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BIOHYDROGEN PRODUCTION – AN OVERVIEW OF THE FACTORS AFFECTING, MICROBES AND ENZYMES INVOLVED

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Abstract– Hydrogen is the lightest element and is an excellent energy carrier due to high energy intensity. Hydrogen can be produced in a number of ways like physical, chemical and biological methods. Since the physical and chemical method of hydrogen production is energy intensive, research is currently concentrated on the biological methods. Among the biological method dark fermentation has gained greater attention because of its independence to light and the ability to use various substrates for fermentation. In this review, there is detailed information about biohydrogen production by dark fermentation using microorganisms including, the hydrogen producing anaerobic and facultative anaerobic bacteria, the enzymes involved, the factors affecting the process such as temperature, pH, substrates, hydraulic retention time and hydrogen partial pressure. Also a brief discussion about the metabolic engineering aspects in biohydrogen production and a note on microbial electrolysis cell has been discussed.

INTRODUCTION

Hydrogen is a clean energy where the result of its combustion is only water droplets, hence it is also called as "Clean fuel". Other advantages of hydrogen are it has high energy yield (~122 kJ/g), low density and it has the ability to store more energy than the hydrocarbon fuels (Kapdan and Kargi, 2006). The physical and chemical methods of hydrogen production are costly and energy intensive which leads to the need for alternate methods of hydrogen production. Hence the biological methods gained its importance since they are eco-friendly. The Microorganisms play a key role in the production of hydrogen in biological methods where they use the carbon sources from the biomass and evolve hydrogen in return. Biohydrogen production can be carried out by the biological methods like Direct and Indirect Biophotolysis, Photo fermentation, Dark fermentation, Integrated dark and photo fermentation and Biocatalysed electrolysis. Among these methods, Dark fermentation is under limelight due to the advantage that it does not require light and its ability to use a variety of carbon sources. Hydrogen generation via fermentation is more suitable for the production of cleaner energy and the more efficient treatment of organic waste. Waste organic biomass is considered as the most appropriate renewable source for conversion to produce biofuels due to its high energy potential and abundancy (Sarangi and Nanda, 2020).

Biohydrogen production by dark fermentation

Dark fermentation is a complex process where the organic substrate is fermentative converted by diverse groups of bacteria which hydrolyse the complex organic polymers to organic acids and

alcohols. Biohydrogen production by dark fermentation involving either facultative or strict anaerobes has the ability to produce hydrogen as an intermediate product while converting organic substrates into volatile fatty acids and alcohol.

In dark fermentation the organic matter is degraded in an anaerobic bioreactor containing microorganisms to produce methane and carbon dioxide as the final products, where hydrogen is produced as an intermediate product. The microorganisms which ferment the organic matter are bacteria like hydrolytic, acidogenic, acetogenic, homoacetogenic bacteria and also methanogenic archaea. The biohydrogen production can be carried out by either pure or mixed culture. Mixed culture is advantageous because it does not need a sterile process. (Chang *et al.,* 2011; Tapia-Venegas *et al.,* 2015).

Dark fermentation is advantageous over other biological method because a wide range of organic compounds can be used as the substrate; it does not require light and also produces a comparatively high rate of hydrogen (Cardoso *et al.,* 2014). On the other hand, the limitations of dark fermentation are its low yield due to the formation of by-products like alcohol and volatile fatty acids (Pandu and Joseph, 2012). Recently, Lay *et al*. (2019) developed a new biologic method where they used the recycled rice husk as the support carrier thereby enriching the biohydrogen production with subsequent higher yield of about 200 ml/l.

Biochemistry of dark fermentation in biohydrogen production

Khanna and Das, (2012) have said that biohydrogen production takes place in two catabolic steps, which are decarboxylation of pyruvate into acetyl CoA which generates the reduced ferrodoxin which in turn acts as the electron donor for hydrogenase, the hydrogen catalyzing enzyme in organisms such as in *Clostridium sp* (Eq 1 and 2). The other pathway is the conversion of pyruvate and CoA into formate and Acetyl CoA (Eq 2 and 4). Here the cleavage of formate is catalysed by the enzyme Formate Hydrogen Lyase (FHL) and this mechanism takes place in Facultative anaerobic organisms such as *Klebsiella sp*.

Pyruvate + CoA + 2Fd(ox) → Acetyl CoA + 2Fd(red) + CO_2 .....(1)

2Fd(red) + 2H+ → 2 Fd(ox) + H_2 .....(2)

Pyruvate + CoA → Formate + Acetyl CoA .....(3)

Formate → CO_2 + H_2 .....(4)

Carbohydrate, especially glucose is the main carbon source for the biohydrogen production. The fermentation of glucose gives acetic acid or butyric acid as the end product along with biogas, when the metabolic pathway favours the production of acetic acid, 4 moles of hydrogen is produced from 1 mole of glucose (Eq 5) whereas only 2 moles of hydrogen is produced when the pathway favors the production of butyric acid(Eq 6) (Ghimire, 2015)

$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$ .....(5)

$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$ .....(6)

Dark fermentation and associated microbes

Hydrogen production by dark fermentation involves both strict anaerobes and facultative anaerobes. They can be spore forming and non-spore forming bacteria. When a mixed culture is used for hydrogen production, both type of bacteria are present.

Anaerobic bacteria

Clostridium sp. is the most common hydrogen producers, some of them are *Clostridium butyricum, C. acetobutyricum, C. beijerinckii, C. thermolacticum, C. Saccharoperbutylacetonicu, C. tyrobutyricum, C. thermocellum, C. paraputrificum*. The Clostridia species produce hydrogen gas during their exponential growth phase (Chong *et al*., 2009). Lin *et al*. (2007) compared *C. butyricum* ATCC19398, *C. acetobutylicum* M121, *C. tyrobutyricum* FYa102 and *C. beijerinckii* L9 strain for the hydrogen yield where all of them produced a significant amount of hydrogen gas and *C. beijerinckii* L9 yielded highest hydrogen gas of about 2.81mmol/mmol glucose. Collet *et al*.(2004) found that a significant amount of hydrogen gas was evolved during acetate fermentation of *C. thermolacticum*. Chen *et al*. (2005) isolated *C. butyricum* CGS5 from sewage sludge and analysed its hydrogen producing ability from sucrose based medium and found that the maximum hydrogen gas produced was 2.78 mol H_2/ mol sucrose. Wang *et al*. (2003) reported the production of biohydrogen by the organism *C. bifermentans* and the biohydrogen produced was from 4.6 to 15mmol H_2/g-dried solids. Shin *et al*. (2004) isolated *Thermoanaerobacterium thermosaccharolytium, Desulfotomaculum geothermicum* and *Thermotogales* strain from food waste and reported that they are hydrogen producing

organisms. Other bacteria called *Ethanoligenens harbinense B49* was studied for its ability to produce hydrogen with glucose as the sole carbon source and obtained the hydrogen yield of about 2.26 mol H_2/ mol glucose (Xu *et al*., 2008).

Facultative anaerobes

Facultative anaerobic bacteria produce energy by aerobic respiration in the presence of oxygen and in the absence of oxygen, they are capable of switching to the anaerobic fermentation process. *Enterobacter sp.* is the most common hydrogen producer among the facultative anaerobic bacteria (Chong *et al*., 2009). Chen *et al.* (2006) reported the production of biohydrogen by the facultative anaerobic bacterium *Klebsiella pneumonia* where the yield was 2.86 mol H_2/mol glucose. Kotay and Das (2007) isolated the strain *Bacillus coagulans* from digested activated sewage sludge and investigated for its ability to produce biohydrogen and they have found that the bacteria produces 2.28 mol H_2/mol glucose. Shin *et al*. (2007) examined the hydrogen producing ability of the organism *Enterobacter asburiae* and found that the yield was 174 ml/l/hr. The bacteria *Enterobacter cloacae* IIT-BT 08 was analysed for its ability to produce hydrogen on lignocellulosic solid matrices and a significant amount of hydrogen production was reported (Kumar and Das, 2001). In another study, Nath *et al.*(2005) used a different strain of *Enterobacter cloacae* which is *Enterobacter cloacae*strain DM11 along with photosynthetic bacteria *Rhodobacter sphaeroides* strain O.U.001 and produced a significantly high amount of biohydrogen.

Sawers, (2005) have said that production of hydrogen is common among *E.coli* and other *Enterobacteriaceae* and have studied about format and its dissociation into carbon dioxide and hydrogen by the enzyme formate hydrogen lyase. The hydrogen producing *Bacillus sp.* was isolated from food waste and confirmed by PCR- DGGE analysis (Shin *et al*., 2004). The facultative anaerobes *Lactobacillus delbrueckii* NBRC13953 was co-cultured with the photosynthetic bacterium, *Rhodobacter sphaeroides* where they produced hydrogen with the yield of 7.1 mol H_2/ mol of glucose (Asada *et al*., 2006).

Enzymes influencing biohydrogen production

According to Kim and Kim, (2011) enzymes play a key role in catalyzing the process of biohydrogen in dark fermentation. Hydrogenases are the enzyme that catalyzes hydrogen formation from the proton. Hydrogenases are of three types

1. [NiFe]-hydrogenase
2. [FeFe]-hydrogenase
3. [Fe]-hydrogenase

Among the above [NiFe]-hydrogenase constitute the larger number of hydrogenases and it is less sensitive to oxygen. It is more active in hydrogen oxidation and considered to be involved in hydrogen consumption. The active site of this enzyme has an iron and nickel ions connected by four cysteine residues to the protein. [FeFe]-hydrogenases are the enzymes involved in the evolution of hydrogen and they are found in anaerobic prokaryotes and in some eukaryotes. These are monomeric and contain only catalytic subunit which varies in size. They have a conserved domain called H cluster which contains the active site. The third type of hydrogenase is the [Fe] - hydrogenase and it catalyzes the CO_2 reduction with H_2 to methane. It is a metal-free hydrogenase called Iron-sulfur –cluster free hydrogenase.

Other than hydrogenase there are enzymes which also catalyze the hydrogen formation pathway, they are involved in three different reactions

1. Pyruvate: formate Lyase (PFL) and Formate Hydrogen Lyase(FHL): The PFL enzyme splits pyruvate into acetyl CoA and Formate. Later FHL catalyses the formation of H_2 and CO_2 from formate (Hallenbeck, 2005).
2. Pyruvate: Ferrrodoxin oxidoreductase(PFOR) and Fd-dependent hydrogenase (HydA): Under anaerobic conditions, PFOR catalyses the oxidative decarboxylation of pyruvate into acetyl CoA and CO_2, here the electrons are transferred to Fd_{ox}, then the reduced ferredoxin Fd_{rd} transfers one electron to proton to form H_2 in the presence of the enzyme HydA (Hallenbeck, 2005). This type of reaction usually takes place in *Clostridium sp.* (Mathews and Wang, 2009).
3. NAD(P)H: Ferrodoxin oxidoreductase (NFOR) and HydA: Here Ferrodoxin is reduced by the NAD(P)H, the reduced ferrodoxin then transfers an electron to proton to form H_2 by the enzyme HydA (Wang *et al*., 2010).

Factors affecting dark fermentation

Biohydrogen production by dark fermentation is affected by various factors such as temperature, pH, Substrates, hydraulic retention time and hydrogen partial pressure (Balachandar *et al*., 2013).

Temperature and pH

Temperature is one among the most widely studied parameter for the efficient biohydrogen production and it plays a key role in the fermentation process. The choice of temperature greatly depends on the type of microorganisms used like Psychrophilic ((0–20 °C), mesophilic (20–42 °C), or thermophilic (42–75 °C). In a fermentation process with mixed culture, the optimal temperature varies widely due to the presence of a complex bacterial population (Balachandar *et al*., 2013).

pH is also an important factor to be considered in the biohydrogen fermentation conditions. Khanal *et al*.(2003) have reported that the production of hydrogen takes place during the exponential growth phase, during the stationary phase the gas production shifts to solvent production. This accumulation of solvent brings the pH down to 4.5 or below which induces the shift. Hence if pH is not in the optimal range, there will be a microbial population shift which eventually affects the hydrogen production. Hawkes *et al*. (2002) reported that pH range between 5.5 and 6.7 gives a successful operation.

Wong *et al.* (2014) said that mostly hydrogen production was reported in the Mesophilic range between 20-45 °C because most of the organisms such as *Clostridium* and *Enterobacter* and *Bacillus* sp. which are hydrogen producers grow in this temperature range. Some of the examples are, Kim *et al.*(2008) have performed the bio hydrogen production with a constant temperature of 35 °C ± 1 °C and pH at 5:3 ± 0:1 throughout the process and obtained good results. In a similar study the bioreactor was kept at the temperature ranging from 33 °C to 41 °C whereas the maximum yield was obtained at 39± 1 °C and pH was optimized as 4.2 where the maximum value of hydrogen production and yield was obtained (Mu *et al*., 2006). Zhang *et al*. (2007a) maintained a temperature of 37 °C and pH of 5.5 for the production of biohydrogen from wastewater seed sludge. Hydrogen production was carried out with the immobilized sewage sludge in three-phase fluidized bed reactor with temperature and pH maintained at 35 °C and 5.8 and 6.8 respectively.

Hydrogen production at higher temperatures is also reported. Kotsopoulos *et al*. (2009) reported the highest hydrogen production of 2.47±0.15 mol H_2/mol glucose using a mixed culture system until the year 2005, where the temperature was hyper-thermophilic (70 °C) and the pH was maintained at 7-7.2 feeding glucose as the substrate.

Substrates

Dark fermentation has gained its fame for the advantage that the process uses organic matter to produce biohydrogen. A variety of substrates have been tried for the production of this clean energy by various researchers. Li and Chen, (2007) produced biohydrogen by dark fermentation of steam exploded corn straw using the organism *Clostridium butyricum AS1.209.* Likewise pretreated Corn stover also has been used for the production of biohydrogen and obtained the hydrogen yield of about 2.84 and 3.0 mol from the mixed sugars in the hydrolysate of neutral and acidic steam exploded corn stover biomass respectively (Datar *et al*., 2007). Acidification pre-treated corn-stalk have also been used for the production of biohydrogen and reported that the acidified corn stalk produced a significantly higher amount of hydrogen than the raw corn-stalk (Zhang *et al.*, 2007b).

Karlsson *et al.* (2008) experimented the biohydrogen production using a mixture of substrates such as slaughterhouse waste, food industry residue, and manure along with hydrolyzing yeast. Ivanova *et al*.(2009) have produced biohydrogen using the air-dried samples of sweet sorghum, sugarcane bagasse, maize leaves, wheat straw and silphium by the extremely thermophilic bacterium *Caldicellulosiruptor saccharolyticus* and they concluded that wheat straw was the best substrate with the highest yield of about 3.8 mol H_2/mol glucose. The biological hydrogen production ability was investigated for the substrates such as the organic solid wastes including rice, cabbage, carrot, egg, lean meat, fat and chicken skin The hydrogen percentages of the total biogas produced were 33.9-55.1%, 27.7-46.8% and 44.0-45.6%, using cabbage, carrot and rice respectively. The researchers have also concluded that carbohydrates produce a higher amount of biohydrogen than the proteins (okamoto *et al*., 2000). Researchers also used food waste as the substrate for the biohydrogen production and to study the factors affecting the process of fermentation (Lay *et al*., 2005). Food waste and sewage sludge was co-ingested in the fermentation process for the efficient biohydrogen production and obtained the maximum biohydrogen production of about 122.9 ml/g COD consumed (Kim *et al.*, 2011). In another study, researchers have used the kitchen waste as the substrate for the biohydrogen production and heat

treated biogas plant slurry as the inoculum under solid phase anaerobic digestion (Jayalakshmi *et al.*,2009). Recently in a study, biohydrogen was produced using duck weed as the substrate for dark fermentation and simultaneously used the fermentative waste for the production of microalgal lipids (Mu *et al*., 2006).

Many numbers of research is being carried out on the production of biohydrogen using animal wastes, which include the production of biohydrogen with the dairy cow waste slurry as the substrate, using the microflora naturally present in the slurry as inoculum, produced an amount of 392 and 248 ml H_2/l slurry at 60 and 75 °C respectively (Yokoyama *et al*., 2006). Fan *et al*. (2006) have reported the efficient conversion of wheat straw into biohydrogen by cow dung compost and obtained of about 56% of biohydrogen of the total biogas produced. Experiment has been done on biohydrogen production using cattle wastewater as the substrate and the inoculum from the sewage treatment plant sludge, cow dung compost, chicken manure compost and river sludge produced 309 ml H_2/mol hydrogen (Tang *et al.,* 2008). In some experiments the substrates such as cattle manure and waste is pre-treated before use, in such research, the acidification pre-treated dairy manure was used as the substrate for the biohydrogen production and obtained 31.5 ml H_2/ gr of total volatile substance (Xing *et al*., 2010). Pig slurry was also used as the potential biohydrogen producing substrate under hyper-thermophilic temperature and biohydrogen was produced (Kotsopoulos *et al*., 2009). Xylan, the biopolymer found in plants were also used as substrate for biohydrogen production using mixed bacterial cultures isolated from elephant dung (Saripan and Reungsang, 2014).

Hydraulic Retention Time (HRT)

HRT is the average length of time that the biohydrogen-producing substrate with inoculum should remain in the bioreactor for adequate reaction to take place and for subsequent biohydrogen production. HRT plays an important role in increasing the yield of biohydrogen from anaerobic fermentation. To attain a successful operation, HRT of 8 to 12 hrs is used generally (Hawkes *et al*., 2002) but the optimal HRT for the biohydrogen production differs from each other depending on the substrate and the bioreactor type (Zhu *et al*., 2008). Salem *et al*. (2018) investigated the effect of HRT on biohydrogen production with substrates such as Sucrose, Potato and Bean and they concluded that Sucrose and Potato showed higher hydrogen yield when HRT was decreased to 18hrs whereas bean showed higher yield when HRT was increased to 24hrs.

Hydrogen partial pressure

Hydrogen evolution pathway is sensitive to hydrogen accumulation and they are also subjected to end product inhibition. As the concentration of hydrogen increases, there happens a metabolic shift which favors the production of more reduced substrates such as lactate, acetone, ethanol, butanol or alanine and subsequent decrease in hydrogen production (Levin *et al*., 2004). Van Niel *et al*. (2002) studied the effect of hydrogen concentration on hydrogen production and growth of the extremely thermophilic *Caldicellulosiruptor saccharolyticus* in the chemostat. They observed that hydrogen was the most severe inhibitor of hydrogen production and growth when allowed to accumulate in the culture. They also reported that the hydrogen concentration of 5-10mM hydrogen in the gas phase leads to a metabolic shift to the formation of lactate. Mandal *et al*. (2006) reported that when the partial pressure of hydrogen was reduced from 760mm Hg to 380mm Hg, the hydrogen yield was increased from 1.9 mol H_2 / mol glucose to 3.9 mol H_2 / mol glucose using the organism *Enterobacter cloacae*.

Metabolic engineering approach for efficient yield

The major drawback in biohydrogen production by dark fermentation is its low yield, hence metabolic engineering is an efficient way to improve microbial hydrogen production. Metabolic engineering in biohydrogen production involves modification or elimination of carbon metabolic pathways, incorporation of a non-native pathway which favors hydrogen production and improving the hydrogen-producing enzymes in the microorganisms (Oh *et al*., 2011).

Maeda *et al.* (2007) manipulated the Formate hydrogen Lyase (FHL) gene in *E.coli* K-12, by controlling the regulators of the gene such that to increase the hydrogen production. By this approach they were able to get a 141 fold higher yield of hydrogen. Akhtar and Jones, (2008) have developed a synthetic operon which can simultaneously express gene encoding hydrogenase and hydrogenase maturation factors from a single plasmid. Yoshida *et al.* (2005) constructed a FHL over expressing strain by over expressing the FHL

activator gene and inactivation of FHL repressor. This modification resulted in 2.8 fold increase in biohydrogen production. Liu *et al*. (2008) inactivated the gene of acetate kinase of *Clostridium tyrobutyricum* which gave an 1.5 fold increase in hydrogen production. A New study revealed that the overexpression of the hydrogenase gene (*frhAGB*) in *Thermococcus onnurineus*, enhanced its oxygen tolerance. The recombinant strain was able to grow in the presence of oxygen and produce significant amount of biohydrogen (Lee *et al*., 2019). Other than engineering the organism, there was a research where an invitro synthetic enzymatic pathway was developed for complete conversion of glucose and xylose to H-$_2$ and CO_2. Researchers combined more than 10 purified enzymes into an artificial enzymatic system for action upon the substrate which greatly fastens the fermentation process. Glucose and xylose were converted as such to produce 2 hydrogen per carbon (Rollin *et al*., 2015).

Microbial Electrolysis Cell

Microbial electrolysis Cell (MEC) is an approach for the production of biohydrogen from various organic sources where the bacteria which are electrochemically active oxidize the organic matter and generate electron, proton and CO_2 (Kadier *et al*., 2016a). MEC are modified version of Microbial fuel cell (MFC) which is used for generation of bioelectricity. MEC has an anode and cathode arranged in single or double chambers, where the difference is that the double chambered MEC has a proton exchange membrane separating the chamber and for proton transfer (Khan *et al*., 2017). A typical MEC consist of an anode, cathode, a membrane, electrochemically active microorganisms and the power supply. At the anode, the microorganisms decompose the substrate into $CO_{2,}$ electron and proton. The electrons are transferred to the anode by the bacteria and the protons are released to the MEC solution, the electron then travels to cathode through the electronic wire with the help of power supply and combine with the protons in the solution and hydrogen gas is evolved (Kadier *et al*., 2016b). Thus the production and yield of biohydrogen can be increased by the integration of dark fermentation and microbial electrolysis cell.

CONCLUSION

Many countries started using the alternate fuel which is ecofriendly. Hydrogen is one such clean fuel which has a broad scope. Hydrogen production by biological process like dark fermentation is very promising way of production among all the other methods. However this process has been studied only at the laboratory level, whereas pilot scale studies are required for its wide usage. For scaling up and commercialization, the above mentioned parameters are to be widely studied and analysed.

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