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Review

Hydrogen production in microbial electrolysis cells with biocathodes

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Electroautotrophic microbes at biocathodes in microbial electrolysis cells (MECs) can catalyze the hydrogen evolution reaction with low energy demand, facilitating long-term stable performance through specific and renewable biocatalysts. However, MECs have not yet reached commercialization due to a lack of understanding of the optimal microbial strains and reactor configurations for achieving high performance. Here, we critically analyze the criteria for the inocula selection, with a focus on the effect of hydrogenase activity and microbe–electrode interactions. We also evaluate the impact of the reactor design and key parameters, such as membrane type, composition, and electrode surface area on internal resistance, mass transport, and pH imbalances within MECs. This analysis paves the way for advancements that could propel biocathode-assisted MECs toward scalable hydrogen gas production.

Highlights

Efficient hydrogen production in biocathode-driven microbial electrolysis cells (MECs) can be obtained by electroautotrophic microbes with the ability to regenerate biocatalytic activity.

Inoculation and enrichment of a pure defined culture in the cathode are critical for high performance in hydrogen production.

Zero-gap MECs with anion exchange membranes (AEMs) enhance hydrogen production due to low internal resistance and better pH balance.

Engineered microbes and optimized microbe–electrode interactions can further increase MEC performance and accelerate its commercialization.

Importance of bioelectrochemical hydrogen production

Hydrogen (H_2) gas has a critical role in achieving a global decarbonized energy system and serves as a key precursor for ammonia production in the Haber–Bosch process [1]. While H atoms are abundant on Earth, predominantly in the form of water, its conversion into H_2 gas via the H_2 evolution reaction (HER) poses significant challenges and requires substantial energy input [2]. Current worldwide H_2 production [~95 million metric tons (MMT) in 2022] results in the release of 1100–1300 MMT of CO_2 equivalent (~11.5–13.6 kg CO_2 kg^{-1} H_2) due to the use of a fossil fuel supply. Thus, several innovative green technologies are being developed to produce H_2 while substantially mitigating CO_2 emissions [3]. Among various methods for H_2 generation, abiotic **water electrolysis** (see Glossary) powered by renewable electricity has emerged as an environmentally sustainable option. This process relies solely on electricity as an energy source and pure water as a reagent. Abiotic water electrolysis can be used as sustainable H_2 production technology, but it still has a high energy requirement compared with conventional routes from fossil fuels. For instance, the energy requirement for water electrolysis [i.e., proton exchange membrane (PEM)] could be as large as 118 kJ/kg H_2 at standard temperature and pressure [4], which is ~3.5 times higher than the value for methane steam reforming (~34 kJ/kg H_2 [5]). Thus, to reduce the energy demand, abiotic water electrolysis requires precious metal catalyst-modified electrodes at acidic conditions.

Microbial electrolysis cells (MECs) offer a potentially more energy-efficient approach to H_2 production compared with traditional water electrolysis methods [6,7]. Typically, in an MEC, exoelectrogenic bacteria present at the anode oxidize organic substrates, generating electrons that are used at the cathode to produce H_2. When utilizing acetate as a substrate, each mole consumed results in the generation of 8 moles of electrons at the anode (Equation S1 in the supplemental information online). The electrons are then used at the cathode to produce 4 moles of H_2 (Section S1.1 in the supplemental information online). MECs can be constructed with at least one electrode, either the anode or the cathode, being biotic [8–10] (Figure 1). MECs

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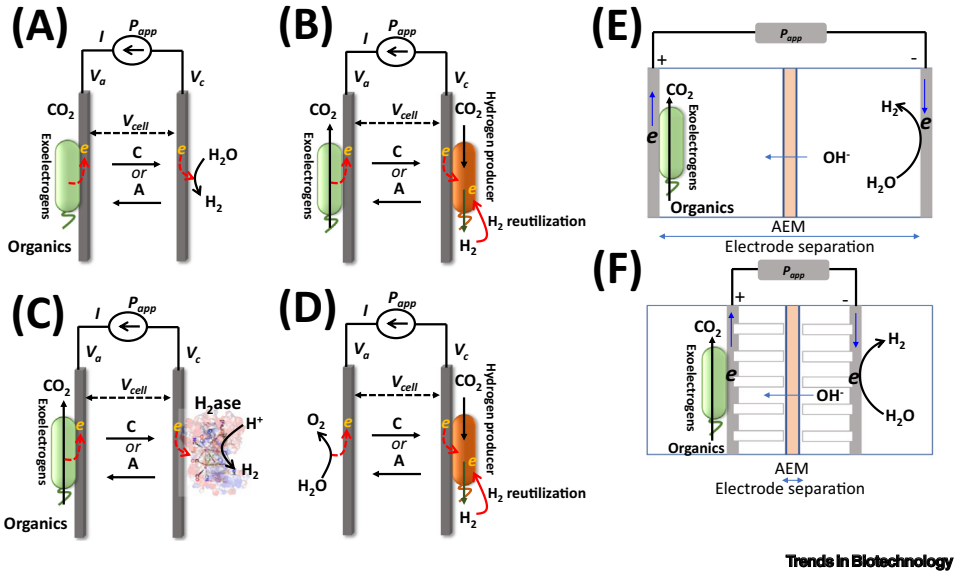

Figure 1. Microbial electrolysis cells (MECs) with different cathode configurations (with or without membrane) and a schematic of dual-chamber MECs. (A) Abiotic cathode, (B) biocathode, (C) hydrogenase (H_2ase)-modified cathode, (D) MEC coupled with an abiotic anode and a biocathode (cation and anion transport between the anode and cathode chambers is denoted by 'C' and 'A', respectively). (E) A standard MEC with anode and cathode chambers. (F) Zero-gap MEC; highly porous electrodes are pressed on either side of the membrane (anion exchange membrane, AEM) to significantly reduce the electrode separation and, thus, the internal resistance.

operate at near-neutral pH, with a cell voltage requirement as low as 0.118 V (with a bioanode and **biocathode**), which is much lower compared with abiotic electrolysis (1.23 V) [11]. However, in MECs, larger applied potentials are often necessary to overcome activation, ohmic, and concentration losses caused by poor catalytic activity, high solution resistance, and mass transport limitations (Section S1.2 in the supplemental information online) [12].

The use of efficient electrocatalysts for cathodes with high activity in an optimized reactor architecture can minimize these losses [13,14]. Unfortunately, the development of such electrocatalysts often relies on precious metals, such as platinum, palladium, and gold [15–17]. These electrocatalysts are susceptible to contamination, inactivation, and performance degradation over time, leading to a significant increase in overpotential [18,19]. Biocathodes in MECs leverage the advantageous electroautotrophic metabolism of microbes to selectively generate H_2. Moreover, biocathodes can be fabricated at a low cost and their activities can be sustained through microbial growth, enabling high catalytic performance at ambient temperatures and pressures. While it is true that microbes consume energy and cannot be classified as true catalysts while producing H_2, they have an important role in MECs by facilitating electron transfer reactions through various enzymes and avoiding the use of precious metals (Box 1). Thus, utilizing biocathodes is a promising approach to minimize electrical energy losses and enhance MEC efficiencies. By reducing the overpotential compared with an abiotic cathode, biocathodes contribute to overall energy reduction, a crucial aspect of sustainable and energy-efficient processes [20]. The cathode biofilm of mixed-culture MECs comprises various **autotrophic bacteria**, which can utilize inorganic carbon sources, such as CO_2, and electricity for their growth. To catalyze HER and produce H_2, these microbes use self-expressed **hydrogenases (H_2ases)** within their cells [21]. Nevertheless, they also require a constant and continuous supply of inorganic carbon for their growth.

Glossary

Autotrophic bacteria: a group of microorganisms capable of producing organic compounds using inorganic substances as a source of energy.
Biocathode: a cathode functionalized with electroactive bacteria capable of catalyzing electrochemical reactions.
Bioelectrochemical systems (BESs): a unique class of electrochemical devices capable of wastewater treatment, chemical production, and energy production using the action of electroactive bacteria-integrated electrodes.
Coulombic efficiency (CE): ratio of electrons incorporated in a desired product during an electrochemical reaction to the total number of electrons available in a substrate.
Direct electron transfer (DET): a mechanism of electron transfer to or from solid electrodes in electroactive microbes through the involvement of conductive appendages, mediators, or soluble electron shuttles.
Direct interspecies electron transfer (DIET): a mechanism of electron transfer among the different microbial species or strains via direct electrical connections without the need for intermediates or soluble electron shuttles.
Electroactive bacteria: a group of microorganisms that can transfer electrons to or from solid electrodes.
Extracellular electron transfer (EET): a process of microbial electron exchange outside the cell membranes.
Hydrogenases (H_{2ases}): a unique set of enzymes produced by certain microorganisms that catalyze the reversible reaction for interconversion of H_2 and protons in biological systems.
Inoculum: a culture of microorganisms that is used to initiate biological reactions in a system.
Mediated electron transfer (MET): a mechanism of electron transfer to or from solid electrodes in electroactive microbes using mediators or soluble electron shuttles, such as 2,6-anthraquinone disulfonate, riboflavin, thionine, and methylene blue.
Microbial electrolysis: a process that utilizes electroactive bacteria to drive electrochemical reactions to H_2 gas through water electrolysis.
Polymer dots (Pdots): water-dispersed nanoparticles comprising π-conjugated polymers varying in size from 1 to 100 nm.

Box 1. Mechanistic overview of biocatalytic action in biocathodes

Electroautotrophs at the biocathode accept electrons from the electrode through different **extracellular electron transfer (EET)** mechanisms, including **direct electron transfer (DET)**, **direct interspecies electron transfer (DIET)**, or **mediated electron transfer (MET)** [97–101]. In DET, the electrons are directly transferred from the solid electrode to the microorganisms through cell proteins (mainly cytochromes) or nanowires. Nanowires electrically bridge the cathode with the bacteria and transfer the electrons from the cathode to the bacteria [102]. In DIET, an exoelectrogenic bacterium, such as *Geobacter sulfurreducens* or *Shewanella* sp. first accepts the electrons from an electrode and then transfers them to a different bacterium [103]. Some electroactive microbes secret mediators, such as phenazine derivatives secreted by *Pseudomonas aeruginosa* [104], and flavins secreted by *Shewanella* [105], using these soluble mediators to accept or release electrons to an electrode. In addition, the H_2 produced during electrolysis at the cathode can act as an electron shuttle for MET. The EET in **electroactive bacteria** has been described in previous literature [106]. Here, we will briefly mention the electron transport chain within the microorganism and the impact of H_2ase in converting periplasmic protons into H_2.

Upon accepting electrons from poised cathodes (see Figure S1 in the supplemental information online), the microbes generate reducing power (NADH) [28,44,107–109], and then transfer the electrons to the H_2ase reaction center, which produces H_2 [110,111]. The H_2ase participates in the interconversion of H^+ to H_2 (see Figure S2 in the supplemental information online).

[Fe-Fe] H_2ase is responsible for catalyzing HER in many prokaryotes and eukaryotes [21] and has been reported in biocathode-based HER in MECs [10]. [Fe-Fe] H_2ase contains a ubiquitous $[4Fe–4S]^{2+}$ protein cluster (either high-potential iron-sulfur protein, HiPiP or ferredoxin, F_d) linked with a [2Fe] cluster $[(CysS)(NC)(OC)Fe[(SCH_2)_2NH]Fe\text{-}(CO)_2(CN)\}^{2-/3-}]$ via a Cys-S– ligand. In particular, the F_d proteins cluster catalyzes one electron transfer process ($[4Fe–4S]^{2+} \leftrightarrow [4Fe–4S]^{+}$) at a reduction voltage window of –0.25 V to –0.40 V [112]. Therefore, it acts as an important redox cofactor for electron transport to the [2Fe] cluster. With at least three active sites, [Fe-Fe] H_2ase efficiently reduces protons into H_2 at a rate of ~8.00 μmol H_2 min^{-1} mg^{-1} protein [113,114]. Despite several dehydrogenation/hydrogenation relays in the HER catalytic cycle, the [2Fe] cluster remains stable [115].

Water electrolysis: chemical process to split water molecules into H_2 and oxygen gas by utilizing an electric current.

In this review, we comprehensively examine recent advances in the field of biotic H_2 production with abiotic and biotic cathodes in MECs. Our primary focus is on the influence of reactor configuration, different inocula, and enzymes derived from microbes on MEC performance [22–26]. We analyze, interpret, and compare the findings from various studies investigating H_2 production in MECs with biocathodes and assess performance parameters such as current density and H_2 production rate, in terms of the cathode surface area and applied voltage. Furthermore, we evaluate the impact of microbial communities (both pure and mixed cultures), reactor configurations (single and dual chamber), and applied voltages to identify limitations in system performance. Finally, we provide valuable insights for future studies aiming to enhance the performance of these systems.

Biocathode in MECs

Pure cultures

Although both pure and mixed-culture bacterial strains have been used for biocathode development in MECs for H_2 production (Table S1 in the supplemental information online), slightly more than half of the studies reviewed here have used pure cultures (Figure 2). Select species of *Desulfovibrio*, Gram-negative sulfate-reducing bacteria from the *Thermodesulfobacteriota* [27–30], are highly appropriate for MEC applications due to the specific activity of their H_2ases. Among the *Desulfovibrio* spp, three exhibit notably high H_2ase activities in MECs, namely 17 000 U mg^{-1} in *Desulfovibrio fructosovorans*, 8200 U mg^{-1} in *Desulfovibrio vulgaris*, and 6900 U mg^{-1} in *Desulfovibrio desulfuricans* [31]. These activity rates surpass those of most other species [32–34], making *Desulfovibrio* spp a preferred choice for MEC applications. The reported H_2ase (FdhA/HytABCDE) activity was even higher in *Clostridium autoetahnogenum* (18 000 U mg^{-1}) [35], an anaerobic bacterium that conducts syngas fermentation (e.g., ethanol from carbon monoxide) [36,37]. This bacterium has been used for the conversion of CO_2 to organic acid in microbial electrosynthesis (MES) systems [38,39], but has not been applied to MECs for H_2 production. *Shewanella oneidensis*, a Gram-negative γ-proteobacterium, also has the H_2ase operon; however, the specific activity of purified enzyme from this bacterium was found to be low (0.06–2000 U mg^{-1}) [40] (Figure 3).

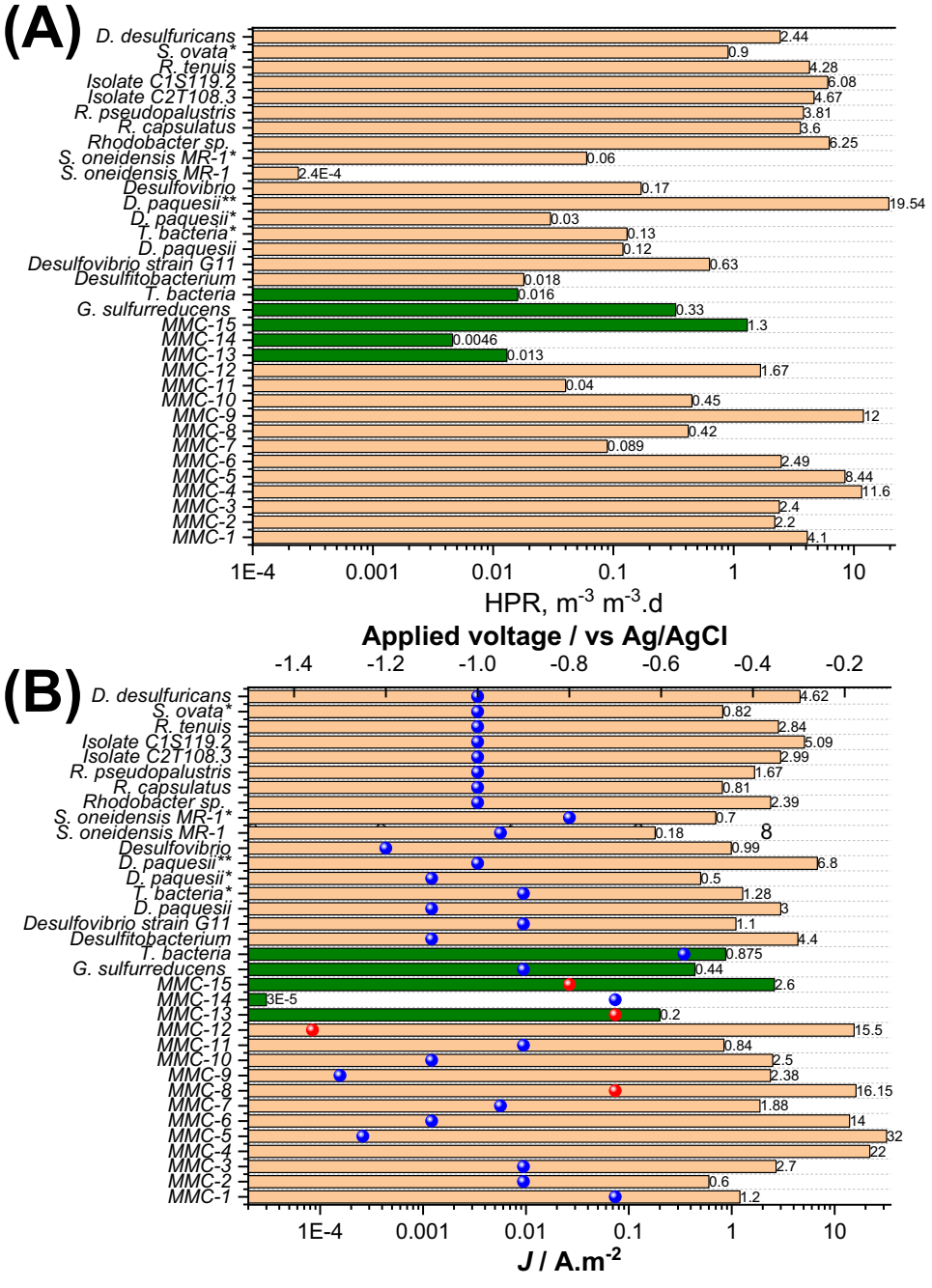

Figure 2. Summary of results obtained from different microbial electrolysis cells (MECs) using either mixed or pure culture strains: (A) Hydrogen (H_2) production rate (HPR) (specific HPR values are shown at the end of each bar), and (B) current density (*J*) production at different applied potentials (specific *J* values are shown at the end of each bar). *Second study with the same strain. **Third study with the same strain. Light-brown and green bars represent dual and single-chamber MECs, respectively. Blue spheres demonstrate applied voltages versus a Ag/AgCl reference electrode, whereas the red spheres show the absolute voltage applied across the electrodes. Abbreviations: *D. desulfuricans, Desulfovibrio desulfuricans; D. paquesii, Desulfovibrio paquesii; D. vulgaris*, *Desulfovibrio vulgaris*; *G. sulfurreducens, Geobacter sulfurreducens; R. capsulatus, Rhodobacter capsulatus; R. pseudopalustris, Rhodopseudomonas pseudopalustris; R. tenuis, Rhytidoponera tenuis; S. oneidensis, Shewanella oneidensis; S. ovata, Sporomusa ovata*; *T. bacteria*, thermophilic bacteria.

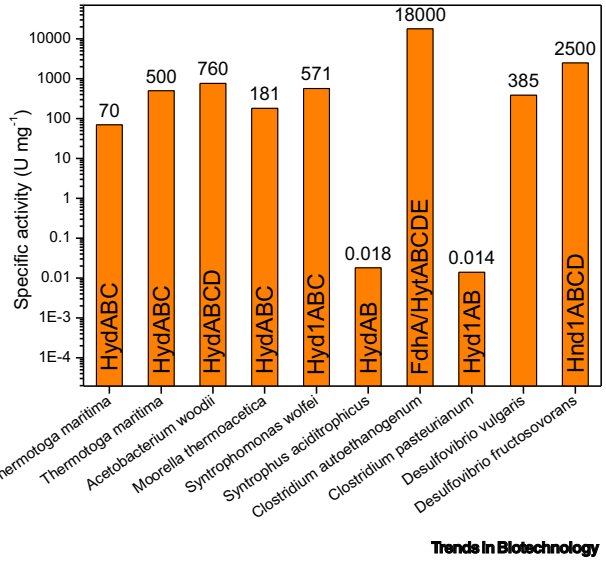

Figure 3. Specific activity of hydrogenases (H_2ases) in different bacteria. H_2ase activity was measured in units per mg of protein isolated from the respective microbes. The names of the H_2ases are given in the bars for each species. Figure created based on data from [32–35,123–127].

In MECs, *Desulfovibrio paquesii* DSM 16681 demonstrated the highest H_2 production rate: 19 m^3 m^{-3} d^{-1}, at a corresponding current density of 6.8 A m^{-2} [41]. Purple bacteria species, such as *Rhodobacter* sp. DSM 5864, *Rhodobacter capsulatus* DSM 152, and *Rhodopseudomonas pseudopalustris* DSM 123, have also produced somewhat lower H_2 production rates of 6.25, 3.6, and 3.81 m^3 m^{-3} d^{-1}, respectively [41]. Much lower H_2 production rates have been obtained from other microorganisms, including *Shewanella oneidensis* MR-1 (0.06 m^3 m^{-3} d^{-1}) [42,43], *Geobacter sulfurreducens* strain PCA (0.33 m^3 m^{-3} d^{-1}) [44], and *Sporomusa ovata* DSM 2662 (0.9 m^3 m^{-3} d^{-1}) [41]. The strains *S. oneidensis* MR-1 and *G. sulfurreducens* are model exoelectrogenic microorganisms mainly studied to produce electricity in microbial fuel cells (MFCs) [45], whereas *S. ovata* has been widely used as an electroautotroph in MES to reduce CO_2 to volatile fatty acids via the Wood–Ljungdahl pathway (Section S1.3 in the supplemental information online) [46–48]. While these pure culture microbes have shown sustainable current densities, such as 0.6–0.7 A m^{-2} for *S. oneidensis* [43], 0.44 A m^{-2} for *G. sulfurreducens* [44], and 0.8 A m^{-2} for *S. ovata* [41], the corresponding H_2 production rates of 0.06, 0.33, and 0.9 m^3 m^{-3} d^{-1}, respectively, were low. The possible reasons for low H_2 productivity could be a low H_2ase activity or a poor reactor configuration and operational parameters. Nevertheless, a few studies have examined the specific concentration of H_2ase based on total cell protein in different bacteria. This metric could be utilized to select more efficient pure culture microbes for future applications of biocathode-based MECs (Table S2 in the supplemental information online).

Mixed cultures

Despite the high H_2 yields using pure cultures, studies utilizing pure cultures often face the challenge of maintaining microbial purity and the requirement for specific media and operating conditions to sustain active pure cultures. Therefore, many studies have been conducted using mixed cultures containing many microbial species that establish syntrophic relationships to achieve the H_2 gas production rate [49,50]. The main challenge of mixed cultures is that many other products can be produced in addition to H_2 gas. Operation of mixed culture reactors is straightforward since no specific medium or growth conditions are typically required, compared with the more stringent conditions needed for pure cultures. In anaerobic sludge, there is usually a diverse population of bacteria, including *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*, and

archaea, such as *Methanosaeta* and *Methanosarcina*, all potential candidates for H_2 production in MECs [51]. Thus, to achieve high HER rates at the cathode, specific protocols and enrichment strategies (Section S1.4 in the supplemental information online) need to be used to selectively enrich the chemolithotrophs as well as to avoid H_2 consumption through methane generation by archaea [52].

Efficient anaerobic microbial consortia suitable for MEC applications have been obtained from diverse environmental sources, including sediments [9,53], wastewater treatment plants [54], and microbial mats (e.g., Hot Lake, Oroville, north-central Washington, USA) [55]. These consortia can then be enriched with desired species to optimize their performance in MECs. In many H_2 production studies, the enriched mixed consortium was derived from operating **bioelectrochemical systems (BESs)**, such as MFCs or MECs (Table 1). This approach allowed for the selection of microorganisms with the ability to interact effectively with the electrodes. In certain cases, mixed cultures were utilized to rapidly establish a biofilm on the anode in standard MFCs, followed by reversing the polarity to switch a bioanode to a biocathode [39,56]. The biofilm formed on the anode primarily comprises exoelectrogens, and certain microbial species within this community can switch their metabolism from heterotrophic to autotrophic mode (e.g., *Desulfovibrio*, *Dehalococcoides*, and *Clostridium* spp) when specific operating parameters are imposed. In this scenario, when the electron and carbon sources are changed from organic compounds to H_2 or a specific potential is applied along with CO_2, these exoelectrogens adjust their metabolic pathways to adapt to the new operating

Table 1. Enrichment of mixed culture microbes in MEC biocathodes

Reactor type (single- and dual-chamber)	Mixed inoculum source	Dominant species/phyla	Biocathode development	Refs
Dual	Dechlorinating enriched bacteria from sediments	Dominant bacteria: *Desulfitobacterium* spp, *Dehalococcoides* spp	Biocathode developed in MEC	[53]
Dual	Inoculum from running MFCs	Dominant bacteria: *Hoeflea* sp., *Aquiflexum* sp. Dominant phyla: *Actinobacteria*, *Bacteroidetes*, *Proteobacteria*	Biocathode developed in MEC	[86]
Single	Inoculum from running MFCs operated under thermophilic conditions	Dominant bacteria: *Thermincola potens* strain JR, *Clostridium* sp. Dominant phyla: *Firmicutes*, *Coprothermobacter*, *Nitrospirae*, *Chloroflexi*, *Thermotogota*	Biocathode developed in MEC	[129]
Dual	Effluent from MECs	Dominant bacteria: *Desulfovibrio vulgaris*, *Desulfitobacterium hafniense*, *Rikenella microfusus* Dominant phyla: *Proteobacteria*, *Firmicutes*, *Bacteroidetes*	Bioanode reversed to biocathode	[27]
		Dominant bacteria: *Clostridium cylindrosporium*, *Desulfotomaculum* sp. Dominant phyla: *Firmicutes*, *Proteobacteria*, *Bacteroidetes*	Biocathode developed in MEC	[132]
	Pond sediment	Dominant bacteria: *D. vulgaris* Dominant phyla: *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*	Biocathode developed in MEC	[9]
	Anodic inoculum of MFC	Dominant bacteria: *Pseudomonas*, *Petrimonas*, *Geobacter*, *Azospirillum* Dominant phyla: *Proteobacteria*, *Chloroflexi*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*	Bioanode reversed to biocathode	[133]
	Generally, mixed culture source enriched in previous MEC	Dominant bacteria: *Desulfovibrionales, Campylobacterales*, *Rhizobiales*, *Clostridiales*, *Bacteroidales* Dominant phyla: *Proteobacteria*, *Chloroflexi*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*	Biocathode of running MECs	[134]
	Anodic inoculum of MFC	Dominant bacteria: *Desulfovibrio* sp., *Pseudomonas* Dominant phyla: *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Chloroflexi*	Biocathode developed in MEC	[10]

conditions. This specific enrichment strategy favors the enrichment of exoelectrogens for quick biofilm development at the cathode, but does not preferentially enrich H_2-evolving biocatalysts.

Analysis of microbial communities in mixed-culture biocathodes revealed the prevalence of certain bacterial phyla, including *Firmicutes*, *Proteobacteria* (or *Pseudomonadota*), and *Thermodesulfobacteriota* (Table 1). Within these three bacterial phyla, various genera and species have been identified, including *Clostridium* sp., *S. oneidensis* MR-1, *Pseudomonas aeruginosa*, *Escherichia coli*, *G. sulfurreducens*, and *R. palustris*, which have been commonly observed in different BESs used for waste-to-energy conversion [57–59]. However, in mixed-culture biocathodes, it becomes challenging to differentiate between microorganisms responsible for H_2 production and others within the biofilm that might consume H_2.

Hydrogenase-based biocathodes

The direct utilization of H_2ases on the cathode has the potential to achieve high selectivity and yields for H_2 production (Box 2) [60]. The use of enzymes for electrochemical H_2 production is not truly an MEC since whole microorganisms are not used, but the concept is reviewed here due to the biotic nature of the cathodes. The enzyme-based approach faces several limitations, with the main one being poor stability over time. Researchers have explored various carbon, metal, and semiconductor-based materials to interface H_2ase enzymes for sustainable H_2 production on the biocathode (see Figure IA in Box 2). The surface properties of the electrodes have a crucial role in enabling enzyme immobilization and facilitating improved electron transport [61]. The electrode surface can be tailored for improved performance by manipulating surface roughness, charge density, and hydrophilicity/hydrophobicity to optimize enzymatic activity [62]. For instance, to attach an H_2ase with a larger subunit diameter of ~4 nm, the surface roughness of an electrode must be >5 nm [63]. Similarly, the surface charge can also impact the interaction of H_2ase with the electrodes. Typically, the surface charge of H_2ase is –11 mV and, therefore, the interacting surface needs to have a net positive surface charge to facilitate electrostatic interaction. **Polymer dots (Pdots)** have been used to achieve a surface charge density of ~+31 mV, thus, could be efficiently used for H_2ase self-assembly [64].

The exploitation of pure H_2ase-immobilized electrodes on a large scale is still in its infancy due to the sensitivity of the enzyme to O_2 and the cost of H_2ase purification and immobilization. Free oxygen attacks the iron (Fe) and sulfur (S) metal centers in the ferredoxin (F_d) cluster and destabilizes the whole structure via the formation of reactive oxygen species (ROS), leading to degradation of both the enzyme and the overall performance (see Figure IB in Box 2). Therefore, in aerobic conditions, the half-life of most of the H_2ase used was <100 h, except for the H_2ase obtained from *Pyrococcus furiosus*, which was stable up to 208 h [65] (see Figure IC in Box 2). Under anaerobic conditions, H_2ase obtained from *Alcaligenes eutrophus* was found to be stable for over 5 h of incubation time [66]. A more detailed summary of the half-life period of H_2ase from different microorganisms is provided in Table S3 in the supplemental information online. The application of H_2ase-modified biocathodes in MECs could be promising for sustainable high-rate H_2 production without the need to supply nutrients or CO_2 (as required for microbe-based biocathodes). However, until the issue of maintaining enzyme stability over time is solved, the use of enzymes in practical applications will have to wait.

Design and configuration of MEC reactors

Reactor design

The reactor configuration (single versus dual chamber) and MEC internal resistance can affect microbial population dynamics at the electrodes. Different reactor configurations have been used for H_2 production in MECs, such as H-cell dual-chamber systems with large electrode

Box 2. Immobilization of H_2ase on different electrodes

H_2ase has been successfully immobilized on different materials, such as pyrolytic graphite edge [116], metallic single-wall carbon nanotubes [117], glassy carbon, and carbon felt [118] (Figure I). These materials have high surface areas and good biocompatibility of the carbon matrix with the enzymes. For example, Hambourger *et al.* purified [FeFe]-H_2ase *CaHydA* from *Clostridium acetobutylicum* on a carbon-felt electrode [118]. Cyclic voltammetry analysis confirmed that the [FeFe]-H_2ase *CaHydA* has a high HER catalytic activity that can achieve as much as ~40% of the current produced by a Pt cathode at a 333 × lower enzyme surface loading (~3.3 pmol cm^{-2}) compared with the loading rate applied for Pt on the cathode (~1.1 nmol cm^{-2} Pt-catalyst) [119,120]. Immobilization of H_2ase on gold electrodes has also been reported to produce H_2 at 1.3 × 10^{-3} mmol H_2 min^{-1} mg^{-1} (or 85 mol H_2 min^{-1} mol^{-1}) catalyst in a single-chamber electrochemical cell [61]. Due to the excellent electronic features of gold, but its high cost, gold electrodes are mainly used for theoretical calculations of HER rate kinetics of biocatalysts rather than for larger scale applications [60]. Recently, a [Fe-Fe] H_2ase was inserted inside black TiO_2 nanotubes (opening diameter of 140 nm and a tube length of ~1 μm) to facilitate direct electron transfer from the TiO_2 matrix to the *Fd-HydA* catalyst center for HER [121]. Both forward and backward (anodic and cathodic) CV scans showed large corresponding oxidative and reductive currents compared with pristine TiO_2, confirming the successful immobilization of *Fd-HydA* on the TiO_2 skeleton. The successful immobilization of [Fe-Fe] H_2ase on TiO_2 could enhance its usability as a self-biased photoelectrode for several fuel cell-based applications in the future [62].

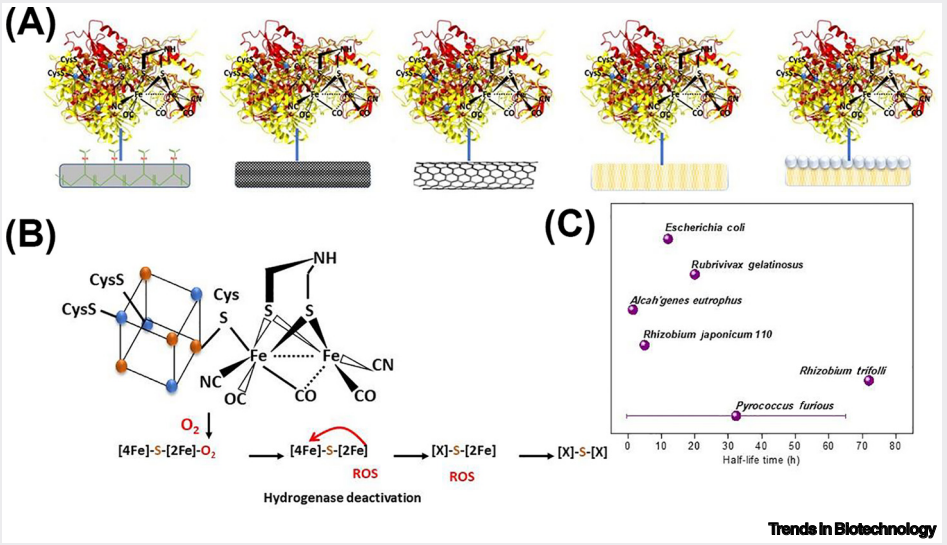

Figure I. Immobilization of hydrogenase (H_2ase) on electrodes and their stability under a stress condition (oxidative stress). (A) immobilization of H_2ase onto different electrode surfaces; (B) degradation of F_d cluster in H_2ase due to oxygen contamination; (C) half-life of different H_2ases in an oxygen environment. [FeFe] H_2ase protein structure shown in (A) is from *Thermotoga maritima*, reproduced from [122] under the terms of the Creative Commons Attribution License. Abbreviation: ROS, reactive oxygen species.

spacing or zero-gap electrolyzers with anode and cathode separated by only a thin polymer membrane (Figure 1F) [8,67]. Zero-gap systems are typically preferred from an electrochemical perspective as they minimize internal resistance and increase the specific surface area, defined as the area of the electrode over the volume of the MEC (A_s, cathode surface area m^2/reactor volume, m^3), increasing volumetric H_2 productivity while minimizing the reactor footprint (Section S1.5 in the supplemental information online) [68]. Deposition of the catalyst layer directly on the membrane to develop a membrane electrode assembly (MEA) can further decrease spacing and minimize the contact resistance [69]. Cloth separators (such as J-cloth) [70] have been used due to their low cost, while ion exchange membranes are preferred for their high conductivity and selectivity toward specific ions (cations, CEM; anions, AEM) [71,72]. Previous studies investigated the impact of membrane charge on MEC performance and indicated that AEM-MECs typically produce more H_2 compared with other types of membranes due to the lower internal resistances (~0.55 mΩ m^{-2} versus 0.65 mΩ m^{-2} for

CEM-MEC). This better performance of AEMs was suggested to be due to improved control of the local pH of the bioanode [8,72].

Maintaining a near-neutral pH is critical for enhancing the viability of the microbial cultures on both electrodes. The HER at the cathode produces hydroxide ions, resulting in the alkalinization of the catholyte, while oxidation of the organic matter or the oxygen evolution reaction at the anode produces protons, leading to acidification of the anolyte [73]. An increase in pH gradient between anode and cathode in two-chamber MECs results in large overpotentials, reducing cell efficiency, while a low bioanode pH adversely impacts maximum current generation from the microbial biofilm. While CEMs are selective for cations and AEMs for anions, these membranes do not discriminate between protons, hydroxide, or the other ions present in solution. Therefore, other cations, such as Na^+ and K^+, which are more abundant than H^+ in typical media, are transported to the cathode through the CEM, which results in the development of large pH differences between the electrodes. AEM also facilitate the transport of anions other than hydroxide if saline or buffered catholytes are used; however, as the catholyte pH rapidly increases, more hydroxide ions will be transported to the anode, balancing proton production by the biofilm. If buffer solutions, such as HPO_4^- or HCO_3^-, are used at the cathode with AEMs, these negative ions could be transported to the anode, increasing the buffer capacity of the anolyte, and helping maintain a near-neutral pH. Therefore, AEMs are preferred in double-chamber MECs over CEMs and typically result in higher performance.

While H-type cells are suitable for pure culture and laboratory studies by isolating the microorganisms present in one or both chambers, these reactors typically have high internal resistances due to the large spacing between the electrodes and the constricted diameters of the side arms connecting the two chambers. High internal resistance in these systems can dramatically limit performance. A previous study indicated that the diameter and length of the tube separating the two chambers in an H-cell MFC were responsible for the large ohmic resistance of the reactor, contributing to more than 80% of the overall internal resistance [74]. A similar impact of the reactor architecture on the internal resistance can be expected for MECs. To reduce ohmic resistance and improve electrochemical efficiency, the electrode spacing needs to be reduced or the solution conductivity should be increased to the maximum possible extent [75]. Typical media for BES have low solution conductivity (~3 mS cm^{-1}), and the tolerance of typical exoelectrogens for high salinity (>30 mS cm^{-1}) is low, indicating that H-type configurations have limited applicability outside the laboratory because of the poor reactor design, resulting in high internal resistance that limits maximum achievable H_2 production rates, such as 0.018 [53], 0.26 [76], 0.12 [28], and 0.45 [20] $m^3.m^{-3}.d^{-1}$, at reasonable applied cell voltages in MECs (Table S1).

Single-chamber MECs, where anodes and cathode are in the same chamber and not physically separated, avoid the utilization of expensive membranes and separators but offer poor control over the microbial communities in the system. Additionally, if an abiotic anode is used, oxygen contamination [77] or chlorine gas evolution can disrupt biocathode performance [78]. For H_2 production, microbes on the cathode can be extremely sensitive to oxygen (which can result in degradation of key enzymes), thus, even a small concentration of dissolved oxygen can hinder cell growth or even damage the biofilm. Therefore, dual-chamber MECs have been proven more efficient in converting electrons into H_2 [10,28]. Another challenge with single-chamber MECs is the low purity of the H_2 produced at the cathode. Single-chamber systems typically have a **coulombic efficiency (CE)** of <50%, compared with nearly 100% for double-chamber reactors. In single-chamber MECs, microbes on the anode or in solution can scavenge and consume the H_2 produced at the cathode, reducing CE. A potential solution to this issue is to increase the spacing between the electrodes, although this will also increase the internal resistance of the cell. Inhibitors can be added to limit H_2 consumption, which not only represents an

additional cost, but the inhibitor will also need to be removed downstream of the cell if the effluent is not reused. Therefore, there is a tradeoff between single and double-chamber systems: single chamber reactors avoid the additional costs of the membrane and its potential degradation but typically generate lower performance due to the large electrode spacing and low purity of the H_2 gas produced. Double chamber reactors, on the other hand, incur in the additional cost of the membrane and separator, and the potential risk of its fouling over time. The absence of a single preferred configuration, coupled with the large variety of electrode types, shapes, and **inoculum** used, hamper the identification of an optimal architecture for bioelectrochemical H_2 generation. Previous studies suggested that the cathode-specific resistance (mΩ m^2) can be used to compare the biocathode performance independently by the cell design and configuration, but only a few studies reported the biocathode-specific resistance[8,79].

Electrode surface area

The successful interaction between electroautotrophic microbes and electrodes has a crucial role in achieving high H_2 production. This interaction is influenced by various factors, including the type of bacteria, the material and surface characteristics of the electrode, and the operating conditions [80]. Several studies showed that the surface area of the cathode is a critical factor governing the performance of MECs in terms of H_2 production [81]. Using an electrode with a small surface area can lead to limited growth of the biofilm on the surface, resulting in low current uptake and, consequently, a low H_2 yield. Numerous studies have shown that increasing the specific surface area of the cathode within the range of 3–50 m^2 m^{-3} is generally associated with higher current density and H_2 production rates, both in single and dual-chamber systems (Figure 4). Therefore, engineered electrode materials and surfaces decorated with nanoparticles, aiming to promote the deposition of a thick biofilm on the cathode were used [49,62,82]. Nevertheless, further research is required to optimize the surface area of the electrode and identify novel nanomaterials that can enhance the specific surface area of the cathode within the constraints of a limited geometric surface area. Biocompatible nanoparticles, such as magnetite, titanium dioxide, and Fe-manganese oxides, can assist electron transfer from cathode to electroautotrophs, which can result in increasing the current density and H_2 production [83–85].

Concluding remarks and future directions

Microbial electrolysis is a sustainable technology with low energy demand that can complement abiotic electrolysis in the H_2 economy by sustainably generating H_2 gas from organic wastes. MECs with biocathodes can operate at 33.2–117 kWh kg^{-1} H_2 by leveraging H_2-generating

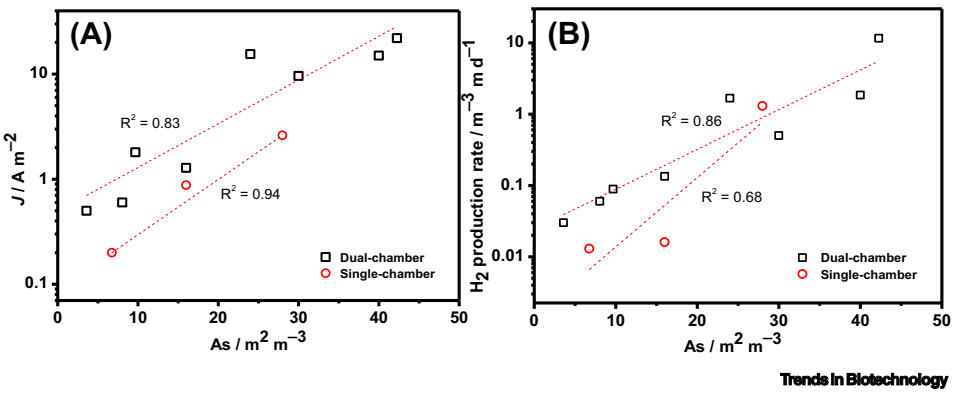

Figure 4. Analysis and trends of hydrogen (H_2) production in different microbial electrolysis cells (MECs) (single and dual-chamber; only representative studies were selected for analysis). (A) Current density versus specific surface area and (B) H_2 production rate versus specific surface area. Figure created using data from [9,29,43,86,88,128–131].

Outstanding questions

What are the fundamental mechanisms governing electron transfer from the electrode through the microorganisms for H_2 gas production and how can these mechanisms be better understood and controlled to improve overall system performance and efficiency?

What could be the most effective strategy to improve the interaction between the electrode and the microbes to minimize energy loss during electron exchange between them?

Can gene-editing technology based on CRISPR be used to obtain a robust microbial strain with enhanced capability to catalyze HER?

How can the practical applications of biocathode-driven MECs be advanced? What are the key steps needed to transition from laboratory-scale studies to large-scale implementation for sustainable H_2 production on a commercial scale?

microorganisms at the cathode, reducing energy consumption compared with abiotic cathodes (170–995 kWh kg^{-1} H_2) [86]. However, using microorganisms at the cathode increases the complexity of the system, resulting in performance variability due to microbial population dynamics, reactor configuration, and operational conditions [87]. The composition of the liquid fuel (wastewater or synthetic media) providing the substrate to the microorganisms can also impart performance variability. Despite these limitations, biocathode-assisted MECs in laboratories are being adopted for commercial applications at larger, more useful scales [88–91]. Most of the pilot scale studies have been conducted using reactors in the range of 10–100 L, which are still too small for commercial-scale implementation of MECs (Table S1). Thus, more efforts need to be directed toward the development of robust and efficient large-scale MECs to enable a rapid transition to commercial applications (see Outstanding questions). The primary challenge in the pilot-scale operations is improving the microbial current uptake, which translates directly into improved H_2 production. Other challenges are reactor design (minimizing internal resistance and preventing oxygen contamination), development of inexpensive electrode materials, and operational issues, such as maintaining culture purity, biofouling, sludge management, and H_2 purity.

The development of a robust electroactive microbial consortium (via enrichment and isolating microbial strains with improved cellular H_2ase expression) could enhance H_2 production in MECs [62]. Genetic engineering in electroactive microbes could be an invasive technique to obtain the desired metabolic response, as has been achieved in cyanobacteria and microalgae [92,93]. For example, the inactivation of uptake hydrogenase (Hup), responsible for unidirectional uptake of H_2, in a model cyanobacterium *Anabaena* sp. PCC 7120 enhanced H_2 productivity by four- to sevenfold compared with wild-type [93]. The possibility of CRISPR-edited hybrid microbial strains can be explored to facilitate the development of better performing biocatalysts (although ethics and policies could be a challenge [94]). One possibility could be to leverage the electroactivity of cyanobacteria in MECs to improve H_2 production in both light and dark conditions [95,96]. Further, genetic engineering in cyanobacteria could impose a more robust and efficient system for H_2 production apparatus, regardless of light availability or the presence of oxygen [93]. This concept is an example of the exciting possibilities in synthetic biology and biotechnology for enhancing H_2 production with biocathodes in MEC operation.

Advances in MEC reactor architecture and design are needed for field applications and could result in optimal electrochemical performance. Using zero-gap electrolyzers with biocathodes can dramatically decrease the internal resistance of the cell while minimizing reactor footprint (electrode surface:volume ratio) [68]. While enabling better performance results from lower internal resistance, the presence of a membrane in these zero-gap systems increases capital costs and could result in reduced stability due to chemical or microbial fouling. Using zero-gap systems represents an additional challenge due to the feed water composition, which can clog the anode chamber due to suspended solids, requiring influent pretreatment. Caution must be used in the selection of the membrane, which primarily controls ion transport and can have a large impact on pH balance between the anode and cathode chambers. Further research should be conducted to investigate the utilization of cost-effective catalysts to enhance microbe–electrode interactions for improved MEC performance. Biocompatible transition metal oxides, especially, those containing Fe-metal nodes, could be effective in improving electron exchange between the cathode and the microbes.

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Declaration of interests

None declared by authors.

Supplemental information

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