HYDROGEN PRODUCTION VIA DARK FERMENTATION: A REVIEW OF INFLUENTIAL FACTORS

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Biohydrogen is a promising low-carbon energy source due to its high energy density, and emerging technologies have been studied to achieve highly efficient and competitive H_2 production. The biological hydrogen production involves microbe-assisted bioconversion, either in the presence or in the absence of light, called photo-fermentation or dark fermentation, respectively. Biohydrogen production using fermentative conversion of organic carbon in the absence of light, *i.e*., dark fermentation, has gained great interest during the last few decades. The mechanistic understanding of various metabolic pathways involved in dark fermentative hydrogen production is well understood and reviewed here. Further, the hydrogen yield is affected by a number of factors during the fermentation of organic substrates by either pure or mixed microbial cultures, and some of the pertinent factors have been discussed in this review. This review aims to present the current state of knowledge on the dark fermentation process, focusing on the use of waste materials as substrates.

Keywords: dark fermentation, biohydrogen, facultative anaerobes, strict anaerobes

INTRODUCTION

The expansion of the global economy and population amplifies the call for sustainable energy as fossil fuels, which presently contribute to nearly 80% of the world's energy, teeter on the brink of exhaustion. The combustion of fossil fuels also involves several environmental issues, such as global warming, the depletion of the ozone layer, and acid rain.[1] The imperative to address both escalating energy demand and negative environmental effects necessitates a comprehensive transformation of the global energy framework.

Hydrogen, distinguished by its remarkably high energy content per unit weight and minimal pollution emissions, has become the ideal alternative to fossil fuels, promising to revolutionize the energy landscape. Hydrogen emerges as a carbon-neutral fuel, releasing only water and heat energy upon combustion, thus assuming a pivotal role within the decarbonization strategy.[2] This strategic integration of hydrogen aligns with the pursuit of a low-emission global economy and the attainment of climate neutrality through the intricate process of energy transition. Distinguished by a remarkable calorific value, reaching 141 MJ/kg higher heating value, hydrogen stands as the utmost among known commercial fuels. A single kilogram of hydrogen bears equivalence to roughly 2.75 kilograms of gasoline.[4] This exceptional energy potency positions hydrogen as a formidable competitor against other alternative energy sources, including wind, solar, tidal, and geothermal energy.

Presently, the primary process for hydrogen production involves the reforming of natural gas. Currently, approximately 96% of the existing hydrogen supply is derived from the steam reforming of coal or natural gas, a method known as black and grey hydrogen. This approach has resulted in the emission of a staggering 900 million metric tons of CO_2 annually into the

atmosphere from global hydrogen production.[5] To truly position hydrogen as a viable solution, a crucial transformation involving the replacement of black and grey hydrogen with a low-carbon alternative is required. Among the diverse hydrogen production pathways currently under scrutiny, the notion of green hydrogen from biomass has garnered noteworthy attention.[6] The most auspicious technological approaches rooted in renewable sources center around biomass feedstocks for hydrogen production. These encompass non-food agricultural crops, residues from agriculture and forestry, and municipal solid waste. Extensive endeavors have been channeled into the evolution of thermochemical processes, encompassing the gasification and pyrolysis of biomass for hydrogen production.[7] Furthermore, biological pathways for producing hydrogen have also been identified, including heterotrophic photo-fermentation and dark fermentation (anaerobic fermentation by microorganisms in the absence of light).[8] This review explores the potential for harnessing biological processes to usher in a new era of hydrogen generation.

OVERVIEW OF BIOLOGICAL PROCESSES FOR HYDROGEN PRODUCTION

The process of dark fermentation is an anaerobic method for biohydrogen production, utilizing lignocellulosic biomass as a renewable feedstock. As shown in Figure 1, lignocellulosic biomass, composed primarily of cellulose, hemicelluloses and lignin, undergoes pretreatment and hydrolysis to release fermentable sugars, which are converted into hydrogen under anaerobic conditions via specific microbial metabolic pathways. The pretreatment is a crucial step in anaerobic digestion of lignocellulosic biomass, as it exposed the cellulose fiber by disrupting the lignocellulosic structure.[9] Different methods of pretreatment or their combinations are used to open up the complex structure of biomass. Effective pretreatment should consume less energy to delignify and reduce the crystalline structure of cellulose effectively. It should be economical, reduce the particle size of biomass, be able to act on different types of lignocellulosic biomass. Effective pretreatment facilitates the efficient conversion of biomass into useful by-products, such as hydrogen, through the process of dark fermentation. Table 1 depicts different methods of pretreatment commonly used.

The process of fermentation is the production of energy from the oxidation of organic molecules by the microbes using an endogenous electron acceptor. The different biological processes for hydrogen production have been shown in Figure 2. The fermentation can be categorized into two types depending upon the necessity of light, *i.e.* dark fermentation and photo-fermentation. The breakdown of organic material during the process of dark anaerobic fermentation produces hydrogen, along with several organic acids and alcohols. In photo-fermentation, organic acids are broken down to produce CO_2 and H_2 with the help of light-dependent sulfur and non-sulfur purple bacteria.

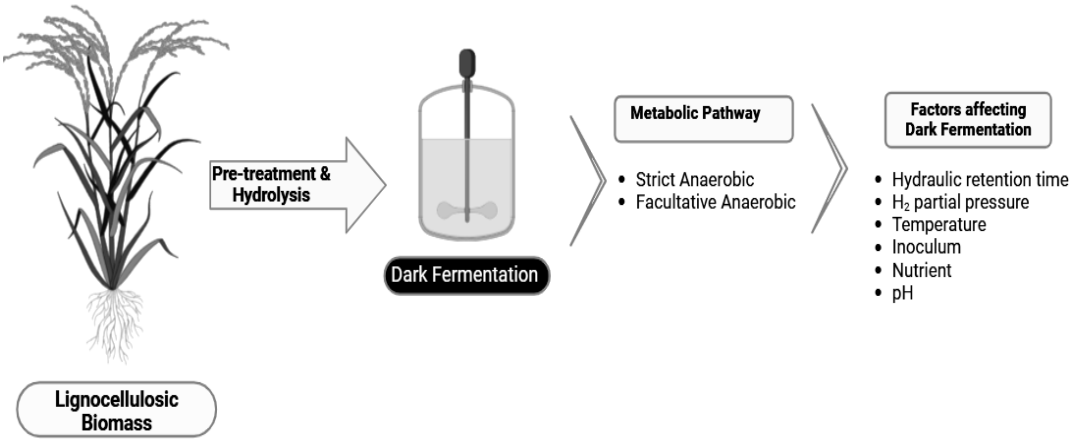

Figure 1: Schematic representation of hydrogen production from lignocellulosic biomass via dark fermentation, highlighting pretreatment, metabolic pathways, and key influencing factors

Table 1
Summary of pretreatment methods used in lignocellulosic biomass conversion for hydrogen production

Pretreatment	Pretreatment conditions	Affected components	Features	Refs.
Physical methods: 1. Ball milling 2. Irradiation 3. Extrusion	Particle size reduced to ≈6-33 mm	Cellulose, hemicelluloses, lignin	Low energy consumption	9, 10
Chemical methods: 1. Acid 2. Alkali 3. Solvent extraction 4. Ionic liquid 5. Ozonolysis	Use of chemicals in varied concentrations (0.5-3%) as per the treatment	Cellulose, hemicelluloses, lignin	Enhanced cellular digestion, reduction in lignin content, high glucose yield	9,10
Biological methods: 1. Fungi 2. Bacteria 3. Enzyme	Extracellular enzymes released by bacteria, like cellulases, peroxidases; bacteria hydrolyze the polymer	Cellulose, hemicelluloses and lignin	Ecofriendly, low energy and low cost, chemical-free transformation of lignin and hemicellulose solubilization	11

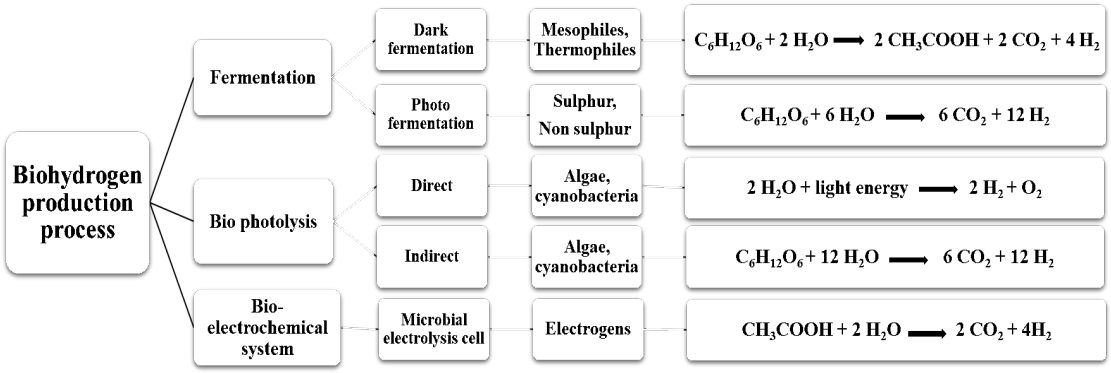

Figure 2: Different processes for biological hydrogen production

Combining the two fermentation processes could increase the amount of biohydrogen produced.[12] The process by which oxygenic photosynthetic microorganisms synthesize hydrogen from sunlight and water is known as bio-photolysis or water-splitting photosynthesis. Green algae and cyanobacteria are examples of photosynthetic microorganisms that participate in direct bio-photolysis, where they absorb solar radiations and use nitrogenase or hydrogenase to evolve hydrogen.[13]

At 680 nm light energy, water splits into protons, electrons, and oxygen. Through PS II and PS I, the electrons are transferred in a sufficient amount to the oxidized ferredoxin (Fd) for its reduction. The reduced ferredoxin then reduces the hydrogenase enzyme responsible for the production of hydrogen. Indirect bio-photolysis is the two-step photosynthetic conversion of light energy to chemical energy in the form of carbohydrates.[14] The first stage involves the fixation of carbon dioxide into carbohydrate i.e., starch and glycogen in cyanobacteria and green algae, respectively, using light energy and along with production of O_2. The second phase is the conversion of carbohydrates to CO_2 and H_2 in an anaerobic environment. The electrons produced through catabolism of carbohydrates are ultimately transferred to Fe-Fe hydrogenases for the reduction of protons to hydrogen.

Dark fermentation

Dark fermentation represents a biological process in which obligate and facultative

anaerobes produce hydrogen, when light and oxygen are absent. It is basically, an acidogenic step of anerobic digestion, which involves the conversion of simple sugars generated by hydrolysis of complex carbohydrates into hydrogen, CO_2 and short chain acids, such as acetic acid, butyric acid.[15] Hydrogen is produced as an intermediate product of anaerobic digestion and the methanogenesis step is required to be stopped in order to produce hydrogen as the primary product. Various pretreatments, such as heat, alterations in pH and chemicals, are able to repress the methanogenic activity during biohydrogen production.[16] The most extensively used method to suppress hydrogen-consuming bacteria and enhance spore-forming hydrogen producers is heat treatment. However, heat treatment does not eliminate the homo-acetogenic bacteria that convert the H_2/CO_2 mixture into acetate. The growth of these bacteria can be suppressed by the removal of CO_2 from the medium with the addition of alkaline chemicals, such as potassium hydroxide.[17]

Molecular basis for hydrogen production – hydrogenases

Hydrogenases are the important enzymes involved in variety of biological processes that consume or produce hydrogen. They are most commonly distributed among microorganisms. They are of two fundamental types with distinct active sites, *i.e*., FeFe-hydrogenase and NiFe-hydrogenase. These enzymatic agents facilitate a reversible reaction:

$$2H^+ + 2e^- \longrightarrow H_2 \quad (1)$$

The reversible heterolytic cleavage of molecular hydrogen is catalysed by hydrogenases.[18] When suitable electron donors are available, these hydrogenases can evolve molecular hydrogen. Otherwise, hydrogen thus produced can be oxidized to protons.[19] Hydrogenases that are located in the periplasm or membrane are often linked to H_2 uptake, while those present in the cytoplasm are generally linked to H_2 evolution. Anaerobic fermentative microorganisms, cyanobacteria and algae can produce hydrogen biologically, due to reversible nature of hydrogenases. Hydrogenases are the enzymes that contain metal ion as prosthetic group and are classified into three types on the basis of metal atom present at the enzyme active site, *i.e*., Fe-Fe hydrogenases, Ni-Fe hydrogenases and those containing only Fe, as shown in Figure 3.[20] The most effective enzymes known for hydrogen production are [FeFe]-hydrogenases. These enzymes can be either monomeric, like in *Clostridium*, or multimeric, with three and four subunits in the case of *Thermotoga maritima* and *Thermoanaerobacter tengcongensis*, respectively.[21]

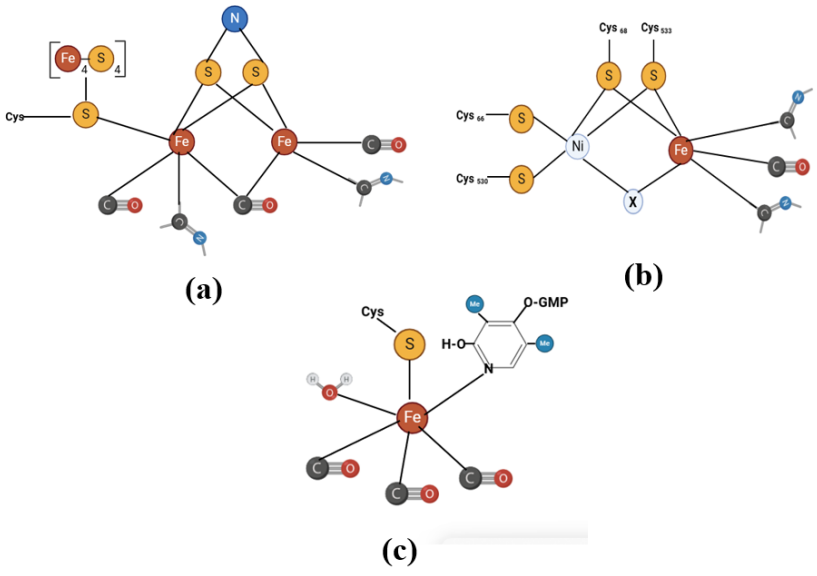

Figure 3: Structures of hydrogenase enzymes: (a) [NiFe]-hydrogenase, (b) [FeFe]-hydrogenase, (c) Fe-hydrogenase

The structural organization of [FeFe]-hydrogenases shows the presence of modular domains. The F cluster, known as accessory cluster, functions as intra as well as inter molecular electron transfer centres and is linked to the catalytic cluster called H-cluster. The H-cluster is composed of [4Fe-4S] cubane, which is linked to a 2Fe unit and further coordinated by five diatomic CN^- and CO ligands, along with non-protein dithiolate ligands.[22] *Scenedesmus obliquus*, *Chlorella fusca* and *C. reinhardtii* contain the most basic [FeFe]-hydrogenases that have been characterized. These organisms' express enzymes that contain only H-cluster and lack the F-cluster domains.[23,24]

METABOLIC PATHWAY OF HYDROGEN PRODUCTION FROM CARBOHYDRATES

The contemporary understanding of fermentation via glycolysis has elucidated the mechanism by which hydrogen is produced from glucose. Pyruvate, ATP, and NADH are produced, when glucose or other carbon sources from plant biomass or waste products reach the primary glycolytic pathway.

Strict anaerobe dark fermentation

Under strict anaerobic conditions, pyruvate is oxidatively decarboxylated into acetyl-CoA along with the reduction of ferrodoxin through catalytic action of pyruvate ferredoxin oxidoreductase PFOR). Fd-dependent hydrogenases then transfer the electrons from the reduced ferrodoxin to the protons to generate hydrogen.[25] This is the most common hydrogen-producing reaction for *Clostridium* species. In addition to this, NADH generated during glycolysis could also generate hydrogen, but this requires very low partial pressure of hydrogen (less than 60Pa) in the dark fermentation process.[26] For this, the sequential reactions are catalyzed by two enzymes *viz*., NADP(H): ferredoxin oxidoreductase (NFOR) and Fd-dependent hydrogenase (FDH). NADP(H) formed during glycolysis reduces the oxidized ferredoxin and transfer of electrons from reduced ferrodoxin to the protons to generate hydrogen. It has been reported that certain thermophilic bacteria and several species of *Clostridium* exhibit this kind of response.[27]

Clostridium butylicum is an obligate anaerobe and can ferment a wide range of substrates into products like acetone, butanol, and ethanol. The metabolic pathways for glucose breakdown and production of various metabolites by *C. acetobutylicum* have been elaborated in Figure 4. There are two different phases of fermentation.[25] The first phase, known as acidogenic phase, is characterized by fast growth and better hydrogen production and production of short chain acids, such as acetic acid and butyric acid. The second phase, known as solventogenic phase, involves comparatively low hydrogen yield, slow growth and production of organic solvents. The utilization of either of these pathways is dependent upon ATP and NADPH levels.[28] Reduced ferrodoxin is oxidized to produce hydrogen during acidogenic metabolism by [Fe-Fe] hydrogenase, whereas oxidized ferrodoxin is reduced back by NADH-ferrodoxin reductase that converts NADH to NAD required to drive glycolysis. The presence of diverse end-products, including several acids such as butyric acids, propionic and acetic acid, and solvents, such as acetone, ethanol and butanol, leads to a reduction in the yield of hydrogen during fermentation process. The formation of acetic acid results in a decrease from twelve moles of molecular hydrogen (Eq. 2-4) to four moles. Likewise, only two moles of hydrogen are produced from one mole of glucose, when butyric acid is the ultimate end-product.[29] In practice, the final product comprises a mixture of various metabolites, thereby, further diminishing the hydrogen yield to approximately 1 to 2.5 moles for one mole of glucose utilized.

$$C_6H_{12}O_6 + 6\,H_2O \rightarrow 6\,CO_2 + 12\,H_2 \quad (2)$$

$$C_6H_{12}O_6 + 6\,H_2O \rightarrow 2\,CO_2 + 2\,CH_3COOH + 4\,H_2 \quad (3)$$

$$C_6H_{12}O_6 + 6\,H_2O \rightarrow 2\,CO_2 + CH_3CH_2CH_2COOH + 2\,H_2 \quad (4)$$

Facultative anaerobic dark fermentation

Both aerobic and anaerobic environment can support the growth of facultative anaerobes. Strict anaerobes are killed by oxygen, although facultative anaerobes are less susceptible to it. Under aerobic conditions, they growth very quickly, and when oxygen becomes scarce, they switch from aerobic to anaerobic metabolism. Facultative anaerobes are therefore, considered to be better fermenting microbes as they can be grown to a high cell density in the presence of oxygen and produce hydrogen at a faster rate, when the oxygen supply becomes limited.[30] The presence of oxygen prevents the generation of hydrogen, as this promotes the electron transfer to

oxygen, even in the case of facultative anaerobes. However, enzymes involved in the production of hydrogen regenerate quickly, when oxygen is removed from the fermenting medium.

Under aerobic conditions, pyruvate is assimilated through the catalytic action of pyruvate dehydrogenase (PDH) and acetyl CoA formed enters into Kreb cycle or is excreted as acetate.[31] NADH produced in glycolysis/Kreb cycle can be used to produce ATP via oxidative phosphorylation process and the primary enzyme NADH oxidase also helps to regenerate NAD^+ from NADH. However, pyruvate is cleaved in the presence of pyruvate formate lyase (PFL) under oxygen limiting conditions, where it generates acetyl coenzyme A (AcCoA) and formic acid. ATP required to support fermentative reactions are generated by substrate-level phosphorylation and this involves upregulation of most of the glycolytic genes. The formation of acetate from acetyl CoA generates ATP through the action of phosphotransacetylase and acetate kinase. The regeneration of NAD^+ is also required to maintain the glycolytic flux and is achieved by conversion of acetyl CoA into various end-products, such as succinate, formate, lactate and ethanol.[32] Succinate production from phosphoenolpyruvate (PEP) through the sequence of reactions catalyzed by PEP – carboxylase, dehydrogenase, fumarase and reductase – requires two molecules of NADPH. Similarly, conversion of one molecule of pyruvate to lactate and ethanol requires one and two molecules of NADH, respectively. *E. coli* thus produces several acids, such as succinate, formate, lactate, acetate and ethanol. The enzyme complex formate:hydrogen lyase (FHL) cleaves formate under acidic conditions into H_2 and CO_2, so as to lower the concentration of formic acid in the cell.[33] Reduced hydrogen yields have been reported because of incomplete degradation of formate and induction of lactate dehydrogenase under these conditions. Thus, some of the reduction potential power of pyruvate is lost because of its subsequent conversion into lactate. The enteric bacteria, thus, carry out a mixed-acid fermentation and produce a variety of end-products, as shown in Figure 5.

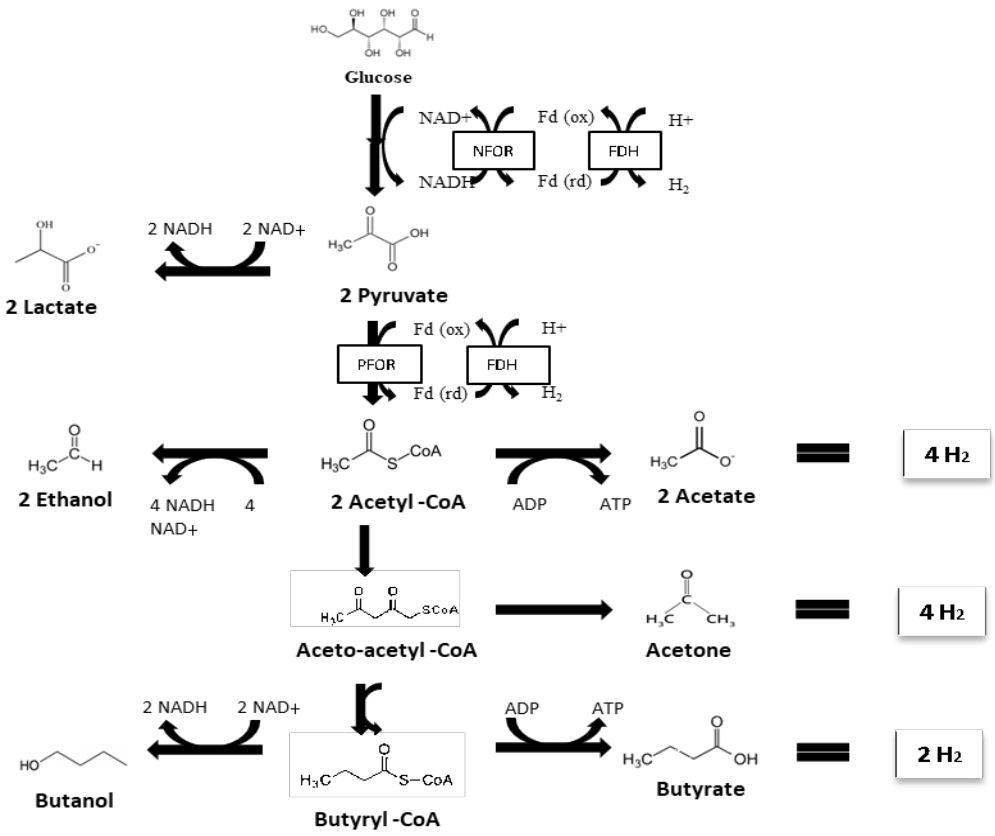

Figure 4: Simplified metabolic pathways for glucose breakdown and production of various metabolites in *C. acetobutylicum* (abbreviations: NFOR: NADH: ferredoxin oxidoreductase, FDH: ferredoxin-dependent hydrogenase, PFOR: pyruvate: ferredoxin oxidoreductase (Fdox), oxidized ferredoxin (Fd rd): reduced ferrodoxin)

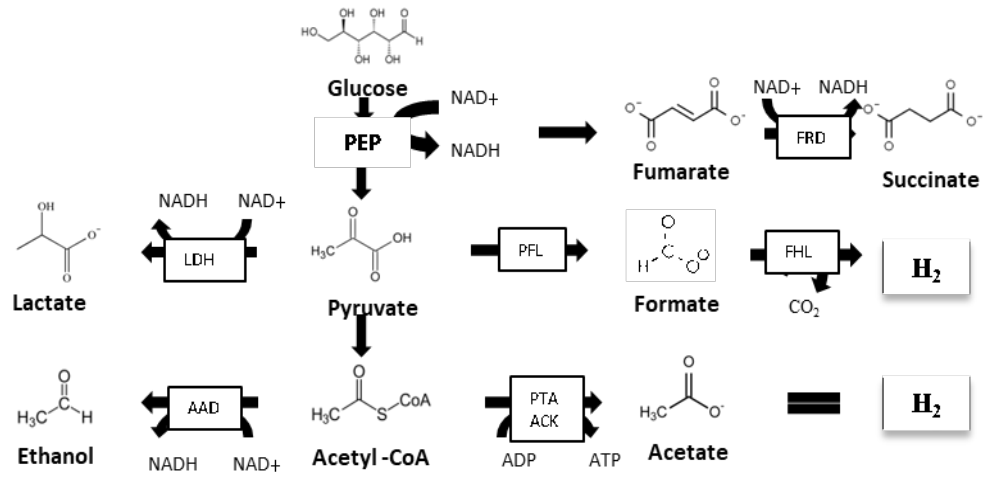

Figure 5: Mixed acid fermentation in *E. coli* (abbreviations: PEP: phosphoenolpyruvate, ACK: acetate kinase, PTA: phosphotransacetylase, LDH: lactate dehydrogenase, PFL: pyruvate formate lyase, FHL: formate:hydrogen lyase complex, AAD: aldehyde alcohol dehydrogenase, FRD: fumrate reductase)

The relative proportions of end-products depend upon the oxidation potential of the substrate. The amount of reduced product balances the fermentation by regenerating the NAD^+.[32] The enteric-type mixed fermentation of glucose leads, thus, to maximum 2 moles of H_2 per mole of glucose.

FACTORS AFFECTING DARK FERMENTATION PROCESS

Several factors influence the efficiency of hydrogen production during the dark fermentation process, including inoculum type, hydrogen ion concentration (pH), temperature, hydrogen (H_2) partial pressure, hydraulic retention time (HRT), and nutrient availability. The choice of inoculum, whether a pure culture or mixed microbial consortia, plays a critical role, as it determines the metabolic pathways and microbial interactions that impact hydrogen yield. The pH of the medium affects microbial activity and enzyme function, while temperature influences reaction rates and microbial growth. Hydrogen partial pressure, if too high, can inhibit hydrogen production by creating unfavorable conditions for the microbes. HRT, the time that substrates remain in the reactor, also affects hydrogen yields, as a balanced retention time is essential to avoid substrate washout or accumulation. Nutrient availability is crucial for supporting microbial growth and metabolic functions. Table 2 illustrates the effect of inoculum type and operational parameters on dark fermentative hydrogen production, using either pure or mixed microbial consortia. This table highlights the interplay of inoculum and operating conditions, emphasizing how optimized parameter combinations can lead to enhanced hydrogen yields in dark fermentation.

Inoculum type

Biohydrogen can be produced by a variety of fermentative bacteria, including aerobic bacteria (*Bacillus*), facultative anaerobes (*Enterobacter*, *Escherichia coli*, *Rhodopseudomonas*, and *Citrobacter*), and stringent anaerobes (*Clostridium*). The most extensively researched of these microbes are the bacteria of the genus *Clostridium*.[34] Strict anaerobes can generate four moles of hydrogen per mole of glucose, although facultative anaerobic bacteria can produce up to two moles of H_2. Because strict anaerobic microbes are quite sensitive to oxygen, so even minute quantity of oxygen in the fermentation medium completely inhibits the formation of hydrogen.[35] However, facultative anaerobes quickly consume oxygen, thereby creating anaerobic conditions in the medium immediately. Therefore, it is believed that facultative anaerobes produce more biohydrogen at a lower cost than stringent anaerobes. For the production of biohydrogen, strict or facultative anaerobic bacteria might be sourced either from organic waste, such as bovine dung, sewage sludge, soil and compost or pure bacterial cultures, such as *Clostridium*, *Enterobacter*, and *Escherichia coli*.[34]

The production of biohydrogen from pure bacterial cultures obtained via isolation and strain

improvement has been thoroughly researched. However, pure bacterial cultures are highly vulnerable to contamination and thus, maintenance of aseptic fermentation conditions is crucial for the feasibility of the dark fermentation process.[36] Due to lower operating and maintenance expenses, less upstream processing and suitability for wider range of feedstocks, mixed cultures are mostly preferred over pure cultures.[37] Mixed microbial cultures must be pretreated (by either heat or some chemical treatment) in order to suppress several hydrogen consuming microbes and activate hydrogen producers (mostly *Clostridium* sp.).[36]

Hydrogen ion concentration in the medium

Hydronium ion concentration is crucial to the dark fermentation processes, because it has a significant impact on the hydrogen yield. The ideal pH range for the synthesis for dark fermentative process is between 4.5 and 9. The variability in optimum pH could be due to differences in type of inoculum and their enrichment methods, type of substrate and optimum organic loading rate. The maintenance of pH at an optimal level is very important as hydrogen production is accompanied by the formation of several organic acids (lactic, butyric, propionic and acetic acid) that lower the pH of the fermentation medium, thereby, inhibiting the activity of hydrogenases.[38] The undissociated form of the acids (acetic and butyric acids) also causes the shift in bacterial metabolism from hydrogen production to solventogenic phase.[39] These acids are able to cross the bacterial cell membrane and dissociate in the cell at the higher internal pH. The uptake of protons uncouples the proton motive force and increases ATP requirements of the cell to maintain the neutral intracellular pH. The uptake of acids also decreases the available coenzymes/phosphate pools that ultimately stops the influx of glucose via glycolysis.[40] The pH, therefore, plays an important role for regulation of various metabolic pathways and metabolites derived from oxidation of glucose/pyruvate ultimately determines the overall hydrogen yield. The pH can also affect the type of the acids that are produced during hydrogen production. The concentration of undissociated forms of acetic or butyric acid was found to be much higher at low pH values, whereas, an increase in pH resulted in higher concentrations of propionic lactic acids and ethanol. The higher amounts of the undissociated form of acids at the lower pH cause inhibition of hydrogen generation.

Temperature

Temperature is an important parameter and dark fermentation depends on the type of fermentative microorganisms, such as thermophilic (42–75 °C), mesophilic (20–42 °C) and psychrophilic (0–20 °C), as this profoundly affects their growth as well as metabolism.[41] The temperature can affect the activity of hydrogen evolving bacteria by influencing the activity of several important enzymes like hydrogenases. The optimum temperature range for effective dark fermentation by mesophilic bacteria is 37–45 °C. Thermophiles in the fermentation medium are more tolerant of high temperature. They possess great potential for hydrogen generation as the reaction conditions involving conversion of glucose into biohydrogen are thermodynamically more favorable under high temperature conditions. Thermophiles have the ability to efficiently convert the complex organic substrates into simpler one. The optimum reactor performance could be achieved at thermophilic temperature (55 °C), with the high degradation of cellulose and stability of the fermentation medium after temperature shock.[42] Thermophiles are also found to be more resistant to environmental contaminations, as compared to the mesophiles. Moreover, thermophilic conditions are more advantageous for generation of hydrogen due to decreased solubility of hydrogen in the liquid medium.[43] However, fermentation at high temperatures requires high thermal energy, which further increases the cost of hydrogen generation.

H_2 partial pressure

The biological hydrogen production in the dark fermentation process is highly sensitive to the partial pressure of hydrogen, a critical rate-limiting factor. With the increase in the level of dissolved hydrogen in the fermentation medium, hydrogenase activity (transfer of an electron from an intracellular electron carrier to H^+) decreases due to feedback inhibition.[44] The reduction of oxidized Fd occurs more favorably than the oxidation of reduced Fd, along with reversible oxidation of the hydrogenase enzyme. The increased hydrogen concentration shifts the metabolic pathways to other metabolites, such as ethanol, alanine, acetone, butanol and lactate.

Table 2
Effect of inoculum type and operational parameters on dark fermentative hydrogen production by using pure/mixed microbial consortia

Inoculumn	Substrate	Temperature	pH	H_2/mole of substrate	Reference
Caldicellulosiruptor saccharolyticus DSM8903	Sucrose	70	7.0	5.9	[51]
T. neapolitana DSM4359	Glucose	65	7.0	1.84	[52]
Citrobacter sp. Y19	Glucose	36	7.0	2.49	[53]
Klebsiella oxytoca HP1	Glucose	65	7.0	1.0	[54]
C. beijerinckii L9	Glucose	35	7.2	12.81	[55]
Clostridium butyricum, Rhodopseudomonas palustris	Starch	35	7.0	3.09	[56]
Citrobacter freundii 01, *Enterobacter aerogenes* E10 and *Rhodopseudomonas palustris* P2	Sugar cane distillery effluent	37	7.0	2.76	[57]
T. thermosaccharolyticum PSU-2	Sucrose	60	6.25	2.53	[58]
T. saccharolyticum JW/SL-YS485	Xylose	55	6.2	0.88	[59]
T. maritima DSM3109	Glucose	65	6.5	1.67	[60]
Clostridium thermocellum	Corn stalk	30	7.4	0.45	[61]
Rhodobacter sphaeroides	Organic waste water	30	7.0	1.81	[62]
Clostridium and *Klebsiella*	Cheese whey waste water	30	5.0	1.1	[63]
Mixed culture dairy waste water treatment plant)	Lactose	30-35	7.0	4.84	[64]
Mixed culture	Palm oil mill effluent	55	5.5-6.5	2.99	[65]
Aneorobic granulated mixed consortium	Brewery waste water	37	5.5	1.5	[66]
Biomass from fermentation	Dairy industry waste water	24-30	3.7-4.3	2.56	[67]
Mixed culture	Beverage waste water	37	5.5	3.76	[68]
Enterobacter LBTM2	Sugarcane bagasse hemicellulose hydrolysates	35	6.5	0.46	[69]
Anaerobic consortia; *Clostridium*; *Klebsiella*; *Enterobacter*; lactic acid bacteria)	*Arundo donax* (giant reed) hydrolysate	35	6.5	0.30 ± 0.05	[70]

It is very important to remove excess of hydrogen from the system to maintain continuous hydrogen production. The reduction in partial pressure of hydrogen by air exhaust through glass syringe and nitrogen purging into the headspace increases the efficiency of hydrogen production by 54%.[45] Lowering the partial pressure also drastically affects the composition of soluble products, as well as ecological factors. Sparging of nitrogen (N_2) gas into the reactor headspace to maintain the low partial pressure of hydrogen increases hydrogen production rate from 1.446 mL hydrogen min^{-1} g^{-1} biomass to 3.131 H_2 mL hydrogen min^{-1} g^{-1} biomass.[46]

Hydraulic retention time (HRT)

HRT is a crucial factor for biohydrogen reactor performance. A prolonged fermentation period is detrimental to the synthesis of hydrogen due to the metabolic switch from acidogenesis to methanogenesis. A shorter HRT (2–10 h) aids in limiting the development of bacteria that produce methane. The ideal HRTs with a variety of substrates are between 8 and 14 hours for good hydrogen yields.[47] However, in order to achieve the best H_2 yield, a number of variables must be considered, such as type and composition of the substrate, type of microorganism, rate of organic loading, and the redox conditions of the system.

Nutrients

Nutrients, such as carbon, nitrogen, phosphorus and metal ions, are required by the hydrogen producing bacteria during the dark fermentation process. Nitrogen sources are required in the bulk and can be provided by protein, nitrate, nitrite, as well as ammonium salts. The demand for phosphorus is met by phosphates. The ammonium salts also act as buffer for organic acids generated during the dark fermentation process.[43] Metal ions are important to activate several enzymes and coenzymes involved in microbial metabolism and are thus, essential for cell growth. Fe is very important for the functioning of hydrogenase as the enzyme has a bimetallic Fe–Fe center surrounded by Fe-S protein clusters. Iron also acts as an active site for the ferrodoxin, which transports the electrons to the hydrogenase.[48] The effect of Fe supplementation on fermentative dark hydrogen production was studied by several researchers. Wang and Wan[49] found a maximum cumulative hydrogen of 302.3 mL and yield of 311.2 mL/g glucose at Fe^{2+} concentrations of 300 and 350 mg/L, respectively. With little to no propionic acid generated, the mixed cultures mostly produced ethanol, acetic acid, and butyric acid as soluble end products. Minor elements, such as manganese, nickel, molybdenum, copper, magnesium, iron, sodium, zinc, potassium, iodine, cobalt, ammonium and calcium, also play an important role in the performance of hydrogen production by mixed flora with *C. pasteurianum* as dominant one. Mg, Fe, Na and Zn were found to be crucial for hydrogen production.[50] The nutrient formulation prepared by using these metal ions resulted in maximum hydrogen productivity of 3.43 moles hydrogen $mole^{-1}$ sucrose.

CONCLUSIONS AND RECOMMENDATIONS

The dark fermentation technology has an excellent future potential for biohydrogen production, as renewable biomass can be used as a feedstock. Microbial communities involved in the breakdown of complex organic substrates into biohydrogen are phylogenetically and functionally diverse. A deep insight into the microbial communities and various biochemical processes involved in the dark fermentation process is important for obtaining a robust and efficient biohydrogen production system. Further research and development on optimization of substrate utilization, enrichment of microbial community and operational parameters, such as pH, temperature and H_2 partial pressure, is required to improve the biohydrogen yield by the dark fermentative process. There is need to explore the diverse microbial community of hydrogen producers, as well as substrates. Moreover, to establish dark fermentative hydrogen production as a viable and competitive large-scale technology, advanced research is essential to address both technological and financial challenges. A continuous bioprocess is necessary for industrial production and economic considerations. Therefore, additional research on continuous dark fermentation techniques is necessary to ensure the long-term operational

viability of these systems. It is anticipated that, with minor adjustments to the process parameters, industrial-scale dark fermentative systems would resemble anaerobic digestion processes in terms of both design and configuration in the future. It is possible to adapt the two-stage anaerobic digesters that produce methane for use in the dark fermentation process, which creates new possibilities for the production of biohydrogen from renewable biomass. Furthermore, combining the dark fermentation process with other biotechnological procedures may enhance its energy benefits and enable the extraction of additional beneficial substances from bio-based technologies. Additional advantages of integrated bioprocesses include full conversion of waste streams and VFAs created from acidogenic processes into useful chemicals, which reduce overall process running costs.

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