A critical review on factors influencing fermentative hydrogen production

Richa Kothari1, Virendra Kumar1, Vinayak V.Pathak1, Shamshad Ahmad1, Ochieng Aoyi2, V.V.Tyagi3

1Bioenergy and Wastewater Treatment Laboratory, Department of Environmental Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow (U.P.), India- 226025, 2Centre for Renewable Energy and Water, Department of Chemical Engineering Vaal University of Technology, South Africa, 3Department of Energy Management, Shri Mata Vaishno Devi University, Katra, Jammu and Kashmir, India-182320

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Types of fermentative hydrogen production processes
   3.1. Dark fermentation
   3.2. Photo fermentation
4. Types of bacteria used in fermentative biohydrogen production
   4.1. Facultative anaerobic bacteria
   4.2. Strictly anaerobic bacteria
5. Factors affecting biohydrogen production
   5.1. Substrate composition
      5.1.1. Carbohydrate
      5.1.2. Lipids
      5.1.3. Protein
      5.1.4. Cellulose and lignocelluloses materials
      5.1.5. Pure/synthetic substrate
      5.1.6. Waste materials as substrate
   5.2. Temperature
   5.3. pH
   5.4. Volatile Fatty acids
   5.5. Hydrogen Partial Pressure
   5.6. Enzymes
      5.6.1. Formate hydrogen Lyase (FHL)
      5.6.2. Hydrogenase
   5.7. Substrate inhibition
   5.8. Inhibition by macronutrients
   5.9. Inhibition by metals
6. Reactors for biohydrogen production
7. Strategies employed to enhance biohydrogen production
   7.1. Pretreatment of bacterial inoculums
      7.1.1. Heat-shock treatment
      7.1.2. Chemical treatment
   7.2. Application of co-culture
   7.3. Engineering tools involved in process
      7.3.1. In pathway
      7.3.2. On enzyme hydrogenase
      7.3.3. In microbes
Influencing factors for fermentative hydrogen production yield

8. Second stage processes: advance approach
   8.1. Photo fermentation
   8.2. Microbial Fuel Cell (MFC)

9. Conclusion

References

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 greenhouse 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 (H₂), 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 H₂ production via combined fermentation process. Though photosynthetic fermentation process shows higher theoretical H₂ 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 H₂ 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 H₂ 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% CO₂ could be produced at the expense of 10% of the energy value of produced H₂. 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 greenhouse gases and toxic byproducts; hence it seems to be more promising energy carrier than methane (Table 1) (6). Application of H₂ 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
Influencing factors for fermentative hydrogen production yield

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

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

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. *Clostridium sp.*). With the evolution of molecular hydrogen (H₂) (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, 1 mol of glucose yields 4 mol H₂ by acetate pathway and 2 mol of H₂ through butyrate pathway, respectively (15):

\[ C_6H_{12}O_6 + 2H_2O + H_2 \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \]  (1)

\[ C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2COOH + 2CO_2 + 2H_2 \]  (2)

\[ \text{Pyrurate} + \text{CoA} + 2\text{Fd}_{(ox)} \rightarrow \text{Acetyl-CoA} + \text{Fd}_{(ad)} + \text{CO}_2 \]  (3)

\[ 2H^+ + \text{Fd}_{(ad)} \rightarrow H_2 + 2\text{Fd}_{(ox)} \]  (4)

\[ C_6H_{12}O_6 + 12H_2 + h_1 \rightarrow 2H_2O + \text{CO}_2 \]  (5)

\[ (\text{CH}_2\text{O})_2 \rightarrow \text{Ferredoxin} \rightarrow \text{Nitrogenase} \rightarrow H_2 \]  (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 capsulata*, *Rhodopseudomonas palustris*, *Rhodospirillum rubrum* etc.) by utilizing the organic carbon of feedstock/substrates. These bacteria possess 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 O₂ present in the system and maintain anaerobic conditions throughout the process.
Influencing factors for fermentative hydrogen production yield

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

<table>
<thead>
<tr>
<th>Processes</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermal Processes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural Gas Reforming</td>
<td>Most viable means of hydrogen production in present scenario due to presence of potential infrastructure support.</td>
<td>Capital cost is high in this technology.</td>
<td>Improve catalyst efficiency and reduction in process of cost is required; development of low-cost, efficient; separation/purification mechanism is also needed.</td>
</tr>
<tr>
<td>Bio-Derived Liquids Reforming</td>
<td>Futuristic fuel with existing infrastructure.</td>
<td>The technology is expansive and requires extensive optimization. Feedstock quantity and quality parameters affect the process.</td>
<td>Low temperature and liquid phase catalysts is needed. Characterization of biomass is required.</td>
</tr>
<tr>
<td>Coal and Biomass Gasification</td>
<td>Low-cost fuel technology.</td>
<td>Feedstock impurities with carbon content affect the system’s efficiency.</td>
<td>Feedstock storage, preparation, and handling is the major hurdles. Emissions control measures are also required.</td>
</tr>
<tr>
<td>Thermo chemical Production Routes</td>
<td>Clean and sustainable route for energy production using solar and nuclear energy in integration with chemicals.</td>
<td>It has durability but in long term application mode as well as it is energy intensive technology.</td>
<td>Developments of thermo-chemical storage and heat transfer devices are needed.</td>
</tr>
<tr>
<td>Electrolytic Processes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water Electrolysis (Splitting Water Using Electricity)</td>
<td>This is pollution free device used to produce energy in more sustainable way.</td>
<td>Solar system can provide a better efficient system, but high cost involves.</td>
<td>Improved photocatalyst with multifunctional materials at low cost to assure uniform quality production designs is required.</td>
</tr>
<tr>
<td>Photolytic Processes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photo electrochemical Hydrogen Production route</td>
<td>Clean and sustainable technology with low temperature requirements.</td>
<td>Solar based technologies require high cost.</td>
<td>Long term technology for sustainable development.</td>
</tr>
<tr>
<td>Biological Processes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Direct bio photolysis</td>
<td>Integrated approach for H₂ production using sunlight with water.</td>
<td>Light intensity should be high and O₂ act as inhibitor in the reaction.</td>
<td>More R &amp;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.</td>
</tr>
<tr>
<td>Indirect bio photolysis</td>
<td>Use of blue green algae for hydrogen production from water.</td>
<td>Uptake of hydrogenates is removed.</td>
<td></td>
</tr>
<tr>
<td>Photo fermentation</td>
<td>Different range of light spectrum can be optimized to enhance the yield. A wide spectral energy can be used by photosynthetic bacteria</td>
<td>Nitrogenase enzymes get inhibited in presence of small amount of O and unionized ammonium₂. Light conversion efficiency is low.</td>
<td></td>
</tr>
<tr>
<td>Dark fermentation</td>
<td>Light independent process, several metabolites are produced in process as by-product, various substrate can be used.</td>
<td>This technology has relatively low H₂ yield, process, which makes the process thermodynamically unfavorable.</td>
<td></td>
</tr>
<tr>
<td>Two stage fermentation</td>
<td>Can relatively high H₂ yield, first stage metabolites can be converted to H₂ and CH₄</td>
<td>It requires continuous light source in Dark+ photo fermentation and pH control in anaerobic digestion processes</td>
<td></td>
</tr>
</tbody>
</table>

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 H₂, 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, CO₂ and H₂.
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 CO₂ 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 H₂ 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...
Influencing factors for fermentative hydrogen production yield

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 H₂ 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.
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 less expensive as well as technically features (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.

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, \textit{Thermoanobacterium} sp. and in mesophiles, \textit{Clostridium} sp. and \textit{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 acidogenic 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...
Influencing factors for fermentative hydrogen production yield

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

a) Acetic acid production from glucose
\[
\text{C}_6\text{H}_12\text{O}_6 + 2\text{H}_2\text{O} + \text{H}_2 \rightarrow 2\text{CH}_3\text{COOH} + 2\text{CO}_2 + 4\text{H}_2 \quad (7)
\]

b) Butyric acid production from glucose
\[
\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow \text{CH}_3\text{CH}_2\text{COOH} + 2\text{CO}_2 + 2\text{H}_2 \quad (8)
\]

c) Lactic acid production from glucose
\[
\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 2\text{CH}_3\text{CH(OH)}\text{COOH} \quad (9)
\]

d) Succinic acid production from glucose
\[
\text{C}_6\text{H}_{12}\text{O}_6 + 2\text{CO}_2 + \text{H}_2 \rightarrow 2(\text{CH}_2\text{COOH}) + 2\text{H}_2\text{C} \quad (10)
\]
e) Formic acid production from carbon dioxide and hydrogen
\[
\text{CO}_2 + \text{H}_2 \rightarrow \text{HCOOH} \quad (11)
\]

f) Ethanol from glucose
\[
\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 2\text{CH}_3\text{CH}_2\text{OH} + 2\text{CO}_2 \quad (12)
\]

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-
Influençando fatores para produção fermentativa de hidrogênio

25%) (84,85). Concentração de etanol é também importante na estimativa de metabolitos líquidos, como alta concentração de etanol na fração de concentração (23-40%) foram alcançadas em 30-45°C, que reduz a produção de hidrogênio. Produção de etanol consuma elétrons e favorece a propionate formação por diretamente utilizando H₂, que diminui o rendimento de H₂ (86).

5.5. Pressão Parcial de Hidrogênio

Pressão parcial de hidrogênio (HPP) é a concentração de gás de hidrogênio produzido dentro do bioreator durante a produção de hidrogênio fermentativo e em excesso inibe o processo (8). A pressão parcial de hidrogênio além de 60 Pa não favorece a geração de gás e leva à síntese da álcool. Aumento na HPP leva à redução do H⁺/H₂ ratio and inhibits the flow of electron so that the electrons from reduced ferredoxin to molecular H₂ 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 50 kPa at 60 °C, 20 kPa at 70 °C 2 kPa 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. Enzimas

Enzimas desempenham um papel principal na produção fermentativa de hidrogênio. A variação levemente na operação e amostra de hidrogênio fermentativo produzido afetam sua atividade muito. Dois tipos de enzimas são importantes na produção de hidrogênio fermentativo. Estes são Formate Hydrogen Lyase (FHL) e (Fe-Fe) hydrogenase; os detalhes dessas enzimas são dados abaixo:

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 CO₂ and H₂. During the reaction, formic acid acts as an electron donor and proton are the only electron acceptor and thus leads to the formation of H₂.

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 *Thermotoga maritima* and *Thermoanaerobacter tengcongensis* 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 hydrogenases with different modular structure (99).

5.7. Inibição do substrato

Fermentação requer um maior carga orgânica para carregar o operação, embora a 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 H₂ 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 H₂ 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 N₃HPO₄ was found suitable to achieve highest hydrogen production, and addition or reduction of 30% N₃HPO₄ 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) hydrogenase 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 Ni²⁺ 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 Ni²⁺ 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,
Influencing factors for fermentative hydrogen production yield

1.2.9 mol H$_2$/mole hexose by UASB from coffee drink manufacturing wastewater. Anaerobic sequencing bioreactor produces 2.5.3 mol H$_2$/mole, sucrose from carbohydrate-rich wastewater, anaerobic fluidized bed reactor produces 4.2.6 mol H$_2$/mole 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, upgradation 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 possess 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.
Influencing factors for fermentative hydrogen production yield

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 (CHCl₃), potassium nitrate (KNO₃), iodopropane (C₃H₅I) and carbon dioxide (CO₂). These chemicals act upon specific sites of the hydrogen consuming bacteria and inhibit their growth. Chloroform (CHCl₃) 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, CO₂ 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, iodopropionate, 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
Influence factors for fermentative hydrogen production yield

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

<table>
<thead>
<tr>
<th>Cultural Conditions</th>
<th>Cultures</th>
<th>T (°C)</th>
<th>Substrate</th>
<th>Substrate concentration (g/l)</th>
<th>pH</th>
<th>Hydrogen Yield (mol/mol)</th>
<th>Hydrogen Production rate (l/l/d)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-cultures involves strict or obligate and facultative anaerobes.</td>
<td>C. butyricum and E. coli</td>
<td>37</td>
<td>Glucose</td>
<td>3.0</td>
<td>6.5</td>
<td>2.09</td>
<td>0.41</td>
<td>(158)</td>
</tr>
<tr>
<td></td>
<td>Enterobacter aerogenes and C. butyricum</td>
<td>37</td>
<td>Starch</td>
<td>-</td>
<td>6.5</td>
<td>2.0</td>
<td>-</td>
<td>(159)</td>
</tr>
<tr>
<td></td>
<td>Enterobacter aerogenes and C. butyricum</td>
<td>37</td>
<td>Sweet potato</td>
<td>-</td>
<td>5.25</td>
<td>2.7</td>
<td>-</td>
<td>(159)</td>
</tr>
<tr>
<td></td>
<td>B. thermoamylovorans and C. beijerincki L9</td>
<td>40</td>
<td>Brewery yeast waste</td>
<td>18.75</td>
<td>-</td>
<td>91.6 (ml H₂) from a 80-ml co-culture</td>
<td>-</td>
<td>(160)</td>
</tr>
<tr>
<td>Cellulose degrading anaerobes and high hydrogen producers via fermenting simple sugars</td>
<td>Thermoanaerobacterium-thermosaccharolyticum QD17 and C. thermocellum JN4</td>
<td>60</td>
<td>Cellulose</td>
<td>5.0</td>
<td>4.4</td>
<td>0.8</td>
<td>0.01</td>
<td>(161)</td>
</tr>
<tr>
<td></td>
<td>Clostridium butyricum-NRRL 1024 and Clostridium pasteurianum-NRRL B-598</td>
<td>30</td>
<td>Wheat starch</td>
<td>10</td>
<td>5.5</td>
<td>109 ml H₂ g TS</td>
<td>1.8</td>
<td>(162)</td>
</tr>
<tr>
<td></td>
<td>C. acetobutylicum x9 and Ethanionogenes herbine</td>
<td>37</td>
<td>Microcrystalline cellulose</td>
<td>10</td>
<td>5.0</td>
<td>1.32</td>
<td>11.06</td>
<td>(163)</td>
</tr>
<tr>
<td></td>
<td>C. thermocellum and C. thermosaccharolyticum</td>
<td>55</td>
<td>Corn stalk waste</td>
<td>10</td>
<td>7.2</td>
<td>-</td>
<td>0.34</td>
<td>(164)</td>
</tr>
<tr>
<td></td>
<td>C. thermocellum DSM1237 and C. thermopalmanur DSM 5974</td>
<td>55</td>
<td>cellulose</td>
<td>9</td>
<td>7</td>
<td>1.36</td>
<td>0.42</td>
<td>(165)</td>
</tr>
<tr>
<td>Acidic hydrogen producing microorganisms and high hydrogen producers</td>
<td>Enterobacter aerogenes W23 and Candida matosa HY 35</td>
<td>35</td>
<td>Glucose</td>
<td>5</td>
<td>6.5</td>
<td>2.59</td>
<td>6.27</td>
<td>(166)</td>
</tr>
</tbody>
</table>

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 O₂ 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. Elshamouby 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.
Influencing factors for fermentative hydrogen production yield

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-CoA. The metabolic engineering required to increase yield of $\text{H}_2$ is so for possible at the pyruvate step (132).

Three types of biochemical reactions are involved in the generation of $\text{H}_2$ biologically. First one is found in the family of Enterobacteriaceae (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-CoA and formate in anaerobic condition whereas FHL cleaves formate to $\text{H}_2$ and CO$_2$. The second type of $\text{H}_2$ producing reaction involves pyruvate ferredoxin oxidoreductase (PFOR) and Fd-dependent hydrogenase (hyd-A) (98).

Then, in third type reaction of hydrogen production, NAD (P)H is utilized by bacteria to evolve $\text{H}_2$. 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 $\text{H}_2$ by increasing flow of electron to $\text{H}_2$ 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 $\text{H}_2$ as per the presence of facultative and strictly anaerobic bacteria. But in several extreme thermophiles 3.3. to 4 mol $\text{H}_2$/mol of glucose can be achieved naturally (136). These bacteria utilize both NFOR and PFOR for $\text{H}_2$ production (136,137). From the thermodynamic perspective, the $\text{H}_2$ 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 $C$. acetobutyllicum 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 $C$. acetobutyllicum to increase the $\text{H}_2$ production is regulated by antisense RNA. Bacterial strains of Clostridium are found to possess great potential for breaking cellulose in to hydrogen such as $C$. cellulyticum and $C$. populeti. The property of these strains i.e. cellulose degrading pathway can be expressed in $C$. acetobutyllicum to achieve highest hydrogen yield. The heterogenous expression of pyruvate decarboxylase and alcohol dehydrogenase from Zymomonas mobilis can be used to increase the cellulose degrading ability of $C$. cellulyticum. 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 $\text{H}_2$/mol of glucose (~33% of substrate concentration) is possible during dark fermentation but only 2 mol $\text{H}_2$/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\textsubscript{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 hydrogeen 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
Influencing factors for fermentative hydrogen production yield

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.

REFERENCES


Influencing factors for fermentative hydrogen production yield


20. Wong YM, Juan JC, Gan HM, Austina CM:Draft genome sequence of *Clostridium perfringens* strain jic, a highly efficient hydrogen producer isolated from landfill leachate sludge, *Genome Announcements* 2:64-14 (2014) DOI: 10.1016/j.ijhydene.2010.03.008


Influencing factors for fermentative hydrogen production yield

Energy, 39, 12524-12531 (2014)  
DOI: 10.1016/j.ijhydene.2014.05.081

DOI: 10.1016/j.ijhydene.2013.10.046

DOI: 10.1016/j.ijhydene.2014.02.139

DOI: 10.1063/1.4863085

DOI: 10.1016/j.procbio.2012.12.007

DOI: 10.1080/10643389.2011.604258

DOI: 10.1002/btpr.1644

DOI: 10.1016/j.fuel.2013.09.077

DOI: 10.1016/j.ijhydene.2016.07.244

DOI: 10.1007/s12010-011-9488-4

DOI: 10.1016/j.bbrc.2013.09.016

DOI: 10.1016/j.ijhydene.2014.02.050

DOI: 10.1016/j.ijhydene.2013.01.031

DOI: 10.1016/j.ijhydene.2014.02.077

DOI: 10.1016/j.foodcont.2013.05.027

DOI: 10.1016/j.ijhydene.2012.06.010

49. Ainala S. K, Seol E, Sekar B. S, Park S: Improvement of carbon monoxide-dependent hydrogen production activity in Citrobacter amalonaticus Y19 by over-
DOI: 10.1016/j.ijhydene.2014.04.148

DOI: 10.1016/j.ijhydene.2005.11.018

DOI: 10.1016/j.ijhydene.2010.03.084

DOI: 10.1016/j.ijhydene.2014.04.083

DOI: 10.1016/j.ijhydene.2012.06.022

DOI: 10.1016/j.jtice.2010.04.005

DOI: 10.1186/s13068-015-0322-z

DOI: 10.3390/ijms16048266

DOI: 10.1016/j.foodchem.2006.10.042

DOI: 10.1007/s00726-011-1151-4

DOI: 10.1016/j.biortech.2013.03.109

DOI: 10.1016/j.msec.2005.01.018

DOI: 10.1016/s0960-8524(03)00195-0

DOI: 10.1016/j.biortech.2011.04.059

DOI: 10.1.063/1.4.941649

DOI: 10.1016/j.biortech.2005.01.036

DOI: 10.1016/j.biortech.2005.01.036

Influencing factors for fermentative hydrogen production yield


82. Wang J, Wan W: Combined effects of temperature and pH on biohydrogen


Influencing factors for fermentative hydrogen production yield


Influencing factors for fermentative hydrogen production yield


132. Elsharnouby O, Hafez H, Nakhla G, Naggar M. H. E:A critical literature review on
Influencing factors for fermentative hydrogen production yield

DOI: 10.1016/j.ijhydene.2013.02.032

DOI: 10.1128/jb.00612-10

DOI: 10.1016/s0360-3199(02)00131-3

DOI: 10.1016/j.ijhydene.2009.05.053

DOI: 10.1016/j.jbiotec.2004.11.002

DOI: 10.1080/09593330903710244

DOI: 10.1093/nar/gkn751

DOI: 10.1111/j.1751-7915.2011.00282.x


DOI: 10.1016/j.mib.2011.01.004

DOI: 10.1128/aem.01484-10

DOI: 10.1073/pnas.1003584107

DOI: 10.1016/j.biortech.2011.06.018

DOI: 10.1002/bit.21297

DOI: 10.1016/j.ijhydene.2010.01.084

DOI: 10.1016/j.ijhydene.2009.02.078


production from cellulose in a co-culture of Clostridium thermocellum and Clostridium thermopalmarium, Bioresour Technol 101, 4029-33 (2010)
DOI: 10.1016/j.biortech.2010.01.042

DOI: 10.1016/j.ijhydene.2006.07.010

DOI: 10.1073/pnas.0706379104

Key Words: Biohydrogen, Fermentation, Substrate, Wastewater, Inhibitory Factors, Review

Send correspondence to: Richa Kothari, Bioenergy and Wastewater Treatment Laboratory, Department of Environmental Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow (U.P.), India- 226025, Tel: 522-2440822, Fax: 522-2440821, 2441888, E-mail: kothariricha21@gmail.com