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Review and comparison of biohydrogen-producing methods

Przegląd i porównanie metod produkcji biowodoru

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ABSTRACT: Biohydrogen is a promising renewable energy carrier that can be produced through various biotechnological methods, including dark fermentation, photofermentation, biophotolysis, and microbial electrolysis cells (MECs). Among these, dark fermentation stands out due to its operational simplicity, scalability, and compatibility with the existing biogas technologies. While photofermentation offers higher substrate-to-hydrogen conversion efficiency, its dependence on light and a complex reactor design limits its practical application (above 100 ml/L·h). MECs provide high-purity hydrogen but are hindered by low yields and high energy input requirements. Biophotolysis suffers from low hydrogen production rates (1–8 ml/L·h) and enzyme inhibition by oxygen. Although all methods can utilise organic waste and wastewater, dark fermentation presents the most practical solution for decentralised hydrogen production, particularly in agricultural and industrial settings. Despite lower hydrogen purity and the need for gas purification, its advantages in cost, reactor availability, and microbial flexibility make it a viable option for sustainable energy generation. Biohydrogen generated via dark fermentation can be used as fuel for proton exchange membrane fuel cells and solid oxide fuel cells after purification. This method offers hydrogen generation rates high enough (as high as 500–1000 ml/L·h) to supply enough hydrogen for small fuel cell stacks (200–2000 W), and the size of the bioreactor is still reasonable.

Keywords: biohydrogen, waste, circular economy, dark fermentation, photofermentation, biophotolysis, microbial electrolysis cells.

STRESZCZENIE: Biowodór jest obiecującym odnawialnym nośnikiem energii, który może być wytwarzany różnymi metodami w tym fermentacją ciemną, fotofermentacją, biolizą oraz w technologii mikrobiologicznych ogniw elektrochemicznych (MEC). Spośród nich fermentacja ciemna wyróżnia się prostotą operacyjną, skalowalnością oraz kompatybilnością z istniejącymi technologiami biogazowymi. Chociaż fotofermentacja zapewnia wyższą efektywność konwersji substratu do wodoru, jej zależność od światła oraz złożona konstrukcja reaktorów ograniczają zastosowanie praktyczne (powyżej 100 ml/L·h). Ogniwa MEC umożliwiają uzyskanie wodoru o wysokiej czystości, jednak ograniczają je niskie uzyski oraz wysokie zapotrzebowanie energetyczne. Bioliza charakteryzuje się niskimi szybkościami produkcji wodoru (1–8 ml/L·h) oraz inhibicją enzymów przez tlen. Mimo że wszystkie metody mogą wykorzystywać odpady organiczne i ścieki, fermentacja ciemna stanowi najbardziej praktyczne rozwiązanie dla zdecentralizowanej produkcji wodoru, szczególnie w sektorze rolniczym i przemysłowym. Pomimo niższej czystości wodoru oraz konieczności jego oczyszczania, jej zalety w zakresie kosztów, dostępności reaktorów oraz elastyczności mikrobiologicznej czynią ją realną technologią zrównoważonej produkcji energii. Wodór wytwarzany w fermentacji ciemnej może być wykorzystywany jako paliwo w ogniwach paliwowych PEMFC oraz stałotlenkowych ogniwach paliwowych (SOFC) po procesie oczyszczania. Metoda ta umożliwia uzyskanie szybkości produkcji wodoru na poziomie 500–1000 ml H_2/L·h, co pozwala na zasilanie małych zestawów ogniw paliwowych (200–2000 W), przy jednoczesnym zachowaniu rozsądnych rozmiarów bioreaktora.

Słowa kluczowe: biowodór, odpady, gospodarka o obiegu zamkniętym, ciemna fermentacja, fotofermentacja, biofotoliza, mikrobiologiczne ogniwa elektrolityczne.

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Introduction

Hydrogen is increasingly seen as a key energy carrier in the transition towards low-carbon energy systems. According to the International Energy Agency, global hydrogen demand has reached around 97 Mt per year, and nearly 95% is still produced using fossil-based technologies, mainly steam methane reforming (SMR) and coal gasification (IEA, 2024). Although these processes are well established and economically efficient, they generate significant CO_2 emissions, making their decarbonisation essential for achieving climate neutrality (Bhuiyan and Siddique, 2025).

Today, low-carbon hydrogen is mainly produced via water electrolysis powered by renewable electricity ("green hydrogen"), biomass gasification, and fossil-based hydrogen combined with carbon capture and storage (Bhuiyan and Siddique, 2025; Sharma et al., 2025). Among these options, electrolysis is widely considered the most promising long-term pathway. However, its expansion is still limited by high electricity demand, dependence on renewable energy availability, and the need for substantial infrastructure development (Bhuiyan and Siddique, 2025). Biomass gasification can convert lignocellulosic materials into hydrogen, but it requires energy-intensive pre-treatment and high-temperature operation, which reduces its overall efficiency and economic attractiveness (Sharma et al., 2025).

In this context, biohydrogen produced through biological processes is attracting growing interest as a complementary option. It includes dark fermentation, photofermentation, biophotolysis, and microbial electrolysis cells (MECs), all of which use organic substrates such as wastewater, agricultural residues, and food industry wastes (Nagarajan et al., 2017; Ahmad et al., 2024; Emetere, 2025). These processes operate under mild conditions and naturally fit within the circular bioeconomy approach by linking waste treatment with energy recovery (Ahmad et al., 2024; Sharma et al., 2025). Despite many years of research, biohydrogen technologies remain at a relatively early stage of development, and their industrial implementation is still limited (Ananthi et al., 2024; Emetere, 2025).

Biohydrogen is not intended to replace established hydrogen production routes such as SMR or electrolysis. Its main limitations include relatively low yields, operational instability, sensitivity to process conditions, and the production of gas mixtures containing CO_2 and other impurities, which require additional purification steps (Yusuf et al., 2015; Rahman et al., 2016; Tripathy et al., 2025). These issues significantly limit its use in large-scale, centralised hydrogen systems.

Because of this, the most realistic role for biohydrogen appears to be in small-scale, decentralised applications. This includes wastewater treatment plants, agro-industrial facilities, and organic waste management systems, where hydrogen can be recovered as a by-product of biological degradation (Ananthi et al., 2024; Emetere, 2025). In such cases, its value lies more in improving resource efficiency and supporting circular economy strategies than in serving as a primary hydrogen supply source.

Rather than competing with green hydrogen or other mature low-carbon technologies, biohydrogen is likely to complement them. Integrated into hybrid energy systems, it may support distributed hydrogen production, waste valorisation, and partial decarbonisation of organic waste streams, particularly in regions where access to renewable electricity is limited (Ananthi et al., 2024; Sharma et al., 2025).

This paper reviews and compares the main biological hydrogen production pathways, focusing on their principles, key limitations, and realistic application potential. Special attention is given to their role in circular economy systems and their possible contribution to future hydrogen-based energy structures.

Biohydrogen production using biophotolysis methods

Biophotolysis is a process of generating hydrogen that utilises the energy of photons. This process is present in cyanobacteria and is similar to photosynthesis as it uses its mechanisms. Biophotolysis can be direct or indirect, and in both cases, photosystems PS I and PS II take part. During the operation of these photosystems, an electron carrier – ferredoxin (Fd) – is reduced by an electron. To prevent the depletion of the pool of oxidised forms of Fd available for PS I and PS II, its regeneration is necessary. Regarding direct biophotolysis, Fd is oxidised by hydrogenase enzymes, which reduce protons

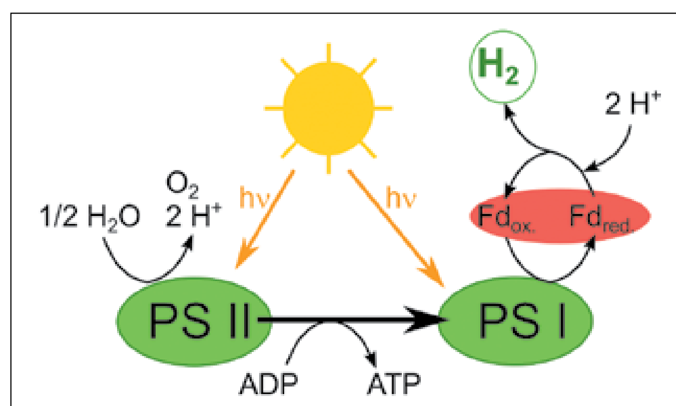


Figure 1. Diagram of direct biophotolysis, where: hν – light; PS I – photosystem I; PS II – photosystem II; $Fd_{ox.}$ – oxidized form of ferredoxin; $Fd_{red.}$ – reduced form of ferredoxin; ADP – adenosine diphosphate; ATP – adenosine triphosphate, a biological energy carrier (Li et al., 2022; Arimbrathodi et al., 2023; Gómez Hernández et al., 2026)

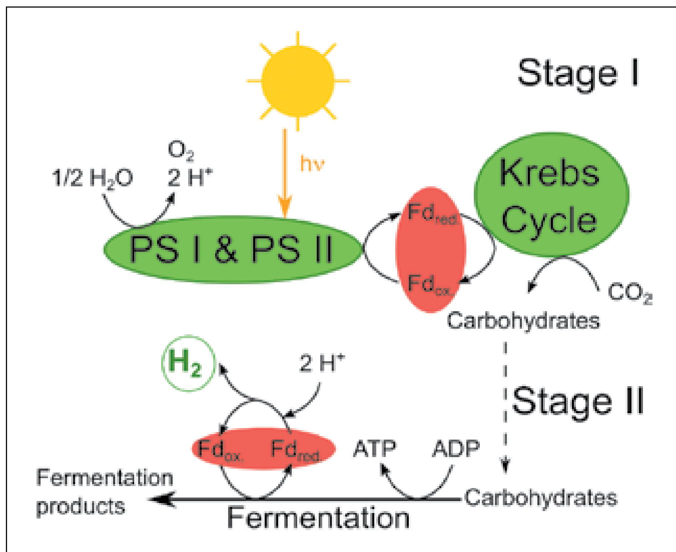


Figure 2. Diagram of indirect biophotolysis, where: hν – light; PS I – photosystem I; PS II – photosystem II; $Fd_{ox.}$ – oxidized form of ferredoxin; $Fd_{red.}$ – reduced form of ferredoxin; ADP – adenosine diphosphate; ATP – adenosine triphosphate, a biological energy carrier (Li et al., 2022; Arimbrathodi et al., 2023; Gómez Hernández et al., 2026)

to molecular hydrogen. Meanwhile, concerning indirect biophotolysis, the process takes place in two stages. In the first stage, the reduced form of Fd obtained from the photosystems is used to fix CO_2 and synthesize carbohydrates, similar to classical photosynthesis. During the second phase, the carbohydrates generated in Stage 1 are fermented reducing Fd, which is then regenerated. The regeneration of Fd is carried out by the enzyme hydrogenase, leading to the formation of molecular hydrogen (Li et al., 2022; Arimbrathodi et al., 2023; Gómez Hernández et al., 2026). The metabolic pathways described above are illustrated in Figures 1 and 2.

During the biophotolysis process, the only substrates needed are water, light, CO_2 (in the case of indirect biophotolysis), and macro- and micro-elements (required for algae). Theoretically, biophotolysis, and especially direct biophotolysis, does not require the addition of substrates (such as carbohydrates), contrary to the other methods described.

The main limitation of this method is its dependence on light. Despite this, many researchers have analysed the possibility of using this method for biohydrogen production, especially due to the fact that direct biophotolysis seems very attractive because of its simplicity. Unfortunately, direct biophotolysis has one major limitation: hydrogenase, the enzyme responsible for hydrogen production, is inhibited by oxygen. As mentioned earlier, oxygen is one of the main products of direct biophotolysis. To overcome this obstacle, some researchers in implementation-focused studies propose conducting the process under conditions deficient in such elements as sulphur, potassium or nitrogen to limit the generation of oxygen. Regarding the species Scenedesmus obliquus, potassium may be substituted with sodium in the feed, leading to the deactivation of PS II, which is responsible for the generation of oxygen (Papazi et al., 2014). Another method used to reduce oxygen generation is to grow microbial cultures in conditions under which the sources of the electrons for hydrogen generation are saccharides instead of water molecules (as it is in classical photosynthesis) (Faraloni et al., 2025). The results of selected studies found in the literature on biohydrogen production via biophotolysis are shown in Table 1.

According to the data presented in Table 1, the rate of hydrogen production was very low in all cases and mostly fell within the range of 1–8 ml of biohydrogen per hour from every 1 L of working volume of the bioreactor. Considering that providing

Table 1. Examples of representative research results concerning biohydrogen generated via biophotolysis reported in the literature (Maswanna et al., 2018; Ruiz-Marin et al., 2020; Touloupakis et al., 2021)

Microorganism	Conditions	Illumination conditions	H_2 yield [ml/L]	H_2 production rate [$ml \cdot L^{-1} \cdot h^{-1}$]	Substrate and medium	Source
Chlorella vulgaris BEIJ strain G-120	Illuminated	150 $\mu mol \cdot m^{-2} \cdot s^{-1}$	896	4.98	–	(Touloupakis et al., 2021)
	Dark	None	405	2.08		
Chlorella vulgaris immobilised	Cell growth in Stage 1, H_2 production in Stage 2 anaerobic conditions	60 W lamp 140 $\cdot m^{-2} \cdot s^{-1}$	60.4	1.63	Wastewater + glucose	(Ruiz-Marin et al., 2020)
Scenedesmus obliquus immobilised			128	8.53		
Tetraspora sp. CU2551 immobilised	Anaerobic	29 $\mu E/m^{-2} \cdot s^{-1}$	307	2.84	S-deprived	(Maswanna et al., 2018)
Synechocystis sp. PCC 680 immobilised	Anaerobic dark conditions	60 mmol photons /$m^{-2} \cdot s^{-1}$	14.5	0.09	Nitrogen-free medium BG110-Tris	(Touloupakis et al., 2016)

light energy either from artificial sources or the sun during the process was necessary, whether using sunlight energy from photovoltaic (PV) systems to power the electrolyser would be more efficient is worth reconsidering. For example, a 1 MW electrolyser is capable of generating 200 m^3 (200·10^3 L) of hydrogen per hour. In comparison, Ruiz-Marin et al. (2020) used a 60 W lamp and reached the highest hydrogen production value of merely 8 ml (8·10^{-3} L) of biohydrogen per hour from every 1 L of bioreactor working volume (see Table 1, Ruiz-Marin et al., 2020). By multiplying the system of such bioreactors to reach the light source energy demand of 1, only 133 L/h of hydrogen would be generated, and the working volume of bioreactors would reach 16.7 m^3. Moreover, during the biophotolytic process, an additional substrate, a carbon source, would be needed so that hydrogen generation occurs mainly via Stage 2 of indirect biophotolysis. This strategy is used in most research papers, while strategies that prioritise the mechanism of direct biophotolysis are seldom employed. This is probably because oxygen is generated during photosynthetic activities, inhibiting the hydrogenase enzyme.

Hydrogen production with the use of microbial electrolysis cells

Another interesting technology is MECs. It is similar to standard electrolysis, but in MECs, microorganisms that are capable of releasing electrons into the environment act as catalysts on at least one of the electrodes. A characteristic feature of an MECs is that the system utilises small amounts of energy from additional power sources and the chemical energy of organic substrates to create electrons can reduce protons into molecular hydrogen. The oxidation of the substrate takes place on the anode side, while the reactions generating hydrogen occur on the cathode side. As electrons migrate through an external electric circuit, protons can freely migrate through the electrolyte if an additional membrane separating the anode area from the cathode area is used then it has to allow proton transport.

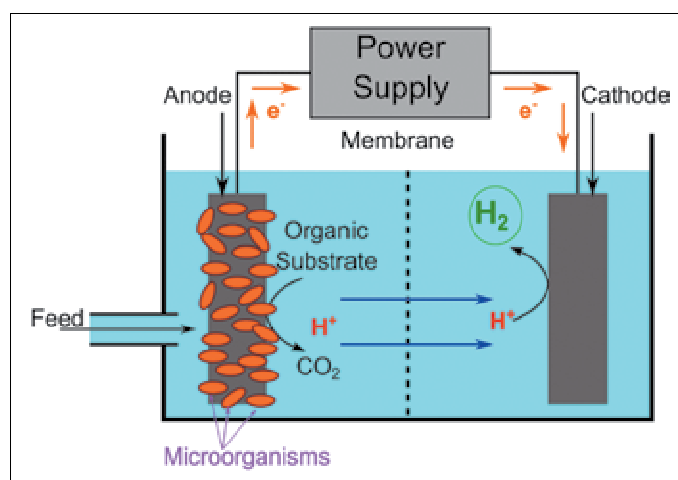


Figure 3. Diagram of a simple microbial electrolysis cell

The anode is made of a conductive material – often a porous transition metal, such as Ni – and is placed in an environment rich in electrogenic bacteria. The anode is often covered and overgrown by these bacteria. The cathode is usually made of a conductive material covered with platinum. However, Jeremiasse et al. (2010) successfully developed MECs using cathodes made of transition metals employed biocathodes, bacteria that reduce hydride ions while consuming electrons. In the case of a biocathode, the hydrogen production rates and volume can be increased by enriching the microbial culture with hydrogen-reducing bacteria prior to the main operation stage of the MECs (Jafary et al., 2017). The hydrogen production rate relies on factors similar to those observed in standard fermentation. In addition, when the system includes a separate cathode chamber, pure hydrogen can be generated (Jafary et al., 2017; Jeremiasse et al., 2010; Logan et al., 2008; Mitov et al., 2017; Wang et al., 2017). A diagram of a simple MEC is presented in Figure 3.

Table 2. Research results concerning biohydrogen generated via MECs

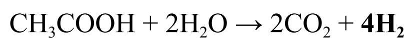
Microorganism	Substrate	Voltage [V]	H_2 production rate [ml·L^{-1}·h^{-1}]	Source
Enriched wastewater inoculum	Acetate	0.45	15.40	(Liu et al., 2005)
Enriched isolate from sulphate-rich paper-mill wastewaters	Acetate	0.5	0.83 (with potential for improvement up to 27.5)	(Rozendal et al., 2006)
Microorganisms present in wastewater substrate	Wastewater	0.5	0.01	(Ditzig et al., 2007)
Enriched isolates from soil and wastewaters	Acetate	0.5	1.10	(Cheng and Logan, 2007)
Microbial consortia used earlier in an acetate-fed microbial fuel cell	Acetate	0.8	3.12	(Call and Logan, 2008)
		0.6	1.99	
		0.4	1.02	
Microbial consortia from previous experiments	Acetate	1	0.30	(Rozendal et al., 2007)
Shewanella oneidensis MR-1 (ATCC)	Acetate	0.6	0.69	(Hu et al., 2008)

Selected examples of the use of MECs and the corresponding results from the literature are presented in Table 2.

One of the advantages of this technology is that using separate chambers for the anode and cathode enables pure hydrogen generation. Unfortunately, this method is characterised by the slow generation of hydrogen despite the consumption of electric and chemical energy (see Table 2). However, energy consumption for hydrogen generation is much lower than in traditional electrolysis (Liu et al., 2005).

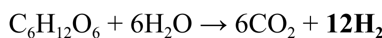
Biohydrogen production through photofermentation

Photofermentation is a promising biotechnological process for generating biohydrogen. This process is carried out by photosynthetic bacteria, especially *Rhodobium*, *Rhodobacter*, *Rhodospirillum* and *Rhodopseudomonas*. Hydrogen generation is catalysed by the nitrogenase enzyme, whose primary function is to fix molecular nitrogen into ammonia. Under stress conditions, especially nitrogen deficiency, this enzyme catalyses the formation of molecular hydrogen. Bacteria that possess such a catalytic activity utilise sunlight energy (via bacterial photosystems and biological energy carriers) to generate electrons with energy high enough to perform energy-demanding reactions, such as nitrogen fixation. The electrons for this reaction come from organic compounds, usually short-chained organic acids, which are by-products of the metabolism of those bacteria. Other substrates, such as glucose, can also be utilised (Azwar et al., 2014; Yusuf et al., 2015; Li et al., 2022). In the case of an acetic acid substrate, the overall reaction is described by the following formula:



$$CH_3COOH + 2H_2O \rightarrow 2CO_2 + \mathbf{4H_2}$$

In the case of glucose as a substrate, the reaction of its decomposition to CO_2 and hydrogen during photofermentation is described by the following formula:



$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + \mathbf{12H_2}$$

Figure 4 presents a simplified diagram of photofermentation.

Although the above reactions represent the theoretical stoichiometry of substrate conversion, in real systems, photofermentation is accompanied by competing metabolic pathways. Part of the carbon flux is directed towards biomass synthesis and residual metabolite formation, which limits hydrogen yields. Therefore, the efficiency of the process strongly depends on operational conditions, such as light intensity, substrate type, and nitrogen availability (Hitam and Jalil, 2023).

In Table 3, examples from the scientific literature on the utilisation of photofermentation for biohydrogen production

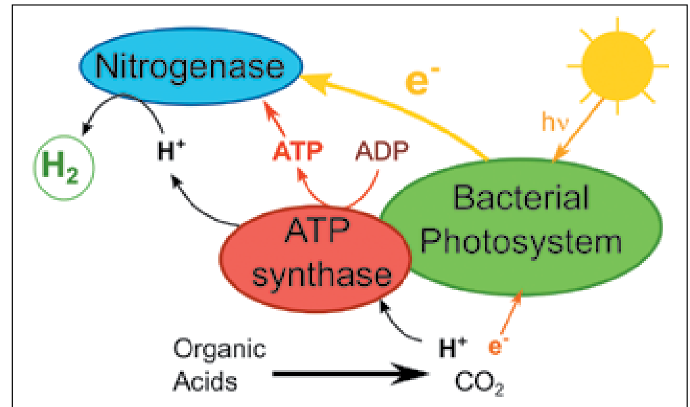


Figure 4. Simplified diagram of photofermentation (Li et al., 2022)

are presented. Table 3 shows that biohydrogen production is largely affected by numerous factors and methods of cultivation, as described in the cited papers. Assawamongkholsiri et al. (2018) and Seifert et al. (2010) reported that inoculum concentration affects bioreactor operation: increasing the inoculum concentration shortens the lag phase. Futhermore, Assawamongkholsiri et al. (2018) indicated that above a certain inoculum concentration, biohydrogen generation is negatively affected. This effect is attributed to the possibility that an excessively high number of microorganisms may limit light penetration throughout the working volume of the bioreactor.

Seifert et al. (2010) analysed a wide range of factors that influence the biohydrogen generation process: substrate pretreatment methods (especially sterilisation), light intensity, inoculum concentration and substrate concentration. An interesting observation was that the presence of suspended solid particles positively affected biohydrogen production, which was attributed to the fact that the solid phase also acted as an additional source of organic substrates. Moreover, the researchers stated that the substrate must have been properly diluted; otherwise, it could negatively impact biohydrogen production or even inhibit it completely. The reason may be that concentrated wastewaters contain ammonium ions, which have an inhibitory effect on nitrogenase activity. Additionally, excessive dilution of the substrate results in lower biohydrogen yields. These findings lead to the conclusion that determining the optimal substrate concentration for the selected systems is necessary (Seifert et al., 2010a, 2010b).

While analysing substrate concentration, Zhu et al. (2018) observed a correlation between substrate concentration, shaking of the bacterial culture, and biohydrogen production. At low substrate concentrations, an increase in culture shaking led to a decrease in biohydrogen production. A different situation was observed regarding high substrate concentrations a rise in shaking intensity increased biohydrogen production.

Among other factors influencing biohydrogen production, pH and illumination should be mentioned. The use of

Table 3. Examples of the representative results from research on biohydrogen generated via photofermentation

Microorganism	Substrate	Illumination [W/m^2]	Hydrogen yield [ml/L]	H_2 production rate [ml·L^{-1}·h^{-1}]	Batch time [h]	Source
Rhodobacter sphaeroides	Sodium lactate	200	265	–	100	(Zhu et al., 2007)
	Sodium lactate + 2.4 mg/L Fe^{2+}		2293	–		
Rhodopseudomonas aecalis RLD-53	Sodium acetate + 10% CO_2 atm.	4000 lux 60 W lamp	1740	–	192	(Liu et al., 2009)
	Sodium acetate + 40% CO_2 atm.		400	–		
Rhodobacter sphaeroides RV	Acid hydrolysed wheat starch	3000 lux	1012	6.28	–	(Kapdan et al., 2009)
Rhodobacter sphaeroides	Sugar wastewater	7500 lux	2614	5.24	552	(Assawamong-kholsiri et al., 2018)
Consortium HAU-M1 isolated from silt, sewage, pig manure and cow dung	Corn stover powder 4 g	6000–7000 lux	Ca. 1330 0 rpm	Ca. 30 0 rpm	120	(Zhu et al., 2018)
	Corn stover powder 4 g		Ca. 940 160 rpm	Ca. 38 160 rpm		
	Corn stover powder 10 g		Ca. 2080 0 rpm	Ca. 33 0 rpm		
	Corn stover powder 10 g		Ca. 3400 160 rpm	Ca. 125 160 rpm		
Rhodobacter sphaeroides	Brewery wastewater 5% *v/v*	116	1400	42	120	(Seifert et al., 2010a)
	Brewery wastewater 10% *v/v*		2240	61	80	
	Brewery wastewater 20% *v/v*		520	40	120	
Rhodobium marinum	Soy sauce wastewater	60	2670	38.14	–	(Anam et al., 2012)
Rhodobacter sphaeroides O.U. 001 (ATCC 4919)	Dairy waste	9000 lux	3620	56	–	(Seifert et al., 2010b)
Purple non-sulphur bacteria (PNSB): *Rhodobacter sphaeroides* NCIMB 8253	Brewery wastewater + pulp and paper mill effluent	7000 lux	17244*	708*	–	(Hay et al., 2017)
	Brewery wastewater		10000*	385*	–	
	Pulp and paper mill effluent		9590*	527*	–	
Rhodobacter sphaeroides NCIMB 8253	Palm oil wastewater + pulp and effluents from the paper industry	7000 lux	8724	763	–	(Budiman and Wu, 2016)

* The working volume of the bioreactor was below 100 ml, and extrapolating yield and production rate to a working volume of 1000 ml may lead to results in which practical issues not present in small bioreactors would be omitted, such as providing uniform light distribution inside the bioreactor.

raw wastewaters, not subjected to any pre-treatment, whose pH = 4, led to no biohydrogen production. However, when the same system was operated with an additional mechanism that injected portions of 0.5 M NaOH solution every 12 h to stabilise the pH, a satisfactory biohydrogen yield of 3600 ml/L was achieved (Seifert et al., 2010b).

As photofermentation is dependent on light, increasing illumination intensity has a positive impact on biohydrogen

production. Nevertheless, excessive light intensity can lead to a decrease in biohydrogen production due to damage done to the bacterial cells caused by light (Seifert et al., 2010b).

Assawamongkholsiri et al. (2018), researchers found differences in the consumption rates of various fatty acid substrates by microorganisms during culture incubation. Some substrates were completely and rapidly depleted, while others remained detectable throughout the entire incubation period. Further analysis of this aspect may help in designing an optimal substrate composition to avoid the effects of bottlenecks and deficits of crucial substrates during incubation.

The results achieved in the studies cited above demonstrate significantly higher biohydrogen production rates and yields compared to those observed in the previously described processes.

Biohydrogen production through dark fermentation methods

Biohydrogen production methods that utilise dark fermentation are based on the fact that through some microbial anaerobic metabolic pathways, hydrogen is generated as one of the by-products. Similar to biophotolysis, hydrogen production is meant to recycle biologic electron carriers that take part in the following cycles of metabolic pathways. Glycolysis is one of the most fundamental anaerobic pathways for utilising the chemical energy of glucose. During this process, electron carriers are reduced and must be recycled. Aerobic organisms regenerate those electron carriers using oxidative phosphorylation pathways, which are found in aerobic bacteria and mitochondria. Anaerobic organisms must find other pathways; the most common include the reduction of pyruvate, a product of glycolysis, in a wide range of fermentative pathways. In some cases, fermentation oxidises pyruvate and generates more reduced electron carriers, which are oxidised by enzymes catalysing the reduction of protons to molecular hydrogen.

This situation is present in the case of acetate fermentation and butyrate fermentation (Yusuf et al., 2015). The reaction formulas for these fermentations are presented below.

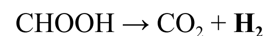
Acetate fermentation:

$$C_6H_{12}O_6 \rightarrow 2CH_3COOH + 2CO_2 + \mathbf{4H_2}$$

Butyrate fermentation:

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + \mathbf{2H_2}$$

Some microorganisms can also perform in interesting pathways if they possess an enzyme called formate hydrogen lyase (FHL). In this pathway, pyruvate created by glycolysis is converted into formate, which in turn undergoes a reaction catalysed by FHL:



$$CHOOH \rightarrow CO_2 + \mathbf{H_2}$$

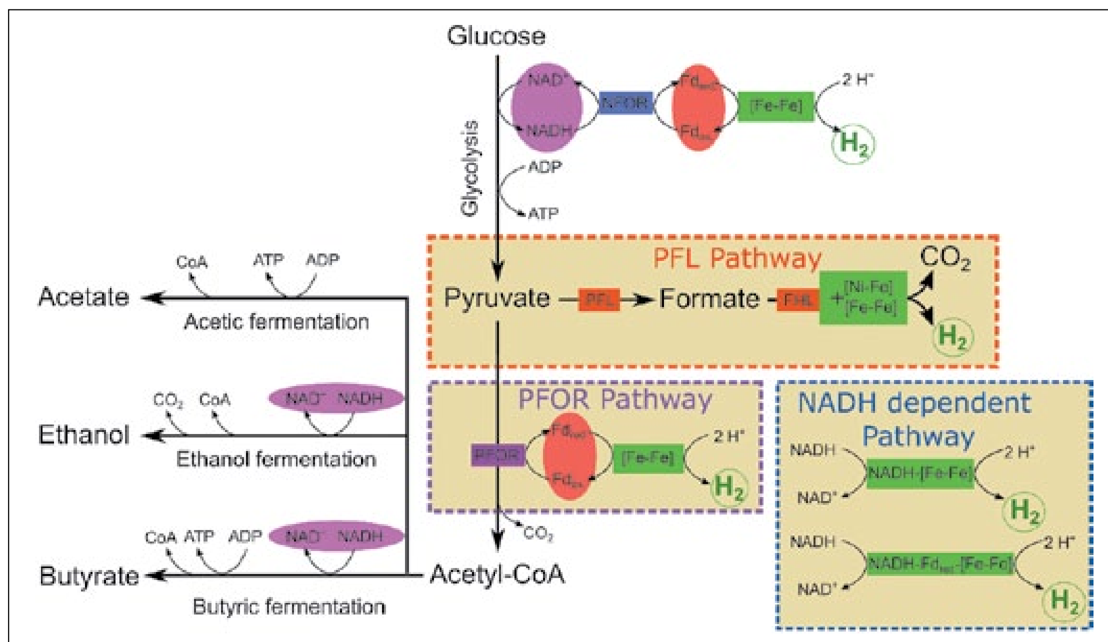


Figure 5. Diagram of dark fermentative metabolic pathways generating hydrogen. Where: Fd_{ox}/Fd_{red} – oxidised/reduced forms of a ferredoxin, a biological electron carrier; NAD+/NADH – oxidised/reduced forms of biological electron carrier; ATP – adenosine triphosphate, a biological carrier of energy; ADP – adenosine diphosphate, a product of the release of energy upon ATP hydrolysis; CoA – Coenzyme A; PFL – enzyme pyruvate formate lyase; FHL – enzyme formate hydrogen lyase; PFOR – enzyme puruvate ferredoxin oxidoreductase; NFOR – enzyme NAD+ ferredoxin oxidoreductase; [Fe-Fe] – enzyme hydrogenase with Fe-Fe active sites; [Ni-Fe] – enzyme hydrogenase with Ni-Fe active sites; NADH-[Fe-Fe] – enzyme NADH-dependent Fe-Fe hydrogenase; NADH-Fd_{red}-[Fe-Fe] – enzyme reduced ferredoxin-dependent Fe-Fe hydrogenase (Łukajtis et al., 2018; Das and Basak, 2021; Albuquerque et al., 2024)

The reaction formulas shown above are only basic ones. Real-world biochemical processes are much more complicated and multi-staged, utilise a wide range of additional co-factors and substrates, and are catalysed by enzymes. A simplified diagram of these fermentations is shown in Figure 5.

Glucose is the most desired substrate for metabolism. Most substrates used for biohydrogen production (e.g., agricultural waste, food industry waste, and wastewater) contain only small amounts of glucose, they are mainly composed of complex compounds, such as cellulose, starch, proteins, and fats. These compounds are gradually decomposed by microorganisms during the incubation of microbial consortia and release simpler nutrients that are easily consumed by microbes.

As inferred from the fermentation formulas, the highest yield of biohydrogen obtained from 1 mol of glucose (as the substrate unit) is generated during acetate fermentation. A parameter that compares the yield of hydrogen per unit of substrate is one of the most important factors when comparing results obtained from different solutions (e.g. microbial consortia, substrate, conditions or technology). Because of such differences between fermentative pathways, a thorough selection and optimisation of microorganism species and strains are necessary to ensure that acetic fermentation processes dominate over their counterparts. In any case, the desired hydrogen yields are in the range of 2–4 mols of hydrogen per 1 mol of glucose (Yusuf et al., 2015; Rani et al., 2024). In research papers biohydrogen or hydrogen-rich biogas production, most of the attention is paid to the possibilities of utilising waste or by-products from agriculture, food processing, waste biomass and municipal wastewaters (Karadag et al., 2014; Hosseini et al., 2015; Nikolaidis and Poullikkas, 2017; Rani et al., 2024).

Thanks to the similarity between dark fermentative biohydrogen technologies and biogas production, the development of biohydrogen bioreactors and production processes may be significantly easier compared to other biohydrogen production methods. A simplified diagram of an exemplary dark fermentation bioreactor for biohydrogen is shown in Figure 6.

One of the most fundamental advantages of this technology is its environmental friendliness due to the use of natural substrates (biological wastes) and the mild conditions of the process (30°C–40°C temperatures, sometimes 50°C–70°C; pH near neutral). Thus, fitting the conditions of the culture to achieve optimal results is always possible.

The findings of Wang et al. (2018) help point out the factors (other than microbial strain selection) that determine the efficiency of biohydrogen production, such as temperature, pH, initial substrate pre-treatment, and control over the composition of the atmosphere above the culture. The authors concluded that to optimise the process, constant monitoring and reacting to changes in those parameters via automation technologies

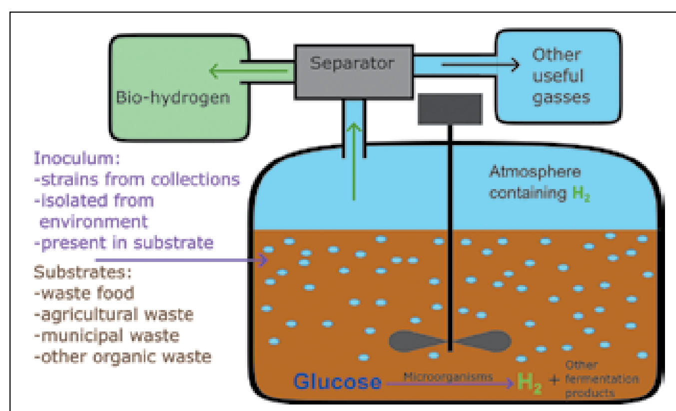


Figure 6. Diagram of an exemplary dark fermentation biohydrogen bioreactor

is necessary (Wang et al., 2018). Table 4 presents the studies selected from the literature on biohydrogen production via dark fermentation.

Data from Table 4 enable the deduction of which factors impact biohydrogen production and in what way. As observed, both the yields and hydrogen production rates achieved in the analysed literature fall within a wide range of values. Similar to photofermentation, these values are significantly higher than those obtained in biophotolysis and MECs.

One of the most important factors influencing the performance of a selected biohydrogen production system is the choice of microorganisms. In the literature on biotechnological methods of hydrogen production, two main approaches are described for obtaining and screening the microorganisms:

- The use of a well-defined inoculum (from microorganism banks or based on earlier research) possessing microorganisms with proven hydrogen-producing activity (Zhang et al., 2013; Al-Shorgani et al., 2014; Saratale et al., 2014; Wu et al., 2017).
- The use of environmental consortia enriched with biohydrogen-producing strains under selective conditions (Yang et al., 2007; Li et al., 2010; Bansal et al., 2013; Wongthanate et al., 2014; Ramos and Silva, 2017).

The literature indicates that wastewater sludge and sediments from wastewater treatment facilities are usually a good source of microorganisms for biohydrogen production. An inoculum selected for biohydrogen production may only contain a single, specific strain – either to allow thorough characterisation of that strain or when attempting to exclude potential undesired interactions with other microorganisms. A different approach is to use microbial consortia and/or additives to the culture medium (such as enzymes) to achieve a synergy effect and increase hydrogen production (Zhang et al., 2013; Saratale et al., 2014; Hassan and Morsy, 2015; Wu et al., 2017). Such consortia can be artificially designed using reference strains from microbial banks, but most often, they are obtained through

Table 4. Research results concerning biohydrogen generated via dark fermentation

Microorganism	Substrate	Additional information	H_2 yield [ml/L]	H_2 production rate [$ml \cdot L^{-1} \cdot h^{-1}$]	Source
Municipal wastewater sludge isolate; enriched and immobilised	Sucrose	Continuous bioreactor, best results at HRT = 2 h	–	1800	(Karadag et al., 2014)
Municipal wastewater sludge isolate; pretreated with heat	Waste pastry	*Aspergillus awamori* and *Aspergillus oryzae* used for pre-treatment of raw substrate; continuous bioreactor	–	255.3	(Wang et al., 2018)
Enterobacter sp. CN1	Agar	Agar substrate that has been enzymatically saccharified	5170	36.0	(Wang et al., 2018)
	Galactose		3757	26.0	
Anaerobic digester sludge isolate; pre-treated with heat	Tofu processing waste	Continuous bioreactor, best results at HRT = 4 h	–	500	(Lin et al., 2009)
Anaerobic sludge from fruit processing wastewater isolate	Solution of dried ultrafiltration whey permeate from a dairy plant	Continuous bioreactor with reduced pressure, OLR* 35 COD**/m³ · d	–	59.2	(Han et al., 2016a)
		Continuous bioreactor with no pressure control, OLR 35 COD/m³ · d	–	29.2	
		Continuous bioreactor with reduced pressure, OLR 30 COD/m³ · d	–	53.3	
		Continuous bioreactor with no pressure control; OLR 30 COD/m³ · d	–	43.8	
A mixture of sludges from a wastewater processing plant and a wastewater treatment lagoon from a cattle farm; pre-treated with heat	Liquid swine manure supplemented with glucose	37°C, HRT*** = 16 h, pH = 4.7	–	14.4	(Wu et al., 2017)
		37°C, HRT = 16 h, pH = 5.0	–	92.5	
		37°C, HRT = 16 h, pH = 5.3	–	25.0	
Sludge from a vinasse treatment bioreactor. Pre-treated with heat to promote thermophilic microorganisms	Vinasse	55°C, HRT = 0.5	–	710	(Kim and Lee, 2010)
		55°C, HRT = 4.0	–	360	
		65°C, HRT = 4.0	–	290	
		75°C, HRT = 4.0	–	8	
	Cheese whey	55°C, HRT = 0.5	–	5360	
		55°C, HRT = 4.0	–	1010	
		65°C, HRT = 4.0	–	1380	
		75°C, HRT = 4.0	–	280	
Escherichia coli XL1-Blue *Enterobacter cloacae* DSM 16657 Sewage sludge from a wastewater treatment plant; pretreated with heat	Beverage wastewater	80 h, sewage sludge	1138	38	(Kisielewska et al., 2015)
		80 h, sludge + *Escherichia coli*	1338	73	
		80 h, sludge + *Enterobacter cloacae*	1219	50	
		80 h, sludge + both strains	1281	63	
Enterobacter cloacae IIT-BT 08	Cane molasses	2 L batch, 37°C, 100 rpm	1280	–	(Li et al., 2010)
	Distillery effluent	2 L batch, 37°C, 100 rpm	1156	–	
	Starchy wastewater	2 L batch, 37°C, 100 rpm	472	–	
	Cane molasses + groundnut de-oiled cake	50 L batch, 37°C, 100 rpm	2900	270 (140 avg.)	
	Cane molasses + groundnut de-oiled cake	10000 L batch, 37°C, 100 rpm	7600	560 (270 avg.)	

cont. Table 4

Microorganism	Substrate	Additional information	H_2 yield [ml/L]	H_2 production rate [$ml \cdot L^{-1} \cdot h^{-1}$]	Source
Enterobacter aerogenes RM 08 MTCC2822; pretreated with heat	Rice mill wastewater	1 L batch, 33°C, acid hydrolysis	2813	103.8	(Ramos and Silva, 2017)
		1 L batch, 33°C, enzymatic hydrolysis	3887	117.7	
		1 L batch, 33°C, acid+ enzymatic hydrolysis	4091	134.6	

[*] OLR: organic load rate;
[**] COD: chemical oxygen demand;
[***] HRT: hydraulic retention time.

a proper selective treatment of environmental isolates. These consortia are usually characterised by good biohydrogen production parameters. However, in some rare cases, such as in the use of co-cultures, they generate less hydrogen. Even so, they can replace other usually costly (financially and energetically) technological processes, such as the hydrolysis of cellulose or other complex compounds (Hassan and Morsy, 2015).

Microbial consortia isolated from the environment are often enriched by the conditions of the preliminary culture (temperature, pH, feed composition, etc.). The goal of this strategy is to selectively promote the growth of these microbial strains, which are characterised by high biohydrogen production activity. This enrichment of the inoculum can be achieved by physical and chemical pre-treatment before the proper culture (i.e. heating and changing the pH) (de Amorim et al., 2012; Kisielewska et al., 2015). Notably, microorganisms generating hydrogen via dark fermentation are anaerobic, and in some cases, even low oxygen content (above 2%) may lead to a significant decrease in hydrogen production (Yang et al., 2007; Kim and Lee, 2010; Li et al., 2010; de Amorim et al., 2012; Bansal et al., 2013; Wongthanate et al., 2014; Kisielewska et al., 2015; Gomez-Romero et al., 2016; Kanniah Goud et al., 2017; Ramos and Silva, 2017). According to several researchers, the best strategy for enrichment is thermal treatment, which is a quick and simple way to remove undesired groups of microorganisms, especially methanogenic bacteria, preserving those capable of generating hydrogen (Mu et al., 2007; Li et al., 2010).

In some research studies, in which environmental isolates were used, a strain composition of the consortium was also analysed using genetic methods by sequencing the gene coding for 16S rRNA subunit. This helped identify which strains were active in the analysed consortium (Yang et al., 2007; Davila-Vazquez et al., 2009; Pessiot et al., 2012). The bacterial strains and species most often found in hydrogen-generating consortia isolated from the environment are *Clostridium* sp. (especially *Clostridium beijerinckii* or *Clostridium acetobutylicum*), *Klebsiella* sp., *Enterobacter* sp., *Kosmotoga* sp. and *Lactobacillus* sp. In addition, even the species commonly used in microbiological research, *Escherichia coli*, has the ability to generate hydrogen (Fernandes et al., 2013; Saratale et al., 2014; Vendruscolo, 2015; Etchebehere et al., 2016; Gomez--Romero et al., 2016).

Depending on the microbial species and strain used, temperature and pH must be maintained in the range optimal for hydrogen production. The temperature is mainly dependent on the requirements of the selected microorganisms. In the case of mesophilic microorganisms, optimal temperatures are usually in the range of 20°C–40°C. Microorganisms that prefer lower temperatures (i.e. below 15°C) are called psychrophiles. Microorganisms that favour high temperatures are called thermophiles, and they prefer temperatures above 40°C, up to 122°C (Ananthi et al., 2024). In the literature on biohydrogen production, researchers mostly used microorganisms that are mesophiles or thermophiles. Usually, hydrogen production rates are much higher when utilising thermophiles (Ramos and Silva, 2017). Moreover, the high culture temperature creates unfavourable conditions for common microorganisms that may contaminate the bioreactor, particularly during the addition of new substrates or feed.

In the case of mesophilic microorganisms, increasing the temperature above optimal values leads to a decrease in hydrogen production. Chaudhary et al. (2015), cultured bacteria at 35°C, 40°C, 45°C and 50°C, generating 92, 86, 52 and 36 ml of biohydrogen after 48 h, respectively. The cultures were incubated in 250 ml vessels. Similar conclusions were made by Ziara et al. (2019), who analysed the influence of both temperature and pH on biohydrogen production. Biohydrogen production was most intensive in a temperature range of 35°C–45°C and a slightly alkaline pH (Ziara et al., 2019).

In the case of pH, the most commonly reported values were in the range of 7–5. In such conditions, the highest rates of hydrogen production and its share in the gas mixture are achieved. Slightly acidic pH values may result from medium

acidification by metabolic by-products, such as volatile fatty acids (VFAs) (Li et al., 2010; Wongthanate et al., 2014).

The literature reports that boosting hydrogen production rates is possible by increasing the concentration of the nutrients and substrates in the culture. However, this can lead to an increase in VFA content in the gas mixture created in the bioreactor (Davila-Vazquez et al., 2009; Lin et al., 2009; de Amorim et al., 2012; Kisielewska et al., 2015).

Kisielewska et al. (2015) demonstrated that reduced pressure conditions significantly increase the hydrogen production rate, the hydrogen content in the gas mixture, and the hydrogen yield per substrate unit. Compared to the control bioreactor, the system operating under reduced pressure showed higher hydrogen production efficiency, with increases ranging from 20% to 100%, depending on the organic loading. Under optimal conditions, the hydrogen production rate reached 7.1 L per day. The authors also reported that pressure reduction mitigated the negative effects of increased substrate concentration (Kisielewska et al., 2015).

Biohydrogen production is heavily influenced by the methods of substrate pre-treatment before fermentation. Thermal treatment and hydrolysis should be emphasised here (Kim and Lee, 2010; Saratale et al., 2014; Han et al., 2016; Wu et al., 2017). Substrate pre-treatment aims to modify the structure and composition of a substrate so that it can undergo fermentation more easily. Additionally, it allows for the removal of other competitive microorganisms that can result in undesired reactions. Hydrolysis can be carried out using physical (Kim and Lee, 2010; Redwood et al., 2012; Si et al., 2016) or biotechnological (Han et al., 2016a, 2016b; Wu et al., 2017) methods. Such a procedure usually leads to an increase in both hydrogen production rates and hydrogen content of the gas mixture in the bioreactor.

Biotechnological processes can be split into batch and continuous cultures. In batch cultures, fermentation is carried out until the end (of a substrate) or upon arriving at a specific point in time (especially if the desired product is degraded as fermentation is prolonged). A new batch of substrates and fresh inoculum is supplied to the bioreactor only after emptying and cleaning the bioreactor (Zhang et al., 2013). In the case of continuous cultures, a fresh stream of substrates is constantly supplied to the bioreactor, and the spent or depleted medium is removed. The product is usually isolated from the spent medium.

In the continuous method, one of the most important parameters is hydraulic retention time (HRT), which describes a medium flow intensity through the bioreactor. Usually, a decrease in this parameter leads to an increase in the hydrogen production rate and hydrogen content in the gas mixture but a decrease in the substrate utilisation factor (Kim and Lee, 2010; Gomez-Romero et al., 2016; Ramos and Silva, 2017). Unfortunately, after crossing a certain threshold of HRT, a drastic decrease in hydrogen production is observed (Kim and Lee, 2010; Fernandes et al., 2013; Gomez-Romero et al., 2016; Han et al., 2016b). This phenomenon is caused by the flushing out of microorganisms from the bioreactor (Davila-Vazquez et al., 2009). To address this issue bioreactors with immobilised microorganisms were developed. In these systems, a further decrease in HRT leads to an increase in the hydrogen production rate (Lin et al., 2009; Fernandes et al., 2013; Han et al., 2016a). Crucially, the porosity of the bed must be ensured with immobilised microorganisms, enabling the free diffusion of substrates and metabolites to prevent the accumulation of metabolic products that may inhibit biohydrogen production or consume it in other metabolic pathways (Fernandes et al., 2013). Even if the risk of microbial flush-out is eliminated, decreasing the HRT still has some drawbacks. While it does increase hydrogen production rates even more, it also dramatically decreases the substrate utilisation factor because most substrates have no opportunity to be utilised by microorganisms before they are removed from the bioreactor (Lin et al., 2009; Kim and Lee, 2010; Ramos and Silva, 2017). A comparison between these two methods, batch and continuous, leads to the conclusion that, while continuous methods generate a high and stable stream of hydrogen, batch methods maximise substrate utilisation.

Some researchers have analysed integrated systems, in which biohydrogen was generated as a result of several processes that took place in their individual bioreactors. All these bioreactors were connected in a series so that effluents from one bioreactor became feed and substrates for another. If necessary, separation steps between bioreactors were added to the system to protect microbial consortia in subsequent bioreactors from harmful by-products. Usually, such systems are used to hydrolyse complex substrates when hydrolysing microorganisms have requirements (temperature, pH, etc.) different from biohydrogen-producing microorganisms. This strategy helps achieve promising results (Redwood et al., 2012). Other applications of a series of bioreactors can be used to generate maximum amounts of usable products from a substrate. Similar to the two-step fermentation system, which was used to generate biohydrogen and biomethane, the researchers used two 1.8 L bioreactors in this setup. The first bioreactor was fed with thermally hydrolysed corn stalks and generated hydrogen. The second bioreactor was fed with used effluents from the first bioreactor and generated methane, allowing the system to achieve the production rates of hydrogen at 2.34 L/d (54 ml/L·h) and methane at 2.54 L/d (59 ml/L·h) (Si et al., 2016; Yeshanew et al., 2016). Similar two-step systems can be used to combine two different fermentative technologies that is

dark fermentation and photofermentation. These solutions take advantage of the fact that the photofermentation process uses organic acids as substrates, which are also major by-products of dark fermentative biohydrogen production. In such hybrid systems, dark fermentation is performed in the first bioreactor, and the effluent rich in organic acids acts as feed for the photofermentative bioreactor. The described systems allow for maximum substrate utilisation (Meky et al., 2020; Zhang et al., 2018, 2024).

Li et al. (2010) managed to obtain promising results for larger-scale biohydrogen production after initial research and the optimisation of culture conditions. By properly adjusting pH, swine manure could be used to generate 2.2 L of hydrogen per day from each 1 L of working volume (0.092 L/L·h). The authors used a 4 L bioreactor and were able to convert each 1 L of manure into 1.48 L of hydrogen (Li et al., 2010). By using thermophilic bacteria, higher incubation temperatures and decreased HRT values, hydrogen production rates as high as 5.36 L/L·h can be obtained in a continuous bioreactor (Ramos and Silva, 2017).

Biohydrogen production through dark fermentation offers several advantages over the previously described methods, such as not requiring illumination of the microbial culture and relying common on metabolic pathways, unlike the case of biophotolysis or photofermentation. Similar to other described methods, dark fermentation can also utilise waste organic material and wastewaters as substrates. In addition, media and substrates do not need to be transparent to light, which is a key limitation of light-dependent technologies. Another advantage of dark fermentation technologies for biohydrogen production is their similarity to biogas production. This similarity makes potential scale-up and commercialisation easier and more cost-effective, thanks to decades of experience in their use and the availability of ready-to-use hardware and technologies on the market (Yusuf et al., 2015; Rahman et al., 2016; Tripathy et al., 2025).

The major disadvantage of dark fermentative biohydrogen technologies is the need to purify and enrich the obtained hydrogen because the gas mixture generated by microorganisms contains additional by-products of their metabolism. Similar to other described technologies, dark fermentation also shares a common disadvantage with most biotechnological methods: low hydrogen production rates compared to a conventional method, such as waste gasification.

Dark fermentation produces not only hydrogen but also a range of soluble by-products; the main ones are VFAs. Focusing only on hydrogen production is not enough to make the process economically viable, as the whole product spectrum must be examined. VFAs are not just waste; they are valuable resources that can be recovered and used in different ways for example, to produce bioplastics (PHA), and bio-based chemicals or as carbon sources for other biological processes. Aside from making the overall process more profitable, this also fits the idea of a circular bioeconomy. VFA recovery is becoming increasingly important, and without it, scaling up dark fermentation would be difficult.

Hybrid biological hydrogen production systems that combine dark fermentation with photofermentation is a promising approach to improve overall hydrogen yields. In this configuration, organic acids produced during the dark fermentation stage are further converted into hydrogen by photoheterotrophic bacteria under light conditions, which enables more complete substrate utilisation (Hallenbeck and Benemann, 2002; Nath and Das, 2004). As a result, the overall energy recovery from organic substrates can be significantly higher compared to single-stage dark fermentation systems (Kapdan and Kargi, 2006). Although such hybrid systems offer clear thermodynamic and yield advantages, their practical application is still limited by operational complexity, the need for controlled illumination, and sensitivity to environmental conditions. Nevertheless, ongoing research continues to optimize these systems due to their strong potential for improving biological hydrogen production efficiency (Das, 2001; Levin, 2004).

Comparison of biohydrogen production methods

Biohydrogen can be produced using biological methods or MECs. In general, however, these approaches still achieve lower production rates and yields than conventional technologies, despite offering some important advantages. At present, MECs are not widely used in practice, mainly because of their relatively low hydrogen production rates, the need for substrates and additional electrical energy, and their overall complexity compared to conventional electrolysers, which are much more efficient and easier to operate. Among the biological approaches, photofermentation and direct biophotolysis are both light-dependent. Photofermentation is attractive because of its high substrate to hydrogen conversion efficiency and its ability to reach hydrogen production rates above 100 mL/L·h, and in some cases even several hundred mL H_2 per litre of reactor working volume per hour. Direct biophotolysis, in contrast, does not require external substrates, but its practical use is limited by low production rates and sensitivity to oxygen, which inhibits the hydrogenase enzymes involved in hydrogen production. A key challenge in light dependent processes is the need to ensure proper illumination throughout the entire reactor volume, which makes reactor design more complicated and increases the costs. The use of artificial light further raises energy consumption, and relying on sunlight

may create competition for space with PV systems, which are often more economically attractive.

Dark fermentation offers a simpler and more flexible alternative. Since it does not require light, it can rely on well-established and widely available reactor designs. Hydrogen production depends on the operating conditions, substrate type (often waste or wastewater), and the microbial community. Although production rates can be similar to those achieved in photofermentation, this method has some drawbacks, including lower substrate conversion efficiency and the formation of byproducts such as VFAs which require additional purification. Taking all this into account, dark fermentation and photofermentation seem to be the most promising biological methods in terms of hydrogen production rate. However, the lack of dependence on light gives dark fermentation a clear practical advantage. It is easier to implement, simpler to scale up, and can benefit from existing experience and infrastructure developed for biogas production. In contrast, photofermentation often requires the design of new types of reactors, which increases both costs and technical challenges. Another important difference between these methods lies in the microorganisms involved. Processes such as MECs, photofermentation, and biophotolysis typically require specific strains with specialised metabolic pathways and carefully controlled conditions. Dark fermentation is more flexible in this respect, as hydrogen-producing pathways are found in a wider range of microorganisms, including thermophilic species with high production potential (Ramos and Silva, 2017). Hydrogen purity is also crucial for practical applications. MECs can produce high-purity hydrogen, especially when the cathode and anode chambers are separated. In most other biological methods, hydrogen is part of a gas mixture. For example, photofermentation mainly produces hydrogen and CO_2, which are relatively easy to handle and may even be suitable for direct use in some fuel cell systems if contaminants are absent. Direct biophotolysis produces a mixture of hydrogen and oxygen, which raises safety concerns and reduces efficiency. In dark fermentation, the presence of VFAs and other volatile compounds means that the gas usually requires purification, particularly for use in proton exchange membrane fuel cells (PEMFCs), although it may still be suitable for solid oxide fuel cell (SOFC) systems. Despite their potential, all biological methods are still limited by relatively low hydrogen production rates. For instance, a 200 W PEMFC stack requires around 120 L of hydrogen per hour at full power. Under optimal thermophilic conditions, very high production rates (up to about 5 L/L·h) are possible, which may allow the use of relatively small reactors. However, such performance requires very short hydraulic retention times and large amounts of substrate. Under more typical conditions, much larger reactor volumes are needed. This leads to a practical limitation: ensuring a sufficient and continuous supply of substrate. In household applications, this can be difficult to achieve. In contrast, industries such as the food sector produce large amounts of wastewater, making them better suited for biohydrogen production. In such cases, hydrogen generation can be combined with waste treatment, improving the overall economic viability of the process. For smaller-scale applications, batch systems may be more suitable, as they can better handle irregular substrate supply. Similar solutions have been used for many years in biogas production, but hydrogen demand in households is not constant and varies throughout the day, which makes storage an important issue. One possible solution is to combine production with storage technologies, such as metal hydrides, to ensure a stable hydrogen supply for fuel cell systems.

Final conclusions

Biohydrogen can be produced through several biological routes, including dark fermentation, photofermentation, direct biophotolysis, and microbial electrolysis cells (MECs). These methods share the advantage of operating under mild conditions and using organic waste or wastewater as feedstock, but their hydrogen production rates remain lower than those of thermochemical processes such as biomass gasification. Among them, MECs can produce very high-purity hydrogen, but they are complex, require additional electrical input, and still suffer from low overall yields. Photofermentation achieves the highest substrate-to-hydrogen conversion efficiency, but it depends heavily on a stable light supply, which makes reactor design more complicated and increases energy costs. Direct biophotolysis is even more limited due to strong oxygen sensitivity and very low hydrogen output. In contrast, dark fermentation is the simplest and most robust option. It does not require light, is compatible with existing biogas infrastructure, and can be easily scaled. Its main drawbacks are lower conversion efficiency and the formation of by-products, but these are outweighed by its practicality and flexibility, including the possibility of using thermophilic microorganisms to improve performance. In terms of gas quality, MECs provide the cleanest hydrogen, while photofermentation and biophotolysis generate gas mixtures that are easier to purify than those from dark fermentation, which often contain volatile fatty acids and other impurities. However, these mixtures can still be used in applications such as solid oxide fuel cells if harmful compounds, especially sulfur-based ones, are removed. A major limitation across all biological methods is the relatively low hydrogen production rate. For instance, supplying a 200 W PEM fuel cell requires around 120 L of hydrogen per hour, which demands either highly ef-

ficient small-scale reactors or much larger systems. Overall, although none of the technologies is yet fully competitive with conventional hydrogen production, dark fermentation offers the best balance between simplicity, scalability, and integration potential, particularly in waste-rich sectors such as agriculture and the food industry. It therefore remains the most promising option for decentralized biohydrogen production despite its lower efficiency.

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