

Review

Mechanistic Insights and Emerging Hybrid Strategies of Magnetite Nanoparticles for Enhanced Dark Fermentative Biohydrogen Production

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Abstract

Dark fermentative biohydrogen (bio-H₂) production is a promising renewable energy technology for converting organic waste and wastewater into clean fuel. However, practical application is limited by low H₂ yield, volatile fatty acid (VFA) accumulation, incomplete substrate utilization, electron diversion to competing pathways, prolonged lag phases, and inhibitory byproducts from lignocellulosic pretreatment. Magnetite nanoparticles (Fe₃O₄ NPs) have attracted attention as redox-active additives because of their electrical conductivity, reversible Fe²⁺/Fe³⁺ cycling, magnetic recoverability, biocompatibility, and microbial interaction potential. This review examines the physicochemical properties of Fe₃O₄ NPs and their proposed roles in dark fermentative bio-H₂ production. Particular emphasis is placed on Fe₃O₄-mediated extracellular electron transfer (EET) in fermentative communities, which differs from direct interspecies electron transfer (DIET) in methanogenic systems. Fe₃O₄ NPs may enhance bio-H₂ production by facilitating electron transfer, supporting hydrogenase activity, regulating redox balance, promoting acetate- and butyrate-type pathways, and enriching H₂-producing bacteria such as *Clostridium* spp. Hybrid systems combining Fe₃O₄ with biochar, activated carbon, reduced graphene oxide, bimetallic nanocomposites, or immobilization matrices may further improve microbial retention and process stability. Remaining challenges include aggregation, dosage-dependent toxicity, recovery, environmental fate, mechanistic uncertainty, and scale-up feasibility.

Keywords: dark fermentation; biohydrogen; electron transfer; hydrogenase; magnetite nanoparticles; hybrid materials

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1. Introduction

Hydrogen (H₂) is a high-energy fuel with a high gravimetric energy density and zero direct carbon emissions during use. However, most industrial H₂ is still produced from fossil fuel-based processes, which are associated with substantial greenhouse gas emissions [1]. Therefore, sustainable and low-carbon H₂ production technologies are urgently needed. Biological H₂ production has attracted increasing attention because it can convert organic wastes, wastewater streams, and lignocellulosic residues into renewable energy under relatively mild operating conditions [2]. Among biological H₂ production routes, dark fermentation (DF) is considered one of the most practical approaches because it does

not require light energy, can operate with simple reactor configurations, and can utilize a broad range of organic feedstocks, including glucose, starch, food waste, lignocellulosic biomass, and industrial effluents [3,4].

Nanotechnology has emerged as an effective platform for circumventing the biochemical and biophysical limitations of DF. The application of nanoparticles (NPs) to DF systems has attracted significant scientific attention due to their unique physicochemical properties, including high surface-area-to-volume ratios, quantum size effects, tunable electronic properties, and catalytic activity, which collectively enable them to interact with microbial metabolism at the molecular scale [5,6]. The inclusion of these metals increases catalytic activity and enzyme stability, which improves hydrogen generation. To overcome low H₂ yields in DF, metal and metal oxide NPs have been added to increase intracellular electron transport, activate hydrogenase enzymes, and encourage selective H₂-producing microorganisms [7,8].

NPs, especially those containing iron, nickel, or cobalt, have clearly shown the ability to boost dark fermentative H₂ production by increasing electron transfer, altering microbial populations, and redirecting metabolic pathways. However, their effects are largely dose-dependent, with high doses causing toxicity and reducing performance. Therefore, improving this method will require greater mechanistic insight, precise control of NP physicochemical properties, and validation under continuous operation to establish efficacy and scalability [9–11].

Among various nanomaterials, iron-based nanoparticles and carbon-based conductive materials have been widely investigated for improving DF bio-H₂ production because iron is closely associated with redox-active enzymes, hydrogenase activity, and microbial electron transfer. In addition to magnetite nanoparticles (Fe₃O₄ NPs), other conductive materials such as activated carbon, biochar, carbon nanotubes, and hematite (Fe₂O₃) have also been explored to enhance extracellular electron transfer, microbial attachment, and H₂ production. Nevertheless, Fe₃O₄ has attracted particular attention because of its mixed-valence Fe²⁺/Fe³⁺ structure, conductivity, relatively low cost, magnetic recoverability, and biocompatibility. For example, Fe₃O₄ supplementation has been reported to improve continuous H₂ production from rice straw hydrolysate at an optimal dose, while excessive dosing inhibited H₂ production, highlighting the importance of dose optimization [12].

This review critically discusses the physicochemical properties, mechanistic roles, microbial interactions, process enhancement effects, and emerging hybrid strategies of Fe₃O₄ NPs in dark fermentative bio-H₂ production. Unlike previous reviews that broadly summarized nanomaterial-assisted biohydrogen production, this review focuses specifically on Fe₃O₄ NPs and integrates their redox chemistry, enzyme-stimulation effects, microbial interactions, VFA redistribution, process performance, and hybrid material strategies in DF systems. Particular attention is given to Fe²⁺/Fe³⁺ redox cycling, extracellular electron transfer (EET), hydrogenase stimulation, microbial community regulation, substrate utilization, VFA distribution, and the distinction between Fe₃O₄-mediated EET in DF and direct interspecies electron transfer (DIET) in methanogenic systems.

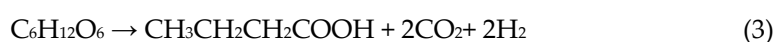
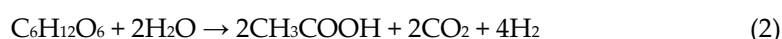
2. Fundamentals of Dark Fermentative Bio-H₂ Production

2.1. Principles and Major Metabolic Pathways of Dark Fermentation

DF pathways are commonly classified according to the dominant soluble metabolites, including acetate, butyrate, propionate, ethanol, and lactate-type fermentation. Acetate- and butyrate-type pathways are generally favorable for H₂ production because they are directly coupled with proton reduction, whereas propionate, lactate, and solvent-forming pathways consume reducing equivalents or divert substrate-derived electron away from H₂ evolution. The complete oxidation of glucose to H₂ and CO₂ can be represented by the following theoretical reactions:



Equation (1) represents the complete theoretical maximum H_2 yield assuming complete oxidation of glucose. However, this reaction is not achieved in conventional DF because part of the substrate is converted into soluble metabolites such as VFAs and alcohols. In practice, the off-gas generated during DF is mainly composed of H_2 and CO_2 . Reported gas compositions vary with substrate type, inoculum, pH, HRT, reactor configuration, and operating conditions, but H_2 commonly accounts for approximately 40–60%, while CO_2 accounts for most of the remaining gas phase. Under well-controlled H_2 -producing DF conditions, CH_4 is generally absent or present only at trace levels because methanogenic activity is suppressed [13]. The major biochemical reactions occurring during glucose-based DF are summarized as follows:



Equation (2) represents acetate-type fermentation, which is the most favorable route for H_2 production and can theoretically yield up to 4 mol H_2 /mol glucose, whereas Equation (3) represents the butyrate-type fermentation pathway, which is also favorable for 2 mol H_2 /mol glucose [14,15]. In contrast, Equation (4) represents the propionate-type fermentation pathway, which consumes reducing equivalents and is therefore associated with reduced net H_2 production. Equation (5) represents the ethanol-type (solvent-forming) pathway, which diverts reducing equivalents toward ethanol formation without net H_2 release; Equation (6) represents the lactate-type fermentation pathway, which similarly competes with H_2 production by channeling electrons into lactate formation rather than H_2 generation; and Equation (7) represents a stoichiometric conversion of lactic acid to propionic acid, acetic acid, CO_2 , and H_2O during DF— a non- H_2 -producing competitive pathway that reduces bio- H_2 yield [16–18]. The major metabolic pathways involved in dark fermentative H_2 production, including glycolysis, pyruvate conversion, and formation of acetate, butyrate, ethanol, lactate, and propionate, are illustrated in Figure 1.

Although the complete oxidation of glucose can theoretically yield 12 mol H_2 /mol glucose, this is not achievable in conventional single-stage DF because part of the substrate is converted into soluble metabolites such as acetate, butyrate, lactate, ethanol, and propionate, resulting in lower H_2 yield [19]. Excessive accumulation of VFAs and solvents lowers pH, disturbs intracellular redox balance, inhibits hydrogenase activity, and reduces process stability. Therefore, controlling metabolite distribution is essential for maximizing H_2 production [20,21].

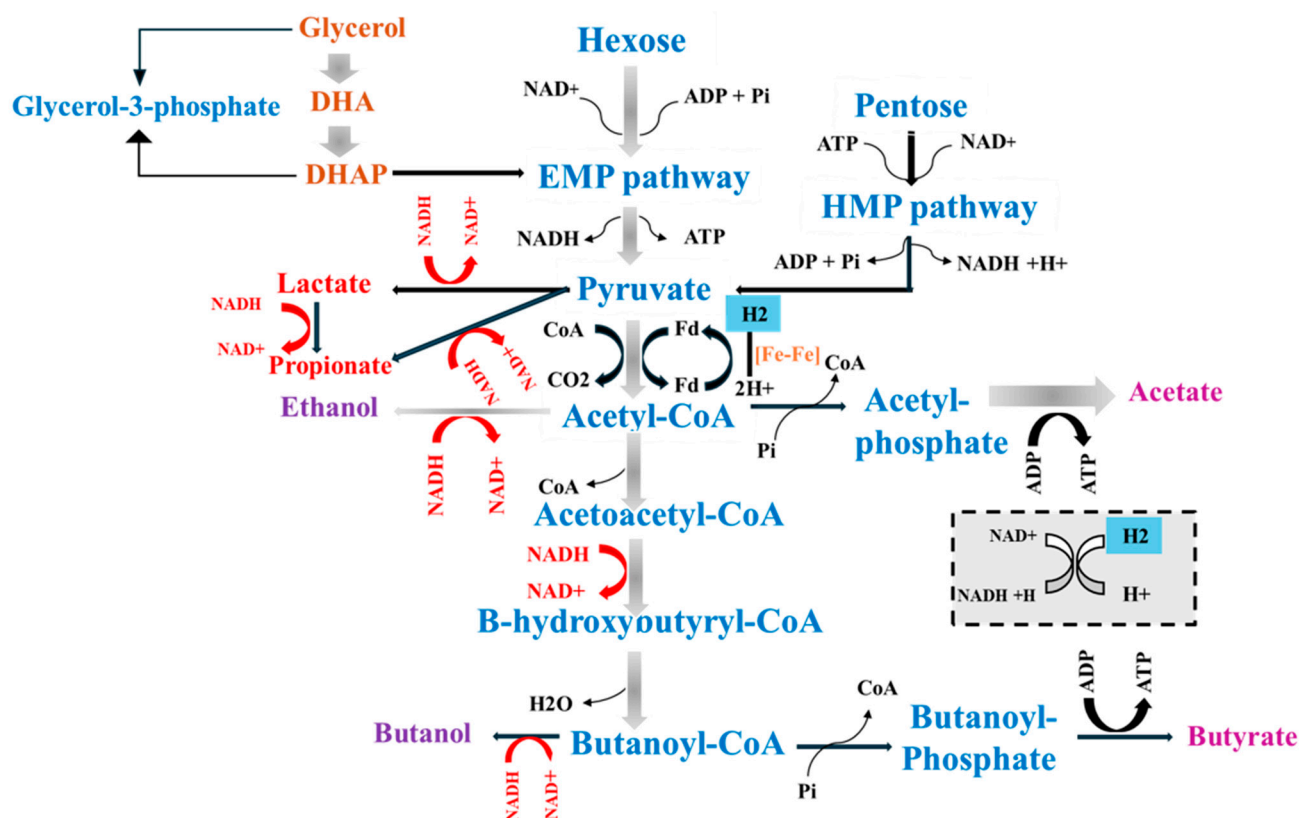


Figure 1. Major metabolic pathways and electron flow involved in dark fermentative bio- H_2 production from carbohydrate substrates.

Another major limitation is the lag phase, during which H_2 -producing bacteria adapt before active H_2 evolution begins. Acidic conditions prolong this phase and reduce H_2 production rates. Incomplete substrate utilization remains a significant concern, particularly when complex and lignocellulosic feedstocks are employed. Because of its resistant structure, lignocellulosic biomass (LCB) is not biodegradable and must be pretreated to improve substrate accessibility. However, strong thermochemical pretreatment techniques, including dilute-acid pretreatment (e.g., H_2SO_4), hydrothermal/liquid hot water pretreatment, steam explosion, alkaline thermal pretreatment and microwave-assisted pretreatment, frequently yield inhibitory chemicals, such as short-chain carboxylic acids, furfural, 5-hydroxymethylfurfural (5-HMF), vanillin, and syringaldehyde, which negatively affect microbial physiology and fermentation performance [22].

2.2. H_2 -Producing Microorganisms

The performance of DF bio- H_2 production depends on the composition, activity, and stability of H_2 -producing microbial communities. Microbial augmentation can boost microorganism resistance to environmental stress, enabling stable reactor operation under a relatively shorter hydraulic retention time (HRT) than a non-augmented system. In continuous DF processes, reducing HRT increases the organic loading rate (OLR) and reactor throughput but may also increase the risk of biomass washout and process instability [23]. The inoculum structure and dynamics, as well as interactions among distinct microbial populations, are significant factors of substrate conversion efficiency and H_2 yield [24]. H_2 -producing bacteria (HPB) are divided into three classes based on their metabolic properties. The first type includes facultative anaerobic bacteria like *Bacillus* and *Enterobacter*. They create H_2 through formate cleavage routes mediated by formate hydrogen lyase systems [25]. The second group consists of obligate anaerobic fermentative bacteria, such as

Clostridium butyricum and related species, that produce H₂ during pyruvate oxidation through ferredoxin-dependent hydrogenase pathways. The third type includes thermophilic H₂-producing bacteria, which thrive at high temperatures and have higher production rates and resistance to contamination [26].

Mixed microbial consortia with multiple H₂-producing species are preferred over pure cultures due to their ability to broaden the range of utilizable substrates, increase metabolic cooperation, and improve process resilience. As a result, synergistic interactions among distinct microbial groups can enhance substrate degradation efficiency and maximize bio-H₂ production.

2.3. VFA Accumulation

The accumulation of VFAs causes increased NADH⁺, upsetting the intracellular redox balance, decreasing hydrogenase activity, and limiting H₂ generation [27]. Yin et al. [15] Undissociated acetic acid (UA) was found as the principal inhibitor of VFAs' H₂ generation during fermentation. When UAs enter the cell, they break down into H⁺ ions and acid radicals in the cytoplasm, lowering intracellular pH, affecting enzyme performance, limiting hydrogenase function, and resulting in decreased H₂ generation. The accumulation of UAs can also affect bacteria's membrane potential, hindering cellular transport and energy generation. Controlling UA concentrations via various methods such as in situ pH adjustment, VFA extraction, or dilution of fermentation broth can reduce acetic acid's inhibitory effects on H₂ production. The inhibition mediated by UAs is connected to substrate inhibition and pH changes. Thus, maintaining an optimum substrate concentration or organic loading rate can help minimize the UA-induced inhibition [28]. DF performance can be greatly impacted by a few other processes in addition to traditional inhibitory variables such as high VFA buildup and HCM. Hazardous substances are produced during the processing of biomass, like phenolic derivatives (vanillin, syringaldehyde) [29]. Furans such as 5-hydroxymethylfurfural (5-HMF) and furfural, as well as weak acids, can drastically reduce microbial activity by compromising cell membrane integrity and blocking important enzyme processes.

2.4. H₂-Consuming Microbes

Methanogens are the primary H₂ consumers in anaerobic settings. During anaerobic digestion, H₂ is created during acidogenesis and acetogenesis and later consumed during methanogenesis. Methanogenic archaea use H₂ and CO₂ to produce CH₄. To maximize H₂ yield, methanogens are often inhibited during bio-H₂ DF [30]. Contaminants like methanogens can outcompete H₂-producing bacteria such as *Clostridium* and *Enterobacter* by exploiting substrates like acetate and butyrate, redirecting the process towards CH₄ generation instead of H₂ [31].

Methanogens compete with other hydrogenotrophic microorganisms, including propionate-producing bacteria and reductive acetogens, which consume H₂ through alternative metabolic pathways. These competing pathways act as alternative H₂ sinks and can reduce the amount of H₂ available for methanogenesis. However, propionate-producing bacteria and reductive acetogens generally exhibit a lower affinity and efficiency for H₂ uptake than methanogens. Consequently, they rarely become dominant H₂ consumers unless methanogenic activity is selectively inhibited or suppressed [32,33].

2.5. Electron Loss Through Lactate Production and Its Inhibitory Accumulation

A central challenge is that reducing equivalents generated during glycolysis are not efficiently channeled to H₂. Instead, electrons are "lost" to competing by-products. Two main electron carriers in DF are ferredoxin (Fd) and reduced nicotinamide adenine dinu-

cleotide (NADH), both of which are complicatedly involved in electron bifurcation. Electron bifurcation involves both exergonic and endergonic electron transfer reactions to minimize energy loss, but previous studies incorporating these reactions into DF metabolic models have significant limitations [34].

Lactate production primarily occurs through the activity of lactic acid bacteria (LAB), which utilize NADH to reduce pyruvate to lactate. Unlike H₂-producing microorganisms, LAB generally does not evolve H₂ because they lack functional hydrogenases or possess negligible hydrogenase activity. Consequently, reducing equivalents that could otherwise be transferred to hydrogenases for H₂ evolution are instead consumed during lactate formation, decreasing theoretical hydrogen yield [35].

Beyond electron competition, excessive lactate accumulation can destabilize dark fermentation by lowering the fermentation pH and promoting conditions favorable for LAB while inhibiting hydrogen-producing bacteria. Nevertheless, the inhibitory effect of lactate is complex and depends on several factors. Previous studies reported that lactate concentrations up to 55 mM did not inhibit H₂ production [36], while no adverse effect was observed even at approximately 300 mM when lactate was produced endogenously during the dark fermentation of pre-fermented food waste [37]. In contrast, the addition of only 44 mM exogenous L-lactate reduced H₂ production by approximately 35% [38], suggesting that the mode of lactate formation and its enantiomeric form substantially influence its inhibitory effect. Furthermore, increased LAB activity has been associated with instability of the dark fermentation process [39].

3. Physicochemical Properties of Fe₃O₄ Relevant to Bio-H₂

Fe₃O₄ is a well-studied conductive iron oxide for improving dark fermentative bio-H₂ generation due to its high electrical conductivity, reversible Fe²⁺/Fe³⁺ redox cycling, nanoscale surface characteristics, and magnetic behavior. These features boost extracellular electron transfer (EET), activate hydrogenase, regulate microbial metabolism, and promote microbial aggregation.

3.1. Conductivity

Several studies have explored the use of conductive NP (CNP) to enhance DF, increase H₂ yield (HY), and profile acids [40–43]. Iron-containing CNP significantly improved H₂ generation, attributed to the delayed release of iron ions from CNP to the broth [44]. Fe₃O₄ is more popular than other iron oxides or ferrite spinel oxides due to its higher magnetic characteristics, electronic conductivity, and biocompatibility. Fe₃O₄ has a cubic inverse spinel crystal structure with Fe²⁺ cations occupying 25% of the octahedral interstitial sites, Fe³⁺ cations filling 25% of the octahedral sites and 12.5% of the tetrahedral sites, and 32 O²⁻ anions in the unit cell [45]. This mixed-valence Fe²⁺/Fe³⁺ configuration directly contributes to the magnetic and electrical properties mentioned below. Fe₃O₄ NPs have different electrical and magnetic properties due to the transition of ions from Fe²⁺ to Fe³⁺ [46]. Conductivity at the nanoscale is heavily influenced by morphological characteristics. Cuboidal Fe₃O₄ NPs synthesized with triphenylphosphine demonstrated ultrahigh electrical conductivity (1.02 × 10^{−4} S/cm at 10 Hz) compared to spherical ones. Low-frequency conductivity and dielectric properties are linked to surface disordering and oxidation [47].

3.2. Redox Properties (Fe²⁺/Fe³⁺ Cycling)

Fe₃O₄ has a unique Fe²⁺/Fe³⁺ redox cycling capability that has been linked to increased bio-H₂ production, according to recent research and review studies. The coexistence of Fe (II) and Fe (III) in the inverse spinel structure allows magnetite to operate as a reversible electron mediator, facilitating extracellular electron transfer (EET), maintaining intracel-

lular redox balance, and promoting hydrogenase activity during DF [22]. In dark fermentative systems, Fe_3O_4 can alternate between Fe^{2+} and Fe^{3+} oxidation states, allowing electrons produced during substrate degradation to efficiently shuttle between microbial cells and metabolic pathways. This redox cycle increases electron availability for proton reduction and H_2 evolution. Fe_3O_4 switches metabolic flux from reduced products like ethanol to acetate-type fermentation pathways, which are thermodynamically more favorable for H_2 production. A recent important study indicated that Fe_3O_4 boosted intracellular NAD(H) content and raised the NADH/NAD⁺ ratio during glucose and xylose fermentation. The creation of Fe (III)Fe (II) electron pairs during extracellular iron oxide respiration, suggesting active redox cycling in the fermentation system. As a result, H_2 yield increased because electrons were more efficiently transported into H_2 -producing pathways rather than competing metabolic reactions [48].

According to Ren et al. 2022, iron-containing enzymes like ferredoxin and [Fe-Fe] hydrogenase can use Fe^{2+} produced by Fe_3O_4 as a micronutrient and cofactor. Meanwhile, Fe^{3+} works as an electron acceptor, maintaining redox balance inside microbial cells. The reversible $\text{Fe}^{2+}/\text{Fe}^{3+}$ transformation decreases the oxidation-reduction potential (ORP), enabling suitable anaerobic conditions for H_2 -producing bacteria, particularly *Clostridium* species [49].

Several analytical approaches have been used to evaluate the redox behavior and electron-mediating potential of Fe_3O_4 -based materials. X-ray photoelectron spectroscopy (XPS) is commonly used to determine the surface oxidation states of iron and to estimate $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratios on magnetite surfaces. Changes in the relative intensity of Fe^{2+} and Fe^{3+} peaks before and after fermentation may indicate changes in surface iron chemistry and possible electron exchange between microorganisms and Fe_3O_4 particles. However, such results should be interpreted carefully because XPS primarily provides surface-level information and does not alone confirm the occurrence of extracellular electron transfer under DF conditions [50,51].

Cyclic voltammetry (CV) and related electrochemical techniques can provide additional information on the redox reversibility, conductivity, and electron-transfer kinetics of Fe_3O_4 -based materials. The appearance of oxidation and reduction peaks with higher current responses suggests that Fe_3O_4 can function as a redox-active and conductive mediator. Nevertheless, direct verification of Fe_3O_4 -mediated electron transfer in DF systems requires the integration of electrochemical analyses with fermentation performance, hydrogenase activity, NADH/NAD⁺ ratio, ORP, VFA distribution, and microbial community data [52–54].

The NADH/NAD⁺ ratio is particularly useful for evaluating intracellular redox regulation. An increased NADH/NAD⁺ ratio indicates enhanced reducing power and greater electron availability for proton reduction, whereas inefficient NADH reoxidation can redirect electrons toward reduced by-products such as lactate, ethanol, and propionate. Therefore, combining NADH/NAD⁺ analysis with hydrogenase activity and metabolite profiling can provide stronger evidence for Fe_3O_4 -induced metabolic redirection toward H_2 -producing pathways [55–57]. Table 1 provides comparison of the previous study Influence of Fe_3O_4 and other iron oxide nanoparticles on dark fermentative.

Table 1. Influence of Fe₃O₄ and other iron oxide nanoparticles on dark fermentative bio-H₂ production.

Additive	Dose	Substrate and Inoculum	Operation Conditions	H ₂ Yield/Productivity	Dominant Microbes	Reference
Fe ₃ O ₄ NPs	50 mg/L	Glucose, Mixed anaerobic sludge	Continuous EGSB reactor; mesophilic (35 ± 1 °C); working volume: 5.5 L	Maximum H ₂ productivity: 4.95 LH ₂ /d at 50 mg/L Fe ₃ O ₄ NPs (40–60 nm), 53.73% higher than the control (3.22 L H ₂ /d).	<i>Acetanaerobacterium</i> , <i>Ethanoligenens</i>	[58]
Fe ₃ O ₄ NPs	200 mg/L	Glucose and pretreated starch, <i>Enterobacter aerogenes</i> ATCC 13408	Batch, mesophilic (37 °C); working volume: 200 mL	H ₂ yields from glucose and pretreated starch increased by 17.0% and 63.1%, respectively, at the optimized concentration (200 mg/L)	<i>Enterobacter aerogenes</i> ATCC 13408	[59]
Fe ₃ O ₄ NPs	150 mg/L	Date fruit waste, <i>Enterobacter aerogenes</i> ATCC 13408	Batch, mesophilic (37 °C); working volume: 100 mL	Maximum H ₂ yield of 238.7 mL/g was achieved at 150 mg/L nanocomposites, representing a 65.7% increase over standalone Fe ₃ O ₄ NPs and a threefold increase compared with the control (78.4 mL/g).	<i>Enterobacter aerogenes</i> ATCC 13408	[60]
Fe ₃ O ₄ NPs	800 mg/L	Glucose, Anaerobic sludge	Batch, mesophilic (37 °C); working volume: 100 mL	Maximum H ₂ production of 273.90 ± 1.67 mL was achieved at 0.8 g/L Fe ₃ O ₄ NPs, representing a 128.12% increase over the control.	<i>Clostridium butyricum</i> , <i>C. tertium</i>	[61]
Fe ₃ O ₄ NPs	50 mg/L	Glucose + starch, Anaerobic digestate	Batch, thermophilic (60 °C); working volume: 200 mL	H ₂ production rate increased by 34% at 50 mg/L Fe ₃ O ₄ NPs under thermophilic conditions.	Not reported	[62]

3.3. Particle Size and Surface Area

Previous research has shown that Fe₃O₄ physicochemical properties, particularly particle size and surface area, play an important role in increasing dark fermentative bio-H₂ production. Nanoscale Fe₃O₄ particles have a greater specific surface area, higher reactive sites, and enhanced interaction between microbial cells and conductive materials. This facilitates extracellular electron transfer and increases hydrogenase activity. Different investigations have shown that smaller Fe₃O₄ particles have stronger enhancing effects due to higher surface reactivity and improved microbial contact. For example, Fe₃O₄ NPs with an average size of roughly 20 nm improved H₂ production from glucose by *Enterobacter aerogenes* ZJU1, obtaining 260.9 ± 6.3 mL H₂/g glucose, or a 21.1% increase compared to the control [57]. Similarly, Fe₃O₄ NPs with particle sizes ranging from 13.5 ± 3.7 nm considerably increased H₂ generation from rice straw hydrolysate, yielding 83.20 ± 2.19 mL/g substrate [63]. In another study, Fe₃O₄ NPs of 40–60 nm boosted H₂ yield in anaerobic sludge systems to 12.97 mL H₂/g-VSS, a 43.31% increase [58]. In a cascading DF–AD system, Cheng et al. investigated H₂ and CH₄ co-generation using Fe₃O₄ NPs of approximately 20 nm. Although this system differs from single-stage DF, the study provides useful evidence that nanoscale magnetite can influence microbial electron transfer and syntrophic metabolism in the downstream methanogenic stage, while also improving H₂ generation during the DF stage [52].

The effect of particle size was also found in wastewater and lignocellulosic substrate fermentation systems. Recent research mostly focuses on nano-sized iron oxides (<50 nm). Elreedy et al. [41] observed that adding nano-hematite (α -Fe₂O₃, <100 nm) to industrial effluent boosted bio-H₂ generation by 41%. Similarly, Zhang et al. [63]. These data indicate that reducing Fe₃O₄ particle size increases surface area and active redox sites, which improves microbial adhesion, biofilm development, electron shuttling, and substrate conversion efficiency. However, extremely small nanoparticles can agglomerate at high concentrations, limiting their effective surface area and perhaps generating inhibitory effects. Thus, adjusting particle size and dosage is critical for increasing bio-H₂ generation efficiency in DF systems. The surface area of Fe₃O₄ is a crucial physicochemical property that impacts H₂ production. Fe₃O₄ NPs having a high specific surface area offer more active sites for microbial adhesion, electron exchange, and catalytic processes, increasing H₂ generation efficiency. In general, particle size reduction raises the surface area-to-volume ratio, which promotes the interaction between microbial cells and conductive particles [64]. Nano metal oxides greatly improve bio-H₂ production compared to bulk-form additions. Higher specific surface areas and quantum size effects enhance electron absorption, facilitating effective electron exchange between NPs and hydrogenase [65].

4. Mechanisms of Fe₃O₄-Enhanced Bio-H₂ Production

Fe₃O₄ NPs enhance dark fermentative bio-H₂ production through several interrelated mechanisms, including electron-transfer facilitation, redox regulation, enzyme stimulation, microbial attachment, and metabolic pathway modulation. In DF, these effects should be distinguished from the classical direct interspecies electron transfer (DIET) mechanism widely reported in methanogenic anaerobic digestion systems. Because methanogens are generally suppressed in DF to prevent H₂ consumption, direct evidence for DIET as a dominant mechanism in single-stage DF bio-H₂ systems remains limited [66]. Therefore, Fe₃O₄-mediated enhancement in DF is more appropriately described in terms of extracellular electron transfer (EET), electron shuttling, Fe²⁺/Fe³⁺ redox cycling, and conductive surface-mediated electron redistribution rather than methanogenic DIET. DIET should be discussed mainly in the context of cascading DF–AD or H₂/CH₄ co-generation

systems, where magnetite may promote electron exchange between syntrophic bacteria and methanogenic archaea during the downstream methanogenic stage [57,67].

4.1. Fe_3O_4 -Mediated Electron Transfer, Redox Regulation, and Enzyme Stimulation (Hydrogenase Activity)

In DF, the beneficial role of Fe_3O_4 is primarily associated with enhanced electron transfer within fermentative microbial consortia and stimulation of redox-active enzymes rather than methanogenic syntrophy [68]. Fe_3O_4 can provide conductive surfaces for microbial attachment and biofilm formation, decrease local electron-transfer resistance, and participate in reversible $\text{Fe}^{2+}/\text{Fe}^{3+}$ redox cycling. These properties facilitate extracellular electron transfer and electron redistribution within H_2 -producing biofilms, thereby improving the transfer of reducing equivalents generated during substrate degradation toward proton reduction and H_2 evolution [57].

Another important mechanism is the gradual release of bioavailable Fe^{2+} from Fe_3O_4 under mildly acidic fermentative conditions. Fe^{2+} can serve as an essential micronutrient for iron–sulfur cluster-containing proteins, including ferredoxin and [FeFe]-hydrogenase. The incorporation of Fe into ferredoxin and hydrogenase active centers can enhance hydrogenase and dehydrogenase activities, improve intracellular redox balance, and promote the conversion of reducing equivalents from NADH and reduced ferredoxin into H_2 . Therefore, Fe_3O_4 supplementation can redirect electron flow away from competing reduced products such as lactate, ethanol, and propionate and toward H_2 -producing acetate- and butyrate-type pathways [69].

For example, Fe_3O_4 addition has been reported to increase hydrogenase and dehydrogenase activities, which was attributed to the release of ferrous ions required for the formation of iron–sulfur clusters in these enzymes [70]. Under mildly acidic fermentative conditions, Fe_3O_4 can partially dissolve or undergo surface corrosion, releasing Fe^{2+} that may be incorporated into [4Fe–4S] clusters of ferredoxins and the H-cluster of [FeFe]-hydrogenase. In addition, the high surface area and conductive properties of Fe_3O_4 NPs may facilitate electron transfer between reduced ferredoxin, NADH-dependent redox reactions, and hydrogenase-mediated proton reduction. The overall mechanism by which Fe_3O_4 NPs enhance dark fermentative bio- H_2 production, including stimulation of hydrogenase activity, acceleration of electron transfer, redox regulation, and modulation of metabolic pathways, is illustrated in Figure 2.

4.2. Microbial Community Shift and Enrichment of H_2 -Producing Bacteria

Fe_3O_4 supplementation can reshape fermentative microbial communities by enriching H_2 -producing and acidogenic bacteria while reducing the relative contribution of non- H_2 -producing or H_2 -consuming populations [71]. In mixed-culture DF systems, *Clostridium*-related taxa are often regarded as key H_2 -producing bacteria because they are closely associated with acetate- and butyrate-type fermentation and possess hydrogenase-mediated H_2 -evolving pathways. Several studies have reported that Fe_3O_4 addition increased the relative abundance of *Clostridium* spp. and improved H_2 production performance, suggesting that magnetite can create a more favorable ecological niche for fermentative H_2 producers [12].

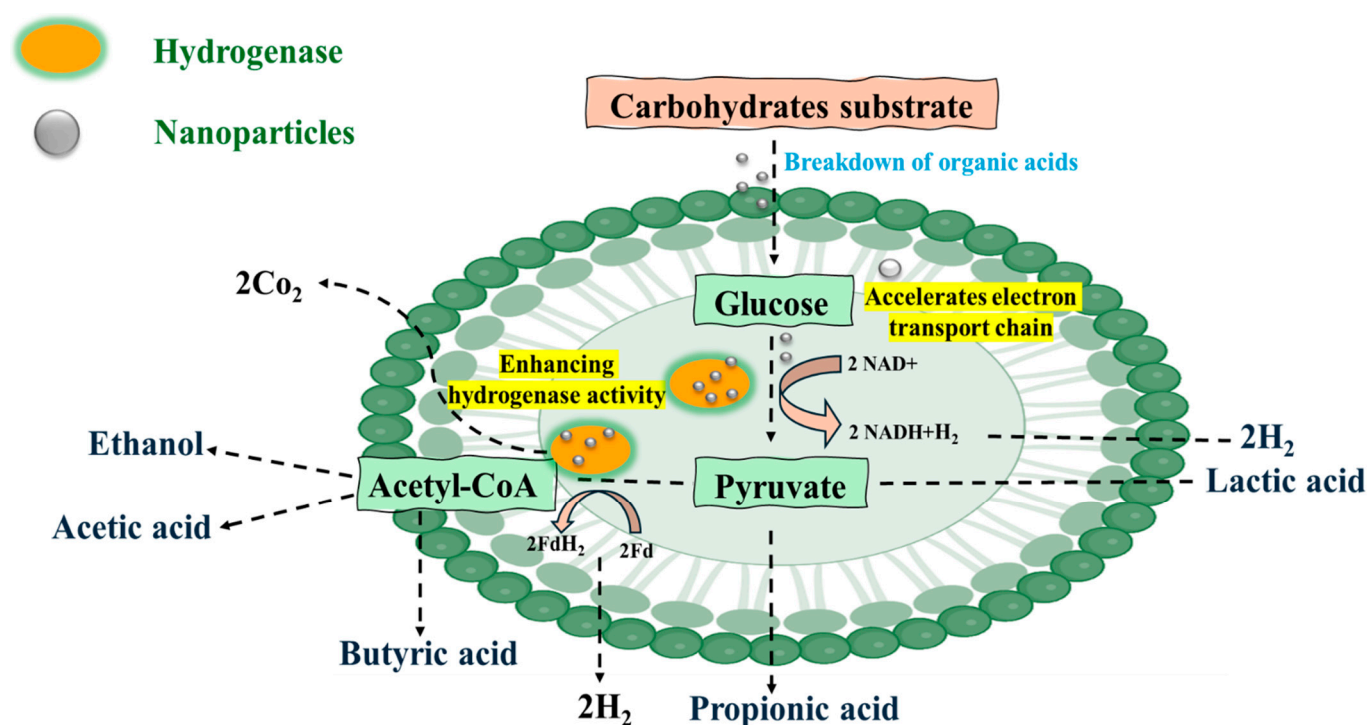


Figure 2. Proposed mechanisms of Fe_3O_4 NP-mediated enhancement of dark fermentative bio- H_2 production through hydrogenase stimulation, accelerated electron transfer, redox regulation, and metabolic pathway modulation.

However, the microbial response to Fe_3O_4 is strongly dependent on substrate type, inoculum source, reactor configuration, and NP dosage. For example, in some continuous systems, Fe_3O_4 supplementation improved H_2 production while shifting the fermentation pattern toward ethanol- or acetate-related pathways rather than simply increasing butyrate-type fermentation. This indicates that Fe_3O_4 does not always induce the same metabolic response across different DF systems. Therefore, microbial community data should be interpreted together with VFA distribution, hydrogenase activity, ORP, substrate conversion, and H_2 yield rather than based on taxonomic shifts alone. Hybrid Fe_3O_4 -based materials can further influence microbial community structure by providing conductive surfaces for microbial attachment, biofilm formation, and electron redistribution. Fe_3O_4 -rGO nanocomposites, for instance, have been reported to increase the relative abundance of *Firmicutes* and *Chloroflexi* while maintaining microbial diversity, as indicated by comparable Shannon and Chao1 indices. These results suggest that Fe_3O_4 -based conductive composites may stabilize mixed microbial consortia and support functional H_2 -producing groups under DF conditions [72].

Nevertheless, Fe_3O_4 -induced microbial shifts are not universally beneficial. In apple pulp waste fermentation, Fe_3O_4 -supported systems showed dominant genera such as *Sporolactobacillus*, *Clostridium*, and *Coprothermobacter*, indicating that both H_2 -producing and non- H_2 -producing fermentative bacteria may coexist depending on the feedstock and operating conditions [73]. Since lactate-producing bacteria can divert carbon and reduce equivalents away from H_2 evolution, the enrichment of such populations should be evaluated carefully. Overall, Fe_3O_4 -mediated improvement in DF bio- H_2 production is most convincing when microbial enrichment of H_2 -producing taxa is accompanied by higher hydrogenase activity, enhanced acetate/butyrate formation, reduced lactate or propionate accumulation, and improved H_2 yield.

4.3. Distinction Between EET in DF and DIET in Methanogenic Systems

Magnetite (Fe_3O_4) NPs, owing to their high electrical conductivity and magnetic recoverability, have been widely recognized as promising conductive materials for promoting DIET. Numerous studies have demonstrated that Fe_3O_4 supplementation enhances methane (CH_4) production by facilitating DIET in methanogenic anaerobic digestion (AD) systems [74,75]. For comparison, biogas from methanogenic anaerobic digestion is mainly composed of CH_4 and CO_2 , typically containing approximately 50–70 vol% CH_4 and 30–50 vol% CO_2 . H_2 is usually present only as a trace intermediate because it is rapidly consumed by hydrogenotrophic methanogens [76]. This gas composition differs from H_2 -producing DF, in which H_2 and CO_2 are the principal gaseous products. It is important to distinguish Fe_3O_4 -mediated electron transfer in DF from DIET-driven syntrophy in methanogenic AD. While both phenomena involve conductive iron as a mediator of microbial electron exchange, they operate through fundamentally different mechanisms, involve different microbes, and serve opposing metabolic objectives. Conflating these two processes risks misattributing the mechanistic basis of H_2 enhancement in DF systems.

The systematic mechanisms governing the response of the intracellular electron transfer chain associated with H_2 production to MNPs remain insufficiently characterized. By comparison, within the context of AD, Fe_3O_4 has been well documented to effectively promote DIET between syntrophic partners in methanogenic systems [77,78]. However, most of these investigations have focused exclusively on single-stage AD configurations; for example, Yin et al. conducted combined electrochemical and metagenomic analyses of the microbial community in the presence of ferrous oxide during single-stage methanogenesis. In contrast, in two-stage H_2 and CH_4 co-generation systems, the feedstock, conductive materials (CMs), and a fraction of the microbial population entering the second-stage AD are inherited directly from the effluent of the first-stage fermentation, a coupled configuration that remains comparatively underexplored from a mechanistic electron-transfer perspective [79].

DIET is the dominant electron transfer paradigm in methanogenic AD and cascading DF–AD configurations, where conductive materials promote electron exchange between syntrophic fatty-acid-oxidizing bacteria and methanogenic archaea. Electrochemical analysis confirms that MNPs change the extracellular electron transfer pathway from indirect interspecies H_2 transfer to DIET in the methanogenic stage, responsible for increasing CH_4 production, a mechanistic shift that is entirely absent in the H_2 -producing DF stage where the same MNPs operate through a fundamentally different electron routing mechanism. Because methanogens are H_2 -consumers, their enrichment via DIET is directly counterproductive in H_2 -targeted DF systems. Therefore, while DIET-related findings from methanogenic AD studies corroborate the intrinsic conductivity and electron-mediating properties of Fe_3O_4 , they must not be presented as the operative mechanism of H_2 enhancement in DF. The governing process in DF is EET-mediated through $\text{Fe}^{2+}/\text{Fe}^{3+}$ redox cycling, enhanced PFOR-ferredoxin-[FeFe]-hydrogenase electron flux, NADH/NAD⁺ rebalancing, and surface-facilitated extracellular electron conduction with a mechanistically distinct H_2 -production pathway that supports rather than competes with fermentative H_2 production [80–82]. This distinction is schematically illustrated in Figure 3. In DF bio- H_2 systems, Fe_3O_4 mainly supports H_2 evolution through redox regulation, hydrogenase activation, and fermentative electron redistribution. In methanogenic AD systems, Fe_3O_4 mainly promotes interspecies electron transfer between syntrophic bacteria and methanogenic archaea, ultimately enhancing CH_4 formation.

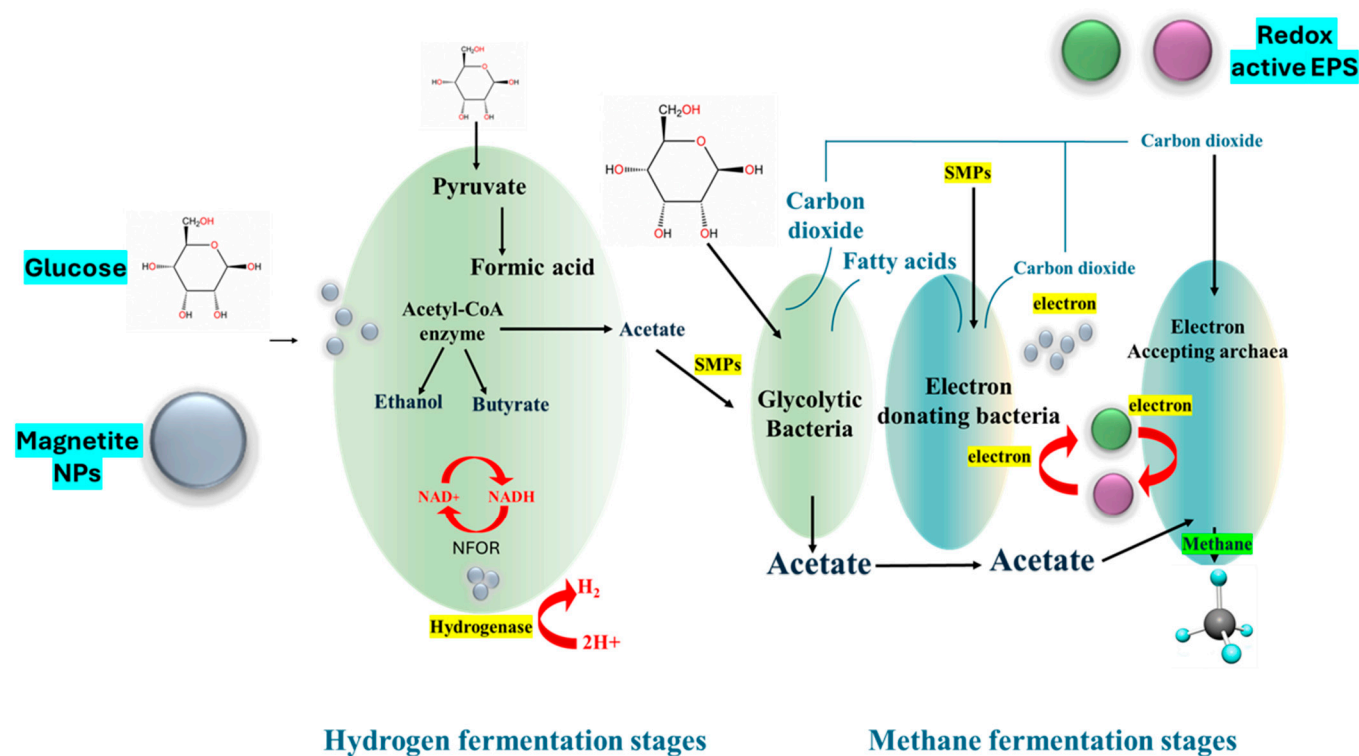


Figure 3. Comparison of Fe_3O_4 -mediated electron-transfer mechanisms in dark fermentative bio- H_2 production and methanogenic anaerobic digestion.

5. Effects of Fe_3O_4 on Process Performance

5.1. Production Rate and Hydrogen Yield

The effect of Fe_3O_4 on H_2 production performance depends strongly on particle size, surface area, dosage, substrate type, inoculum source, and reactor configuration. At appropriate concentrations, Fe_3O_4 NPs can increase H_2 production by improving electron transfer, supplying bioavailable iron for hydrogenase-related enzymes, and promoting favorable metabolic pathways. For example, green-synthesized Fe_3O_4 NPs have been reported to increase cumulative H_2 production from lignocellulosic hydrolysate, with the highest enhancement observed at an optimized NP dosage [63]. Similarly, magnetite addition improved bio- H_2 production from apple pulp waste, with the best performance obtained at 100 mg/L Fe_3O_4 , corresponding to a substantial increase compared with the control [73]. These results indicate that Fe_3O_4 can enhance both H_2 yield and production rate when applied within an appropriate concentration range.

However, the stimulatory effect of Fe_3O_4 is not linear with dosage. Excessive Fe_3O_4 loading can reduce performance because of NP aggregation, mass-transfer limitation, oxidative stress, or metabolic imbalance. Therefore, the relationship between Fe_3O_4 dosage and H_2 production should be evaluated using both performance indicators, such as H_2 yield and production rate.

5.2. Lag Phase Reduction

The lag phase (λ) in DF represents the initial adaptation period during which H_2 -producing bacteria become metabolically active before measurable H_2 evolution occurs. This parameter is an important kinetic indicator because it reflects the time required for microbial acclimation, enzyme activation, and initiation of fermentative H_2 production. The modified Gompertz model is commonly used to estimate the lag phase:

$$H(t) = P \cdot \exp\{-\exp[(R_{\max} \cdot e / P)(\lambda - t) + 1]\} \quad (8)$$

where λ represents the lag phase duration (h), P denotes the maximum H_2 production potential (mL), and R_{max} corresponds to the maximum H_2 production rate (mL/h). Fe_3O_4 supplementation can shorten the lag phase mainly by accelerating microbial acclimation, improving enzyme activation, and enhancing electron-transfer efficiency rather than by promoting methanogenic DIET. Current research has begun to disclose the biotechnological potential and differential role of iron-based additives on H_2 production kinetics, as well as their influence on functioning microbial communities [83,84]. For example, nZVI assesses a high reduction and chemical activity in the H_2 production process, making the growth of microorganisms more quickly but has no significant impact in the lag phase while Fe_3O_4 can increase the acetate pathway and shorten lag phase [85]. The inclusion of iron oxide NPs significantly reduced the lag phase from 13 h in the control experiment to under 6 h. The shorter lag phase resulted in a higher maximum H_2 production rate compared to the control. Thus, these results have revealed that the NPs have a bigger influence on the kinetic of H_2 synthesis than the total biogas production caused by glucose utilization [86].

Fe_3O_4 or other iron-based nanoparticles have been reported to increase H_2 -producing microorganisms, particularly *Clostridium* spp., while enhancing the expression of the *hydA* gene, which encodes [FeFe]-hydrogenase. This enhancement of hydrogenase activity, together with an improved NADH/NAD⁺ balance, promotes the more efficient utilization of reducing equivalents for proton reduction, thereby enhancing bio- H_2 production. Consequently, Fe_3O_4 -supplemented systems often show a faster onset of H_2 evolution, higher maximum H_2 production rates, and shorter lag phases in kinetic models such as the modified Gompertz model [87]. Similarly, kinetic modeling using the Gompertz and modified Logistic models has shown that Co- Fe_3O_4 NPs can reduce lag phases and increase H_2 production rates. At an optimal dose of 300 mg/L, Co- Fe_3O_4 NPs increased H_2 yield and productivity compared with the control, with acetate- and butyrate-type pathways dominating H_2 evolution. These results suggest that the lag-phase reduction caused by Fe_3O_4 -based additives is mainly associated with faster microbial adaptation, improved redox balance, enhanced hydrogenase activity, and enrichment of H_2 -producing bacteria [88].

5.3. Substrate Utilization

Iron-based NPs, especially Fe_3O_4 , improve metalloenzyme activity and feedstock utilization, leading to increased H_2 production during DF. Fe_3O_4 generally improves substrate conversion efficiency, although just a few studies have reported quantifiable substrate usage measurements. Most studies, however, have consistently found increased complete sugar and VFA intake in Fe_3O_4 -supplemented systems. Fe_3O_4 promotes microbial metabolism by gradually releasing bio-available Fe^{2+} and Fe^{3+} ions, which stimulate important enzymes involved in bio- H_2 generation. Iron ions serve as cofactors for hydrogenase and other redox-active metalloenzymes, increasing electron transfer and enhancing fermentative activity. Nonetheless, high concentrations of iron-based NPs may have an inhibitory effect due to aggregation, oxidative stress, or metabolic imbalance, lowering microbial activity and H_2 production efficiency [12]. The feasibility and efficiency of Fe_3O_4 NPs for improving biohydrogen production from glucose anaerobic DF were studied in an EGSB reactor. A maximum hydrogen production of 12.97 mL H_2 /g-VSS was obtained with 50 mg/L Fe_3O_4 NPs (40–60 nm). Furthermore, the optimum dosage resulted in H_2 generation rate of 4.95 L H_2 /d, 53.73% higher than the control (3.22 L H_2 /d). Fe_3O_4 NPs improved coenzyme activity and electron transfer efficiency, whereas Fe^{2+} by-products increased soluble microbial products and promoted ethanol-producing bacteria growth [40].

5.4. VFA Distribution

Microorganisms produce a variety of metabolic byproducts during DF, including VFAs, polyhydroxyalkanoates (PHAs), and bioalcohols. Excessive accumulation of these metabolites might limit microbial metabolic activity, resulting in lower biogas and bio- H_2 generation efficiency [89,90]. Among these metabolites, VFAs are regarded as essential intermediates in anaerobic fermentation and are frequently formed during the acidogenesis of wastewater substrates. Acetic, propionic, butyric, and valeric acids are the most common VFAs produced during anaerobic treatment of dairy effluent [91]. Previous research found that VFA generation increased when HRT decreased and organic loading rate rose. In addition, HRT had a substantial impact on the makeup of acidogenic microbial consortia, as well as the distribution and concentration of VFAs produced during fermentation. The effects of iron-based nanoparticles on VFA generation have also been widely studied. Jin et al. [92] found that excessive addition of nanoscale zero-valent iron (nZVI) (>10 g/L) hindered VFA synthesis during anaerobic fermentation. This inhibition was linked to an increased intracellular demand for iron uptake and transport. Previous research indicated that bacterial uptake of iron-containing substances needs ATP-binding cassette (ABC) transporters, which utilize ATP and reduce equivalents such as NADH during the membrane transport process [58]. Zhang et al. [93] studied how vegetable characteristics affected acid generation during anaerobic fermentation. The results revealed that acetic acid and propionic acid were the primary products of acid generation via anaerobic fermentation of potato peels. However, unlike the results of this investigation, the concentration of propionic acid at the end of the experiment was significantly higher. This is most likely because the metabolic pathway of acid generation was altered by the addition of Fe_3O_4 , which transforms major products into acetic and butyric acid [94]. Overall, Fe_3O_4 NPs enhance DF performance through multiple mechanisms, including H_2 production rate, substrate utilization, and metabolic regulation. The key effects of Fe_3O_4 on process performance are summarized in Table 2.

Table 2. Summary of the effects of Fe_3O_4 nanoparticles on DF process performance.

Process parameter	Effect of Fe_3O_4	Mechanism/Outcome
H_2 production rate	Increased at optimum Fe_3O_4 dosage	Fe_3O_4 promotes extracellular electron transfer (EET), provides bioavailable Fe^{2+}/Fe^{3+} for hydrogenase activation, and enhances electron transport efficiency, leading to increased H_2 production rates and cumulative H_2 production.
H_2 yield	Increased under optimum NP concentration	Enhanced redox balance increased reducing equivalent availability (NADH), and activation of [FeFe]-hydrogenase promotes effective substrate-to- H_2 conversion, resulting in higher H_2 production.
Lag phase	Shortened	Improved microbial acclimation, enhanced hydrogenase activity, and enrichment of H_2 -producing bacteria accelerate the onset of fermentation and increase the maximum H_2 production rate.
Substrate utilization	Improved	Fe_3O_4 promotes metalloenzyme activity and microbial metabolism, enhancing glucose/COD utilization, substrate conversion efficiency, and fermentative performance.
VFA distribution	Shift toward acetate-butyrate pathway	Fe_3O_4 transfers metabolic flux from reduced by-products (e.g., propionate and ethanol) toward acetate- and butyrate-type fermentation, enhancing electron recovery for H_2 generation and improving process efficiency.
High Fe_3O_4 dosage	Inhibitory	Excessive Fe_3O_4 leads to nanoparticle aggregation, oxidative stress, and mass-transfer limits, interrupting microbial metabolism and reducing H_2 generation and substrate consumption.

6. Interaction with Other Additives/Hybrid Systems

6.1. Magnetite Coupled with Carbon-Based Conductive Materials (Biochar, Activated Carbon)

Fe₃O₄ combined with carbon-based conductive materials such as biochar, activated carbon, and graphene-based materials has emerged as an effective hybrid strategy for improving dark fermentative bio-H₂ production. Carbonaceous materials provide porous structures, large specific surface areas, abundant surface functional groups, and favorable sites for microbial attachment and biofilm formation [95,96]. These properties can improve biomass retention, substrate accessibility, and intercellular electron exchange within fermentative biofilms, thereby enhancing process stability and H₂ production performance [97].

Biochar and activated carbon are particularly attractive because of their low cost, high porosity, redox-active surface moieties, and scalability. When combined with Fe₃O₄, these materials can form a conductive hybrid network that improves extracellular electron transfer and electron redistribution within H₂-producing fermentative consortia. In this context, the role of Fe₃O₄–carbon hybrids in DF should be described as conductive electron mediation and biofilm support rather than methanogenic syntrophic interaction. Such hybrid systems can reduce electron-transfer resistance, facilitate hydrogenase-associated redox reactions, promote microbial attachment and biofilm formation, and create favorable microenvironments that enhance the activity of H₂-producing bacteria, ultimately improving bio-H₂ production performance [43].

Several studies have demonstrated the effectiveness of Fe₃O₄–carbon hybrid materials in DF systems. Fe₃O₄ NPs embedded in granular activated carbon derived from coconut shells increased the hydrogen productivity rate by 65.49% compared with non-magnetite granular activated carbon in thermophilic DF, indicating that magnetite-loaded carbon carriers can promote microbial activity and electron transfer [98]. Fe₃O₄/date seed activated carbon nanocomposites also enhanced H₂ production from date fruit waste using *Enterobacter aerogenes*, achieving a maximum H₂ yield of 238.7 mL/g. In this system, activated carbon likely acted as both an adsorbent and buffering support, improving medium stability and strengthening the stimulatory effect of Fe₃O₄ NPs [42]. In addition, Fe₃O₄-rGO nanocomposites showed better performance than bare Fe₃O₄ NPs, producing 225.60 mL H₂/g glucose compared with 198.30 mL H₂/g glucose for Fe₃O₄ alone. This improvement was attributed to the high conductivity and large surface area of rGO, which facilitated electron transfer and enhanced hydrogenase activity.

6.2. Fe₃O₄ Integration with Other Metal Nps for H₂ Enhancement

Supplementing the fermentation medium with these materials allows for a greater spectrum of biochemical processes and enzyme activity, resulting in a higher overall H₂ output. The new focus on synthesizing bimetallic NPs, such as iron-transition metal nanocomposites, provides a viable strategy that may result in improved physicochemical qualities and lower microbial stress when compared to co-addition of individual NPs. However, despite the potential advantages of these materials, studies specifically examining the synergetic effects of iron and other transition metals on H₂ remain sparse [99,100]. A deeper understanding of these interactions is crucial for advancing the application of NPs in efficient fermentative H₂ production.

A recent study found that hydrothermally produced Co-Fe₃O₄ NPs with a cobalt-to-iron ratio of 15:85 outperformed pure Fe₃O₄ NPs. At an ideal dose of 300 mg/L, Co-Fe₃O₄ NPs boost H₂ yield by 41.78% and productivity by 46.13% above the control [88]. To overcome these hurdles, emerging studies propose the synergistic coupling of photocatalysis with nanomaterials. Nano-sized photosensitizing materials, such as N-TiO₂ and N-Fe₃O₄, have shown encouraging results when combined with phototrophic and DF bacteria

[101,102]. In contrast, the $\text{Fe}_3\text{O}_4 + \text{NiO}$ mixture yielded a CHP of 261.67 ± 2.65 mL and a H_2 yield of 1.39 ± 0.26 mol H_2 /mol glucose, representing a 75.95% increase in the H_2 yield over that of the control [43]. The addition of TiO_2 and Fe_3O_4 NPs in a hybrid dark/photo fermentation reactor enhanced H_2 yield 2.1 times compared to control. NPs operate as scaffolds for enzyme and substrate binding, increasing the rate of enzyme-catalyzed processes.

6.3. Fe_3O_4 -Assisted Immobilization Effects

Nano-based immobilization approaches show significant interest in bio- H_2 production with the application of nanosized biocatalysts, which stimulates the active sites of microbial enzymes and, in response, boosts H_2 synthesis [103]. A recent study highlights the benefits of employing NPs as catalysts to modify biochemical pathways and improve product quality [104]. Researchers used immobilized *Clostridium beijerinckii* NCIMB8052 to fix bacterial cultures and produce bio- H_2 using a layer-by-layer technique. This involved functionalizing Fe_3O_4 NPs with alginic acid polyelectrolytes and chitosan. Carbon-based nanocomposites, such as the synergistic combination of Fe_3O_4 and Powdered Activated Carbon (PAC), provide another highly scalable smart material. PAC has a very porous matrix that protects bacteria from hazardous inhibitory chemicals (such as high levels of undissociated volatile fatty acids). Co-immobilization of Fe_3O_4 and PAC within protective hydrogel matrices (e.g., Polyvinyl Alcohol-Sodium alginate [PVA-SA]) has recently been shown to not only boost metabolic activity but also create an artificial micro-niche in which glucose is rapidly converted into H_2 , acetic acid, and butyric acid with minimal lag phase [43].

Graphene oxide (GO) and its reduced form (rGO) have high electrical conductivity, a large surface area, and several functional groups (such as hydroxyls and epoxides) that act as ideal anchoring sites for microbial adhesion. The combination of magnetite and rGO (Fe_3O_4 -rGO nanocomposites) results in a continuous, long-range conductive network. When the nanocomposites are disseminated in a DF bioreactor, the rGO sheets behave as macroscopic electron highways. The attached magnetite inhibits the rGO sheets from restacking while also providing iron cofactors. Research reveals that Fe_3O_4 -rGO nanocomposites greatly outperform pristine Fe_3O_4 in intensifying butyrate-type fermentation and modifying H_2 -producing cell shape, especially enriched strains like *Clostridium-sensu-stricto-1* [72].

Rambabu et al. [59] found that adding 150 ppm of Fe_3O_4 NPs increased bio- H_2 output from date fruit waste by 62.2% compared to without NPs. Cell immobilization is another potential strategy to address the difficulties while simultaneously increasing bio- H_2 production. It is a process of entrapping the microalgal cells into or onto a stable support. Using a layer-by-layer technique, Fe_3O_4 NPs were functionalized with chitosan and alginic acid polyelectrolytes to encourage bacterial adhesion. When *Clostridium beijerinckii* was cultivated with these NPs, the lag growth phase was shortened and the overall amount of bio- H_2 produced in 100 mL, 250 mL, and 3.6 L reactors was higher than when the organisms were suspended freely. With an energy conversion efficiency of $10 \pm 3\%$ and a substrate conversion efficiency of $52 \pm 18\%$, the highest H_2 output was 2.1 ± 0.7 mol H_2 /mol glucose [103]. The previous study is compared with the Fe_3O_4 -assisted immobilization systems using a comprehensive Table 3.

Table 3. Fe₃O₄-assisted immobilization systems and their effects on dark fermentative bio-H₂ production.

Immobilization System	Microbial Culture/Inoculum	Feedstock	Condition	Key Findings	Reference
Chitosan + alginic acid layer-by-layer on magnetite NPs	<i>Clostridium beijerinckii</i> NCIMB8052	Glucose	Batch DF reactor; mesophilic (35 ± 1 °C); working volume: 250 mL	Shorter lag phase, Substrate and energy conversion efficiencies of 52 ± 18% and 10 ± 3%, respectively, with H ₂ production comparable to literature values.	[104]
MNPs embedded in coconut shell GAC (Ni: Fe composite)	Thermophilic mixed sludge	Synthetic wastewater	Batch DF reactor; Thermophilic (60 °C); working volume: 200 mL	MGAC increased H ₂ productivity by 65.49%, achieving an optimum HPR of 20.33 ± 0.32 mL H ₂ /L·h.	[98]
PVA + Sodium alginate (Fe ₃ O ₄ +PAC nanocomposite)	Mixed microorganisms (anaerobic sludge)	Glucose	Batch DF reactor; Mesophilic DF (37 °C); working volume: 100 mL	H ₂ production increased by 115.2% compared with the control and without immobilization (Powdered form of Fe ₃ O ₄ +PAC), lag phase was 60.75% shorter than that of the control.	[43]
Alginate (Biochar and Fe ₃ O ₄ NPs)	<i>Clostridium tertium</i> IGP01	Cacao Pod Husk	Batch DF reactor; Mesophilic DF (37 °C); working volume: 100 mL	Bio-H ₂ production increased by >2-fold and 76.4 ± 4.3% over the control, with reduced 5-HMF and enhanced carbonyl, quinone, and hydroxyl functional groups after immobilization.	[105]

7. Future Perspectives

Fe₃O₄ NPs have shown considerable potential for enhancing dark fermentative bio-H₂ production, but several scientific and engineering challenges must be resolved before practical application. First, a deeper mechanistic understanding of Fe₃O₄–microbe interactions is required. Future studies should combine metagenomics, transcriptomics, proteomics, metabolomics, electrochemical analysis, hydrogenase activity assays, NADH/NAD⁺ measurements, and ORP monitoring to clarify how Fe₃O₄ regulates extracellular electron transfer, intracellular redox balance, enzyme activation, and metabolic pathway distribution.

Second, the physicochemical properties of Fe₃O₄ NPs, including particle size, morphology, crystallinity, surface charge, surface functionalization, and dosage, should be systematically optimized. Although nanoscale Fe₃O₄ provides high surface reactivity and strong microbial interaction, excessive NP loading can cause aggregation, toxicity, oxidative stress, or mass-transfer limitation. Therefore, future research should define safe and effective dosage windows under different substrates, inoculum, and reactor configurations.

Third, long-term stability, magnetic recovery, reusability, and nanoparticle fate should be evaluated under continuous and pilot-scale fermentation conditions. Most current studies remain limited to batch-scale experiments, which do not fully represent industrial operating environments. The environmental fate, biosafety, and potential release of Fe₃O₄-based nanomaterials should also be assessed to ensure sustainable application.

Fourth, hybrid systems combining Fe₃O₄ with biochar, activated carbon, graphene derivatives, bimetallic NPs, and polymeric immobilization matrices offer promising opportunities to improve electron transfer, microbial retention, and process robustness. In addition, integration of DF with photo-fermentation, microbial electrolysis cells, or photocatalytic processes may further improve overall H₂ recovery and energy efficiency. Finally, techno-economic analysis and life cycle assessment are needed to evaluate the economic feasibility and environmental sustainability of Fe₃O₄-assisted bio-H₂ production systems. Interdisciplinary research integrating nanotechnology, microbiology, electrochemistry, and reactor engineering will be essential for translating laboratory-scale findings into scalable bio-H₂ production technologies.

8. Conclusions

Dark fermentative bio-H₂ production is a promising technology for sustainable H₂ generation because of its operational simplicity, rapid H₂ evolution, and ability to utilize a wide range of renewable biomass and organic waste substrates. However, practical applications are constrained by low H₂ yields, prolonged lag phases, incomplete substrate utilization, volatile fatty acid accumulation, and inefficient electron utilization.

This review shows that Fe₃O₄ NPs can address these limitations through multiple physicochemical and biological mechanisms. The mixed-valence Fe²⁺/Fe³⁺ structure, electrical conductivity, high surface area, magnetic recoverability, and redox activity of Fe₃O₄ can facilitate extracellular electron transfer, stimulate hydrogenase and dehydrogenase activities, regulate intracellular redox balance, promote microbial attachment, and enrich H₂-producing bacteria. Fe₃O₄ supplementation has been shown to improve H₂ yield and production rate, shorten lag phases, enhance substrate conversion, and shift VFA distribution toward H₂-producing acetate- and butyrate-type pathways.

This review demonstrates that Fe₃O₄ NPs can effectively enhance dark fermentative bio-H₂ production through multiple complementary mechanisms. Their mixed-valence

$\text{Fe}^{2+}/\text{Fe}^{3+}$ structure, electrical conductivity, redox activity, high surface area, and extracellular electron transfer, stimulate hydrogenase and dehydrogenase activities, improve intracellular redox balance, promote microbial attachment, and enrich H_2 -producing microorganisms. As a result, Fe_3O_4 supplementation consistently increases H_2 yield and production rate, shortens lag phases, improves substrate conversion, and shifts fermentation toward acetate- and butyrate-type pathways that favor H_2 production.

The review also highlights that the role of Fe_3O_4 in DF differs fundamentally from its function in methanogenic anaerobic digestion. In DF, Fe_3O_4 primarily promotes H_2 production by regulating electron flow and microbial metabolism, whereas in methanogenic systems conductive materials mainly facilitate direct interspecies electron transfer to methanogens, enhancing methane formation. Furthermore, recent advances involving Fe_3O_4 -based hybrid materials and immobilization strategies demonstrate additional improvements in electron-transfer efficiency, microbial retention, process stability, and nanoparticle recovery.

Despite these promising advances, further work is needed to optimize nanoparticle properties and dosage, verify long-term performance under continuous operation, and evaluate environmental safety and economic feasibility before Fe_3O_4 -assisted dark fermentation can be widely implemented for sustainable biohydrogen production.

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