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REVIEW ARTICLE

Microbial cell immobilization in biohydrogen production: a short overview

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ABSTRACT

The high dependence on fossil fuels has escalated the challenges of greenhouse gas emissions and energy security. Biohydrogen is projected as a future alternative energy as a result of its non-polluting characteristics, high energy content (122 kJ/g), and economic feasibility. However, its industrial production has been hampered by several constraints such as low process yields and the formation of biohydrogen-competing reactions. This necessitates the search for other novel strategies to overcome this problem. Cell immobilization technology has been in existence for many decades and is widely used in various processes such as wastewater treatment, food technology, and pharmaceutical industry. In recent years, this technology has caught the attention of many researchers within the biohydrogen production field owing to its merits such as enhanced process yields, reduced microbial contamination, and improved homogeneity. In addition, the use of immobilization in biohydrogen production prevents washout of microbes, stabilizes the pH of the medium, and extends microbial activity during continuous processes. In this short review, an insight into the potential of cell immobilization is presented. A few immobilization techniques such as entrapment, adsorption, encapsulation, and synthetic polymers are discussed. In addition, the effects of process conditions on the performance of immobilized microbial cells during biohydrogen production are discussed. Finally, the review concludes with suggestions on improvement of cell immobilization technologies in biohydrogen production.

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Biohydrogen; cell immobilization; process parameters; microorganisms

Introduction

One of the most effective ways of reducing the reliance on fossil fuels and minimizing environmental pollution is the introduction of clean and sustainable energy resources. Over the past few decades, hydrogen has captured increasing global attention as an alternative to fossil fuels owing to its several merits such as zero-carbon emissions, high heating value (122 kJ/g), its abundance, and diverse storage forms [1]. Hydrogen is used in many industrial applications such as ammonia synthesis, methanol production, removal of impurities in oil refineries, steel processing, production of electronic devices, desulfurization, and reformation of petroleum distillates [2]. The global annual production of hydrogen is currently projected at 62 million tons, with an annual growth rate of 8–10% [3]. Among the industrial hydrogen production processes, steam reforming of methane is an extensively used method. It produces up to nearly 50% of hydrogen compared with 30% of hydrogen obtained from catalytic reforming, 16% from coal gasification, 3.9% from water electrolysis, and 0.1% from other methods [3]. However, these processes are energy intensive and contribute to greenhouse gas emissions. Therefore, researchers are focusing on biological methods like biophotolysis, dark, and photo-fermentation to produce cleaner hydrogen energy [3]. Biophotolysis and photo-fermentation processes generate high biohydrogen yields; however, they require an illumination source and use limited substrates for photosynthetic microorganisms which might escalate the process costs at large-scale production [3]. Dark fermentation is more favored because it requires less energy, uses diverse feedstocks (including waste materials), operates at ambient temperature and pressure, and offers the most environmentally benign pathway of producing hydrogen. It usually proceeds via the acetic and butyric fermentation pathways as shown in the following equations:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (2)$$

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Obtaining high yields is still a major challenge in dark fermentation processes [4]. The experimental yields are lower than the theoretical yields due to the presence of biohydrogen-competing pathways that lowers the overall efficiency. In addition, other biohydrogen-producing pathways co-produce ethanol resulting in a low stoichiometric yield of 2 mol H_2/mol glucose (Equation (3)). Similarly, it has also been shown that some biohydrogen-producing bacteria, such as *Clostridium articum* and *Clostridium barkeri,* are capable of using hydrogen for their metabolic activities to generate undesirable products such as propionic acid (Equation (4)) and lactic acid (Equation (5)). The process is also accompanied by biohydrogen-consuming species, i.e. hydrogenotrophic methanogens, homoacetogens, nitrate-reducing bacteria, and sulfate-reducing bacteria [5]:

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2OH + 2H_2O + CH_3COOH + 2CO_2 + 2H_2 \quad (3)$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad (4)$$

$$C_6H_{12}O_6 \rightarrow CH_3CHOHCOOH + 2CO_2 \quad (5)$$

Over the past decades, there have been an increasing number of publications on biohydrogen optimization strategies via metabolic engineering [6–15], two-stage fermentation processes [16–19], multivariate tools [20–26], and pretreatment methods [27–32] in the open literature. Figure 1 depicts the progression in the number of peer-reviewed articles in scientific journals as obtained from the Web of Science [33]. The progression shows an increase per year with a maximum number of about 497 articles published in 2016.

Nonetheless, this process is still plagued with low yield due to its complexities. Currently, the highest yield documented in the literature is 2.3 mol H_2/mol glucose and is about 50% of the theoretical yield [34]. There is a need for other novel biohydrogen augmentation approaches to overcome this challenge. Recently, there has been an upsurge of interest in the utilization of immobilized microbial cultures due to their advantages such as high substrate conversion efficiency, high metabolic activity, shortened lag phase, increased cell density, ease of handling, reusability, better solid/liquid separation efficiency, and better operational stability [35].

Furthermore, this technology can be incorporated in various biohydrogen reactor types such as a continuous stirred tank reactor [36], fluidized bed reactor [37], carrier induced granular sludge bed reactor [38], up-flow anaerobic sludge bed reactor [39], and trickling biofilter [40]. Microorganisms are immobilized using various biological materials (alginate, agar, cellulose, carrageenan, etc) or synthetic materials (acrylamide, polyurethane, polyvinyl alcohol, polyethylene glycol among others). Cell immobilization technologies are widely used in many industrial processes like wastewater treatment [41], food processing [42], and enzyme enhanced production [43]. Therefore, this review discusses the

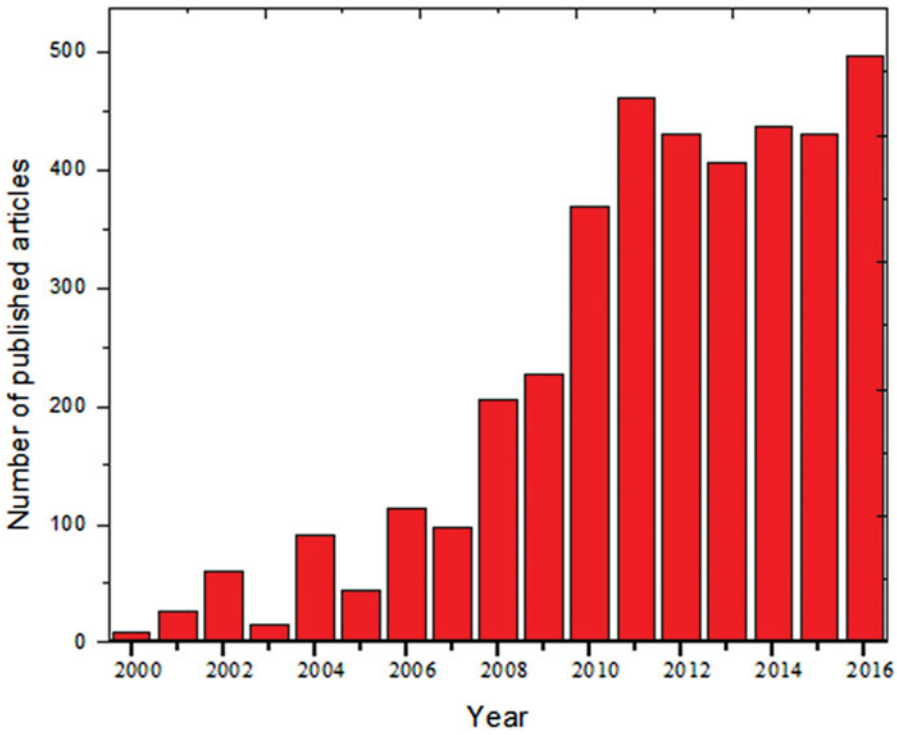

Figure 1. Number of published peer-reviewed articles on dark fermentation [33].

potential of using immobilized microbial cells in biohydrogen production. It provides information on various types of immobilization methods employed in biohydrogen production and discusses the effect of process parameters on the performance of immobilized cells during production. Finally, this review attempts to provide suggested solutions that could be implemented to improve this technology.

Immobilized microorganisms versus suspended cells

Most biohydrogen production studies use suspended cells which are prone to washout during continuous processes. This causes operational instability and reduces its yield [35]. Therefore, immobilized microbial cells are used to prevent this problem. They also possess the following advantages:

- Enhancement of the biohydrogen yield during biohydrogen production.
- Ability to withstand harsh fermentation conditions such as solvents, pH, and toxic metals.
- Potential to increase the substrate conversion efficiency.
- Enhancement of the ability to operate at high organic loading rates and at short retention times.
- Provision of a simple downstream process i.e. this minimizes the need for separation and filtration steps and therefore reduces the process costs.
- Possible reusability of microorganisms.
- Minimization of microbial contaminations.
- Protection of the microbial cells against shear stress caused by stirring.

Carrier and techniques during immobilization

Property of carrier employed for immobilization

A carrier should have the following prerequisites in order to be suitable for immobilization of biohydrogen-producing microorganisms [43]:

- It should have a large surface area and functional groups to which cells adhere.
- It should be non-toxic towards biohydrogen-producing microorganisms.
- It should have a good mechanical, chemical, and thermal stabilities.
- It should be inexpensive, reusable, and amenable to scale-up process.
- It should be resistance towards fermentative by-products.
- It should provide adequate homogeneity within the bioreactor.
- It should have uniform permeability thus enabling free diffusion of nutrients, gases, and cofactors.

Immobilization techniques applied in biohydrogen production

Immobilization of biohydrogen-producing microorganisms is primarily achieved through entrapment, adsorption, encapsulation, and containment within synthetic polymers. These methods of immobilization are illustrated in Figure 2.

Entrapment

This is one of the most simplest and common method whereby biohydrogen-producing cells are entrapped inside a support matrix. The support material creates a protective barrier around the cells ensuring their protection and prolonged use [44]. For an effective bioprocess, the material should have good permeability to enable nutrients from the medium to diffuse and be metabolized by the microbial cells. This method uses various immobilizing materials such as agar, alginate, cellulose, carrageenan, polyacrylamide, polyurethane, polyvinyl, and polypropylene [45]. Among these materials, entrapment using alginate gels is the current most exploited method because it is inexpensive, uses mild fermentation conditions, cells are reusable, and offers a simple process of producing biohydrogen. However, it does not retain its structure at high cations concentrations (K^+, Ca^{2+}, Mg^{2+}). Therefore, several approaches have been included in alginate beads to improve their mechanical stability. For example, Wu et al. [46] obtained a three-fold increase in biohydrogen production when alginate beads were supplemented with chitosan and titanium oxide, giving a biohydrogen production rate of 21.3 mmol L^{-1} h^{-1}. Wu et al. [47] obtained a two-fold increase in biohydrogen production when the calcium alginate beads were supplemented with activated carbon. Singh et al. [48] maximized the production of biohydrogen (0.38 L H_2/g COD) using immobilized *Clostridium* LS2 cells with polyethylene glycol in a continuous process at short hydraulic retention time of 16 h. Ismail et al. [49] reported an enhanced biohydrogen production rate (2.1 NL/L/d) from palm oil effluent using polydimethylsiloxane immobilized cells at short hydraulic retention time (2 h). In another study, Seelert et al. [50] obtained a high yield of 2.1 mol H_2/mol glucose using alginate beads incorporated with metal ions. Supplementary materials such as cations have also been shown to improve the uptake of

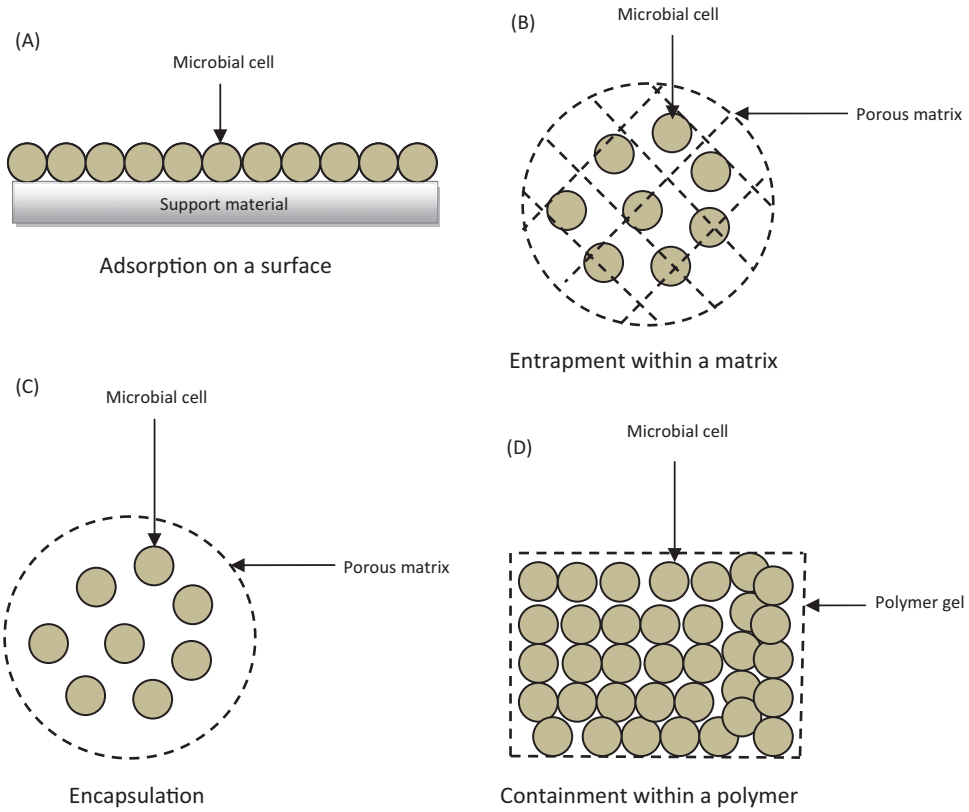

Figure 2. Methods of immobilizing biohydrogen-producing microorganisms (adapted from [35]).

nutrients during biohydrogen production. Second, they plays an important role in biohydrogen-producing dehydrogenase enzymes during dark fermentation [34].

Adsorption

Adsorption involves the attachment of microbial cells to the surface of the support matrix. This method provides a better mass transfer efficiency, improved biomass retention capacity, stable and prolonged biohydrogen production, and substrate utilization at short hydraulic retention times [51]. The interaction between microbial cells and the support matrix is governed by various forces such as Van de Waals force, ionic, hydrophilic, and hydrophobic bonds [45]. Unlike cell entrapment, adsorption is advantageous because nutrients are in direct contact with immobilized cells and therefore it improves the substrate conversion efficiency. Microbial cells are attached using cations, chitosan, activated carbon, and other materials [45]. Activated carbon and cations are the most exploited adsorption materials in biohydrogen production probably because of their simplicity and availability. Han et al. [52] achieved a high biohydrogen production rate of 353.9 ml H_2/L/h using an immobilized sludge rector combined with activated carbon at packing ratio of 15% (v/v), the results were 1.33 times higher than those of 20% (v/v). Moreover, it was also concluded that higher packing ratios retain low cell concentrations at high organic loading rates [52]. Wang et al. [53] attained a maximum biohydrogen production of 2.27 mmol H_2 $L^{-1}h^{-1}$ in a reactor packed with activated carbon material. Wu et al. [47] observed a peak production of 1.22 L H_2 $L^{-1}h^{-1}$ in a fluidized bed reactor that used anaerobic sludge that was supported with activated carbon. Lutpi et al. [54] maximized the biohydrogen yield (2.8 mol H_2/mol hexose) by conducting repeated batch cycles using immobilized cells on activated carbon granules under thermophilic conditions. Moreover, addition of cations (Ca^{2+} and Ni^{2+}) was shown to enhance the production of biohydrogen by 1.4 times [47]. Similarly, Thakur et al. [55] reported a maximum biohydrogen production of 55 and 58 ml H_2 using iron (Fe^{2+}) and cobalt (Co^{2+}), respectively. Lee et al. [56] investigated the effect of different F^{2+}concentrations on biohydrogen production and observed a high production of 24 ml H_2 g^{-1} VSS h^{-1} at 4 g/l $FeCl_2$. Lin and Shei [57] assessed various cations (Fe^{2+}, Ni^{2+}, Mg^{2+}, Na^{+}, Zn^{2+}, K^{+}, Co^{2+}, Cu^{2+}, Mo^{2+}, and Ca^{2+}) on biohydrogen production and found Fe^{2+}, Mg^{2+}, Na^{+}, and Zn^{2+} to be favorable for biohydrogen yields. These cations stabilize the beads and enhance the uptake of nutrients as mentioned earlier.

Other novel adsorption materials have also been used to improve biohydrogen production. For instance, Zhao et al. [58] enhanced the production of biohydrogen using silver nanoparticles. These authors reported a maximum yield of 2.48 mol H_2/mol glucose at a silver concentration of 20 nm L^{-1}. Liao et al. [59] maximized the production of biohydrogen through adsorption of *Rhodoseudomonas palustris* CQK 01 on SiO_2–chitosan medium and was effective for enhancing cell adhesion, reducing biofilms formation, and thus improved its production rate by 80% [59]. Deng et al. [60] used gels cross-linked with saturated boric acid and calcium chloride solution and obtained a hydrogen fraction of 24.2% at biohydrogen production rate of 6.2 mmol H_2/L/h. Mullai et al. [61] reported an enhanced biohydrogen yield of 2.54 mol H_2/mol glucose using a silver nanoparticles concentration of 5.67 mg/l. Beckers et al. [62] reported an increased in the biohydrogen production rate by 113% when iron oxide nanoparticles were used as a carrier. The nanoparticles improved the biohydrogen production by transferring electrons to acceptors during the anaerobic fermentation conditions. This enhances the activity of biohydrogen-producing biocatalysts [62].

Encapsulation

Encapsulation is similar to entrapment i.e. cells are retained within a permeable membrane that allows diffusion of nutrients. This method helps prevent cell leakage, prevent inhibitory materials, minimize contamination, and enhance substrate conversion efficiency. A recent study by Akinbomi et al. [63] assessed biohydrogen production using polyvinylidene fluoride membrane-encapsulated cells from a fruit-flavoured medium of hexanal, myrcene, and octanol. Cell encapsulation increased the biohydrogen yield by 2.7-, 1.3-, and 2.2-folds compared with suspended cells. Stojkovic et al. [64] encapsulated *Chlamydomonas reinhardtii* within TiO_2 shells to enhance the light-to-biohydrogen conversion efficiency. Jeonghee et al. [65] reported stable biohydrogen production using polyvinyl alcohol-encapsulation bioreactor. Woodward et al. [66] developed a novel approach for encapsulating biohydrogen-producing hydrogenase and glucose dehydrogenase enzymes within liposomes. Moreover, Pandey and Pandey [67] enhanced the production of biohydrogen by 35-fold using *Rhodopseudomonas sphaeroides* cells encapsulated within reverse micelles. In a similar study, Pandey et al. [68] obtained a biohydrogen yield that was 4.8 times higher using sodium sulfosuccinate-isooctane reverse micelles. Microorganisms are able to increase biohydrogen production when encapsulated within reverse micelles as a result of their unique properties such as compartmentalization, high porosity, and ability able to create anoxic microenvironments [48].

Containment within synthetic polymers

The use of synthetic polymers is gaining increasing interest in biohydrogen production because these materials are more stable and exhibit a better performance compared to natural polymers. Examples of synthetic polymers include polyacrylamide, polyurethane, polyvinyl alcohol, polyethylene glycol, and polycarbamoyl sulfonate. This is similar to the entrapment method where cells are trapped inside a support material. Synthetic polymers have been used in many studies to maximize biohydrogen production. For instance, Singh and Wahid [60] maximized biohydrogen production yield (0.31 L H_2/g COD) using *Clostridium* LS2 cells entrapped in polyethylene glycol gel. Tian et al. [70] used *Rhodopseudomonas palustris* CQK 01 cells immobilized on polyvinyl alcohol-boric acid gel and obtained a maximum biohydrogen production rate of 3.6 mmol H_2 g^{-1} cell dry weight h^{-1}. The performance of biological polymers (ramie and loofah) and synthetic polymers (acrylic, polyethylene, and polyvinylchloride) was compared by Wongthanate and Polprasert [71]; a two-fold increase in biohydrogen was achieved using polyethylene. Barros and Silva [72] evaluated three support materials i.e. polystyrene, grounded tire, and polyethylene terephthalate for biohydrogen production using three anaerobic fluidized bed reactors, and reported a biohydrogen fraction of 60% and a biohydrogen yield of 2.11 mol H_2 mol^{-1} using grounded tire. Jo et al. [73] reported a maximum biohydrogen production rate of 7.2 l H_2 L^{-1} d^{-1} at 2 h HRT, a hydrogen fraction of 50%, and substrate conversion efficiency of 97.4% using *Clostridium tyrobutyricum* JM1 immobilized on polyurethane-foam support material. Mitchell et al. [74] also enhanced the production of biohydrogen in a batch process using *Clostridium tyrobutyricum* ATCC 25755 cells immobilized on polyurethane matrix. In addition, the authors reported a cumulative biohydrogen production of 110 L. Other synthetic support materials such as polyacrylonitrile have been used for biohydrogen production. Li et al. [75] immobilized biohydrogen-producing cells on polyacrylonitrile composite nanofiber mesh filled with carbon nanotubes and achieved 386 ml H_2. The use of synthetic polymers could pave a way for large-scale biohydrogen production process due to their versatility and mechanical stability. The major milestones attained using immobilized microbial cells are presented in Table 1.

Table 1. Types of immobilization techniques used in biohydrogen production process.

Method	Microorganism	Carrier material	Substrate	Evaluation of method	H_2 production rate	H_2 yield	Reference
Entrapment	Mixed bacteria	Alginate + chitosan + titanium oxide	Sucrose	Three-fold H_2 production increase	21.3 mmol/L/h	–	[47]
Entrapment	Mixed bacteria	Calcium alginate + activated carbon	Sucrose	Two-fold H_2 production increase	–	2.6 mol H_2/mol sucrose	[46]
Entrapment	*Clostridium* LS2	Polyethylene glycol	POME	H_2 increased at short HRT of 16 h and 90% substrate conversion efficiency achieved	–	0.35 L H_2/g COD	[48]
Entrapment	Mixed bacteria	Polydimethyl siloxane	POME	Prolonged H_2 production (150 days), and less production of H_2 inhibiting metabolites	2.1 NL/L/d	–	[49]
Adsorption	Mixed bacteria	Activated carbon	Food waste	H_2 increased by 1.33 times when the continuous immobilized sludge reactor was packed with a packing ratio of 15%, and OLR of 40 kg/m^3/d	353.9 ml/L/h	85.6 ml H_2/g food waste	[52]
Adsorption	Mixed bacteria	Activated carbon	Molasses	Maximum H_2 production at short HRT (5 h) and H_2 content of 62%, pH was stabilized at 4.60	12.27 mmol/L/h	–	[53]
Adsorption	Mixed bacteria	GAC	Sucrose	Repeated batch processes enhanced H_2 production	2.7 mmol/L/h	2.8 mol H_2/mol hexose	[54]
Adsorption	*Klebsiella oxytoca*	Sodium alginate	RME	3% (w/v) sodium alginate enhanced the production of H_2	–	55 ml H_2/g RME	[55]
z	Mixed bacteria	Silver nanoparticles	Glucose	Silver nanoparticles increased H_2 production	–	2.48 mol H_2/mol glucose	[58]
Adsorption	Mixed bacteria	Nickel nanoparticles	Glucose	RSM was used to optimize nickel nanoparticles for maximum H_2 production	–	2.54 mol H_2/mol glucose	[61]
Encapsulation	Mixed bacteria	Polyvinylidene fluoride	Hexanal	H_2 increased by 2.8 times	–	189 ml H_2/g COD	[63]
Encapsulation	Mixed bacteria	Polyvinylidene fluoride	Myrcene	H_2 increased by 1.3 times	–	179 ml H_2/g COD	[63]
Encapsulation	Mixed bacteria	Polyvinylidene fluoride	Octanol	H_2 increased by 2.25 times	–	198 ml H_2/g COD	[63]
Encapsulation	*C. reinhardtii*	TiO_2	Tris-acetate	Double conversion efficiency achieved using TiO_2-encapsulated cells	–	–	[64]
Encapsulation	*R. sphaeroides 2.4.1*	Benzene + sodium lauryl sulfate reverse micelles	Glucose	25-fold H_2 production increase	1.71 mmol/mg protein/h	–	[67]
Encapsulation	*R. sphaeroides 2.4.1*	AOT + isooctane reverse micelles	Glucose	35-fold H_2 production increase	11.5 mmol/mg protein/h	–	[67]
Encapsulation	*R. palustris P4*	AOT + isooctane reverse micelles	Phosphate	H_2 was 3.9 times higher	8.6 mmol/h/mg protein	–	[68]
Entrapment	*Clostridium* LS2	Polyethylene glycol	Endo-media	Optimizing polyethylene glycol concentration, initial biomass concentration, and temperature enhanced H_2 production	7.3L/L-POME/day	0.31 L H_2/g COD	[70]

–: not available; POME: palm oil mill effluent; GAC: granular activated carbon; RME: rice mill effluent; AOT: *N*-ethyl hexyl sodium sulfosuccinate.

Table 2. Parameters affecting biohydrogen production using immobilized microbial cells.

Immobilized cells	Carbon source	pH	Bead size (mm)	Support material	Reactor type	H_2 yield	H_2 production rate	Reference
Anaerobic sludge	Sucrose	5.5–7.0	5.0	PMMA	CSTR	2.25 mol H_2/mol sucrose	238 ml H_2/L/h	[80]
Anaerobic sludge	Sucrose	6.6	2–3	Silicone gel	CSTR	3.5 mol H_2/mol sucrose	15 L H_2/L/h	[122]
Anaerobic sludge	Sucrose	6.8	3–4	Silicone gel	DTFBR	1.20 mol H_2/mol sucrose	2.59 L H_2/L/h	[123]
Anaerobic sludge	Molasses	3.56–4.25	1.5–2	Activated carbon	CSTR	–	3.65 L H_2/l/h	[124]
Anaerobic sludge	POME	4.0–5.0	3.0	PEG	UASBR	–	0.632 L H_2/L/h	[48]
Anaerobic sludge	Sucrose	5.5	1–5	Pumice stone	UASBR	4–5 mol H_2/g sucrose	2.98 L H_2/L/d	[125]
Thermotoga neapolitana	Xylose	7	4.0	Glass beads	CSTR	1.84 mol H_2/mol xylose	5.64 mmol H_2/L/h	[108]
Anaerobic sludge	Glucose	4	5.0	Activated carbon	AFBR	–	2.36 L/L h	[126]
Swine wastewater	Glucose	3.68–4.05	2.8–3.35	Expanded clay	AFBR	2.41 mol H_2/mol glucose	–	[127]
Anaerobic sludge	Glucose	6.0–7.0	4.0	Silicone gel	CSTR	1.54 mol H_2/mol glucose	0.97 L H_2/L/h	[128]
Anaerobic sludge	Glucose	5.5	2.8–3.35	Polystyrene	AFBR	2.59 mol H_2/mol glucose	1.21 L H_2/L/h	[77]
Anaerobic sludge	Glucose	5.5	2.8–3.35	Ground tire	AFBR	2.25 mol H_2/mol glucose	–	[129]
Anaerobic sludge	Glucose	5.5	2.2	PET	AFBR	1.87 mol H_2/mol glucose	–	[129]
Anaerobic sludge	Glucose	3.8	2.8–3.35	Expanded clay	AFBR	2.29 mol H_2/mol glucose	1.28 L H_2/L/h	[130]
Anaerobic sludge	Glucose	4.86–5.53	2.8–3.35	Ground tire	AFBR	2.11 mol H_2/mol glucose	0.36 L H_2/L/h	[73]
Anaerobic sludge	Sucrose	6.8	3	Silicone gel	DTFBR	4.98 mol H_2/mol sucrose	2.27 L H_2/L/h	[36]

–: not available; PMMA: polymethyl methacrylate; PEG: polyethylene glycol; PET: polyethylene terephthalate; CSTR: continuous stirred tank reactor; DTFBR: draft tube fluidized bed reactor; UASBR: upflow anaerobic stirred bed reactor; AFBR: anaerobic fluidized bed reactor.

The process yields were enhanced when these biocatalysts were used in biohydrogen production (Table 2). Figure 2 summarizes the methods used to immobilize biohydrogen-producing microorganisms (Figure 3).

Process conditions and immobilized cells during biohydrogen production

Biohydrogen production using immobilized cells is governed by various process parameters such as carrier type, inoculum rate, carbon source, pH, and bioreactor configuration. Thus, understanding their effect could pave the way for optimizing and controlling the process.

Nature of the carrier

The selection of the carrier material (support) is crucial because it affects overall biohydrogen production performance. Thus, an ideal support should possess the following characteristics: (i) hydrophilic, (ii) non-toxic to microorganisms, (iii) non-biodegradable, (iv) strong mechanical stability, (iv) inexpensive, (v) high biomass retention, and (vi) good permeability. Support materials are classified as either organic or inorganic based on their chemical composition [42,43]. Moreover, organic materials are more abundant and are subdivided into natural or synthetic carriers. Natural carriers include: alginate, agar, collagen, starch materials, clay, activated carbon, agarose, carrageenan, chitosan, and other types of natural materials [44]. Synthetic materials include: polyurethane, acrylamide, polyvinyl alcohol, polyethylene glycol, polycarbamoly sulfonates, polypropylene, and polyacrylonitrile [45]. Meanwhile, materials such as silica, celite, clay, zeolite anthracite, porous glass, and activated charcoal are categorized as inorganic carriers [45]. Natural polymers are mostly used because they are non-toxic, affordable, and easily available. However, they suffer from weak mechanical stability and may be vulnerable to microbial degradation [45]. Thus, synthetic carriers are preferred because they have stronger mechanical stability than their natural counterparts [51].

Several authors have investigated the effect of different support materials on biohydrogen production process. Barros et al. [76] used polystyrene and expanded clay as carriers for biohydrogen-producing anaerobic sludge, and obtained a high biohydrogen yield of 2.59 mol H_2 mol/mol glucose using expanded clay. Wu et al. [47] evaluated the effect of supplementing calcium alginate with three types of support material: activated carbon (AC), polyurethane (PU), and acrylic latex plus silicone (ALSC). Beads enriched with AC produced optimal biohydrogen yield of 2.6 mol H_2/mol sucrose. In addition, Nunes et al. [77] examined four support materials of expanded clay, porous ceramic, polyethylene, and charcoal for biohydrogen production potential using packed bed reactors at an hydraulic retention time (HRT) of 24 h. The maximum biohydrogen production were 3.2, 2.6, 0.4, and 0.05 mol H_2/mol carbohydrates total for expanded clay, porous ceramic, polyethylene, and charcoal, respectively. Recently, Kirli and Kapdan [78] compared different microbial support materials of plastic nylon sponge, plastic scouring sponge pad, and plastic scouring sponge pad with metal mesh for biohydrogen production process; and achieved an optimum yield of 2.1 mol H_2/mol glucose from metal mesh covered with a plastic scouring sponge pad.

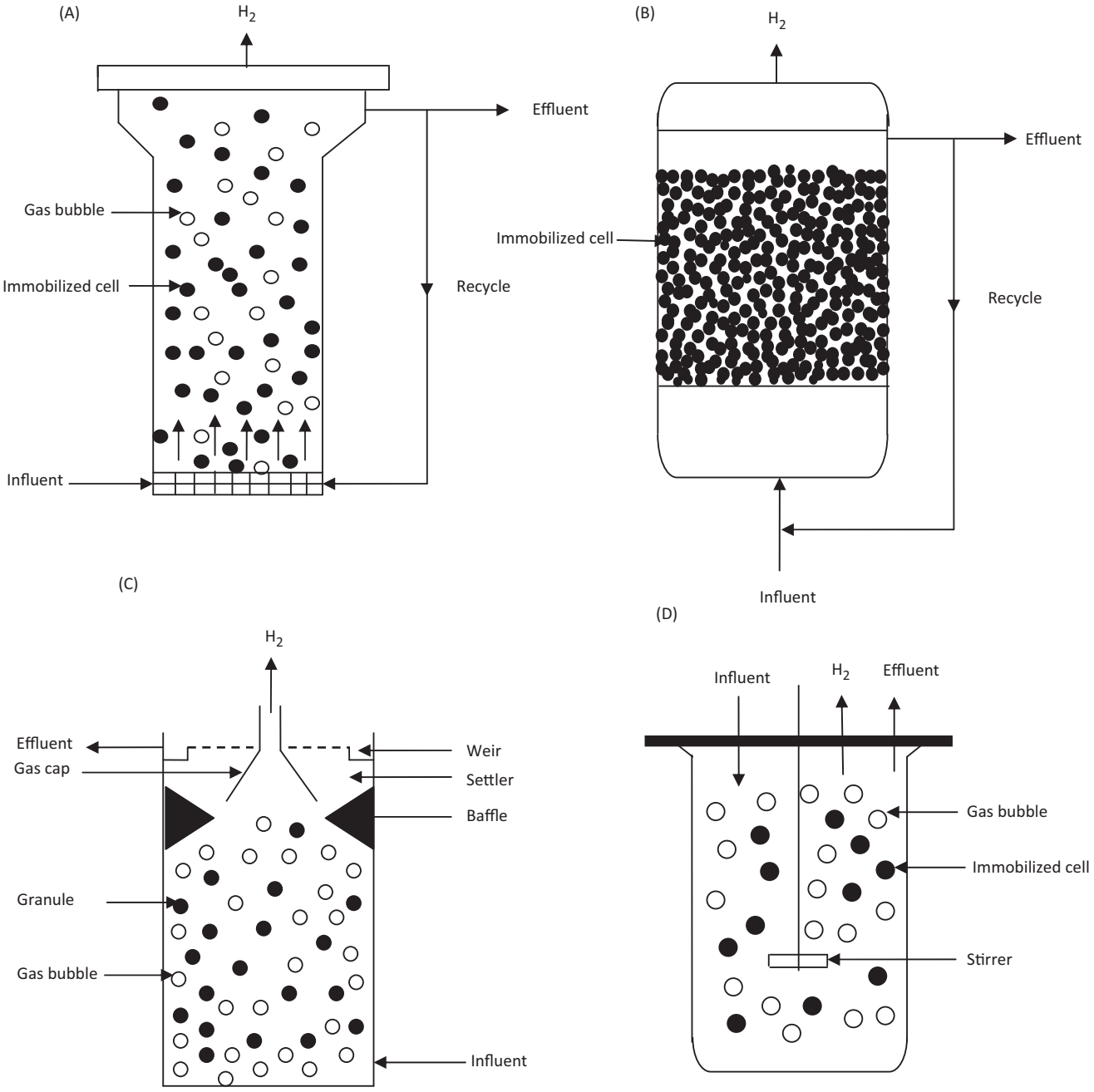

Figure 3. Different schematics of bio-hydrogen bioreactors consisting of immobilized cells are shown, (A) Anaerobic fluidized bed reactor, (B) packed bed reactor, (C) upflow anaerobic sludge bed reactor, and (D) stirred tank reactor (adapted from [108]).

Bead size

Bead size affects the distribution of cells within the bioreactor and in turn influences the substrate conversion efficiency. Various bead sizes have been used in biohydrogen immobilization studies. Wu and Chang [79] observed a stable biohydrogen performance using immobilized-polymethyl methacrylate (PMMA) beads with a diameter of 5 mm. The PMMA beads attained high biohydrogen yield (2.0 mol H_2/g sucrose). They were also supplemented with collagen and activated carbon to improve their pore size, density, and mechanical stability [79]. Xie et al. [80] examined different diameter sizes of activated carbon fibers (2, 4, and 6 mm) and discovered that the biohydrogen yield decreased with an increase in activated carbon size due to an uneven fluidization pattern within the bioreactor. A maximum biohydrogen yield of 2.65 mol H_2/mol acetate which corresponded to a production rate of 28.45 ml H_2/L/h was obtained using a carrier size of 2 mm [80]. The effect of three different polyethylene ethylene glycol bead sizes of 3, 4, and 5 mm on the biohydrogen production rate was assessed by

Singh et al. [81]. A maximum rate of 427 ml/L/h was obtained using a bead size of 3 mm [81]. In other microbial studies, the size of the beads and their morphology were highlighted as the key factors that affected the overall process yield [82]. For example, it was shown that spherical beads with a narrow size of 1.32 to 1.70 mm were best suited for encapsulation of *Lactobacillus acidophilus* DMSZ20079 i.e. the encapsulation efficiency of more than 98% was achieved [82]. These findings demonstrate the importance of using an appropriate bead size for effective biohydrogen production process.

pH of the fermentation medium

pH is one of the most crucial parameters in the biohydrogen production process. It affects the hydrogenase activity, metabolic activity, and substrate hydrolysis [83]. Moreover, it should be operated at optimum conditions in order to suppress the growth of biohydrogen-consuming reactions [84]. This variable has been evaluated in several biohydrogen-producing studies using immobilized microbial cells. Wang et al. [53] reported peak production (12.27 mmol $L^{-1} h^{-1}$) at pH 4.4–4.5 using activated carbon as a support material. It was also shown that pH and hydraulic retention time (HRT) are joint parameters and short HRT of 5 h along with pH 4.4–4.5 inhibited the formation of biohydrogen-consuming methanogenic archaea and hence maximized its production. This occurrence might be attributed to the fact that biohydrogen-competing reactions are usually suppressed at low pH, while short HRTs prevent the growth of biohydrogen-inhibiting microorganisms which usually requires extended incubation periods [85]. Keskin et al. [86] also achieved a five-fold biohydrogen increase when the pH was maintained at 4.5–5.0 in a continuous system packed with ceramic beads. Ren et al. [87] maintained a pH value of 6 during the 20 d period of continuous biohydrogen production process using granular activated carbon material. Nonetheless, other pH values ranging from 4 to 9 have also been reported in the literature due to several contributing factors such as support material type, substrate, microbial consortia, and process conditions employed [88–90].

Carbon source

Monomeric sugars such as glucose [58,61,72,76], sucrose [46], and other hexoses [54] are widely used in biohydrogen immobilization studies. However, these substrates are expensive and might escalate the process costs at large-scale [91]. Therefore, economic viable feedstocks such as biowaste effluents are currently being exploited due to their accessibility and nutritional content. Carbohydrate containing feedstocks are highly favored because they are rich in nutritional composition i.e. 80–95%, volatile solids, and 75–85% moisture and hence enhance the production of biohydrogen [92–95]. Moreover, the microbial hydrogen production process from waste materials presents many opportunities such as clean energy production while mitigating environmental pollution. Thus, many researchers are incorporating biowaste effluents in their biohydrogen augmentation studies using immobilized biocatalysts i.e. palm oil mill effluent (POME), rice mill effluent (RME), and food waste as shown in Table 1. POME is an ideal feedstock because it comprises high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) content of 50,000 and 25,000 mg/l, respectively [96]. Singh et al. [48] used POME for biohydrogen production using mixed sludge immobilized with polyethylene glycol and achieved a better yield of 0.589 H_2 L/(L POME h) that was 1.7 times higher compared with suspended cells. In a similar study using POME, Ismail et al. [49] reported a maximum biohydrogen production rate of 2.1 NL/L/d using anaerobic sludge immobilized with polydimethyl siloxane, and were able to prolong its production for 150 d. RME is also regarded as a suitable substrate because it consists of various organic components such as chemical oxygen demand (2578–6480 mg/l), biochemical oxygen demand (510–6900 mg/l), and total suspended solids (700–3010 mg/l) [51].

Types of microorganisms used in immobilization

Biohydrogen production is carried out using diverse microorganisms which are either pure or mixed cultures. Mixed cultures are better suited than pure cultures because of the following reasons: (i) the levels of contamination is minimized, (ii) there is high microbial diversity, (iii) there is mixed co-fermentation amongst microorganisms, (iv) ideal for continuous processing, and (v) utilization of diverse feedstocks [97,98].

Clostridium and *Enterobacter* species are the mostly used organisms in biohydrogen immobilization studies [51]. *Clostridium* species are Gram-positive, spore-forming, and rod-shaped obligate anaerobes [99]. *Enterobacter* species are facultative, Gram-negative, and rod-shaped organisms; however they produce low yields as compared with *Clostridium* species [100]. A maximum biohydrogen yield of 223 ml H_2/g hexose which corresponded to a production rate of 7.2 l H_2 L^{-1} d^{-1} was achieved from immobilized strain of *Clostridium tyrobutyricum* JM1, using polyurethane

foam as a support material [73]. Moreover, the utilization of immobilized cells enhanced the substrate conversion efficiency to 97.4% [73]. Zhao et al. [101] obtained a 40.8% increase in biohydrogen production from immobilized *Clostridium* T2 strain. Plangklang et al. [102] also used *Clostridium butyricum* TISTR1032 immobilized on sugarcane bagasse. An optimum yield of 1.52 mol H_2/mol hexose and productivity of 3.5 L H_2/L/d were obtained from the process. The use of immobilized *Enterobacter* species is also documented in literature. Kumar and Das [103] enhanced the production of biohydrogen by 2.1 times using immobilized *Enterobacter cloacae* IIT-BT 08 on lignocellulosic solid matrices. The highest biohydrogen production rate achieved was 75.6 mmol/L/h. Reungsang et al. [104] used immobilized *Enterobacter aerogenes* ATCC 13048 on heat-treated UASB granules and observed an optimum production rate of 6.24 mmol H_2/L/h from waste glycerol. Meanwhile, Han et al. [105] immobilized *Enterobacter aerogenes* ATCC 29007 with agar and attained a biohydrogen yield of 0.74 ml H_2/g glycerol.

Non-sporeformers have been studied as well; Ngo and Bui [106] employed immobilized cells of hyperthermophilic *Thermotoga neapolitana* on porous glass beads. The biohydrogen production rate and yield were 5.64 mmol H_2 $L^{-1}h^{-1}$ and 1.84 mol H_2/mol xylose, respectively; and were 1.7-fold and 1.3-fold higher than those of suspended cells. *Klebsiella* sp. TR17, a Gram-negative and facultative anaerobic organism was immobilized with pretreated methanogenic granules and produced a high biohydrogen rate and yield of 242.15 mmol H_2/L/d and 44.27 mmol H_2/g glycerol, respectively [107]. In another study, two strains of *Brevundimonas diminuta* (B1 and B2) were immobilized on calcium alginate for biohydrogen production. The production of biohydrogen was elevated to 1200 ml H_2/culture and 1300 ml H_2/culture for B1 and B2, respectively. Biohydrogen-producing microorganisms are constantly being enumerated from diverse environments and are characterized using various molecular techniques such as 16 S rRNA PCR-DGGE, FISH, and Microarrays [1].

Type of bioreactor configuration during immobilization

Among the bioreactors configurations reported in the literature, continuous stirred tank reactors (CSTRs) are widely used because they enhance biomass retention, substrate utilization, and biohydrogen yields [108]. Other bioreactor types such as a packed bed reactor (PBR), anaerobic fluidized bed reactors (AFBR), and upflow anaerobic sludge bed reactors (UASBR) are used as well because of their ability to retain large amounts of biomass; hence microorganisms are well retained in these reactors in the form of biofilms/granules [109,110]. They also improve mass transfer and extend the fermentation periods [109]. Better biohydrogen performance was reported using these reactors. For instance, Pekguzel et al. [111] maximized biohydrogen production using thermophilic cultures immobilized on raschig rings in a PBR; the biohydrogen production rate was 7–11 times higher compared to suspended cells [111]. Amorim et al. [112] reported an enhanced biohydrogen yield of 1.9 mol H_2/mol glucose and production rate of 2.04 L/h/L at HRT of 2 h in an AFBR. Andreani et al. [113] used AFBR supported with bamboo stems to generate a biohydrogen rate of 1.1 L/L/d. pH was successfully maintained within the optimal range of 4.5–6.0 at organic loading rates of 15–28 g. L^{-1} d^{-1} [113]. In another study, Wu et al. [114] reported an optimum yield and production rate of 2.67 mol H_2/mol sucrose and 0.93 L/h/L, respectively, in an AFBR immobilized with a mixture of acrylic latex, silicone, sodium alginate, and activated carbon. Evaluation of two reactor types (CSTR and AFBR) was conducted by Zhang et al. [115]; CSTR showed a better biohydrogen production performance i.e. a maximum biohydrogen yield of 1.88 mol H_2/mol glucose was obtained, whereas AFBR produced only 1.71 mol H_2/mol glucose [115]. It has been shown that low biohydrogen yields in AFBRs are primarily caused by pH imbalance and heterogeneous distribution of microbial cells [116]. To overcome this challenge, it was suggested that a recirculation flow be used during biohydrogen production process [51]. AFBRs are able to prevent cell washout during continuous bioprocesses because of this feature [116]. Moreover, incorporating various designs in AFBRs was shown to improve the biohydrogen process yield i.e. AFBRs with tubular, tapered, and rhomboid shape configuration were used to improve the biohydrogen process conversion efficiency by Kumar and Das [117]. The rhomboid shaped reactor produced optimal biohydrogen production rate of 1.60 L H_2/L/h, compared with tubular and tapered shaped reactors which produced 1.46 and 1.40 L H_2/L/h, respectively.

UASBR systems have been applied during biohydrogen production due to their good biomass retention ability and enhanced biohydrogen production [116]. They are an alternative to AFBRs and consist of an influent port that is positioned at the bottom of the reactor [50]. Various support materials such as alginates, expanded clay, ceramic balls, activated carbon, ceramic rings, and stones are used in these reactors [46,47,51,55,86,118]. Keskin et al. [86] used ceramic balls as a support material for biohydrogen production using

UASBR and CSTR. The UASBR showed better performance with a biohydrogen production rate of 2.7 H_2 L/L/d at HRT of 3 h. Meanwhile, the CSTR produced a biohydrogen production rate of 0.5 H_2 L/L/d at HRT of 24 h. Moreover, the UASBR was more resistant to cell washout as compared with CSTR [86]. Veeravalli et al. [119] investigated the effect of an organic loading rate on biohydrogen production using granulated UASBR and attained a high biohydrogen yield of 1.64 mol H_2/mol glucose at 12.8 g COD L^{-1} d^{-1}. Increasing the organic loading rate from 8.6 to 12.8 g COD/L/d favored biohydrogen-producing hydrogenase enzymes and suppressed the biohydrogen-consuming methanogenic archaea [119]. It is important to maintain a large population of biohydrogen-producing microorganisms to ensure high process yields. Hence, some studies assessed the microbial composition in these reactors during the biohydrogen production process i.e. microbial community analysis was carried out in a granulated UASBR by Ning et al. [120]. The dominant bacterial cultures were *Variovorax paradoxus* SJ100, *Variovorax* CN3b, *Clostridium* HPB-4, and *Janthinobacterium* WPCB148 [120,121]. In addition, all the process parameters affecting biohydrogen production using immobilized microbial cells are summarized in Table 2.

Conclusions and future outlook

Cell immobilization could play a pivotal role in biohydrogen process development by overcoming the low yields which hinders its large-scale production. These cells are beneficial in the biohydrogen production processes because they increase microbial concentrations, increase operational stability, enhance substrate conversion efficiency, stabilize pH, extend the fermentation periods, minimize contamination, protect cells against toxic fermentation by-products, and maximize biohydrogen yields. This technology possesses these benefits, many technical issues needs to be addressed such as finding inexpensive and stable support carriers, most support materials are less permeable, and uneven fluidization pattern is often experienced. To advance this technology and overcome these limitations, several recommendations are proposed for future studies, which include:

- Developing more stable immobilization matrices. For instance, nano-scale materials (for example, nanofibres, and nanotubes) have been shown to be advantageous in various applications as a result of their large surface area and high porosity. Hence they could play a pivotal role in biohydrogen process advancement.
- Incorporating genetically engineered strains which are tailored for cell immobilization will improve biohydrogen production performance. However, factors such as mechanical stability, reusability of cells, resistance against pH decrease, and inhibition to toxic by-products needs to be taken into account when modifying such microorganisms.
- Conducting more biohydrogen scale-up processes using immobilized microbial cells to fully understand the process constraints and implement innovative improvement strategies.
- Multidisciplinary collaboration of biological, material, and chemical sciences will progressively advance this technology and brings about new insights.

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