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A critical review on factors influencing fermentative hydrogen production

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1. ABSTRACT

Biohydrogen production by dark fermentation of different waste materials is a promising approach to produce bio-energy in terms of renewable energy exploration. This communication has reviewed various influencing factors of dark fermentation process with detailed account of determinants in biohydrogen production. It has also focused on different factors such as improved bacterial strain, reactor design, metabolic engineering and two stage processes to enhance the bioenergy productivity from substrate. The study also suggest that complete utilization of substrates for biological hydrogen production requires the concentrated research and development for efficient functioning of microorganism with integrated application for energy production and bioremediation. Various studies have been taken into account here, to show the comparative efficiency of different substrates and operating conditions with inhibitory factors and pretreatment option for biohydrogen production. The study reveals that an extensive research is needed to observe field efficiency of process using low cost substrates and integration of dark and photo fermentation process. Integrated approach of fermentation process will surely compete with conventional hydrogen process and replace it completely in future.

2. INTRODUCTION

Limited Fossil fuel resources and their large scale consumption causes an accelerated serious problem of peril of their exhaustion and also pollution, particularly emission of greenhouse gases, which are the major cause of global warming and atmospheric pollution. The future vision for global bio-energy market mainly comprises of renewable energy sources like biohydrogen, biofuel and biogas. Among these, biohydrogen (biomass originated) provides a long-term sustainability for economic development as a future fuel with zero-pollution (1). Hydrogen becomes a promising bioenergy fuel since it is clean, renewable and contains high energy value without contributing to green house effect. In the environment, resources such as water, solar, biomass etc. are linked together and it requires extensive research to fulfill future energy demand by using these resources worldwide. In this regard researchers have developed keen interest for biohydrogen (H_2), an intermediate product during the process of anaerobic digestion. The Anaerobic digestion process is a well known technology and practiced at industrial level for methane production (2-4).This process involves three steps to get final output in terms of methane i.e. acidogenesis (formation of lower molecular weight organic acids), acetogenesis (formation of acetate) and eventually methanogenesis (5).

Recent advances in biohydrogen production are mainly concerned with photo fermentation (PF) and dark fermentation process (DF), however some authors have also achieved enhanced rate of H_2 production via combined fermentation process. Though photosynthetic fermentation process shows higher theoretical H_2 yield, it is found to be impractical for implementation at industrial level due to low utilization efficiency of light and high cost input in suitable reactor design. However, DF process offers high rate of H_2 production with simple operation (3,4). In a series of progress in biohydrogen production processes, combined fermentation process has emerged as an advance biohydrogen production process. For instance, Sargsyan *et al*. (6), achieved three fold higher rate of H_2 production with mixed bacterial culture in combined fermentation process than pure culture. Effluent from DF stage provides an adequate organic substance for photo fermentation stage; however it also produces inhibitory substance like ammonium which retards the photo fermentation process by inhibiting the nitrogenase activity of photosynthetic bacteria. Hence, the nutrient composition should be supplied with optimum concentration under controlled environmental variables. Moreover to maintain the temperature range during fermentative process, a conceptualized study based on fermentative hydrogen production by using waste heat released from power plant was conducted. The study concluded that by supplying waste heat at a temperature level of about 80 °C, hydrogen gas containing 10% CO_2 could be produced at the expense of 10% of the energy value of produced H_2. Thus cost involved in temperature maintenance can be minimized by combining fermentation process with waste heat. Biological hydrogen production has no negative externalities like production of green house gases and toxic byproducts; hence it seems to be more promising energy carrier than methane (Table 1) (6). Application of H_2 in fuel cell shows higher efficiency (35-45%) than in internal combustion engine (25-30%) without co-generation, which proves its potential to be used as better renewable energy fuel in the future (7). In anaerobic digester, hydrogen production rate is 10 fold higher than theoretical methane production rate but

Table 1. Assessment of energy and carbon emission for commercially viable fuels (11,12)

Fuel type	Energy/unit mass, (MJ/Kg)	Energy /Vol. (MJ/L)	Carbon emission (Kg C/Kg fuel)
Hydrogen (gas)	120	2	0
Hydrogen (liquid)	120	8.5	0
Coal(anthracite)	15-19	-	0.5
Natural gas	33-50	9	0.46
Diesel	42.8	35	0.9
Biodiesel	37	33	0.5
Ethanol	21	23	0.5

it is rapidly consumed by methanogenic bacteria (8), in which, acidogenesis and acetogenesis are carried out in separate vessel. However, it is easy to generate desired biogas containing either H_2 or CH_4 depending on operation parameters of the process (9). Ding and Wang (10) in their experimental study found that the highest yield mean volumetric hydrogen and methane potential would be in a stoichiometric ratio of 2:2 to 2:3. Hydrogen has been identified as a potential alternative to fossil fuels among all other renewable energy sources due to its carbon neutral and high energy value properties with various production routes from various types of substrates. Among these, some are totally pollution free processes like water electrolysis, photo-electrochemical and biological ways of production as described in Table 2 with summarized remarks.

This review throws light on, effective utilization of low-cost substrate and an approach to the use of co-substrates for fermentative hydrogen production using substrate-strain system with inexpensive energy generation. It also focuses on the effective pre-treatments methods of inoculums and substrates for higher hydrogen yield. The impact of reactor used in the process, with particular dimension/ structure is also highlighted in the article. This review article helps in exploring the present/current status and scientific advancement that have been made to improve the fermentative routes of biohydrogen production particularly with the process factors, substrate-strain system, advancement in reactors pre-treatment of bacterial inoculums, concept of co-culture and impacts of engineering tools involved in the dark fermentation process and also gives an outline about the second stage processes integration, an advanced approach for energy production.

3. TYPES OF FERMENTATIVE HYDROGEN PRODUCTION PROCESSES

The conversion process of substrate to hydrogen may proceed in the dark or with assistance of light. Chemical reactions involved in both processes are differentiated according to their mode of working. The core components involved in biohydrogen production are substrate and microorganism. The interaction between these two components is responsible for many chemical reactions to occur that are discussed.

3.1. Dark fermentation

Dark fermentation process is characterized by the degradation of low molecular weight compounds (e.g. glucose) to simple organic acids (such as acetic acids) by the activity of some fermentative bacteria (e.g. *Clostridum sp*.) with the evolution of molecular hydrogen (H_2) (13,14). The fate of the dark fermentation process for the quantity of hydrogen produced is dependent on the bacteria involved in the process and formation of acids. Theoretically, in the dark fermentation process, 1mol of glucose yields 4 mole H_2 by acetate pathway and 2 mol of H_2 through butyrate pathway, respectively (15):

$$C_6H_{12}O_6 + 2H_2O + H_2 \longrightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1)$$

$$C_6H_{12}O_6 + 2H_2O \longrightarrow CH_3CH_2COOH + 2CO_2 + 2H_2 \quad (2)$$

$$\text{Pyruvate} + CoA + 2Fd_{(ox)} \longrightarrow \text{Acetyl-CoA} + Fd_{(rd)} + CO_2 \quad (3)$$

$$2H^+ + Fd_{(rd)} \longrightarrow H_2 + 2Fd_{(ox)} \quad (4)$$

$$C_6H_{12}O_6 + 12H_2 + h\nu \longrightarrow 2H_2O + CO_2 \quad (5)$$

$$(CH_2O)_2 \xrightarrow{NADPH} \text{Ferredoxin} \longrightarrow \text{Nitrogenase} \longrightarrow H_2 \quad (6)$$

3.2. Photo fermentation

The photo fermentation process shows dependency on light to produce biohydrogen as it is mediated by some phototrophic bacteria (like *Rhodobacter spheroids, Rhodopseudomonas capsulate, Rhodopseudomonas pulustris, Rhodospirillum rubrum* etc.) by utilizing the organic carbon of feedstock/ substrates. These bacteria posses enzymes like nitrogenase and hydrogenase to ferment the substrate into biohydrogen, carbon dioxide and organic acids. Besides this, these bacteria lack photosystem II which helps in eliminating O_2 present in the system and maintain anaerobic conditions throughout the process.

Table 2. Summarized remarks with advantages and disadvantages of hydrogen production processes.

Processes	Advantages	Disadvantages	Remarks
Thermal Processes			
Natural Gas Reforming	Most viable means of hydrogen production in present scenario due to presence of potential infrastructure support.	Capital cost is high in this technology.	Improve catalyst efficiency and reduction in process of cost is required; development of low-cost, efficient; separation/purification mechanism is also needed.
Bio-Derived Liquids Reforming	Futuristic fuel with existing infrastructure.	The technology is expansive and requires extensive optimization. Feedstock quantity and quality parameters affect the process.	Low temperature and liquid phase catalysts is needed. Characterization of biomass is required.
Coal and Biomass Gasification	Low-cost fuel technology.	Feedstock impurities with carbon content affect the system's efficiency.	Feedstock storage, preparation, and handling is the major hurdles. Emissions control measures are also required.
Thermo chemical Production Routes	Clean and sustainable route for energy production using solar and nuclear energy in integration with chemicals.	It has durability but in long term application mode as well as it is energy intensive technology.	Developments of thermo-chemical storage and heat transfer devices are needed.
Electrolytic Processes			
Water Electrolysis (Splitting Water Using Electricity)	This is pollution free device used to produce energy in more sustainable way.	Solar system can provide a better efficient system, but high cost involves.	Improved photocatalyst with multifunctional materials at low cost to assure uniform quality production designs is required.
Photolytic Processes			
Photo electrochemical Hydrogen Production route	Clean and sustainable technology with low temperature requirements.	Solar based technologies require high cost.	Long term technology for sustainable development.
Biological Processes			
Direct bio photolysis	Integrated approach for H_2 production using sunlight with water.	Light intensity should be high and O_2 act as inhibitor in the reaction.	More R &D is needed for efficient functioning of microorganism for long term development. Zero-cost method for development of microbes using wastewater as a nutrient resource. Development of efficient bioreactor is required.
Indirect bio photolysis	Use of blue green algae for hydrogen production from water.	Uptake of hydrogenates is removed	
Photo fermentation	Different range of light spectrum can be optimized to enhance the yield. A wide spectral energy can be used by photosynthetic bacteria	Nitrogenase enzymes get inhibited in presence of small amount of O and unionized ammonium$_2$. Light conversion efficiency is low.	
Dark fermentation	Light independent process, several metabolites are produced in process as by-product, various substrate can be used.	This technology has relatively low H_2 yield, process, which makes the process thermodynamically unfavorable.	
Two stage fermentation	Can relatively high H_2 yield, first stage metabolites can be converted to H_2 and CH_4.	It requires continuous light source in Dark+ photo fermentation and pH control in anaerobic digestion processes	

4. TYPES OF BACTERIA USED IN FERMENTATIVE BIOHYDROGEN PRODUCTION

Fermentative bio-hydrogen production occurs in an anaerobic condition, where bacteria degrade organic substrate by oxidation of organic materials/substrates, to provide metabolic energy to the cell with the generation of Adenosine Triphosphate (ATP). In this process, electrons are released. As oxygen is absent in anoxic condition, these electrons are accepted by some other compounds such as protons, which are reduced to molecular hydrogen and maintain electronic neutrality in the cell. This process of fermentative hydrogen production is dominated by the bacteria involved in the process comprising mainly of two types (a) facultative and (b) strictly anaerobic bacteria.

4.1. Facultative anaerobic bacteria

Facultative anaerobic bacteria are gram-negative, rod shaped, with relatively simple nutrient requirements (16). The anaerobic bacteria that can produce H_2, particularly belong to the family *Enterobacteriaceae* such as *Escherichia* (*E. coli*), *Enterobacter (E. aerogenes)*. These bacteria ferment sugars to a variety of end products like acetate, formate, lactate, succinate, ethanol, CO_2 and H_2.

Table 3. Bacterial strains used for biohydrogen production

Pure bacterial strains	Substrates	References
Clostridium sp. *Clostridium butyricum* CWBI1009, *Clostridium perfringens,* *Clostridium perfringens* ATCC 13124 *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564) *Clostridium butyricum* EB6 *Clostridium strain* BOH3	Glucose, Xylose, Rice bran, Pome sunflower stalks xylan	(32- 37)
Enterobacter sp. H1, *Enterobacter cloacae* IIT-BT 08 *Enterobacter* sp. H_2 *Enterobacter aerogens* ATCC 13048 *Enterobacter aerogens* *Enterobacter aerogens* MTCC 2822	Glycerol, Microalgal biomass Distillery effluent Glucose Biomass waste Cheese whey	(22,38-41)
Escherichia Coli *Escherichia coli* (XL1-Blue) *Escherichia coli* strain HD701 *Escherichia coli* WDHL *Escherichia coli* O157:H7	Glucose, oil palm frond juice and sewage sludge, glucose and glycerol, glycerol Molasses	(42-46)
Citrobacter sp. Y19 *Citrobacter* sp. CMC-1 *Citrobacter* sp. S-77 *Citrobacter freundii* CWBI952 *Citrobacter amalonaticus* Y19 *Citrobacter freundii*	Glucose , glucose, xylose, galactose, mannose, arabinose and rhamnose, oxidation of carbon monoxide (CO),	(47-50)
Klebsiella pneumoniae ECU-15 *Klebsiella* sp. *Klebsiella* sp. HE1 *Klebsiella oxytoca* HP1 *Klebsiella pneumoniae* TR17 *Klebsiella* sp. TR17	Glucose, Glycerol ,xylose and bamboo stalk hydrolysate, crude glycerol of biodiesel plant Glycerol	(51-55)

The degradation of organic matter in anaerobic environments by these microbial consortia involves the cooperation of population of microorganisms that generate a stable, self-regulating fermentation process. The facultative bacteria form pyruvate through glycolysis from carbohydrate rich substrates; these bacteria generate acetyl-co A and formate through the enzyme Pyruvate Formate Lyase (PFL). Formate is then converted to hydrogen and carbon dioxide through enzyme activity (17).

4.2. Strictly anaerobic bacteria

Strictly anaerobic or obligate bacteria live in completely anaerobic environment. Even a little amount of oxygen becomes too toxic for their growth. In obligate anaerobic bacteria *Clostridium* sp has been widely studied for hydrogen production. Genus *Clostridia* is a group of spore forming bacteria, which have the ability to sustain in adverse conditions (18, 19). All *Clostridia* lack-cytochrome system. In the anaerobic oxidation of carbohydrates, electrons are generated and they need to be disposed off, to maintain electrical neutrality of the cell. In the oxidative breakdown of carbohydrate, NADH-ferredoxin reductase functions as an electron carrier and facilitates the oxidation of pyruvate to acetyl Co-A and CO_2 as well as to reduce proton to molecular hydrogen. The EMP or glycolytic pathway is applied to convert glucose into pyruvate and further to Acetyl Co-A associated with the transformation of NADH form NAD^+, in reoxidation of NADH under anaerobic condition, in the presence of ferredoxin oxidoreductase and hydrogenase. *Clostridium sp*. can produce 2 mol of hydrogen when 1 mole of n–Butyrate is the end product and 4 mole of H_2 with 2 mol of acetate from 1 mole of hexose (17).

Table 3, representing the pure strains of bacteria belongs to the facultative and strictly anaerobic type, which have been used by various researchers in previous studies with different substrates.

5. FACTORS AFFECTING BIOHYDROGEN PRODUCTION

5.1. Substrate composition

In most of the studies for biohydrogen production, simple sugars such as glucose or sucrose have been used as model substrates. Only a few studies (20, 21), have looked into agricultural waste, industrial waste such as distillery wastewater (22), dairy industry wastewater, food processing and beverage industry (23,24), rice slurry waste water (25) etc. Besides the industrial waste water, agriculture waste such as wheat straw, corn straw (26), sugar cane molasses (27) are

also used for anaerobic fermentation. These forms of organic material seem to be the potential substrates for sustainable biohydrogen production. The constituents of all these substrates are carbohydrates, proteins and lipids which are the deciding components of biohydrogen yield from different substrates.

5.1.1. Carbohydrate

Carbohydrate rich substrates are the primary choice of hydrogen producing microbes. Waste from food processing industry such as potato processing industry, rice slurry, sugar and distillery industry, are rich in carbohydrate and have shown to be suitable for anaerobic fermentation (23-25).

During hydrolysis of carbohydrate rich substrates, hydrolytic bacteria produce simple sugars such as sucrose, glucose, xylose and hexose (28) and these simple sugars are further consumed by the anaerobic bacteria to produce biohydrogen.

5.1.2. Lipids

Source of lipids are food waste, food processing waste, oils and dairy products (29). Lipid hydrolysis is performed by the lipase enzyme found in some bacteria. Lipid hydrolysis results in generation of free fatty acids and glycerol that can be hydrolyzed to acetyl-CoA, acetate and hydrogen from NADH oxidation during the β-oxidation pathway (30, 31).

The process of hydrogen production from lipid hydrolysis is slower than carbohydrate hydrolysis. Hydrolysis of a lipid is inhibited by the accumulation of volatile fatty acids produced which causes decrease in pH of the medium (56).

5.1.3. Protein

Proteins are the polypeptides of amino acids. The source of protein for biohydrogen production consists of food waste, food processing waste from cheese whey, casein, fish meat chicken egg etc (57). In the hydrogen production from proteins, bacteria convert it into polypeptides and amino acids by protease enzymes (58), further amino acids are broken down to volatile fatty acid, carbon dioxide and hydrogen. However, there are very few studies on the use of proteinaceous substrates as biohydrogen production feedstock except by Xiao *et al*., (59) who proposed the pathway for biohydrogen production from protein.

5.1.4. Cellulose and lignocelluloses materials

Plants and agricultural biomass including fruits and vegetable waste are the good sources of cellulose, hemicelluloses and lignocelluloses materials. They contain different kinds of sugars that can be used for biohydrogen production (59). The only problem with this biomass lies in the fact that cellulose is hardly degradable by microbes due to its crystalline structure (60). It requires some pretreatments such as steam explosion, chemical treatment, acidic or alkaline treatment to break rigid structure of cellulose hemicelluloses and lignocelluloses materials to release sugars (61).

5.1.5. Pure/synthetic substrate

Biohydrogen production has not been applicable for industrial level till now and most of the studies are still going on for biohydrogen production at lab scale with pure substrate such as use of glucose, cellulose cellobiose, arabinose, starch, xylose, sucrose and glycerol. Among these, glucose, sucrose and starch are more common substrates of interest while some researchers have also worked with glycerol (shown in Figure 1A). However, uses of pure substrates for biohydrogen production are more expensive. Figure 1 (A & B) shows the graphical representation of research accomplished on pure and waste substrates according to literature availability.

5.1.6. Waste materials as substrate

Besides pure substrate many other materials such as industrial wastewater, sludge, municipal solid waste, agriculture waste, domestic wastewater (62), paper mill wastewater (63), molasses based wastewater, glycerol based waste water (64), chemical wastewater, dairy industry process waste, distillery wastewater (22,65) have been well studied for biohydrogen production.

5.2. Temperature

Temperature is an important operational parameter for fermentative hydrogen production as the anaerobic bacteria are more sensitive to temperature regime. The anaerobic fermentation process lies among four temperature ranges, ambient (15-30°C), mesophilic (30-39°C), thermophilic (50-64°C) and hyperthermophilic (>64°C) (5).Change in temperature ranges highly affects the H_2 production rate in general and consumption of substrate in the process, biohydrogen yield, formation of metabolites in the form of volatile fatty acids and presence of microbes in the system. Although several studies have been done for biohydrogen production with the variations in temperature but mesophilic temperature is more favorable condition in all other aspects of temperature due to its technical features as well as being less expensive (66-68). However, as per our review on data, one negative aspect of this condition is that, it also favours the growth of some non-hydrogen producing microbes.

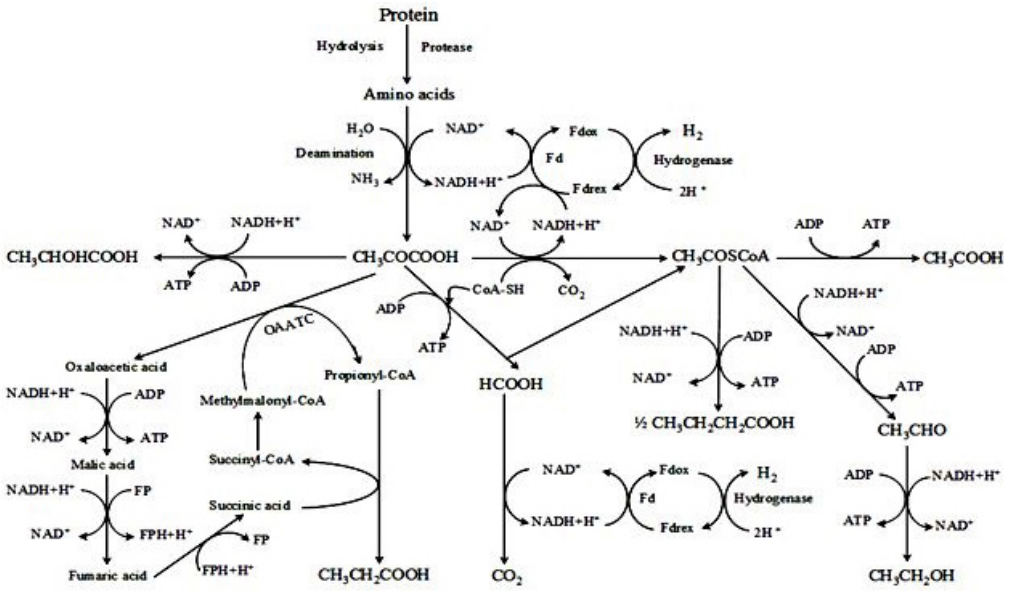

Figure 1. Substrates used for biohydrogen production (A) Pure substrates, (B) Waste materials.

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Studies have revealed that thermophilic and hyper-thermophilic bacterial cultures are more proficient in hydrogen production than mesophilic. The highest yield reported by the thermopiles is 4 mol H_2/mol glucose which is very close to the theoretical yield (69). In case of agricultural biomass, it has been recently reported that mesophilic bacteria are unable to use cellulose directly for hydrogen production; an addition of exogenous cellulase enzyme is required for bacterial hydrolysis. On the other hand, thermophilic anaerobic bacteria effectively utilize cellulose without the addition of exogenous cellulase (70). In thermophilic condition hydrolysis rate of substrates is also high (71). After extensive literature survey, it has been found that among thermophiles, *Thermoanobacterium* sp. and in mesophiles, *Clostridium* sp. and *Enterobacter* sp. are the most popular species of bacteria for hydrogen production (reviewed in Figure 2).

5.3. pH

There is a significant effect of pH on fermentative hydrogen production as it is a deciding factor for the acidic and alkaline condition, limits the growth of bacteria and governs the concentration of the solvent in the system (Table 4). Ma *et al*., (56) reported that the optimum range of pH for the hydrogen production is 5.5.-6.5,and at this pH maximum gas production and least solvent production occurs. Another significance of the pH is its effect on the activity of enzyme (Fe-Fe) hydrogenase, as low pH affects this enzyme's activity and inhibits the hydrogen production (72, 73). Another reason behind the inhibition of hydrogen production at low pH, is the presence of a number of protons generated by the breakdown of organic acids. These ions have the ability to enter in the cytoplasm of bacteria via cell membrane and inhibit their growth.

5.4. Volatile Fatty acids

Volatile fatty acids are produced in the form of different solvents in fermentative hydrogen production process (57, 83). Most of the fatty acids are produced by the hydrolysis process in the acidiogenic phase. These acids include acetic acid, propionic acid, isobutyric, butyric acid, lactic acid and ethanol. Their concentration distribution and fractions can be used to monitor the fermentative hydrogen production system. In anaerobic treatment process, the drop in pH occurs

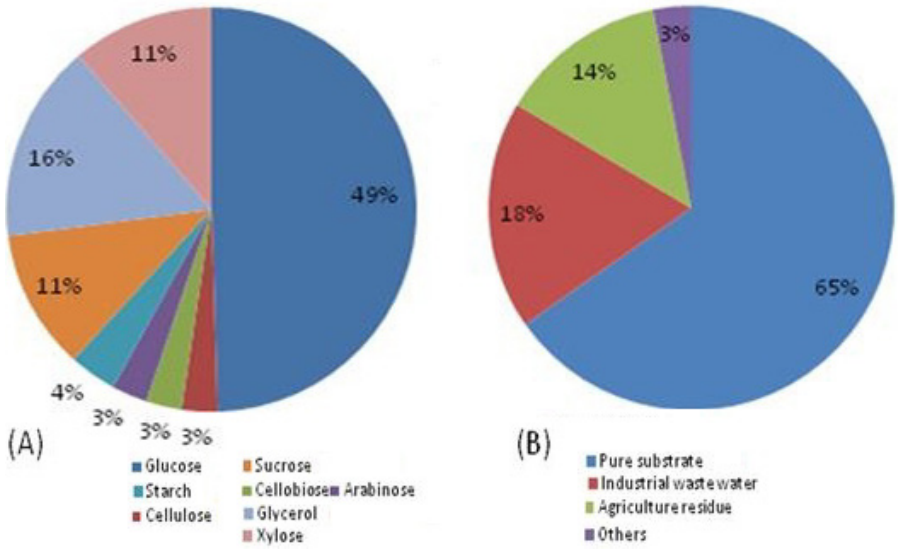

Figure 2. Bacterial strains used for biohydrogen production (A) Thermophilic, (B) Mesophilic.

Table 4. Hydrogen production from various substrates at optimized pH conditions

Substrates	Optimum pH	References
Glucose	6.0	(59)
Cheese Whey	6.0	(75)
Glucose and Peptone	7.0	(50)
Glucose	6.5	(76)
Glucose	6.5	(77)
Agro industrial waste	6.65	(78)
Mixture of olive mill wastewater, cheese whey and liquid cow manure	6.0	(79)
Organic fraction of cafeteria food waste	5.5	(80)
Anaerobic digested sludge	7.1	(81)
Food industry waste	7.0	(82)

due to either accumulation of VFA or excessive generation of CO_2 or both.

The processes occur for the formations of these acids from glucose are as follows (86)

a) Acetic acid production from glucose

$$C_6H_{12}O_6 + 2H_2O + H_2 \longrightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (7)$$

b) Butyric acid production from glucose

$$C_6H_{12}O_6 \longrightarrow CH_3CH_2COOH + 2CO_2 + 2H_2 \quad (8)$$

c) Lactic acid production from glucose

$$C_6H_{12}O_6 \longrightarrow 2CH_3CHOCOOH \quad (9)$$

d) Succinic acid production from glucose

$$C_6H_{12}O_6 + 2CO_2 + H_2 \longrightarrow 2(CH_2COOH) + 2H_2O \quad (10)$$

e) Formic acid production from carbon dioxide and hydrogen

$$CO_2 + H_2 \longrightarrow HCOOH \quad (11)$$

f) Ethanol from glucose

$$C_6H_{12}O_6 \longrightarrow 2CH_3CH_2OH + 2CO_2 \quad (12)$$

The identification of volatile fatty acids formed during the process gives valuable information about the type of metabolic pathway followed by the bacteria.

The VFA generation in fermentative hydrogen production process is also affected by change in temperature as at the higher temperature (45°C), acetate and butyrate concentration is higher (26-30%) than the mesophilic temperature (30-35°C) concentration of acetate, propionate and butyrate (20-

25%) (84,85). Ethanol concentration is also important in the estimation of liquid metabolites, as high ethanol concentration fraction (23-40%) were achieved at 30-45°C, which reduces the hydrogen production. Ethanol production consumes electron and favours the propionate formation by directly utilizing $H_{2,}$ which decreases the yield of H_2 (86).

5.5. Hydrogen Partial Pressure

Hydrogen partial pressure (HPP) is the concentration of hydrogen gas produced within the reactor during the biohydrogen production process and in excess it inhibits the process (8). The hydrogen partial pressure beyond 60 Pa does not favour the gas production and leads to the synthesis of alcohol. Increase in HPP leads to the lowering of H^+/H_2 ratio and inhibits the flow of electron so that the electrons from reduced ferredoxin to molecular H_2 via the hydrogenases system also get inhibited (15, 86). Several studies have shown the significant relation between the reactor temperature and hydrogen partial pressure. Favorable pressure reported so far for biohydrogen production are 50kPa at 60 °C, 20kPa at 70 °C 2kPa at 98 °C (87-90). The effect of hydrogen partial pressure on biohydrogen production can be decreased by sparging of inert gas like nitrogen in the reactor (91-93).

5.6. Enzymes

Enzymes play a major role in the fermentative hydrogen production process. A slight variation in the operating condition affects their activity very much. Two key enzymes take part in dark fermentative hydrogen production. These are Formate hydrogen lyase (FHL) and (Fe-Fe) hydrogenase; the details of these enzymes are given below:

5.6.1. Formate hydrogen Lyase (FHL)

In most of the facultative anaerobic bacteria, hydrogen production is catalyzed by the enzyme formate hydrogen lyase (FHL) (94). In anaerobic condition under acidic environment, formic acid is converted into hydrogen. This reaction is catalyzed by the formate hydrogen lyase. The FHL consists of formate dehydrogenase, hydrogenase and electron transfer carrier, responsible for formic acid oxidation to CO_2 and H_2. During the reaction, formic acid acts as an electron donor and proton are the only electron acceptor and thus leads to the formation of H_2.

5.6.2. Hydrogenase

Hydrogenase enzymes are classified into three groups based on the number and identity of the metal in their active sites such as (Ni-Fe), (Fe-Fe) and Fe- hydrogenase (95-97).On the basis of their active sites they all contain Fe and Co as a ligand to the Fe atom. Among all these, Fe-Fe hydrogenase is known to be the most potent in terms of fermentative hydrogen production. These enzymes are monomeric as in *Clostridium* or multimeric as in *Thermotogamaritima* and *Thermoanaerobactertengcon-genesis* that consist of three and four subunits, respectively. The (Fe-Fe) hydrogenases are organized into modular domains. The accessory cluster known as the F cluster, functions inter and intra molecular electron transfer centers (98). The accessory cluster is linked electronically to the catalytic cluster known as the H cluster. The (Fe-Fe) hydrogenase have the activity about 10-100 times higher than others hydrogenase. Genomic analysis shows that there is a great deal of varieties in (Fe-Fe) hydrogenase with some *Clostridia* containing a large number of hydrogenase with different modular structure (99).

5.7. Substrate inhibition

Fermentation requires a higher organic loading rate to carry out an energy efficient operation, though initial substrate concentration is also an important factor to activate the germination process and in prevention of re-sporulation. However, initial substrate concentration within optimum range enhances the hydrogen production in dark fermentation process. While high substrate concentration may cause unfavorable conditions for the process, by causing variation in the pH, concentration of VFAs and hydrogen partial pressure of the reactor. Hence, an optimum range of initial substrate concentration is required to minimize the substrate inhibition. Inhibition by the substrate concentration has been reported in literature but most of the reported studies mainly focus-on the carbohydrate sources (such as glucose, sucrose, starch or xylose), and only few studies have explored the use of real organic waste and wastewater as a substrate for dark fermentation process (103-104). Majority of these studies were performed in batch mode with an initial substrate concentration of 1-50 g (COD)/L and suggested that concentration beyond 20 g (COD)/L may decrease H_2 production via substrate inhibition (105). However, a defined level of substrate inhibition is not consistent in dark fermentation process, since various factors are responsible to cause this inhibition.

Substrate inhibition can be avoided by controlled addition of substrate such as fed batch reactor operation, which maintains the high biomass concentration in reactor. In contrast to this approach, high organic load can be handled in granular sludge based dark fermentation process. Optimization of microbial community is also significant to reduce the substrate inhibition such as, presence of aerobic bacteria like Bacillus sp. *Enterobacter aerogens*, etc. can stimulate the microbial activity of hydrogen producers.

5.8. Inhibition by macronutrients

The inorganic constituents, like N and P are essential macronutrients for the growth of bacteria but, they can also cause inhibitory action to reduce the biohydrogen production process in some circumstances. For instance, high ammonical nitrogen concentration and high C/N ratio in feedstock inhibit the dark fermentation process (105). Nitrogen induced inhibition is more frequent in case of organic substrate obtained from animal manure which contain high concentration of ammonical nitrogen that interfere with intracellular pH and inhibit the enzymatic activity required for biohydrogen production process (106). In dark fermentation process, ammonical nitrogen remains in ionized state due to acidic pH, which is less toxic in nature hence there should be other inhibitory mechanism in this case. Anaerobic fermentative microorganisms consume carbon 25-30 times faster than nitrogen, hence balancing of appropriate carbon to nitrogen ratio (C/N ratio) in the feedstock is highly required to avoid N induced inhibition. Despite this, wide range of optimum C/N ratio (5-200) have been reported in the literature for dark biohydrogen fermentation due to differences in operating conditions such as temperature, pH, inoculums, substrate etc (107).

Phosphate is another major inorganic macronutrient and constituent in pH buffer for microbial metabolism which affects the H_2 production in dark fermentation process. Thus, an optimum range of phosphate concentration is required to enhance the hydrogen production by reducing the lag phase of bacteria. According to a study, an optimized concentration of 600 mg/L of phosphate using N_2HPO_4 was found suitable to achieve highest hydrogen production, and addition or reduction of 30% N_2HPO_4 causes 40% reduction in hydrogen production (108). In case of dark fermentation process, the recommended dose of C/P ratio is about 130, however this optimum C/P ratio is influenced by presence of other inorganic constituent .Thus, to maximize the hydrogen production process proportion of carbon, nitrogen and iron should be optimized in selected feedstock.

In case of organic waste and wastewater, sulphate occurs, which can be reduced through sulfate reducing bacteria (SRB) to sulphide under anaerobic condition. Moreover, biodegradation of sulphur containing protein such as cysteine, and methionine also produce sulphide. Higher sulphide concentration is toxic and causes inhibition in hydrogen production in dark fermentation process by reducing the bioavailability of essential macronutrients and trace metals. In case of dark fermentation process lower pH (<6) is maintained to carry out the fermentation process, which causes the formation of hydrogen sulphide. Hydrogen sulphide has high ability to penetrate the microbial cell membrane and denature the cell protein. Sulphide mediated inhibition is less reported in case of dark fermentation process and a concentration beyond 100 mg/L can completely inhibit the dark fermentation process. On the other hand, an optimized concentration (< 25 mg/L) is essential to enhance the yield of dark fermentation process (109).

5.9. Inhibition by metals

Growth of microbial cell, enzymatic activity and metabolic pathway involved in hydrogen producing bacteria is highly influenced by low concentration of trace metals (110). However higher concentration of these metal ions is also responsible for inhibition of hydrogen production process involved in dark fermentation. High concentration of metal reduces the availability of nutrients and causes the destruction of membrane function (111). Effect of Fe and Mg has been explored by several researchers, because the presence of both is essential for hydrogenase enzyme (84). Hydrogenase, which is capable of catalyzing the oxidation of hydrogen or reduction of proton, can be classified in to (Ni-Fe) hyrogenase and (Fe-Fe) hydrogenase (100). (Ni-Fe) hydrogenase is widely distributed in bacteria, whereas (Fe-Fe) hydrogenase is restricted to some specific bacteria (101). This (Ni-Fe) hydrogenase is made up of two subunits (one small and one large) and contains 1 atom Ni and 12 atoms of Fe/molecule and contains clusters of Fe-S (102). In hydrogenase catalyzed hydrogen production process, electrons are transported through intra-molecular electron transport chain to the active site where proton is reduced and hydrogen is produced (103). Since Ni and Fe both are the fundamental elements of hydrogenase, concentration of both these metal may significantly influence the fermentative biohydrogen production process. Higher concentration of Fe was observed to form cell clumps, which limits the mass transfer. Limited iron concentration (< 10 mmol/L) for *Clostridium pasteurianum* was found to change the pattern of product during glucose fermentation and lactate was reported as a major product (112). On the other side with iron concentration up to 25mmol/L, metabolism of *C. acetobutyricum* was acidogenic and hydrogen was formed as the major metabolite. Trace concentration of Ni is required to activate the function of (Ni-Fe) hydrogenase, thus it is conducive for fermentation of hydrogen production (104). Influence of Ni^{2+} concentration on biohydrogen production was investigated by Wang and Wan (84). They achieved maximum hydrogen production (296.1ml/g-glucose) at 0.1. mg/L of Ni^{2+} concentration.

6. REACTORS FOR BIOHYDROGEN PRODUCTION

In recent years, several different reactors came in existence for biohydrogen production as shown in Table 5. With the advancement of technology,

Table 5. Different reactors available for biohydrogen production

Reactor type	Substrate used	OLR	Yield (H_2)	HRT/SRT	References
CSTR	Kitchen waste	20 g COD/ld	2 mmol/g(COD)	4d	(105)
	Rice winery wastewater,	34 g COD/ld	2.14mol/mol(hexose)	1d	(106)
	Corn starch	26.7 g COD/ld,	0.92 mol H_2/glucose	18h	(107)
	Fruit waste water	5-15 kgCOD/m^3d	5.4-4.2mol/kg(COD)	15-5h	(108)
UASB	Citric acid wastewater	10.0 to 75.0 kg COD/m^3 d	0.84 molH_2/mol hexose	12h	(109)
	Sludge of coffee drinking wastewater	-	1.78 mol H_2 to 2.76 l H_2/l	12h	(110)
	Coffee drink manufacturing wastewater	20 g COD/ld	1.29 mol H_2/mol hexose	6h	(112)
	Cheese whey	5- 20 g COD/ld	0.38 and 0.36 l H_2/l	6h	(113)
AnSBR	Alcohol waste water	68 kg/m^3 d	130ml H_2/g COD	21.3h	(114)
	Chemical waste water	6.3 kg COD/m^3 d	0.297 - 0.483 mol H_2/kg COD	24h	(66)
	Synthetic Sample	75 g-COD/ld	60–74 mLH_2/g COD	8h	(115)
	Carbohydrate rich substrate	22.5 g COD/ld	2.53 molH_2/mol sucrose	16h	(116)
	Synthetic wastewater	2.36 l/l h	4.34 mmol-H_2/g VSS h	4h	(117)
AnFBR	Sewage sludge	20 g COD/l	4.26 ± 0.04 mol H_2/mol sucrose	6h	(118)
	Glycerol waste	0.70 g/l d	-	-	(119)
	Extracted sunflower flour	9.3 g COD/l d	-	1.1d	(120)
EGSB	Starch waste water	1.0 g-starch/l d	1.64 l/l d	4h.	(121)
	Molasses	8 kg COD/m^3 d	3.47 mol/mol sucrose	1-6h	(122)
	Brewery wastewater sludge	5 g COD/l.	500 ml/d	13h	(123)
	Brown Sugar	97.2kg COD/m^3 d.	5.73l / l d	2h	(124)

these have become more specific to enhance the yield with utilization of different waste at various loading rate and hydraulic retention time. Among the reactors used for biohydrogen production, Continuous Stirred Tank reactors (CSTR) and Upflow anaerobic Sludge Blanket (UASB) reactor are still in more preference for industries due to high yield and less retention time and application of wide range of wastewater. The maximum yield reported are, 2.1.4 mol/mol hexose by CSTR while, 1.2.9 mol H_2/mole hexose by UASB from coffee drink manufacturing wastewater. Anaerobic sequencing bioreactor produces 2.5.3mol H_2/mol, sucrose from carbohydrate-rich wastewater, anaerobic fluidized bed reactor produces 4.2.6 mol H_2/mol sucrose from sewage sludge, and extended granular sludge bed reactor (EGSB) can produce 3.4.7 mol/mol sucrose from molasses.

7. STRATEGIES EMPLOYED TO ENHANCE BIOHYDROGEN PRODUCTION

Several attempts have been made to address the factors responsible for low H_2 production such as pretreatment technologies, process optimization and biochemical engineering *etc.* Various reviews do consider the pretreatment for enhancement of H_2 production, however very few authors have reviewed pretreatment of inoculum as well as substrate for improvement in H_2 production. Recent advances such as metabolic engineering, suppression of inhibitory factors, up gradation of H_2 through gene insertion are found as potential methods to induce the H_2 production through fermentation process. Different aspects of improvement methods are as follows:

7.1. Pretreatment of bacterial inoculums

The principle of inoculum pretreatment involves those hydrogen producing bacteria, which posses the ability to sporulate when passed through stress conditions of pH, temperature, radiation etc. and their resulting spore should be more resistant, so that they can survive in severe conditions during pretreatment. In contrast, methanogens are susceptible to severe conditions; consequently consumption of H_2 production in fermentation process is reduced due to inhibition of H_2 consumers and thus improves the biohydrogen yield. On the other hand, it is critically argued that pretreatment of inoculum also suppress non sporulating hydrogen producing bacteria (HPB) such as *Enterobacter aerogens* and some hydrogen consuming bacteria (HCB) like *Clostridium*

aceticum and *Clostridium thermoautotrophicum* which could survive in such extreme condition. Therefore, to remove the hydrogen consuming bacteria from the process, various pre-treatments of bacterial inoculums are employed such as heat pretreatment, alkali pretreatment, acid pretreatment, ultrasonic, chloroform, chemical treatment and combined treatment, *etc*. These are the most frequently used pretreatment methods to increase H_2 production yield (125-127). Among these, heat shock treatment and chemical inhibitor treatment are considered to be more effective and taken as a part of this section to study more specifically.

7.1.1. Heat-shock treatment

Heat shock treatment (HST) is employed for pretreatment of mixed bacterial culture to enrich the sporulating HPB. Most of the researchers have employed HST at 90-100°C for 15 to 20 minutes. However some researchers also observed HST by simultaneous increasing the temperature from 50 to 100 °C. These conditions cause the elimination of futile microorganism from reactor. Some hydrogen producing bacteria like *Bacillus* sp. and *Clostridium* sp. have the capacity to form spore under unfavorable conditions such as high temperature, presence of toxicant and change in nutrients. They can germinate again when environmental conditions become benign to them (128). This fact has been used in several studies to remove the hydrogen consuming and methane producing bacteria for specific studies. Besides inhibition of hydrotrophic and methanogenic bacteria Dong *et al.* (129) reported inhibition of acetoclastic methanogens. In another study, a lactic acid bacterium was also found to be inhibiting at 90°C for 20 minutes (130). Some bacterial strain detects changes in temperature and change accordingly to survive in such harsh conditions. Consequently, endosperm form endures the high temperature range. Thus HST is also referred to selective enrichment of HPB having capability to survive on high temperature. In contrast to the above findings, some researchers have reported reduction in biohydrogen yield on HST such as inhibition of non-sporulating Enterobacter sp. Some researchers (131,132) have also reported that HST has temporary effect on suppression of HCB because methanogens grow again under suitable condition. Nonetheless, high energy input and partial improvement in biohydrogen makes the technology debatable. Hence, economic feasibility and technical viability of HST process needs to be optimized prior to the commercialization.

7.1.2. Chemical treatment

Inoculum pretreatment by using chemicals, mostly employed, acid/alkali treatment and addition of methanogen inhibitors such as chloroform ($CHCl_3$), potassium nitrate (KNO_3), iodopropane (C_3H_8I) and carbon dioxide (CO_2). These chemicals act upon specific sites of the hydrogen consuming bacteria and inhibit their growth. Chloroform ($CHCl_3$) prevents the functioning of corrinoid enzymes and inhibits the function of methyl co-enzyme M reductase that performs methanogenesis in methanogenic bacteria (133). Addition of acid and alkali cause lower pH and high pH respectively whereby methanogen and other non sporulating bacteria are unable to survive, consequently only spore formulating HPB survive with this change. This is because methanogens are susceptible to change in pH and their growth occurs within the pH range of 6.7.-7.5. However, some authors have reported that acid pretreatment supports some hydrogen consuming bacteria like *Propionibacterium acenes* (134), therefore acid pretreatment only suppress some selective HCB. Most common acids used in pretreatment are sulphuric acid, nitric acid, perchloric acid, hydrochloric acid in a range of 0.1to 6 M varying from pH 2 to pH 4, while most common alkali includes sodium hydroxide in a range of 1 to 8 M. The enhancement in H_2 yield after acid/alkali pretreatment should be compared with solubility of metabolites in fermentation process.

Other chemical processes such as ozonolysis are mainly reported for substrate pretreatment and need to be further investigated for microbial pretreatment. Some researchers have criticized ozonolysis process due to high cost expenditure therefore, further investigation of ozonolysis for microbial pretreatment and its economic feasibility is required. Some researchers have observed that inoculation of micro flora in substrate containing high organic load restrains the growth of methanogens or hydrogen consuming bacteria. Substrate containing high organic load causes load shock to the micro flora and this process is referred to as load shock treatment (LST). Compounds such as formate, VFA, CO_2 are formed with high organic load, which causes decrease in pH and inhibition of methanogenic activities. Though, this process is effective for improvement of hydrogen yield but its long term sustainability should be further investigated. In addition to these chemical pre treatment methods, various chemical inhibitors like bromoethenesulphonate (BES), chloroform, iodopropane, acetylene, linoleic acids, nitro compound etc. are widely investigated for improvement of biohydrogen production. BES has been widely employed for inhibition of methanogens in fermentative hydrogen production process. The mechanism behind this inhibition is explained (135-136). Researchers have explained that BES is a chemical analogue of CoM, which helps to inhibit the transfer of methyl group and its reduction in methane. The optimum range of BES varies from 10 to 50 ml/L and the duration of pre treatment ranges from 10 minute to 24 hour. Similar mechanism is also proposed for chloroform induced

Table 6. Various pure strains bacterial co-cultures for biohydrogen production

Cultural Conditions	Cultures	T (°C)	Substrate	Substrate concentration (g/l)	pH	Hydrogen Yield (mol/mol)	Hydrogen Production rate(l/l/d)	References
Co-cultures involves strict or obligate and facultative anaerobes,	*C.butyricum* and *E.coli*	37	Glucose	3.0	6.5	2.09	0.41	(158)
	Enterobacter aerogens and *C.butyricum*	37	Starch	-	6.5	2.0	-	(159)
	Enterobacter aerogens and C. butyricum	37	Sweet potato	-	5.25	2.7		(159)
	B.thermoamylovorans and C. beijerinckii L9.	40	Brewery yeast waste	18.75	-	91.6 (ml H_2) from a 80-ml co-culture	-	(160)
Cellulose degrading anaerobes and high hydrogen producers via fermenting simple sugars	*Thermoanaerobacterium-thermosacchrolyticum* GD17 and *C. thermocellum* JN4	60	Cellulose	5.0	4.4	0.8	0.01	(161)
	Clostridium butyricum-NRRL 1024 and Clostridium pasteurianum-NRRL B-598	30	Wheat starch	10	5.5	109 ml H_2 g TS	1.8	(162)
	C. acetobutylicum x9 and *Ethaniolegenes herbinese*	37	Microcrystalline cellulose	10	5.0	1.32	11.06	(163)
	C. thermocellum and *C. thermosacchro-lyticum*	55	Corn stalk waste	10	7.2	-	0.34	(164)
	C. thermocellum DSM1237 and *C. thermopalmarium* DSM 5974	55	cellulose	9	7	1.36	0.42	(165)
Aciduric hydrogen producing microorganisms and high hydrogen producers	*Enterobacter aerogens* W23 and *Candida matosa* HY 35	35	Glucose	5	6.5	2.59	6.27	(166)

inhibition of methanogenic bacteria. Chloroform limits the activity of corrinoid enzyme and causes the reduction of methyl group to methane. Despite having potential to inhibit the methanogenic activity of various chemical inhibitors, most of these chemicals are not experimentally evaluated to improve the fermentative biohydrogen production.

7.2. Application of co-culture

There are many advantages of using co-culture of bacteria over the single strain for hydrogen production. From the economic point of view, co-cultures provide better anaerobic conditions for strictly anaerobic hydrogen producers and eliminate the need of an expensive reducing agent to remove O_2 present in the reactor. Similarly, presence of single strain of hydrogen producing bacteria, can only occupy the single feature such as strictly anaerobic bacteria cannot survive in the presence of even at slight amount of oxygen while facultative can do. However, a combination of different bacteria that can retain several hydrolytic enzymes and the co-culture of bacteria, which can exist in wide range of acidic and alkaline conditions, give the feature of enhanced hydrogen yield in co-culture system. Elsharnouby *et al*. (132) in his study provides a platform for classification and combinations of possible co-cultures of different bacteria. Firstly, co-culture of facultative and strictly anaerobic bacteria to eliminate the oxygen toxicity. Secondly, co-culture of cellulose degrading bacteria with high yield producing bacteria and thirdly, co-culture of Aciduric hydrogen producing bacteria with high hydrogen producing bacteria, as given in Table 6. This table explains the potential of co-culture with other important operational parameters, after reviewing the several research reviews and experimental studies available on scientific database.

7.3. Engineering tools involved in process

Recently through the observation of various studies available on biohydrogen production, it is found that use of biochemical engineering is an efficient tool to enhance biohydrogen production in the anaerobic system. With some limitations, these tools can be applicable only in certain areas of the process such as, in biohydrogen production pathway, in enzyme hydrogenase and in some microbes which can be used in the process after some genetical modifications.

7.3.1. In pathway

In the fermentative hydrogen production, glucose is oxidized in two steps:1) glyceraldehydes 3-phosphate to 1,3-biphosphoglycerate and., 2) Pyruvate to acetyl –Co A. The metabolic engineering required to increase yield of H_2 is so for possible at the pyruvate step (132).

Three types of biochemical reactions are involved in the generation of H_2 biologically. First one is found in the family of *Enterobactereacae (*134,135), where it employs two major enzymes viz. Pyruvate formate lyase (PFL) and Formate hydrogen lyase (FHL) to mediate biohydrogen production (101). PFL acts upon splitting of Pyruvate into acetyl-Co A and formate in anaerobic condition whereas FHL cleaves formate to H_2 and CO_2.The second type of H_2 producing reaction involves pyruvate ferredoxin oxidoreductase (PFOR) and Fd-dependent hydrogenase (hyd-A) (98).

$$Pyruvate + CoA \xrightarrow[PFL]{} Acetyl - CoA + Formate$$
$$Formate + \xrightarrow[FHL]{} H_2 + CO_2 \quad (13)$$

$$Pyruvate + CoA + 2Fd_{ox} \xrightarrow[PFOR]{} Acetyl - CoA + CO_2 + 2Fd$$
$$2Fd_{rd} + 2H^+ \xrightarrow[HydA]{} 2Fd_{ox} + H_2 \quad (14)$$

$$Glucose + 2NAD^+ \xrightarrow[EMP]{} 2\ Pyruvate + 2NADH$$
$$2NADH + 4Fd_{ox} \xrightarrow[NFOR]{} + 2NAD^+ 4Fd_{rd} \quad (15)$$
$$4Fd_{rd} + 4H^+ \xrightarrow[HydA]{} 4Fd_{ox} + 2H_2$$

Then, in third type reaction of hydrogen production, NAD (P)H is utilized by bacteria to evolve H_2. This reaction is catalyzed by two major enzymes: NAD (P)H-ferredoxin oxido-reductase (NFOR) and HydA (133). So far, all the systematic and quantitative analysis of pathway approach to evolve more H_2 by increasing flow of electron to H_2 producing pathway by increasing substrate utilization efficiency and investigation of more efficient and oxygen resistant enzymes. The metabolic engineering in native hydrogen producing pathway mainly focuses on the increase of yield by maximum utilization of carbon source. This includes over expression of several enzymes and redirection of carbon flux by eliminating competitive reaction in production pathway.

The process discussed in equation 13-15 gives the maximum theoretical yield of 2 or 4 mole H_2 as per the presence of facultative and strictly anaerobic bacteria. But in several extreme thermophiles 3.3. to 4 mol H_2/mol of glucose can be achieved naturally (136). These bacteria utilize both NFOR and PFOR for H_2 production (136,137). From the thermodynamic perspective, the H_2 production from NAD(P)H is unfavorable but the high yield indicates that NFOR and HydA function efficiently in some thermophilic bacteria at elevated temperature.

7.3.2. On enzyme hydrogenase

Many early attempts to express (Fe-Fe) hydrogenases in *E.Coli* by over expression of hydA from an organism such as *Clostridium* were unsuccessful and have remained unreported. Later it was shown that in order to co-express maturation gene hydE, hydF and hydG that are required for H-cluster maturation, insertion of the organism does not possess these enzyme (138,139). On the other hand, heterologous expression of hydA is simpler and possible without the heterologous expression of the accessory genes if these are encoded by the host genome. Some recent works reported for the expression hydrogenase gene hydA in *Enterobacter colace* IIT BT08, expressed high hydrogen yield from the strain of *E. aerogens* (ATCC 13408) which doubled the hydrogen yield (140).

7.3.3. In microbes

Various efforts have been made to enhance the biohydrogen production via genetic engineering application. Genetically modified bacteria such as *E. Coli (*140,141*) Clostridium* sp. (142,143) and some species of *Enterobacter* (144) were successfully used for high yield of biohydrogen. In this perspective *C. acetobutylicum* and *E. coli* are ideal strains because of the availability of appropriate genetic tools for gene knockout and gene over expression. The genetic expression in *C. acetobutylicum* to increase the H_2 production is regulated by antisense RNA. Bacterial strains of *Clostridium* are found to possess great potential for breaking cellulose in to hydrogen such as *C. cellulolyticum* and *C. populeti*. The property of these strains i.e. cellulose degrading pathway can be expressed in *C. acetobutylicum* to achieve highest hydrogen yield. The heterogonous expression of pyruvate decarboxylase and alcohol dehydrogenase from *Zymomonas mobilis* can be used to increase the cellulose degrading ability of *C. cellulolyticum.* Thus the approach of metabolic engineering enables the researchers to develop efficient strains to improve the biohydrogen production. However, practical viability of these improved strains should be investigated for their long term sustainability.

8. SECOND STAGE PROCESSES: ADVANCE APPROACH

Theoretically, a maximum 4 moles of H_2/ mol of glucose (~33% of substrate concentration) is possible during dark fermentation but only 2 mol H_2/ mol of glucose (~17% substrate conversion) is achieved during the process due to low conversion efficiency of the substrates. Recently, researchers are seeking for many hybrids, approaches to improve the hydrogen yield by

combining the dark fermentation with photo-fermentation/ Methanogenesis/microbial electrolysis of cells to get more substrate degradation efficiency (145,146).

8.1. Photo fermentation

The approach for the two phase bioenergy production system i.e. combination of dark and photo fermentation process with the aim of complete degradation of substrate to get maximum yield of 12 molH_2/mol hexose near to theoretical yield has been reported by various researchers (147,148). Improvement in biohydrogen yield from photo-fermentation process employed the various type of photo-bioreactor design (PBR) such as groove-type photo-bioreactor, multi-layered photo-bioreactor, flat panel, rocking photo-bioreactor. Besides photo-bioreactor design, operating modes of PBR also affects the yield of biohydrogen production. In this regard, sequencing batch reactor process offers benefit like high biomass retention process and ensures the maintenance of high biomass concentration. Xie *et al*., (149) have used sequencing batch reactor for first time to carry out the photo-fermentation process and expressed enhanced biohydrogen production. On the other hand, culture mode also determines the efficiency of photo-fermentation process. The most common culture mode utilized in photo-fermentation process is batch culture, however semi-continuous and continuous culture has also been studied by some researchers. In case of batch culture, decline of cells causes low biomass density and affects biohydrogen production yield. The semi continuous mode of culture has been found as the most favorable culture mode for photo-fermentation process but its optimization is still needed for further investigation (150).

8.2. Microbial Fuel Cell (MFC)

Bioelectrochemical processing provides a potential green technology for biohydrogen production, which comprises electrochemically active bacteria for conversion of organic matter into biohydrogen or other wide range of chemicals such as methanol, formic acid, methane, acetate, hydrogen peroxide *etc*. Likewise the photo-fermentation process, MFC technology is also considered as secondary stage process to achieve more energy recovery and high extent of substrate degradability. In MFC, the anode respiring bacteria oxidize the organic substrate and released electrons travel to cathode by employing an external circuit, thus power is produced in this process. It is a well established fact that MFC is considered as efficient and cleanest technology for biohydrogen production, despite the fact that MFC technology is still in developing stage and needs more advancement and innovation. The major obstacles in commercialization of MFC are low biohydrogen yield, high internal resistance, complicated design and high expenditure (151). Recently various researchers have introduced significant advancement in MFC application to improve the hydrogen yield as well as cost reduction by employing micro-fluidic MFC (152), integrated approach for pollution reduction and energy production, nano technology, low cost material in MFC design, use of active bio-cathodes etc. In comparison to conventional MFC design, micro-fluidic MFC is efficient, inexpensive and produces high energy output (152). Basically, it is a small carbon neutral device consisting of self organized bacteria to oxidize organic substrate. Small size of micro-fluidic MFC offers various advantages such as high surface to volume ratio, quick response to reactant, compatibility with easy micro-fabrication etc. Further improvements like cell culture optimization and electrode surface modifications in micro fluidic MFC are proposed by researchers to reduce the operational cost and increase the energy output MFC technology coupled with wastewater treatment is found more promising than its single application for energy production. Most of the recent studies are focused on anodic treatment of pollutants like azo dyes, polyaromatic hydrocarbons (PAHs), derivatives of benzene and other aromatic compounds (153,154). The MFC was found to have potential to remove pollutants such as COD, and ammonia by 89% and 98% respectively (153).However, its efficiency for digestion of solids is generally low. On the other hand, cathodic treatment for wastewater, heavy metals, organic substance like chlorobenzene and trichloroethylene have also been well explored by various researchers (155). Though, combine wastewater treatment and energy production shows advantages over conventional MFC application, it needs pilot scale study to explore field challenges. Reliance on unsustainable materials for MFC operation is another challenge in the way of commercialization of MFC technology. The development of low cost material competitive to platinum can improve the sustainability of MFC application (156). Application of ceramic material as a part of MFC is a pioneer research in this direction , which was further explored to improve the efficiency of earthen pot based MFC design (157). These development have demonstrated efficient MFC application, however important considerations like using sustainable materials for MFC design and their assessment should be taken into account.

9. CONCLUSION

The study concludes that after critical review of extensive literature on the production features of biohydrogen by the dark fermentation process, it is the most feasible approach than other established technologies of hydrogen production. But simultaneously, it also faces many challenges like efficient use of substrates, suitable microbes, bioreactors, process parameter optimization *etc.* which make–this technology very challenging. The

study also revealed that there are so many options such as engineering in enzyme hydrogenase, use of genetically modified bacteria and integration of second stage approach with fermented substrates that can become a remedy for appropriate use of this technology at a large scale. The use of waste materials for hydrogen production is another very valuable idea than use of pure substrates for hydrogen production in terms of energy recovery and treatments options. The identification of efficient bacteria which require least pre-treatments and use of pure facultative anaerobic bacteria to reduce the use of oxidative chemicals and co-culture application is also a more elegant approach to reduce the cost of this process. Hence, with the proper implementation of emphasized factors and simplifying the strategies to enhance biohydrogen production, this technology has the potential to be used for clean environment and future energy demands.

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