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Advanced strategies for enhancing dark fermentative biohydrogen production from biowaste towards sustainable environment

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Abstract

As a clean energy carrier, hydrogen is a promising alternative to fossil fuel so as the global growing energy demand can be met. Currently, producing hydrogen from biowastes through fermentation has attracted much attention due to its multiple advantages of biowastes management and valuable energy generation. Nevertheless, conventional dark fermentation (DF) processes are still hindered by the low biohydrogen yields and challenges of biohydrogen purification, which limit their commercialization. In recent years, researchers have focused on various advanced strategies for enhancing biohydrogen yields, such as screening of super hydrogen-producing bacteria, genetic engineering, cell immobilization, nanomaterials utilization, bioreactors modification, and combination of different processes. This paper critically reviews by discussing the above stated technologies employed in DF, respectively, to improve biohydrogen generation and stating challenges and future perspectives on biowaste-based biohydrogen production.

Keywords: Biohydrogen production; Biowaste; Dark fermentation; Purification; Advanced technology

1. Introduction

Currently, fossil fuels are the world's primary form of energy. The demand for energy has hugely increased due to the rapid growth of the global population and higher living standards. It is established that the total energy consumption will increase by 40% by 2040 (Cronshaw & Economics, 2015). Fossil fuels, including

74 coal, oil and natural gas, are non-renewable resources and take millions of years to be
75 produced again. Fossil fuels cause serious environmental problems like climate
76 change such as CO_2 emissions and other greenhouse gases (GHGs) (Dincer & Acar,
77 2015). In this case, the focus of current research moves to the exploration of
78 renewable and green energy sources. Hydrogen is deemed to be an alternative energy
79 source that can overcome the problems caused by fossil fuels. The energy content of
80 hydrogen (149.1 kJ/g) is much higher than that most of common fossil fuels (Suzuki,
81 1982). Hydrogen is a clean fuel and harmless to the environment and human health in
82 comparison with fossil fuels as water is the only by-product (Baykara, 2018).
83 Additionally, hydrogen has emerged as an ideal fuel and outperforms other
84 hydrocarbon-based biofuels in combustive engines (Dicks et al., 2004). Vehicles with
85 hydrogen fuel-based engines and fuel cells can reduce their fuel gas emissions and
86 achieve the real 'greenization' of vehicles.

87 Hydrogen can be generated from fossil fuels, biomass and water through
88 thermochemical, electrochemical and biological methods. Currently, hydrogen is
89 mainly produced from fossil fuels through the steam reforming of natural gas,
90 gasification, electrolysis of water and as a co-product from a few industrial processes
91 (Chandrasekhar et al., 2020). However, the thermo- and electro-chemical processes
92 for hydrogen production consume a lot of energy and cause serious environmental
93 issues. Hydrogen produced from the fossil fuel-based petroleum or coal is
94 nonrenewable and is accompanied by release of greenhouse gases into the
95 environment (Mishra et al., 2019). Although hydrogen production from water

96 electrolysis might be the cleanest technology, its commercial application is limited by
97 the high cost of electricity, accounting for 80% of the total operating cost of
98 hydrogen production (Armor, 1999).

99 By contrast, hydrogen produced from renewable biomass using microbes at
100 ambient temperatures and pressures using biological processes is less energy intensive
101 and environmentally friendly, and referred to as biohydrogen (Das & Veziroğlu,
102 2001). Biowastes, such as agricultural residuals, food waste, sewage sludge and
103 wastewaters can be used as feedstocks for biohydrogen production. Proper
104 management of these biowastes is essential to avoid further environmental pollution.
105 The conventional disposal of solid biowastes mainly includes landfills, incineration
106 and biomethanization, which requires large areas of land, is expensive, and releasing
107 greenhouse gases into the environment. Alternatively, the conversion of biowastes to
108 biohydrogen is a green and sustainable strategy that can resolve problems of energy
109 shortages, waste management, environmental pollution and global warming, which
110 plays a major role in achieving the circular bioeconomy and sustainable economic
111 development (Sharma et al., 2020).

112 Anaerobic fermentation (AF) is one of the most common biological technologies
113 for biohydrogen production from biowastes using microorganisms. AF is divided into
114 dark fermentation (DF), photo fermentation (PF) and integrated dark-photo
115 fermentation depending on the necessity of light for the microorganisms (Hay et al.,
116 2013). In the DF process, biohydrogen is produced through the acidogenesis of
117 biowaste hydrolysates with fatty acids and other metabolic intermediates as by

118 products in the absence of light. The fatty acids can be further degraded through
119 acetogenesis to hydrogen and acetate (Nandi & Sengupta, 1998). Hydrogenase plays
120 an important role in promoting the reaction of proton and electron for biohydrogen
121 production in the DF process. For the biohydrogen production in the PF process,
122 photosynthetic bacteria are responsible for the conversion of simple sugar like glucose
123 and fatty acids to biohydrogen by using sunlight as energy and the nitrogenase as
124 catalyst (Mishra et al., 2019). DF and PF can be combined to a hybrid system.

125 DF is the most studied method of biohydrogen production from biowastes among
126 biological processes because it is independent of light and suitable for a wide range of
127 biowastes (Sekoai et al., 2020). The biohydrogen production in DF is complex
128 process. Pure cultures, such as *Clostridium*, *Enterobacter* or *Escherichia coli*, as well
129 as mixed cultures like anaerobic sludge, bovine manure or organic compost, can be
130 used as inoculum in DF process. Operating parameters, such as pH, temperature, and
131 biohydrogen partial pressure also can influence the biohydrogen production in DF
132 (Soares et al., 2020).

133 However, only part of substrates can be converted in the fermentative process,
134 resulting in low yields of biohydrogen production. In comparison with the commercial
135 hydrogen production from fossil fuels, relative low hydrogen yields, production rate,
136 as well as high costs prevent the large-scale application of fermentative biohydrogen
137 production. To solve these challenges, various studies on fermentative biohydrogen
138 production have been conducted. To enhance biohydrogen production yields,
139 advanced approaches such as genetic engineering to change metabolic pathways to

increase substrate utilization and improve the electron flux used to the reduction of protons, nanotechnologies to improve biohydrogen production rate by enhancing the catalytic activity of enzymes, and combination of different processes to increase biohydgeon production synergistically. This review evaluated various advanced strategies in the DF process, as well as advanced purification methods of the fermentative biohydrogen to improve the production and application of biohydrogen.

2. Biowastes used for biohydrogen production

Biowastes rich in carbohydrates in the form of single sugars, starch and cellulose are renewable and promising feedstocks for biohydrogen production (Banu et al., 2020). It is reported that the biohydrogen production potential from carbohydrate-based waste was around twenty times higher than that from fat and protein-based wastes. Karadag et al. (2014) indicated that the low biohydrogen production (14–156 mL H_2/g COD) from dairy wastewater might attributed to partial consumption of biohydrogen during protein degradation. Carbohydrate-rich wastes like agricultural and food processing wastes, industrial wastewater, and waste sludge from wastewater treatment plants have high potential to be used for biohydrogen production (Kapdan et al., 2006). Microorganisms in biological processes can use these biowastes as carbon sources for their metabolisms and produce biohydrogen. Therefore, the conversion of biowastes to biohydrogen through biological processes emerges as an environmentally friendly and economical strategy for achieving both biowaste treatment and valuable biohydrogen production, and boosting the development of the circular economy.

Abundance of agricultural wastes, such as rice straw and husk, wheat straw, corn

stover, cobs and bran, as well as sugarcane bagasse, etc., are produced every year from the growing and processing of agricultural products and will continue to be produced in the future owing to population growth and economic advancement. The agricultural wastes are renewable, easily available, non-toxic, and environment friendly. However, complex carbohydrates like cellulose, hemicelluloses and starch in agricultural wastes are difficult to be degraded by microorganisms for biohydrogen production directly. These high molecular weight compounds require to be pretreated and hydrolyzed to get monomer unit like glucose and maltose that can easily accessible by hydrogen-producing microorganisms (Kumari et al., 2018). Pretreatment methods, including physical, chemical, and biological strategies have been extensively reviewed previously (Singh et al., 2021). Chemical pretreatment is the most-effective one to degrade complex carbohydrates among these methods, however, high costs of chemicals and the produced inhibitors limit its industrial application. Although enzymes are promising to reduce the production of inhibitors, the high cost of the present commercial enzyme is another obstacle for the large-scale application economically (Saravanan et al., 2021). Therefore, further studies on the development of low-cost methods for the effective pretreatment and the production of economic enzymes are important in the future. Compared with cellulose and hemicelluloses, the pretreatment of starch is comparatively simple. Starch can be hydrolyzed into smaller subunit by physical, thermal, chemical and biological (enzymatic) or combination of these methods (Kumar et al., 2019). Food wastes produced from food processing industrial, such as corn, wheat, rice, banana and

potato processing industry, contain huge amount of starch are potential sources of biohydrogen (Das et al., 2021). Moreover, food wastes generated from industrial effluent are usually homogenous and contain high amount of sugar could be used for biohydrogen production directly (RedCorn et al., 2018).

Industrial wastewater generated from the sugar industry and food processing rich in easily biodegradable carbohydrates are considered as ideal feedstocks for biohydrogen. Sugar molecules including glucose, maltose, and sucrose could be directly metabolized by the biohydrogen producing microbes without any pretreatment (Arimi et al., 2015). Although wastewater from food processing industries was able to be used for biohydrogen production directly, the high organic contents in wastewater may reduce the biohydrogen yield. Dilution or other pretreatment might be necessary to enhance biohydrogen production (Ntaikou et al., 2010). It demonstarted that biohydrogen production from tofu processing effluent was increased 2.8-fold after the dilution of the wastewater with tap water and then mixed with 0.5% HCl for 5 min. Cappelletti et al. (2011) also discovered that a higher hydrogen production (2.41 mol H_2/ mol glucose) from cassava processing wastewater could be achieved by reducing the organic load of the raw wastewater.

For wastewater originating from oil refineries and containing a mixture of carbohydrate, lipids and other organic compounds, can also serve as sources of biohydrogen production (Usman et al., 2019). Ntaikou et al. (2009) reported that the content of carbohydrates in olive mill wastewater can reach 60% of its total dry weight. The biohydrogen production from palm oil mill wastewater (20 g COD/L)

206 was conducted using immobilized and suspended-cell culture in upflow anaerobic
207 sludge blanket reactors (Singh et al., 2013). The author indicated that higher hydrogen
208 production rate was obtained in the immobilized-cell containing reactor at hydraulic
209 retention time (HRT) of 2 h.

210 Waste activated sludge also has been considered as potential sources for
211 biohydrogen production, due to its high organic content and huge quantity
212 (Assawamongkholsiri et al., 2013). It is estimated that the global waste activated
213 sludge production was 0.1-30.8 kg per person per year (Kumar, 2018). Organic
214 components of waste activated sludge mainly include sludge flocs, extracellular
215 polymeric substances and the materials inside of the microbial cell membranes (Li et
216 al., 2015). The direct biohydrogen production rate from raw sludge was very poor
217 because of the minimal release of soluble organics in the raw sludge (Yin & Wang,
218 2015). To increase the conversion efficiency of waste activated sludge to
219 biohydrogen, prior pretreatment of the sludge is required to release the organics into
220 the solution so that biodegradability is improved, and thus eventually increase
221 biohydrogen production (Liu et al., 2017). Several methods, including ultrasound,
222 thermal, chemical, biological, and a combination of these methods have been devised
223 for the pretreatment of the sludge (Wang et al., 2014). The co-fermentation of waste
224 sludge with other organic wastes, such as agricultural wastes, food waste, forestry
225 wastes, grass residuals and wastewaters, has been considered an effective method to
226 enhance biohydrogen production (Yang et al., 2017). Yang et al. (2019) noted that the
227 co-fermentation of sewage sludge with grass residue and fallen leaves could

228 significantly enhance the biohydrogen production rate. Furthermore, a synergistic
229 effect of the co-fermentation on biohydrogen production was observed.

230 **3. Advanced technologies for enhancing biohydrogen production**

231 **3.1. Advanced technologies in dark fermentation process**

232 Dark fermentation (DF) is one of the most promising clean technologies for
233 biohydrogen production as it can convert various biowastes into biohydrogen under
234 mild fermentation conditions, whereas, low biohydrogen yields limit the industrial
235 application of dark fermentation. During the DF process, only part of the substrates
236 can be converted to biohydrogen, and most of them (60–70%) remain in the form of
237 volatile fatty acids (VFAs) and alcohols. The maximum biohydrogen yield can be 4
238 mol /mol glucose if acetic acid is the only by-product. As reported, the yield of
239 biohydrogen was only 1- 3 mol /mol glucose by DF in most cases Sekoai et al., 2020).
240 Thus, several advanced technologies including novel microbial culture selection,
241 genetic engineering, cell immobilization and nanotechnology have been conducted in
242 recent years to enhance biohydrogen production in the DF process.

243 **3.1.1 Microorganisms selectin in DF process**

244 In the DF process, the efficiency of microorganisms exerts a great influence on
245 biohydrogen production yields. The DF process could be operated using different
246 types of microorganisms, including wild-type mixed culture, pure culture and co-
247 culture (Lee et al., 2011). Traditional mixed culture used in the DF process contain
248 not only hydrogen producers but also hydrogen consuming microorganisms (e.g.,
249 acetotrophic and hydrogenotrophic methanogens and sulfate-reducing bacteria), so as

the produced biohydrogen can be further consumed by the hydgrogen consumer. Therefore, thermal or chemical treatment processes were usually used to deactivate the hydrogen consuming microorganisms (Reddy et al., 2017). Several researchers focused on the isolation of novel bacteria strains for improving biohydrogen production from various substrates (Show et al., 2012).

The limitation of biohydrogen consumption in the mixed culture can be solved by using the pure culture. Microorganisms including *Clostridium* sp., *Enterobacter* sp., *Klebsiella* sp., *Citrobacter* sp. and *Bacillus* sp. are known to be super biohydrogen producers in DF systems (Bravo et al., 2015; Lertsriwong & Glinwong, 2020). For example, a research by Nizzy et al. (2020) found that it is feasible for biohydrogen production from sago industrial wastewater using new isolated pure culture of *Clostridium sartagoforme* NASGE 01 and *Enterobacter cloacae* NASGE 02 from sago industrial effluent. Up to 56.7% of the substrate could be degraded by *Clostridium sartagoforme* NASGE 01 with the maximum biohydrogen yield of 1.26 mol H_2/mol glucose. Lertsriwong and Glinwong (2020) also successfully screened new microbial species (*Bacillus coagulans* MO11 and and *Clostridium beijerinckii* CN) with effective biohydrogen producing ability from molasses and ethanol refinery wastewater. Two pure *Bacillus cereus* strains (*Bacillus cereus* RTUA and RTUB strains) with multi-enzyme capabilities were isolated from anaerobic digester and proved to be potential candidates for biohydrogen production from different substrates in one recent study (Saleem et al., 2020). However, it is difficult to maintain a pure culture without contamination due to various pollutants from wastewaters and

biowastes. The strict and sterile conditions for the pure culture consumed more energy and led to high operating costs. Comparatively, co-cultures, which are a combination of different pure hydrogen producers, constitute a promising method to solve limitations of the wild-type mixed culture and pure culture process to improve biohydrogen production.

As reported by Mthethwa et al. (2019), the co-culture with the mixture of different biohydrogen producers including *Enterobacteriaceae, Gammaproteobacteria, Betaproteobacteria, and Clostridium histolyticum* obtained higher biohydrogen yield (2.3 mol H_2/mol glucose) from *Pistia stratiotes* hydrolysate than that of the pure culture *Bacillus cereus (*2.21 mol H_2/mol glucose*)*, *Bacillus anthracis (*1.10 mol H_2/mol glucose*)* and *Enterobacter cloacae (*1.97 mol H_2/mol glucose*)*. The co-culture exhibited a synergistic effect on biohydrogen production and was more stable compared to the mixed or pure cultures (Abreu et al., 2016). The use of defined cultures through controlling the bacterial composition could control metabolic pathways and products, thereby increasing the biohydrogen yields (Ozmihci & Kargi, 2011). The use of defined cultures through controlling the bacterial composition was also able to control metabolic pathways and products, thereby increasing the biohydrogen yields (Ergal et al., 2020). Based on prior physiological and biotechnological knowledge from meta-data analysis, a novel precision artificial mixed culture was developed by selecting microorganisms with specific metabolic and economic functions to break the limitation of biohydrogen production (4 mol H_2/mol glucose) (Ergal et al., 2020). The authors indicated that the

294 defined artificial microbial consortia contained two hydrogen-producing species -
295 *Enterobacter aerogenes* and *Clostridium acetobutylicum* - which increased the
296 biohydrogen yield to 5.6 mol/mol glucose, 40% higher than the Thauer limit. They
297 also exhibited a higher biohygrogen production rate than mono-cultures of
298 *Enterobacter aerogenes* and *Clostridium acetobutylicum*.

299 **3.1.2 Genetic engineering in DF process**

300 Genetic engineering as an effective technology to improve biohydrogen production
301 has received increasing attention recently (Mohanraj et al., 2019). Metabolic reactions
302 mainly occurred in facultative anaerobes *Escherchia coli* and anaerobic *Clostridium*
303 sp., representing two basic metabolic pathways for biohydrogen production with
304 different side products (Majidian et al., 2018). The substrate-like glucose was
305 degraded to pyruvate in the first step, and then pyruvate was degraded through the
306 pyruvate:formate lyase (PFL) pathway in *Escherchia coli* and pyruvate:ferredoxin
307 oxidoreductase (PFOR) pathway in *Clostridium* sp., respectively (Hallenbeck, 2009).
308 Ni-Fe hydrogenase and Fe-Fe hydrogenase are used to catalyze the biohydrogen
309 production in these two pathways (Mohanraj et al., 2019). Therefore, most mutations
310 for improving biohydrogen production take place in *Escherchia coli* and *Clostridium*
311 sp. by: 1) inactivating uptake of hydrogenase (*hyd1, hyd2*) to prevent hydrogen
312 oxidation; 2) inactivating lactate dehydrogenase (*ldhA*) to eliminate a drain on
313 pyruvate; 3) inactivating fumarate dehydrogenase (*frdBC*) to eliminate side reaction
314 thereby increasing pyruvate; 4) inactivating formate-hydrogen lyase (FHL) repressor
315 (*hycA*) to increase in FHL; as well as 5) overexpressing FHL complex (*fhlA*) and

316 hydrogenase (*hydA*) (Hallenbeck & Ghosh, 2012; Mohanraj et al., 2019). Examples of
317 genetic engineering for improving biohydrogen production are summarized in Table
318 1.

319 *Insert Table 1*

320 For instance, as reported by Poladyan et al. (2018), the mutations in *Escherichia*
321 *coli* genes led to the inactivation of uptake hydrogenase (*hyd1*, *hyd2*) and then
322 achieved double the amount of biohydrogen produced from brewery waste compared
323 to the wild type. Cha et al. (2013) stated that the deletion of *Caldicellulosiruptor*
324 *bescii* lactate dehydrogenase by a mutation method increased biohydrogen production
325 by 21-34% in comparison with the wild type, by shifting the metabolic pathway from
326 the production of lactate to acetate and hydrogen. Wang et al. (2011) isolated a
327 dominant hydrogen producer, *Clostridium perfringens* and increased hydrogen yield
328 and acetate and butyrate concentrations by 51%, 26%, and 57%, respectively, through
329 a double mutation to delete the *plc* gene (encoding an alpha toxin protein) and *ldh*
330 gene (encoding lactate dehydrogenase). Hallenbeck and Ghosh (2012) and Majidian et
331 al. (2018) reviewed that moderate increase of biohydrogen yields (20 - 45%) could be
332 achieved by inactivating the uptake hydrogenases, lactate dehydrogenase and
333 fumarate dehydrogenase in *Escherichia coli*, with the value ranging from 1.37 to 2.11
334 mol H_2/mol glucose. The overexpression of hydrogenase in *Clostridium* obtained the
335 biohydrogen yield of 1.8 – 2.4 mol H_2/mol glucose.

336 Additionally, induction of certain microbial mutations in the co-culture improved
337 the microbial performance and then increased the biohydrogen yield (Ramprakash &

338 Muthukumar, 2014). Veeramalini et al. (2019) studied the biohydrogen production
339 from brewery effluent using mutated co-culture of *Rhodobacter* M 19 and
340 *Enterobacter aerogenes*, and concluded that the immobilized strains mutated by
341 ethidium bromide enhanced around 30% hydrogen production than that of wild
342 strains. However, the rising biohydrogen yields via increasing the activity of enzymes
343 in particular pathways is only effective when the number of specific enzymes is
344 limited. This scenario depends on the amount of degradable substrate generated from
345 different culture conditions (Hallenbeck & Ghosh, 2012).

346 **3.1.3 Immobilization technologies in DF process**

347 Continuous biohydrogen production from large amounts of organic waste is
348 necessary to make the whole process industrially worthwhile. During biohydrogen
349 production, maintaining the microbial cell inside reactors is important to maximize
350 the microbial efficiency, because the suspended biomass can be easily washed out
351 from the reactor at a short hydraulic retention time. In recent years, cell
352 immobilization in the DF process emerged as an effective technology to increase
353 biohydrogen production by keeping a high cell concentration in the reactor and
354 enhancing the reactor's stability (Kumar et al., 2016). Based on recent reports, the
355 biohydrogen generation yield and rate in the system with immobilized cell was more
356 than the system with suspended microbial cells (Kumar et al., 2016; Sekoai et al.,
357 2020). The method of immobilizing microorganisms in biohydrogen production
358 system mainly includes adsorption, entrapment and encapsulation (Kumar et al.,
359 2016) as shown in Fig. 1.

Insert Fig. 1

Adsorption is one of the simplest and the most commonly used methods of cell immobilization. Microbial cells can be adsorbed on the surface of inorganic and polymer matrixes through the mechanism of electrostatic interaction, hydrophilic and hydrophobic interactions, as well as Van der Waals force. Referring to the method of entrapment, hydrogen production cells are trapped inside the porous matrix, which can provide better biomass transfer between substrates and microorganisms (Kumar et al., 2016). Encapsulation is processed by encapsulating microbial cells inside a semi-permeable membrane like the polyvinylidene fluoride (PVDF) membrane (Akinbomi et al., 2015). It is important to select suitable support materials for the immobilization system. The materials selected for immobilization should have the properties of mechanical, chemical and thermal stability, non-toxicity, cost effectiveness, reusability, porous structure, high specific surface area, and uniform permeability.

Carbon-based matrices such as traditional activated carbon (AC), novel carbon nanotubes, carbon cloth, carbon fiber and biochar have been extensively studied for their applications in the immobilization system of biohydrogen production (Boshagh et al., 2019; Cheng et al., 2019). Adsorption of microbial cells on AC is a common immobilization method, because AC has properties of less toxicity, higher surface area and effective porosity. The porous structure of AC can support the growth of microbial cells, help to maintain cell viability and increase cell density. Additionally, carbon materials with the property of conductivity can enhance direct interspecies electron transfer (Zhao et al., 2016). Zhang et al. (2017) indicated that the use of AC

as a carrier of microorganisms in DF provided a stable environment for the rapid growth and metabolism of bacteria, and increased the biohydrogen production by 259% compared with the process without AC being supplied. Li et al. (2020) stated that the addition of rice straw-derived biochar (3 g/L) could increase biohydrogen production by 118.4% and 79.6% in ethanol-type and butyrate-type fermentations, respectively, because of its advantages of boosting cell immobilization and thereby enhancing cell growth and substrate consumption. The use of carbon nanotubes as support materials of microorganisms achieved faster and higher biohydrogen production in upflow anaerobic sludge blanket reactors than conventional activated carbons (Liu et al., 2012).

Natural biopolymers like alginate and agar are much used support matrices in the DF process because of their high accessibility, low cost, non-toxicity, biocompatibility and large-surface area (Astrilia Damayanti et al., 2018). A recent study by Park et al. (2020) demonstrated that the reactor seeded with alginate immobilized sludge confirmed more active biofilm formation and higher biohydrogen production at HRT of 3h by increasing the hydrogen-production and decreasing the hydrogen-consuming pathway. However, natural carriers like alginate suffer from the drawbacks of poor mechanical and chemical stability and reduced porosity. Consequently, they are unsuitable for long-term industrial use. To overcome the limitation of alginate matrixes and achieve long-term application, alginate is incorporated with other materials like some synthetic polymers, activated carbon and metal.

Polyvinyl alcohol (PVA) as a non-toxic synthetic polymer is also widely employed. It is proved that the mixture of PVA and sodium alginate had high activity and retained good mechanical stability. Yin et al. (2018) indicated that the immobilization of sludge in PVA gels could remain active after ten repeated batch operations, which showed that the sludge immobilization not only increased biohydrogen yield but also achieved continuous biohydrogen production during long-term operation. As reported recently, the attachment of microbial cells on granular activated carbon (GAC) could achieve a consistent biohydrogen production rate at higher temperature, and further using alginate as an immobilized bead material promoted the growth of hydrogen-producing bacteria. GAC can act as a support for the alginate and maintain the stability of beads (Dzul Rashidi et al., 2020). However, it is noted that the amount of alginate used for microbial immobilization should be controlled, as a larger concentration of alginate could prevent the growth of microbial cells and limit biohydrogen production (Dzul Rashidi et al., 2020). A novel hybrid immobilization material with the combination of calcium alginate, activated carbon, silica gel and chitosan was also developed and applied in continuous biohydrogen production. It is observed that the hybrid immobilization carrier with high efficiency and stability, can be used repeatedly in the reactor (Sivagurunathan et al., 2014).

Previous researchers found that agricultural wastes and other biomass, such as bamboo stems, coconut coir and corn stalk, also can be employed as support matrices of biohydrogen-producing bacteria, due to their advantages of biodegradability, renewability, and biocompatibility (Sekoai et al., 2020). For instance, utilization of

corn stalk as a support matrix proved to be better than fiber material (polyester fiber) and AC. Immobilized bacteria could use starch as the carbon source directly to enhance biohydrogen production significantly (Ma et al., 2017; Wang et al., 2018). Therefore, further research is required for studying the potential of using agriculture wastes as immobilized materials, considering the dual benefits of resource recovery and low-cost materials production.

3.1.4 Nanotechnologies in DF process

With the advances being made in nanotechnology, different types of nanomaterials with large surface area, high adsorption capacity and high electro-conductivity such as metallic nanoparticles, metal oxide nanoparticles, nanocomposites, and graphene-based nanomaterials, have been used in the fermentative process for improving biohydrogen production (Sekoai et al., 2020). Nanomaterials used in individual, dual and multiple forms can play significant roles in DF by acting as support matrices of microbial and enzyme immobilization, cofactors on the active site of hydrogenase, as well as enhancers of electrons transfer between the nanoparticle and enzyme. The end result is better biohydrogen production (Elreedy et al., 2019; Srivastava et al., 2020; Taherdanak et al., 2016). As reported by Seelert et al. (2015), the immobilization of *Clostridium beijerinckii* NCIMB8052 on magnetite nanoparticles reduced lag phase of microbial growth and then enhanced the biohydrogen production. Moreover, nanomaterials can be used for cellulase enzymes' immobilization to enhance hydrolysis of lignocellulosic waste in the pretreatment process thereby accelerating biohydrogen production of DF (Srivastava et al., 2017). For instance, Amin et al.

448 (2018) showed that the immobilized laccase supported on modified
449 Fe_3O_4@SiO_2@Kit-6 magnetite nanoparticles enhanced the delignification of olive
450 pomace biowaste and had high recyclability and stability. Similarly, the study by
451 Shanmugam et al. (2020) reported that *Trichoderma asperellum* laccase immobilized
452 on chitosan-coated Fe_3O_4@SiO_2 nanoparticles performed better in the delignification
453 of the lignocellulosic biomass (84.46%). They also had a longer utilization cycle than
454 the free enzyme, resulting in higher biohydrogen production.

455 In DF, protons are reduced to molecular hydrogen under the catalysis of Fe-Fe
456 hydrogenase and Ni-Fe hydrogenase (Taherdanak et al., 2015). Therefore, the
457 addition of certain amounts of iron and nickel nanoparticles can especially enhance
458 the activity of hydrogenases and further increase how much biohydrogen is produced.
459 This has been demonstrated in some studies. For example, Yu et al. (2014) showed
460 that the hydrogen yields could be improved by 16% and the lag time reduced by 36%
461 with the addition of Fe^0 powder in the process of biohydrogen fermentation of
462 dewatered sludge. A study by Yang and Wang (2018) also found that Fe^0
463 nanoparticles improved hydrogen yield and hydrogen production rate by 73.1% and
464 128.3%, respectively, in comparison with the control experiment of grass
465 fermentation. The improvement of hydrogen production by Fe^0 nanoparticles
466 supplementation was attributed to the following: improved microbial activity;
467 changed dominant hydrogen producer from *Enterobacter* sp. to *Clostridium* sp.;
468 induced metabolic pathway towards more hydrogen production; accelerated electron
469 transfer between ferredoxin and hydrogenase; and promoting the activity of key

enzymes (Yang & Wang, 2018; Yu et al., 2014).

Taherdanak et al. (2016) evaluated effects of Fe^{2+} and Ni^{2+} ions versus Fe^{0} and Ni^{0} nanoparticles on the performance of biohydrogen production in the mesophilic DF process, and indicated that the yield of biohydrogen rose by 55%, 37% and 15% under the effects of Ni^{2+} ions, Fe^{0} nanoparticles and Fe^{2+} ions, respectively, by reducing the formation of hydrogen inhibitors. In contrast, the supplementation of Ni^{0} nanoparticles showed an insignificant effect on the hydrogen yield in this study. The study by Mullai et al. (2013) demonstrated that the biohydrogen yield was improved by 22.7% when adding Ni^{0} nanoparticles in the bioreactor. Sun et al. (2019) also demonstrated that the co-addition of Ni^{0} nanoparticles and biochar (BC) could enhance biohydrogen production through acetate pathway during the DF process. Carbon-based materials like activated carbon and biochar have complementary roles with nanoparticles, which can synergistically improve the activity of microorganisms and enzymes with co-addition of nanoparticles (Yang & Wang, 2019). As expected, the co-addition of biochar and Fe^{0} nanoparticles revealed a synergetic effect on the enhancement of biohydrogen production from grass fermentation, because of their complementary functions and more Fe^{2+} being released from the Fe^{0} nanoparticle-biochar micro-electrolysis (Yang & Wang, 2019).

It is reported that the co-addition of different nanoparticles also played a synergistic role in increasing the production of biohydrogen compared to the sole addition(Yang & Wang, 2019). Both Gadhe et al. (2015a) and Gadhe et al. (2015b) documented that co-addition of hematite (Fe_2O_3) and nickel oxide (NiO) nanoparticles

was more effective for improving biohydrogen production than the sole supplementary of nanoparticles, due to the an enhanced activity of ferredoxin oxidoreductase, ferredoxin and hydrogenases. Another study by Elreedy et al. (2019) found that higher biohydrogen production was observed by the application of dual (α-Fe_2O_3 + NiO, α-Fe_2O_3 + ZnO, and NiO + ZnO) and multi-nanoparticles (α-Fe_2O_3 + NiO + ZnO) in comparison to individual nanoparticles, by enhancing the growth of *Clostridium* species and the activity of hydrogenase. It is subsequently expected that novel multifunctional nanocomposites could be developed in the future.

The role of nanoparticles in biohydrogen production can be influenced by concentrations of nanoparticles added to the DF process (Mohanraj et al., 2014). Thus, to maximize biohydrogen production, desired concentrations of nanoparticles should be selected due to the toxicity of nanoparticles to bacteria. Mishra et al. (2018) found that the biohydrogen yield could be increased 1.51-fold and 1.61-fold by adding 1.5 mg/L of nickel (NiO) and 1.0 mg/L of cobalt oxides (CoO) in anaerobic digestion of palm oil mill effluent, respectively. However, 63% and 83% reductions in bacterial cell growth were observed after the application of 3.0 mg/L of the nanoparticles.

4. Advanced technologies for fermentative biohydrogen purification

In the fermentation process, carbon dioxide (CO_2) as well as other compounds to lower extent, such as nitrogen, hydrogen sulfide, water vapor and methane are coproduced with biohydrogen (Aasadnia et al., 2021; Muin et al., 2020). Therefore, biohydrogen purification is a major and a challenging task for its various potential

514 applications, because high-purity hydrogen (>99.99 vol%) is required for the success
515 of fuel cell technology. In addition, reduction of H_2 and CO_2 partial pressure in the
516 fermentation reactor could also improve the production of biohydrogen (Bakonyi et
517 al., 2017). Different methods, such as chemical absorption, cryogenic separation,
518 adsorption and membrane separation, have been developed to purify the biohydrogen.
519 Chemical absorption has been regarded as a suitable technique for biohydrogen
520 purification, considering biohydrogen is generally produced at the temperature
521 between 30 and 60 °C and atmospheric pressure (Muin et al., 2020). For instance, a
522 two-stage chemical absorption system using methyldiethanolamine (MDEA) activated
523 with piperazine and NaOH has successfully purified the fermentation product of CO_2
524 and H_2 mixture up to 99 mol% hydrogen purity (Azira & Aisah, 2019). However,
525 high-costs of chemicals and corrosion issues are some of the barriers to its use.
526 Comparatively, cryogenic separation is a clean and environmentally friendly method
527 for hydrogen purification without chemical addition and production (Aasadnia et al.,
528 2021). The cryogenic separation process is carried out at high pressure and low
529 temperature (−250 °C) to cool of the gas mixture to separate and purify H_2, because
530 H2 has relatively high volatility in comparison with CH_4, CO, and N_2. This process
531 can achieve a high H_2 recovery rate, but standard H_2 purity (85–99%) does not satisfy
532 the application requirements (Du et al., 2021). In addition, energy intensive and
533 numerous instruments requirement are also challenges of this method
534 (Chozhavendhan et al., 2020). Moreover, the trace amount of H_2S that existed in the
535 fermentation product will solidify at the cryogenic condition and then lead to clogging

536 of the system and damage to rotating equipment.

537 Pressure swing adsorption (PSA) process is a most studied and effective technology
538 to produce high purity hydrogen (>99%), which based on the adsorption capacity of
539 solid adsorbents and the used technical process (Golmakani et al., 2017). In the PSA
540 process, the purification of hydrogen is achieved through the selective adsorption of
541 gases at a high pressure, while reducing adsorbed impurities by lowering the pressure,
542 simultaneously (Chozhavendhan et al., 2020). Developing novel and effective
543 adsorbent materials is important for improving hydrogen purification via PSA process
544 (Sircar et al., 2009). The study by Kuroda et al. (2018) found that hydroxyl aluminium
545 silicate clay was a novel adsorbent in PSA for CO_2 and H_2S separation from
546 multicomponent gas mixtures for biohydrogen purification by using low energy input.
547 Metal-organic frameworks (MOF's) are a relatively new class of microporous
548 materials, which have promising properties for adsorption of CO_2. Delgado et al.
549 (2017) evaluated and compared different agglomerated MOFs in PSA for separation
550 of CO_2 and biohydrogen purification and concluded that UTSA-16 presents the higher
551 performance for biohydrogen purification than HKUST-1 and ZIF-8, attributing to its
552 high selectivity towards carbon dioxide, and to its high volumetric heat capacity.
553 Hybrid processes of PSA and other methods with advantages of both separation
554 methods were also developed to make biohydrogen production economically
555 attractive. For instance, a hybrid PSA and membrane system produced high purity H_2
556 with a 29% higher H_2 recovery than a system only using PSA (Lin et al., 2020).
557 Unfortunately, the PSA method normally requires high pressure and temperature to

558 achieve high hydrogen purity (>99.9 %), which is energy-intensive. In addition, the
559 recovery of biohydrogen in diluted fermentative mixtures is low and cost-intensive,
560 because the most economically feasible PSA process feed streams have to be already
561 compressed at 15–30 bar and contain 75–90 vol.% hydrogen (Ohs et al., 2019; Xiao et
562 al., 2020). Commonly, cryogenic separation and PSA are designed primarily for large-
563 scale hydrogen production and might inappropriate for relatively small-scale
564 biohydrogen purification (Kazakov et al., 2020).

565 Comparatively, membrane technology is flexible and scalable in responding to the
566 variation of plant capacity in the purification of biohydrogen from fermented gas
567 mixtures without significant changes in production cost (Bakonyi et al., 2013b; He et
568 al., 2021). The hydrogen purification using membrane also have advantages of lower
569 operating costs, ease of installation and operations as well as minimal footprints
570 compared to conventional separation techniques (Bakonyi et al., 2018; Sharip et al.,
571 2019). Moreover, membrane technology can be easily coupled with other separation
572 processes to enhance the efficiency and economics of separation process, even can
573 couple with the fermentation bioreactors to form an integrated bioprocess (Bakonyi et
574 al., 2017; Bakonyi et al., 2015). The selection of suitable membranes is crucial to
575 provide a cost-effective process for biohydrogen purification, which depends on
576 membranes selectivity and permeance (He et al., 2021). The permeance and
577 selectivity characteristics of different membrane materials for H_2 and CO_2 separation
578 at different conditions have been summarized by previous reports (Mohamad et al.,
579 2016). Currently, the most reported gas separation membranes for hydrogen

580 purification include metallic membranes, polymeric membranes, microporous
581 inorganic membranes, MOF membranes, and mixed matrix membranes (Mohamad et
582 al., 2016). Among them, metallic membranes (e.g., palladium and its alloys) are
583 usually operated in steam reforming process to continuously remove the hydrogen
584 produced in water–gas shift membrane reactors at high temperatures (>350 °C), which
585 are impractical for the purification of biohydrogen produced in biological processes at
586 ambient conditions (He et al., 2021).

587 Polymeric membranes can be easily fabricated and upscaled at a low cost, and they
588 were tested for the separation of fermentative biohydrogen (Mohamad et al., 2016).
589 However, it is noted from Table 2 that the hydrogen purity (67-96%) of polymeric
590 membranes was too low for its further utilization. The main reason is the low
591 selectivity (<10) due to the smaller kinetic diameter and lower solubility of the
592 hydrogen molecule in the polymeric matrix results (Yin & Yip, 2017). Moreover,
593 most of current studies were carried out under ideal laboratory conditions, but the
594 complex composition of fermentative gases, H_2S in particular, can significantly
595 influence the polymeric membrane performance (Bakonyi et al., 2016).

596 *Insert table 2*

597 Carbon membrane, which prepared by the carbonization of polymeric precursors,
598 displayed high biohydrogen purity related to H_2/CO_2 separation. A two-stage carbon
599 membrane system operated by He et al. (2021) indicated that the carbon membrane
600 was techno-economically feasible for biohydrogen purification with a lower specific
601 cost of \$0.06/N m^3 to achieve the biohydrogen purity of 99.5 vol% compared to PSA.

602 The authors also found that carbon membranes can also tolerate impurities (such as
603 H_2S) when exposed to fermentation gases. Graphene-based membranes also showed
604 interest in the gas separation field, because their ultra-low thickness results in minimal
605 transmission resistance and maximum penetration flux (Du et al., 2021). In one of
606 recent studies, a novel graphene oxide-poly (dimethyl siloxane) membrane has been
607 produced and applied for the biohydrogen purification (Nigiz & Hilmioglu, 2020). It
608 is reported that the CO_2/H_2 selectivity could increase from 7.10 to 11.7 when loading
609 0.5 wt% of graphene oxide.

610 In addition to these carbon-based membranes, several advanced membranes also
611 displayed excellent gas separation performance with high selectivity of for H_2 and
612 CO_2. For example, a carbon molecular sieve membrane prepared from cellulose
613 hollow fiber precursors showed the H_2/CO_2 selectivity of 36.9 and high - purity
614 hydrogen (>99.5%) at 10 bar and 110 °C from a steam methane reforming process
615 (Lei et al., 2021). Metal organic frameworks (MOFs) based mixed matrix membranes
616 also showed a great potential for hydrogen purification. An extremely high H_2/CO_2
617 selectivity (53.1) has been reported using an ultrathin MOF/polymer mixed matrix
618 membrane by loading 20 wt% of the MOF powders, because of the incorporation of
619 MOFs and ultrathin nanolayer (Zhao et al., 2019). Two-dimensional (2D)
620 nanomaterials also were reported as attractive membrane materials for high-
621 performance hydrogen separation considering their unique physical and chemical
622 properties (Yang et al., 2021). Ma et al. (2021) indicated that a thin film composite
623 membrane (TFCM) with two-dimensional (2D) MOF nanosheets gutter layer

exhibited excellent H_2/CO_2 selectivity (12.3–12.6) and long-term stability comparing with the traditional TFCMs, which contain polymer gutter layers. Therefore, these advanced membranes may also highly effective in biohydrogen purification from fermentation mixed streams, which is necessary for further investigation. Moreover, to achieve commercial application of membrane for biohydrogen purification, more and in-depth studies requires to be conducted about advanced membrane fabrication, membrane performance under various conditions, and techno-economic feasibility.

5. Future perspectives

In recent years, challenges of resources and energy depletion, environmental pollution and climate change have promoted studies on the conversion of renewable biowastes to eco-friendly energy source. Biohydrogen produced from various carbohydrate rich biowastes via biological processes have been regarded as a promising strategy for biowastes management and clean energy production simultaneously. Though it is possible to product biohydrogen through DF processes, it is still a major challenge for the production of biohydrogen at an adequate scale to meet the increasing energy demand worldwide. For example, the low conversion efficiency of substrates and accumulation of VFAs in the DF process, the low biohydrogen production yields and rates, as well as the high overall cost of production and purification, are all bottlenecks for limiting their large-scale application. As reviewed in this article, advanced technologies applied in DF processes have high potential to overcome the challenge of these conventional bioprocesses and achieve higher biohydrogen production. However, most studies were only operated in a

laboratory scale so far, and more researches are still needed to solve various issues presented in different bioprocesses in the lab to optimize the biohydrogen production and reduce the whole operating costs before the large-scale application.

Recent advances in genetic engineering suggested that it is possible to create mutant microbial strains to produce biohydrogen at high yields. However, more and deeper investigations are necessary to explore the effect of genetic engineering on the enhancement of biohydrogen production, because there are still many unknowns and uncertainties in the field of genetic engineering. Immobilization technology plays an important role in enhancing biohydrogen production by increasing the microbial concentrations and system stability. It is necessary to develop more stable and inexpensive support carriers in the future study. The application of nanomaterials in bioprocesses is an effective technology to enhance the biohydrogen production through their effects on enzymes in microorganisms. Further studies are necessary to investigate influences of the dosage, size, type, shape and toxicity of nanoparticles on the process of biohydrpgen production. Currently, most of nanoparticle are synthesized via chemical methods, leading to high cost and hazardous effects to the environment and human health. Therefore, the identification of cheap and green nanomaterials is required in the future, such as their production from microorganisms and plants.

Microorganisms play a significant role in bioprocesses for biohydrogen production. Considering the difference of substrates, it is important to screening specific biohydrogen producing bacteria to achieve higher biohydrogen production. Further

study about the definition of mixed cultures is also necessary due to their critical to increase the conversion efficiency of complex substrates and stability of the system. Various operating parameters like substrate concentrations, pH, temperature, etc., have major effects on biohydrogen production. The development of mathematical tools like response surface methodology (RSM) to optimize the operating conditions of the bioprocess is also important to improve biohydrogen production and reduce the operating costs. The information about techno-economic analysis and cost comparison of different processes is significant for the large-scale biohydrogen production as well.

6. Conclusions

Multiple advantages can be achieved for biohydrogen production from the carbohydras rich biowastes. As reviewed in this study, the application of advanced technologies, such as genetic engineering, cell immobilization, nanotechnology in DF, as well as membrane technology for purification, are a promising method to improve biohydrogen production cost effectively while achieving the goal for meeting the increasing energy demand globally. However, to achieve large-scale biohydrogen production cost effectively and environmentally friendly, more and in-depth studies as recommended in this review are necessary in the future.

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Figure caption

Fig. 1 Immobilization methods of microbial cells in dark fermentation

Table caption

Table 1 Examples of genetic engineering for improving biohydrogen production

Table 2 Membrane technologies for biohydrogen purification

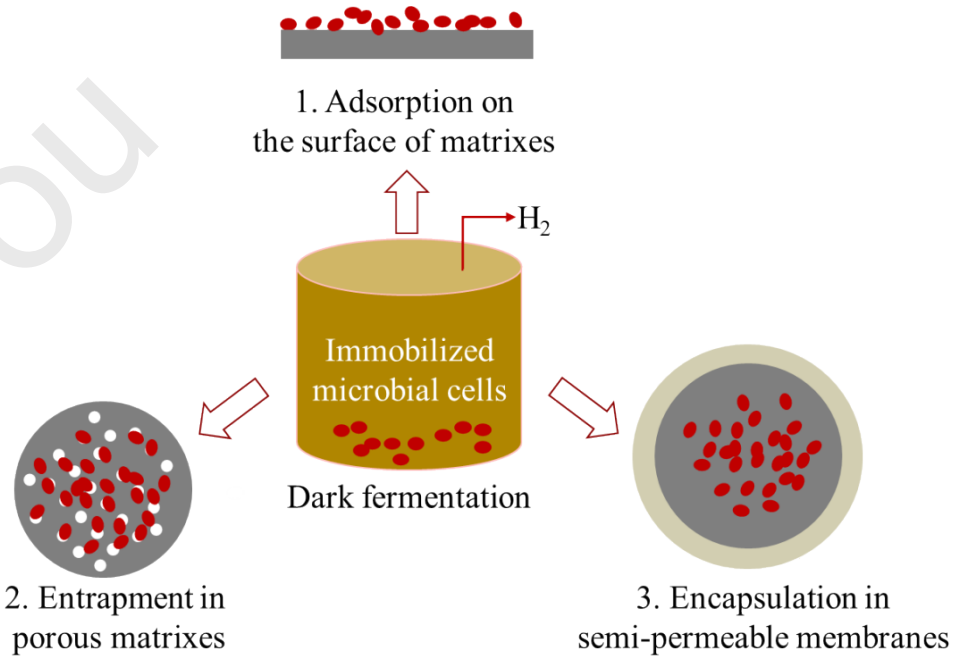

Fig. 1 Immobilization methods of microbial cells in dark fermentation

Table 1 Examples of genetic engineering for improving biohydrogen production

Strains	**Genetic engineering strategy**	**Biohydrogen yield**	**Reference**
Escherichia coli SR13	Inactivating formate hydrogen lyase (FHL) repressor (*hycA*) and overexpression FHL activator (*fhlA*)	2.8-fold increase compared to the wild-type strain	(Yoshida et al., 2005)
Escherichia coli strain SR15	Inactivating FHL repressor and overexpressed FHL	0.7 times over the wild-type strain	(Yoshida et al., 2006)
Escherichia coli	Eliminating uptake hydrogenases, competing metabolites, knocking out repressor, over-expressing inducer,	41-fold increase	(Maeda et al., 2007b)

	decreasing competing formate consumption, and preventing formate export.		
Escherichia coli K-12KEIO	Inactivating negative regulator for FHL, uptake hydrogenase 1 (*hyaB*) and 2 (*hybC*), fumarate reductase	141-fold increase from formate, 1.5 times increase from glucose	(Maeda et al., 2008)
Escherichia coli	Inactivating FHL repressor encoded by *hycA*, overexpressing the activator encoded by *fhlA*, deleting deleting *hyaB* and *hybC*, deleting *frdC* and *ldhA*	2-fold increase from glucose	(Maeda et al., 2007a)
Escherichia coli	Activating pentose-phosphate pathway through deletion of phosphoglucose isomerase and overexpression of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase	1.2 times over the wild strain	(Sundara et al., 2017)
Escherichia coli K–I2 BW25113	Inactivation of *hyaB* and *hybC*	2-fold increase using brewery waste	(Poladyan et al., 2018)
Escherichia coli	Heterologous expression of HupSL hydrogenase from Rhodobacter sphaeroides	20.9-fold increase of biohydrogen production	(Lee et al., 2010)
Enterobacter cloacae	over-expression of Fe-hydrogenase (*hydA*) gene	95% increase over the parental strain	(Zhao et al., 2010)
Enterobacter aerogenes	Knocking out of gene *hycA* (encoding the FHL repressor protein) and *hybO* (encoding the small subunit of the uptake hydrogenase)	0.52 times increase over the wild type	(Zhao et al., 2009)

Enterobacter aerogenes	Homologous overexpression of NAD synthetize gene *hadE*) and deletion of phosphoenolpyruvate carboxylase gene (*ppc* and *hybO*)	1.49 times increase over the control strain	(Wang et al., 2013)
Clostridium pasteurianum	Overexpression of hydrogenase (*hydA*)	1.7-fold increase from crude glycerol	(Sarma et al., 2019)
Clostridium pasteurianum	Overexpression of Glycerol dehydrogenase (*dhaDI* and *dhaK*)	1.5-fold increase from crude glycerol	(Sarma et al., 2019)
Clostridium tyrobutyricum JM1	Homologous overexpression of the [FeFe]-hydrogenase gene	1.5-fold increase	(Jo et al., 2010)
Enterobacter cloacae IIT-BT 08	Homologous overexpression of [FeFe]-hydrogenase *(hydA)* gene	1.2-fold increase	(Khanna et al., 2011)
Caldicellulosiruptor bescii	Deletion of lactate dehydrogenase gene (*ldh*)	21-34% increase of biohydrogen production from lignocellulosic biomass	(Cha et al., 2013)

Table 2 Membrane technologies for biohydrogen purification

Membrane	Conditions	CO_2/H_2 selectivity	H_2 purity	References
Polyvinylidene difluoride (PVDF) membrane	50% H_2, 50% CO_2, prepared using 18 wt% polyme, feed pressure of 3 bar	/	85%	(Rohani et al. 2021)
Polyvinylidene Difluoride-co-Polyethylene Glycol Membrane	PVDF coated with polyethylene glycol (PEG) (10%), feed pressure of 3 bar	3.3	96%	
Polydimethylsilixone (PDMS) membrane	39% H_2, 49% CO_2, 8% N_2, feed pressure of 1–8 bar at 28 °C	<1.19	/	(Mohamad et al., 2016)
Polysulfone (PSF) membrane	39% H_2, 49% CO_2, 8% N_2, feed pressure of 1–8 bar at 28 °C	1.54–3.32	77%	(Mohamad et al., 2016)

Polydimethylsilixone (PDMS) membrane module in cross-flow design	51.3% H_2, 47% CO_2, 1.7% unknown trace gases, feed pressure 3 bar at 25°C	3.7	67%	(Bakonyi et al., 2015)
Polyetherimide (PEI) coated nanofiber bio-cellulose membrane	3 wt.% PEI coating, feed pressure of 3 bar at 25 °C	0.15	/	(Wu et al. 2017)
composite polyimide membrane in hollow-fiber configuration	65% H_2, 35% CO_2, feed pressure of 2.2 bar at 55 °C	1.62	/	(Bakonyi et al. 2013a)
Silicone hollow-fiber membrane	33%–60% H_2, feed pressure of 1.5 bar at 35 °C	4.4	80%	(Koroglu et al. 2019)
Polysulfone membrane	39% H_2, 49% CO_2, 8% N_2, feed pressure of 5 bar at 28 °C	2.9	90%	(Hamid et al. 2019)
Polyimide membrane		3.1	63%	
Polysulfone-polyimide membrane		4.4	80%	
Two-stage carbon hollow fiber membrane system	60% H_2,40% CO_2, feed pressure of 5–6 bar at 50°C	/	99.5	(He et al., 2021)
Graphene oxide (GO) incorporated poly (dimethyl siloxane) (PDMS) nanocomposite membrane	0.5 wt.% of GO loading, 0.2 Mpa of the trans-membrane pressure	11.7	/	(Nigiz and Hilmioglu 2020)

Highlights

- Biowastes rich in carbohydrates are sustainable sources for biohydrogen production.
- Advanced methods employed in bioprocesses to enhance hydrogen yield were critically discussed.
- Advanced strategies were proposed for biohydrogen purification.
- Challenges and perspectives concerning biohydrogen production were stated.