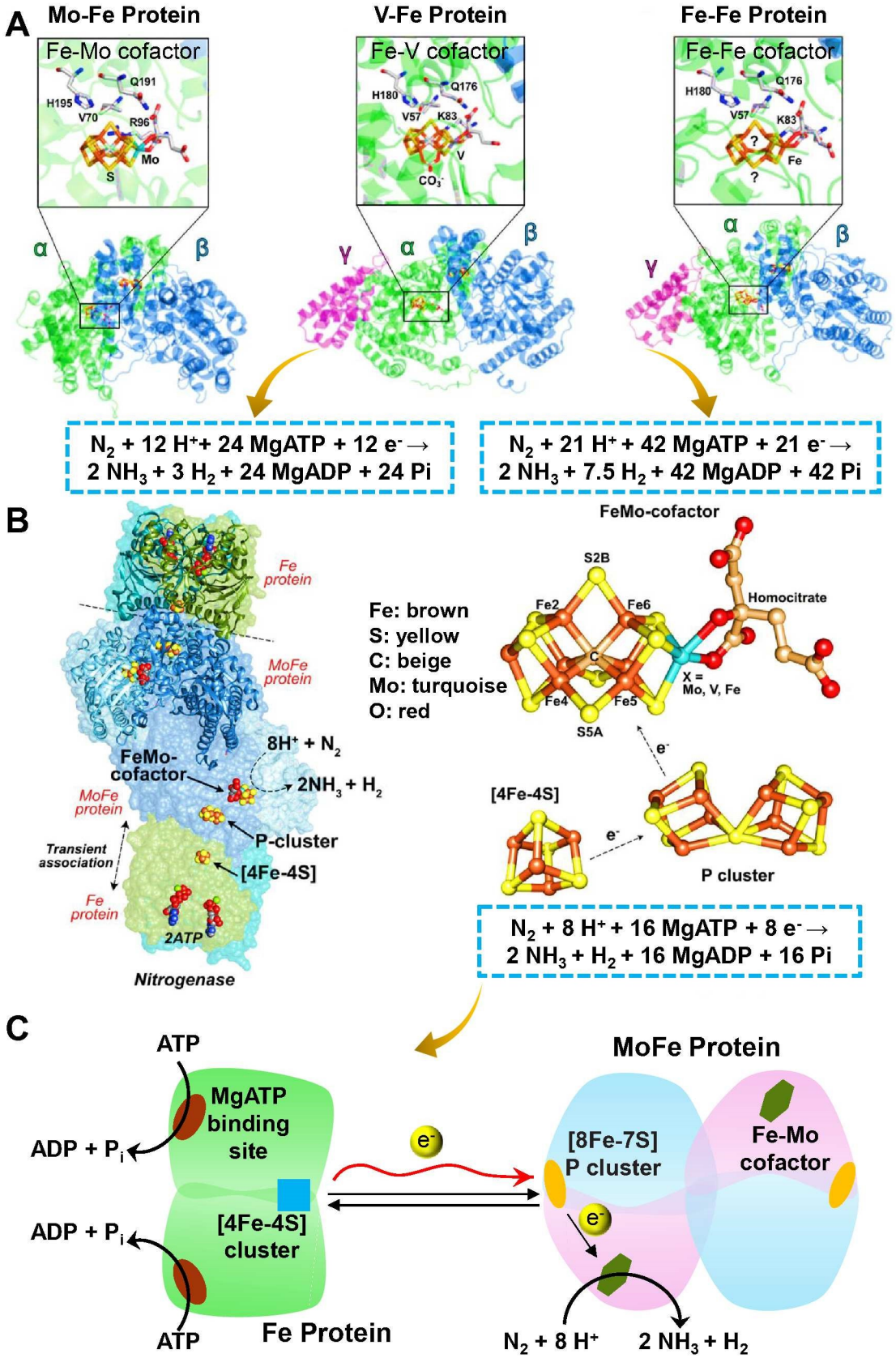

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Fig. 2 (A) Overview structures of contrasting isoforms for three types of nitrogenase [63]. The structure of one catalytic half of the Mo-Fe protein is a $\alpha_2\beta_2$ heterotetramer, with the Fe-Mo cofactor residing in the α-subunit (green) and P cluster located at the interface between the α-subunit and β-subunit (blue). The Fe-Mo cofactor, which is a [7Fe-9S-Mo] cluster with a central carbide and homocitrate moiety, is coordinated by the residues His195, Gln191, Val70, and Arg96. The V-Fe protein has a similar catalytic half structure as the Mo-Fe protein except for the Fe-V cofactor. In contrast to the Fe-Mo cofactor, which has a bridging sulfide atom, the Fe-V cofactor is somewhat elongated, contains a carbonate ligand, and is coordinated by the residues HIS180, Gln176, Val157, and Lys83 (a [7Fe-9S-V] cluster). In addition, the γ subunit (magenta) is in close proximity to the α-subunit. However, the homology structure of the Fe-Fe protein is currently unknown, and one presumable catalytic half model is a $\alpha_2\beta_2\gamma_2$ heterohexamer and the γ subunit (magenta) probably also contacts the α-subunit. (B) Schematic of Mo-dependent nitrogenase isolated from *Azotobacter vinelandii* and corresponding crystal structure of Fe: MoFe protein complex [57]. (C) Ammonia synthesis process involving the electron transfer and Mo-Fe protein [4]. Electrons are transferred from the [4Fe-4S] (Fe_4S_4) cluster of the Fe protein to the Fe-Mo cofactor (or Fe-V, Fe-Fe, not shown) via a [8Fe-S7] (Fe_8S_7) cluster (P cluster), both of which are contained within the Mo-Fe protein (or V-Fe, Fe-Fe protein).

Bioelectrochemical enzymatic fuel system for ammonia production

Nitrogenase has been recently employed to design bioinspired nitrogen reduction electrocatalysts in engineered systems for ammonia or biofuel production (Table 2). For instance, a bioelectrochemical enzymatic fuel system, as a bioelectrocatalytic cell in which redox enzymes are immersed in the electrode chambers or immobilized on the electrodes, could be an impressive substitute for NH_3 synthesis at ambient conditions (Fig. 2C). Additionally, reversible electron mediators have been utilized to facilitate the sluggish electron transfer between enzymes and electrodes [64]. Milton et al. initially reported that methyl viologen (N, N'-dimethyl-4,4'-bipyridinium, MV) as an electron shuttle could be the exclusive electron donor or acceptor to mediate N_2 to reduce NH_3 in the bioelectrochemical enzymatic fuel system [18]. This process coupled the anodic hydrogenase (from *Desulfovibrio gigas*) enzyme to oxidize molecular H_2 (also as the electron mediator) with the cathodic MoFe nitrogenase (from *Azotobacter vinelandii*) enzyme for nitrogen reduction. The ammonia production rate of 2.431 ± 0.009 $ug{\cdot}h^{-1}{\cdot}mg^{-1}$ Mo-Fe protein and corresponding Faradaic efficiency of 26.4 ± 0.1% were achieved with 48.0 ± 3.1 $\mu A{\cdot}cm^{-2}$ current density (that is a current flow of 60 mC discharge across the cell) [18].

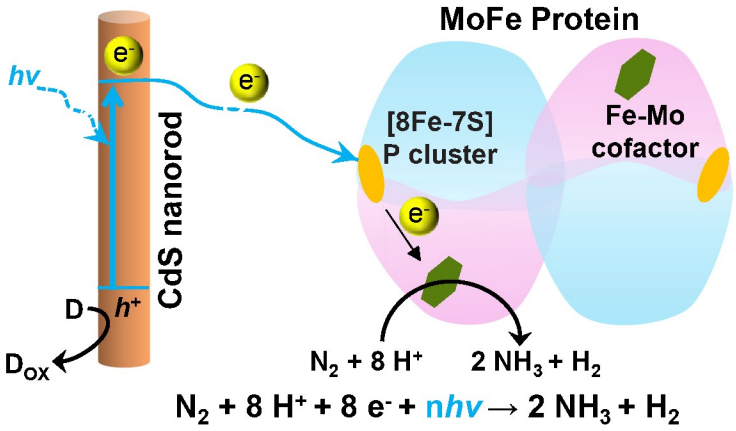
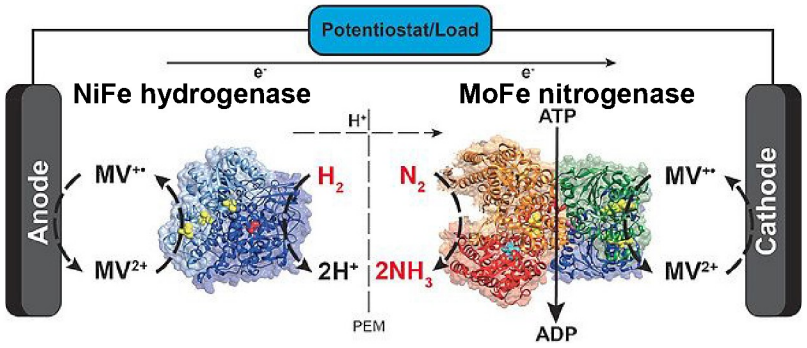
Since electron mediators with a highly negative reducing potential were necessary for turnover in a nitrogenase bioelectrocatalytic system, ATP generated from creatine phosphokinase/creatine phosphate was supplied as the driving power in this study [65]. Thus, ATP investment for nitrogenase turnover and anaerobic operation are two critical bottlenecks of the bioelectrochemical enzymatic fuel system. Milton et al. further developed an aerobic bioelectrosynthetic ammonia production process to address the anaerobic issue. It was the sole one that could accomplish the change of "conformational switch" and ultimately prevent nitrogenases from oxidative damage by regulating the [2Fe-2S] cluster's potential [66]. Additionally, the same research group further established an ATP-free DIET-based bioelectrochemical enzymatic fuel system for N_2 fixation by immobilizing MoFe nitrogenase in a pyrene-modified linear polyethyleneimine (serving as a cross-linked hydrogel) adhered to a carbon paper electrode [67]. This was the first documentation of e-BNF without ATP. Previously, Roth et al, successfully uncoupled the Mo-Fe protein of nitrogenase for substrate reduction ($H^+ + C_2H_2 \rightarrow H_2 + C_2H_4$) from ATP hydrolysis and protein-protein interactions driven by light using a surface-immobilized Ru-photosensitizer as an electron-trigger [68]. This finding disproves the conventional knowledge that ATP hydrolysis is essential for nitrogenase catalysis and provides fresh perspectives on the complex mechanisms underlying BNF. Intriguingly, Brown et al., successfully coated nanocrystals of cadmium sulfide (CdS) on the Mo nitrogenase and achieved photosensitive to capture light instead of ATP hydrolysis to facilitate enzyme-catalytic N_2 reduction into NH_3 [4].

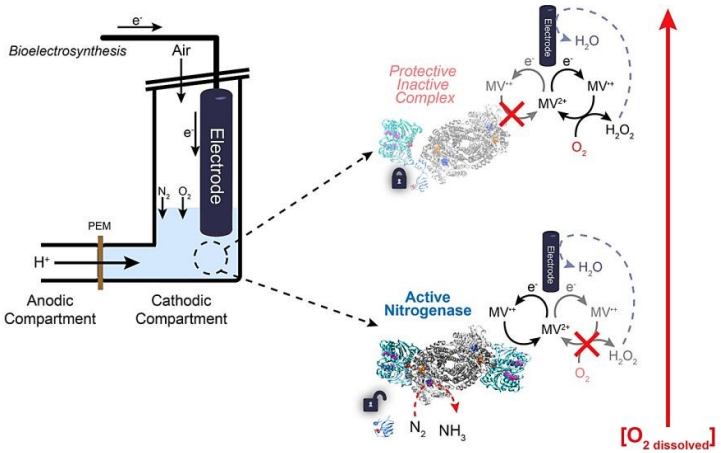
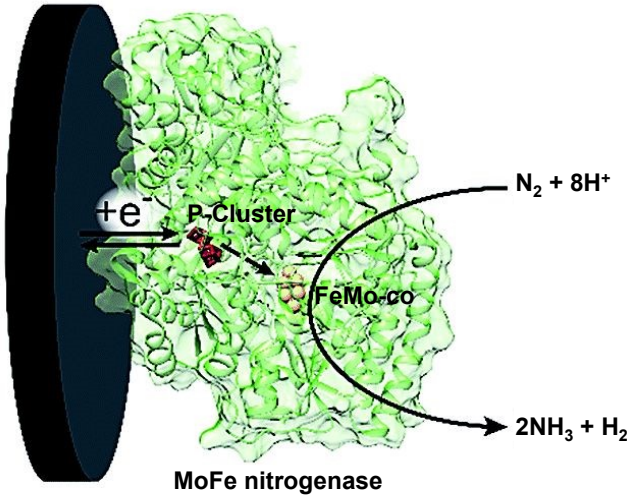
Microbial electrocatalysis

Microbial electrocatalysis provides the following merits over conventional biological routines for ammonia synthesis: (i) it can generate H_2 from waste streams at high efficiency, serving as an alternative H_2 source for N_2 fixation; (ii) it could manipulate the redox metabolism and promote the biosynthesis of ammonia by in-situ generating reducing power of NADH within the cell through interactions with an electrode; (iii) it can take advantage of the membrane and electrochemistry technologies for separation, such as the potential for ions separation, thus, those by-products may be efficiently separated and eventually recovered by arranging the membranes and reactor architecture in microbial electrochemical systems; (iv) renewable energy such as wind and solar power could be a direct driving force for ammonia synthesis, conclusively forming a fully energy-self-sufficient system.

An innovative microbial electrosynthesis platform employing renewable energy is proposed to integrate carbon capture and ammonia production process [14]. To begin, an external voltage supply causes the water-splitting reaction in the anode to generate O_2 and protons. Protons are transported across the separator (such as ion exchange membranes, size-selective membranes, stacks of membranes, or the cloth [54]) to the cathode, where they combine with electrons transferred from the anode via an external circuit to form H_2, which is then further converted into biomass at the cathode together with CO_2 and N_2. The synthesized NH_3 can be either assimilated into biomass or dispersed extracellularly, depending on the operational conditions (Fig. 3A). For instance, potential control, the inclusion of an NH_4^+ assimilation inhibitor, etc., can generate different forms of ammonia. Renewable energy sources, such as natural intermittent solar power and triboelectric nanogenerator, could be employed to power the ammonia synthesis, thereby achieving a truly fossil-free system [69]. Furthermore, nitrogen fixation in such a system is driven by nitrogen-fixing microorganisms utilizing a biocathode, rather than expensive abiotic catalysts.

Table 2. State-of-the-art studies for enzymatic electrocatalysis ammonia synthesis under ambient conditions.

Schematic (Electrode bioenzyme cascade)	Reactor operation *	Faradaic efficiency (%)	NH_3 production rate ($\mu g \cdot h^{-1} \cdot mg^{-1}$ protein)	Ref. [Year]
	Single-chamber sealed vial with biohybrid complexes (MoFe protein adsorbed onto CdS nanocrystals), 0.3 mL; pH = 7; room temperature; anaerobic	Light-driven (No external electricity input)	321.3 ± 56.1	4 [2016]
	Double-chamber septum-sealed vial with a proton exchange membrane, a glassy carbon (anode) and a Toray carbon paper (cathode); pH = 7; 25 °C; anaerobic	26.4 ± 0.1% (Electricity generation)	2.431 ± 0.009	18 [2017]

	A custom-made glass H-cell with a proton exchange membrane, a platinum mesh electrode (anode), and a cylindrica reticulated vitreous carbon foam electrode (cathode); 3 mL; pH = 7; 30 °C; aerobic	NA (Cathode potential −0.76 V vs. SHE)	2.491 ± 0.837	66 [2017]
	A three-electrode setup with a platinum mesh (anode) and a Toray carbon paper (cathode); pH = 7; 25 °C; anaerobic	NA (Cathode potential −0.512 ± 0.008 vs. SHE)	18.7 ± 3.4 μg·mg^{-1}	67 [2018]

* Reactor operation includes reactor configuration (in the presence or absence of membrane), electrode materials, operational pH, and temperature.

** Faradaic efficiency for enzymatic electrocatalytic nitrogen reduction reaction is defined as the quantity of electric charge used for synthesizing ammonia divided by the total charge passed through the electrodes during the electrolysis process.

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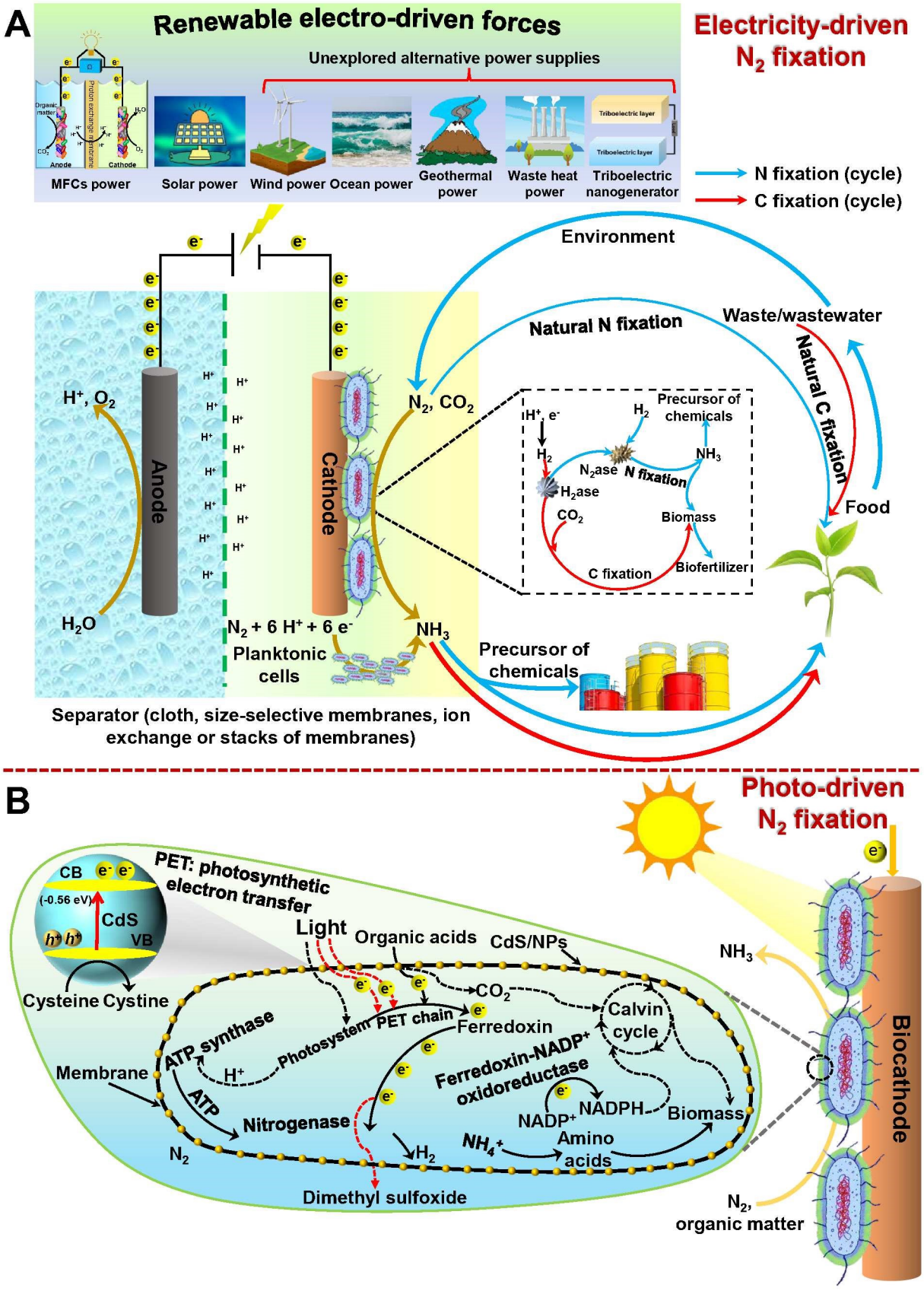

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Fig. 3 (A) Schematic of microbial electrosynthesis for nitrogen cycle using electrons derived from renewable powers. Exoelectrogenic bacteria-based microbial fuel cells (MFCs) generating electricity from organic wastes [70], and salinity gradient-based microbial reverse-electrodialysis systems generating electricity from biomass and salinity [71], are deemed as a potential renewable energy resource technology to drive bioelectrocatalytic ammonia. However, low power density and voltage stability are the main obstacles to the former; membrane fouling, and pumping loss are the main limitations of the latter. Solar-based driven microbial electrochemical systems recently have been demonstrated that the day/night intermittent solar light power can effectively enhance biogas production and regulate electron transfer protein [72, 73], while it is still in fancy. Thus, renewable electricity production and transportation issues and stationery and portable energy needs would be the major barriers. Emerged self-powered triboelectric nanogenerators can transform ambient small-scale mechanical movements into electricity through a coupling of contact electrification and electrostatic induction via the intermittent contact-separation and/or shifty motions occurring at two triboelectric layers with diverse capacities of obtaining/losing electric charges [74]. For example, stir energy waste in bioelectrocatalytic systems that is always lost can be harvested via triboelectric nanogenerators to power the e-BNF process. In this context, how to use the actual power output (e.g., alternating current) will be the first challenge to address. (B) Conceptual photosynthetic microbial-hybrid heterotrophic system for enhancing BNF and carbon neutralization (coupling of wastewater treatment) under ambient conditions, adapted with permission from Wang et al., [75]. *Rhodopseudomonas palustris*, a genetically tractable model bacteria, is commonly employed for photoheterotrophic growth using energy from light and organic matter. In principle, to maintain redox homeostasis and allow continuous organics assimilation, electrons derived from the oxidation of carbon substrates should be allocated entirely via the direct generation of biomass, activity of Nase (generating H_2 and intracellular ammonium), or refixation of produced CO_2 [76]. In addition, the cellular electrons also can be further oxidized by extracellular electron acceptors (e.g., dimethyl sulfoxide). Thus, the Nase play a vital role in nutrient acquisition and electron pool formation.

Intracellular biomass ammonia production

The ammonia synthesized from microbial electrocatalysis can be categorized into intracellular (biomass) or extracellular (cell-free ammonia). Microbes, according to existing studies, are more easily capable of producing intracellular ammonia (Table 3). In most cases, an optimized C/N/P ratio of 100: 5: 1 is necessary for biological wastewater treatment. However, some specific wastewaters, such as the paper mill, pulp winery, and cassava starch wastewater, usually possess a large amount of chemical oxygen demand and are deficient in reduced ammonia (i.e., high C/N ratio). Thus, immoderate use of ammonia fertilizer could lead to excessive N discharge in the environment. To circumvent these obstacles, for the first time, Wong et al. developed a bioelectrochemical anode inhabited by substantial nitrogen-fixing bacteria, dominated by the electrochemically active genus of *Clostridia* that is capable of fixing N_2, for anaerobically treating nitrogen-deficient wastewater (the primary N-deficient substrate was glucose $C_6H_{12}O_6$) [77]. This study also explained the underlying syntrophic correlations between anodophilic fermentative diazotrophs (mostly *Clostridia*) and anodophiles (mainly *Geobacter*). Fermentative bacteria or anodophilic glucose oxidizing bacteria directly utilized the anode as the ultimate electron acceptor to break down glucose into CO_2, or indirectly fermented glucose into volatile fatty acids (main acetate), which would yield 38 and 4 ATP per mol, respectively. Anodic respiration of acetate generated 12 ATP per mol as well. Then, to maintain substantial nitrogen fixation, producing ATP was necessary, which required 16 ATP to fix 1 mol N_2. Thus, this work implied more energetically favoring glucose fermentation and synchronously facilitating nitrogen-fixation activity with the input of electricity. In addition, Betancourth et al. further explored a novel strategy of microbial nitrogen fixation that supplemented free-living nitrogen-fixing bacteria (dominated by the species of *Clostridium pasteurianum*) sourced from mixed sludge in bioreactors to treat the nitrogen-deficient wastewater system in the absence of electrode [15]. Consequently, such bioreactors fixed appreciable quantities of nitrogen at a rate of 11.8 mg $N{\cdot}L{\cdot}d^{-1}$ while simultaneously achieving 73 ± 4.8% of chemical oxygen demand removal efficiency. However, more research into intracellular ammonia production still needs further investigation. For instance, the elemental composition of the dewatered sludge in the form of intracellular ammonia biomass generated from this process, the influence of hydraulic retention time, and sludge retention time on the selection of nitrogen-fixing bacteria, could be a subject of investigation in future studies to assess its suitability as a fertilizer. Though promising, it would be difficult to control for the synthesis of desirable reduced N and C compounds since there may be symbiotic connections within a microbial community where the compounds generated by one bacterium are likely to be consumed by another and vice versa.

Table 3. State-of-the-art studies for microbial electrocatalytic ammonia synthesis under ambient conditions.

Inoculum	Reactor operation *	Electrode materials		Applied voltage	Cathode medium	Faradaic efficiency ** (%)	NH_3 (or NH_4^+) production rate ($mg \cdot L^{-1} \cdot d^{-1}$)	Ref. [Year]
		Anode	Cathode					
Intracellular (biomass) ammonia								
Soil microorganisms (anode, dominating genus *Clostridia*)	Double chamber; continuous; 0.4 L; pH = 6.68 ± 0.43; 43 ± 3 °C	Granular graphite	Granular graphite	+0.2 V vs. Ag/AgCl anode potential	Glucose, N_2	39	NA	77 [2014]
Xanthobacter autotrophicus	Single chamber; 0.10 L; pH = 7; 30 °C	Cobalt phosphate coated on 316 stainless steel mesh	ROS-resistant cobalt-phosphorus alloy coated on carbon cloth	3 V	H_2, CO_2, N_2	4.5	14.4 ± 1 $mg \cdot L^{-1}$ (increased total N content in the medium)	14 [2017]
Rhodopseudomonas palustris (cathode)	Double chamber; 0.10 L; pH ~ 7.5; 20–25 °C	16-gauge platinum wires	16-gauge platinum wires	−1.4 V vs. SHE (constant potential)	N_2, CO_2, HCO_3^-	8.5	2.76	78 [2019]
Extracellular (cell-free) ammonia								
Xanthobacter autotrophicus	Single chamber; 0.10 L; pH = 7; 30 °C	Cobalt phosphate coated on 316 stainless steel mesh	ROS-resistant cobalt-phosphorus alloy coated on carbon cloth	3 V	H_2, CO_2, N_2	2.5	11 ± 2 $mg \cdot L^{-1}$ ***	14 [2017]
Pseudomonas stutzeri	Double chamber; 0.06 L; pH = 7; 30 °C	Carbon cloth	Carbon cloth	−0.3 V vs. SHE	N_2, HCO_3^-	20	2.32 $mg \cdot L^{-1}$	17 [2021]
An engineered, non-diazotrophic *Synechococcus elongatus* PCC 7942	Double chamber; 0.03 L; pH = 7.0	Platinum mesh	Carbon paper	−0.85 V vs. saturated calomel electrode	N_2 (methyl viologen as a mediator)	6.85	1.47 ***	79 [2021]
An engineered, non-diazotrophic *Synechococcus elongatus* PCC 7942	Double chamber; 0.03 L; pH = 7.0	Platinum mesh	Carbon paper	−0.9 V vs. saturated calomel electrode	N_2 (no diffusible mediator)	23.3	6.03 ***	80 [2021]
Activated sludge from a	Single	Pt mesh	Carbon felt	1.5 V	CH_3COONa, N_2	60 $mA \cdot m^{-2}$	0.63	81

local coking wastewater treatment plant (dominating genus *Clostridia*)	chamber; 0.2 L; pH = 7.0; 40 °C	(current density)	[2022]

* Reactor operation includes reactor configuration (in the presence or absence of membrane), batch or continuous operation mode, operational pH, and temperature.

** Faradaic efficiency for microbial electrocatalytic nitrogen reduction reaction is defined as the quantity of electric charge used for synthesizing ammonia divided by the total charge passed through the electrodes during the electrolysis process.

*** It represents that the irreversible glutamate synthetase inhibitors (such as phosphinothricin and L-methionine sulfoximine) were added to the electrolyte to block the ammonia assimilation pathway in the e-BNF process.

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Given that e-BNF requires reducing power or energy, the utilization of phototropic diazotrophs bestows an innovative proposition for permanently generating ATP and imbibing energy from light, which entails facilitating nitrogen fixation. *Rhodopseudomonas palustris*, a typical purple phototrophic α-proteobacteria, has displayed extraordinary versatile metabolic potentials in aerobic chemoheterotrophy (or chemoautotrophy) and anaerobic photoheterotrophy (or photoautotrophy). Such the ability could support own growth by using multiple obtainable inorganic or organic carbon, nitrogen sources (as the electron donor, acceptor, or energy), and/or light (as the energy or trigger of electrons) [82], and thus, has been extensively employed in wastewater treatment, environmental remediation, and high-value-added commodities production. Soundararajan et al. developed an e-BNF system by incorporating *R. palustris* and water electrolysis (as the H_2 source in the cathode) to implement N_2 and CO_2 fixation driven by low energy infrared photons (wavelength > 750 nm, taking into account that the plants often absorb wavelengths beyond 750 nm for food production). Moreover, the process reached 2.76 $mg \cdot L^{-1} \cdot d^{-1}$ of intracellular (biomass) nitrogen fixation rate [78]. Intriguingly, Wang et al. successfully coated the surface of living bacteria (i.e., *R. palustris*) with biocompatible cadmium sulfide nanoparticles (CdS/NPs) without penetration into the cell. The coated cells achieved significant BNF with extra 153% intracellular biomass and 6.73% photosynthetic efficiency under a heterotrophic condition using malate as the carbon source [75]. In such a system, the electrons and ATP for nitrogenases to fix nitrogen were induced by solar light through the CdS/NPs loaded on the cell surface. The generation and transduction of electrons were dependent on the number of NPs and the cross-membrane of cells. Meanwhile, the Calvin cycle contributed 28.1% to the increase of solid biomass. Inspired by these two studies, integrating nitrogen fixation with such biohybrid cells, which are directly powered by solar light and the electrons harvested by anodic exoelectrogens from multitudinous industrial wastewaters, along with CO_2 capture, could offer a facile and captivating route to solar-to-NH_3 conversion for a multifaceted range of industrial application (Fig. 3B). Depth-annotated understandings of this process are needed for further optimization and exploration.

Extracellular cell-free ammonia production

Using N_2, CO_2, and H_2 (the electron donor) as the feedstock, Liu et al. designed a hybrid inorganic bioelectrochemical system to synthesize intracellular ammonia in biomass or even extracellular ammonia after adding the ammonia assimilative inhibitor [14]. This approach can allow for CO_2 capture and N_2 fixation at the same time. The same group further developed such a system by constructing silicon-based microwire array electrodes to mimic a symbiotic root nodule associative diazotrophs and achieved 6.6 mg $N_2 \cdot g^{-1}$ dry $biomass \cdot h^{-1}$ free ammonia which exceeded the natural counterparts by two orders of magnitude [83].

Another group demonstrated the first study of extracellular ammonia (NH_4^+) production by using a microbial biocathode (as the only electron donor) and *Pseudomonas stutzeri* A1501 (as the cathodic catalyst) instead of using any assimilative inhibitors, precious catalysts, or genetic manipulation [17]. The endogenous excretion of phenazine-1-carboxylic acid by *P. stutzeri* played a pivotal role in settling mediated extracellular electron transfer from electrodes to cells, which accumulated to a specific concentration before the electron uptake could commence, resulting in the current curve's 2-day lag time. Electrons are transported to the inner membrane via phenazine-1-carboxylic acid. Then the proton motive force moves electrons to the respiratory chain with complex III. They are used to create reducing power (NADH and $FADH_2$) and then given downstream to the membrane-bound Rnf complex and the nitrogenase enzyme complex to reduce N_2 to NH_3. It was also reasonably illustrated why *P. stutzeri* in e-BNF produces far more ammonia than that required by its assimilation for biomass growth. The ammonia transport AmtB protein is still expressed as usual despite under redox potentials of e-BNF. This research extends great promises for green ammonia production and hints at inspecting newfangled syntrophic microbial nitrogen-fixing mode enormously in the natural environment.

Major challenges and prospects

The conventional methods of chemical commodity production are associated with the challenges of fossil fuel consumption, greenhouse gas emission, and capital intensiveness. Hence, the development of green ammonia synthesis technologies with competent energy efficiencies and economic merits will be a game-changer for the ammonia industry. It is also expected that the bioinorganic e-BNF process through bioelectrocatalysis for ammonia production will eventually be beneficial for sustainability and carbon neutrality (Table 4).

Biocatalysts, electron transfer mechanisms, system configurations, renewable power sources, electrode materials, operating parameters (e.g., temperature, oxygen, solution chemistry, syngas compositions, etc.,) and downstream recovery and purification are anticipated to have multiple effects on the practical application of e-BNF for green ammonia synthesis, in terms of economy, sustainability, and environmental impacts. It should be noted that e-BNF as an emerging field of research, and technology development is still in its infancy. Several technical and economic challenges still need to be addressed before it becomes comparable with the commercialized conventional technologies. First, the synthetic rate of ammonia through bioelectrocatalytic approaches is much lower than the traditional Haber-Bosch process. Second, low titers are the common limitation of all the BNF processes. Third, the main energy input of e-BNF is electricity; thus, the operating costs depend on the sustainable nature of energy sources. Fourth, the costs of N_2 gas, one of the main feedstocks for the e-BNF, are rarely mentioned in previous studies. N_2 is a valuable commodity that is usually obtained through the distillation of liquid air. Thus, the economic and sustainability of the bioelectrocatalytic processes are also subject to the availability and renewability of N_2, which should be considered in future development. Furthermore, while most of the studies focus mainly on developing alternative synthesis approaches and improving ammonia yield, the sustainability, economic and environmental impacts of the emerging technologies essential for practical implementation are rarely studied. The new technology development should consider environmental impacts apart from the economic benefit. The potential strategies are discussed as follows to elicit additional innovations and advance bioelectrocatalytic green

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ammonia synthesis toward large-scale application.

Table 4. Evaluations of the state of three types of (bio)electrocatalytic nitrogen fixation towards green ammonia under ambient conditions.

Technologies	Advantages	Challenges
Electrocatalysis	Low energy consumption; Low CO_2 emission; High conversion efficiency; Simple configuration design; Easily operate by regulating electrode potentials/voltages and reaction parameters	Low kinetics of N_2 adsorption and splitting; Competition between N_2 reduction reaction and H_2 evolution; High electrode overpotentials; Low production rates; Unsatisfactory Faradaic efficiency
Enzymatic electrocatalysis	Green catalysts; Moderate conditions; No need for functional-group activation, protection, and deprotection; Extracellular ammonia generation	Need of strict anaerobic environment; System instability; Poor NH_3 synthesis efficiency
Microbial electrocatalysis	Green and stable catalysts; Wide range of products; Mild conditions; No need for nitrogenase purification; Manipulable microbial metabolic activities; Intracellular/extracellular ammonia generation	Microbial-produced ammonia is easily assimilated so extracellular ammonia is difficult to be recovered; Need of ammonia assimilation inhibitors for extracellular NH_3; High electrode overpotential; Requirement of microbial adhesion

Synthetic biology may bring more efficient biocatalysts

Synthetic biology is an interdisciplinary field of biology, chemistry, and engineering that blends biology's inquisitive character with engineering design concepts. The majority of synthetic biology activities have been directed toward producing novel medicines, natural products, biochemicals, and bioenergy at a low cost [26]. Biocatalysts, as the key driver of the e-BNF process, need to be more efficient and selective. Introducing synthetic biology (or protein engineering) may help satisfy this goal. Transferring modified minimal *nif* gene cluster from *Paenibacillus* sp. WLY78.727 into *Synechococcus elongatus* PCC 7942 can embark on non-diazotrophic cyanobacterium extracellular NH_3 secreted continuously in the e-BNF system via regulating the heterologous expression of the nitrogenase cluster [79]. Furthermore, synthetic biology is pervasively performed by controlling communication networks, modulating gene expression via exogenous inputs, and designing syntrophic relationships to build and govern microbial consortia [84]. Thus, engineering species with specific genes could be strategies to form synergetic interactions with other microorganisms, perhaps resulting in entirely new ammonia-producing communities with greater capacities and resilience [85]. Admittedly, the novel syntrophic communities may execute diverse activities and potentially surpass constraints encountered by a single species. For instance, if one of the two species generates acetate from syngas or mainly poisonous carbon monoxide, a second bioengineered nitrogen-fixing bacteria to "eat" acetate may be introduced to alleviate the toxicity of substrates. In addition, the physical biology of the microbe-material interface for energy harvest and chemical synthesis is another mysterious direction [86]. This novel hybrid interface provides a fantastic chance to build efficient solar-to-chemical conversion. Solar-induced bioavailable reducing equivalents such as electrons (current) and various small molecule mediators (e.g., H_2, formate) could access native or engineered metabolic specialization and force enzyme- or microbe-based cells to reduce exhausted gases and waste streams into value-added chemicals. Even though numerous effective material-microbe combinations are emerging, the inherence of these interactions and driving fundamentals underlying their optimization, for instance, the material biocompatibility to promote beneficial physical interactions between inorganic catalysts and microbes, abide unexplored.

Another interesting candidate for e-BNF might be the recently discovered cable bacteria group. Cable bacteria are multicellular filamentous bacteria capable of transferring electrons over centimeter distances by coupling the oxidation of H_2S in anoxic sediment layers to the reduction of O_2 at the top of the sediment [87]. Cable bacteria belong to the Desulfobulbaceae, a family dominated by anaerobic sulfate reducers [88], and are classified into the two genera *Candidatus* Electronema and *Candidatus* Electrothrix [89]. Cable bacteria have been found globally in freshwater and marine sediments, where they play important roles in several biogeochemical cycles [90]. Their long-distance electron transfer mechanisms have not been resolved yet but may involve a novel Ni-S protein [91]. Interestingly, all cable bacteria studied so far have the genetic potential to fix CO_2 and N_2, with CO_2 fixation proven experimentally [92], and a few studies have even reported cable bacteria attached to anodes of benthic fuel cells [93, 94]. However, efficient nitrogen fixation remains to be shown, and the absence of pure cultures and need to grow cable bacteria in sediment-containing gradient systems are still obstacles that should be addressed before these fascinating and promising organisms can be fully exploited for e-BNF.

Boosting electron flow with reactor technology and material science

The direct contact of microbes and electrodes will enable the fastest electron transfer, thereby maximizing energy conversion efficiency. There are several questions to which need to pay specific attention: (i) materials: which electrode materials are more efficient? (ii) kinetics: how fast can NH_3 be synthesized with the selected electrode materials? (iii) mechanism: how do diazotrophs interact with other microbes on the electrode? The success of e-BNF is subject to several

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physical, chemical, and biological parameters. To accelerate NH_3 synthesis in different environmental conditions, we first must select the best-suited electrode materials with optimized geometry and size per biofilm formation, electron transfer efficiency, and resilience to corrosion, aging, and other negative impacts. Subsequently, efforts should be devoted to investigating and optimizing the microbe-electrode interfaces at varied operating conditions (applied voltages, gas compositions, medium properties, electrode area/reactor volume ratio, and temperature, pH, water content, and O_2 level).

Another important field is to develop innovative and viable bio-inorganic chemo-autotrophic or syntrophic microbial electrosynthesis reactor technology platforms to boost the conversion of nitrogen to ammonia via renewable energy. Since both bioelectrocatalytic and electrocatalytic ammonia synthesis involves electrochemical reactions, thus, they are engaged to securely grant that the essential redox equivalents are reliably from the electricity derived from renewable power resources [26]. And the nature of the electron sources also determines the economy and sustainability of the processes. Longer lifespan, higher power density, and a solid electrical affinity between biomolecules and electrode surfaces are some of the primary obstructions that must be resolved. Focusing on high energy density by raising the degree of oxidation of fuel to capture a greater quantity of electrons is one of the opportunities for enhancing ammonia yield. In addition, 3D printing can start from either 3D electrode architecture or electroactive biofilm formation, which is likely to bring more possibilities for large-scale ammonia production [95]. Recently, Chen et al. developed an aerosol jet 3D-printing method to fabricate libraries of micropillar array electrodes ranging from height and submicrometre surface which can harbor photosynthetic cells to harness electricity, that significantly achieved a high biophotoelectrochemical performance [96].

Mathematical modeling assists in better understanding and prediction for bioprocess

Manipulating microbes with math would attract more attention from computer scientists, systems theorists, and mathematicians. Mathematic modeling or machinery learning could be an important tool for assisting in the optimization of the e-BNF processes. Advanced numerical approaches extend our understanding and prediction of biological system behavior, and there are plenty of mathematical tools that enable us, for example, to investigate the behavior of biological systems defined by a model, including their stability and responses to external stimuli [97]. It is feasible to impose a desired behavior in the functional species and hence optimize the biological process, ultimately enhancing and triggering ammonia yield and revenue. However, due to the inherent uncertainties of biological systems, microbial interspecies interactions, and the interface of microbes-materials affected by various factors [98], it is reasonable to imagine that mathematical models employed to regulate e-BNF systems may be inaccurate or fail to explain certain activities or correlations between microbes with an electrode (weak electric field). Mathematical models are predictive, but at the end of the day, it has to be validated with actual experiment. A resilient, stable but not complicated algorithm model would be helpful for a better understanding of how functional microbiome communities switch from inferior states of bioreactor performances to enriched status, which one day can help us figure out ways to help them switch back to a working state [99].

Controlling a colony of all kinds of microorganisms adds complexity [100]. For example, mixed culture species might compete for a finite matrix, presumably bringing about the extinction of one of the microbial species and thereby lowering the richness and diversity of microbial ecosystems [101, 102]. Control algorithms aimed at regulating such a co-population must consider the relationship between two species while making decisions that would secure their survival and, as a result, benefits from bioengineers. In addition, exogenous mediators for promoting indirect electron transfer in bioelectrocatalysis will also bring the underlying obstacle: downstream removing and recycling redox mediators from fermentation broth [53]. Thus, whether importing the intelligent algorithm into bioelectrocatalysis can effectively regulate or circumvent the above challenges, more efforts should be devoted.

In conclusion, the bioinorganic e-BNF process for ammonia synthesis under mild conditions driven by externally renewable electrons and the green and renewable biocatalysts will offer multiple bonuses for simultaneous biological ammonia synthesis, greenhouse gas sequestration, and storage of fluctuant solar energy or other renewable energy (Fig. 4). It also could intrigue novel research interests and launch further applications. In addition, the power-to-ammonia concept may represent a highly interdisciplinary research area tightly connected with microbiology, biochemistry, electrochemistry, and material science. Hence, e-BNF creates a bridge between different disciplines, bringing a range of opportunities for applied and fundamental research in the years to come.

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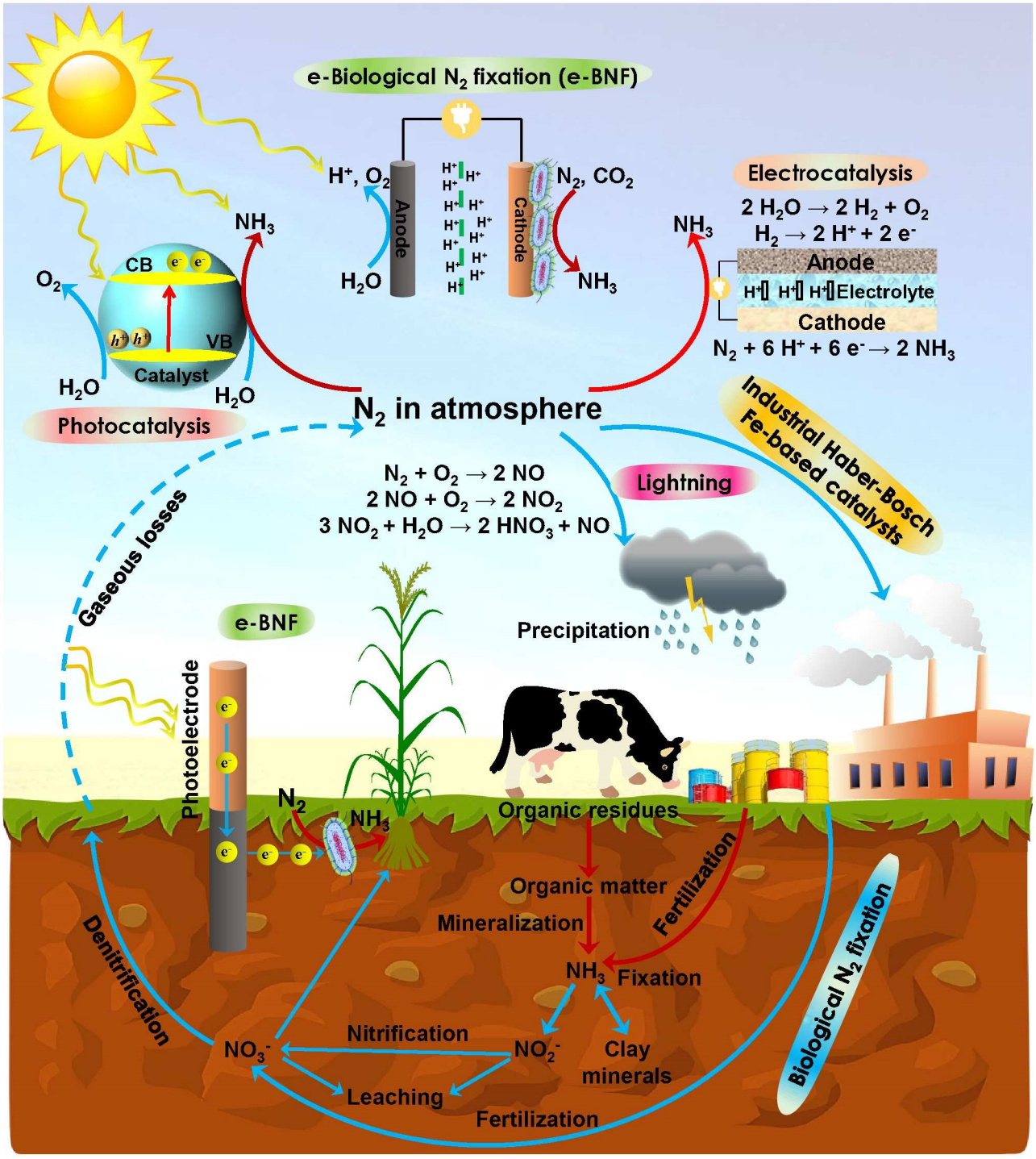

Fig. 4 Conceptual diagram of nitrogen fixation processes and nitrogen resource cycle, including industrial, biological, lightning, photocatalytic,

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and (bio)electrocatalytic processes. Nature-inspired e-BNF can be proposed into two types: ex-situ green ammonia synthesis combining anode water electrolysis and cathode biosynthesis; and in-situ green ammonia synthesis using a novel bioinorganic hybrid photo-electrode. Both external driving power sources are from renewable energy, for example, solar light, wind, and tidal power.

Author Contributions

B.W, Y.F.Z, and S.D.M conceived the topics of the perspective. B.W drafted the manuscript. The authors read, edited, and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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