Highlights

- Biohydrogen production through dark fermentation (DF) has been extensively reviewed.
- Current and future status of DF-based biorefinery concepts have been discussed.
- Two-stage anaerobic digestion is the sustainable option for DF system upscaling.
- Energy recovery, techno-economic and life cycle analyses are pointed out.
- Present scenario of the DF-based biorefinery concept is evaluated using SWOT analysis.
Graphical abstract

Policy and legislation interventions

Biohydrogen

Future biorefinery concepts

Current biorefinery status

Global opportunities
Indian scenario
SWOT analysis

Production
Upgradation
Storage

Energy recovery
Techno-economic analysis
Life cycle analysis

Research advances
Research gaps
Research directions
Biohydrogen production through dark fermentation from waste biomass: Current status and future perspectives on biorefinery development

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aTinku C. D’ Silva and Sameer A. Khan have equal contributions.
Abstract

Green and clean hydrogen production has become a significant focus in recent years to achieve sustainable energy fuel needs. Biohydrogen production through the dark fermentation (DF) process from organic wastes is advantageous with its environmentally friendly, energy-efficient, and cost-effective characteristics. This article elucidates the viability of transforming the DF process into a biorefinery system. Operational pH, temperature, feeding rate, inoculum-to-substrate ratio, and hydrogen partial pressure and its liquid-to-gas mass transfer rate are the factors that govern the performance of the DF process. Sufficient research has been made that can lead to upscaling the DF process into an industrial-scale technology. The article also discusses the possible hydrogen purification and storage techniques for achieving fuel quality and easy accessibility. However, the DF process cannot be upscaled at the current technology readiness level as a stand-alone technology. Hence, it requires a downstream process (preferably anaerobic digestion) to improve energy recovery efficiency and economic viability. The article further tries to unfold the opportunities, challenges, and current/future research directions to enhance hydrogen yield and microbial metabolism, depicting the commercialization status for biorefinery development. Finally, the current progress gaps and policy-level loopholes from the Indian perspective are highlighted by analyzing the strengths, weaknesses, opportunities, and threats.

Keywords: Biohydrogen production, Biorefinery concept, Dark fermentation, Biohydrogen purification, Biohydrogen storage

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List of Abbreviations

AD – Anaerobic digestion

ASBR – Anaerobic sequencing batch reactor

C/N ratio – Carbon-to-nitrogen ratio

CDC – Carbide-derived carbon

COD – Chemical oxygen demand

COF – Covalent organic frameworks

CSABR – Continuous stirred anaerobic bioreactor

CSTR – Continuous stirred tank reactor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>CW</td>
<td>Cardboard waste</td>
</tr>
<tr>
<td>DF</td>
<td>Dark fermentation</td>
</tr>
<tr>
<td>DOE</td>
<td>Department of Energy</td>
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<tr>
<td>FW</td>
<td>Food waste</td>
</tr>
<tr>
<td>HRT</td>
<td>Hydraulic retention time</td>
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<tr>
<td>ISR</td>
<td>Inoculum-to-substrate ratio</td>
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<tr>
<td>LCA</td>
<td>Life cycle analysis</td>
</tr>
<tr>
<td>LH$_2$</td>
<td>Liquid hydrogen</td>
</tr>
<tr>
<td>LOHC</td>
<td>Liquid organic hydrogen carriers</td>
</tr>
<tr>
<td>MOF</td>
<td>Metal-organic frameworks</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide + hydrogen</td>
</tr>
<tr>
<td>NFOR</td>
<td>Nicotinamide adenine dinucleotide + hydrogen: ferredoxin oxidoreductase</td>
</tr>
<tr>
<td>OFMSWs</td>
<td>Organic fraction of municipal solid wastes</td>
</tr>
<tr>
<td>P2M</td>
<td>Power to methane</td>
</tr>
<tr>
<td>PHAs</td>
<td>Polyhydroxyalkanoates</td>
</tr>
<tr>
<td>PSA</td>
<td>Pressure swing adsorption</td>
</tr>
<tr>
<td>SOFC</td>
<td>Solid oxide fuel cells</td>
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<tr>
<td>SWOT</td>
<td>Strengths, weaknesses, opportunities, and threats</td>
</tr>
<tr>
<td>TEA</td>
<td>Techno-economic analysis</td>
</tr>
<tr>
<td>TPD</td>
<td>tonnes per day</td>
</tr>
<tr>
<td>TS</td>
<td>Total solids</td>
</tr>
<tr>
<td>TSA</td>
<td>Temperature swing adsorption</td>
</tr>
<tr>
<td>VS</td>
<td>Volatile solids</td>
</tr>
</tbody>
</table>
1. Introduction

Based on the United Nations’ 7th and 13th sustainable development goals of “affordable and clean energy and climate action,” most nations are targeting towards adopting renewable energy production to fulfill the energy demand. Hydrogen is the cleanest fuel available on earth, with no environmental harm. It possesses the highest energy content (~120-145 MJ/kg) [1] and can be produced through different routes (Fig. 1). By 2050, the global hydrogen market is expected to reach up to $1.6 trillion [2,3]. Based on the methods used for production, hydrogen is classified into different categories as described through the colour codes (Table 1) [4–7]. Biohydrogen production from organic waste biomass has more prospects in terms of economic viability and environmental sustainability [8–10]. Among them, the DF process is more advantageous with no photosynthetic reactions involved and can be applied in a simple reactor design. Additionally, the DF process can potentially yield maximal biohydrogen yield with lower input energy [11,12].
Fig. 1 (a, b). Available hydrogen production methods [1] (a) and colour classification (b).
Despite having all the positive attributes, the development of the DF process is still limited to laboratory and pilot-scale studies [13]. There are still engineering gaps between the laboratory-scale upscaling of the DF technology to an industrial full-scale biorefinery system. Various studies have intensively discussed the concept of the DF process and the basics involved [12–17]. Review articles that dealt with comprehensive information on the different hydrogen production, upgradation, and storage techniques have also been published. However, those studies possess limited knowledge of biohydrogen production through DF, its upgradation, and storage for biorefinery development [18,19]. This article tries to comprehensively review the topics of biohydrogen production, upgradation, and storage as an integrated biorefinery system.

Initially, the basic principles and governing factors of DF are discussed, followed by the methods to improve the quality of biohydrogen produced for fuel applications through various biohydrogen purification and storage techniques. Finally, multiple aspects pertaining to developing a biorefinery concept, techno economics, environmental sustainability, recent advances, future research directions, and policy interventions in context with the Indian scenario are also discussed.

2. Literature review methodology

Research on biohydrogen production through DF has been picking up its pace substantially. The Scopus data was first assessed for writing this review article, which was retrieved from the database using the keywords DF, biohydrogen production, two-stage anaerobic digestion (AD), biohydrogen upgradation/purification, storage, and biohydrogen biorefinery concept. As shown in Fig. 2a, Scopus data revealed more than 3,100 publications, including research/review articles, books, book chapters, conference proceedings, dissertation thesis, web information, etc. About 252 publications were shortlisted for further reviewing according to the list's relevance, year, and...
content details (Fig. 2b). The publication years of the shortlisted articles were 2018 to 2022 (39%), 2013 to 2017 (29%), 2008 to 2012 (18%), 2003 to 2007 (10%) and older than the year 2002 (4%). A significant proportion of literature published in the last decade reflects the importance of reviewing these articles and consolidating the findings from these studies.

Fig. 2. Research evolution over dark fermentation and two-stage anaerobic digestion (a) and the publication year of selected publications (b) (Scopus data, dated 13\textsuperscript{th} March 2023).
3. Dark fermentation process

3.1 Principle and general concept

It is well understood that anaerobic fermentation of organic substrates, using specific microbes for biohydrogen production, is called dark fermentation. A wide range of organic substrates rich in carbohydrates, proteins, lipids, and cellulose/hemicellulose contents are used for producing biohydrogen through DF [20,21]. Figure 3 depicts these pathways involved in biohydrogen production from glucose. Biohydrogen production depends on the essential enzymes, hydrogenases. It is to be noted that the nitrogenase enzyme complex also displays hydrogenase activity [22,23]. The hydrogenase enzymes catalyze the hydrogen molecules into protons and electrons. The hydrogenase enzymes are classified into three groups: (a) [Ni-Fe]-hydrogenase, (b) [Fe-Fe]-hydrogenase, and (c) [Fe]-hydrogenase [24].

These enzymes take part in two major pathways of DF. First is the acetate pathway that theoretically yields around 4 mol of H₂ per mol of glucose. Second, the butyrate pathway produces 2 mol of H₂ per mol of glucose [12,25–27]. At the initial stages of the DF process, nicotinamide adenine dinucleotide + hydrogen (NADH) is formed by the oxidation of the organic substrates into pyruvate. It may be utilized by microbial species having NADH: ferredoxin oxidoreductase (NFOR), producing reduced ferredoxin [15,28,29]. Later, pyruvate is converted into acetyl-CoA and formate by pyruvate formate lyase or acetyl-CoA and reduced ferredoxin via pyruvate-ferredoxin oxidoreductase (PFOR), producing H₂ [30,31].

In the process of glucose glycolysis, excess production of NADH would be occurred because of limited electron transport chain in fermentative bacteria. Usually, NADH/NAD⁺ ratio is sufficiently maintained through oxidation of NADH and H⁺ into NAD⁺ during acidogenesis stage. The inadequate oxidation of NADH results in surplus NADH, and H⁺. The fermentative bacteria
attempts to oxidize the excess NADH producing hydrogen to maintain regular metabolism [32]. Other than that, during acetogenesis, acidogenic bacteria (e.g., *Syntrophomonas wolfei*, *Syntrophbacter wolinii* etc.) could convert propionic acid, butyric acid, ethanol, and other organics into acetic acid and hydrogen [33–36]. For cellulosic and hemicellulosic materials, the arabinose, xylose, glucose, and galactose form glyceraldehyde-3-P and further get converted to pyruvate and follow the same pathway as in the case of glucose and more information is available in Bhatia et al. [37].

In the case of complex materials, the pathway for biohydrogen production is via the deamination of amino acids (proteins) and β-oxidation of long-chain fatty acids (lipids). Hydrogen could be also generated via two different pathways from the degradation of pyruvate, an important intermediate produced from the glycolysis of carbohydrates and deamination of amino acids. The degradation of pyruvate produces acetyl-CoA via decarboxylate with reduced ferredoxin produced, which donate electrons to protons for generating hydrogen. This pathway is predominantly used for hydrogen production by *Clostridium* sp [38]. On the other hand, facultative anaerobes, such as *Enterobacter* and *Klebsiella* takes the formate cleavage pathway [39,40]. However, emulsified lipids may hinder the mass transfer between the microbes and other utilizable metabolites during lipid degradation. The microbial metabolism for biohydrogen production through protein and lipid degradation are well explained in Dong et al.[20], Fu et al. [41] and Xiao et al. [42].

Nonetheless, the uncontrolled production of acids beyond a permissible limit can adversely affect the DF process and the H₂ yield due to the sensitivity of hydrogenases to low pH. Microbial intermediate products are produced during metabolic activities apart from acetic acid and butyric acids such as ethanol, fumaric, lactic, propionic acids, and polyhydroxy butyrate. The overall set
of reactions involved in the DF reaction can be represented as given below in the Equations (1-11) for glucose glycolysis pathway [12,26].

\[ C_6H_{12}O_6 + 2NAD^+ \rightarrow 2CH_2COCOO^- + 4H^+ + 2NADH \quad \text{(1)} \]

\[ CH_3COCOO^- + CoA - H \rightarrow \text{acetyl CoA} + HCOO^- (PFLP) \quad \text{(2)} \]

\[ HCOO^- + H^+ \rightarrow CO_2 + H_2 \quad \text{(3)} \]

\[ CH_3COCOO^- + CoA + \text{Ferredoxin (Fd)ox} \rightarrow \text{acetyl CoA} + Fd_{\text{red}} + CO_2 (PFORP) \quad \text{(4)} \]

\[ Fd_{\text{red}} + 2H^+ \rightarrow Fd_{\text{ox}} + 2H_2 \quad \text{(5)} \]

\[ \text{Acetyl CoA} + H_2O \rightarrow CH_3COO^- + H^+ + \text{CoA} - H \quad \text{(6)} \]

\[ \text{Acetyl CoA} + 2NADH + 2H^+ \rightarrow CH_3CH_2OH + \text{CoA} - H + 2NAD^+ \quad \text{(7)} \]

\[ NADH + H^+ \rightarrow NAD^+ + H_2 \quad \text{(8)} \]

\[ C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 12H_2 \quad \text{(9)} \]

\[ C_6H_{12}O_6 + 6H_2O \rightarrow 2CO_2 + 2C H_3COOH + 4H_2 \quad (\Delta G^0 = -206.3 \text{ kJ/mol}) \quad \text{(10)} \]

\[ C_6H_{12}O_6 + 6H_2O \rightarrow 2CO_2 + 2C H_3CH_2CH_2COOH + 2H_2 \quad (\Delta G^0 = -254.8 \text{ kJ/mol}) \quad \text{(11)} \]
**Fig. 3.** Pathways involved in the DF process using glucose for biohydrogen production.
Notably, biomass conversion to biohydrogen through DF completely depends on microbial activity. The contribution of anaerobes such as *Bacillus, Klebsiella, Enterobacter, Clostridium,* etc., for biohydrogen production, has been well-known in the laboratory and full-scale DF microbiota [43–46]. Researchers have used pure microbial cultures or mixed cultures to enrich the specific hydrogen-producing microbial species [47]. Another method is to pre-treat the mixed culture consortia primarily to inhibit the hydrogen-consuming bacteria, such as homoacetogens, hydrogenotrophic methanogens, lactic acid-producing bacteria, propionate-producing bacteria, and sulfate reducers [12]. Hence, diverse pretreatment techniques such as physical (heat shock, ultrasonication, ultraviolet irradiation, aeration, freeze, and thaw, etc.) and chemical (pH pretreatment, chemical activation, and inhibition) are applied [47]. Further, the pretreated inoculum having hydrogen-producing consortia is enriched using macro and micronutrients consisting of trace elements (Fe, Mg, Mo, Ca, Na, Zn, Si, Cu, etc.) [48,49]. The metal ions such as Fe\(^{+}\), Ni\(^{+}\), Mg\(^{2+}\), Cu\(^{+}\), and Zn\(^{+}\) have been shown to positively affect the Ni-Fe, Fe-Fe hydrogenase, and Acetyl-CoA synthase enzymatic activities [50]. The continuous feeding of macro and micronutrients flourishes the activity of hydrogen-producing bacteria in a parental reactor, which can be used further in inoculating DF reactors [51]. However, a long-term operation of the DF reactor may prevail in conditions suitable for culturing hydrogen-producing bacteria. Thus, a lower hydraulic retention time (HRT) is preferable, i.e., below 4 days (on average, even below 2 days), and a high feeding rate must be maintained [12,52].

### 3.2 Suitable feedstocks, characteristics, and biohydrogen production potential

The biohydrogen production rate and yield depend heavily on the type and characteristics of the substrates/feedstocks used. It can vary from the organic fraction of municipal solid wastes (OFMSWs), wastewater sludges, and livestock waste to industrial wastes and effluents. This
section discusses the different waste biomasses used for biohydrogen production and their characteristics. Biomass consists of various macromolecules such as carbohydrates, proteins, lipids, cellulosic and hemicellulosic contents that can be utilized for dark fermentation microbial metabolism for biohydrogen production. Table 1 shows the theoretical biohydrogen potential of various molecules available in biomass resources. However, the experimental yields are reported much lower than the theoretical yield since the metabolic pathways vary according to the microbes involved and the environmental conditions applied [53]. The protein and lipids degradation through anaerobic microbial metabolism is not an easy task for direct hydrogen production. This is because of the low carbon-to-nitrogen (C/N) ratio for proteins [54] and the high C/N ratio for lipids [55] and their complex molecular structures. The biohydrogen production potential of carbohydrate-rich wastes is thus observed to be 20 times higher than that of protein-rich wastes [56].

**Table 1.** Theoretical biohydrogen production potential of various monomers and macromolecules [20,37]

<table>
<thead>
<tr>
<th>Monomer/Macromolecule</th>
<th>Theoretical biohydrogen yield per mol of monomer/macromolecule</th>
<th>Theoretical biohydrogen yield per gram of monomer/macromolecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>4 mol</td>
<td>498 mL</td>
</tr>
<tr>
<td>Xylose</td>
<td>3.33 mol</td>
<td>497 mL</td>
</tr>
<tr>
<td>Mannitol</td>
<td>5 mol</td>
<td>615 mL</td>
</tr>
<tr>
<td>Glycerol</td>
<td>3 mol</td>
<td>730 mL</td>
</tr>
<tr>
<td>Carbohydrates*</td>
<td>8 mol</td>
<td>996 mL</td>
</tr>
<tr>
<td>Proteins</td>
<td>2 mol</td>
<td>105 mL</td>
</tr>
<tr>
<td>Lipids</td>
<td>2 mol</td>
<td>56 mL</td>
</tr>
<tr>
<td>---------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>Cellulose</td>
<td>2 mol</td>
<td>276 mL</td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>2 mol</td>
<td>339 mL</td>
</tr>
</tbody>
</table>

*Theoretical biohydrogen yield of carbohydrates was considered twice the amount of glucose yield.

The molecular weight of macromolecules considered: Glucose: 180 g/mol, Xylose: 150 g/mol, Mannitol: 182 g/mol, Glycerol: 92 g/mol, Proteins: 425 g/mol, Lipids: 800 g/mol, Cellulose: 162 g/mol, and Hemicellulose: 132 g/mol.

Biohydrogen yields of various waste biomass through the DF process are summarized in Table 2. One such biomass is the OFMSWs, which can be further classified according to their origin, such as food processing industries, wholesale markets, restaurants/canteens, households, etc. [57]. The OFMSWs are rich in polysaccharides, such as cellulose, hemicellulose, starch, lipids, proteins, etc. These wastes are promising and potential sources for biohydrogen production due to their abundant availability at a cheaper cost. The OFMSWs have reported a hydrogen yield of 14 – 238 mL/g. substrate of hydrogen through DF process [57–59].

Organic matter-rich wastewater from various industries such as palm oil and olive oil mill, brewery, and dairy can also be utilized for biohydrogen production [29]. Hence, the biohydrogen yield of wastewater from different industries, such as sugar, starch, beverage, palm oil mill, etc., have been investigated [58,60–68]. Besides the conventional carbohydrate-rich wastes, byproducts from other biofuel production processes were also explored for biohydrogen production. Glycerol, the primary by-product of biodiesel production, is an example that possesses a biohydrogen production potential of up to 7 mmol/g. glycerol. This was much higher compared to the other substrates such as glucose (2 mmol/g. glucose), galactose (2 mmol/g. galactose), gluconate (1 mmol/g. gluconate), sorbitol (5 mmol/g. sorbitol), mannitol (5 mmol/g. mannitol) and fructose (2 mmol/g. fructose) using the facultative anaerobic bacterial strain of Enterobacter aerogenes [39].
Plant-originated non-food/feed residues such as straws, stems, stalks, leaves, energy crops, processed wastes, etc. can also be used for biohydrogen production. Besides the agricultural residues, all energy plants (willow, poplar, miscanthus) and waste from the paper and wood industries can be used for biohydrogen production [69]. Eskicioglu et al. [70] observed potential substrates in lignocellulosic biomass subjected to hydrothermal pretreatment. The lignocellulosic biomass can be enlisted as sorghum, fir bark, corn stover, rice, and wheat straw. However, other substrates such as edible and non-edible de-oiled cakes, seeds of invasive and wildly growing plants/trees, various agricultural biomasses, etc., reported good methane yields during AD [71–78], could also be investigated for assessing biohydrogen potential through DF.

Animal manure-based biohydrogen production using the DF process has also been studied [79–81]. Recently, liquid swine manure was examined for continuous biohydrogen production at different dilution rates of 0.5 to 2%. The liquid swine manure was mixed with 10 g glucose/L to balance the carbon and nitrogen ratio and reduce ammonia inhibition. Thus, liquid-based substrates are also suitable for biohydrogen production but have lower HRTs (< 1 d) than solid biomass to obtain maximal biohydrogen production [82]. Besides the above-mentioned organic sources, sewage sludge has also been investigated for biohydrogen production due to the rich composition of peptides and carbohydrates [83,84]. However, the presence of methane-forming microbes in animal manure and sewage sludge limits its usage in DF without effectively inhibiting the metabolic pathways of hydrogen-consuming bacteria [47,85].

In general, biohydrogen yield relies on the solubilization efficiency of the substrates used. Easily soluble substrates such as fruits, vegetable wastes, starchy materials, and different wastewaters could result in enhanced hydrolytic rate and subsequently in biohydrogen production. In turn, pretreatments should be employed to exploit microbial activity when utilizing lignocellulosic
biomass [86]. Different pretreatment methods could be adopted, from mechanical, chemical, and thermal to biological, with variants and combinations available and are extensively reported and reviewed elsewhere [57]. Co-fermentation of different biomass is also a preferred strategy to enhance the biohydrogen yield and maintain the process parameters so that the co-substrates complement each other during DF. Recently, Silva et al. [87] evaluated the hydrogen yield of food waste with glycerol as a co-substrate at a mixing ratio of 1 – 3%. Co-fermentation with 3% glycerol improved the biohydrogen yield by two-fold the yield of food waste alone [87]. Tarazona et al. [88] optimized that a maximal biohydrogen yield can be obtained if the carbohydrate to protein to lipid ratio in substrates is maintained as 1:0.4:0.4 (15, 6, and 6 g/L, respectively). This is where the role of co-fermentation strategy arises where different substrates can be fermented together for generating maximum hydrogen production. A wide variety of substrates suitable for biohydrogen production has been enlisted in detail by Hay et al. [53]. Nevertheless, the biohydrogen yield from all the enlisted substrates generally relies on the operational configuration and other governing factors. The following section highlights how different operational parameters govern the biohydrogen yield and production rate by controlling the biochemical processes.
Table 2. Various waste biomass and their biohydrogen production potential through dark fermentation

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reactor configuration and operational conditions</th>
<th>Biohydrogen yield (mL/g_{substrate})</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Organic fraction of municipal solid waste</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food waste (pasta, bread, fruit, vegetable, fish, and meat)</td>
<td>Batch, Temperature: 36°C</td>
<td>25</td>
<td>[89]</td>
</tr>
<tr>
<td>Residential home food waste</td>
<td>Batch, Temperature: 50°C, pH: 7.5</td>
<td>14</td>
<td>[90]</td>
</tr>
<tr>
<td>Fruit waste</td>
<td>Batch</td>
<td>179</td>
<td>[91]</td>
</tr>
<tr>
<td>Date fruit waste</td>
<td>Batch, Temperature: 37°C, pH: 6.5</td>
<td>239</td>
<td>[92]</td>
</tr>
<tr>
<td>Kitchen waste</td>
<td>Inclined plug flow reactor, pH: 5.5</td>
<td>10</td>
<td>[59]</td>
</tr>
<tr>
<td>Kitchen garbage</td>
<td>Continuous stirred tank reactor (CSTR), Temperature: 55°C, pH: 5.0</td>
<td>25</td>
<td>[93]</td>
</tr>
<tr>
<td><strong>Industrial waste and effluents</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Source</td>
<td>Method</td>
<td>Parameters</td>
<td>COD (kg/ton)</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------------------------</td>
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<td>--------------</td>
</tr>
<tr>
<td>Palm oil mill effluent</td>
<td>Batch</td>
<td>Temperature: 38°C, pH: 5.9</td>
<td>108</td>
</tr>
<tr>
<td>Brewery plant wastewater</td>
<td>Batch</td>
<td>Temperature: 35°C, pH: 5.5</td>
<td>249</td>
</tr>
<tr>
<td>Waste glycerol</td>
<td>Upflow anaerobic sludge blanket reactor (UASB), Temperature: 37°C, pH: 5.5</td>
<td>78</td>
<td>[65]</td>
</tr>
<tr>
<td>Citric acid wastewater</td>
<td>UASB</td>
<td>Temperature: 36°C, pH: 7.0</td>
<td>104</td>
</tr>
<tr>
<td>Cassava starch wastewater</td>
<td>Batch</td>
<td>Temperature: 30°C, pH: 5.5</td>
<td>196</td>
</tr>
<tr>
<td><strong>Agricultural/agro-industrial/ energy crop wastes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn stover</td>
<td>CSTR</td>
<td>Temperature: 55°C</td>
<td>61</td>
</tr>
<tr>
<td>Agave bagasse</td>
<td>CSTR</td>
<td>Temperature: 55°C, pH: 7.0</td>
<td>121</td>
</tr>
<tr>
<td>Cashew apple bagasse</td>
<td>Batch</td>
<td>Temperature: 38°C</td>
<td>336</td>
</tr>
<tr>
<td>Untreated rice straw</td>
<td>Batch</td>
<td>Temperature: 75°C, pH: 7.5</td>
<td>51</td>
</tr>
<tr>
<td>Material</td>
<td>Batch, Temperature:</td>
<td>pH</td>
<td>Value</td>
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<tr>
<td>----------------------------------</td>
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<tr>
<td>Untreated rice straw</td>
<td>55°C, 6.5</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>Untreated Wheat straw</td>
<td>60°C, pH: 7.0</td>
<td></td>
<td>79</td>
</tr>
<tr>
<td>Untreated barley hulls</td>
<td>60°C</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>Untreated Switchgrass</td>
<td>65°C</td>
<td></td>
<td>310</td>
</tr>
<tr>
<td>Untreated cornstalk</td>
<td>35°C, pH: 6.5</td>
<td></td>
<td>87</td>
</tr>
<tr>
<td>Untreated sugarcane bagasse</td>
<td>70°C</td>
<td></td>
<td>252</td>
</tr>
<tr>
<td>Untreated corn leaves</td>
<td>70°C</td>
<td></td>
<td>224</td>
</tr>
<tr>
<td>Delignified wood fibers</td>
<td>60°C</td>
<td></td>
<td>288</td>
</tr>
<tr>
<td>Untreated soyabeans straw</td>
<td>35°C, pH: 7.0</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Wheat straw (pretreated with white-rot fungi)</td>
<td>40°C, pH:6.5</td>
<td></td>
<td>79</td>
</tr>
<tr>
<td>Waste Type</td>
<td>Pretreatment Process</td>
<td>Batch, Temperature:</td>
<td>pH</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>-----------------------------------------------</td>
<td>---------------------</td>
<td>----</td>
</tr>
<tr>
<td>Corn stalk (pretreated with fungi)</td>
<td></td>
<td>60°C, pH: 7.0</td>
<td>80</td>
</tr>
<tr>
<td>Rice straw (pretreated with NH₄OH &amp; H₂SO₄)</td>
<td></td>
<td>75°C, pH: 7.5</td>
<td>60</td>
</tr>
<tr>
<td>Animal waste</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cattle wastewater</td>
<td></td>
<td>45°C, pH: 5.5</td>
<td>278 mL/g</td>
</tr>
<tr>
<td>Liquid swine manure</td>
<td>Anaerobic sequencing batch reactor (ASBR), Temperature: 37°C, pH: 5.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dairy manure</td>
<td>Continuous stirred anaerobic bioreactor (CSABR), Temperature: 36°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cattle manure</td>
<td></td>
<td>78°C</td>
<td>8</td>
</tr>
<tr>
<td>Buffalo sludge</td>
<td></td>
<td>39°C, pH: 70</td>
<td>1</td>
</tr>
</tbody>
</table>
3.3 Key factors involved

3.3.1 pH

Several process parameters affect the DF process. These include pH, temperature, HRT, feeding rate, hydrogen partial pressure, etc. [29,51,112]. Among them, the pH value is a primary DF process parameter. The pH maintained in the DF process controls the enzymatic and microbial activity involved. Moreover, an appropriate hydrogen ion concentration regulates microorganisms' metabolic pathways, morphology, and cell structure. This directly influences the hydrogen yield and the metabolic pathways/metabolic by-products involved (e.g., organic acids such as acetic, lactic, butyric, and propionic acids). The excess organic acid production reduces the slurry's operational pH inside the reactor. A pH level below the value of 5 can directly affect the intracellular pH limiting the activity of the microbes involved. According to Li and Chen [113], an initial pH of around 7 to 7.5 is optimal for the DF of corn stover pretreated by steam explosion. A study has reported that based on the substrates, the optimal initial pH can vary accordingly, e.g., livestock wastes, agricultural wastes, and food wastes have an optimal initial pH of 7.0, 6.5 – 7.0, and 5.0 – 6.0 values, respectively [114]. Nevertheless, operational pH may be different from the initial pH, depending on the biochemical process involved. It is reported that DF requires an optimal operational pH in the range of 5.0 to 7.0 for optimal microbial growth and activity [115].

3.3.2 Temperature

The hydrogen yield of the DF process is also governed by the operational temperature. Compared to mesophilic temperature, the thermophilic conditions have been advantageous for biohydrogen yield [116] and volatile fatty acids (VFAs) production due to improved thermodynamics and enzymatic activity [117–119]. Biohydrogen yields of 33.16 mL/g. volatile solids (VS) were achieved at thermophilic conditions (55°C); meanwhile, the mesophilic operation (37°C) yielded
30.36 mL/g. VS from rice crop residues at a 10% total solids (TS) feeding rate [120]. A more recent study reported a very low biohydrogen yield of 2.13 mL/g. VS during mesophilic conditions (which could be due to the varied microbial routes involved) and 64 mL/g VS under thermophilic conditions at a feeding rate of 6% TS [121]. The study claimed that the thermophilic conditions stimulate the microbes involved resulting in increased biohydrogen and VFAs production compared to mesophilic conditions. As a result, the study observed higher butyric acid rate production under thermophilic conditions.

On the contrary, Azbar et al. [61] have reported a lower biohydrogen production at thermophilic conditions (8 mmol/g. COD) than in mesophilic conditions (9 mmol/g. COD) from cheese whey wastewater. Similarly, in another study, the hydrogen yields were reported to be better at lower mesophilic temperatures (25℃), and hydrogen productivity was higher at higher mesophilic temperatures (40℃) while fermenting marine macroalgae (S. japonica) [122]. A maximum hydrogen yield of 179 mL/g. VS was obtained within 5 days of operation using the prescribed macroalgae at a feeding rate of 35 g/L. The contradiction between the results could be due to the difference in the inoculum, operational conditions, substrate characteristics, and reactor configurations or the competition of hydrogen-consuming microbial consortia. However, the researchers have mostly recommended thermophilic conditions over mesophilic conditions for better biohydrogen and VFAs productivity. Other benefits of maintaining thermophilic conditions are improved substrate degradation, increased hydrogenase enzymatic activity, and decreased growth of hydrogen-consuming bacteria (hydrogenotrophic methanogens, homoacetogens, and associated acetoclastic methanogenic activity) [123]. But the major constraint with the thermophilic biohydrogen production through DF is energy efficiency, a detailed discussion is given in section 5.3.
3.3.3 Substrate concentration or feeding rate

The substrate concentration or the feeding rate is crucial for the DF process. A higher feeding rate is generally prescribed in the literature to keep active acidogenesis/fermentation consistent. A daily feeding rate as low as 1% TS can yield moderate hydrogen productivity; however, a higher substrate feeding rate may enhance hydrogen production. At a feeding rate of 1% TS, Wu and Chang [80] have reported a hydrogen yield of ~3 mol H₂/mol sucrose. Likewise, the DF of glucose has produced 1.84 mol H₂/mol glucose at 1% TS [81]. The VFAs are known to impact both productivity and hydrogen yield. Liu and Shen [124] investigated the performance of batch reactors at varied substrate (starch) concentrations of 2 to 32 g/L. The study observed a maximum hydrogen yield of 194 mL H₂/g starch at a 2 g starch/L concentration. Furthermore, as the starch concentration increased to 32 g/L, the hydrogen yield decreased to 86 mL H₂/g starch. The hydrogen production rate differed from the hydrogen yield profile. The hydrogen production rate recorded a maximum of 237 mL/g VSS.d at 24 g/L, while further reduced at 32 g/L. De Amorim et al. [94] noted similar observations while treating glucose at a concentration of 2 g/L at an HRT of 2 h. The studies have suggested that there is a narrow line of substrate concentration to minimize the gap between hydrogen yield and production rate. Solid-state fermentation is also a feasible strategy for efficient hydrogen production that reduces the requirement for water and the volumetric working capacity of the reactor at higher loading (>15% TS). However, a significant load increase may give rise to technical issues such as clogging in the case of full-scale applications and hence require sophisticated system design.

3.3.4 Hydraulic retention time

The hydrolysis rate of the substrates that advance the biochemical process is influenced by the initial substrate characteristics, the feeding rate, and the time given for sufficient substrate
degradation (Fig. 3a). Thus, the HRT is a parameter that influences the production of various VFAs and the H₂ production. Moreover, multiple studies have utilized HRT to control the growth of hydrogen-consuming bacteria (homoacetogens and hydrogenotrophic methanogens) and aceticlastic methanogens inside the DF reactor. This can be done because hydrogen-producing bacteria grow faster than hydrogen-consuming bacteria. The lower HRT reduces the proliferation of hydrogen-consuming bacteria and also could result in washout under continuous operation conditions, hence a better hydrogen production rate [12,52].

Although lower HRTs improve the biohydrogen yield and production rate, optimizing HRT always depends upon the substrate to be treated. Since DF involves several biochemical processes, HRT alone cannot be decisive in the fate of the DF reactor performance [125]. Thus, some researchers have investigated the combined effects of HRT with operational pH and temperature. Hyperthermophilic (70 °C) operation of DF-based CSTR treating domestic organic wastes yielded a stable biohydrogen production of 21 mL H₂/g VS_{added} at a pH value of 5.5 and HRT of 3 d, even though the maximum yield obtained was 107 mLH₂/g. VS_{added} at a pH value of 7 [126]. In another study treating glycerol in a CSTR, Silva-Illanes et al. [127] observed that HRT influenced hydrogen yield and production rate more than pH. At an optimal HRT of 12 h and pH of 5.5, the study recorded 0.58 mol of hydrogen per mole of glycerol.

In contrast, a lower HRT of 2 h disrupted the microbial activity due to lower microbial abundance (volatile suspended solids) while treating galactose, which optimized a better hydrogen yield at an HRT of 6 h in a continuous reactor [128]. Another study reported a tolerance level of 1.5 h HRT while treating glucose [129]. The pH and temperature influence the nitrogenase and hydrogenase enzymatic activities, affecting the biohydrogen yield. The nitrogenase activity increased at a temperature of around 30 °C and pH around 7.1 – 7.3, while hydrogenase enzymatic activity was
observed to be optimal at a higher temperature, in the range of 55–70 °C with pH in the range of 6.5-7.5 [10].

3.3.5 Hydrogen partial pressure

The continuous biohydrogen production might increase hydrogen partial pressure inside the DF reactor. The solubility of hydrogen in the aqueous environment is extremely poor (Henry’s law constant of $7.8 \times 10^{-4}$ mol/L. atm). This may positively affect the hydrogen production rate further since it has been reported that the lower partial pressure enables the hydrogen mass transfer from the aqueous phase to the gaseous phase at ease as per Henry’s law [130,131]. The excess hydrogen hampers the oxidation and reduction of ferredoxin by hydrogenase, affecting hydrogen production [132]. According to Lee et al. [133], reducing the hydrogen partial pressure enhances hydrogen productivity. The study noticed that at a permissible limit of H$_2$ partial pressure, a maximal hydrogen yield of 5 mol H$_2$/mol sucrose was achieved with a production efficiency of 56%. Correspondingly, a reduction in hydrogen partial pressure from 760 mmHg to 380 mmHg achieved a maximum yield of 3.9 mol H$_2$/mol$_{glucose}$ (51% increase) [131]. Later, Junghare et al. [134] claimed increased production yield at an H$_2$ partial pressure of 76 mmHg relative to 254 mmHg. The claim was supported by Beckers et al. [135], who reported lower hydrogen yields at a partial pressure of 135 mmHg and a substantial increase at negative atmospheric pressure (668 mmHg). Hence, the hydrogen partial pressure should be maintained closer to atmospheric pressure, as shown in Fig. 3(b). Various researchers have suggested an external stirring or applying gas permeable membranes, or vacuum pumps to remove dissolved H$_2$ from the mixed liquor and improve liquid-to-gas mass transfer [133,136]. The best way to maintain the partial pressure of hydrogen could be to transfer the produced gas from the reactor to another collection tank at regular intervals [12,52].
3.3.6 Inoculum

The type of microbial culture used for the DF start-up process is crucial in hydrogen productivity. Certain obligate and facultative anaerobes have been found to support biohydrogen production during DF [19]. Pure cultures of robust hydrogen-producing bacteria are generally recommended for DF start-ups, although DF is expensive under sterile conditions. Thus, using mixed culture directly or under selection pressure, i.e., inhibiting hydrogen-consuming bacteria, is also recommended [12]. Alternatively, direct use of acidogenic culture is also a possibility [137]. Hence, anaerobic digestates, sewage sludge, and other anaerobic effluents are also suggested as good sources of hydrogen-producing microbes required to start the DF process.

The inoculation of the DF reactor using anaerobic granular sludge has been highly beneficial, yielding better biohydrogen and providing a protective environment against sudden environmental shocks and changes. The inoculum type also assists the oxidation-reduction potential directly
involved with bioprocesses carried out by the microorganisms [138]. Thus, an optimal value exists for the inoculum-to-substrate ratio (ISR) based on the substrate type utilized. Lower ISR reduces the fermentation activity, whereas higher ISR increases the inter-microbial competition, which could eventually lead to the growth inhibition of the hydrogen-producing microbial cells [51]. A maximal biohydrogen yield of 62.5 mL H$_2$/g VS was achieved in a DF reactor treating OFMSW under the optimized conditions of 6 g VS/L d feeding rate, 55 °C temperature, and ISR of 0.5 for an operational period of 4 d. The ISR of 0.25 resulted in a low hydrogen yield relative to the results at an ISR of 0.5 [51]. This is because of the competition within the microbial community, which may result in an incomplete substrate-to-hydrogen conversion. It could also be due to the change in the type of fermentation. For instance, if the substrate loading is increased (lower ISR) then due to the higher rate of substrate consumption, the rate of acid production will be higher. The higher rate of acid production will in turn result in a faster drop in the pH with pH being lower for lower ISR. This lower pH in turn affects the microbial community characteristics, probably favoring the predominant occurrence of lactic acid fermentation with low or no H$_2$ production.

Increasing the ISR beyond 0.5 might negatively impact hydrogen production. Higher ISR implies high microbial biomass concentration limited substrate accessibility within the reactor, thus limiting the substrate consumption rate. It is also conceivable that the fast-growing hydrogen-consuming microorganisms predominate the microbial community under those conditions. Alavi-Borazjani et al. [51] suggested that substrate concentration is the predominant factor governing the DF process parameters, followed by ISR and temperature.

In addition, the overall efficiency of the DF system is directly governed by the initial microbial enrichment and long-term natural shift in the microbiome involved [139]. It has been validated that there should be a permissible limit, i.e., 2.5:1.0, between the abundance of hydrogen-
producing microbes to the lactate-producing microbes. pH is the primary controlling parameter for this microbial shift, e.g., fermenting non-sterile food waste in a continuous reactor inoculated using *Clostridium butyricum* sp. [140]. An increase in the optimal ratio could disrupt the system's efficiency, adversely affecting biohydrogen production. A review article by García-Depraect et al. [141] suggests that although lactate-producing microbes are regarded as one of the most common root causes for performance failure in DF systems, they can also support enhancement in hydrogen production. This generally occurs when there is a positive interaction between the hydrogen-producing microbes and the lactate-producing microbes. For example, Cheng et al. [142] observed that the lactate-producing bacterial species *Bifidobacterium* sp. enhanced the hydrolysis of the substrate (starch), releasing VFAs favorable for hydrogen-producing bacterial species of *Clostridium* sp. However, there is more need to explore the biomechanism between these interspecies activities for deducing its applicability in the DF process.

Apart from that, it is known that the inoculum to be used for the startup of the DF reactor is expected to be enriched in hydrogen-producing bacteria, either spore-forming bacteria such as *Clostridium* species, known as conventional hydrogen producers, or non-spore-forming hydrogen producers microbes such as *Firmicutes* and *Prevotella* species [143]. Along with *Clostridium* species (*Clostridium butyricum*, *Clostridium pasteurianum*, and *Clostridium beijerinckii*, etc.), *Enterobacter aerogenes* species are also known for giving high biohydrogen yield [144,145]. *Enterobacter aerogenes* yielded 24.7 mL/L h at an optimum concentration of 32.5 g/ L cheese whey at 31°C and 6.5 pH [145]. *Clostridium butyricum* has outranked other species for giving a better biohydrogen production rate from glucose (3.90 mL H2/g glucose at 10 g/L of glucose) [144]. Most recently, Campos et al. [146] utilized four lignocellulosic plant-based microbial communities, i.e., *Clostridium, Lactobacillus, Enterobacter*, and *Pichia* (fungus), through a
consolidated bioprocessing approach. In the study, at a feeding rate of 10 g/L.d, the fermentation of lignocellulosic biomass such as corn stover, wheat straw, sugarcane bagasse, and agave bagasse produced a hydrogen yield of up to 2.5 L H₂/kg d. Likewise, another method of inoculum development using immobilization and natural fermentation without external inoculation was established by Liete et al. [147] and later used by Fernandes et al. [148] and Zavala-Méndez et al. [149]. The cited studies have used either synthetic or real agro-industrial wastewater for natural inoculum development in anaerobic packed bed reactors within one week of operation. Dauptain et al. [150] investigated the role of utilizing untreated activated sludge collected from a full-scale wastewater treatment plant as an inoculum for the DF process treating seven different substrates of corn silage, Tunisian dates (pitted), sorghum, OFMSWs, microalgae (Scenedesmus quadricauda and Pediastrum), sewage sludge (from same inoculum source), and food waste. The enriched indigenous bacterial consortia consisting of Clostridial and Enterobacter sp. had a stronger influence on the overall biohydrogen yield irrespective of the substrate used.

In general, the microbial consortia for the DF process could be developed and stabilized through an appropriate selection of inoculum for start-up, reactor configuration, packing materials, HRT, and feeding rate [139]. Another strategy that could be followed is the inoculation of the specific active inoculum consisting of hydrogen-producing species at regular intervals. Researchers commonly named this strategy as bio-augmentation, in which the hydrogen-producing microbial consortia are inoculated inside the DF reactor at a given point of time, thereby making their way towards increasing the hydrogen yield. The mechanism behind this strategy is that adding inoculum at regular intervals reinforces the active hydrogen-producing species to dominate inside the reactor over a long-term operational period [151]. Deep insights into the microbiological
aspects of DF are available in Dzulkarnain et al. [152]. Table 3 shows the optimal operating conditions for the DF process developed from this study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Optimal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.0–7.0</td>
</tr>
<tr>
<td>Temperature</td>
<td>Mesophilic: 25 – 40 °C,</td>
</tr>
<tr>
<td></td>
<td>Thermophilic: 55 – 70 °C</td>
</tr>
<tr>
<td>Daily feeding rate</td>
<td>Liquid state fermentation: &gt;1% TS – 10% TS</td>
</tr>
<tr>
<td></td>
<td>Solid state fermentation: &gt;15% TS – 20% TS</td>
</tr>
<tr>
<td>Hydraulic retention time</td>
<td>For liquid wastes: &gt;1.5 h - &lt;12 h</td>
</tr>
<tr>
<td></td>
<td>For solid wastes: 1 to 3 d</td>
</tr>
<tr>
<td>Hydrogen partial pressure</td>
<td>Closer to atmospheric pressure</td>
</tr>
<tr>
<td>ISR</td>
<td>~ 0.50*</td>
</tr>
<tr>
<td>Inoculum type</td>
<td>Thermally or chemically pretreated anaerobically treated effluents/digestate or pure culture of obligate or facultative anaerobes</td>
</tr>
</tbody>
</table>

*This will depend upon the substrate utilized.

4. Biohydrogen as an energy fuel: opportunities and challenges in upgrading and storage techniques

4.1 Biohydrogen polishing and upgrading

From reviewing various literature, it was understood that the biohydrogen produced from the DF process consists of incombustible gas such as CO₂ and trace amounts of hydrogen sulfide, moisture, etc. Hence, hydrogen enrichment/upgrading is as crucial as its sustainable production. It is also to be noted that H₂ can be further utilized as energy fuel in specific applications only if the purity is at least around 99.99% [153]. Even though no studies have claimed biohydrogen upgradation from the DF process so far, hydrogen produced from other conventional techniques
has been subjected to various hydrogen upgradation methods. The primary impurity to be
eliminated from the biohydrogen mixture is CO₂, so these methods could also be applicable for
biohydrogen upgradation. Figure 4a depicts the various hydrogen purification techniques
available. They can be generally classified into two according to the upgradation principle
adopted: (a) physical and (b) chemical. At present, physical purification techniques such as
pressure swing adsorption (PSA), temperature swing adsorption (TSA), cryogenic and membrane
separation techniques are generally considered the established upgrading technologies in
chemical and petrochemical refineries [154–156]. The PSA technology is commonly used to
separate hydrogen from SMR off-gas mixture (Fig. 4b). This technology can lower the
concentrations of unwanted impurities within the permissible level and is reported to achieve a
maximum H₂ upgrading of up to 99.99% from the off-gas mixture that contains a trace amount
of impurities. Since PSA is entirely dependent upon the compressibility of the gas components at
different pressures, the performance of the technology is governed by factors such as inlet
pressure, purge gas pressure, and gas composition. Hence, PSA could only be utilized for
biohydrogen production if optimized to remove excess carbon dioxide from the gas mixture.
Otherwise, pretreating the gas mixture is a prerequisite to removing the hydrogen sulfide and
moisture before feeding it into the PSA reactor.

Similar to PSA, TSA is also a technology that could reduce the concentration of impurities in the
gas mixture. The principle of TSA is based on the adsorption of gas molecules through
increasing temperature. However, in the case of TSA, the slow heating and cooling rates require
more cycles per unit of gas mixture for enhanced removal performance. Thus, applying TSA is
even more restricted for removing the gas impurities at low concentrations than PSA.
On the other hand, cryogenic distillation technology is an alternative widely applicable technology for separating gas mixtures. In the cryogenic process, the gas mixture is separated by maintaining a low temperature, thus utilizing the varied boiling temperature characteristics of the components of the gas mixtures. Since biohydrogen is known for its highly volatile nature and impurities such as carbon dioxide, an additional component of the methane wash column is required to eliminate these gas mixtures. Methane wash columns are known to remove the carbon dioxide from gas mixture efficiently comprising hydrogen, carbon dioxide, and carbon monoxide [157]. The major challenge with the cryogenic separation is that the hydrogen recovery performance has been moderate, with a maximum recovery of 95%. Moreover, the PSA and cryogenic separation technologies are either cost- or energy-intensive.

In another approach, membrane separation of the gas mixture has been widely recommended for its low energy consumption, low cost, and suitability for continuous operation, as shown in Fig. 4 (c) [158]. In membrane separation, direct production and separation of gas mixtures are possible using membrane-based reactors. The membranes are flexible enough to be fixed inside the specially designed reactors and only pass the required gas molecules from the mixture. Membrane-based reactors are known for reduced investment costs, improved selective separation, and upgrading performance [159]. Membrane-based reactors have improved performance during hydrogen production through SMR at high temperatures and pressure [160]. At the same time, eliminating CO₂ from the biogas mixture obtained from the DF requires modifications since the biological process is closer to ambient environmental conditions. Hence, specific membranes (e.g., polymers) must generally be manufactured according to the biogas composition and characteristics, with improved resistance to impurities, economic viability,
longevity, and robust design. Zeolite-based membrane system has been employed in a study by Sanchez et al. [9] for a DF-based biorefinery system. Recently, membrane-based systems with novel materials or modified versions of existing membranes have been employed to improve the selective separation of hydrogen gas or impurities [153,161–163]. Upscaling the process requires flexible and affordable membrane modules to separate the biohydrogen produced through DF effectively. The liquid-to-gas mass transfer rate is insufficient in membrane-based systems, which can affect the performance of the DF reactor. Thus, effective, and continuous withdrawal of biohydrogen in membrane-based systems is expected with sufficient liquid-to-gas mass transfer efficiency. More detailed information regarding liquid-to-gas transfer efficiency and its effects on the DF process for biohydrogen production and purification are available in Nemestóthy et al. [164].

The biological process of microalgae-based CO₂ absorption has also become a promising technique for hydrogen upgradation. During photosynthesis, the microalgae metabolize the CO₂ and thus upgrade the gas mixture. A closed-loop cycle of biohydrogen, biogas, and simultaneous microalgal growth and biogas upgradation can be developed through this technique [165]. However, the major disadvantage of this technique is that photosynthesis results in the simultaneous production of H₂ and O₂, which is dangerous and requires sophisticated equipment for the timely separation of H₂. All these technologies have also been reported to purify the biomethane from a biogas mixture [166,167]. Thus, it could also play an instrumental role in the purification of biohydrogen.
<table>
<thead>
<tr>
<th>Upgradation technique</th>
<th>Principle</th>
<th>Performance</th>
<th>Benefits</th>
<th>Drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pressure swing adsorption</td>
<td>Based on physical adsorption</td>
<td>Moderate</td>
<td>• No requirement of water</td>
<td>• Removal of H₂S required</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• No requirement of chemicals</td>
<td>• Complex system</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• No requirement of water</td>
<td>• High investment cost</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Removal of H₂S required</td>
<td>• High investment cost</td>
</tr>
<tr>
<td>Temperature swing adsorption</td>
<td>Based on temperature-based adsorption</td>
<td>Moderate</td>
<td>• No requirement of chemicals</td>
<td>• Extended no. of cycle operation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• No requirement of water</td>
<td>• Complex system</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Removal of H₂S required</td>
<td>• High investment cost</td>
</tr>
<tr>
<td>Membrane separation</td>
<td>Permeation</td>
<td>High</td>
<td>• Compact and simple process</td>
<td>• Removal of H₂S required</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• No requirement of chemical</td>
<td>• High investment cost</td>
</tr>
<tr>
<td>Cryogenic separation</td>
<td>Compression and condensation</td>
<td>High</td>
<td>• No requirement of chemicals</td>
<td>• Removal of H₂S required</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• The fuel at the outlet is available in a compressed state, hence can be directly stored</td>
<td>• High investment cost</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• High energy demand</td>
<td></td>
</tr>
<tr>
<td>Microalgae-based absorption</td>
<td>Photosynthesis</td>
<td>Moderate</td>
<td>• Simple and economical</td>
<td>• Performance is dependent upon photosynthetic rate and microalgal growth rate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Microalgal biomass could be further utilized for biofuel production</td>
<td>• Simultaneous production of H₂ and O₂ during photosynthesis requiring sophisticated separation technologies enhances additional costs</td>
</tr>
</tbody>
</table>
Fig. 4. Hydrogen upgradation methods (a), PSA technology concept (b), and membrane separation technology concept (c).
4.2 Biohydrogen storage and transport

Succeeding the biohydrogen upgradation, the hydrogen gas at the outlet will be high in purity for further applications. However, the concern is with its storage and transportation, which has been a rapidly developing topic in recent years. Various agencies and institutes investigated the possibilities of feasible hydrogen storage systems. The United States Department of Energy (DOE) has set the target for an on-board hydrogen storage system, including volumetric density, gravimetric density, and cost, as mentioned in Table 5. Another parameter that must be standardized is the fueling time, i.e., the time taken to store the hydrogen in a vehicle. It was estimated that the fueling time should be less than 3 min. for filling hydrogen fuel in the vehicle to run a distance of 450 km [168].

Numerous developments have been made to use hydrogen for fuel applications, improving its storage capacity. This was based on considering two critical characteristics of the hydrogen molecule: specific energy and energy density. Pure hydrogen fuel has a high heating value of 120 MJ/kg, almost three times that of gasoline, having 44 MJ/kg. A lower density and volumetric energy density make hydrogen storage impossible under normal temperature and pressure conditions, which questions its economic feasibility. Thus, a cost-effective hydrogen storage method is what researchers are aiming for. Currently, there are various hydrogen storage technologies based on different principles, as summarized in Fig.5. Broadly, it can be categorized into three: (1) Physical methods, in which hydrogen is stored in its purest form, either liquid or compressed gas, without any chemical bonding; (2) Adsorption, where hydrogen is adsorbed or adhered by weak Van Der Waal’s force on the surface of an adsorbent with high surface area; (3) Absorption, where hydrogen atom form a strong chemical bond with another element [168–172].
Table 5. The year-wise target set for the on-board hydrogen storage system by USDOE

[170,173] (Also retrieved from: https://www.energy.gov/eere/fuelcells/hydrogen-storage)

<table>
<thead>
<tr>
<th>Target for storage system</th>
<th>Volumetric density</th>
<th>Gravimetric density</th>
<th>Cost</th>
<th>Operating conditions</th>
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</thead>
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<tr>
<td></td>
<td>kWh/L system</td>
<td>kWh/kg system</td>
<td>$/kWh</td>
<td>Pressure (MPa) (min./max.)</td>
</tr>
<tr>
<td>2010 (target set in 2003)</td>
<td>1.5</td>
<td>0.045</td>
<td>2</td>
<td>0.060</td>
</tr>
<tr>
<td>2015 (target set in 2003)</td>
<td>2.7</td>
<td>0.081</td>
<td>3</td>
<td>0.090</td>
</tr>
<tr>
<td>2010 (target set in 2009)</td>
<td>0.9</td>
<td>0.028</td>
<td>1.5</td>
<td>0.045</td>
</tr>
<tr>
<td>2015 (target set in 2009)</td>
<td>1.3</td>
<td>0.040</td>
<td>1.8</td>
<td>0.055</td>
</tr>
<tr>
<td>2017</td>
<td>1.3</td>
<td>0.040</td>
<td>1.8</td>
<td>0.055</td>
</tr>
<tr>
<td>2020</td>
<td>1.0</td>
<td>0.030</td>
<td>1.5</td>
<td>0.045</td>
</tr>
<tr>
<td>Ultimate (2020)</td>
<td>1.7</td>
<td>0.030</td>
<td>2.2</td>
<td>0.065</td>
</tr>
</tbody>
</table>
4.2.1 Physical methods

4.2.1.1 Compressed hydrogen

Storing hydrogen at high pressures, generally called compressed hydrogen, is the physical way to store the hydrogen gas in a high-pressure vessel (10,000 psi). For vehicular or mobile applications, it is beneficial that the fuel should have a high energy density, be cheaper, lighter, and suitable for onboard delivery systems. Compressing the hydrogen at higher pressure parallelly increases gravimetric and volumetric energy density. Shortly this storing pressure is expected to be increased to 70 MPa or 700 bar or higher, and maybe up to 1000 bar for vehicular applications. Hydrogen density increases from 0.1 to 40 g/L when pressure increases from 1 to 700 bar, while volumetric energy density increases from 0.0033 to 1.32 kWh/L [168,171,174].

Fig. 5. Various technologies for hydrogen storage (taken from [168]).
Currently, there are five types of pressure vessels for compressed gas storage, as shown in Table 6. Type I is the metallic type, and storage pressure is 20–30 MPa, which is used in most industrial applications, but it has a low gravimetric density of about 1% (0.01 kg H₂/kg system). Type II has higher storage than type I due to partial carbon fiber covering, whereas Type IV uses polymer liner and has better gravimetric performance [168]. Compressed hydrogen is used in nearly 80% of hydrogenation processes worldwide for storage and transportation. It is stored between 200 and 500 bar in cylinders or bundle tubes on tube trailers and transported on trucks. The amount of hydrogen that can be stored in the trailer at 200 bar is 420 kg. This capacity increases to 666 kg of hydrogen using composite material. At 500 bar, the jumbo trailer can store up to 1100 kg of hydrogen [168,174].

**Table 6. Pressure vessel types (taken from [168])**

<table>
<thead>
<tr>
<th>Type</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Material</td>
<td>Complete metallic</td>
<td>Metallic enclosure with some fiber overwrap</td>
<td>full composite over-wrap with a metallic liner</td>
<td>full composite over-wrap, polymer liner, and metal boss</td>
<td>Complete composite</td>
</tr>
<tr>
<td>Pressure limit</td>
<td>≤ 50 MPa</td>
<td>Not limited</td>
<td>≤ 45 MPa</td>
<td>≤ 100 MPa</td>
<td>Under consideration</td>
</tr>
<tr>
<td>Suitable Application</td>
<td>Stationary</td>
<td>Stationary</td>
<td>Industrial and vehicular</td>
<td>Vehicles for industrial purposes (at high pressures)</td>
<td>--</td>
</tr>
</tbody>
</table>

Vehicles such as Hyundai Tucson and Toyota Mirai have variants consisting of compressed hydrogen technology with a volume capacity of 140 L and 122.4 L. Among them, Toyota Mirai
has a hydrogen storage capacity of 5.7 wt.% [174]. These vehicles can store hydrogen at 70 MPa in a full tank, covering a distance of 426 km and 500 km, respectively. Although a simple technology, the compression process is gravimetrically and volumetrically inefficient. Energy consumption during isothermal compression from 0.1 MPa to 80 MPa is 2.21 kWh/kg. In another scenario, it is mentioned that power consumed during pressurizing the hydrogen gas at 700 bar is 10% of the energy content of the gas. [168,169].

4.2.1.2 Liquified hydrogen

Liquifying the gaseous fuel or hydrogen is another way to increase the volumetric energy density and capacity. On liquefaction of hydrogen at 1 atm and 20 K, volumetric capacity reaches 70 g/L, whereas compressed hydrogen at 350 bar and 700 bar is 24 g/L and 40 g/L, respectively. Liquid hydrogen (LH2) tanks consist of metallic double-walled containers with a vacuum between the walls for thermal insulation. The LH2 can be stored in a more efficient way for large volumes. The LH2 is successfully transported through trucks with a capacity of 60000 L. The main application for LH2 is in space and flight, where volumetric capacity and gravimetric density are more important than power consumption. The required power for liquefaction is nearly 35% of the energy content of stored hydrogen. The worldwide installed capacity of the liquefaction plants is 355 tonnes per day (TPD). The world’s largest liquefaction plant has a 34 TPD capacity. The main issue is boil-off hydrogen (above 20 K temperature, LH2 starts to boil and convert to gas), even in highly insulated tanks. This can create dangerous situations in closed spaces. [168,170,172].

4.2.1.3 Cryo-compressed hydrogen

This technology combines cryogenic and compression, which lessens energy losses. In this method, hydrogen is pressurized between 250 to 350 atm at cryogenic temperature because hydrogen gas becomes denser than LH2 above 15 MPa and near liquefaction temperature. The
volumetric density can reach up to 87 g/L at a pressure of 240 bar and a temperature of 20 K [168,170,175]. Cryo-compressed hydrogen at 276 bar and 20 K exceeds DOE 2017 target as it provides a gravimetric density of 5.8 wt. % and 43 g H₂/L. Researchers from the Lawrence Livermore National Laboratory, United States showed that the longest drive recorded with cryo-compressed hydrogen is 660 miles on a single tank. No evaporative loss was recorded when the vehicle was parked for 8 d [170]. Manufacturing cost decreased to 8$/kWh from $12/kWh for a system equipped with 10.4 kg of usable hydrogen [176].

4.2.1.4 Adsorbent-based storage system

Physical adsorption or adsorbent-based storage system is a reversible process where gas and solid particles interact through Van Der Waals forces. Various materials are used for hydrogen storage based on adsorption. Most materials are carbon-based materials such as activated carbons, activated carbon fibers, fullerenes, carbon nanotubes, carbon nanofibers, carbide-derived carbons, graphite, graphene, etc. Other porous materials used for hydrogen storage are zeolites, metal-organic frameworks (MOF), covalent organic frameworks, and polymers of intrinsic microporosity. Some of these materials have good hydrogen storage capacity, fast kinetics, and better reversibility [168,175,177,178].

Activated carbon has adsorption capacities in the range of 1–7 wt.% at 77 K at 1-20 bar pressure. At ambient temperature with a pressure between 2–4 bar, gravimetric capacities come down in the 2-3 % range. Super activated carbon at 77 K and 296 K stores up to 5 wt.% and 1.3 wt. % respectively. Casa-Lillo et al. [179] studied hydrogen storage capacity on activated carbon or carbon fiber up to a pressure of 70 MPa. The highest value for hydrogen adsorption capacity was 1 wt.% at 10 MPa. Carbon nanotubes provide high-density hydrogen storage with about 5-10 wt.% [168]. Gupta et al. [180] found carbon nanofibers adsorbed about 17 wt. % of hydrogen at 12 MPa
at room temperature. Dillon et al. [181] worked with single-walled carbon nanotubes containing less than 0.2% nanotubes, showing the adsorption capacity for hydrogen of 5 and 10 wt.%. Another work by Chambers et al. [182] was performed on carbon nanofiber. In the study, the authors manufactured herringbone carbon nanofiber, which showed a hydrogen adsorption capacity of 67.55 wt.% and 53.68 wt.% on platelet carbon nanofiber at room temperature and pressure of 11.2 MPa. Romanos et al. [183] used a nanoporous graphene monolith for hydrogen storage and achieved a gravimetric storage capacity of 10.7 g H₂/ kg material. Carbon is obtained by separating it from metal carbide, known as carbide-derived carbon (CDC) [177]. Singer et al. [184] developed CDC using Polytetrafluoroethylene for adsorbing hydrogen gas. The study achieved excess hydrogen adsorption volumetric capacity of 21 g/L with a total volumetric capacity of 29 g/L at 77 K, and 4 MPa. Yeon et al. [185] prepared the CDC using ceramic-titanium carbide plates, showing that hydrogen was adsorbed with a volumetric capacity of 35 g/L at -196 °C and 60 bar.

Hydrogen can also be stored using an electrochemical technique. Electrochemical hydrogen storage values are in the range of 0.27 – 6.1 wt.%. In this technique, the electrodes are made from a mixture of carbon, metals, and organic binder. This electrode is then cathodically charged with hydrogen, and hydrogen is obtained anodically [178]. Other carbon material fullerenes, such as C₆₀ buckyballs, exhibited no hydrogen storage capability; theoretically, the chances of forming HC₆₀ complexes are very narrow [178]. Dillon et al. [181] performed a theoretical study on scandium and fullerene. The result showed that scandium could bind to the twelve five-membered rings in C₆₀. The predicted hydrogen capacity for reversible systems was approximately 7 wt. % with C₆₀[ScH₂(H₂)₄]₁₂ complex between scandium and fullerene. Komatsu et al. [186] encapsulated the hydrogen molecule in a fullerene C₆₀. Covalent organic frameworks (COF) are
held by covalent bonds (C-C, C-O, B-O, Si-C) with high porosity and low crystal density. These have crystalline frameworks with high surface area. These can be either 3D or 2D structures, and 3D structures have 3 times the storing capacity of the 2D structure. COF-102 with 3D structure shows a gravimetric capacity of 9.95 wt.% at 77 K and 100 bar. In place of phenylene, using diphenyl (COF-102-2), triphenyl (COF-102-3), naphthalene (COF-102-4), and pyrene (COF-102-5), COF-102-3 can achieve an adsorption capacity between 6.5 – 26.7 wt.% at 77 to 300 K and 100 bars [171].

Besides carbon material, MOF and zeolites are also being investigated for hydrogen storage. After observing more than 4000 MOF, it was concluded that the range of the specific surface area of zeolite is 3100 – 4800 m²/gm. MOF-5 (Zn₄O (BDC)₃ (where BDC is 1,4-benzene di-carboxylate) has a hydrogen adsorption capacity of 4.5 wt. % at the cryogenic condition and 1 wt.% at the ambient condition of 1 bar and 20 bar, respectively [187,188]. It has been reported that the hydrogen uptake capacity of materials such as MOF-5 and IRMOF-8 can be increased upto 8 times by dissociative chemisorption [168]. Zeolite can be defined as crystalline alumino-silicate with evenly distributed pre-size and refined structure. Hydrogen encapsulation, i.e., hydrogen is forced into the porous structure of zeolite at a high pressure of 900 bar, and temperature can reach up to 3500 C. The system can be enclosed at room temperature [187]. Langmi et al. [189] have worked with four zeolites, i.e., NaA, NaX, NaY, and NaCsRHO, for hydrogen adsorption. NaY showed the highest specific surface area of 725 m²/g and had a hydrogen capacity of 1.81 wt.% at 15 bar and -196⁰ C.

4.2.2.2 Chemical methods

This storage system is based on bond formation with hydrogen; it can be either an ionic, covalent, or metallic bond. Two major hydrogen storage technologies based on bond formation are chemical
hydride and metal hydride-based storage systems. Absorption and desorption processes are included to make the system’s overall operation reversible. Various techniques, such as thermolysis, hydrolysis, and ammonolysis, are employed to desorb hydrogen. These techniques require additional system components and reduce the hydrogen density [168].

4.2.2.1 Chemically bonded hydrogen

Chemical hydrides store hydrogen by forming a chemical bond, and hydrogen can be generated through a chemical reaction. Some papers suggest that metal hydride comes under the category of chemical hydrides. Others represent it as a non-metal hydride. Some consider chemical hydride as the material used for hydrogen storage that cannot be regenerated. Here non-metal hydrides are treated as chemical hydrides. The most crucial difference is that chemical hydrides are in a liquid state under normal conditions. This simplifies the transport and storage, and mass transfer can be observed during the hydrogenation and dehydrogenation processes. Material that stores hydrogen is ammonia, ammonia borane, formic acid, methanol, carbohydrates, synthetic hydrocarbon, and liquid organic hydrogen carriers (LOHC) [168,171,172,176]. Ammonia has 17.8 wt.% or 10.7 kg H₂/100 L hydrogen storage density. Ammonia borane has a slightly high hydrogen content of 19.6 wt.% [168]. Formic acid has 53 g/L hydrogen content at room temperature and atmospheric pressure with a gravimetric density of 4.3 wt.%. Carbohydrates (polymeric C₆H₁₀O₅) can be hydrogen carriers with 14.8 wt.% capacity on complete conversion [171]. Gaseous hydrocarbons (C₁ – C₃) and liquid hydrocarbons (C₄ – C₁₀) can both be used for hydrogen production through auto thermal reforming and steam reforming and partial oxidation reforming with some by-products [176]. The simplest alcohol, methanol, contains hydrogen 12.5 wt.% and 99 kgH₂/m³ gravimetrically and volumetrically, respectively. The most common LOHC types are methylcyclohexane and toluene, dibenzyl toluene and perhydro-dibenzyl toluene and N-ethyl
carbazole and dodecahydro-N-ethyl carbazole with 6.1 wt.%, 6.2 wt.%, and 5.8 wt.% of gravimetric hydrogen, respectively [172].

4.2.2.2 Absorption-based storage system

Some metals can absorb hydrogen at low temperatures and moderate pressure. Metal hydrides are formed when transition metal and their alloys react with gaseous hydrogen to form metal hydrides. The advantage of this system is that it is the safest technique to store hydrogen at low operating temperatures. On the other hand, the major disadvantages are that the onboard hydrogen storage system is quite heavy, has low reversibility, and requires high dehydrogenation temperature.

Metal-based hydrides are categorized into elemental, intermetallic, and complex hydrides [98,168].

Elemental hydrides are promising hydrogen storage materials derived from metals such as Mg, Na, Li, Ca, and Al. These hydrides include one metal with hydrogen, best described with the MHx formula, where M is a metal [176]. MgH₂ has a gravimetric density of 7.6 wt. % whereas Magnesium based alloys show nearly 5 wt.% of hydrogen storage capacity [168,175]. Aluminium hydride or alane (AlH₃) have 10.1 wt. % gravimetric and 7.47 kg H₂/100 L volumetric hydrogen storage capacities, but due to instability, it is stored at high pressure, which is in the range of GPa. Other elemental hydrides are LAH₂, YH₂, and ZrH₂, which are stable, whereas NiH and FeH are unstable and require high pressure [168].

Intermetallic compounds or interstitial hydride contains at least two metals along with hydrogen. They can absorb and desorb hydrogen under mild conditions [176]. The general formula for interstitial hydride is AₓBₓHₓ, various forms being AₓBₓ are AB, AB₂, A₂B, A₃B, AB₅, and A₂B₇, where A and B are transition or earth metals. The material TiFe shows hydrogen absorption up to 1.9% with the possibility of reversibility. ZrFe₂ has 1.7 wt.% of hydrogen storage capacity at 20
Solid solution alloys are also used for hydrogen storage and are generally based on vanadium, which is also included in this category. It shows a gravimetric density of 4 wt.% [168].

Complex metal hydrides contain metallic cations and anionic groups that make partial covalent bonds with hydrogen [168,176]. Under this category, amide-hydride (e.g., LiNH₂) system, alanates (e.g., LiAlH₄), borohydrides (e.g., LiBH₄), and some metal amine complexes (M(NH₃)ₙXₘ, where M is a cation and X is anion) are included [98,168]. Lithium nitride (Li₃N) has been utilized to store a maximum hydrogen capacity of 11.5 wt.% of gravimetric density and 7.35 kg H₂/100 L of volumetric density and dehydrogenate successfully. Lithium borohydride (LiBH₄) has a complicated hydrogenation process and high decomposition temperature but with a gravimetric storage capacity of 18.5 wt.% at room temperature. Lithium alanate (LiAlH₄) at high pressure and temperature shows 10.6 wt. % of hydrogen storing capacity [168].

5. Evaluating the sustainable application of the dark fermentation process as a biorefinery

5.1 Biorefinery concept

The scalability of DF-based biorefinery relies on the biohydrogen productivity and subsequent utilization of the derived VFAs. Bio-electrochemical systems, microbial fuel cells, photo fermentation, etc., are recent technologies evaluated as a downstream process for utilizing the VFAs [190]. The decision to select the post-utilization of VFAs could be based on the microbes used and the primary composition of the VFAs produced. For example, if the acetate-based pathway is involved in the DF process, AD could be the go-to downstream technology to utilize VFAs to produce biogas [19]. If the butyrate-based pathway is engaged, the solventogenic process could be followed where the VFAs are converted to acetone, butanol, and ethanol in the ratio of 3:6:1 [191]. However, the solventogenic process involves energy and cost-intensive recovery and purification processes that may disrupt the overall techno-economics. Thus, with
the current technology readiness level, AD technology is more feasible for establishing the DF-based biorefinery system. The integration of the DF process with AD has several advantages. The process can produce biohydrogen and biomethane simultaneously. These biofuels can be utilized separately or as a combination named biohythane. In addition, excess hydrogen can even be used for in-situ microbial methane enrichment through two-stage AD. Such a concept has been discussed by D’Silva et al. [12]. Integration of in-situ microbial methane enrichment with the DF process has been discussed further in section 6. Moreover, two-stage AD has been known for its better biomass degradation efficiency at a higher feeding rate [192]. In addition, the performance of the two-stage AD can be consistently maintained by strategizing specific operational conditions separately for DF and AD reactors [193–197]. A possible concept of two-stage AD for easily soluble substrates (kitchen wastes and other substrates rich in carbohydrates) is represented in Fig.6. However, lignocellulosic biomass can also be treated using two-stage AD. The difference in treating lignocellulosic biomass using two-stage AD is the pretreatment requirement, which may also require higher HRT and lower feeding rate than easily soluble materials. The research on two-stage AD is currently focused on long-term operation, techno-economics, energy efficiency, and strategizing operation and maintenance and process monitoring [63,198].
5.2 Pilot-scale experiences

The commercial viability of a process can only be validated through pilot-scale experiences. This includes the viability in terms of energy and mass balances, techno-economics, and life cycle analysis. In addition, it is also essential to solve some practical challenges such as collection, transportation, and storage of substrates to be treated, material handling and operation and maintenance, and developing a proper process workflow [199,200]. Even though there have been various types of bioreactors developed and investigated, such as CSTR, anaerobic fluidized bed reactor, anaerobic sequencing batch reactor, up-flow anaerobic sludge blanket (UASB), and membrane bioreactor in lab-scale studies [13], the CSTR mainly was preferred as the DF under

**Fig. 6.** The concept of two-stage AD [52].

**Hydrogen, Carbon dioxide rich gas mixture**

- pH: 5.00 – 6.00
- HRT = 2 – 4 d

**Methane, Carbon dioxide rich gas mixture**

- pH = 6.00 – 8.00
- HRT = 8 – 15 d

**Dark fermentation**

(hydrolysis, acidogenesis, acetogenesis)

**Anaerobic digestion**

(methanogenesis)
mesophilic conditions in pilot-scale studies with pH maintained around 4.5 - 6.5 [201]. The pH is
maintained by adding acid/alkali chemicals at regular intervals, or the effluent from the
methanogenic reactor is recirculated again to the DF reactor [193]. This approach is more
suitable for the two-stage AD system that has been inoculated by mixed cultures. Such an
approach has been strategized from the concept of ‘mixed culture biotechnology’ developed by
Kleerebezem and Van Loosdrecht [202]. Through this concept, unknown mixed cultures are used
for the bioprocess development of the DF process based on natural selection by controlling the
operational conditions or by using natural inoculum from diverse sources.

The DF reactor was initially inoculated using the anaerobic digestate pretreated thermally or
chemically to inhibit hydrogen-consuming microbes and generally kept under thermophilic
conditions. These temperature ranges help hydrolysis and abridge the microbial activity suitable
for biohydrogen production [203]. So far, based on the experiences from pilot-scale studies,
Ueno et al. [204] observed that 1 kg of COD equivalent available in the substrate was
transformed to biohydrogen, i.e., about 1 kg of COD equivalent is required to produce 3.7 to 6.6
m³ of biohydrogen (1.5 to 2.4 mol H₂/mol. hexose) at an HRT between 0.6 to 1.2 d.

Different from that, recently, a pilot-scale DF study of 10 m³ capacity (CSTR) situated at the
Indian Institute of Technology Kharagpur, India treating cane molasses and groundnut de-oiled
cake together has reported a maximum hydrogen yield of 16.2 mol hydrogen per kg of COD
removed (which is equivalent to 0.4 m³ of H₂ per kg of COD) [43]. However, the study has
observed much-improved performance in the pilot-scale reactor than in the bench-scale reactor
(50 L capacity). At the same time, earlier, a two-stage AD plant (UASB-based DF reactor with a
working capacity of 0.4 m³ and anaerobic digester with an operational capacity of 2.5 m³) was
developed, namely “Innovative Hydrogenation & Methanation Technology (HyMeTek)” at Feng
Chia University, Taiwan [205]. The system treating food industry wastewater (60 g COD/ L) has reported a hydrogen production rate of 3 m³/m³. d and a yield of 1.5 mol hydrogen/ mol hexose at an HRT of 9 h and a methane production rate of 0.86 m³/m³. d and yield of 27 to 56 mL/g. The study also suggested expanding the downstream processes, such as carbon-capturing using a membrane bioreactor for treating the digested effluent and a microalgal photobioreactor to capture the carbon dioxide from the gaseous mixture produced from the DF. This way, the AD plants improve the functionality and zero carbon emission targets from the biorefinery concept.

5.3 Energy recovery

Energy recovery is a governing factor for the techno-economic feasibility of a system. A major benefit of integrating the DF process with AD is the maximal energy recovery compared to single-stage AD, irrespective of the type of feedstock used and operational parameters [125,206,207]. The authors of the cited literature reported an increased methane yield between 11 to 21% for two-stage AD over single-stage AD. The total energy recovered from the substrate in the form of H₂ has been reported as around 41% for the acetate pathway and 27% for other mixed culture pathways. Exergy analysis of the proposed biorefinery concept will be instrumental in identifying the irreversible processes within the system. So far, various studies have only investigated energy efficiency based on the energy value of hydrogen and methane.

The total energy recovered from the two-stage AD can be determined by calculating the energy produced in the form of hydrogen and methane. About 1.8 MJ/kg. VSadded of hydrogen and 12.3 MJ/kg. VSadded of methane (a total energy recovery of 14.21 MJ/kg. VSadded) was recovered in a two-stage AD treating manure and market wastes which were 8–43% higher energy recovery than one-stage [208]. Likewise, a total energy recovery of 7.1 MJ/kg. VSadded was achieved in a two-stage AD-treating alkali (NaOH) -pretreated wheat straw [209]. However, the study
observed no significant difference between one-stage and two-stage AD systems. The results were 3% higher energy recovery than one stage system treating alkali-pretreated wheat straw and 23% higher energy than one stage treating untreated wheat straw. In another study, a 19% increase in energy yield was observed in a two-stage AD treating (1.64 MJ) thin stillage compared to single-stage AD (1.38 MJ) [207]. At the same time, Luo et al. [210] reported a stabilized two-stage AD at a feeding rate of 0.05 kg VS/ Ld treating stillage. Total energy of 11.8 MJ/kg was recovered from the system, with about 0.7 MJ/kg from biohydrogen production and 12.4 MJ/kg from biomethane production. A higher total energy yield of 22 MJ/kg. VS (H$_2$ yield of 76 L/kg. VS and CH$_4$ yield of 598 L/kg. VS) was obtained during the two-stage AD of food waste [57].

Fu et al. [211] investigated the performance of two-stage AD treating vinasse. The study obtained a cumulative hydrogen and methane yield of 14.8 and 274 L/kg. VS$_{\text{substrate}}$ with energy recovery of 10.54 MJ/kg VS (13% higher than single-stage AD). A hydrogen yield of 106 L/kg VS and a methane co-production efficiency of 125% were achieved in a two-stage system during the co-digestion of food waste, corn straw, and chicken manure [212]. Ramos et al. [213] simulated upscaling estimation for a two-stage AD system treating vinasse wastewater. According to the study, the best scenario for treating the vinasse wastewater is maintaining thermophilic conditions for the acidogenic reactor and mesophilic conditions for the methanogenic reactor, achieving a maximum energy yield of 7 MJ/kg COD$_{\text{removed}}$.

However, some researchers have disagreed with these claims [214]. From their studies, they have observed that there are no significant differences in overall energy recovery between one-stage and two-stage AD systems. The common root cause being suggested is the accumulation of intermediate metabolites such as VFAs, phenols, amino acids, ketones, and amines which makes
the two-stage system inefficient. The low pH effluent consisting of a high concentration of intermediate metabolites from the DF reactor may weaken the microbial activity and diversity in methanogenic reactors. Therefore, process efficiency and stability must be ensured to recover higher energy from two-stage AD. It is generally directly linked with the substrate type, feeding rate, HRT, bioreactor used, and energy input required for the operation [215,216].

5.4 Techno-economic analysis (TEA)

The techno-economics of any biorefinery system depends on the profit from the output over the investment. Thus, it relies on how biohydrogen and biomethane fuels produced are applied. Hsu et al. [217] evaluated the techno-economics of such a biorefinery concept by treating condensed molasses in a DF reactor with a working capacity of 50 m³ and an anaerobic digester having a capacity of 300 m³, followed by chemical scrubbing for biogas purification and recovering hydrogen, methane, and carbon dioxide. The techno-economic analysis (TEA) showed that the internal rate of return of the system was 33%, with a payback period of about 3.2 years. More recently, Mahmod et al. [218] studied the techno-economics of a two-stage AD for treating palm oil mill effluent, having a plant capacity of 700 m³ (for DF) and 7000 m³ (for AD). The plant was designed for thermophilic conditions (50°C) at an HRT of 1 d for DF and 10 days for AD. The TEA projected a payback period of 8 years, a return on investment of 20%, an internal rate of return of 21.50%, and a net present value of around 46.25 million USD. The study also recommended that the substrate quality and selling price of the fuel products influence the dynamics in the economics of the proposed two-stage AD system. Bastidas-Oyanedel and Schmidt [219] compared the TEA of food waste valorization through single-stage and two-stage AD systems. Within a timeframe of 20 years, the return on investment increased from 36% to 73%, and payback time was reduced from 15 years to 8 years in two-stage AD systems. Sanchez
et al. [9] showed that the biohydrogen production cost from DF of agricultural wastes is between 2.30 and 2.50. Similarly, hydrogen production through DF using food waste cost 0.54 – 3.20 USD/m$^3$ [13,50,220]. The reported production cost of biohydrogen from various substrates is summarized in Table 7.

Integrating the DF process with AD might reduce the overall production cost of biohydrogen. Moreover, the studies suggested that solely producing hydrogen from DF through waste biomass is influenced by the substrate cost, system establishment cost, and cost inclusive of collection, transportation, and distribution. Since waste biomass is available cheaply, the substrate cost can be vastly reduced. Rajendran et al. [221] have calculated that the two-stage AD requires only a 3% excess capital investment compared to single-stage AD for a 1000 – 1100 m$^3$ working volume digester. Moreover, the techno-economics of a two-stage biorefinery system is mainly governed by several factors such as reactor configuration, hydrogen/methane productivity, transportation, collection, processing, and pretreatment of the substrate and substrate quantity to be treated, plant capacity, energy input required, etc. [221,222]. However, DF-based biorefineries can be feasible over conventional techniques only if economic and environmental benefits are considered [9].

Table 7. Cost economics of biohydrogen production through the DF process

<table>
<thead>
<tr>
<th>Substrate type</th>
<th>Biohydrogen production cost (USD/m$^3$)</th>
<th>References</th>
</tr>
</thead>
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<td>Food wastes</td>
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<td>[13]</td>
</tr>
<tr>
<td>Food wastes</td>
<td>0.54</td>
<td>[223]</td>
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<td>Food wastes</td>
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<td>[50]</td>
</tr>
<tr>
<td>Molasses</td>
<td>1.80</td>
<td>[220]</td>
</tr>
<tr>
<td>Agricultural wastes</td>
<td>2.70</td>
<td>[224]</td>
</tr>
<tr>
<td>Beverage wastewater</td>
<td>2.70</td>
<td></td>
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<tr>
<td>Agricultural wastes (wheat straw)</td>
<td>2.30–2.50</td>
<td>[9]</td>
</tr>
</tbody>
</table>

5.5 Life cycle analysis (LCA)
Life cycle analysis (LCA) is an essential factor that determines the fate of an industrial-scale biorefinery establishment. One study has evaluated the environmental concerns involved in the two-stage biorefinery concept for two different substrates, i.e., food waste and wheat straw, and compared it with single-stage AD and diesel-based energy generation [225]. The study observed that a two-stage biorefinery could remarkably reduce the associated environmental problems (carcinogens and ecotoxicity). They also reported that the two-stage hydrogen and methane-producing biorefinery concept using wheat straw increases the energy returns over a single-stage AD process. Isola et al. [226] investigated the LCA of a portable two-stage AD treating food waste (FW) and cardboard waste (CW) (at the best co-digestion (FW: CW) ratio of 65:35). The portable two-stage AD exhibited performance equivalent to full-scale reactors yielding 37% COD of energy in the form of biogas. The study cited that the primary contributing parameter for the life cycle of a two-stage AD is the temporal variation of the feedstock. Likewise, Coats et al. [227] evaluated the LCA of a two-stage AD coupled with algae production. The study analysed that the system can substantially reduce the greenhouse gas emissions contributing to climate change by up to 60% compared to the anaerobic lagoon process. Sun et al. [228] studied the LCA of biohythane production through two-stage AD treating microalgae. The study found that the net greenhouse gas emissions of biohythane production consisting of upgradation, energy, and nutrient recovery systems were 18% higher than that of a system without a hydrogen fermentation system. Apart from energy recovery, the study recommended that nutrient recovery is an essential component that must be considered in a biorefinery concept to improve the LCA of a two-stage AD system. Schramm, [229] investigated the LCA of a two-stage AD-treating OFMSWs. The results from the study indicated that the DF process treating OFMSWs initially provided a better energy balance for the whole system. Further, the utilization of VFAs in the
succeeding AD reactor delivers the lowest impact on the environment per kJ of energy produced than the conventional AD systems. Very recently, Camacho et al. [230] claimed that the substrate treated is the major parameter that governs the carbon neutrality of the overall DF biorefinery system. The study found that it is much more energy-positive and sustainable to utilize the sugar beet molasses as a suitable feedstock for hydrogen production than cheese whey and co-fermentation of wine vinasses and wastewater treatment plant sludge. The outcome of all the studies, in general, was that the energy and nutrient recovery along with almost equivalent greenhouse emissions paved way for considering two-stage AD as a sustainable way to treat waste biomass over conventional AD.

6. Recent advances and future research directions

Dark fermentation for biohydrogen production is an exciting topic with huge prospects. However, the stability and long-term operation of the process still pose challenges [18]. Microbiological investigations using mixed culture inoculum to initiate the DF process are to be targeted further for fast start-up and long-term sustainable operation. Most recent biohydrogen potential investigations are based on batch study assessments. More long-term continuous studies are required for further development of the biorefinery concept. The feasibility of integrating microbial fuel cells, photo fermentation, microalgal ponds, and bioelectrochemical systems with the two-stage AD need to be investigated further [231,232]. This might make the biorefinery system more reliable and enhance the synthesis of various products. For example, producing biobutanol apart from biohydrogen and biomethane [209,210] or improving both fuels' productivity [233,234].

The future concept of a microalgae-based biorefinery unit is shown in Fig. 7 [231,233]. Integrated DF and photofermentation techniques are not economically viable as of current
research developments, as per Ahmad et al. [235] and Urbaniec et al. [236]. The study by Ahmad et al. examined the possibility of treating liquid pineapple wastes through DF and photofermentation for biohydrogen production. The results indicated that a rate of interest between 2 to 20% varies the payback period between 9.90 to greater than 20 years, which is not reasonably feasible in terms of investment. However, there have been reports of better techno-economic viability of DF plants integrated with polylactic acid fermentation [219]. With different findings being reported by various researchers, more investigations to optimize such concepts with respect to product yield, techno-economics, and life cycle analysis are required for conclusive validations. As in Fig. 7, interventions of different processes for producing various value-added products, such as polyhydroxyalkanoates (PHAs), biodiesel, biobutanol, acetic acids, etc., may reduce the investment cost and thus improve economic viability.

Researchers have recently utilized various strategies, such as adding biochar, nanoparticles, etc., to improve the biohydrogen yield and microbial metabolism [13,237]. Nanoparticles (NPs), specifically inorganic nanoparticles such as nickel, titanium oxide, silver, and iron, have enhanced biohydrogen production [238]. However, the dosage quantity must be optimized according to the substrate type and inoculum. On the other hand, some researchers have incorporated carbon materials such as biochar, hydrochar, etc., produced from various substrates into the DF process. These carbon materials, rich in microbial abundance and activity-enhancing properties such as porosity, high specific surface area, neutral pH, and trace elements, have been reported to boost the hydrolysis and acidogenesis rates, subsequently supporting biohydrogen production [237]. Different trace elements, such as Fe$^{2+}$, could stimulate the Fe-based hydrogenase reactions during the DF, resulting in biohydrogen production [239], but this requires further investigation.
In the case of upgradation techniques, water scrubbing technology has been neglected for biohydrogen purification. However, regarded as having much more economical and less environmental effects for biomethane upgrading [240], biohydrogen purification through water scrubbing could be a solution that can be further researched. Biohythane is a suitable fuel that could be directly used as a vehicular fuel. Hence, two-stage AD could be focused on producing biohythane. It can be directly utilized as an alternative to compressed natural gas, especially in vehicles that improve upgraded biomethane energy density enhancing its applicability. Still, the challenge is that the economical and environmentally friendly purification and storage systems are lacking and require much research focus shortly. The separated bio-CO$_2$ could be utilized for agricultural crop production, harvested crop storage, other industrial applications, etc. Kumar et al. [241] have successfully demonstrated using bio-CO$_2$ for wheat grain storage. The results suggest that bio-CO$_2$ enhanced shelf life and controlled pests.

Recently, Adlak et al. and Khan et al. have successfully stored enriched biomethane in activated carbon-filled cylinders at lower pressures (<70 bar) [242–244]. The same concept may be adaptable to hydrogen storage, as discussed in section 4.2.1.4 but requires extensive investigation for biorefinery development. The large-scale H$_2$ storage and transport systems are underdeveloped, expensive, and energy intensive. Another way to solve biohydrogen storage and transportation problems involves converting biohydrogen to methane. A massive advantage of utilizing methane as a storage and transport medium is the existence of efficient and advanced storage and transport pipeline systems already developed. Hydrogenotrophic methanogens reduce the carbon dioxide (CO$_2$) to CH$_4$ when appropriate reducing power, i.e., H$_2$ or low redox potential electrons, are available. The energy conversion of H$_2$ and CO$_2$ into CH$_4$ is called Power-to-Methane (P2M) [245].
P2M could be achieved in two ways: (a) within the AD reactor called in-situ P2M, or (b) in a separate AD reactor, i.e., ex-situ P2M, or in combination. The key methanogens involved depend on how the P2M process is achieved, i.e., mixed anaerobic communities are required for in-situ P2M. At the same time, pure cultures are essential for ex-situ P2M, which could be enriched from full-scale anaerobic digestion plants [246,247]. Further, the converted methane from H₂ could be either utilized directly to replace natural gas or converted back to hydrogen. The pathway for methane to hydrogen conversion could be methane-electricity generation-water electrolysis [248] or through methane reforming using solid oxide fuel cells (SOFC) [249]. This could minimize the requirement for hydrogen-based storage and transport systems and avail the already available natural gas-based storage and transport systems as an alternative reducing the huge initial investment costs and the overall carbon footprint. The concept can be instrumental for the future “low carbon hydrogen transport.” However, these concepts, including biohydrogen upgradation, storage, transport, P2M, and SOFC technologies, are still at primary scale investigations and require extensive pilot-scale evaluations, TEA, and LCA studies.
Fig. 7. Integration of different biofuel and biochemical recovery technologies with two-stage AD biorefineries (adapted and modified from Sitthikitpanya et al. [233]).
Policy interventions for introducing biohydrogen into the energy fuel market: An Indian perspective

Hydrogen production is necessary to mitigate greenhouse gas emissions, tackle climate change issues, and minimize the overutilization of fossil fuels. So far, the existing hydrogen production techniques are more based upon SMR or else with electrolysis-dependent systems. Especially the developed countries (primarily Western countries) have initiated indigenous hydrogen production, fulfilling energy security and tackling climate change [250]. Afro-Asian countries need to pick up their pace in adopting hydrogen as a clean fuel through various international/national policy developments and tie-ups. Recently, Govt. of India unveiled a National Hydrogen Mission to build India as a global hub in hydrogen production. The mission aims to achieve “green hydrogen” production focusing on energy self-reliance, self-sufficiency, and clean energy transition.

Renewable hydrogen production through the biological process of DF, bio photolysis, and photofermentation should also get the attention it deserves in the “Green hydrogen” platform with its benefits. This makes the self-reliant biohydrogen production and increases the green growth and jobs that the National hydrogen mission aims to. In addition, the National Hydrogen Mission can be merged with the missions such as Swachh Bharat Abhiyaan (a solid waste management scheme) and Sustainable Alternative towards Sustainable Transportation (SATAT) (a clean vehicular energy scheme based on compressed biomethane), making it engaged in more widened perspectives along with solid waste management, clean energy, and transportation. Capacity building across the nation is crucial and decisive from a political, technical, and economical aspect for successfully establishing biorefineries along with other hydrogen production technologies.
The decisions may be considered after the conclusive evidence elucidated from the managerial decision-making approaches such as strengths, weaknesses, opportunities, and threats (SWOT) analysis [16,251]. Likewise, Das et al. [252] conducted a SWOT analysis to determine the feasibility of the biological biogas upgradation systems. Similarly, Table 8 shows the SWOT analysis results for the two-stage AD-based biorefinery concept discussed in this review article.

From Fig. 8 (a, b), it can be seen that the research publications from different countries on biohydrogen production through DF and two-stage AD. Asian countries have been primarily interested in research developments on these topics. However, there is a lack of knowledge dissemination or collaboration between the countries specifically working on DF and two-stage AD, as seen in Fig. 8 (a, b). Hence, more international partnerships and industrial symbiosis are required to boost the development of biorefinery concepts, which depend highly on intergovernmental decisions and policy frameworks. Moreover, the enlisted weaknesses and threats must be adequately addressed.
Fig. 8. Research across the world over dark fermentation (a) and two-stage anaerobic digestion (b) (Scopus data, dated 28th April 2022).
Table 8. SWOT analysis of the two-stage anaerobic digestion-based biorefinery concept according to this review

<table>
<thead>
<tr>
<th>Strength</th>
<th>Weaknesses</th>
<th>Opportunities</th>
<th>Threats</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Abundant availability of waste biomass</td>
<td>• Collection, transportation, and segregation of the waste biomass resources</td>
<td>• Achieving the hydrogen fuel demand</td>
<td>• Varied performance based on substrate composition and type, inoculum type, and microbes involved</td>
</tr>
<tr>
<td>• Eco-friendly and sustainable technology compared to other techniques</td>
<td>• Adopting the technology without downstream technologies is not feasible economically</td>
<td>• More research and developments (collaborations and partnerships) within the countries between academic institutions and industries and between the countries.</td>
<td>• Lack of policy framework promoting “biohydrogen” production</td>
</tr>
<tr>
<td>• Simple, adaptable technology with less complexity</td>
<td>• Start-up and long-term stable operation require rigorous optimization methods</td>
<td>• Valorization of biohydrogen, biocarbon dioxide, biomethane, VFAs, and bio-slurry replacing conventional energy fuels/chemical fertilizers</td>
<td>• Lack of economical techniques for hydrogen purification, storage, and transportation.</td>
</tr>
<tr>
<td></td>
<td>• Lack of adequate pilot-scale experiences</td>
<td>• Proper treatment of waste biomass contributes to sustainable waste management</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Low productivity in terms of energy recovery</td>
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</table>
8. Conclusions

Tapping the biohydrogen from waste biomass through DF possesses immense potential globally. Still limited to the laboratory and pilot-scale studies, there is a push to develop biorefinery concepts based on DF. Thus, research has focused over the last two decades on investigating the potential of DF for biohydrogen production. From this review, several notable conclusions were elucidated as given below:

• There is a requirement for long-term studies at a pilot-scale level based on DF from various waste biomass for stable operation, by-product production, and microbiological aspects, which is still lacking.

• Microbial consortia used for DF startup are crucial for biohydrogen productivity and VFAs production.

• Biorefinery concepts solely based on DF are not viable for upscaling regarding techno-economics and biomass utilization.

• So far, two-stage AD stands out as the most suitable option for simultaneous biohydrogen and biomethane production even though other technologies, such as photo fermentation, bioelectrochemical systems, etc., are being investigated lately. The research on the latter technologies must be established regarding technical and economic feasibility and life cycle analysis.

• Two-stage AD can utilize the waste biomass resources to the maximum potential in terms of energy recovery, techno-economics, and life cycle analysis.

• The effect of adding nanomaterials and other bio-additives to the DF and AD reactor requires more investigations at pilot-scale studies in terms of performance, environmental sustainability, and techno-economics.
• Hydrogen purification and storage require further investigation into sustainable and cheaper mechanisms with lesser complexity.

• Biohydrogen production requires a synergistic push from a policy aspect, developing more international collaborations, industrial-academia symbiosis, etc.

CRediT authorship contribution statement


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