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Review

A Game Changer: Microfluidic Technology for Enhancing Biohydrogen Production—Small Size for Great Performance

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Abstract: One of the approaches widely used today to intensify processes is their miniaturization. Small, compact, portable devices that can be used directly in the field will become popular in the near future. The use of microstructured devices is becoming more widespread in diagnostics, analytics, and production, so there is no doubt that the same approach is being applied to energy production. The question is whether it is possible to create an energy production system that has all the external characteristics of a miniaturized device but is sustainable, durable, environmentally friendly, based on renewable sources, and cost-effective. The first challenge is to choose a production route, an energy source that has the required characteristics, and then to adapt this production on a microscale. Among the different energy sources, biohydrogen meets most of the requirements. The carbon emissions of biohydrogen are much lower, and its production is less energy-intensive than conventional hydrogen production. Moreover, it can be produced from renewable energy sources. The challenge today is to make this process sustainable due to the low substrate conversion, production rate, and yield. Microfluidic systems are one of the technologies that could address the above shortcomings of the current biohydrogen production processes. The combination of microdevices and biohydrogen production opens up new possibilities for energy production. Although this area of research is growing, the focus of this review is on the possibility of using microfluidics for biohydrogen production.

Keywords: microfluidics; biohydrogen; sustainably; environmentally friendly; renewable processes

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1. Introduction

Accelerating fossil fuel depletion, market instability, and negative environmental impacts are just some of the reasons why the Kyoto Protocol [1] and the recent Paris Protocols [2] call for the use of clean, green, and renewable energy sources. Biofuels (bioethanol, biodiesel, biohydrogen, etc.) are considered an acceptable alternative to fossil fuels, whose use reduce carbon dioxide emissions, make many countries independent of major fossil producers, and stabilize energy prices, which are extremely important nowadays. Biofuels can be obtained from different sources and by applying different technologies. Among them, hydrogen is considered one of the most promising alternative energy solutions and is expected to bring a revolution to the energy supply of the 21st century [3]. This is because hydrogen has a high energy content (143 kJ/g) [4]; does not release CO_2 and other toxic gasses (CO, NO_x, SO_x, etc.); and the energy produced by H_2 is 2.75-fold higher than that of hydrocarbon fuels [5]. Hydrogen does not exist in nature but can be produced by chemical and biological methods. The main disadvantages of the conventional, chemical method of hydrogen production are the investment and overall costs, high energy consumption during production, and low process efficiency [6,7]. To overcome the above problems, and

to justify and improve the process of hydrogen production itself ecologically and economically, intensive work is being done to improve existing and develop new technologies and processes. Biological processes for hydrogen production have become accepted as a good alternative to chemical processes. The hydrogen produced in such processes is referred as to biohydrogen. Biological processes require less energy and operate in mild reaction conditions (atmospheric pressure and mild temperature). The most favorable method for biohydrogen production is fermentation [8] (dark fermentation or photo-fermentation or hybrid dark and photo-fermentation). Unfortunately, regardless of the production method, the substrate conversion, production rate, and yield are still low. To overcome these problems, new approaches are being explored, such as the better use of light [9], genetic modification of microorganisms used in the process [10], and process optimization [11]. Another direction is to develop new reactor designs or even to change the scale of operation, i.e., to move from a macro- to microscale. Microfluidic systems are one of the new technologies that could overcome the above shortcomings of the current biohydrogen production processes. The combination of biohydrogen production and device miniaturization could represent a significant step into the future of medicine, sensors, small-scale local biofuel production facilities, and domestic energy supply. However, as with any new technology, there are obstacles that must be overcome in order for this technology to come to life. The development of integrated systems is always a challenge. The number of cells per unit volume and the availability of reduced media will likely lead to completely different cell behavior. The developments of a modular system, analytics, a completely new fluid behavior at the microscale, material selection, etc. are just some of the challenges that will be addressed in this manuscript.

In this review paper, the focus is on the possibility of using microfluidics for biohydrogen production. First, we explain the basic principles of hydrogen and biohydrogen production. Then, the advantages of microfluidics and their properties that can be used for a better process performance are discussed. Finally, the recent achievements in the field of combining microfluidics and biohydrogen production are explained, and a brief outlook on the future of biohydrogen production is given.

2. Hydrogen Production—A Challenge with Undefined Colors

Hydrogen is a chemical that is used in various processes today. It can be used as a feedstock in various processes such as ammonia and fertilizer production, methanol and polymer synthesis, petroleum refining (hydrocracking and hydrotreating), the pharmaceutical industry, etc. It is estimated that 55% of the hydrogen produced is used for ammonia production, 25% for refineries, and about 10 % for methanol production. The remaining 10% is used for other applications. Despite the fact that hydrogen is mainly used as a raw material, it has great potential as an energy source [4]. Nowadays, due to the global energy crisis, there is an increased need for the development of new energy sources. According to Zhang et al. [7], hydrogen is considered the cleanest and most promising energy source of the 21st century, and according to Horvath et al. [12], hydrogen fuel cells are most likely to replace fossil internal combustion engines in 2040. Different sources [13–15] assume different demands for hydrogen, but a rough estimate is shown in Figure 1.

It is also believed that the use of hydrogen can significantly support the decarboxylation process, with a focus on decarboxylation in the transportation, industrial, and heating sectors. Switching to cleaner fuels such as biofuels, nuclear energy, and hydrogen could lead to a 22% reduction in greenhouse gas (GHG) emissions [12]. Some of the advantages and disadvantages of hydrogen as a fuel are listed in Table 1.

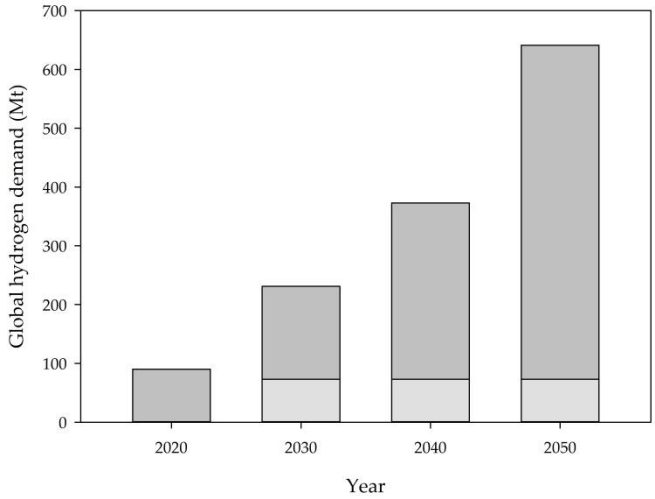

Figure 1. Estimations of the hydrogen demand for the period from 2020 to 2050 (□ minimum and □ maximum projected demands) (adopted from [13,15]).

Table 1. Advantages and disadvantages of hydrogen as a fuel [6,7].

Advantages	Disadvantages
produced from various sources	dependence on fossil fuels to drive some processes
high energy conversion	investment and overall costs
environmentally friendly	storage and transport
renewable	highly flammable
regeneration	
zero carbon emission	
reduces carbon footprint	
versatility of use	

Since hydrogen is the most abundant element on Earth, it is accessible and renewable. The major drawback is that it does not occur naturally as a molecule, so it must be produced from various sources. Hydrogen can be produced by different thermodynamic; electrochemical; and biological processes, such as electrolysis, pyrolysis, oil reforming, gasification, fracking, photo-fermentation, dark fermentation, etc., and from various sources such as natural gas, coal, renewable electricity, biomass, etc. [16–19]. To distinguish hydrogen produced by different processes, it is assigned different colors (Table 2). The color classification of hydrogen can vary depending on the study [20–23], but the main colors are green, blue, gray, and turquoise [18]. Some processes produce carbon, while others are carbon-free. Until a few years ago, 95% of the hydrogen produced worldwide was gray hydrogen produced by steam methane reforming (SMR), autothermal reforming (ATR) of natural gas, and by the partial oxidation (POX) of coal or heavy oil [24]. Each process produces syngas (a mixture of H_2 and CO) from which carbon monoxide must be removed, resulting in significant CO_2 production that is released into the atmosphere [25]. Since the GHG emissions from this process are very high (about 530 Mt/a), nonrenewable sources are depleted, and this process is very energy-intensive, so research is being conducted into alternative processes. For this reason, other hydrogen colors such as blue, turquoise, and especially green hydrogen are becoming increasingly important [16]. The basic idea is to use renewable energy sources (RES) in combination with energy efficient technologies to produce hydrogen in a process with low GHG emissions. When CO_2 is captured and stored (Carbon Capture and Storage (CCS) process) by downstream processes as part of the SMR process, the hydrogen produced is referred to as blue hydrogen. Greenhouse gas emissions from this process are low but only if the captured CO_2 is permanently stored [25].

Table 2. The colors of hydrogen. Inspired by [18].

Gray	Blue	Turquoise	Green
Hydrogen produced by steam **methane reforming** or **coal gasification** using **natural gas** or **coal**.	Hydrogen produced by **steam methane reforming** or **gasification with carbon capture** and **storage** using **natural gas**.	Hydrogen produced by **methane pyrolysis** from **natural gas**.	Hydrogen produced by polymer **electrolyte membrane water electrolysis** using **water**.
GHG footprint: high	**GHG footprint**: low	**GHG footprint**: carbon free	**GHG footprint**: carbon free

To produce hydrogen without carbon emissions, methane pyrolysis is used. This is a process of methane cracking in which H_2 and C are formed [26,27]. Since no CO_2 is produced in the process, this turquoise hydrogen is considered to pave the way for the energy transition [28]. Another carbon-free technology for hydrogen production is electrolysis. In this process, water is split into O_2 and H_2 by an electric current. If the electric current comes from renewable sources, the hydrogen produced is called green hydrogen [29,30].

As with any process, the use of a particular process and substrates determines the final price of the product. Depending on the production process and the origin of the substrate, the price of hydrogen varies (Figure 2). Currently, the price of gray hydrogen is about 0.8–2 EUR/kg, while green hydrogen costs about 3–5 EUR/kg [20]. Newborough and Cooley [25] estimated in 2020 that green hydrogen will soon be cheaper than blue due to falling costs in renewable electricity and electrolysis. Then, it will be cheaper than gray hydrogen and, eventually, cheaper than natural gas. Unfortunately, the economic crisis in 2022 has shown that their predictions were too optimistic. Unless new solutions are found to reduce production costs, 95% of the hydrogen produced worldwide will continue to be gray hydrogen.

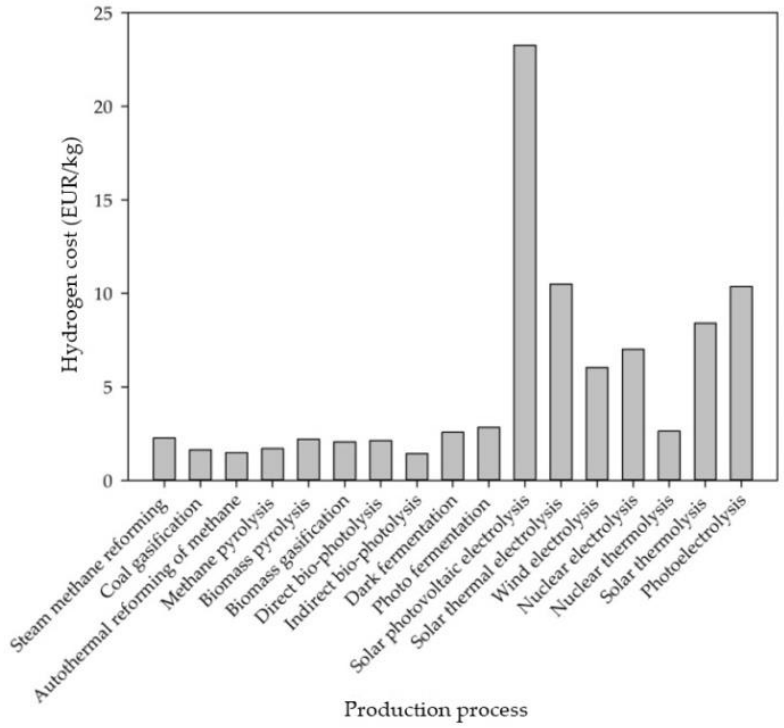

Figure 2. Hydrogen costs based on different production processes [7].

In addition to the hydrogen types mentioned above, there are also some types that have not yet been assigned a color [31–34]. This is mainly due to the fact that these production technologies are still in the development and research phase, and it will take some time before they reach the level of development of today's technologies. The use of biomass and biohydrogen is certainly one of the most interesting approaches to hydrogen production.

Biohydrogen Production

Biohydrogen ($BioH_2$) is the hydrogen produced in biological processes by metabolism. Compared to black/grey hydrogen production, $BioH_2$ production is much cleaner and more sustainable, because carbon emissions are much lower, and the process is less energy-intensive. In addition, these processes use renewable substrates, which makes them interesting. The idea of $BioH_2$ production is not new, and the synthesis routes are well-known. What is new and challenging is the industrial sustainability of this process, since the substrate conversion, production rate, and yield are low [35]. The process would be economically justified if the substrate conversion reached 60–80% [36].

There are several pathways for $BioH_2$ production [4,37,38], such as photo-fermentation (direct photolysis, indirect photolysis, and light fermentation); microbial electrolysis; and anaerobic fermentation (dark fermentation) (Figure 3). All of these methods are considered environmentally friendly.

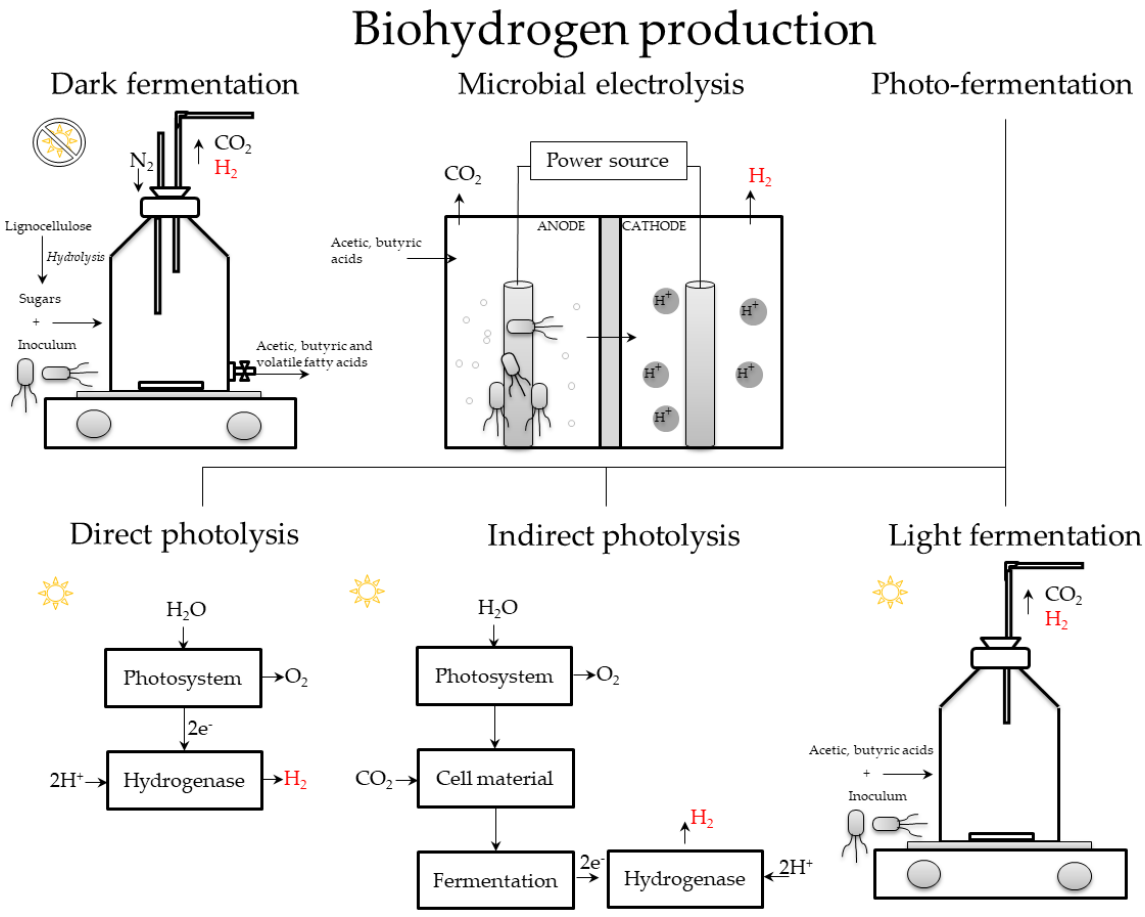

Figure 3. Different $BioH_2$ production processes.

Photo-fermentation (PF) can be divided into two processes. The first is biophotolysis, which is based on the production of $BioH_2$ from water and various raw materials using mainly green algae and cyanobacteria [39]. Biophotolysis can be further divided into direct and indirect photolysis. Direct photolysis is used to convert water into $BioH_2$ and O_2 in the presence of light and CO_2 [40]. In indirect photolysis, there are two distinct phases. In the first phase, CO_2 and H_2O are converted into organic products and O_2 in the presence of cyanobacteria. In the second phase, the organic products formed are converted by the cyanobacteria into $BioH_2$, CO_2, and other metabolites. The main disadvantages of these processes are the formation of O_2, which can inhibit the existing strains, and the formation of an explosive mixture of O_2 and H_2. In addition, indirect photolysis requires the inhibition of hydrogenases to prevent the degradation of $BioH_2$ [39]. The second

process is photo- or light fermentation, in which $BioH_2$ is produced from organic substrates together with alcohols, acetone, and CO_2. Purple non-sulfur photosynthetic bacteria such as *Rhodospirillum, Rhodopseudomonas*, and *Rhodobacter* are used to produce $BioH_2$ in the presence of light. As far as the efficiency of the process is concerned, the yields obtained are comparable to those of biophotolysis [41].

Dark fermentation (DF) is based on the growth of anaerobic bacterial strains (*Clostridia, Escherichia coli, Enterobacter, Citrobacter, Alcaligenes*, and *Bacillus*) [42] in the dark fermenter and the conversion of carbohydrates into $BioH_2$ and other valuable components such as acetic acid, butyric acid, and volatile fatty acids [43].

The disadvantage of both fermentation processes is the resulting biogas, which is a mixture of CO_2 and $BioH_2$. To obtain $BioH_2$, the CO_2 must be removed so that it is not released into the atmosphere. Additionally, DF is faster compared to other processes, but the amount of H_2 produced is small, because many other byproducts are produced during the process. In addition to CO_2, various acids (butyric acid, lactic acid, and acetic acid); alcohols (methanol, butanol, and acetone); and gasses such as methane and hydrogen sulfide can also be produced.

Although the amount of $BioH_2$ that could be produced during fermentation can be theoretically calculated using mass balances and depending on the substrate chosen, the theoretical amount cannot be achieved due to the many byproducts that are produced. For example, if glucose is used as a substrate and acetic acid is produced during DF, 4 mol H_2/mol glucose can theoretically be obtained, but in practice, this number is lower and usually in the range of 1–2.5 H_2/mol glucose [41].

The last process is microbial electrolysis cells (MECs). This is a combination of microbial metabolism and electrochemistry. In this process, the selected strain or a mixture of strains convert organic material into CO_2, electrons, and protons (H^+). The generated electrons are transferred to the anode, while H^+ remains in the electrolyte solution. Under the influence of the external electric circuit, electrons move from the cathode to the anode, where they combine with H^+ to produce $BioH_2$ [39]. MEC is still a new technology compared to other $BioH_2$ production processes. Therefore, many shortcomings and obstacles still need to be addressed. First and foremost, the production cost must be reduced, the mechanism of the electron transfer must be fully understood, the methanogenic activity must be reduced, the electrode material must be selected to be cheap but efficient, the microorganisms must be selected to achieve a high yield, etc. An overview of some recent $BioH_2$ production technologies can be found in Table 3.

Table 3. Overview of $BioH_2$ production technologies.

Process	Substrate	Microorganism	$BioH_2$ Yield	Reference
Direct photolysis	Chlorophyll a + b	*Synechocystis* sp. PCC 6803	4.44 μmol H_2/mg chlorophyll	[44]
	Chlorophyll a + b	*Desertifilum* sp. IPPAS B1220	38.014 μmol H_2/mg chlorophyll	[44]
	Chlorophyll a + b and 10 mM 3-(3,4-dichlorophenyl)-1, 1-dimethylurea	*Desertifilum* sp. IPPAS B1220	57.77 μmol H_2/mg chlorophyll	[44]
Indirect photolysis	Crude glycerol	*Cyanothece* sp. ATCC 51142	74.2 mL H_2/L glycerol	[45]
	Starch	*Chlamydomonas reinhardtii* D240, D239-40, D240-41	388–490 mL H_2/L starch	[46]
	Crude glycerol	*Chlorella* sp.	11.65 mL H_2/L glycerol	[47]
Photo-fermentation	Corn stalk	Mixed strains (*Rhodospirillum rubrum, Rhodopseudomonas capsulata, Rhodopseudomonas palustris, Rhodobacter capsulatus, Rhodobacter sphaeroides*)	160.4 ± 2.7 mL H_2/g corn stalk	[48]
	Corn stover	Photosynthetic bacteria HAU-M1 and dark fermentative bacteria *Enterobacter aerogenes*	36.08–141.42 mL H_2/g total solids	[49]
	Acetate	*Rhodopseudomonas palustris* CGA009	2.31 mol H_2/mol acetate	[50]
	Brewery wastewater	*Rhodobacter sphaeroides* 158 DSM	408.33 mL H_2/L wastewater	[51]
	Mixed substrate (biosuccinate effluent)	*Rhodobacter sphaeroides* KKU-PS1	1217 mL H_2/L biosuccinate	[52]
	Cellulose	*Cellulomonas fimi* ATCC 484 and *Rhodopseudomonas palustris* GCA009	44 mmol H_2/L cellulose	[53]
	Brewery wastewater and pulp and paper mill effluent	*Rhodobacter sphaeroides* NCIMB 8253	0.69 mol H_2/L medium	[54]
	Palm oil mill effluent and pulp and paper mill effluent	*Rhodobacter sphaeroides* NCIMB8253	9.98 mol H_2/L medium	[55]
Dark fermentation	Cashew apple bagasse	*Clostridium roseum* ATCC 17797	1.89 mL H_2/g cashew apple bagasse	[56]
	Coconut husk	*Enterobacter aerogenes* NBRC 13534	0.279 mol H_2/mol reducing sugar	[57]
	Potato and glucose	*Rhodopseudomonas palustris*	7.35 mmol H_2/substrate	[53]
	Cassava	*Enterobacter aerogenes* ATCC 13408	124.3 mL H_2/substrate	[58]
	Corn stew	Sludge	1287.06 mL H_2/g total organic carbon	[59]
	Sago wastewater	*Enterobacter aerogenes*	7.42 mmol H_2/g glucose	[60]
	Coffee silverskin	Indigenous microflora	24.1 mL H_2/g COD (chemical oxygen demand)	[61]
	Glucose	Mixed culture	198.3 mg H_2/g glucose	[62]
	Waste activated sludge	Mixed culture	10.73 mL H_2/g volatile suspended solids	[63]

The efficiency of all the above processes depends mainly on the metabolic pathways of the microorganisms used for production. Therefore, the selection of suitable microorganisms is of crucial importance [64]. The most commonly used microorganisms for $BioH_2$ production are bacteria such as *Clostridum* spp., *Enterobacter* spp., *Escherichia coli*, *Klebsiella* spp., and *Bacillus* spp. [65–69]. Since the use of wild types results in low yields, genetic manipulations are performed to increase productivity.

In addition to the microorganisms, the choice of substrate is another important factor contributing to the efficiency of the process. The most important requirement is that the substrate be rich in carbohydrates [70]. Costly substrates such as glucose and sucrose have been thoroughly researched [71–73], but today, the lignocellulosic biomass is receiving more and more attention. Lignocellulosic biomass is the most abundant renewable source of organic carbon on Earth. It is mainly composed of cellulose (33–40%), hemicellulose (20–25%), and lignin (15–20%) and is considered one of the most important feedstocks for biofuel production due to its price and availability. Most of the lignocellulosic biomass is generated as waste from the agriculture, food, and wood industries (e.g., grass, straw, and wood). Although $BioH_2$ production from lignocellulosic feedstocks is promising, it has not yet been commercialized [74–77].

The use of lignocellulosic feedstocks for $BioH_2$ production requires their treatment, which includes three phases: pretreatment, hydrolysis, and, if necessary, detoxification of the resulting hydrolysates. The pretreatment processes are used to modify the composition and structure of the feedstock. These include separation of the lignin and modification of the lignocellulosic structure, hydrolysis of the hemicellulose, decrystallization of the cellulose, and creation of a surface accessible to enzymes. In addition to enzymes, steam, organic solvents, acids, alkalis, ammonia, sulfur dioxide, ionic liquids, gamma rays, or ultrasound can be used for pretreatment. Pretreatment of the feedstock is followed by hydrolysis of the structural carbohydrates (saccharification), fermentation of the hydrolysate, and, finally, the separation of $BioH_2$. The hydrolysis of cellulose to fermentable sugars is most commonly performed by enzymes isolated from the molds *Trichoderma reesei* and *Aspergillus niger*. Since the hydrolysis process can be inhibited by reaction products and intermediates, saccharification and fermentation are carried out simultaneously after hydrolysis [78].

Other interesting and promising substrates include animal waste, kitchen waste, wastewater, and sewage sludge.

In addition to the choice of microorganisms and substrate, the pH, temperature, and light intensity, the substrate concentration, use of pure or mixed strains, reactor design and type (batch: fed batch and continuous), and process type also affect $BioH_2$ production [79]. Since there are many parameters that can affect the process, the amount of $BioH_2$ produced varies significantly between processes, as shown in Table 3.

In addition, as mentioned earlier, the yield of $BioH_2$ is low due to the many byproducts produced by the above processes. By combining several processes, the yield of $BioH_2$ can be significantly increased [80]. Usually, this involves a combination of DF and photo-fermentation, which can be carried out as a two-stage sequential process or as a single-stage process [5]. In the two-stage sequential process, the first stage is usually the DF. In the second stage, the acids produced as byproducts in the DF are used as feed for hydrogen production by photo-fermentation. The disadvantage of this process is that two reactors and pretreatment prior to photo-fermentation are required. When the combined process is carried out as single-stage fermentation, only one bioreactor is used. The main challenge in this process is to create conditions suitable for both DF and photo-fermentative bacteria.

An integrated system can also be built by combining DF and MEC to produce $BioH_2$, or DF can even be coupled with other processes to utilize the remaining organic matter and obtain valuable products (Figure 4) [81].

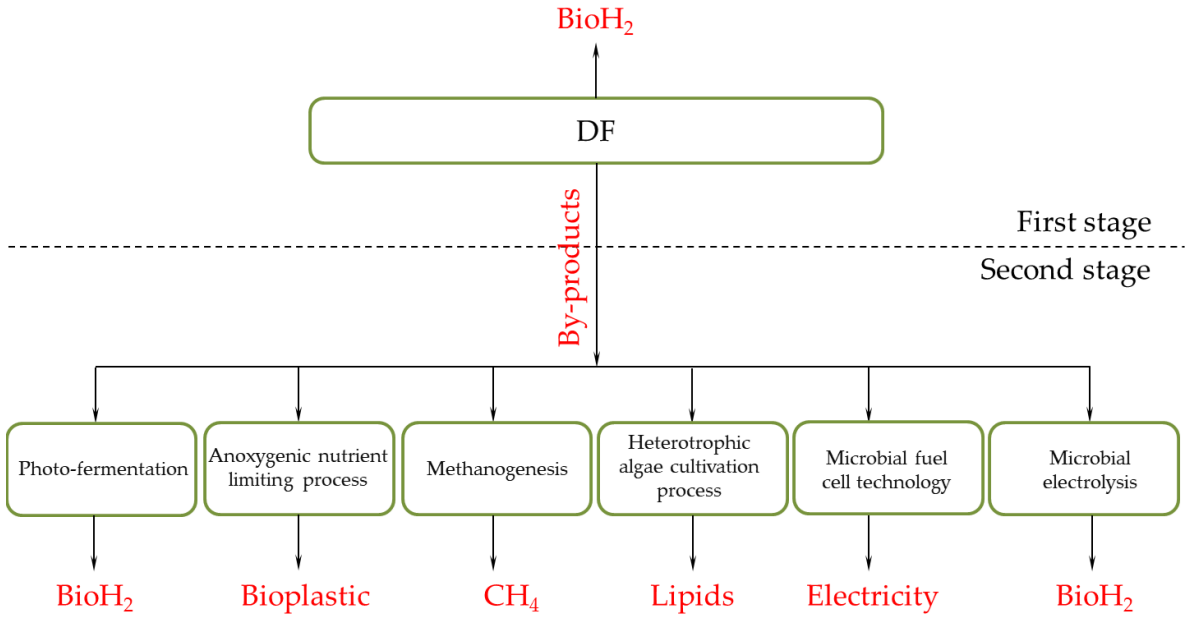

Figure 4. Various secondary bioprocesses coupled with DF to obtain valuable products [81].

3. Microfluidic Technology for Enhancing Biohydrogen Production

Miniaturization, microengineering, and the development of microfluidics in general have brought significant breakthroughs to science, analytics, medicine, manufacturing, etc. in the 21st century [82]. Starting from early research in the 1980s and microfluidics, where a simple straight line was chiseled into a plate with no concrete idea of the final application, we now see microdevices that can be used for cancer cell diagnosis [83], point-of-care testing [84], or even replicating different organs [85]—an organ-on-a-chip concept. Rapid development and prototyping, the advent of 3D printing, new materials, and precise engineering are just some of the new technologies that have enabled this tremendous explosion of microfluidic applications worldwide.

Due to the small channel size (on the order of micrometers), many advantages have emerged over similar macro-sized devices. A large surface-to-volume ratio, short diffusion paths, laminar flow, fast and efficient mass, and heat transfer are some of the main advantages of microreactor systems successfully used in the field of synthesis and production. The main difference between micro- and macroscale processes is the fluid behavior. Low Reynolds numbers, high Peclet numbers, and surface tensions combined with the above properties are the driving forces for microfluidic high performances. Additionally, compared to reactions carried out in conventional reactor systems, higher conversions and productivities have been observed [82]. Some of microfluidic advantages in comparison to classical reactor systems are listed in Table 4.

The application of various microreactor systems to intensify the hydrogen production process has been the subject of numerous studies [86,87], and the results obtained show that the use of microfluidic technology is justified in terms of conversion and productivity. One of the greatest advantages of microfluidics is the possibility of integrating different processes and technical solutions even on a single chip. In hydrogen production, the integration of micromixers to improve the mass transfer or microseparators for gas separation could make a significant difference. Due to the excellent mass/photon transfer, optomicrofluidics could also provide better control of light during the process. In these reactors, the photocatalytic surface area is much larger compared to conventional reactor systems.

So far, microreactors have been used in the water–gas shift (WGS) reaction to adjust the H_2/CO ratio in the final gas mixture [87,88] and to produce hydrogen from hydrocarbons (mainly methane and propane [89]), alcohols (mainly methanol and ethanol), and from other substrates such as ammonia and dimethyl ether [90].

When it comes to the application of microfluidics in biotechnology and $BioH_2$ production, microfluidics can be used for strain screening, process optimization, and media engineering, which could lead to better process performances [91]. Due to the small size, microfluidics can be used to develop low-cost, portable, accurate, and robust portable $BioH_2$ energy sources such as microbial electrochemical cells (MXC), microbial electrolysis cells (MEC), or microbial fuel cells (MFC) [92]. Among them, microfluidic MFC (MMFC) are of particular interest. MMFC are based on the use of microorganisms as biocatalysts for energy production from organic substrates or biomass [93]. The use of microorganisms instead of noble metals as anodes significantly reduces the cost, opens up new possibilities for design and integration on a single chip, and makes the overall process more environmentally friendly [84–97] due to the mild operating conditions. Unfortunately, these systems have not yet gained widespread acceptance, because they are limited by their low power density.

Table 4. Advantages of microreactors in comparison to conventional reactors.

Property	Advantage of Microreactors			
Surface-to-volume (S/V) ratio	• usually ranges from 10^4 to 10^8 m^{-1} (in comparison: $S/V_{\text{fed-batch reactor}}$ = 1 m^{-1}, $S/V_{\text{continuously stirred tank reactor}}$ = 1 m^{-1}, $S/V_{\text{milireacor}}$ = 10^3 m^{-1}) • responsible for intensive mass and heat transfer in microfluidics, which leads to a higher reaction rate and consequently to considerable savings in energy and raw material consumption			
Dimensionless numbers	• dimensionless numbers provide insight into the physical phenomena occurring in microreactors that are substantially different in comparison to conventional reactors			
		Conventional reactor	Microreactor	Change
	Bond number (ratio of gravitational forces and surface tension)	$3 \cdot 10^{-2}$	10^{-3}	Reduced
	Eötvös number (similar to the Bond feature; the difference is that the characteristic dimension can be length)	$3 \cdot 10^{-2}$	10^{-3}	Reduced
	Weber number (ratio of internal force and surface tension force)	$8 \cdot 10^{-3}$	10^{-7}	Reduced
	Reynolds number (ratio of inertial force and viscous force)	10^6	1	Reduced
	Capillary number (viscosity to surface ratio tension)	10^{-2}	10^{-4}	Reduced
	Froude number (ratio of inertial and gravitational force)	$2 \cdot 10^{-1}$	10^{-4}	Reduced
	Ohnesorge number (ratio of viscous force to to the square root of the product of internal and surface forces tension)	10^{-5}	10^{-2}	Increased
	Suratman number (surface tension ratio according to the momentum transfer)	10^7	10^3	Reduced
Diffusion time	• Diffusion time is the ratio of the square of the path and the diffusion coefficient so by reducing the size of the process equipment of microfluidics results in the very short time needed for the molecule to diffuse in the process space			
Surface tensions	• Macroreactor: gravity and pressure play important role in fluid dynamics • Microreactor: capillary forces and surface tensions play important role in fluid dynamic			

3.1. Small Size for Large Properties

As mentioned earlier, the characteristics of microfluidics differ from those of macrosystems. The main advantages of microfluidics that could be used for biohydrogen production relate to flow and transport phenomena, a large surface-to-volume ratio, and a short diffusion path. Whether it is cell cultivation, cell immobilization, photo-fermentation, downstream processing, or screening, microfluidics offers many advantages that can be used for successful $BioH_2$ production at the microscale or to optimize production on the macroscale by collecting information on cell behaviour, light irradiation, etc.

3.1.1. Cell Cultivation in Microfluidics

Whether it is direct photolysis, indirect photolysis, or photo-fermentation, dark fermentation cells play the most important role in $BioH_2$ production. In these processes, miniaturized devices can be used as a screening platform to optimize the physical and nutrient requirements. When working with microfluidics, smaller amounts of substrates/chemicals are used. This is very useful when screening or collecting information, because the media must be prepared. In addition, the physical and chemical properties are the same when

using multiple microsystems produced by precise engineering, allowing the comparison of results with high accuracy [98]. The use of microfluidics also requires a small footprint; as there is little waste, less heating is required due to excellent heat transfer, and the cleaning is minimal. Microfluidics also enables multiparametric studies by combining a wide network of channels, mixers, sensors, light sources, etc. Since microfluidics allows the monitoring and analysis of the growth and work of a single cell, growth kinetics can be accurately described.

For example, microfluidic bioreactors are an ideal tool for studying fuel-producing cells in a microenvironment [91]. Under micro conditions, it is easy to control the pH, temperature, and oxygen content. In addition, microbioreactors are heated uniformly at every point of the reactor due to good heat transfer (better thermal management). Microbioreactors have shorter hydraulic residence times: the operation is isothermal, and the reaction time is shorter [99]. The use of a microbioreactor could increase the $BioH_2$ production rate, reduce microbial contamination, and improve heterogeneity [100].

Microfluidics can also be used to screen strains. In this way, the desired strains can be separated from contaminants [101] or better-performing strains can be isolated for further applications. This allows a better control of biocatalyst growth and selection of the best microorganisms. In addition, only a small starting amount of microorganisms is required, which also reduces the cost of process development. When using microbioreactors for cell cultivation, cells usually grow in a single layer. This effect can contribute to a better understanding of biofilm formation [102] and interactions between cells (quorum sensing) [103].

There are several types of microfluidic technologies most commonly used for cell cultures: microfluidics with mechanical traps [104,105], microfluidics for droplet formation [106–110], and microfluidics with microchambers [111–115] (Figure 5).

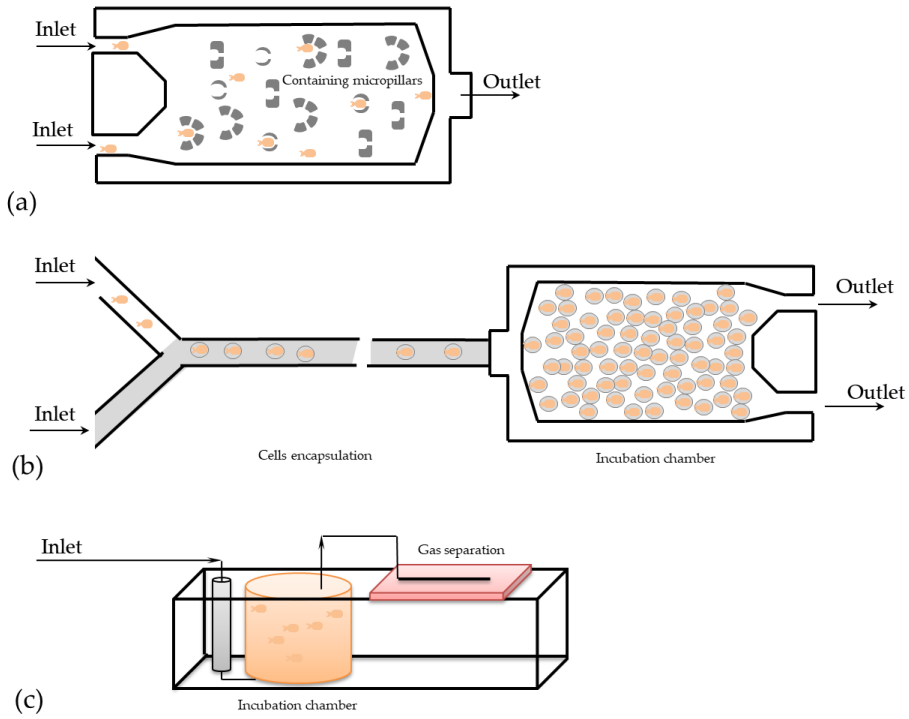

Figure 5. Schematic diagram for (**a**) microfluidics with different mechanical traps/pillars, (**b**) microfluidics for droplet formation, and (**c**) microfluidics with microchambers.

Microfluidics with mechanical traps/pillars are used to retain cells in flow-through systems. These systems are best suited for the study of single cells, as they allow continuous cell monitoring with a microscope. In droplet systems, cells are entrapped in a solvent. These systems allow single or multiple cells to be confined in a specific environment. This

approach can mimic a batch system to define the optimal process conditions. Finally, in microfluidics with microchambers, the cells are free in a confined environment. Which system will be chosen depends on what information or products are to be obtained.

In the previously mentioned systems, the cells are used in a suspended form. However, microfluidics, especially microfluidics with pillars, can also be used to immobilize cells (Figure 6).

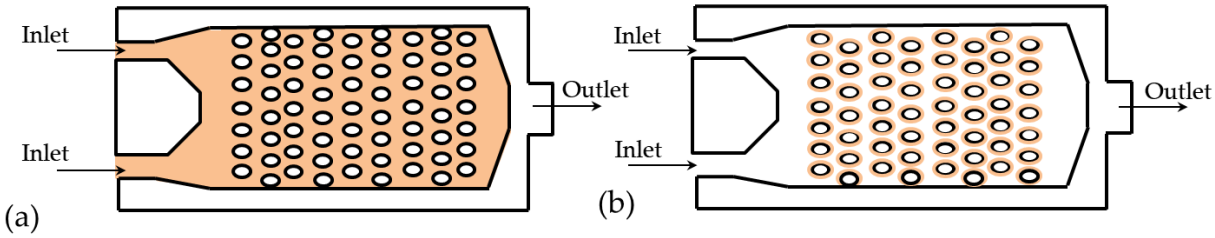

Figure 6. Distribution of biofilm in a microbioreactor: (**a**) suspended biofilm and (**b**) immobilized biofilm. Inspired by [116].

According to Sekoai et al. [100], cell immobilization has numerous advantages over suspended cells. Immobilized cells can withstand harsh fermentation conditions, downstream processes are simpler, and cells can be reused, because their activity is extended. On the other hand, the main disadvantage of such systems is that they are very difficult to remove from microfluidic device once their activity decreases.

3.1.2. Light Irradiation in Microfluidics

In hydrogen production by direct photolysis, one of the major drawbacks of conventional macrosystems is that they require a high light intensity and a small surface area [39]. By using microfluidics, the surface area-to-volume ratio is significantly increased, and less light intensity is required due to the intensive mass transfer in microfluidic devices. According to Alias et al. [117], due to the small size of a microfluidic device, the effects of light self-shading on cell cultures are minimal. This allows for the acquisition of detailed information on the response of cells to illumination conditions. Most microfluidic devices used for cell cultures are made from glass, which means that the intensity of the light inside is identical to the intensity of the external source, allowing precise control.

To date, most research has focused on the impact of the light, design, and fabrication of a microfluidic photobioreactor on algal cultures for biofuel production [118–121].

However, in a study by Velasquez-Orta et al. [122], the authors showed that algae can serve as a renewable source of electricity production in MFC if they are additionally improved to make them competitive with alternative energy technologies. Bioelectricity production from *Chlorella vulgaris* and *Ulva lactuca* was investigated in a single-chamber MFC. The maximum power densities obtained with both the single-cycle and multiple-cycle methods were 0.98 W/m^2 with *C. vulgaris* and 0.76 W/m^2 with *U. lactuca*.

3.1.3. Laminar Flow

Microfluidic fuel cells (MFFCs, Figure 7), also known as co-laminar flow-based fuel cells, also use one of the fundamental properties of microchannels for their function. When fluids are introduced into the microchannel, the flow is predominantly laminar due to the small diameter of the channel [82]. The laminar flow is very predictable and allows the fluids to flow side by side in straight, parallel lines. There is no mixing, and mass transfer occurs solely by diffusion. MFFCs do not require a membrane to separate the anolyte and catholyte [102] compared to macrosystems. The use of MFFCs reduces the costs and avoids membrane degradation. MFFCs are interesting, because they are continuously operating systems with a constant substrate supply and power generation, which makes them more valuable compared to batteries.

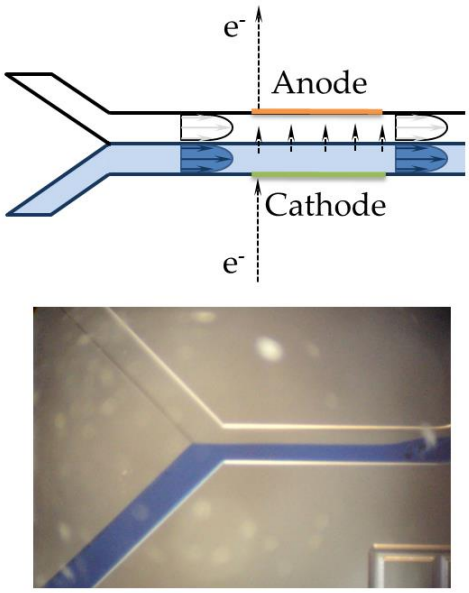

Figure 7. Microfluidic fuel cell (MFFC).

3.1.4. Integration of Different Processes and Technical Solutions

As mentioned earlier, one of the biggest advantages of microfluidics is the possibility to integrate different processes and technical solutions even on a single chip [94]. The development of the so-called Micro Total Analysis System (μTAS), which can perform different operations such as preparation, production, separation, and analysis on a single chip, can become a valuable option for biofuel production. Due to their small size, they have the potential to become fully portable, small-scale energy production devices. Although this idea will only be realized in the future, some steps have already been taken in this direction.

Based on this, the development of so-called microfluidic microbial electrochemical cells [123] (MMXCs) or microfluidic microbial fuel cells (MFMFCs) could bring up a significant change in continuous BioH_2 production [92]. In the work of Shirkosh et al. [124], the authors also showed how easy it is to integrate engineered solutions to increase the power density. The authors simply integrated a magnetic field into MFMFCs and managed to increase the power density by more than 2.4 times using a Zn anode (Figure 8). This simple approach would be challenging if implemented on a large scale.

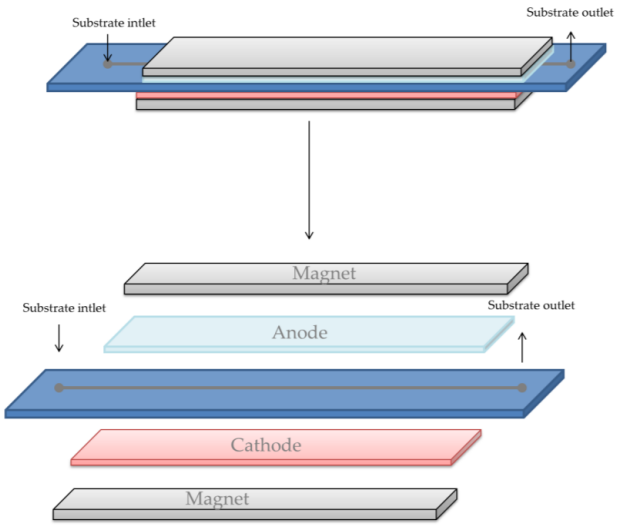

Figure 8. Microfluidic microbial fuel cell (MFMFC). Inspired by [124].

3.1.5. Numbering Up

Increasing the production capacity and increasing the scale of microfluidics was solved by connecting individual units in a series or parallel. Following this concept, compact microplants could be built to achieve the capacity of $BioH_2$ production for industrial applications [125–127]. This approach is much simpler, less time-consuming, and cheaper than the classical scale-up. Another advantage of numbering up is the smooth operation of continuous processes in case one of the units fails. In this case, it is possible to replace a chip without interrupting the processes running in the parallel units. Moreover, in a traditional scale-up, each increase in scale leads to a new design, new calculations, and a new adaptation of the process, which is time-consuming. In microfluidics, once the process is described in a single chip, the capacity can be increased by combining the same units.

3.2. Microfluidic Production of $BioH_2$

As far as microfluidics is concerned, while reactors have become smaller and smaller, many accompanying devices such as pumps, downstream analytical devices, power supplies, etc. have remained unchanged in size for a long time. Therefore, to truly implement the concept of miniaturization, these areas had to be considered as well. Micropumps for microfluidic devices [128,129], green micro total analysis systems (GμTAS) [130], which are a further step compared to the classical μTAS but still a strong alternative to the traditional macroscale analysis systems, etc. are some of the new directions of total miniaturization. In addition, over the years, more and more work has been done on the development of portable green energy sources [86]. When we already use microdevices that fit in the palms of our hands, no one wants to carry around/use devices that are hundreds of times larger than themselves for the sole purpose of powering them. Moreover, with the development of microbiosensors, medical diagnostic devices, and microimplants, a small, reliable power source was needed. Thus, the idea was born to develop a microfluidic technology for on-site or on-board applications. The idea was based on the generation of hydrogen for distributed consumption by fuel cells. Kolb et al. [86] emphasized that microstructured reactors for decentralized and mobile fuel processing could be used as hydrogen sources for duel cells. For powering microdevices such as microbiosensors, medical diagnostic devices, and microimplants, most of the conventional methods of hydrogen production are not suitable. Therefore, not only are new raw materials and new substrate sources being sought but also new technologies and approaches that can improve hydrogen production. Microfluidic fuel cells could be the next step. A good example of the application of a microfluidic $BioH_2$ fuel cell ($MBioH_2FC$) to power microdevices is the work of Mardanpour et al. [131]. The aforementioned authors developed a microfluidic microbial electrolysis cell (MMEC) with a nickel electrode and glucose and urine as substrates, with *E. coli* as the biocatalyst. They succeeded in producing a power of 2.2 $\mu W/cm^2$ and 1.4 μL $BioH_2$/μL substrate per day. The authors concluded that this is a promising technology for medical applications for several reasons: a significant generation of biohydrogen, use of cheap substrates, minimal consumption of expensive materials, and simple design. In this study they used *E. coli*, which lives in the lower intestines of humans. However, they emphasized that further research is needed to use non-pathogenic microorganisms in medical devices.

In the work by Fadakar et al. [92], the authors coupled a microfluidic microbial electrochemical cell (MXC), including MMFCs and a microfluidic microbial electrolysis cell (MEC), as a self-powered $BioH_2$ generator. By using a non-phatogenic *E. coli* strain as the biocatalyst, they were able to achieve the maximum hydrogen production rates of 46 and 28 ppm/h for glucose- and urea-based substrates, respectively.

In the work presented by Delavar and Wang [132], the authors produced biohydrogen in a microbiorector during photo-fermentation. They used microbioreactors for rapid screening of the process. They studied the effects of illumination, glucose concentration, and rate on biohydrogen production. The authors found that there was a threshold for the illumination level. Below this threshold, the amount of $BioH_2$ increased when the illumination level was increased. Above this threshold, productivity decreased when the

illuminance was increased. They also examined the effect of the glucose concentration and concluded that, as the concentration increased, the $BioH_2$ concentration and extraction efficiency increased, but the $BioH_2$ yield decreased. When the velocity was examined, it was found that a lower inlet viscosity resulted in a higher $BioH_2$ production rate and concentration but decreased the extraction efficiency and yield. Finally, the authors presented a mathematical model based on the lattice Boltzman method that takes into account the bacterial growth, light energy conversion, and light transmittance during biofilm growth. The proposed mathematical model could improve the design of future microbioreactors.

The same authors [116] also studied the effects of pH on anaerobic bacteria on $BioH_2$ production during dark fermentation in a microbioreactor. The results showed the importance of acidity and pH on $BioH_2$ production and extraction rates.

Gele et al. [133] performed a comparative study of three types of zinc anodes in a MMFC. They performed their experiments in a spiral microchannel with a channel width of 500 µm and an internal volume of 16.5 µL using *Shewanella oneidensis MR-1* as the biocatalyst and oxalate as the substrate. They compared zinc foil, externally connected stainless-steel zinc foil, and modified zinc foil as anode electrodes and concluded that the zinc oxide nanorods could not serve as an effective way to improve the MMFC performance.

4. Obstacles to Be Overcome

As can be seen, there are very few examples of the application of microfluidics in the production of $BioH_2$. Although microfluidics was initially hailed as a revolutionary tool that would overcome many obstacles in many fields, it is now viewed much more cautiously and critically [134,135]. This is probably because many obstacles still need to be overcome for microdevices to become attractive for $BioH_2$ production in a broader range of applications.

4.1. Cell Cultivation

The first challenge concerns the behavior of cells for $BioH_2$ production in the microchannels and microchambers. In the transition from a macroenvironment to microenvironment, many standardized approaches should be changed. According to Halldorsson et al. [136], the switch to microdimensions has strong impacts on the process: a different culture surface, reduced media volume, different rates, different medium exchange, etc. This will lead to a large number of different devices being developed before the right one is found.

In microfluidic cell cultivation, pH regulation is always a challenge. If CO_2 removal is not controlled, the pH will deviate significantly from the physiological range. The question is how to remove CO_2 and obtain $BioH_2$.

Another question is how to culture cells under flow conditions. A large number of cells in a small volume requires constant media exchange in the chamber, as the nutrients are rapidly consumed.

Moreover, the lifetime of such devices is short compared to classical systems due to biofouling, air entrapment, etc. [135].

4.2. Modular Systems vs. Integrated Systems

One of the promising directions of $BioH_2$ production is to use microfluidic flexibility in the design to develop an integrated system, leading to better productivity and efficiency. For example, dark fermentation could be performed in one microbioreactor, followed by the photo-fermentation of organic acids in a second microfluidic unit connected in a series using combined cocultures. While this sounds theoretically possible, the commercialization of such a system is still a problem. Most of today's microfluidic devices are complex, with specific purposes, with holders and connectors, tubing, and many other accessories required for assembly. Many of them combine all the necessary functions on a single chip. However, manufacturing and developing such microfluidics is expensive and time-consuming. Once the microfluidic is made, it is also difficult to make further adjustments. For this reason, when building a two-stage $BioH_2$ production system, a modular system

would be a better choice. Modular systems allow for much greater flexibility in design, leading to the development of devices with the desired characteristics. With these systems, it would be easy to replace a specific unit rather than redesign an entire system, as is the case with fully integrated systems. An example of such devices is LEGO microfluidics, which can have both fluidic and active functions (detection, sorting, etc.) [137]. According to Mark et al. [138], the development of more generic platforms with the ability to integrate multiple functions will increase the impact of microfluidics in the global market and make the overall system more attractive to non-microfluidics users.

4.3. Sensors

To successfully use a microfluidic system for $BioH_2$ production, integrated sensors should be used. In addition to simple detection, the sensors should also have quantitative detection properties. The most commonly used sensors in microfluidics are optical and electrochemical sensors, both of which can be used for the detection of $BioH_2$. In the work of Luong et al. [139], the authors developed a class of lightweight optical hydrogen sensors based on a meta-surface of Pd nano-patchy particle arrays that meet the increasing requirements for a safe hydrogen sensing system without the risk of sparking. In addition to the Pb layer, a WO_3 layer can also be used. The advantages of the different optical sensors were discussed in the work by Zhang et al. [140].

Nowadays, the challenge is not in the development of the sensors but in their miniaturization, adaptation to microfluidics, and integration.

4.4. Microscale CCS

$BioH_2$ is considered environmentally friendly, and it is widely believed that CO_2 produced in this way can be released into the atmosphere. However, CO_2 can be captured with CCS, leading to the production of negative-carbon hydrogen. The challenge is to transfer the current CCS technology to the microscale. One possible solution is gas–liquid absorption. In the article of Ganapathy et al. [141], the authors proposed carbon capture in a multiport microscale absorber. The absorber consisted of 15 straight parallel channels. The authors tested the absorption of CO_2 mixed with N_2 in aqueous diethanolamine and reported that a high absorption efficiency of almost 100% was observed under certain operating conditions. Although this approach is promising, new approaches for CO_2 removal should be developed.

4.5. Selection of the Substrate

Special attention should also be given to the study of substrates (i.e., biomass) that can be used for $BioH_2$ production. Although many will agree that renewable materials such as lignocellulose and wastes are the preferred substrate source, there is the question of available quantities. Although microfluidics requires only small amounts of substrates, there is general concern about whether the available quantities are sufficient. According to the European Commission, Directorate General for Energy [142], potential biomass resources amount to about 10% of the EU energy consumption. There is also a general concern about whether there will be a biomass in the future. In the Xu et al. [143] paper, the authors ask a valid question—what will happen to the biomass for large-scale bioenergy production in the context of global warming? As crop yields decline and the food industry is affected, the availability of a biomass will rapidly decrease. Therefore, new substrates and new technologies with negative emission should be explored.

4.6. Selection of the Material for the Production of Microdevices

Another problem is the choice of material for microfluidic production. The most commonly used microdevices are made of polydimethylsiloxane (PDMS). It is said that the use of PDMS has accelerated the development and application of microfluidics [134]. This material is easy to handle, cheap, and available. In cell cultivation, this material is chosen, because it is gas-permeable. It allows O_2 permeation through the material to the

buffer to oxygenate the medium and releases CO_2 generated in the system. On the other hand, this property is undesirable in $BioH_2$ production, because it would lead to losses during production. As a possible solution, microfluidics made of glass or silicone can be used, but their use in microfluidic production requires special manufacturing processes that make the whole production process more expensive. For this reason, a new material for microfluidic devices for $BioH_2$ production should be investigated that is easy to handle, cheap, and not gas-permeable.

4.7. Changing the User's Perspective

For non-microfluidic experts, using microfluidics is still a challenge. Usually, their assembly is not easy. Avoiding clogging and damage, setting the right process conditions, etc. require knowledge. It is also difficult to convince people, and it takes an exceptional performance of microdevices to encourage them to switch from conventional macroprocesses and technologies to microfluidics. The initial equipment costs are always a challenge when replacing something old with something new [144]. Users are also skeptical about the quantities of products that can be achieved in microfluidics, so a better understanding of the "numbering up" concept is needed.

5. Outlook and Conclusions

At this point, it can be said that microfluidics will find its place in power generation, just as it has found its place in many other fields. Considering its characteristics, such as reduction of the reaction time, reduction of the energy required for operation, small number of samples needed, small amount of waste generated, and high precision, the future of microfluidics in power generation is more than promising. At the same time, $BioH_2$ can be produced using immobilized cells or enzymes, in which the mass transfer is significantly reduced through the use of microreactors, thus intensifying the processes.

It is very likely that, in the near future, we will see microdevices that can power devices much larger than themselves, but their focus and use will likely be on powering biosensors, implantable medical devices, and as environmental monitoring systems. Given the different approaches to $BioH_2$ production and the adaptability of microfluidics, the possibilities are very different. We can only look forward to seeing what the future holds.

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