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RESEARCH ARTICLE

BIOLOGICAL HYDROGEN PRODUCTION FROM RENEWABLE RESOURCES USING MICROBES

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ABSTRACT

Hydrogen is the fuel for the present and future, due to its recyclability and nonpolluting nature. Hydrogen generates no carbon based pollutants but produces water when it used as fuel. In comparison with fossil fuel, hydrogen has a higher energy yield also. Microbial hydrogen production provides some advantageous over the chemical process, it is clean, efficient and environmental friendly. Photochemical and fermentative systems are the two main system of microbial hydrogen production. Various types of biomass are considered as the source for bio-hydrogen generation such as sago industry waste, dairy wastes, palm oil mill effluent, rubber industrial effluent, agricultural waste and rice mill effluent can be used for hydrogen production through suitable bioprocess techniques. This review article summarizes types of biowastes, bio-processing strategies, types of microbes, microbial cultures to be used and the recent developments are discussed with their relative advantages.

INTRODUCTION

Today the global energy requirements are mainly focused on fossil fuels but the heavy dependence on fossil fuels has caused growing environmental concerns worldwide due to the release of carbon dioxide in the atmosphere resulting in global warming and climate change which may cause acidification of oceans, rising sea levels, extreme weather events, food shortages and biodiversity loss (**Balat and Balat, 2009**). Many approaches have been developed to generate alternative source of energy. Hydrogen is one of the most promising fuels for the future which is a clean energy source, ecofriendly and sustainable. Combustion of hydrogen results in pure water instead of CO_2 emissions, hence it does not contribute to green house effect. When the oil crisis broke out in 1970s, the technology started receiving attention, especially in biohydrogen production by photosynthetic process and fermentation. At present 40% of hydrogen is produced from natural gas, 30% from heavy oils and naphtha, 18% from coal, 4% from electrolysis and about 1% is produced from biomass (**Melis *et al.,* 2000**). However, the feasibility of a future hydrogen economy depends entirely on the development of efficient, large scale, pollution free and sustainable energy production systems.

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Based on the National Hydrogen program of the United States, the contribution of hydrogen to total energy market will be 8–10% by 2025 (**Armor, 1999**). Bio-hydrogen can be produced by aerobic, anaerobic and photosynthetic microorganisms using carbohydrate rich and non-toxic raw materials (**Kapdan and Kargi, 2006**). Raw material cost is one of the major limitations for hydrogen production. Utilization of some bio waste from various industries could be a source for biohydrogen production leads to the combination of waste treatment and energy production would be an advantage. In contrast, biological hydrogen is generated from biomaterials that are abundant, sustainable and most importantly rich in carbohydrate, such as biomass and agricultural waste through ecofriendly process. Production of biohydrogen from biowaste by microbes make a novel and promising advance to meet the increasing energy needs as an alternate for fossil fuels.

On the basis of these facts, this review focus on possible use of biowastes as the raw material, microbes, fermentation process and the current developments on bio-hydrogen production.

Hydrogen Production Methods

Hydrogen can be generated by several processes namely electrochemical, thermo-chemical, photo-chemical and Biological process. In 2003, Kim investigated a wide variety of gaseous, liquid and solid carbonaceous wastes as renewable sources for formation of hydrogen gas by steam reforming.

Electrolysis should be used in areas where electricity is inexpensive since electricity costs account for 80% of the operating cost of hydrogen production. In this, feed water has to be demineralised to avoid deposits on the electrodes and corrosion (**Armor, 1999**).

Biological hydrogen production can be classified into five different groups such as direct biophotolysis, indirect biophotolysis, biological water gas shift reaction, photo-fermentation and dark fermentation (**Levin *et al.*, 2004**). Among the process of hydrogen production, microbial hydrogen synthesis is gaining momentum because it is an energy saving process (**Nandi and Sengupta, 1998**). Microorganisms are capable of producing hydrogen through fermentation (**Fumiaki *et al.*, 1996**) and photosynthesis (**Hansel and Lindblad, 1998; Matsunga *et al.*, 2000**). Biological processes utilizing microorganisms are promising approaches for cleaner and sustainable production of hydrogen without generating any harmful byproducts (**Das, 2009**). Biological waste and wastewater treatment by anaerobic digestion is an economically and environmentally sustainable technology (**Noike and Mizuno, 2000**).

Microbes in biohydrogen production

Microbial hydrogen production can be either Photosynthetic (light dependent) or Non-photosynthetic (light-independent). Photosynthetic hydrogen production is carried out by algae or photosynthetic bacteria (**Kumazawa and Mitsui, 1981; Miyake and Kawamura, 1987**) and Non photosynthetic or fermentative hydrogen production is performed by facultative anaerobes (**Yokoi *et al.*, 1995**) or obligate anaerobes (**Kim *et al.*, 1999**).

biohydrogen production from water through the use of cyanobacteria species such as blue green algae, *cyanophyceae* or *cyanophytes* (**Kapdan and Kargi, 2006**). Biological water-gas shift reaction is another method of generating hydrogen from water through the use of photoheterotrophic bacteria, belong to the *Rhodospirillaceae* family which use carbon monoxide as the carbon source (**Ni *et al.*, 2006**). The biohydrogen production by *Enterobacter aerogenes* and *Rhodobacter sphaeroides* using Calophyllum inophyllum oil cake under dark and photo fermentation conditions was studies by **Arumugam *et al.* (2014).** Cyanobacteria and green algae produce both hydrogen and oxygen by light-driven biophotolysis processes involving both nitrogenase and hydrogenase enzymes. The oxygen produced in biophotolysis inactivates the hydrogen producing conditions which leading to lower yields of biohydrogen. Fermentative bacteria can produce hydrogen from organic compounds using hydrogenase throughout the day in dark fermentation process but at lower yields. On the other hand, purple non-sulfur photosynthetic bacteria can decompose organic acids by using light energy and nitrogenase in a photofermentation process. The hybrid system is a combination of dark fermentation by fermentative bacteria followed by photofermentation by purple non-sulfur photosynthetic bacteria, wherein the overall hydrogen yield can be enhanced to a great extent (**Basak and Das, 2006**). Various species like *Rhodospirillum rubrum* (**Piyawadee *et al.*, 2005**), *Rhodobium marinum* (**Anam *et al.*, 2012**), *Rhodobacter sphaeroides* (**Pattanamanee *et al.*, 2012; Eroglu *et al.*, 2011**), *Chlorella vulgaris* (**Bala Amutha and Murugesan, 2011**), *Clostridium butyricum* (**Pattra *et al.*, 2011; Wang *et al.*, 2008**), *Enterobacter cloacae* (**Namita *et al.*, 2011; Ghosh *et al.*, 2011**), *Clostridium saccharoperbutylacetonicum* (**Shorgani *et al.*, 2013**), *Bacillus coagulans* (**Ghosh *et al.*, 2011**),

Table 1. Different methods implicated in Microbial conversion of Biomass to Hydrogen

Methods	Microbes	General Reaction	Advantages	Disadvantages
Direct biophotolysis	Green Algae	$2H_2O$ +light→ $2H_2 + O_2$	Hydrogen directly produced from water and sunlight.	Requires high light intensities. Low hydrogen production rate.
Indirect biophotolysis	Microalgae, Cyanobacteria	$6H_2O+6CO_2$+light→$C_6H_{12}O_6+6O_2$	Hydrogen production from water with nitrogenase enzyme. Generate Ammonium at the same.	Lowers the hydrogen production rate and hydrogen yield by degradation of hydrogen through the uptake of hydrogenases.
Photo fermentation	Photosynthetic bacteria	$CH_3COOH+2H_2O$+light→$4H_2+2CO_2$	Hydrogen production from different waste materials by utilizing the light.	Light conversion efficiency is with about 1-5% very low. Oxygen is strong inhibitor of hydrogenase.
Dark fermentation	Fermentative bacteria (*Enterobacter, Clostridia, Thermotoga, Klebsiella*)	$C_6H_{12}O_6+6H_2O \rightarrow 12 H_2 + 6 CO_2$	Hydrogen production in the absence of light. Concurrent production of other value products, such as butyric acid, lactic acid, ethanol, etc.	Comparatively low Hydrogen yields with expensive carbon feedstock like glucose. Product gas mixture contains CO_2 and may contain other toxic gases like H_2S which have to be separated.

Table 1 summarizes the different methods for microbial production of hydrogen. Photoproduction of hydrogen by photosynthetic microorganisms requires the use of a simple solar reactor or artificial illumination, which is low in energy requirements (**Basak and Das, 2007**). In direct biophotolysis, photosynthetic systems of algae or cyanobacteria use solar energy to convert water into chemical energy in the form of hydrogen (**Sautaux, 2010**). Indirect biophotolysis is the two stage process of photosynthesis and aerobic fermentation for

Bacillus subtilis (**Manikkandan *et al.*, 2013**) and *Clostridium fredundii* (**Ghosh *et al.*, 2011**) have been studied for the biohydrogen production.

Factors Influencing Biohydrogen Production:

The efficiency of hydrogen and organic acids production is influenced by several operational parameters such as substrate concentration, pretreatment of substrate, hydraulic retention time (HRT), pH and temperature.

Substrate concentration

Many agricultural and food industry wastes contain starch and cellulose which are rich in terms of carbohydrate contents. Complex nature of these wastes may harmfully affect the biodegradability. Carbohydrate containing solid wastes is easier to process for hydrogen gas formation. The industrial effluents such as dairy industry, olive mill, Sago industry, baker's yeast and brewery wastewaters can be used as raw material for bio-hydrogen production. In an appropriate range, increasing substrate concentration could increase the ability of hydrogen producing bacteria to produce hydrogen during fermentative hydrogen production (**Wang and Wan, 2009**), but substrate concentrations at much higher levels could decrease this ability (**Van Ginkel *et al*., 2001; Lo *et al*., 2008**). Carbohydrates from hydrolyzed biomass have been a potential feedstock for fermentative hydrogen production (**Chong *et al.*, 2013**).

Lignocellulosic material must be pre-treated prior to fermentation to hydrogen in order to remove lignin and hemicelluloses, reduce the cellulose crystallinity and increase the surface area of the material to enhance the release of sugars (**Xia and Sheng, 2004**). Pretreatment is also required to open up the plant cell wall and break up the lignocellulose structure, thus making the carbohydrates more available to either acid or enzyme in subsequent hydrolysis (**Zhou *et al.*, 2012**). In 2006, Cheong and Hansen compared pretreatment methods such as acid, sodium 2-bromoethanesulfonate, wet heat-shock, dry heat-shock and freezing and thawing, respectively, for enriching hydrogen-producing bacteria from cattle manure sludge and then concluded that the acid pretreatment method was the best among the five methods studied. Hu and Chen in 2007 pretreated the sewage sludge and methanogenic granules for enriching hydrogen producing bacteria using acid, heat-shock and chloroform and concluded that the chloroform pretreatment method was the best among the three methods studied.

Table 2. Microbial production of biohydrogen from Biowastes

Biowastes	Photosynthetic Microorganisms	Yield of biohydrogen	Reference
Pine apple waste	*Rhodospirillum rubrum*	247.75 ml H_2/g	Piyawadee *et al.*, 2005
Soy sauce waste water	*Rhodobium marinum*	200 ±67 mL H_2	Anam *et al*., 2012
Bagasse	*Rhodobium marinum*	41 ±16 mL H_2	Anam *et al*., 2012
Oil palm empty fruit bunch	*Rhodobacter sphaeroides*	27.7 mL H_2/L h	Pattanamanee *et al.*, 2012
Corn stalk	*Chlorella vulgaris*	5.534 ml/l	Bala Amutha and Murugesan, 2011
Wheat straw hydrolysate	Thermophilic mixed culture	9.8 mL L^{-1} h^{-1}	Kongjan *et al.*, 2010
Olive mill waste	*Rhodobacter sphaeroides*	40 mL	Eroglu *et al.*, 2011
Sugarcane juice	*Clostridium butyricum*	3.38 mmol H_2/L/h	Pattra *et al.*, 2011
Complex substrate	*Enterobacter cloacae* IIT-BT 08	3.1 molH_2 mol−1	Namita *et al.*, 2011
Molasses	*Clostridium butyricum*	1.63 mol H_2/mol	Wang *et al.*, 2008
Sago Starch	*Clostridium saccharoperbutylacetonicum*	4628mL H_2/L	Shorgani *et al.*, 2013
Molasses	*Enterobacter cloacae, Bacillus coagulans, Clostridium fredundii*	16.66m H_2/mol	Ghosh *et al.*, 2011
Sugarcane bagasse extract	*Bacillus subtilis*	0.49 mol H_2/mol	Manikkandan *et al.*, 2013
Alkali treated sewage sludge	*Clostridium sp.*	2.1 mol H_2/mol	Kim *et al*., 2013
Calophyllum inophyllum Oil cake	*Enterobacter aerogenes and Rhodobacter sphaeroids*	7.95 L H_2/L	Arumugam *et al.*, 2014

Mixed anaerobic micro floras obtained from sewage sludge are well used for fermentative hydrogen production because they contain a variety of hydrogen-producing cultures. All substrates in dark fermentation reactors are not utilized in hydrogen production process. Organics could be incorporated into the biomass and generate various fermentation products, including acetate, propionate, butyrate and ethanol. Therefore, biohydrogen production is strongly correlated with the composition of the microbial community as the amount of predominant hydrogen producers grows. In other words, the existence of carbohydrate consuming but non-hydrogen producers could compete with the hydrogen-producers, thereby decreasing hydrogen yields (**Jo *et al.*, 2007**).

Pretreatment

Pre-treatment of parent inoculums or cultures used in biological hydrogen production process which permits selective enrichment of specific group of bacteria. Pretreatment techniques such as heat-shock, chemical, acid, alkaline, oxygen-shock, load-shock, infra red and freezing have been employed on variety of mixed culture for inhibits the methanogenic bacteria and selective enrichment of acidogenic hydrogen producing inoculums **(Venkata Mohan, 2008).**

pH and Temperature

Hydrogen is produced by bacteria through bio-process under ambient temperature and pH regime, and the yield can be enhanced by the manipulation of other environmental conditions. The initial pH and temperature are the important factors that influence hydrogen production by bacteria (**Wongthanate *et al.*, 2014**). Temperature influences the hydrogen production, metabolite product distribution, substrate degradation and bacterial growth. There are two temperature conditions have been commonly used for hydrogen fermentation such as mesophilic (30-45^{o}C) and thermophilic (50–60^{o}C). In 2009, Akutsu *et al.,* revealed that hydrogen from starch using mixed cultures was successfully produced under the thermophilic condition and stable hydrogen production was not observed under the mesophilic condition.

Maintaining of pH in the acidic range (5.5–6.0) is ideal for effective hydrogen production due to repression of methanogenic bacteria and it indirectly promoting the hydrogen producers within the system. pH also influences the efficiency of substrate metabolism, synthesis of protein and storage material and release of metabolic by-products. This is especially important for fermentative hydrogen production for the activity of acidogenic bacteria (**Venkata Mohan *et al.*, 2007**). As the pretreatment pH and sewage sludge concentration increased, initial pH was increased and the pH was maintained high during fermentation. A proper amount of sewage sludge addition and pH pretreatment level could maximize the hydrogen production potential (**Kim *et al.*, 2013**).

components of nitrogenase are the molybdenumeiron protein and iron. The creation of hydrogen by nitrogenase can be described by the chemical reaction (Eq.1)

$$4ATP + 2H^+ + 2e^- \rightarrow H_2 + 4ADP + 4Pi \quad \text{----------------} \quad (1)$$

Table 3. H_2 production from different substrates with different pH and Temperature

Substrate	pH	Temperature(°C)	Yield of BioH_2	Ref
Apple waste	5.4	35	2.2 molH_2 mol^{-1}	Hwang *et al.*, 2011
Mushroom waste	8.0	55	51 mL H g^{-1}	Chuang *et al.*, 2012
Water hyacinth	8.0	55	17 mL H g^{-1}	Chuang *et al.*, 2012
Oil extraction residue	8.0	55	27 mL H g^{-1}	Chuang *et al.*, 2012
Poultry Slaughter house waste water	9.0	37	22.80 mL H_2/L/h.	Patcharaporn *et al.*, 2011
Pear waste	5.6	35	1.7 molH_2 mol^{-1}	Hwang *et al.*, 2011
Grape waste	5.4	35	0.1 molH_2 mol^{-1}	Hwang *et al.*, 2011
Corn syrup waste	5.5-6.5	37	430 mL H_2/g	Hisham *et al.*, 2009
Oil palm empty fruit bunch	7.0	35	27.7 mL H_2/L h	Pattanamanee *et al.*, 2012
Pharmaceutical Waste Water	6.0	31	3.45 mmol/day	Hema Krishna *et al.*, 2013
Sugacane Bagasse	7.0	36	0.49 mol H_2/mol	Manikkandan *et al.*, 2013
Xylose/Arabinose	6.5	55	2.70 mol-H_2/mol	Saripan and Reungsang, 2013
Arabinose	5.5	70	1.10 molH_2 mol^{-1}	Abreu *et al.*, 2012
Glucose	5.5	70	0.75 molH_2 mol^{-1}	Abreu *et al.*, 2012
Formate	7	30	33.5 mmol/L	Wu *et al.*, 2012
Glucose	6.3	37	5.95 H_2/gVSS h	Cubillos *et al.*, 2010
Acetate	8	30	2.56 mol/mol	Cai and Wang, 2012
Cellulose	5.73	80	19.02 mmol H_2/g	Gadow *et al.*, 2012
Cellulose	5.8	55	15.2 mmol H_2/g	Gadow *et al.*, 2012
Sucrose	6.0	35	2.8 mol H mol^{-1}	Lay *et al.*, 2012
Propionic acid	7	30	34.2mmol/L	Wu *et al.*, 2012
Pyruvic acid	7	30	19.8mmol/L	Wu *et al.*, 2012
Sewage sludge	10	35	2.1 mol H_2/mol	Kim *et al.*, 2013
Food and beverage processing waste water	6.5	35	0.28 L/L	Wongthanate *et al.*, 2014

Table 4. Comparison of Hydrogenase and Nitrogenase enzymes

Properties	Hydrogenase	Nitrogenase
Hydrogen production	Produce	Produce
Hydrogen Uptake	Uptake	Not uptake
ATP dependent	Non-dependent	Dependent
Oxygen Sensitivity	Sensitive	Sensitive
Catalytic rate	High	Low
Metal Components	Ni, Fe, S	Mo, Fe
Subunits	One to Three	Six
Optimal Temperature	55°C	30°C
Optimal pH	6.5 – 7.5	7.1 – 7.3
Stimulators	Absence of Organic compounds	Light
Inhibitors	CO, EDTA, O_2, Some organic compounds	N_2, NH_4^+, O_2, high N:C ratio of hydrogen production.
In Prokaryotes	Present	Present
In Eukaryotes	Present	Absent
Substrates	H^+, hydrogen	ATP, H^+, Nitrogen, Electrons
Products	ATP, H^+, hydrogen, electrons	H_2, NH_4^+

Enzymes involved in biohydrogen production

Biological hydrogen production process such as direct biophotolysis, indirect biophotolysis, biological wateregas conversion, photofermentation and dark fermentation are controlled by enzymes, particularly nitrogenase and hydrogenase enzymes. Three different classes of hydrogenases have been identified so far: [Fe]-hydrogenase, [NiFe]-hydrogenase, and [NiFeSe]-hydrogenase. In major cases, it is evident that [NiFe]-hydrogenase is responsible for hydrogen uptake while [Fe]-hydrogenase catalyses the hydrogen production processes. [Fe]-Hydrogenase is highly sensitive towards oxygen and possesses 100-fold more activity than [NiFe]-hydrogenase (**Basak and Das, 2006**). The main

where ATP is adenosintriphosphate, ADP is adenosindiphos-2 phate and Pi is inorganic phosphate, respectively.

In the majority of photosynthetic microorganisms, hydrogenases exist as acceptor and reversible hydrogenases. The important components of acceptor hydrogenase are NiFe and NiFeS, which consume molecular hydrogen by the reaction (Eq. 2)

$$H_2 \rightarrow 2e^- + 2H^+ \quad \text{----------------} \quad (2)$$

Reversible hydrogenases have the ability to create molecular hydrogen as well as to consume it depe+nding on the reaction conditions (**Bicakova and Straka, 2012**). The properties and comparison of hydrogenase and nitrogenase enzymes are shown in Table 4 (**Basak and Das, 2006; Ni *et al*., 2006**).

Bioreactors for biohydrogen production

Batch bioreactors have been frequently used in biohydrogen production process for determining the biohydrogen potential from organic substrates (**Fernandes *et al*., 2010**). Packed bed reactors (PBR) have been used for the treatment of both dilute and high strength soluble wastewaters (**Perna *et al.*, 2013**). The biohydrogen production yields of 0.65 and 1.04 mol H /mol hexose at HRT of 4.0 h using both fluidized bed and packed bed bioreactors with polyethylene beads as carrier media (**Wu *et al.* 2007**). The biohydrogen production from rice straw hydrolyzate in a continuously external circulating bioreactor (CECBR) with the working volume of 300 mL with a height of 22.5 and a width of 7.5 cm respectively was carried out by Liu *et al.,* 2014. Batch tests were effective in showing the suitability of the selected inoculum/substrate ratios for testing for hydrogen production in the thermophilic range. Semi-continuous trials with supernatant recirculation did not show any significant hydrogen production (**Chinellato *et al.,* 2013**).

In photobioreactors, cyanobacteria and green algae are used in hydrogen production is coupled with photosynthetic water splitting reactions. Thus, it is possible to use solar/light energy for the production of hydrogen from water. The main challenge in photobioreactor design is to create a simple, inexpensive, with high volumetric productivity energy efficient photobioreactor, which is scalable to industrial capabilities (**Markov, 2012**).

Conclusion

Various biological technologies such as direct biophotolysis, indirect biophotolysis, biological water-gas shift reactions, photo fermentation, dark fermentation and a combination of dark/photo fermentation processes have been studied over the past decades for hydrogen production. There are a tremendous number of researches being practicing for the development of hydrogen (H_2) production technologies. In order to decrease the energy demand and environmental pollution, significant development of alternative is important. Biomass and water are considered as, environmental friendly, cheap and renewable resources for the hydrogen production technologies. The future biohydrogen production efficiency is mostly depends on the genetically modified microbes, culture conditions, design of bioreactors and the social acceptance.

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