Practical Aspects of Hydrogen Production by Dark Fermentation – A Review

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***Abstract*— Hydrogen is a clean source of energy and promising alternative to fossil fuels. But, conventional methods of hydrogen production are high energy intensive processes. Hence biological hydrogen production methods that are less energy intensive and more eco-friendly became new trend in researching area of sustainable energy. Among biological methods, dark fermentation method has received considerable attention in recent years as it is comparatively cost effective and independent of light energy. The parameters such as source of inoculums, substrate, pH, temperature, hydrogen partial pressure, presence of nutrients and buffering chemicals and type and configuration of reactor are considered as crucial factors for biohydrogen production by dark fermentation. This paper discusses the behavior of these parameters on biohydrogen production based on previous studies.**

Keywords- biohydrogen; dark fermentation; operational factors; bioreactors

I. INTRODUCTION

Energy is a factor that significantly influencing the development of civilization. In 2014, fossil fuels represented 86% of all energy consumed (32 % crude oil, 30 % coal, 24 % natural gas), remaining energy requirement was provided 7% by hydro electricity, 4% by nuclear energy and 3% by renewable energy. The crisis in the world regarding our fossils fuel reserves and an increase in concern for a healthy environment lead to a search for clean and unconventional sources of energy that can sustain human growth. Alternative fuels such as hydrogen and biodiesel are being investigated for further sustainable transport options that reduce dependence on petroleum and other conventional non-renewable fuels. Hydrogen is poised to become an important future energy carrier owing to its clean, renewable and high energy yield nature.

Hydrogen can be produced by a number of processes including electrolysis of water, thermo catalytic reformation of hydrogen rich organic compounds and biological processes. Both electro-chemical method and thermo-chemical method require massive energy and fossil fuel, also in these methods the cost of hydrogen production is relatively high and the environment is damaged. Nevertheless, applying biotechnology to produce hydrogen possesses environmental protection, energy conservation and renewability [1]. Biological production of hydrogen (biohydrogen) using microorganism is an exciting new area of technology development that offers the potential production of usable hydrogen from a variety of renewable resources. Biological systems provide a wide range of approaches to generate hydrogen, and include direct biophotolysis, indirect biophotolysis, photo fermentation and dark fermentation.

Despite of high energy content of hydrogen, the cost of biological hydrogen production was still not a cost effective when compared to the easy conventional hydrogen production. Hence future research is necessary in biological method to improve the technical feasisibility and scalability of hydrogen production based on renewable energy, higher carbon emission and large investment growth in renewable energies.

II. HYDROGEN PRODUCTION BY DARK FERMENTATION

In anoxic or anaerobic environments, hydrogen is commonly produced during microbial breakdown of organic compounds. The process is termed as 'dark' hydrogen fermentation if organic compounds are the sole carbon and energy source providing metabolic energy. When light is required to provide additional energy, the process belongs to category of photobiological processes. Hydrogen production by dark fermentation is a natural phenomenon but it is often obscured in natural environments due to rapid consumption of hydrogen by other species. The last step in anaerobic fermentation is the conversion of H_2 or VFA to methane. This step can be prevented by inhibiting the growth of hydrogen consuming bacteria. By turning off the last step, both the hydrogen and VFA remain for utilization during the proposed process [2].

This fermentation effluent containing VFA can be effectively utilized in Microbial Electrolytic Cell (MEC) by providing of small external voltage. Integrating dark fermentation with electrochemically assisted microbial fermentation in MEC reactor is such an approach to improve H_2 molar yield. Exoelectron generating bacteria or exoelectrogens are key players in MECs. These are the bacteria which utilize organic rich matter as carbon source, oxidize them and release the electrons outside the cell. Electrons released by exoelectrogenic bacteria in specially designed reactors reduce the protons (released simultaneously during metabolism) to form hydrogen gas when a small voltage is applied through the circuit [3].

III. EFFECTS OF OPERATIONAL FACTORS

Most of the bottlenecks of dark hydrogen fermentation are related to operational factors as Source and type of microorganisms, substrates, temperature, pH, Hydraulic Retention Time (HRT), hydrogen partial pressure etc.

A. Microorganisms

Hydrogen can be produced by strict anaerobes from the *clostridiaceae* family as well as facultative anaerobes. The process can be take place in the presence of pure or mixed culture. A pure culture is characterized by high selectivity and yielding higher hydrogen production efficiency with fewer byproducts. However such a pure culture is susceptible to impurities, which requires aseptic environment and increase the overall cost of the process.

A mixture culture of microorganism can use wider spectrum of substrate and cost of the process can be reduced since no sterilization is necessary. The process also has some downsides. A mixture might contain microorganism which do not produce hydrogen but compete for carbon source. Hence some pretreatment steps are necessary to dominate hydrogen generating. Due to different origins of inocula, substrate type and the process conditions it is hard to determine most efficient way of pretreatment [4]. The inocula that use to seed the experiment can be obtained from common natural sources. Experimental results based on the study using anerobes from cowdung compost revealed that the composts have metabolic functions similar to hydrogen producing clostridium rich sludge. In the study, metabolic repression occurred during the heat shocked anaerobic composts consumed sucrose and no methanogens were observed. The lag in bacterial growth (i.e metabolic repression) is usual when the bacterial cells were transferred into a superior environment [5].

Recent success in genome sequencing and a gene expression analysis has enhanced the ability to transform microorganism for specific metabolic tasks. Redirection of enzyme catalyzed reaction or electron flux through an existing pathway is an approach referred to as metabolic engineering. It is well known that organic acids synthesized during microbial metabolism decrease the H_2 producing efficiency of the microorganism since too low pH condition force the microorganism to reduce the concentration of H^+ there by inhibiting the production of hydrogen. Mutation with defects both in alcohol and organic acid formation pathways can lead to higher yield. In another approach of metabolic engineering, it targets the increase of the hydrogen yield by increasing the substrate utilization efficiency of microorganism. Hence cellulose which is available in plenty can be used as an apt substrate for biohydrogen production [6].

The study on biohydrogen production from food component in batch reactor condition, where hydrogen yield using both granular sludge and suspended sludge was investigated revealed that Bromoethane sulphonate (BES) – inhibited granular and suspended inocula showed higher hydrogen production potential with smaller lag times when compared against heat treatment. Largest difference was observed with suspended inocula at 37^oC. In the case of granular inocula, the differences were larger at thermophilic and hyperthermophilic temperatures [7].

The increase on H_2 production rate is considered to have close connection with the formation of granular sludge. The work using sucrose as carbon source and a carrier-induced granular sludge bed bioreactor showed boosting the hydrogen production. Granular sludge formation led to efficient biomass retention in the bioreactor against an increasing substrate loading rate (i.e., a decrease of HRT from 2.2 to 0.5 hrs) and continuous mode operation. i.e. the quick formation of granular sludge also gains benefits to the bioreactor operation for high-rate biohydrogen production. Although H_2 production rate depend on HRT, H_2 yield did not vary significantly with HRT. HRT when shortened to 0.3 hrs, cell washout occurred leading to a poor H_2 production performance. Maintaining a satisfactorily high yield during high rate H_2 production is quite important to ensure a good H_2 producing performance. The ability to stably operate against a low HRT with effective biomass retention appeared to play a major role in achieving a much higher H_2 production rate as well as comparable H_2 yield [8].

In batch study controlled by a programmable logic controller where paper mill sludge treated with ultra sound for 20 min.used as substrate and pig manure pretreated with infrared radiation for 0.5-1.5 hrs as seed, it was revealed that because of the exhaustion of DO and the decreased pH the facultative aerogens which escaped the pretreatment could not adapt to anaerobic conditions in the reactor system and eliminated gradually. Then microbial flora of acidogenic fermentation which had adapted to the new circumstance began to propagate [9]

In the study conducted by Ruggery et al. (2015), it was observed that acid treatment is highly selective for clostridium spp. with respect to other bacteria. Also biogas produced

becomes free of methane and hydrogen sulphide, indicating the lack of methanogenic activities in the sludge after acid treatment [10].

The study on the effects of combined pretreatment (acid treatment with or without heat shock) for enriching hydrogen producing microbial species in mixed culture revealed that as enrichment pH is lowered, the hydrogen production is enhanced. This result indicate that the spore-forming hydrogen producing bacteria, clostridium species, might prevail over other anaerobic microbial populations, especially hydrogen-utilizing methanogens, due to selective environmental pressure. Other harsh environments such as heat, desiccation, and radiation also contribute to the selective survival of spore-forming bacteria. In the experiment, enrichment at pH 3 enhanced hydrogen production and additional heat treatment produced predominance of hydrogen-producing spore-forming bacteria in the mixed bacterial communities [11].

Anaysis of biogas produced from rice bran de-oiled wastewater by anaerobic fermentation using slaughter house sludge (SHS) as seed indicate that sludge pretreatment methods have different effect on hydrogen yield. In heat treatment process, the production of hydrogen mainly depends on duration of heat treatment of sludge. During heat treatment certain amount of methane was produced indicating the presence of some heat resistant methanogens in the sludge. In acid treatment method, the collected sludge was treated with 0.1 N HCl to maintain pH range 3-4 and then sustained for 24 hrs. The acid treated sludge showed the hydrogen production less. Also it showed less efficiency with respect to substrate removal than the heat treatment method. But the advantage in this method is that no methane is detected. Therefore the combined use of heat treatment and acid treatment is a way of solving a problem [12].

Wu et al. (2009) conducted a comparative study of pretreatment methods (acid alkali treatment, heat digestion and ultrasonic treatment) for hydrogen production by anaerobic fermentation from municipal sludge. When the sludge was treated by acid alkali treatment, most of the organics has been dissolved, some of the hardly dissolved organics as lignose, cellulose and hemicelluloses etc. had structural changes, hydrolyzed by cellulose, ligninase etc. The rate of organic dissolution was faster than bio-degradation and resulted in accumulation of organics in liquor. Therefore sCOD value rose obviously. As plenty of carbon sources were needed to maintain biological metabolism, anaerobic digestion speeded up, the organics were consumed by bacteria as nutrient and COD started to decline. The difference between acid pretreatment and alkali pretreatment was that acid pretreatment provided methanogen a good growth condition because of the acidification of substrate and formation methanogenic phase. After sludge was treated by acid alkali treatment, the period of hydrogen production was shortened. When the sludge temperature is 80-100^{o}C, the amount of dissolved organics increased. The dissolution rate decreased when the temperature rose to 120^{o}C i.e. at that temperature the structure of molecule destroyed and transforming into glucose like simple compounds which degraded by bacteria finally. Therefore sCOD value increased slowly. Heat digestion dissolve the de-lipid of cell, weakened the tolerance ability of the cell wall against heat, promoting the hydrolysis of sludge. The analysis showed that sludge solution was affected by ultrasonic energy experience dynamic processes of vibration, growth, collapse and closure and produce organic matter easily biodegradable such as sugar. After ultrasonication, the permeability of cell membrane and cell wall have changed which inturn increase the biological mass transfer and improved the enzyme activity. It was also revealed that after 28-32 hrs start up, the number of biomass and live bacteria was maximum in the system [13].

Seed inoculation into synthetic glucose solution and then to industrial wastewater after hydrogen production rate stabilization make the success of the acclimatization phase and lack of inhibitors in the wastewater. Statistical analysis between the H_2 produced by acclimatized and non-acclimatized food waste substrates revealed a significant difference in the hydrogen production between them. The significant differences might be because the additional bacteria obtained from acclimatization enhanced the fermentation process in the acclimatized food waste substrates. Also, the bacteria in the system have adapted to the food waste substrate during acclimatization while the bacteria needs to adapt to the environmental conditions in the non-acclimatized food waste substrate and hydrogen producing bacteria were not helped in anyway, therefore only the indigenous microbes performed the fermentation. The food waste having high carbohydrate component which is easily convertible to H_2 showed not quite a significant difference in H_2 production with or without the additional microbe from acclimatization. Along with H_2 gas production, biogas production also increases in acclimatized waste as a result of presence of methanogenic bacteria which were also enhanced acclimatization even though they were affected by preheating [14].

In the study by Wang and Chang (2007) on biohydrogen yield from starch based influent in the system operating on continuous mode using heat treated mixed inocula at controlled pH of 5-5.5, the results showed that the short start up HRT (5.3 hrs) could eliminate a prolonged adaptation period commonly involved in most starch-feeding bio-H_2 producing systems and also enhance the H_2 production rate by increasing the substrate loading rate [15].

B. Substrate

The most efficient substrate for H_2 production by means of dark fermentation are carbohydrate such as glucose, sucrose, starch etc. however pure substrates are very expensive and it remains difficult to economically justify biological hydrogen production from commercially produced pure substrates such

as glucose and sucrose as typically most of the organic matter cannot be converted to hydrogen [26]. So in order for industrial scale H_2 production to be profitable and cheap, waste products such as sewage sludge, solid municipal waste, molasses and wastewater originating from biodiesel productions, olive oil or palm oil products etc. have to be used [4].

It has to be noted that, sufficient carbohydrate concentration is necessary for good hydrogen yield. The study conducted by Kim et al. (2004) detected no hydrogen production in the reactors including blank in which carbohydrate concentration was lower than 2.0 g COD/l. Their study was based on food waste along with sewage sludge as substrate and sludge from anaerobic digester after pretreatment at 90^{o}C for 10 min. as seed with proper nutrient supply. Sewage sludge showed lower hydrogen production potential than food waste. Less hydrogen production from sewage sludge might be caused by hardly hydrolysable organics present in the sludge. And the maximum specific hydrogen production potential of 122.9 ml/g carbohydrate was found at the waste composition of 87:13 (food waste: seawage) and the volatile Solid concentration of 3.0%. But specific hydrogen production rate was not enhanced by addition of sewage sludge. Enhanced hydrogen production by adding sewage sludge can be explained on the basis of enriched protein. Food waste is carbohydrate rich waste while sewage sludge is protein rich waste. The addition of sewage sludge on food waste up to 13 – 19 % could enhance hydrogen production potential due to balanced carbohydrate/protein ratio. Methane was observed in reactor where sewage sludge was added mainly due to methanogenic bacteria in the sludge but, lesser than methanogenic bacteria was externally loaded. As organic acids produced along with H_2, alkaline addition was needed to prevent pH drop. Environmental conditions such as substrate protein, inorganic nutrients and pH should appropriate for spore germination and enzyme activation, so that lag-phase time (**λ**) can be reduced. Food waste should require high alkaline dose due to its low alkalinity of 0.4 g $CaCO_3$ /l [16].

Enhanced biohydrogen production from synthetic protein wastewater by altering protein structure and amino acid through pH control was investigated by Xiao et al. (2014). Under anaerobic fermentation conditions, protein is firstly hydrolyzed to peptides and amino acids and then fermented to volatile fatty acids and hydrogen. Pretreatment of wastewater at pH 12 not only destroy the bonding bridge but changed their conformation. Thus hydrolysis of protein increased from 28.8% to 86.7% after the wastewater was pretreated at pH 12. Since more protein was biohydrolyzed, more amino acids were provided for hydrogen producers and the hydrogen production was increased. At pH 12, there is no protein hydrolysis but protein structure is altered [17].

The limiting step in increasing the rate of hydrogen production is clearly the conversion of solid substrate to soluble compounds that can be taken up by the cells. When the cellobiose was the substrate, the rate of gas production in the fermenter was 6.6 times higher than that with insoluble lignocellulose. Lalaurette et al. (2009) examined the use of two stages - dark fermentation and electrohydrogenesis process for converting lignocelluloses into hydrogen. In the study, they observed that the use of Microbial Electrolytic Cell (MEC) provides an additional advantage of converting certain components (hemicellulose) into hydrogen that the first stage fermentation could not [18].

Mohanakrishna et al. (2010) conducted a batch study on enhancing hydrogen yield from vegetable based waste using domestic sewage as co-substrate in the reactor where anaerobic mixed culture pretreated by heat shock (100^{o}C, 2 hrs), acid treatment (pH 3, 24 hrs) and chemical treatment (2-BESA, 0.2 g/l, 24 hrs) was used as seed. The results showed that supplementation of domestic sewage as co-substrate to vegetable waste showed positive influence on both hydrogen production and substrate degradation. After sewage supplementation, marked improvement in overall process efficiency including system buffering capacity was noticed compared to non-supplementation operation. Supplemented domestic sewage can provide additional micro-nutrients, organic matter and microbial consortia which might have positive synergism with the resident mixed consortia on overall process efficiency. Also period of H_2 evolution was improved after sewage supplementation. They also compared the process efficiency of both substrates with or without pulp of masticated vegetable waste used. Higher substrate degradation observed in the case of non- pulpy waste might be attributed to the relatively easy degradability of the feed due to the absence of cellulosic material. Presence of pulp produce more specific H_2 yield then that by non-pulp operation after co-substrate supplementation when compared to normal operation. Specific Hydrogen Yield (SHY) registered higher values at lower organic load in both compositions with or without pulp. Unlike H_2 production, substrate degradation showed a consistent improvement with increase in organic load [19]. High suspended solid wastewater which contains large particle size and insoluble organic matter require pretreatment in order to enhance the solubilization and high solubilization of solids increase hydrogen production yield. The influence of pretreatment methods differs for various substrates [20].

C. Hydrogen Partial Pressure

Increase in hydrogen partial pressure shifts the metabolic activity of the bacteria towards synthesis of more reduced products, thus decreasing the overall hydrogen yield. As concentration of H_2 increases in the liquid phase, the transfer of e^- from glucose to hydrogen becomes increasingly unfavorable [21]. Also an exorbitant partial pressure level of hydrogen in the headspace of the reactors would inhibit the hydrogen production because the microorganism could switch to alcohol [22]. One approach to reduce hydrogen partial

pressure and in turn to increase hydrogen yield is to strip hydrogen from the liquid using Nitrogen (N_2) sparging. A second method is to apply a vacuum to the headspace, lowering overall pressure in the system. Alternative method of lowering the dissolved hydrogen concentration would be to reduce the rate of hydrogen production, allowing more efficient stripping of the hydrogen from the liquid phase by stirring [21].

D. Temperature

H_2 production can take place in the presence of mesophilic bacteria (clostridium, enterobacter), thermophiles (caldicellulosiruptor, thermoananaerobacterium) or hyper thermophiles (thermotoga). Hyperthermophilic bacteria are less inhibited by the partial pressure of hydrogen and that due to high temperature, fermentation is less susceptible to contamination with other cultures. From engineering point of view, a disadvantage of thermophilic process is that a reactor must be heated up to desired temperature and volumetric production rate of hydrogen is less which would require an application of much bigger reactors than those used for mesophilic fermentation, hence increase cost. In the study on hydrogen production from food wastes using anaerobic sewage sludge as seed under batch condition, higher hydrogen production was observed at 35^{o}C than that at 27^{o}C and 55^{o}C. This might be because it favors the proliferation of the hydrogen producing bacteria, also the temperature made sugar conversion easier for the hydrogenase which inturn increases Hydrogen production from food waste [23]. The study conducted by Moreno-Davila et al. (2011) revealed that at room temperature hydrogen production was not detected. This was explained on the basis of complex or particulate matter that has to be cleaved into smaller molecules before they can be introduced into cell for energy production. Otherwise it will cause biomass problems due to accumulation of solids i.e. time taken for maximum hydrogen production and rate of hydrogen production varies along time and operational parameters [22].

Optimization study on fermentative hydrogen production from agricultural waste concluded that higher hydrogen yield obtained at thermophilic temperature might be due to higher degradation rate of organic substances at high temperature and alleviate inhibition from hydrogen partial pressure [24].

As the characteristics of hydrogen producers varied, optimal temperature for hydrogen production is diverse. In the study on biohydrogen production in an anaerobic batch reactor operated at pH 6 with rice bran de-oiled wastewater as substrate, max. H_2 production and yield were observed at 57^{o}C than that at other temperature conditions. This may be due to the reduction of the solubility of hydrogen at high temperatures. But beyond certain temperatures (i.e. from 57^{o}C to 67^{o}C) the hydrogen percentage gradually decreased. This may be due to the inactivation of some essential enzymes and proteins associated with cell growth or hydrogen production. At higher temperatures the specific VFA/alcohol production rates in the thermophilic condition were slightly greater than the corresponding values in the mesophilic conditions and beyond 57^{o}C the VFA production showed decrement. This confirms that increased operating temperature accelerated the acidogenic reaction rate and substrate removal. Therefore, VFA concentration, distribution and their fraction have been successfully used as indicators for monitoring hydrogen production [24]. The difference in the metabolic products at different temperatures has also been observed and may have been caused by different microbial community. The amount of sCOD and VFA produced was related to the amount of hydrogen that was generated. The amount of sCOD and VFA_{total} was low when H_2 production was less [7]

E. pH

pH is a key parameter to control H_2 production, which affects the activity of hydrogenase, microbial communities, their structure and their metabolism. Results reported by Moreno-Davila et al. (2011) showed that a high yield hydrogen production (5.83 mM/g COD_c) was achieved at fermentation pH 4 from dairy wastewater in packed bed batch reactor using pretreated mixed culture as seed. Although, maximum hydrogen production was similar for 35, 45, and 55^{o}C, but that was achieved at different times as a result of different lag phases for each case. This behavior can be explained due to the formation of semisolid aggregates when pH was adjusted to 4.0 at which max. hydrogen production was achieved, creating conditions with low substrate availability. This conditions decrease as temperature was increased, and accelerating its segregation at high temperatures (35 to 45^{o}C) [22].

At different pH, the hydrogen production from amino acid does not depend on utilization efficiency of substrate but to the different anaerobic biochemical reactions. Under anaerobic conditions the evolution of hydrogen through a NADH pathway is driven by the necessity of reoxidising the residual NADH (reduced form of nicotinamide adenine dinucleotide coenzyme) of metabolic reactions as

$$NADH + H^+ \rightarrow NAD^+ + H_2 \quad (2.1)$$

NADH-Fd reductase and hydrogenase are key enzymes to oxidize NADH and reduced ferredoxin to produce molecular hydrogen and different pH has different effect on the relative activities of these enzymes [17].

In a study conducted by Cheong and Hansen (2006), it was reported that the culture pH of 7 resulted in a significant increase in average cumulative hydrogen production over the culture pH 5 and 6. These results indicated that culture pH affected hydrogen production. They also consider hydrogen production potential may be dependent on an altered or special anaerobic fermentation pathway induced by hydrogen producing bacterial communities enriched during the

acidogenic conversion, although degree acidification has great effect on hydrogen production. The hydrogen production occurred during culture at pH 7 after enrichment at pH 3 with intermediate liquid metabolite contents that has butyrate-acetate pathway and no occurrence of propionate. Butyrate-acetate or ethanol-acetate fermentation has potential for the hydrogen production. However propionate fermentation pathway is not involved in hydrogen production [11].

The study on biohydrogen production from chemically pretreated Palm Oil Mill Effluent (POME) showed that the cumulative hydrogen yield increased when initial pH changed from 4.5-5.5 and obtained maximum yield at 5.5 at temperature 60^{o}C. Hydrogen production yield was high in the pH range of 6-6.5, but this range of pH may promote methanogens thus not selected as optimum pH. The low pH (5.5) and high temperature (60^{o}C) lead to deactivation of methanogens, thus evolved biogas mainly composed of H_2 and CO_2 and free of methane. H_2 production from alkaline pretreated POME was terminated by with the formation of VFA [20].

The study conducted by Cubillos et al. (2010) observed that initial pH values did not have any significant effect on hydrogen yield for initial substrate concentration of 5 and 10 g COD/l. At initial glucose concentration of 3 g COD/l max. yield was observed at initial pH of 6.5 than that observed at 5.5 and 7.5 [25]

Max. Hydrogen production from rice bran de-oiled wastewater using mixed culture was observed at pH 6. This may be due to the suppression of methanogens under acidic conditions. At lower pH, accumulation of acids causes a sharp drop of culture pH and subsequent inhibition of bacterial hydrogen production. Because of increased production of acidic or alcoholic metabolites destroys cells ability to maintain internal pH [12].

F. Organic Loading Rate (OLR)

The hydrogen production increases with the increase of OLR. But hydrogen yield has inverse relation to the glucose feeding rate. The reason for this behaviour in the yield is likely due to end product inhibition by over accumulated (supersaturated) hydrogen gas in the liquid at high organic loading rate. The glucose concentration has a greater effect on H_2 yield than the HRT, hence it is more advantageous to operate biohydrogen reactors at lower HRTs and keep the glucose concentration as low as possible through dilution or recycle. [21]. As HRT decreased, the production of HBu (Butyric acid) decreaed slightly and HAc (Acetic acid) formation increased [8]. This may be one of the facts for increasing hydrogen yield since Acetic acid pathway leads to more hydrogen production than Butyric acid pathway. The optimization study on fermentative hydrogen production from dairy wastewater showed that, the change of COD concentration remarkably affected the hydrogen yield. Although, an increase in COD concentration could enhance the Hydrogen yield, but a higher substrate concentration results in accumulation of the volatile fatty acids, which inhibit the growth of hydrogen-producing bacteria. In addition, the partial pressure of hydrogen in the reactor rose with the increase of substrate concentration and hydrogen production [22].

The performance of MEC using fermentation effluent showed that longer batch cycle times needed for the fermentation effluent of high concentration lead to a lower hydrogen production rate, and allowed more time for growth of methanogens as mixed culture was used as inocula. High organic loading and longer cycle time adversely affect reactor performance. One method to reduce potential for methane production and electron cycling in MEC is that the application of higher voltage which lead to increase the current density and shortens the reaction run time. The fermentation-MEC integrated process also improves significantly the overall hydrogen molar yield to favor its techno-economic feasibility [18].

Analysis of hydrogen production from paper mill sludge revealed that hydrogen yield change considerably with the change of organic loading and then tended to be steady, but the hydrogen production rate decreases when organic load was too large. When organic loading is appropriate, the system maintain good biodegradation conditions (BOD_5/COD > 0.31) because of the dynamic balance of C/N/P and the ammonium nitrogen buffer action against organic acid which prevent change in pH, which in turn helps the hydrogen producing bacteria to keep composition of the bacteria and the characteristics of the cells uniform and to reduce generation time to improve organic matter utilization there by hydrogen yield. When OLR is too low, the hydrogen producing bacteria are inhibited by the lack of a carbon source. High OLR lead to accumulation of VFAs, decreased, dynamic balance of NAD^{+}/NADH become upset, biological activity declined finally enzyme related hydrogen production is inactivated [9].

Samples obtained from different food processing plants (apple processor, potato processor and confections) initially showed that hydrogen production was significantly related to the COD concentration of the wastewater when nutrients were added. But, hydrogen production was not a function of COD and varied widely when using the different waste stream. This may be due to the macromolecular nature of carbohydrate and further work is needed to understand these results. It was also observed that the hydrogen production efficiency from food processing wastewater is lower than that measured for pure carbohydrates on the basis of COD reduction. Lower yield is observed for actual wastewaters than pure compounds, because wastewater components have high molecular weight than simple sugars as well as the particulate nature of many of the wastewater components. Disinfectant using for daily cleaning period at the plant affect the hydrogen production from in-plant wastewater. The effect of intermittened doses of

disinfectants and varying carbohydrate concentration could be ameliorated through the use of an equalization tank [26].

G. Nutrient Solution and Buffering Chemicals

Biohydrogen production requires certain essential micro-nutrients such as Nitrogen, Phosphorus and some trace elements for bacterial metabolism, growth and activity. Nitrogen is an important component of proteins, nucleic acids and enzymes that are of great significance to the growth of hydrogen producing bacteria. But hydrogen production would be inhibited after adding synthetic nutrients if the C/N ratio becomes too low in the reactor i.e. the C/N ratio of substrate used is high then no need of nutrient addition [24]. Nutrient addition would enhance hydrogen production when using wastewater where the main inputs are sugars and not more nutritionally well balanced foods [26]. Hydrogen production without adding synthetic nutrients is cost effective. According to results of study on biohydrogen production from agricultural waste conducted by Chuang et al. (2012), significant factors for hydrogen production from water hyacinth, soybean oil extraction residue and mushroom waste were in the order nutrient addition> temperature> substrate concentration> pH, temperature> nutrient addition> substrate concentration> pH, temperature> nutrient concentration> pH> substrate concentration respectively [24].

Sivaramakrishna et al. (2010) reported the impact of different nitrogen sources on hydrogen production. In the experiment, organic nitrogen sources were yeast extract, tryptone and the inorganic nitrogen sources used were ammonium chloride, urea and ammonium sulphate. The results showed that fermentation with organic nitrogen sources showed better hydrogen production compared to inorganic nitrogen sources. Inorganic nitrogen sources probably contain only the nutrients that satisfy no more than the minimal requirement for increased hydrogen production. Organic nitrogen sources used may be due to the presence of aminoacids, peptides, water soluble vitamins, and carbohydrates. When using different nitrogen sources, lag phase was in the order urea> ammonium chloride> ammonium sulphate> tryptone> yeast extract [12].

The study by Wang and Chang (2008) revealed that the use of phosphate-based buffer, instead of the bicarbonate buffer was useful to prevent abiotic production of additional CO_2 from the reaction of bicarbonate ion with the acidic soluble metabolites. Keeping low CO_2/H_2 ratio not only lowers the production of the major green house gas (CO_2) but could also make H_2 purification easier [8].

The effects of ionic Cr, Cu and Zn on fermentative hydrogen production using mixed microflora were examined by Lin et al. (2007). The results showed that low Cr, Cu concentration slightly stimulated fermentative hydrogen production of the mixed microflora. Zn is also reported as an important nutrient factor enhancing hydrogen production. The heavy metals related to functions in the reactions and transformations of dehydrogenases, dismutase, hydrogenase and methyl transferace [15]. Eventhough at a higher concentration, metal ion may inhibit the activity of hydrogen producing bacteria, a trace level of metal ion is required for fermentative hydrogen production. The effect of iron concentration on hydrogen production by clostridia may be caused by not only as the limiting factor for the growth but also other environmental conditions which iron concentration affects. The mixed culture used as seed sludge contained bacteria which do not have hydrogen production ability along with hydrogen producing bacteria and changes in the iron concentration may have affected the relationship between these anaerobic bacteria [27]. However, too high dose of the ions should be avoided to prevent inhibition problems. But this toxicity may change with respect to substrate used and the toxic comparison basis i.e. whether it is based on acidogenesis/hydrogenesis [15].

IV. BIOREACTORS

Bioreactor configuration is of prime importance in hydrogen production process as it influences the micro environment of the reactor, its established hydrodynamic behavior, the prevailing population of microorganism and their contact with substrate [4]. The decoupling of Sludge Retention Time (SRT) from Hydraulic Retention Time (HRT) in biohydrogen production system facilitate superior sludge settling characteristics of hydrogen producers which indicate the suitability of gravity settlers after Continuous Stirred Tank Reactor (CSTR) to maintain high biomass retention in the system and decrease biomass wash out. It will help to improve hydrogen yield and sustainability of hydrogen production. The use of clarifier after hydrogen reactor helps for decoupling SRT and HRT through sludge recirculation [28].

Decoupling of SRT from HRT in hydrogen bioreactors has been achieved using biofilms on several media. Problem with the development of methanogenic biofilm on the carrier media has adverse impact on the process stability, which is critical for hydrogen production. Membranes are prone to fouling in reductive environment existing in the reactor [28]. Fermentative hydrogen production studied in packed bed batch reactors showed that the use of biofilm system increase the specific activity also this type of system offer a higher operational stability as microbial community is protected by the polymeric matrix and allows tolerating extreme environmental conditions. In the packed bed batch reactor, renewable *o.imbricata* with high grade of reusability and without disposal problems was used as substratum for biofilm formation [22].

Microbial electrolytic cell is a new approach for biological hydrogen production from organic matter using microbes. Six variables such as electrode material, electrode area, substrate, electrode spacing, electrode potential and microbes are reported as influencing parameters for hydrogen production.

The work carried out on dual chambered MECs with cation exchange membrane using wastewater from sugar industry as substrate concluded that external addition of microbes had a visible impact on the production of hydrogen and volume of gases was doubled using *pseudomonas aeruginosa* [29]. The study conducted by Call and Logan (2011) using small-scale membrane-free MECs showed that small electrode spacing and lack of separators reduced factors such as pH gradients and proton diffusion resistance that contribute to high internal resistance. Also cathode screening indicated that the highest current densities could be obtained using stailesssteel mesh cathodes [30].

In Table 1 effect of various substrate, microbial culture and bioreactors are outlined.

TABLE I. HYDROGEN YIELD OF DIFFERENT CARBON SOURCES USING INOCULUMS FROM VARIOUS SOURCE.

substrate	Inoculam type & source	system	Max. hydrogen production	Ref.
Mixture of food waste and sewage sludge	Mixed culture, Anaerobic digester in wastewater treatment plant	Batch	122.9 ml/g carbohydrate COD	16
Sucrose solution	Mixed culture, soybean-meal silo	Batch	131.9 ml/g sucrose	27
Corn-syrup waste	Mixed culture, anaerobically digested sludge from ww treatment plant	CSTR	430 ml/g COD	28
Glucose solution	Agricultural soil	batch	2.8 mol /mol glucose	21
Cassava starch	Mixed culture, final sedimentation tank of municipal ww treatment plant	batch	1.43 mol/mol of hexose	8
Mixed food waste	Mixed culture, anaerobic sewage sludge from sewage treatment plant	Batch	130.95 ml	23
Dairy wastewater	Mixed culture obtained from UASB reactor	Packed bed batch reactors	12.73 m mol/g COD_c	22
Lignocellulosic material	Pure culture, *Clostridium thermocellum*	Batch reactor followed by MEC	750 +/- 180 ml/ g COD	18
Paper mill sludge	Mixed culture, pig manure	Reactor with programmable logic controller	620.8 ml/ g COD	9
Sucrose solution	Mixed culture, cow dung compost	Batch	90 ml/g sucrose	5
Solid organic matter in palm oil mill effluent	Mixed culture, anaerobic sludge from palm oil mill wastewater treatment plant	Batch	5.2 l H_2/l-POME	20
Glucose solution	Mixed culture, sludge treating anaerobic continuous stirrer tank reactor	Batch	1.07 mol H_2/ mol glucose	25
Agricultural waste (water hyacinth (W), oil extraction residue (O) and mushroom waste (M))	Mixed culture, anaerobic sludge from municipal wastewater treatment plant.	Batch	17 ml H_2/g substrate (W), 27 ml H_2/g substrate (O), 51 ml H_2/g substrate (M).	24
Rice bran de-oiled wastewater	Mixed culture, slaughter house sludge manure treatment plant.	batch	2.2 mol H_2/ mol substrate	12

V. CONCLUSION

Biological method of hydrogen production is considered as solution for reducing extensive use of fossil fuels and environmental pollution. In addition, this approach also utilizes organic waste that contributes to large source of biomass, which inturn lead to waste recycling. The low hydrogen yield and production rate are the main limitation of practical application of biohydrogen production Hence the studies focus on optimizing operating conditions and reactor conditions that favor the fermentation process for hydrogen production and this is considered as the major step towards the field application of bio-hydrogen production by sustaining process efficiency and economic viability. Substrate degradation is considered with biohydrogen production when wastewater is used as substrate. Two stage process combining fermentation with electrohyrogenesis has been evaluated for enhancing overall hydrogen yield and substrate degradation as the residual organic fraction in fermentation effluent can be utilized by applying external voltage in MEC reactor.

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