Fermentative hydrogen production with simultaneous wastewater treatment: influence of pretreatment and system operating conditions

S Venkata Mohan
Bioengineering and Environmental Centre, Indian Institute of Chemical Technology, Hyderabad 500 007

Received 15 July 2008; revised 11 September 2008; accepted 22 September 2008

H\textsubscript{2} is a sustainable and viable form of green/alternative energy source. This study reviews the work on H\textsubscript{2} production from wastewater treatment pertaining to inoculum pretreatment and system operating conditions. Process was evaluated and discussed based on pretreatment procedures applied on mixed parent anaerobic culture to selectively enrich acidogenic culture, operating pH and retention time (HRT) in concurrence with wastewater type as substrate.

Keywords: Biohydrogen, Mixed consortia, pH, Retention time, Substrate loading rate, Wastewater treatment

Introduction
Capturing of energy as molecular biohydrogen (H\textsubscript{2}) especially from wastewater treatment process is gaining prominence. H\textsubscript{2} is a sustainable, less polluting and clean renewable energy carrier with high-energy yield (142.35 kJ/g). Various biological routes of H\textsubscript{2} production include biophotolysis, photo-fermentation and dark-fermentation processes or by a combination of these processes\textsuperscript{1-4}. Among them, dark fermentation is considered as a viable method on practical front and is leading to open a new avenue for the utilization of renewable energy sources.

Fermentative conversion of substrate to H\textsubscript{2} is complex biochemical process manifested by diverse group of bacteria by a series of biochemical reactions similar to anaerobic conversion\textsuperscript{5}. Anaerobic conversion requires four major steps and five physiologically distinct groups of microorganisms where hydrocarbons are converted from complex to simple molecules through H\textsubscript{2} and acid intermediates (Fig. 1). Hydrocarbons are ultimately converted to carbon dioxide (CO\textsubscript{2}) and methane (CH\textsubscript{4}). Fermentative/hydrolytic microorganisms hydrolyze complex organic polymers to monomers, and then ferment monomers to a mixture of low-molecular-weight organic acids and alcohols. Obligatory H\textsubscript{2} producing acetogenic bacteria (AB) oxidize fermentation products to acid intermediates and H\textsubscript{2}, which also include acetate production from H\textsubscript{2} and CO\textsubscript{2} by acetogens and homoacetogens and finally acetoclastic methanogens convert organic acids to CH\textsubscript{4} and CO\textsubscript{2} \textsuperscript{5-7}. Many thermophilic systems use syntrophic oxidation of acetate to CO\textsubscript{2} and H\textsubscript{2} by acetogenic or homoacetogenic bacteria coupled to H\textsubscript{2} consumption by hydrogenotrophic methanogens\textsuperscript{5}. H\textsubscript{2}-producing AB grow in syntrophic associations with hydrogenotrophic methanogens (H\textsubscript{2} consuming), which keep H\textsubscript{2} partial pressure low enough to allow acetogenesis to become thermodynamically favorable by interspecies H\textsubscript{2} transfer\textsuperscript{5}. Using mixed consortia as biocatalyst for H\textsubscript{2} production can be a practical and promising option for scaling up of technology especially when wastewater is used as substrate.

This review summarizes the work carried out on H\textsubscript{2} production pertaining to inoculum pretreatment and system operating conditions during wastewater treatment. Process was evaluated and discussed based on pretreatment procedures applied on mixed anaerobic culture to selectively enrich acidogenic H\textsubscript{2} producing culture, operating pH, hydraulic retention time (HRT) and substrate loading rate in concurrence with wastewater used as fermentable substrate.

Pretreatment Methods
Biological H\textsubscript{2} production (acidogenic/acetogenesis) by dark fermentation process shares many common features with methanogenic anaerobic digestion\textsuperscript{5}. Employing
mixed culture is extremely important and well suited to non-sterile, ever-changing, complex environment of wastewater treatment. Typical anaerobic mixed cultures cannot produce H$_2$ as it is rapidly consumed by methane-producing bacteria. Most effective way to enhance H$_2$ production from anaerobic culture is to restrict or terminate methanogenesis process by allowing H$_2$ to become an end product in metabolic flow. Induction of H$_2$ accumulation in fermentative consortia is related with inhibition of H$_2$ consumers (especially methanogens), which is essential for its further scale-up and industrial application. Pretreatment of parent inoculum/culture used in fermentative H$_2$ production process permits selective enrichment of specific group of bacteria. It is important for acidogenic H$_2$ producing process and also inhibits bacteria ([H$_2$ consuming methanogenic bacteria (MB)] that are not required especially when mixed cultures are used as parent inoculum. Physiological differences between H$_2$ producing bacteria (AB) and H$_2$ uptake bacteria (MB) form fundamental basis for methods used for preparation of H$_2$ producing seeds. Spore forming H$_2$ producing bacteria (Clostridium) can form protective spores when they are in a restrictive environment (high temperature, extreme acidity and alkalinity), but methanogens have no such capability. Application of pretreatment prevents competitive growth and coexistence of other bacteria, which are H$_2$ consuming.
Pretreatment helps to accelerate hydrolysis step, thus, reducing impact of rate limiting step and augment anaerobic digestion to enhance $H_2$ generation\textsuperscript{4,10,11,14}. Inoculum selection and its pretreatment have vital role in selecting requisite microflora for efficient $H_2$ production\textsuperscript{4,10,11-14}. Different pretreatment procedures (heat-shock, chemical, acid, alkaline, oxygen-shock, load-shock, infrared, freezing, etc.), have been employed on variety of mixed cultures\textsuperscript{9,8-26} for selective enrichment of acidogenic $H_2$ producing inoculum. Seed preparation affects both start up and overall efficiency of $H_2$ production\textsuperscript{3}.

**Heat-shock Treatment (HST)**

HST facilitates suppression of non-spore forming bacteria and allows growth of spore forming bacteria, which are important for $H_2$ production\textsuperscript{4,10,14,27-32}. HST relies on thermal suppression of methanogenic Archaea and non-sporulating bacteria, thereby enriching culture with sporulating $H_2$ producing bacteria ($Clostridia$)\textsuperscript{33}. It also facilitates suppression of methanogenic activity, which leads to $H_2$ production associated with concomitant volatile fatty acid (VFA) generation. HST parameters\textsuperscript{10} vary with temperatures (80-104°C) and exposure times (15-120 min). It is efficient to remove $H_2$ consuming bacteria while protecting spore forming bacteria and reported\textsuperscript{34} to repress methanogenic activity completely. HST kills vegetative cells of non-sporo-forming microorganisms, some of which consume $H_2$ such as methanogens but in this process it could also kill $H_2$-producing microorganisms ($Enterobacter$ sp.), which are unable to sporulate\textsuperscript{35}. On the contrary, HST may activate dormant spores of strains able to germinate and produce $H_2$ when conditions become optimal, for example, spores of $Clostridial$ species\textsuperscript{36}. In few cases, reduced efficiency in $H_2$ production observed by HST consortia, may be due to destruction of other non-spore forming $H_2$ producing bacteria, which resulted in reduced conversion of substrate into $H_2$\textsuperscript{10,11}. Short stability of HST sludge was observed for $H_2$ production and repeated HST of sludge was used to recover the reactor once in 30 days\textsuperscript{37}. HST is effective for inducing $H_2$ accumulation in batch reactors with mesophilic anaerobic consortia\textsuperscript{38,39-40}.

**Acid Treatment**

Most methanogens are limited to a relatively narrow pH range (6.8-7.2), while most $H_2$ producing bacteria can grow over a broader pH range\textsuperscript{41,42}. Methanogens are repressed by controlling cultivation conditions at low pH (5.5)\textsuperscript{43,44}. Acid treatment is efficient in removing $H_2$ consumer bacteria and also protects spore-forming bacteria by repressing methanogenic activity\textsuperscript{45}. Acidic pH (5.0-5.5) was considered to be ideal for effective $H_2$ production due to its influence on repression of methanogenic activity thus indirectly promoting $H_2$ producers within the system\textsuperscript{10}. Optimum acid treatment is reported at pH 3 for exposure period of 24 h. For pH adjustment, HCl and orthophosphoric acid are employed.

**Load-shock Treatment (LST)**

LST is reported\textsuperscript{38,29,44} more effective than HST due to the presence of higher diversity of microbes, as no physical or chemical treatment was applied. On the other hand, overloading or shock-load treated sludge led to accumulation of organic acids, resulting in decrease of pH\textsuperscript{29,44} from 5.5 to 4.6; hence, not suitable for growth of methanogens. LST facilitates preparation of $H_2$ producing by physical pretreatment by means of direct cultivation of digested sludge without any chemical pretreatment\textsuperscript{10}. LST is reported to be effective compared to base, acid, chemical (BESA) and HST methods for enriching thermophilic $H_2$ producing seeds, as it completely repressed methanogenic activity along with good $H_2$ production\textsuperscript{39}.

**Chemical Treatment**

$H_2$ consuming methanogens in mixed culture could be eliminated by applying inhibiting chemicals [iodopropane, acetylene and 2-bromoethanesulfonic acid (BESA)\textsuperscript{4,8,10,14,16,17,25,41,45,46}. BESA is a structural analog of co-enzyme-M specifically found in methanogens only. Chemical treatment by BESA facilitates selective inhibition of methanogenic activity without disturbing $H_2$ production where co-enzyme M reductase complex, a chief component for methanogenesis, inhibits\textsuperscript{10}. In addition, BESA could inhibit acetate producing process\textsuperscript{47} and long-term operation with BESA addition were not found sustainable and likely to have side effects on $H_2$ producing bacteria\textsuperscript{48} as well as occurrence of BESA-resistant mutants in anaerobic fermentation\textsuperscript{4}. Iodopropane, a corrinoid antagonist, prevents functioning of B12 enzymes as a methyl group carrier\textsuperscript{48}. Acetylene is considered a non-specific inhibitor of methanogenesis\textsuperscript{8,9,49}. Methanogenic species lose their ability to maintain a transmembrane pH gradient, thus, exposure to acetylene results in a decline of methanogenic functions such as ATP synthesis and
methanogenesis\textsuperscript{50}. Inhibitory effects of acetylene on fermentative eubacteria producers of H\textsubscript{2} such as \textit{Enterobacter} sp. and allow a major microbial diversity to produce H\textsubscript{2} without effecting archea and several eubacteria (including Clostridial species)\textsuperscript{8,9,25}. Employing acetylene for pretreatment have several advantages, as it is a cheap gas that will exit of reactor with H\textsubscript{2} rich gas. It does not accumulate in solid or liquid culture and culture does not usually show any significant lag time for H\textsubscript{2} production onset\textsuperscript{25}. Acetylene (1\% v/v) treatment was applied to induce H\textsubscript{2} accumulation from a model paper mill waste in batch reactors seeded with mesophilic anaerobic consortia\textsuperscript{8,25}. Chemical pretreatment method has an advantage of being readily applied and when required.

**Other Treatment Methods**

Alkaline treatment [pH 8.5-12 (using NaOH), exposure period, 24 h] suppresses growth of methanogens and enhances H\textsubscript{2} production\textsuperscript{51,54}. HST represses methanogenic activity completely while base treatment\textsuperscript{14} represses partially\textsuperscript{10} giving low H\textsubscript{2} yield\textsuperscript{55}. Methanogens are obligate anaerobic archaeobacteria; when exposed to an aerobic environment, oxygen lowers their adenylate charge and causes them to die\textsuperscript{41}. Conversely, \textit{Clostridium} and \textit{Enterobacter} are facultative bacteria that can also grow in presence of oxygen\textsuperscript{10}. Application of forced aeration as pretreatment method inhibits activity of H\textsubscript{2} consuming MB. After inoculating anaerobic reactor with an aerated sludge (purged with compressed air for 1 h) acetoclastic methanogens are reported to be effectively inactivated\textsuperscript{56}. However, with this method of seed preparation, a lower H\textsubscript{2} production rate is reported compared to HST treated seed. Improvement in H\textsubscript{2} yields is also reported with sludge pre-treated by freezing-and-thawing\textsuperscript{57}. Application of infrared pretreatment to seed inoculum also inhibits bioactivity of H\textsubscript{2} consumers\textsuperscript{58}. Methanogens from mixed culture could also be eliminated/repressed by maintaining short HRT (2-10 h) as H\textsubscript{2} producing bacteria grow faster than methanogens\textsuperscript{10,29,43,59,60}.

**Combined Treatment**

Each pretreatment applied to prepare acidogenic H\textsubscript{2} producing inoculum has its own efficacy associated with the nature of parent inoculum and wastewater used as fermentative substrate apart from the system operation conditions\textsuperscript{4,12,14}. Acid methods repress methanogenic activity and give low H\textsubscript{2} production than chemical (BESA) and HST methods with concurrent substrate consumption efficiency\textsuperscript{10}. But chemical and HST methods are not easy to implement and involves high cost\textsuperscript{54}. However, H\textsubscript{2} production using anaerobic consortia inhibited by acidogenic operation requires a long acclimatization time (10-30 days)\textsuperscript{25,61,62} whereas LST and HST can be used without a long acclimatization time (<48 h). Chemical (BESA) treatment showed higher H\textsubscript{2} yield followed by HST and acid methods to ferment wastewater\textsuperscript{12} compared to untreated inoculum. Seed obtained by combined HST and acid treatment resulted in a simplified microbial population while seed obtained by chemical treatment had a relatively complex bacterial community, which also suppressed methanogenic activity\textsuperscript{63}. In another study, HST inoculum showed higher H\textsubscript{2} production than acid treated inoculum with chemical wastewater as substrate\textsuperscript{14}. Thus, efficiency of applied pretreatment depends on the nature of wastewater used as substrate, nature of parent inoculum and system operating conditions.

Combination of different pretreatment methods also showed positive effect on H\textsubscript{2} evolution rate\textsuperscript{12,14,16-19}. Integration of acid and chemical (BESA) pretreatment methods evidenced higher H\textsubscript{2} production. Sequentially coupled repeated pretreatments [HST (100°C, 2 h) and acid (pH 3, 24 h)] also showed positive influence on overall H\textsubscript{2} generation when chemical wastewater was used as substrate\textsuperscript{16}. Integration of multiple pretreatment procedures (chemical, HST and acid) showed positive influence on H\textsubscript{2} production with distillery based parent inoculum\textsuperscript{19}. By combining all three methods, acidogenic spore forming bacteria can be enriched which are capable of producing H\textsubscript{2} associated with VFA production as metabolic intermediate in absence of methanogenic activity. Selective enrichment strategy by pretreatment procedure has resulted in enumeration of specific group of morphologically similar group of bacteria\textsuperscript{16,17,19,64}. Efficiency of HST (100°C, 1 h), acid pretreatment (pH 3, adjusted with ortho-phosphoric acid; 24 h) and chemical pretreatment [BESA (0.2 g/l), 24 h] on anaerobic mixed microflora for selectively enriching H\textsubscript{2} producing mixed consortia was evaluated individually and in combination with wastewater\textsuperscript{12,17}, chemical wastewater\textsuperscript{12,16} and designed synthetic wastewater\textsuperscript{16,18} as fermentative substrates. LST combined with pH control (5.5) showed good H\textsubscript{2} production\textsuperscript{10}. HST pretreated inoculum showed higher H\textsubscript{2} production and substrate degradation, whereas, acid pretreated inoculum had less efficiency with chemical wastewater as substrate\textsuperscript{14}. In
another study combining acid and chemical (BESA) methods yielded higher H₂ production efficiency followed by acid and HST, and acid, HST and chemical (BESA) methods when dairy wastewater was used substrate. Integration of multiple pretreatment methods [chemical (BESA), HST and acid] showed positive influence on process efficiency followed by HST, chemical (BESA) and acid treatments, when chemical wastewater was used as substrate. Scanning electron images of mixed consortia after combined pretreatment [HST, chemical (BESA) and acid] showed comparatively similar morphology demonstrating presence of related group of bacteria (Fig. 2). Selective enrichment procedure resulted in enumeration of specific group bacteria, capable of producing H₂. HST combined with chemical (acetylene) treatment has shown positive influence on H₂ production rate; HST combined with chemical (BESA) method (30 min, 121°C) is also reported. H₂ production using anaerobic consortia inhibited by acidogenic operation requires longer acclimatization period (10-30 days). Combined HST and chemical (acetylene) treatment has been observed to reduce acclimatization time (≤96 h).

Molecular H₂ generation was associated with substrate removal through anaerobic metabolic reaction. In spite of good enhancement in H₂ production, marked reduction in substrate degradation efficiency was observed after applying pretreatment which can be attributed to inhibition of MB in anaerobic parent inoculum. Methanogenesis is an essential metabolic route, which metabolizes resulting VFAs generated from acidogenic process to methane. Once methanogenic activity was inhibited due to pretreatment, a marked reduction in substrate degradation was observed. HST and acid treatment showed lower substrate removal compared to an untreated inoculum. In combination, acid and HST combination showed good substrate removal followed by acid and chemical (BESA) combination and acid, chemical (BESA) and HST combination when dairy wastewater was used as substrate. An untreated inoculum showed low H₂ yield in spite of effective substrate removal efficiency.
attributing to onset of solventogenesis leading to possibility of methane formation due to presence of MB.

Factors Influencing Biohydrogen Production

System operating conditions significantly influence overall performance of H₂ production process in association with wastewater treatment. Data enveloping analysis (DEA) performed on H₂ production process illustrated the importance of pH microenvironment, nature of mixed consortia, composition, nature and complexity of fermentative substrate used.

pH

Bacteria respond to changes in internal and external pH by adjusting their activity and synthesis of proteins associated with many different processes, including proton translocation, amino acid degradation, adaptation to acidic or basic conditions and virulence. Depending on organism and growth conditions, changes in external pH can bring about subsequent alterations in several primary physiological parameters, including internal pH, concentration of other ions, membrane potential and proton-motive force. This is especially important for fermentative H₂ production where activity of acidogenic group of bacteria is considered to be crucial and rate limiting. pH plays a critical role in governing metabolic pathways of organism where activity of AB is considered to be crucial. H₂ production occurs in acidification stage of metabolic process. System pH also influences efficiency of substrate metabolism, protein synthesis, synthesis of storage material and metabolic by-product release.

The pH could also stimulate microorganisms to achieve maximum H₂ production ability. Restricted nature of specific group of bacteria at particular pH helps to maintain reactor in H₂ producing conditions during operation. Optimum pH range for MB is between 6.0-7.5, while AB functions well below 6 pH. The pH range of 5.5-6.0 is ideal to avoid methanogenesis and solventogenesis, which is a key factor for effective H₂ generation. Maintaining pH in acidophilic range (5.5-6.0) is ideal for effective H₂ production due to repression in MB, thus indirectly promoting H₂ producers within the system. However, highly acidic pH is detrimental to H₂ production as it inactivates H₂ producing bacteria. Moderately low pH (5.5-6.0) induces a process that protects cell from a subsequent challenge at lower pH (3.4-4.0) by acid tolerance response (ATR). Fermentative conversion of substrate to H₂ can be increased by maintaining operating pH in and around 6 compared to near neutral pH values of 5.5-7.5. Maintenance of acidophilic conditions (pH at 5.5) gave good H₂ production yield and easy to implement. Initial pH values of 5.5-7.5 represent optimum and acceptable pH ranges for H₂ production, whereas H₂ yield sharply drops at pH lower than 5.5 or higher than 7.5. Increase in feeding pH (acidic to neutral) has resulted in suppressed H₂ production. Compared to neutral conditions [15 days (continuous); 18 days (fed-batch)] rapid stabilization of performance (12 days) was observed during acidophilic operation irrespective of the operation mode used. pH control could stimulate microorganisms to achieve maximum H₂ production ability because the activity of hydrogenase was inhibited by low or high pH in fermentation.

It is necessary to avoid the presence of organisms utilizing H₂, particularly methanogens, and this has been achieved in laboratory studies by operating at low pH and/or short retention times, since methanogens are more affected by lower pH and are slow growing than fermentative organisms. In this direction, feeding pH showed considerable influence on both fermentative H₂ production and substrate removal efficiency in biofilm configured reactor. Compared to neutral pH, relatively low substrate degradation efficiency is observed at acidophilic pH (below 6), which reduced methanogenic activity. There is a trade-off between technical efficiency based on H₂ yield and substrate removal at different feeding pH. Neutral pH is ideal for wastewater treatment while acidophilic pH is useful for effective H₂ production. Influence of feeding pH (6 and 7) on molecular H₂ production and substrate degradation was evaluated with anaerobic mixed consortia in biofilm reactor using molasses based wastewater and synthetic wastewater. Acidophilic feeding pH (6.0) documented effective H₂ production while neutral pH (7.0) showed higher substrate degradation rate. Acidogenic operation along with HST and chemical (acetylene) pretreatment methods have been used. Maintenance of acidophilic conditions (pH at 6) along with pretreatment was observed to be effective in H₂ production during treatment of chemical wastewater, molasses wastewater and dairy wastewater. Bio-electrochemical behavior of mixed anaerobic consortia (whole cell) during H₂ production process was evaluated employing cyclic voltammogram (CV) in electrochemical cell [platinum as working
electrode; Ag/AgCl (S) as reference electrode; graphite rod as counter electrode; wastewater as electrolyte] to gain insight into the possible mechanism based on intracellular electron transfer involved in the fermentative metabolic process with the function of pH microenvironment. CV obtained at acidophilic and neutral pH conditions visualized marked variation in forward and reverse scans. In acidophilic condition a clear redox peak was observed at 6 h in the reverse scan (-0.323 V to -0.214 V vs Ag/AgCl) along with poorly defined peak in the forward scan (0.333 V vs Ag/AgCl) and these redox pairs persisted up to 24 h of operation in the range of applied voltage with improved intensity. Redox peak in the reverse scan improved with time while the peak in the forward scan did not show much variation in the intensity. In neutral operation, the voltammogram showed redox peaks in the reverse direction (-0.240 V to -0.060 V vs Ag/AgCl) with exception of 6 h (-0.337 V to -0.252 V vs Ag/AgCl). However, in the forward scan, a peak was first observed at 12 h (0.155 V to 0.170 V) which sustained up to 24 h. Increase in signal/peak intensity was observed up to 24 h and 12 h during neutral and acidophilic operations respectively. The signal obtained at both pH microenvironments corresponds to intracellular electron carrier, NADH (E° = -0.32 V). However, relatively higher energy output and comparatively more or less uniform energy generation are observed in acidophilic microenvironment, which might be attributed to the efficient proton transfer between metabolic intermediates.

VFA and pH are integral expressions of acid-base conditions of any anaerobic process and also an intrinsic index of balance between two of the most important microbial groups (AB and MB). Production of acids gradually reduces buffering capacity of system, which, in turn, resulted in a concomitant decline in the system pH in all experimental variations studied, due to accumulation of organic acids leading to process inhibition. If pH is not maintained in optimum range, cessation of H₂ production will result along with marked shift in microbial population. Higher concentration of soluble metabolite production (VFA) is observed under acidophilic experiments over the corresponding neutral microenvironment and corroborates well with H₂ production observed. During acidophilic operation, soluble metabolites distribution showed acid-forming (formation of acetic acid) metabolic flow, which is considered optimum for effective H₂ production.

**Hydraulic Retention Time (HRT)**

HRT also influences H₂ generation process. Fermentation time is considered as an important operational parameter to restrict the process of

<table>
<thead>
<tr>
<th>Wastewater type, operation details, bioreactor configuration</th>
<th>Organic loading rate kg COD/m³-day</th>
<th>Volumetric H₂ production mmol H₂/m³-day</th>
<th>COD removal efficiency (%)/substrate degradation rate</th>
<th>Specific H₂ yield mol H₂/kg COD₅⁻/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic wastewater [BOD/COD = 0.67; total/working volume, 4/2], Biofilm reactor</td>
<td>4.8</td>
<td>6.97</td>
<td>32.4/1.55</td>
<td>14.35</td>
</tr>
<tr>
<td>Chemical wastewater [BOD/COD = 0.3; total/working volume, 4/2], Biofilm reactor</td>
<td>5.6</td>
<td>3.28</td>
<td>26.7/1.49</td>
<td>7.02</td>
</tr>
<tr>
<td>Molasses wastewater [BOD/COD = 0.35; total/working volume, 1/0.8], Biofilm reactor</td>
<td>7.9</td>
<td>2.70</td>
<td>17.2/1.35</td>
<td>6.06</td>
</tr>
<tr>
<td>Dairy wastewater [BOD/COD = 0.45; total/working volume, 1.8/1.5], Suspended growth reactor</td>
<td>2.83</td>
<td>21.94</td>
<td>57.6/1.63</td>
<td>20.1</td>
</tr>
<tr>
<td></td>
<td>4.6</td>
<td>22.66</td>
<td>52.6/2.41</td>
<td>16.8</td>
</tr>
</tbody>
</table>
methanogenesis during acidogenic \( \text{H}_2 \) production. It facilitates metabolic shift in concurrence with extended fermentation time, nature of inoculum, nature of substrate, applied loading rate and fermentation pH. A number of workers have used short HRT and acidophilic pH. Optimum HRT between 8.0 and 14 h\(^2,39,67,89\) has been reported to yield maximum \( \text{H}_2 \) with inhibition of methanogenesis. Continuous \( \text{H}_2 \) production from a mixed culture was observed at long HRT of 3 days (pH 6.4) without encountering problems with methanogenesis\(^90\). Reducing HRT from 18 h to 12 h improved \( \text{H}_2 \) yield without affecting starch removal efficiency\(^39\). Propionate production was observed to be lower at 12 h, suggesting washout of a non-\textit{Clostridial} propionate-forming community. Maximum \( \text{H}_2 \) yield is reported between 0-14 h of fermentation period in all experimental variations studied in batch mode during dairy, chemical and molasses based wastewater treatment\(^6,17,19,84\). Provision of short HRT’s during reactor operation may wash out slow growing methanogens and can reduce reactor size and capital cost\(^63\).

### Wastewater as Fermentable Substrate

Utilization of wastewater as primary fermentative substrate facilitates wastewater treatment apart from \( \text{H}_2 \) production, which is considered to be economically viable. Industrial wastewaters contain high levels of easily degradable organic material, which results in a net positive energy or economic balance, even when heating of the liquid is required\(^5\). Industrial wastewater as fermentative substrate for \( \text{H}_2 \) production will address most of the criteria for substrate selection viz., availability, cost and biodegradability\(^91\). Paper mill wastewater\(^92\), starch effluent\(^93\), food processing wastewater\(^24,94\), domestic wastewaters\(^74,95\), rice winery wastewater\(^61\), dairy wastewater\(^2,17,95\), chemical wastewater\(^16,18,64,84\), distillery and molasses based wastewater\(^19,96\), wheat straw waste\(^58\) and palm oil mill effluent\(^97,98\) have been studied as fermentable substrate for \( \text{H}_2 \) production along with wastewater treatment.

Comparative performance of bioreactor with respect to \( \text{H}_2 \) production and substrate degradation during treatment of chemical, distillery and wastewaters at IICT, Hyderabad is depicted in Table 1. \( \text{H}_2 \) production with simultaneous wastewater treatment was studied in anaerobic sequencing batch biofilm reactor (AnSBBR; temperature, 28±2°C, acidophilic conditions, pH 6.0) using distillery wastewater as substrate with selectively enriched anaerobic mixed consortia (sequentially pretreated with repeated HST (100°C, 2 h) and acid (pH -3.0, 24 h))\(^19\). Chemical wastewater as fermentative substrate was studied in anaerobic sequencing batch biofilm reactor (AnSBBR, temperature, 28±2°C, acidophilic conditions, pH 6) employing selectively enriched acidogenic \( \text{H}_2 \) producing mixed consortia (repeated HST (100°C, 2 h) and acid (pH -3.0, 24 h) treatment) and observed \( \text{H}_2 \) production along with simultaneous wastewater treatment (substrate/COD removal efficiency, 22 to 17%)\(^16,84\). With dairy wastewater as fermentative substrate relatively good \( \text{H}_2 \) production in conjugation with wastewater treatment was observed in suspended growth reactor operated with sequentially pretreated [HST (100°C, 2 h) and acid (pH 3.0, 24 h)] mixed consortia [temperature, 28±2°C, acidophilic conditions, pH 6.0]\(^17\). Relatively good \( \text{H}_2 \) yield along with concurrent substrate/COD removal efficiency (50 to 64.7%) observed compared to chemical and distillery wastewater as substrates apart from rapid stabilization. Inferior performance observed especially with chemical wastewater may be attributed to the complex nature of substrate, due to its low-biodegradability (BOD/COD=0.3) and its composite nature\(^16,84\). Addition of co-substrate has shown positive influence on both \( \text{H}_2 \) production and substrate degradation\(^14,16\). \( \text{H}_2 \) production efficiency was found to depend on nature and composition of wastewater used as fermentable substrate and its biodegradability, reactor configuration, operation mode of reactor, initial and operating pH and substrate loading rate apart from nature of anaerobic mixed consortia used. Extent of substrate degradation of wastewater under acidogenic microenvironment was also important when \( \text{H}_2 \) production efficiency is concerned. Relatively low COD removal efficiency (between 17 to 57%) was observed during acidogenic process which was attributed due to the persistent of acidophilic microenvironment. Due to the persistence of acidophilic microenvironment associated with soluble acid metabolites production as end-products, process inhibition takes place leading to low substrate conversion efficiency to \( \text{H}_2 \). Nature and composition of wastewater associated with other system operational conditions will govern the extent of the substrate degradation.

### Substrate Loading Rate

Apart from wastewater characteristics, substrate/organic loading rate (OLR) of wastewater had marked influence on \( \text{H}_2 \) production. \( \text{H}_2 \) yields were inversely related to glucose feeding rate, while highest \( \text{H}_2 \) yields

---

**Table 1:**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>HRT (h)</th>
<th>H2 Yield (%)</th>
<th>COD Removal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical</td>
<td>8-14</td>
<td>50-64.7</td>
<td>22-17</td>
</tr>
<tr>
<td>Distillery</td>
<td>8-14</td>
<td>50-64.7</td>
<td>22-17</td>
</tr>
<tr>
<td>Dairy</td>
<td>8-14</td>
<td>50-64.7</td>
<td>22-17</td>
</tr>
</tbody>
</table>

---

**References:**

1. MOHAN et al: BIOHYDROGEN PRODUCTION WITH WASTEWATER TREATMENT 957
were observed at lowest glucose loading rate\textsuperscript{99}. Glucose concentration exceeding 2 g/l (as co-substrate) showed suppression in H\textsubscript{2} production\textsuperscript{18}. Feed consisting of only glucose as substrate showed low H\textsubscript{2} yield, while feed with chemical wastewater admixed either with glucose or with sewage wastewater as co-substrates demonstrated relatively high H\textsubscript{2} yield\textsuperscript{18}. Adding glucose and sewage wastewater as co-substrates along with chemical wastewater showed positive influence on H\textsubscript{2} generation rate\textsuperscript{14,18}. Influence of OLR on fermentative H\textsubscript{2} production was studied using chemical wastewater as substrate\textsuperscript{84} in which a marked reduction in H\textsubscript{2} production rate was observed with increase in OLR [specific hydrogen yield - 13.44 mol H\textsubscript{2}/kg COD\textsubscript{r}-day (6.3 kg COD/m\textsubscript{3}-day), 8.23 mol H\textsubscript{2}/kg COD\textsubscript{r}-day (7.1 kg COD/m\textsubscript{3}-day) and 6.064 mol H\textsubscript{2}/kg COD\textsubscript{r}-day (7.9 kg COD/m\textsubscript{3}-day)]. This may be reasoned for increase in recalcitrant nature of wastewater at corresponding OLR studied. Increase in OLR also showed marginal reduction in COD removal efficiency in spite of constant substrate degradation rate (22.6% - 6.3 kg COD/m\textsubscript{3}-day; 19.8% - 7.1 kg COD/m\textsubscript{3}-day and 17.2% - 7.9 kg COD/m\textsubscript{3}-day). H\textsubscript{2} evolution rate also showed suppression with increase in OLR when dairy wastewater was used as substrate\textsuperscript{17}. Substrate (COD) removal efficiency of 58% to 50% was registered with increase in OLR. Increase in H\textsubscript{2} yield may be due to end product inhibition by over-accumulated (supersaturated) H\textsubscript{2} in liquid at high OLRs\textsuperscript{100,101}. H\textsubscript{2} production rate increased with increase in initial glucose concentration from 0.5-2.0% but dropped at 2.5% indicating substrate inhibition\textsuperscript{102}. Similar observations with H\textsubscript{2} production in concurrence with substrate loading was reported\textsuperscript{15,44,103}. At higher feeding rates, where more H\textsubscript{2} was produced, there would have been more inhibition of hydrogenase\textsuperscript{106}. Decline in process efficiency observed at higher OLRs may also be attributed to inhibition occurred due to higher substrate availability\textsuperscript{104}. However, each wastewater has its own threshold value, which relates to system microenvironment and output required. However, for effective H\textsubscript{2} yield and substrate degradation, significantly diverse system operational conditions are required individually under anaerobic microenvironment. Balancing conditions for combined effective performance and process optimization in this direction are especially important to sustain economic viability. In this direction, a study was performed to evaluate the system performance by combining both output parameters using two diverse mathematical approaches [data enveloping analysis (DEA) and Taguchi design of experimental (DOE) methodology]\textsuperscript{14}. Feed composition showed stronger influence followed by pH and pretreatment with respect to H\textsubscript{2} production. Acidophilic pH responded favorably to H\textsubscript{2} generation. Feed composition showed significant influence on H\textsubscript{2} production and substrate degradation.

**Concluding Remarks**

Reactor configuration, operation mode of reactor, nature and origin of inoculum, pretreatment applied to parent inoculum, operation conditions such as feeding and operating pH, OLR, HRT, etc. significantly contribute to H\textsubscript{2} production and substrate degradation apart from nature and characteristics of wastewater. Amount of substrate degradation is important when process efficiency is considered when dealing with wastewater as fermentative substrate for H\textsubscript{2} production. Balancing the conditions for combined effective performance are especially important for up-scaling the process and to sustain its economic viability. One of the vital aspects to be paid significant attention is non-utilized organic fraction, which remains as a soluble fermentation product from acidogenic H\textsubscript{2} production process. Utilization of residual organic fraction in wastewater associated with VFA mixture as substrate can be explored. Integrating this with acidogenic/photo-biological process for additional biohydrogen production or anaerobic process for producing CH\textsubscript{4} or microbial fuel cell (MFC) for producing bioelectricity can be evaluated.

**Acknowledgments**

Author thanks DBT, New Delhi for financial support. Author also thanks Dr J S Yadav, Director, IICT, Hyderabad, and Dr P N Sarma, Head, BEEC, IICT for encouragement and support, and acknowledges inputs of V L Babu, G Mohananikrishna, S V Raghuvulu and S Srikanth.

**References**

4. Venkata Mohan S, Harnessing of biohydrogen from wastewater treatment using mixed fermentative consortia: process evaluation towards optimization, *in Proc Int Workshop on...*


26 O-Thong S, Prasertsan P & Kare Birkeland N, Selection and implementation of biohydrogen producing seeds and responsible community structures under thermophilic condition, Bioresour Technol, 2008 (in press).


68 Dinopoulou G, Rudd T & Lester J N, Anaerobic acidogenesis of complex wastewater. The influence of operational