Nanoparticles – Enhanced Biohydrogen Production: A Comprehensive Review of Mechanisms, Advances and Challenges

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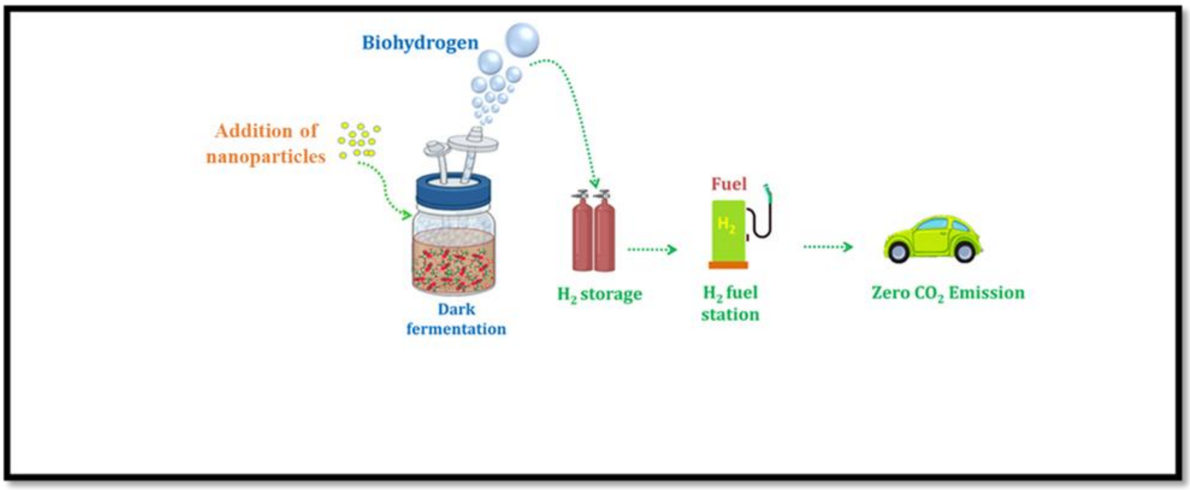
***Abstract*—The rapid increase in global energy demand and the limited sustainability of conventional fossil fuels have accelerated the alteration toward renewable energy sources. Hydrogen is considered a promising alternate energy source due to its high energy yield and ecologically benign nature, producing only water upon combustion. Among several biofuels, biohydrogen is considered as one of the most eco-friendly and efficient options, as it is carbon-neutral and can be synthesized from a wide range of renewable biomass and waste feedstocks. This review highlights recent improvements in dark fermentative biohydrogen production from several biomass sources, with a particular focus on the role of nanomaterial supplementation. Different types of nanoparticles, including titanium dioxide, copper, silver, gold, palladium, iron, nickel, and cobalt nanoparticles, have been examined for their ability to increase hydrogen production efficiency. These nanomaterials improve microbial activity, facilitate electron transfer, and enhance enzymatic functions, thereby significantly increasing hydrogen yield. Furthermore, the review discusses the underlying mechanisms of nanoparticle-assisted enhancement, including microbial community modulation, enzyme activation and improved redox balance. Despite the promising outcomes, challenges related to nanoparticle toxicity, cost and large-scale implementation remain. Future research should concentrate on developing sustainable synthesis techniques, optimizing nanoparticle properties, and evaluating long-term environmental impacts to enable practical applications of this technology.**

***Keywords*—Nanoparticles, Biohydrogen, Biofuels, Fermentation, Enzymes**

Graphical abstract



I. INTRODUCTION

The worldwide energy sector is experiencing a significant transition from conventional fossil fuels namely diesel, coal, petroleum, and natural gas that currently fulfill around 80% of the world's total energy demand, towards renewable and ecologically friendly energy sources (Das & Veziroğlu, 2008; Sohale et al., 2023). This transition is driven by increasing apprehensions regarding the environmental repercussions of fossil fuel usage, which substantially contributes to greenhouse gas release that are accountable for global warming and climate change (Show et al., 2011; Sohale et al., 2023). Alongside carbon dioxide, the combustion of fossil fuels emits several detrimental particle pollutants that deteriorate air quality and adversely affect ecosystems (Li & Fang, 2007). Extensive research and development have been conducted in renewable energy technologies, encompassing sun, wind, tidal, geothermal, and organic biomass (Das & Veziroğlu, 2008). Organic waste biomass is especially promising as it may be transformed into biofuels with high energy content, providing a feasible substitute for fossil fuels in energy generation and as feedstock for chemical industries (Show et al., 2011; Pengadeth et al., 2024).

Biofuels are mostly classified into liquid and gaseous forms, encompassing biobutanol, biodiesel, biomethane, bioethanol, bioalkanes, and biohydrogen (Li & Fang, 2007). These biofuels are regarded as sustainable, ecologically friendly, efficient, and economically viable, as they originate from renewable biological sources and contribute to the minimization of net carbon emissions (Das & Veziroğlu, 2008; Patel et al., 2024). Hydrogen is considerable for its remarkably high calorific value of 141 MJ/kg, the highest of any known commercial fuels (Show et al., 2011). Hydrogen serves as an excellent energy transporter and vector, suitable for direct use as a gaseous fuel in combustion engines or for conversion into hydrocarbon fuels (Das & Veziroğlu, 2008). Moreover, hydrogen can be employed in fuel cell technologies to create energy effectively, yielding only water and heat as byproducts without any detrimental emissions (Show et al., 2011; Patel et al., 2024). This adaptability enables hydrogen to be used into diverse energy systems, ranging from transportation to power generation (Das & Veziroğlu, 2008). The energy density of hydrogen indicates that 1 kilogram of hydrogen possesses energy comparable to approximately 2.75 kg of gasoline, based on its lower heating value of 120 MJ/kg (Show et al., 2011). Owing to these benefits, hydrogen is increasingly acknowledged worldwide as a pivotal fuel of the future, accompanied by escalating investments in its storage, manufacturing, and use technologies (Das & Veziroğlu, 2008; Sohale et al., 2023). The use of hydrogen and other biofuels is important for decreasing reliance on fossil fuels, alleviating environmental degradation, and fulfilling global objectives for sustainable development and clean energy, so facilitating a greener and additional resilient energy future (Li & Fang, 2007; Pengadeth et al., 2024).

Current hydrogen generation primarily uses thermochemical approaches such as steam methane reforming, which, although economically viable, depend on fossil fuels and contribute to greenhouse gas emissions (Das & Veziroğlu, 2008; Sohale et al., 2023). Conversely, biohydrogen production pays a variety of renewable organic materials while generating no greenhouse gas emissions, providing a more ecologically friendly option (Show et al., 2011; Patel et al., 2024). Nonetheless, increasing biohydrogen generation encounters considerable obstacles, such as intricate and expensive pretreatment procedures to extract fermentable sugars from biomass, low yields, and the necessity for augmented microbial efficiency (Li & Fang, 2007; Sohale et al., 2023). Current advancements have investigated molecular biology and genetic engineering to augment hydrogen yields; nevertheless, these approaches are sometimes labor-intensive and costly (Show et al., 2011). Nanotechnology offers a potential and cost-effective alternative by enhancing catalytic efficiency and simplifying processes (Khan et al., 2019; Jannat et al., 2024). Dark fermentation, a biological method in which anaerobic bacteria transform organic substrates into hydrogen without light, is garnering interest for its versatility and capability to utilize diverse waste sources (Show et al., 2011; Pengadeth et al., 2024). Notwithstanding these improvements, industrial hydrogen production predominantly relies on fossil-based methods, while alternative techniques including photocatalysis, water electrolysis, and photo-electrochemical processes are under development but not yet extensively marketed (Das & Veziroğlu, 2008; Sohale et al., 2023). In addition to energy applications, hydrogen is necessary in the chemical industry for the synthesis of methanol, hydrogenation of lipids, aniline, and ammonia production (Li & Fang, 2007). In summary, although biohydrogen production presents a sustainable solution, it is critical to address technological and economic challenges through advancements in biotechnology and nanotechnology to fully harness its abilities as a clean, large-scale hydrogen source (Khan et al., 2019; Patel et al., 2024).

Diverse biomass-to-gas conversion procedures exist for hydrogen production, with selection contingent upon the feedstock type employed (Das & Veziroğlu, 2008). Thermochemical conversion techniques, including gasification, are primarily utilized for fossil fuels but can also directly transform renewable resources such as lignocellulosic biomass, petrochemical feedstocks, and organic-rich sewage sludge into hydrogen (Show et al., 2011; Sohale et al., 2023). In contrast to steam reforming, these thermochemical biomass-to-gas technologies provide faster conversion rates; however, they exhibit worse selectivity and necessitate elevated temperatures, rendering them extra energy-intensive (Li & Fang, 2007). Electrochemical techniques, especially water electrolysis, are extensively employed for hydrogen production; yet, they are often expensive due to significant maintenance requirements stemming from corrosive reaction environments (Das & Veziroğlu, 2008). Conversely, biochemical procedures utilizing microbes and enzymes are more efficient for feedstocks characterized by elevated water content and reduced crystalline cellulose, providing improved selectivity for hydrogen production (Show et al., 2011; Pengadeth et al., 2024). Nonetheless, biological hydrogen production typically experiences reduced productivity and extended reaction durations (Li & Fang, 2007; Sohale et al., 2023). Lignocellulosic biomass has developed as a viable substrate for large-scale biohydrogen production; however, challenges persist, such as incomplete conversion of cellulose to fermentable sugars, restricted accessibility to cellulose fibrils, and the high costs and efficiency constraints of cellulolytic enzymes (Show et al., 2011; Srivastava et al., 2019). Given that cellulolytic enzymes are primary sources for decomposing biomass to liberate sugars for fermentation, augmenting enzyme synthesis and activity is imperative for surmounting these obstacles and enhancing the overall efficacy of biohydrogen generation systems (Li & Fang, 2007).

Biohydrogen generation has experienced notable enhancements due to developments in enzyme synthesis, yield, efficiency, and reaction kinetics, with nanotechnology serving a pivotal role in these biochemical applications (Khan et al., 2019; Ahmad et al., 2023). Nanomaterials, characterized by exceptional features such as elevated electrical conductivity, extensive surface area, and a high surface-to-volume ratio, can significantly enhance biohydrogen production in diverse biological processes, including bio-photolysis, dark fermentation, and photo-fermentation (Jannat et al., 2024). Metallic nanoparticles such as iron and nickel augment output by functioning as enzyme cofactors (Liu et al., 2012; Leroy-Freitas et al., 2024). Moreover, nanomaterials can enhance the enzymatic environment by minimizing oxygen infiltration and adjusting the oxidation-reduction potential, thereby promoting the anaerobic conditions vital for hydrogenase enzyme function, which directly results in elevated biohydrogen production (Qu et al., 2013; García-Depraect et al., 2025). Moreover, nanoparticles are demonstrating efficacy in improving the pretreatment of lignocellulosic biomass by enabling more efficient lignin extraction, thus expediting the process (Wang & Wang, 2019). They enhance cellulose hydrolysis and sugar production by augmenting cellulase enzyme activity, modifying pH stability, and regulating temperature efficacy (Singh et al., 2018). This review seeks to elucidate the different nanoparticles utilized for improved biohydrogen synthesis from distinct source materials, while also examining their fundamental mechanisms of action (Khan et al., 2019; Sohale et al., 2023).

II. METHODS OF BIOHYDROGEN PRODUCTION

Biochemical hydrogen synthesis is rapidly esteemed for its environmental beneficial and capacity to fulfill energy requirements by converting biomass into hydrogen through diverse microorganisms and feedstocks (Das & Veziroğlu, 2008; Show et al., 2011). Principal approaches encompass dark fermentation, photofermentation, photolysis, and microbial electrolysis cells (MECs) (Show et al., 2011; Logan et al., 2008). Lignocellulosic biomass, which is plentiful, renewable, and rich in carbohydrates, serves as a promising substrate; however, it necessitates pretreatment with alkalis, acids, or enzymes to decompose lignin and liberate fermentable sugars (Li & Fang, 2007; Kumar et al., 2020). Dark fermentation utilizes anaerobic bacteria to produce hydrogen from organic waste, whereas photofermentation employs photosynthetic bacteria to convert organic acids into hydrogen, frequently enhancing yields when integrated (Show et al., 2011; Basak & Das, 2007). MECs embody a novel technique in which microbes decompose organic matter, producing protons and electrons that traverse a proton exchange membrane and an external circuit to the cathode, where hydrogen gas is synthesized with a catalyst (Logan et al., 2008; Kadier et al., 2016). This method can employ wastewater, facilitating concurrent hydrogen production and environmental remediation through the synthesis of value-added compounds such as methane and formic acid (Kadier et al., 2016). Despite the great selectivity and sustainability of biochemical approaches, problems persist, including prolonged reaction times, limited productivity, and the necessity for effective pretreatment (Show et al., 2011). Biochemical hydrogen production, particularly using lignocellulosic biomass and modern technologies such as MECs, presents considerable possible as a clean, renewable energy source that facilitates energy generation and environmental conservation (Das & Veziroğlu, 2008; Kumar et al., 2020).

Photofermentation is an alternative method of biohydrogen synthesis, facilitated by photosynthetic bacteria that employ the nitrogenase enzyme in the presence of light (Basak & Das, 2007). Purple non-sulfur bacteria holding the nitrogenase enzyme system enable the photofermentation process (Show et al., 2011). These bacteria can employ reduced organic acids as their primary carbon source and sunlight to produce molecular hydrogen through the nitrogenase enzyme system (Basak & Das, 2007). The integration of light-harvesting pigments like as chlorophylls, phycobilins, and carotenoids facilitates the capture of solar energy, resulting in the dissociation of water into protons, electrons, and oxygen during photofermentation (Show et al., 2011). The catalytic role of nitrogenase enables the reaction of protons and electrons with nitrogen and adenosine triphosphate (ATP) to produce ammonia, hydrogen, adenosine diphosphate (ADP), and inorganic phosphates (Pi) (Basak & Das, 2007). Light energy and biomass, facilitated by the bacterial photosystem, yield two electrons and four ATP molecules, thereby producing hydrogen through the nitrogenase enzyme system (Show et al., 2011).

2.1 Dark Fermentative Biohydrogen Production

Dark fermentation is a biological process that engages anaerobic or facultative anaerobic microbes to produce hydrogen from organic waste materials in the absence of light, typically operating at temperatures between 30 and 80 °C (Show et al., 2011; Li & Fang, 2007). During this procedure, fermentative microbes metabolize diverse substrates such as municipal waste, lignocellulosic biomass, and algae into pyruvate, while hydrogen is produced through the acidogenic phase of glycolysis (Hallenbeck & Ghosh, 2009). The process is widely preferred due to its high hydrogen production rate, broad substrate versatility, and low energy requirements (Show et al., 2011). However, the accumulation of acidic metabolites like ethanol and lactic acid often reduces substrate utilization efficacy and overall hydrogen yield, which remains below the theoretical maximum of 4 moles of hydrogen per mole of glucose (Hallenbeck & Ghosh, 2009). Various fermentative bacteria, including Thermoanaerobacterium, Lactobacillus, Rhodopseudomonas, Clostridium, Enterobacter, and Citrobacter, have demonstrated the capability to convert organic waste into biohydrogen (Li & Fang, 2007; Show et al., 2011). Hydrogen production in dark fermentation is catalyzed by hydrogenase enzymes, which are metal-binding proteins critical to the process; improving the activity of these enzymes can enhance hydrogen yields (Liu et al., 2012). Mechanistically, anaerobic bacteria convert pyruvate into acetyl-CoA via ferredoxin reduction, simultaneously synthesizing ATP and acetate, which are key to cellular energy and metabolic balance during biohydrogen production (Hallenbeck & Ghosh, 2009).

2.2 Principles of Dark Fermentative Biohydrogen Production

Biohydrogen production via dark fermentation is primarily facilitated by strictly anaerobic or facultative anaerobic bacteria in oxygen-free environments, utilizing a various organic compound including sugars, proteins, carbohydrates, and lipids, though the biotransformation of glucose to acetate is commonly used as the standard for estimating theoretical hydrogen yields (Show et al., 2011; Hallenbeck & Ghosh, 2009). The maximum theoretical hydrogen yield from glucose fermentation is 4 moles of H_2 per mole of glucose consumed, achievable via two phases involving the fermentation of glucose to acetate and formate (Hallenbeck & Ghosh, 2009). However, if butyrate is the exclusive fermentation product as a substitute of acetate, the maximum theoretical hydrogen yield reduces to 2 moles of hydrogen per mole of glucose (Hallenbeck & Ghosh, 2009).

The fundamental step in these approaches is the conversion of glucose to pyruvate, typically through the Embden-Meyerhof-Parnas (EMP) metabolic pathway, during which 2 moles of hydrogen can theoretically be produced via the regeneration of NADH ($NADH + H^+ \leftrightarrow NAD^+ + H_2$) (Brindha et al., 2023). Pyruvate is next converted to acetyl-CoA, a critical intermediate whose subsequent metabolic fate determines whether the theoretical hydrogen yield reaches 4 or 2 moles per mole of glucose (Hallenbeck & Ghosh, 2009). Acetyl-CoA formation happens either via pyruvate:ferredoxin oxidoreductase, an enzyme that utilizes ferredoxin as an electron acceptor, or alternative enzymatic routes depending on the bacterium (Brindha et al., 2023). This enzyme is found in various strictly anaerobic and facultative anaerobic bacteria, as well as cyanobacteria (Hallenbeck & Ghosh, 2009).

The re-oxidation of reduced ferredoxin by hydrogenase enzymes produces hydrogen, with one mole of hydrogen produced per mole of ferredoxin oxidized (Liu et al., 2012). If acetyl-CoA is converted to acetate, an additional mole of hydrogen is synthesized from NADH oxidation, resulting in a total of 4 moles of hydrogen per mole of glucose (Hallenbeck & Ghosh, 2009). Conversely, if butyrate is the product, NADH is consumed in its synthesis, restricting hydrogen production to 2 moles per mole of glucose (Brindha et al., 2023). Under specific culture conditions and depending on the microbial species, acetate and butyrate are often produced simultaneously, yielding hydrogen amounts between 2 and 4 moles per mole of glucose (Show et al., 2011). Such mixed fermentation pathways are characteristic of several Clostridium species, including Clostridium butyricum and Clostridium pasteurianum (Li & Fang, 2007). This detailed understanding of metabolic pathways and enzyme functions is important for optimizing biohydrogen production through dark fermentation (Hallenbeck & Ghosh, 2009).

$$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2 \quad \Delta G^O = -206.3\ kJ\ mol^{-1}$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COO^- + 2HCOO^- + 4H^+ + 2H_2 \quad \Delta G^O = -209.1\ kJ\ mol^{-1}$$

$$2HCOOH \rightarrow 2CO_2 + 2H_2 \Delta G^O = -6\ kJ\ mol^{-1}$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COO^- + 2HCO_3^- + 3H^+ + 2H_2 \quad \Delta G^O = -254.8\ kJ\ mol^{-1}$$

$$C_6H_{12}O_6 + 2NAD^+ \rightarrow 2CH_3COCOO^- + 4H^+ + 2NADH\ \Delta G^O = -112.1\ kJ\ mol^{-1}$$

$$pyruvate + CoA + Fd_{ox} \leftrightarrow acetylCoA + CO_2 + Fd_{red}\ \Delta G^O = -19.2\ kJ\ mol^{-1}$$

$$pyruvate + CoA \leftrightarrow acetylCoA + formate \quad \Delta G^O = -19.2\ kJ\ mol^{-1}$$

The alternative route for acetyl-CoA synthesis, enabled by the enzyme pyruvate-formate lyase (PFL), results in the concurrent development of formate and is typical of enterobacteria like Enterobacter aerogenes and Escherichia coli in anaerobic environments (Sanghvi et al., 2024; Teke et al., 2023). The activity of PFL in these bacteria is stringently regulated at the transcriptional level in response to ambient oxygen levels (Rama et al., 2023). In addition to enterobacteria, certain clostridial species also exhibit PFL (Sanghvi et al., 2024). In this route, pyruvate is cleaved into acetyl-CoA and formate, thereby commencing mixed-acid fermentation (Teke et al., 2023).

Glucose fermentation can yield extra metabolites such as succinate, lactate, propionate, and 2,3-butanediol directly from pyruvate, along with ethanol, butanol, and isopropanol derived via acetyl-CoA metabolism (Rama et al., 2023). These byproducts do not produce hydrogen and are hence deemed undesirable during dark fermentation, as their presence diminishes the overall hydrogen output (Sanghvi et al., 2024). Such compounds frequently emerge in mixed cultures or when bacteria have diverse metabolic pathways (Teke et al., 2023). The distribution of fermentation products is mainly influenced by growth conditions; for instance, in E. coli, acidic pH levels below 7 improve lactate synthesis, hence reducing hydrogen yields (Rama et al., 2023). Consequently, enhancing cultural conditions is necessary to direct metabolism towards improving hydrogen synthesis and promoting hydrogen-producing microbes, particularly in mixed cultures (Sanghvi et al., 2024).

III. NANOTECHNOLOGY ON DARK FERMENTATION

Biohydrogen production can attain high yields and low costs by integrating appropriate nanoparticles (NPs) into various processes (Fig.1), with their use in dark fermentation representing an innovative and economical approach (Ihsanullah et al., 2024).

Nanoparticles enhance the ability of microorganisms to synthesis hydrogen by increasing microbial populations and boosting production rates (Sanghvi et al., 2024). Among the most effective are inorganic nanoparticles consist of metals and metal oxides such as silver (Ag), copper (Cu), nickel (Ni), titanium (Ti), palladium (Pd), cobalt (Co), gold (Au), and iron (Fe) (Ihsanullah et al., 2024).

For example, carbon nanoparticles combined with Fe_2O_3 have recently been shown to produce 218.63 mL of hydrogen per gram of glucose during anaerobic fermentation (Fuel, 2023; Ihsanullah et al., 2024). Dark fermentation (DF) itself occurs under anaerobic, light-independent conditions and involves anaerobic bacteria converting organic biomass—such as starch—into pyruvate through glycolysis, which is then transformed into biohydrogen via the acidogenic pathway (Sanghvi et al., 2024). Although DF produces hydrogen quicker than many other methods, enhancing hydrogen concentration can inhibit further hydrogen formation (Teke et al., 2023). Economically viable substrates for DF-based biohydrogen production include food waste, algal biomass, agricultural residues, and municipal solid waste (Rama et al., 2023). Despite its advantages, the main limitation of DF is its comparatively low hydrogen yield, a challenge that can be addressed effectively through the application of nanomaterials to improve microbial activity and process efficiency (Ihsanullah et al., 2024).

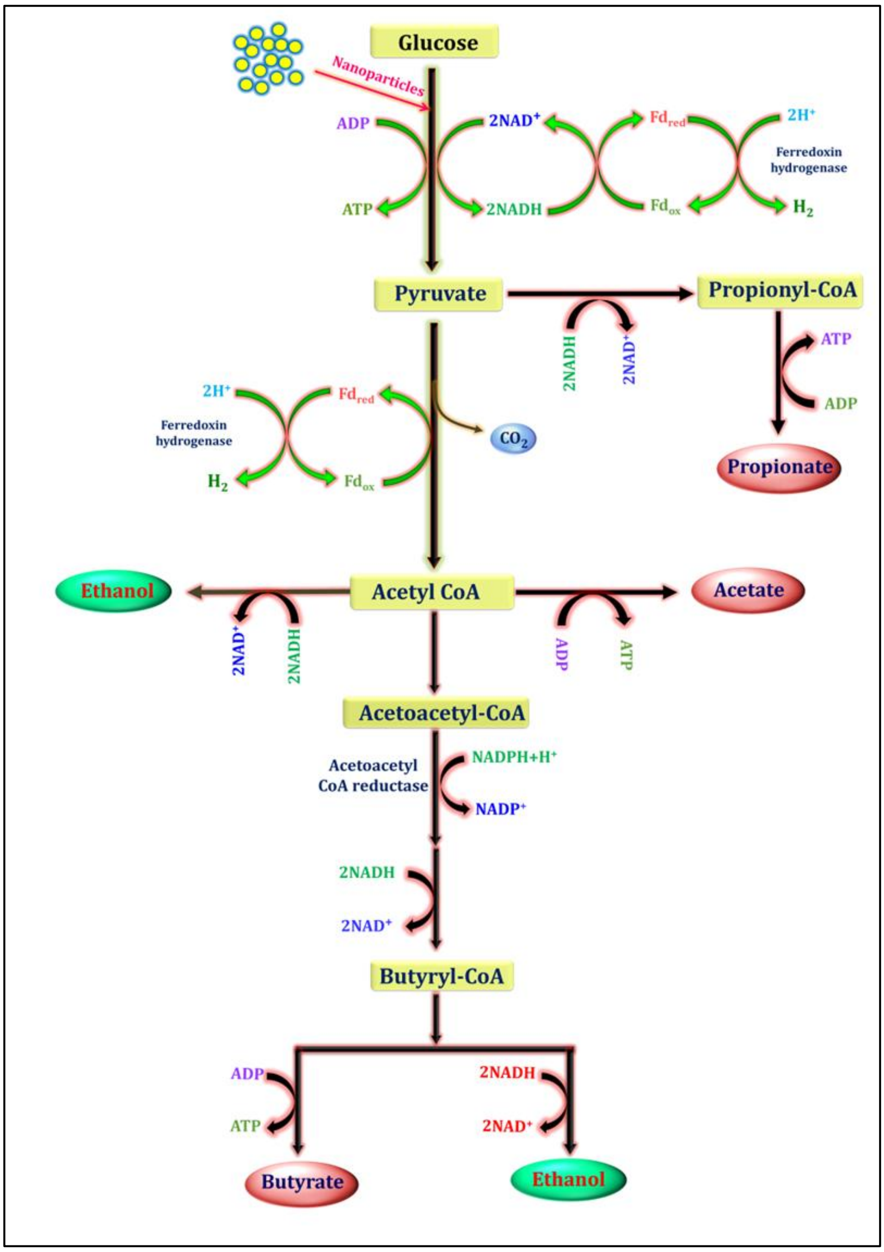


Figure 1. Nano supplemented dark fermentative biohydrogen and byproducts production

3.1 Effect of Nanoparticles in a Dark Fermentative Process

The use of nanoparticles (NPs) has proliferated across various domains, including biofuel production, protein immobilization, and biosensor innovation (Ihsanullah et al., 2024). In biohydrogen generation, nanoparticles augment microbial metabolic activity by promoting effective electron transfer, hence enhancing hydrogen yields even in adverse conditions (Sanghvi et al., 2024). Diverse metal and metal oxide nanoparticles including copper (Cu), silver (Ag), gold (Au), palladium (Pd), iron and iron oxides (Fe-Fe oxide), silica, nickel and nickel oxides (Ni-Ni oxide), titanium dioxide (TiO_2), carbon nanotubes (CNTs), and various nanocomposites have exhibited substantial beneficial impacts on biohydrogen production (Ihsanullah et al., 2024). These nanoparticles improve the effective surface area and demonstrate quantum effects that augment catalytic activity (Fuel, 2023). A crucial enzyme in this procedure, hydrogenase, facilitates the reversible transformation between hydrogen and protons, mitigating the inhibition induced by hydrogen oxidation (Sanghvi et al., 2024). The next sections observe the application of several nanoparticles in hydrogen production and their advantageous effects on improving biohydrogen yields.

3.2 Metal Nanoparticles (Fe, Ni, Co, Cu, Ag)

Metal nanoparticles (MNPs) have emerged as powerful enhancers in dark fermentative biohydrogen production due to their redox activity, exceptional catalytic efficiency and interaction with microbial metabolic systems (Khan et al., 2019; Qu et al., 2013). Among them, nickel (Ni) and iron (Fe) nanoparticles are specifically important because they serve as necessary cofactors for hydrogenase enzymes responsible for hydrogen evolution (Liu et al., 2012). Iron nanoparticles enhance ferredoxin-mediated electron transfer pathways, thereby improving hydrogen yield (Qu et al., 2013). Similarly, nickel nanoparticles contribute to the functional and structural integrity of hydrogenase enzymes, helping enzymatic activity and stability (Liu et al., 2012). Cobalt (Co) nanoparticles impact hydrogen synthesis indirectly by participating in vitamin B_{12}-dependent metabolic pathways, regulating carbon flux in anaerobic microorganisms (Show et al., 2011). Copper (Cu) nanoparticles act as micronutrients at low concentrations, activating microbial growth, but may induce oxidative stress at higher levels (Khan et al., 2019). Silver (Ag) nanoparticles selectively avoid hydrogen-consuming microorganisms such as methanogens, thereby improving net hydrogen production (Qu et al., 2013). The effectiveness of MNPs depends on surface properties, particle size and concentration. Smaller nanoparticles offer higher surface area but may increase toxicity risks (Khan et al., 2019). Additionally, MNPs impact metabolic pathways by altering $NADH/NAD^+$ balance and redirecting carbon flow toward hydrogen-producing pathways (Show et al., 2011). Despite their advantages, nanoparticle accumulation and toxicity remain concerns, requiring additional investigation into safe and optimized applications (Khan et al., 2019).

3.3 Metal Oxide Nanoparticles (Fe_3O_4, TiO_2, ZnO, CeO_2)

Metal oxide nanoparticles (MONPs) have attracted important attention due to their catalytic properties, stability, and tunable surface characteristics (Wang & Wang, 2019). Iron oxide (Fe_3O_4) nanoparticles are widely studied for their magnetic properties, enabling easy recovery and reuse via magnetic separation (Qu et al., 2013). These nanoparticles increase improving microbial respiration, extracellular electron transfer and hydrogen production efficacy (Zhang & Angelidaki, 2015). Titanium dioxide (TiO_2) nanoparticles improve microbial adhesion and biofilm formation, contributing to stable fermentation processes (Wang & Wang, 2019). Under anaerobic conditions, TiO_2 acts as an electron mediator, facilitating redox reactions (Qu et al., 2013). Zinc oxide (ZnO) nanoparticles display antimicrobial properties that help regulate microbial populations (Khan et al., 2019). Cerium oxide (CeO_2) nanoparticles possess antioxidant properties due to their reversible Ce^{3+}/Ce^{4+} redox cycling, which helps mitigate oxidative stress in microbial cells (Khan et al., 2019). MONPs also influence fermentation pathways, promoting butyrate and acetate production allied with higher hydrogen yields (Show et al., 2011). However, issues such as nanoparticle aggregation, environmental risks, and toxicity require careful evaluation (Wang & Wang, 2019).

3.4 Carbon-Based Nanomaterials (Graphene, CNTs, Biochar Nanoparticles)

Carbon-based nanomaterials (CBNs) such as graphene, carbon nanotubes (CNTs), and biochar nanoparticles have attracted important attention due to their biocompatibility and high electrical conductivity (Lovley, 2017). These materials facilitate direct interspecies electron transfer (DIET), which enhances microbial interactions and improve hydrogen production efficiency (Lovley, 2017; Zhang & Angelidaki, 2015). Graphene acts as an effective electron shuttle, promoting rapid electron transfer between microbial cells (Zhang & Angelidaki, 2015). Carbon nanotubes offer a conductive network that enhances microbial adhesion and biofilm formation, improving reactor stability (Khan et al., 2019). These materials also support the development of electroactive bacteria necessary for hydrogen production (Lovley, 2017). Biochar nanoparticles, derived from biomass, offer a supportable alternative by providing porous structures for microbial colonization and acting as electron mediators (Wang & Wang, 2019). They also adsorb inhibitory compounds such as volatile fatty acids (VFA), reducing process inhibition (Show et al., 2011). Additionally, CBNs contribute to buffering ability and microbial community structuring, improving system performance (Zhang & Angelidaki, 2015).

3.5 Magnetic Nanoparticles and Their Functional Roles

Magnetic nanoparticles (MNPs), specifically iron-based nanoparticles, provide multifunctional benefits in dark fermentation systems (Qu et al., 2013). Their magnetic properties permit easy recovery and reuse, making them cost-effective for continuous processes (Wang & Wang, 2019). These nanoparticles enhance microbial immobilization by providing surfaces for biofilm formation, improving reactor stability (Show et al., 2011). MNPs facilitate effective electron transfer between enzymes and microbial cells, thereby enhancing hydrogen production (Zhang & Angelidaki, 2015). They also improve substrate consumption and decrease lag phases in fermentation processes (Qu et al., 2013). Innovative reactor systems, such as magnetically stabilized beds, benefit from the integration of these nanoparticles (Wang & Wang, 2019). Surface functionalization of magnetic nanoparticles can further enhance their interaction with microbial systems and improve catalytic efficiency (Khan et al., 2019). However, challenges such as synthesis cost, potential toxicity, and aggregation must be mentioned for large-scale applications (Khan et al., 2019).

3.6 Biogenic and Green-Synthesized Nanoparticles

Biogenic nanoparticles produced using biological routes offer a sustainable alternative to conventional chemical synthesis approaches (Singh et al., 2018). These nanoparticles are formed using waste biomass, plant extracts or microorganisms, reducing the use of toxic chemicals and energy-intensive processes (Singh et al., 2018). Biogenic nanoparticles possess distinctive surface properties due to biological capping agents, which increase their interaction with microbial systems (Khan et al., 2019). These natural coatings enhance stability and biocompatibility, leading to better microbial activity and hydrogen production (Singh et al., 2018). Green-synthesized nanoparticles exhibit lesser toxicity compared to chemically synthesized nanoparticles, making them suitable for biological applications (Khan et al., 2019). They enhance microbial growth, enzymatic activity, and electron transfer under anaerobic conditions (Show et al., 2011). Additionally, the use of waste biomass contributes to sustainability and circular bioeconomy principles (Singh et al., 2018). However, challenges such as scalability, reproducibility, and variability in synthesis need to be addressed (Singh et al., 2018). Standardization and large-scale validation are necessary for their practical application in biohydrogen production systems.

IV. MECHANISMS OF NANOPARTICLE-ENHANCED BIOHYDROGEN PRODUCTION

Nanoparticles increase biohydrogen production through multiple interconnected mechanisms involving microbial physiology, enzymatic activity, and metabolic regulation (Khan et al., 2019; Zhang & Angelidaki, 2015). Their high surface area and unique physicochemical properties allow them to interact with both intracellular and extracellular components of microbial systems (Qu et al., 2013). These interactions impact key biochemical pathways, including enzyme activation, substrate metabolism, and electron transfer (Show et al., 2011). Nanoparticles can act as micronutrient providers, catalysts, or electron mediators, depending on their composition and structure (Wang & Wang, 2019). The effectiveness of nanoparticles depends on factors such as concentration, surface functionalization, size, and shape (Khan et al., 2019). Smaller nanoparticles exhibit higher reactivity but may also create toxicity risks if not properly controlled. Additionally, nanoparticles can modify microbial community dynamics, support hydrogen-producing species while suppressing competing pathways (Lovley, 2017). Their role extends to mitigating inhibitory situations, such as accumulation of toxic metabolites and oxidative stress, thereby maintaining system constancy (Singh et al., 2018). The combined effect of these mechanisms leads to improved hydrogen yield, production rate, and process efficiency. Understanding these mechanisms is essential for designing optimized nanoparticle-assisted fermentation systems and scaling up biohydrogen production methods (Show et al., 2011).

4.1 Enhancement of Microbial Growth and Cell Viability

Nanoparticles enhance microbial growth by providing necessary trace elements required for metabolic and enzymatic activities (Liu et al., 2012). These elements increase cellular functions such as respiration, energy generation, and biosynthesis. Nanoparticles also rise nutrient bioavailability by facilitating transport across cell membranes (Khan et al., 2019). Enhanced cell viability is observed due to improved protection against environmental stresses, including oxidative damage and pH fluctuations (Singh et al., 2018). Certain nanoparticles, such as zinc and iron-based materials, act as micronutrients that activate microbial proliferation (Liu et al., 2012). Enhanced biomass concentration leads to improved metabolic activity and hydrogen production rates (Show et al., 2011). Additionally, nanoparticles promote biofilm formation and cell aggregation, which progress microbial stability and retention within reactors (Zhang & Angelidaki, 2015). However, excessive nanoparticle concentrations can disrupt cell membranes and create reactive oxygen species, leading to toxicity (Khan et al., 2019). Therefore, optimizing nanoparticle dosage is crucial to maximize growth-promoting effects while minimizing inhibitory effects.

4.2 Improved Electron Transfer and Redox Balance

Efficient electron transfer is a vital factor in biohydrogen production, as hydrogen synthesis depends on the availability of reducing equivalents (Lovley, 2017). Nanoparticles act as electron shuttles, facilitating quick electron transfer between metabolic intermediates and microbial cells (Zhang & Angelidaki, 2015). This better electron flow enhances NADH oxidation and promotes hydrogenase-mediated proton reduction (Qu et al., 2013). Carbon-based nanomaterials and metal nanoparticles are mainly effective in mediating direct interspecies electron transfer (DIET), which enriches syntrophic microbial interactions (Lovley, 2017). Nanoparticles also help maintain intracellular redox balance by regulating NADH/NAD$^+$ ratios, thereby reducing the formation of reduced byproducts such as lactate and ethanol (Show et al., 2011). Improved redox conditions favour pathways that produce hydrogen, such as butyrate and acetate fermentation (Zhang & Angelidaki, 2015). Overall, enhanced electron transfer effectiveness directly correlates with improved hydrogen yield and process performance, making this mechanism a key contributor to nanoparticle-assisted biohydrogen generation.

4.3 Activation of Hydrogenase and Key Metabolic Enzymes

Hydrogenase enzymes play a vital role in biohydrogen generation by catalyzing the conversion of protons into molecular hydrogen (Liu et al., 2012). Nanoparticles enhance hydrogenase activity by supplying important metal cofactors such as iron and nickel in bioavailable forms (Qu et al., 2013). These cofactors are important for the structural integrity and catalytic function of hydrogenase enzymes. Nanoparticles also promote enzyme synthesis by stimulating gene expression related to hydrogen production pathways (Show et al., 2011). In addition, nanoparticles stabilize enzyme structures under stressful conditions, such as unfavorable pH levels or high substrate concentrations (Khan et al., 2019). This stabilization avoids enzyme denaturation and ensures sustained catalytic activity. Nanoparticles can also enhance the activity of other key metabolic enzymes involved in glycolysis and fermentation, thereby improving overall metabolic efficiency (Zhang & Angelidaki, 2015). The increased enzymatic activity leads to maximum hydrogen production rates, making this mechanism fundamental to nanoparticle-mediated enhancement.

4.4 Modulation of Microbial Community Structure

Nanoparticles mainly influence microbial community dynamics in mixed-culture fermentation process (Lovley, 2017). They selectively activate the growth of hydrogen-producing bacteria while suppressing competing microbial species such as methanogens (Qu et al., 2013). This selective pressure increases electron allocation toward hydrogen production rather than methane or solvent formation (Show et al., 2011). Nanoparticles also enhance microbial diversity and promote synergistic interactions among different species (Zhang & Angelidaki, 2015). These interactions increase substrate degradation and metabolic efficiency, leading to improved hydrogen evolution. Additionally, nanoparticles facilitate direct interspecies electron transfer, which supports syntrophic relationships within microbial consortia (Lovley, 2017). However, excessive nanoparticle concentrations may interrupt microbial balance and reduce system performance (Khan et al., 2019). Therefore, careful optimization is required to attain valuable community modulation.

4.5 Mitigation of Inhibitory Metabolites and Stress Responses

The accumulation of inhibitory metabolites such as volatile fatty acids (VFAs) can negatively impact biohydrogen production (Show et al., 2011). Nanoparticles mitigate these properties by adsorbing toxic compounds and reducing their bioavailability (Wang & Wang, 2019). Certain nanoparticles also exhibit antioxidant properties, which help alleviate oxidative stress in microbial cells (Khan et al., 2019). This protection improves cell viability and maintains metabolic activity over extended duration (Singh et al., 2018). Nanoparticles can reduce acid stress and regulate intracellular pH, avoiding metabolic inhibition (Zhang & Angelidaki, 2015). They also increase system resilience by enabling microorganisms to adapt to fluctuating environmental conditions. Overall, mitigation of inhibitory factors enhances process stability, productivity, and long-term operational efficacy in biohydrogen systems.

V. INFLUENCE OF NANOPARTICLES ON PROCESS PERFORMANCE

Nanoparticle supplementation significantly increases key performance indicators in dark fermentation, including production rate, substrate conversion efficiency and hydrogen yield (Qu et al., 2013; Show et al., 2011). These improvements are attributed to enzyme function, boosted microbial activity, and metabolic regulation (Khan et al., 2019). The extent of performance development depends on nanoparticle concentration, type, and operating conditions such as temperature and pH (Wang & Wang, 2019). Systematic evaluation is important to optimize nanoparticle usage and achieve maximum efficacy. Nanoparticles also contribute to better reactor stability and scalability, making them promising tools for industrialized biohydrogen generation (Zhang & Angelidaki, 2015).

5.1 Hydrogen Yield and Production Rate

Nanoparticles significantly enhance hydrogen yield by improving metabolic pathways and enzymatic activity (Qu et al., 2013). Improved hydrogen production rates are observed due to accelerated reaction kinetics and better electron transfer efficiency (Lovley, 2017). Enhanced hydrogenase activity plays a crucial role in boosting hydrogen production (Liu et al., 2012). Nanoparticle-assisted systems often outperform conventional fermentation processes in terms of yield and productivity (Show et al., 2011). However, extra nanoparticle concentrations may lead to diminishing returns or inhibitory effects (Khan et al., 2019). Therefore, optimization of nanoparticle dosage is essential for maximizing performance.

5.2 Substrate Utilization Efficiency

Efficient substrate utilization is crucial for the economic feasibility of biohydrogen generation (Show et al., 2011). Nanoparticles improve hydrolysis and acidogenesis processes, leading to enriched substrate breakdown (Wang & Wang, 2019). Enhanced microbial activity results in maximum substrate conversion rates and reduced residual substrate (Qu et al., 2013). This contributes to better hydrogen yield per unit substrate consumed. Additionally, nanoparticles enable enzyme-substrate interactions, enhancing metabolic efficiency (Khan et al., 2019). Enhanced utilization also reduces generation of waste and increases overall process sustainability.

5.3 Volatile Fatty Acid (VFA) Profile Modulation

Nanoparticles impact the distribution of metabolic end products by shifting metabolic pathways (Zhang & Angelidaki, 2015). They promote pathways that produce acetate and butyrate, which are associated with maximum hydrogen yields (Show et al., 2011). At the same time, nanoparticles suppress unfavourable pathways such as propionate formation, which consumes reducing equivalents

(Lovley, 2017). This optimization of VFA profiles improves hydrogen production efficiency. Understanding VFA distribution is essential for process control and optimization in nanoparticle-assisted systems.

5.4 Process Stability and Reactor Performance

Nanoparticles enhance reactor stability by enhancing microbial resilience and metabolic activity (Khan et al., 2019). Stable operation is maintained even under changing environmental conditions (Wang & Wang, 2019). Better biofilm formation leads to improved biomass retention and reactor efficacy (Zhang & Angelidaki, 2015). Nanoparticles also decrease inhibition caused by toxic metabolites, supporting long-term operation (Show et al., 2011). These improvements are critical for scaling up biohydrogen generation systems. Overall, nanoparticle supplementation significantly increases reactor performance and process reliability.

VI. CHALLENGES, KNOWLEDGE GAPS, AND LIMITATIONS

Despite the promising role of nanoparticles in enhancing biohydrogen production, several challenges and boundaries hinder their large-scale application (Khan et al., 2019; Wang & Wang, 2019). One of the primary concerns is the potential toxicity of nanoparticles to microbial communities, mainly at higher concentrations, which can disrupt cell membranes and create reactive oxygen species (Khan et al., 2019). The lack of standardized guidelines for nanoparticle dosage and exposure conditions further complicates their real-world application (Qu et al., 2013). Another important challenge is nanoparticle aggregation, which reduces their effective surface area and limits their catalytic efficiency (Wang & Wang, 2019). Aggregation also disturbs their dispersion within fermentation systems, leading to inconsistent performance. Additionally, the long-term stability and reusability of nanoparticles under continuous fermentation conditions persist unclear (Zhang & Angelidaki, 2015). Knowledge gaps occur in understanding the exact mechanisms of nanoparticle–microbe interactions at the molecular level (Singh et al., 2018). While several studies report boosted hydrogen production, the underlying biochemical pathways and gene-level responses are not fully elucidated. The effect of nanoparticles on microbial community dynamics and functional diversity in mixed cultures also needs further evaluation (Lovley, 2017). Economic feasibility is another main limitation. The synthesis and functionalization of nanoparticles, particularly advanced nanomaterials such as graphene and carbon nanotubes, can be expensive and energy-intensive (Khan et al., 2019). This increases concerns regarding their scalability for industrial applications. Furthermore, the environmental impact of nanoparticle release, potential ecotoxicity and accumulation is not yet fully understood, posing regulatory challenges (Wang & Wang, 2019). There is also a lack of comprehensive life-cycle assessments investigating the sustainability of nanoparticle-assisted biohydrogen systems (Singh et al., 2018). Variability in experimental conditions across evaluations makes it difficult to compare results and establish universal conclusions. Moreover, maximum study has been conducted at laboratory scale, with limited pilot-scale or industrial-scale validation (Show et al., 2011). Future research should focus on developing ecologically friendly and cost-effective nanoparticles, optimizing operational parameters, and establishing standardized protocols. Integrating advanced analytical tools such as omics approaches could provide deeper insights into metabolic pathways and microbial responses. Addressing these challenges and knowledge gaps is essential for translating nanoparticle-based biohydrogen production from laboratory research to practical, large-scale applications.

VII. FUTURE PROSPECTS

Since energy security is an significant concern for our modern civilisation, it is necessary for us to take action to use renewable energy sources, which should lessen our reliance on conventional fossil fuels. One of the most realistic and environmentally friendly alternatives is the production of biohydrogen from cellulosic biomass, although further research on a commercial scale is necessary for its long-term practical applications. The production technology based on cellulose biohydrogen has been improved. Creating thermophilic microbes, modifying their genes, employing co-culture or mixed-culture microorganisms, using various substrates, and creating effective enzyme systems are a few alternative ways for biohydrogen generation.

Nanomaterials are being used and considered an advanced method in the field of biohydrogen generation technology. Since nanomaterials have effective catalytic activity, they can significantly enhance the process of biohydrogen production from cellulosic biomass. Numerous studies have shown that the use of nanomaterials can effectively enhance the biohydrogen production effeciency in the process of turning biomass into biohydrogen. The major drawback in the application of nanomaterials based biohydrogen production is the cost of preparing nanomaterials. Another issue with this approach is the reuse of the nanomaterials, which are utilised at every step of the procedure. While recycling nanomaterials may be possible through sophisticated filtration techniques, the cost associated with producing nanomaterials may further lower the overall process's cost economy when environmentally friendly green synthesis of nanomaterials are used instead of chemical processes. Still, more research are needed to be further explored to understand the exact mechanisms of nanomaterial influenced biohydrogen production process at biochemical as well as molecular level. From literature review it has noticed that very limited information is available on the nanomaterials assisted dark fermentative biohydrogen production approach and therefore, more research should be carried out to explore this technology.

VIII. CONCLUSION

This review discusses the process of dark fermentative biohydrogen production and explores the potential improvements achieved through the application of various nanoparticles. Since both iron and nickel function as the active centres of hydrogenase enzyme, extensive investigations have focused on the application of metal nanoparticles, particularly those composed of iron and nickel and their oxides, to enhance biohydrogen production via dark fermentation. Furthermore, the production of biohydrogen during dark fermentation can be improved through the use of gold, silver, cobalt, palladium and other nanocomposites. Based on the available information, nanoparticles can effectively increase the biohydrogen production process sustainably, leading to a high yield of biohydrogen on a practical level. Further research is essential to address the existing gap in the practical applications and long-term sustainability of this process. Optimizing nanoparticle dosage, enhancing biocompatibility, and minimizing potential toxicity remain critical areas of investigation. Moreover, the integration of advanced bioprocessing approaches and omics-based methods could provide deeper insights into system performance and microbial interactions. Addressing these challenges will be important for translating nanoparticle-assisted biohydrogen production from laboratory investigations to large-scale industrial applications.

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