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Production of hydrogen by dark fermentation using organic wastes and mixed microbial cultures: facts and challenges

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Abstract
Hydrogen can be produced by a number of processes, including electrolysis of water, thermo catalytic reformation of hydrogen rich organic compounds, and biological processes. Biological production of hydrogen provides a wide range of approaches to generate hydrogen, including direct biophotolysis, indirect biophotolysis, photo-fermentation, and dark fermentation. Hitherto known biological hydrogen production processes have substantial potential for production of hydrogen on larger scales. However to develop a commercially viable technology from these processes requires an in-depth research into various aspects of production processes such as optimization of bioreactor designs, rapid removal and purification of gases, and genetic modification of enzyme pathways that compete with hydrogen producing enzyme systems offer exciting prospects for biohydrogen systems. This paper reviews various biological production processes for hydrogen, potential advantages of fermentative processes, possible utilization of organic fraction of MSW as raw material for hydrogen production.

Keywords: Hydrogen, fermentative, dark fermentation, acedogenic bacteria, anaerobic bacteria.

Introduction
Global energy requirements are mostly met with fossil fuels today, leading to the foreseeable depletion of limited fossil energy resources apart from the increase in pollution levels. In this scenario hydrogen is viewed as a future source of clean energy. However, at present, hydrogen is produced mainly from fossil fuels by well known reductive reactions. Coal gasification and electrolysis of water are other industrial methods for hydrogen production (Das and Verziroglu, 2001) [2]. In this context research efforts in recent times are focused on the production of hydrogen, wherein biological production processes are preferred. Hydrogen is produced in the anaerobic digestion of organic substrates. However production of methane and hydrogen are interlinked in anaerobic digestion and efficient production of hydrogen depends upon controlling of methanogenesis. This paper presents a review of biological hydrogen production processes and delineates the probable research requirements to enhance the hydrogen production.

Hydrogen production by anaerobic fermentative bacteria
Studies on hydrogen production by mixed cultures have attracted research attention only recently. Anaerobic digestion can be used to produce valuable energy from waste streams of natural materials or to lower the pollution potential of a waste stream. There are several groups of bacteria that perform each step, and dozens of different species are needed to degrade heterogeneous compounds completely. Methanogens use CO_2 as the ultimate oxidizing agent of an electron transport chain. Remaining CO_2 will be used as carbon substrate for growth. The reducing agent that drives the electron transport chain is hydrogen, which is also taken up from the growth environment. This hydrogen is the end product of the metabolism of other, heterotrophic microorganisms (acidogens and acetogens), and its utilization by methanogens, maintains a lowered hydrogen partial pressure in the growth environment. This process lies at the heart of some of the best characterized syntrophic relations and is referred to as interspecies hydrogen transfer and it is important because hydrogen utilization by the methanogens reduces the hydrogen partial pressure and this alters the pattern of metabolism of syntrophic hydrogen "donor" partner species. Raising or lowering the partial pressure of hydrogen in their growth environment determines their fermentation pathways and thus affects their ATP production, and growth (J.G. Zeikus, 1977) [34].

Enzyme systems responsible for the production of hydrogen
All processes of biological hydrogen production are fundamentally dependent upon the presence of a hydrogen-producing enzyme. It is hypothetically possible that the quantity or inherent activity of these enzymes could limit the overall process. However, a survey of all presently known enzymes capable of hydrogen production show that they contain complex metallo-clusters as active sites and that the active enzyme units are synthesized in a complex processes involving auxiliary enzymes and protein maturation steps. At present three such enzymes are known as nitrogenase, Fe-hydrogenase, and NiFe hydrogenase (Fauque *et al*. 1988) [4].

Nitrogenase
Nitrogenase is a two component protein system that uses MgATP (2ATP/e^-) and low-potential electrons derived from

reduced ferredoxin or flavodoxin to reduce a variety of substrates. In the absence of other substrates, nitrogenase continues to turnover, reducing protons to hydrogen. This is the basis for hydrogen production by nitrogenase-based systems. In each cycle, MgATP complexed Fe-protein associates with MoFe-protein, 2ATP are hydrolyzed with the transfer of one electron to MoFe-protein and the complex dissociates. Obviously, biosynthesis of these complex metal centers requires a great deal of additional enzymatic machinery, energy, and time. Alternative nitrogenases exist, but given their lower catalytic activities and stabilities they are even poorer candidates for a hydrogen evolution system. Thus, considering the low turnover number, the considerable energy inputs necessary for biosynthesis and the requirement of ATP for catalysis, nitrogenase is not an effective mean, to produce H_2.

Hydrogenases

Molecular hydrogen is a key intermediate in the metabolism of mainly fermentative bacteria. Hydrogenases catalyze the simplest of chemical reactions, the interconversion of the neutral molecule hydrogen and its elementary constituents: two protons and two electrons. Hydrogenases catalyse the reversible oxidation of molecular hydrogen and play a vital role in anaerobic metabolism. There are two types of hydrogenases exist, one is sensitive to hydrogen other is insensitive to hydrogen. This means that this enzyme is of reduced functionality when the hydrogen produced by the hydrogen insensitive hydrogenase that regenerates oxidized ferredoxin used as an oxidizing agent in pyruvate conversion to acetyl CoA is not removed by co-cultured methanogens. If hydrogen sensitive hydrogenase is not working then NAD regeneration must be mainly by the succinate producing pathway. Metal containing hydrogenases are subdivided into three classes: Fe, Ni-Fe hydrogenases and Ni-Fe-Se hydrogenases (Fauque *et al*. 1988) [4]. Hydrogen oxidation is coupled to the reduction of electron acceptors such as oxygen, nitrate, sulphate, carbon dioxide and fumarate, whereas proton reduction (H_2 evolution) is essential in pyruvate fermentation or in the disposal of excess electrons. The Ni-Fe hydrogenases, when isolated, are found to catalyse both H_2 evolution and uptake, with low potential multihaem cytochromes such as cytochrome C_3 acting as either electron donors or acceptors, depending on their oxidation state (Cammack *et al*. 1994) [1]. Many microorganisms have been shown to contain a NiFe or NiFeSe hydrogenase which is usually thought of as functioning as an "uptake" hydrogenase, that is a hydrogenase whose normal metabolic function is to derive reductant from H_2. Electrons derived from H_2 are used, either directly, or indirectly through the quinone pool, to reduce NAD(P). The NiFe hydrogenases are heterodimeric proteins consisting of small and large subunits. The small subunit contains three iron-sulfur clusters-two [4Fe-4S] and one [3Fe-4S]. The large subunit contains a unique, complex nickel iron center with coordination to 2 CN and one CO, forming a biologically unique metallocenter (Pierik *et al*. 1999) [25]. Thus, even working in reverse of its normal function this class of hydrogenase appears to be a better catalyst for hydrogen evolution than nitrogenase (Peters *et al*. 1998; Patrick and Benemann, 2002) [24, 23].

Rates of bio-hydrogen production

A comparison of hydrogen production rates reported for several Biohydrogen systems are presented in Table 1. Light dependent biohydrogen systems (direct photolysis, indirect photolysis, and photo-fermentation) all have rates of hydrogen synthesis well below 1mmol H_2/1/h). Dark fermentation systems produce hydrogen at rates that are well above 1 mmol H_2/1/h). The rates of hydrogen synthesis by an undefined consortium of thermophilic *Clostridium* and by the extreme thermophilic *Caldicellulosiruptor saccharolyticus* are very similar (8.2 and 8.4 mmol H_2/1/h), respectively). A pure strain of mesophilic *Clostridium*, demonstrated very good rates of hydrogen synthesis with xylose as a substrate (21.0 mmol H_2/1/h), and two dark fermentation systems that utilized undefined consortia of mesophilic bacteria had impressively higher rates of hydrogen synthesis (64.5 and 121.0 mmol/H_2/1/h), respectively). Both light dependent and dark fermentation Biohydrogen systems are under intense investigation to find ways to improve both the rates of hydrogen production and the ultimate yield of hydrogen.

Table 1: Biohydrogen Production by Pure/Mixed Cultures

Substrate	Microorganisms	H_2 yield (mol H_2/ mol sub)	Reactor used	Reference
Glucose	*Clostriclium acidisoli* CAC 237756	1.0	Membrance Bioreactor	Sang-Eun *et. al.* (2004)
Sucrose	Mixed microbial culture	2.2	CSTR continuous	Shihwn *et al*. (2003)
Glucose	Mixed cultures (With different pH and heat treatment)	0.96	Respirometric system	Sang-Eun *et al*. (2004)
Sucrose	*Clostricluium butyricum*, *C. butyricum*CG S5	2.78	Batch (CSTR) fermentors	Wen-Ming *et al*. (2004)
Refined Sucrose	Mixed microflora	1.0-1.9	CSTR HRT: 14-15 hrs. N_2 sparging	Hussy *et al.* (2005)
Water extract of sugar beet	Mixed microflora	0.9 1.7	CSTR HRT: 14-15 hrs. N_2 sparging	Hussy *et al.* (2005)
Sucrose	Anaerobic sludge (*Clostridium pasteurianum*)	--	CSTR HRT : 12hr. 35$\pm$ 1^{o}C	Lin and Lay (2005)
Food waste	Mesophilic and thermophilic acidogenic culture & *Bacillus* sp. *Thermosaccharo lyticum* & *Desulfotomaculum*	0.9- 1.8	Continuous stirred acidogenic reactors	Hang-Sik *et al.* (2004)

	geo thermicum		35-55± 1ºC	
Glucose	Anaaerobic Sludge (Heat Treatment)	0.96		Logan *et al*. (2002)
Sucrose	Mixed microbial anaerobic culture	2.2	--	Ginkel *et al*. (2001)
Sucrose	Anaerobic respirometers	0.92	Respirometer	Ginkel *et al*. (2001)
Glucose	Anaerobic mixed culture	0.88-1.00 (MBR)	--	Logan *et al*. (2004)
Glucose	*Clostridium* Sp.	1.15	--	Park *et al*. (2003)
Glucose	Aeromonas	1.13	--	Park *et al*. (2003)
Glucose	*P. phyromonas* sp.	1.08	--	Park *et al*. (2003)
Glucose	Vibrio sp.	1.22	--	Park *et al*. (2003)
Glucose	*Rhodopseudomonas palustris* p4	2.76	--	Oh *et al*. (2003)
Sewage sludge glucose	Mixed microflora (mesophilic) pH 5.7	1.7	CSTR	Lin and Chang (1999)
Glucose	*Enterobacter cloaceae* IIT BT 08	2.3	--	Kumar & Das (2001)
Glucose	*Enterobacter cloacae* DM 11	3.8	--	Kumar *et al*. (2001)
Glucose	*Citrobacter* sp Y 19	2.49	--	Oh *et al*. (2003)
Glucose	Acclimatized sludge	1.66	CSTR	Fang & Lin, (2002)
Cellulose	Anaerobic sludge sludge compost	0.9 2.4	CSTR	Ueno *et al*. (1995)
Cellulose	Sludge compost wastewater	2.59	CSTR	Ueno *et al*. (1996)

CSTR: Continuous Stirred Tank Reactor, HRT: Hydraulic Retention Time

Factors affecting the hydrogen production rates

Effect of methanogens on hydrogen production

Methanogens affect the growth of some but not all hydrogen producing species of microorganism. The growth of *Fibrobacter succinogenes* for example is not affected by the presence of methanogens. This is because hydrogenase activity in *Fibrobacter* is not sensitive to the prevailing hydrogen tension. These microorganisms do not therefore live in syntrophic relations with methanogens but methanogens do use the hydrogen they produce which is available to them as a result of interspecies hydrogen transfer. The hydrogenase is however hydrogen sensitive, it is inhibited by hydrogen. This means that this enzyme is of reduced functionality when the hydrogen produced by the hydrogen insensitive hydrogenase, that regenerates oxidized ferredoxin used as an oxidizing agent in pyruvate conversion to acetyl CoA, is not removed by co-cultured methanogens. If the hydrogen sensitive hydrogenase is not working then NAD regeneration must be mainly by the succinate-producing pathway. This means that succinate production is higher when methanogens are in lower abundance. All these examples suggest that an in depth knowledge of co-culture systems is essential for developing viable bio-production of hydrogen.

Effect of temperature on hydrogen production

Microbial hydrogen generation from biomass occurs at significant rates; however, the presence of other competing bacteria, which consume hydrogen to produce methane, limits the net production of hydrogen. Researchers can eliminate the competing methane-producing, hydrogen-consuming microbes by heat-treating a reactor when hydrogen production slows under normal operation. Because bacteria are fragile organisms, heat treatment kills and controls the competing methane-producing bacteria, but the useful bacteria may also be harmed as a result of heat treatment. Previous studies demonstrated that a hydrogen-producing species of *Clostridium*, a useful bacterium, are spore forming. Bacteria that are capable of going into a spore phase are protected from the heat treatment; thus, the desired bacteria survive and the competing organisms are removed. Using spore-forming bacteria also allows operators to shut down a system, with vegetative cells forming spores during down times and converting back to vegetative cells when the process resumes.

Effect of hydrogen partial pressure on hydrogen production

The hydrogen partial pressure increases to a certain level in the reactor headspace, the culture then switches to alcohol production and produces much less hyderogen. Under these conditions, the H^+/H_2 redox potential is lowered and the flow of electrons from reduced ferredoxin to molecular hydrogen via the hydrogenase system is inhibited. The electron flow would be shifted to the generation of NADPH via the action of the appropriate ferredoxin oxidoreductase, resulting in an increase in the production of butanol and ethanol (Jones and Woods, 1986) [9]. Hydrogen is a key intermediate in the overall anaerobic oxidation-reduction reactions involved in the mineralization of organic matter, and the concentration of H_2 plays a central role in controlling the proportions of the various products generated during fermentation. Many H_2 producing reactions are thermodynamically unfavorable unless the partial pressure of the H_2 is kept low. In natural ecosystem, this is achieved via syntrophic associations between H_2 producing acetogenic and H_2 consuming methanogenic bacteria. By artificially removing H_2, the partial pressure of H_2 can be kept, low in the absence of H_2 consuming methanogenic bacteria.

Effect of pH on hydrogen production

Several researchers have observed that butyric and acetic acids are produced first during the H_2 production by anaerobic mixed cultures, the cultures then undergo a shift and solvents are formed. The pH decrease seems to induce the shift to solvent production, the intracellular pH decreases to lesser extent than the extracellular pH. So, unionized organic acids tend to attain similar concentrations inside and outside. The increase in pH means that there are higher butyrate and acetate concentrations inside than outside the cell. This high intracellular acid concentration seems to be related to the shift in to the solventogenic phase. Therefore, it is important to keep the pH of the fermentation above 5.5 in order to avoid the solventogenic phase. The control of pH is crucial to the H_2 production, due to the effect on the hydrogenase activity also. The generation of hydrogen by fermentative bacteria also accompanies the formation of organic acids as metabolic

products, but these anaerobes are incapable of further breaking down the acids. Acid accumulation causes a sharp drop of culture pH and subsequent inhibition of bacterial hydrogen production (Oh *et al*. 2003) [20].

Effect of substrate on hydrogen production

Carbohydrates are the preferred organic carbon source for H_2 producing fermentations. The maximum yield of 4 H_2 per glucose when acetic acid is the by-product, half of this yield per glucose is obtained with butyrate as the fermentation end product. However, the H_2 production rate with disaccharides and starch was much slower than that with monosaccharides. The maximum H_2 yield and H_2 production rate were estimated to be 2.76 mmol H_2/mmol glucose and 29.9 mmol H_2/g cell h, respectively (Oh *et al*. 2003) [20]. Levin *et al*., (2004) [13] reported that *C. pasteurianum* metabolism of glucose can be shifted away from hydrogen production and towards solvent production by maintaining high glucose concentrations (12.5% w/v), by introducing CO (an inhibitor of hydrogenase) and by iron limitation. This indicates the high concentrations of substrates also affect the hydrogen production rate.

Possible strategies for the enhanced and economic production of hydrogen

Review of literature on hydrogen production by microorganisms, brings to four certain critical facts, which must be considered in developing any commercial hydrogen production technology. These critical facts are:

- Fermentative production can involve many organic substrates, which could be available even as waste, and a verity of microorganisms capable of utilizing these substrates
- In fermentative production of hydrogen, critical studies on specific substrates and the behaviour of co-culture systems are necessary
- Heat treatment or any other means to inhibit hydrogen utilizers is an essential step in development of such systems
- Hydrogen production by the microorganisms is more under reduced partial pressures of hydrogen in the growth environment. The possible strategy for hydrogen production should essentially include some mechanism of hydrogen removal viz., inert gas purging, vacuum, or membrane separation, to maintain the reduced partial pressures of hydrogen in growth environment

There is a need to integrate all these strategies to develop a viable hydrogen production technology. These points are discussed in details along with the available literature in the following sections.

Microbial production of hydrogen from wastes

It is well known that anaerobic bacteria can utilize waste material like municipal solid wastes, industrial effluents, sewage sludge, etc. Unlike other hydrogen forming species such as green algae, production of hydrogen with anaerobic processes is accompanied by the breakdown of organic substrates and appears to be advantageous in converting organic wastes in the environment into more valuable energy resources (Horiuchi *et al*. 2002) [7]. Fermentative hydrogen production can be maximized through the effective coupling of rich sources of electrons and biochemical electron pump along with an active hydrogenase. Many anaerobic organisms can produce hydrogen from organic substances, converting carbohydrates in a fermentative process to organic acids and hydrogen. By using microorganisms in hydrogen production, the process would encourage additional waste recycling, thus reducing waste disposal and treatment burden.

Feasibility of biological hydrogen production from organic fraction of municipal solid waste was reported (Zhu *et al*. 1999) [35]. A high hydrogenic activity for the pretreated digested sludge was obtained at a high food to microorganism (F/M) ratio, however, that for the hydrogen producing bacteria was found at a low F/M ratio (Ueno *et al*. 1995) [30]. However, these examples demonstrate utilization of different waste organic substrate by a verity of microorganisms for the production of hydrogen. It clearly indicates the potential of wastes and microorganisms towards the development of eco-friendly biohydrogen production. Future research demands waste specific studies on hydrogen production and delineating different viable waste-microorganism combinations.

Heat-shock pretreatment to inhibit hydrogen consumers

The heat-shock pretreatment inhibits or kills non spore-forming microorganisms (hydrogen-consuming methanogens) and enriches the culture in spore-forming bacteria (hydrogen-producing acidogens). The anaerobic spore-forming bacteria form an important portion of the acidogenic population performing acetate/butyrate fermentation. Lay and Noike (Lay and Noike, 1999) [12] studied the feasibility of H_2 production from the organic fraction of municipal solid waste by *Clostridium sp.* and a heat-shock pretreated digested sludge. They concluded that the heat treatment of the anaerobic sludge effectively developed its H_2 producing capability. Okamoto *et al*., (2000) also found that heat-shock pretreatment was very effective to enrich the inocula with hydrogen producing bacteria for hydrogen production from organic fraction of municipal solid waste. They found that the pretreatment succeeded to inhibit the methanogenic phase, the H_2 percentage in the biogas was 60% and no methane was observed.

Chemical inhibitors to inhibit hydrogen consumers

Many chemical substances have been reported for specific inhibition of methanogenic archaeal bacteria like 2-bromoethanesulfonate (BES) and 2,4-pteridinedione (lumazine) (Reimann *et al*. 1996) [26]. Sparling and Daniels, (1987) [29] compared the effectiveness of BES and acetylene as methanogenic inhibitors. Acetylene in the headspace was as effective as BES in inhibiting methanogenic activity for hydrogen generation in batch anaerobic composters with an undefined consortium. Acetylene had no effect on the rate and amount of H_2 produced from *Clostridium thermocellum*. Valdez-Vazquez *et al*., (2004a) [32] also used acetylene to inhibit the methanogenic activity during the batch, repeated fermentation of paper wastes.

Reduction in the hydrogen partial pressure in growth environment

Mizuno *et al*., (2000) [19] demonstrated that hydrogen producing mixed culture produces more H_2 when the H_2 is removed by an inert gas. They found an increase in the H_2 yield from 0.85 to 1.43 mol/mol when the reactor was sparged with nitrogen at 15 v/v N_2/H_2. However, this method has a disadvantage, because of the hydrogen will be diluted in the resulting gas mixture, thus its application at industrial scale is not economically feasible or,

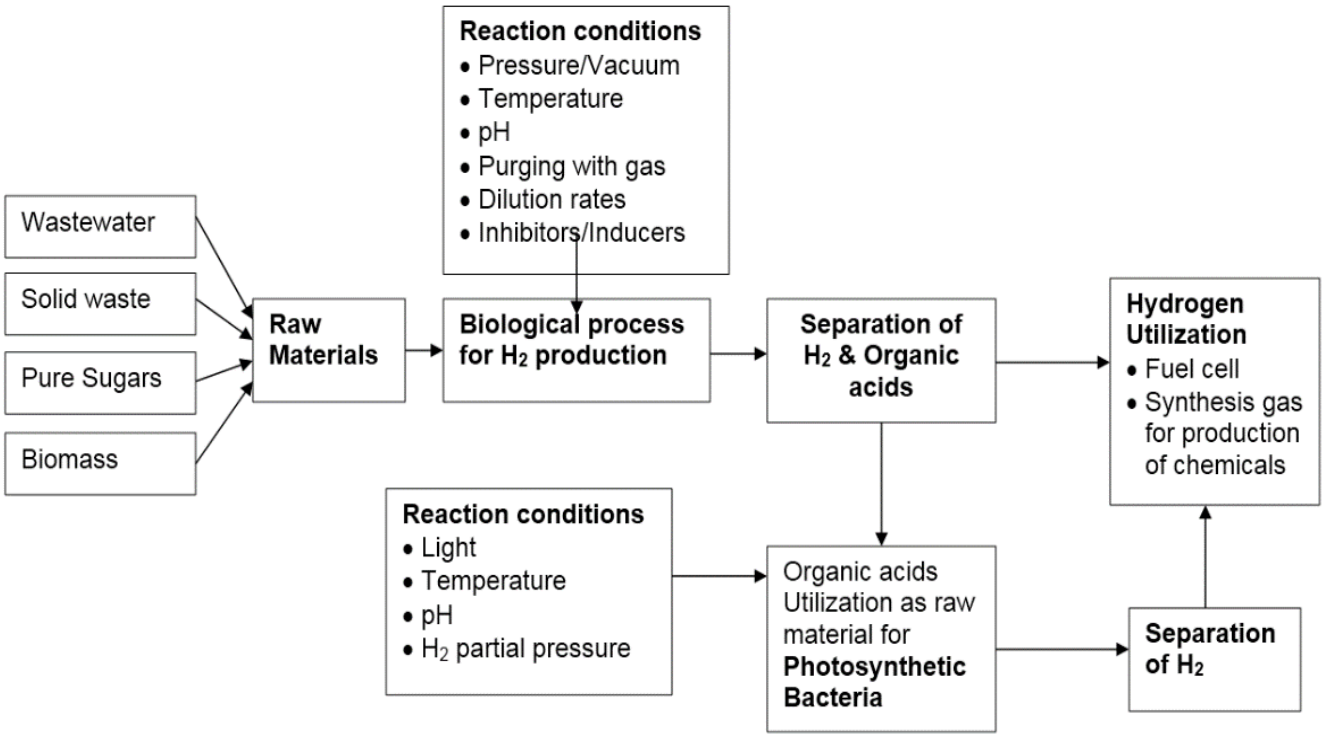
at least, debatable. Logan *et al*., (2002) [17] improved the hydrogen production with two techniques: an intermittent pressure release method and a continuous gas release method using a bubble measurement device. Valdez-Vazwurz *et al*., (2004a) [32] studied that H_2 gas from paper mill wastes using microbial consortia inhibited with acetylene. The headspace of the batch reactors was flushed with N_2 after the H_2 gas pressure was reached to maximum, and subsequently incubated, with no further addition of inhibitor or substrate. It was found that H_2 production resumed after the purging. This procedure was repeated as and when the H_2 pressures in the reactor increased. The total cumulative H_2 harvested was nearly double than that in the first cycle alone. This procedure was named as intermittently vented solid substrate anaerobic H_2 generation.

Membrane bioreactors for the improvement of hydrogen production efficiency

Removal and selective purification of H_2 has been demonstrated using membrane technologies. A hallow fiber/silicon rubber membrane effectively reduced biogas partial pressure in a dark-fermentation system, resulting in a 10% improvement in the rate of H_2 production and a 9% increase in H_2 yield i.e., from 4.7 mmole H_2/g glucose for the reactor without the silicone rubber membrane to 5.14 mmole H_2/g glucose for the reactor with membrane (Liang *et al*. 2002) [14]. Sang-Eun *et al*., (2004) [27] studied a cross-flow membrane coupled to a chemostat, to create an anaerobic membrane bioreactor for biological hydrogen production. The reactor was fed with glucose (10 g/l) and inoculated with a soil inoculum heat-treated to kill non-spore forming methanogens. When operated in chemostat mode at a hydraulic retention time of 3.3 h, 90% of the glucose was removed, producing 2200 mg/l of cells and 500 ml/h of biogas. When operated in membrane bioreactor mode, the solids retention time was increased to 12 h producing a solids concentration in the reactor of 5800 mg/l. This solids retention time increased the overall glucose utilization (98%), the biogas production rate (640 ml/h), and the conversion efficiency of glucose to hydrogen from 22% to 25%.

Future research needs

All the biological hydrogen producing systems are under intense investigation to find ways to improve both the rates of hydrogen production and the ultimate yield of hydrogen. Improvements in gas separation will contribute to significant increases in hydrogen production. As discussed above, the partial pressures of hydrogen in the reactor is an extremely important factor for continuous hydrogen synthesis. The hydrogen concentrations increase, hydrogen production decreases and metabolic activity shifts to pathways that synthesize more reduced substrates. Increase in hydrogen yield could be achieved by shifting the chemical reactions so as to increase the amount of NADH usable for hydrogen evolution. Substantial gains in hydrogen production can be achieved through optimization of bioreactor designs. Finally, hydrogen evolution can be achieved through elimination of uptake hydrogenases, over expression of hydrogen evolving hydrogenases that have themselves been modified to be oxygen tolerant and elimination of metabolic pathways that compete for reducing equivalents required for hydrogen synthesis. A critical review of all these processes indicates that fermentative production of hydrogen has a great potential. This approach has a great promise for commercialization in future. However the fundamental aspects of these processes needs in depth R&D following various approaches, prior to possible commercialization. A diagrammatic representation of these approaches is shown in **figure 1**.



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