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REVIEW PAPER

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## RECENT DEVELOPMENTS IN BIOLOGICAL HYDROGEN PRODUCTION PROCESSES

*Biohydrogen production technology can utilize renewable energy sources like biomass for the generation of hydrogen, the cleanest form of energy for the use of mankind. However, major constraints to the commercialization of these processes include lower hydrogen yields and rates of hydrogen production. To overcome these bottlenecks intensive research work has already been carried out on the advancement of these processes such as the development of genetically modified microorganisms, the improvement of the bioreactor design, molecular engineering of the key enzyme hydrogenases, the development of two stage processes, etc. The present paper explores the recent advancements that have been made till date and also presents the state of the art in molecular strategies to improve the hydrogen production.*

*Key words: biohydrogen; hydrogenases; genetically engineered microorganisms; microbial fuel cell; two-stage process.*

Renewable energy sources have received great attention from the international community during the last decades. These include solar power, wind power, hydroelectricity, biomass etc. Currently, hydrogen is considered as a dream fuel by virtue of the fact that it is renewable, does not evolve the green house gas, has high energy content per unit mass of any known fuel ( $143 \text{ GJ t}^{-1}$ ), is easily converted to electricity by fuel cells and on combustion it gives water as the only byproduct. Presently, 40 %  $\text{H}_2$  is produced from natural gas, 30 % from heavy oils and naphtha, 18 % from coal, and 4 % from electrolysis and about 1 % is produced from biomass [1-2]. The advantages and disadvantages of various  $\text{H}_2$  production processes are outlined in Table 1.

Today, biological  $\text{H}_2$  production processes are becoming important mainly due to two reasons: (i) they can utilize renewable energy resources, and (ii) they are usually operated at ambient temperature and atmospheric pressure. However, the reported biohydrogen production rates, stabilities and efficiency of these processes are yet to be sufficient to make them commercially viable.

The present paper discusses the bottlenecks to overcome the major technical barriers and also deals

with the advancements of biological  $\text{H}_2$  production processes.

Table 1. Advantages and disadvantages of different hydrogen production processes

Process	Advantages	Disadvantages
Thermochemical gasification	Maximum conversion can be achieved	Significant gas conditioning is required. Removal of tar
Pyrolysis	Produces carbonaceous material along with bio-oil, chemicals and minerals	Chances of catalyst deactivation
Solar gasification	Good hydrogen yield	Required effective collector plates
Supercritical conversion	Can process sewage sludge, which is difficult to gasify	Selection of supercritical medium

### PROCESSES AVAILABLE FOR BIOHYDROGEN PRODUCTION

Hydrogen is a natural, though transient, by-product of several microbial driven biochemical reactions, mainly in anaerobic fermentation processes. In addition, certain microorganisms can produce enzymes that can produce  $\text{H}_2$  from water if an outside energy source, like sunlight, is provided to them. Specific ways in which microorganisms can produce  $\text{H}_2$  are described below.

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*i.* Biophotolysis of water using green algae and blue-green algae (cyanobacteria)

- Direct biophotolysis
- Indirect biophotolysis

*ii.* Photofermentation

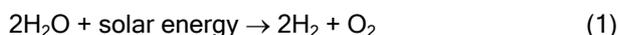
*iii.* Dark fermentation

*iv.* Hybrid systems

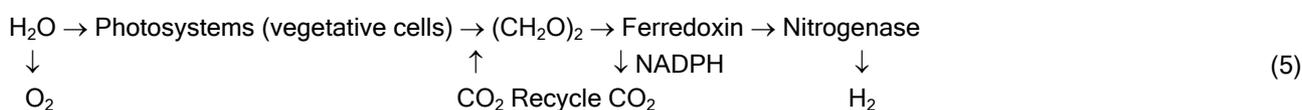
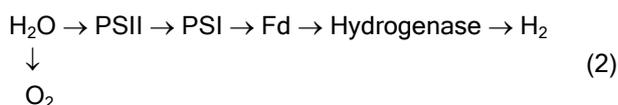
- Using dark fermentative and photofermentative bioreactors
- Using bioelectrochemically assisted microbial bioreactors.

### Direct biophotolysis

A direct biophotolysis of H<sub>2</sub> production is a biological process which utilizes solar energy and photosynthetic systems of algae to convert water into chemical energy.



The two photosynthetic systems responsible for photosynthesis process are: (*i*) photo system I (PSI) which produces reductant for CO<sub>2</sub> and (*ii*) photo system II (PSII) which splits water to evolve O<sub>2</sub>. The two photons obtained from the splitting of water can either reduce CO<sub>2</sub> by PSI or form H<sub>2</sub> in the presence of hydrogenase. In plants, due to the lack of hydrogenase, only CO<sub>2</sub> reduction takes place. On the contrary, green algae and cyanobacteria (blue-green algae) contain hydrogenase and thus have the ability to produce H<sub>2</sub> [3]. In these organisms, electrons are generated when PSII absorbs light energy, which is then transferred to ferredoxin. A reversible hydrogenase accepts electrons directly from the reduced ferredoxin to generate H<sub>2</sub> in the presence of hydrogenase.



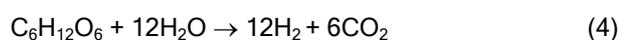
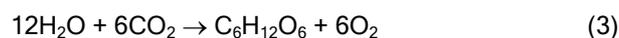
However, it is not yet clear how to manage such a process for the efficient H<sub>2</sub> production. Indeed, when dark adapted (hydrogenase induced) cells are exposed to light, photosynthetic O<sub>2</sub> production commences inhibiting H<sub>2</sub> evolution [8]. Thus, the key issue is to delay the onset of PSII activity during the light driven H<sub>2</sub> production until all the substrate is exhausted.

Since hydrogenase is sensitive to oxygen, it is necessary to maintain the oxygen content at a low level (under 0.1 %) so that the hydrogen production can be sustained [4]. This condition can be obtained by the use of green algae, *Chlamydomonas reinhardtii* which can deplete oxygen during the oxidative respiration [5]. However, the reaction is very short-lived and the rate of the hydrogen production is very low, less than one-tenth than that of other photosynthetic reactions [6].

### Indirect biophotolysis

In indirect biophotolysis, the problem of sensitivity of the H<sub>2</sub> evolving process to O<sub>2</sub> is usually circumvented by separating O<sub>2</sub> and H<sub>2</sub> [7]. In this process, CO<sub>2</sub> is intermittently fixed and released serving as the electron carrier between the O<sub>2</sub> producing (water splitting) reaction and the O<sub>2</sub> sensitive hydrogenase reactions. In such concepts the algae undergo a cycle of CO<sub>2</sub> fixation into storage carbohydrates (starch, glycogen) followed by its conversion to H<sub>2</sub> by dark anaerobic fermentation processes.

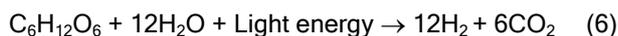
In a typical indirect biophotolysis H<sub>2</sub> is produced as follows:



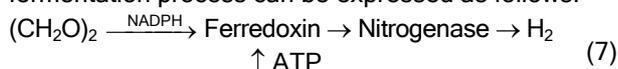
Many types of green algae and cyanobacteria, besides having the ability to fix CO<sub>2</sub> via photosynthesis, also have the ability to fix nitrogen from the atmosphere and produce enzymes that can catalyze the second H<sub>2</sub> generating step. Since these nitrogen fixing enzymes, nitrogenase, are localized within the heterocyst, they provide an O<sub>2</sub> free environment to carry out the H<sub>2</sub> evolution reactions.

### Photofermentation

H<sub>2</sub> production by purple non-sulfur bacteria is mainly due to the presence of nitrogenase under oxygen-deficient conditions using light energy and reduced compounds (organic acids). The reaction is as follows:



The overall biochemical pathways for the photo-fermentation process can be expressed as follows:



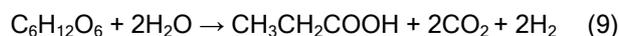
Photosynthetic bacteria have long been studied for their capacity to produce significant amounts of  $H_2$  [9]. The advantage of their use is in the versatile metabolic capabilities of these organisms and the lack of Photo system II (PSII), which automatically eliminates the difficulties associated with  $O_2$  inhibition of  $H_2$  production. Phototrophic bacteria require an organic or inorganic electron source to drive their photosynthesis. They can utilize a wide range of cheap compounds. These photoheterotrophic bacteria have been found suitable to convert light energy into  $H_2$  using organic wastes as substrate [9-11] in batch processes [12], continuous cultures [13], or immobilized whole cell system using different solid matrices like carrageenan [14], agar gel [15], porous glass [11], and polyurethane foam [10].

Major bottlenecks of the process involve low photochemical efficiencies (3-10 %). Moreover, inhomogeneity of the light distribution in the reactor also contributes to lowering of the overall light conversion efficiency. This may be overcome by using co-cultures having different light utilization characteristics. The improvement of a photobioreactor design with the increased light diffusing capability may also improve photoconversion efficiency rates. By this process,  $H_2$  yields may further be improved by maintaining a maximal activity of nitrogenase and a minimal activity of hydrogenase, a favorable molar ratio of the carbon source to nitrogen source and the availability of a uniform distribution of light through the culture [16]. The major hindrance in the practical application of this process is a large surface area required to collect light energy, since the construction of such photobioreactors with a large surface/volume ratio for direct absorption of sunlight is expensive. A possible alternative to this might be the utilization of solar collectors [16].

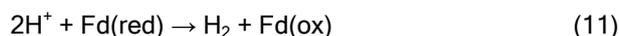
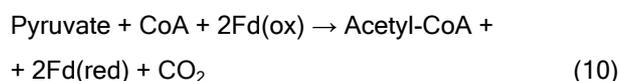
### Dark fermentation

Dark fermentation is a ubiquitous phenomenon under anoxic or anaerobic conditions. The oxidation of the substrate by bacteria generates electrons which need to be disposed off in order to maintain the electrical neutrality. Under the aerobic conditions  $O_2$  serves as the electron acceptor while under the anaerobic or anoxic conditions other compounds, such as protons, act as the electron acceptor and are reduced to molecular  $H_2$  [17,18]. Carbohydrates, mainly glu-

cose, are the preferred carbon sources for this process, which predominantly give rise to acetic and butyric acids together with  $H_2$  evolution [19].



In this process, glucose is initially converted to pyruvate by the glycolytic pathway. Pyruvate is further oxidized to acetyl-CoA, which can be converted to acetyl phosphate and results in the generation of ATP and the excretion of acetate. This oxidation to acetyl-CoA requires a ferredoxin (Fd) reduction. Reduced Fd is oxidized by hydrogenase which regenerates Fd(ox) and releases electrons as molecular  $H_2$  [19,20]. The overall reaction of the processes can be described as follows:



Despite having higher evolution rate, the yield of  $H_2$  from the fermentation process is lower than that of other chemical/electrochemical processes. Theoretical  $H_2$  yield is 4 mol of  $H_2$ /mol of glucose when the end product is acetic acid, while 2 mol of  $H_2$ /mol of glucose will be obtained if the metabolic end product is butyric acid. In practice, the yields are low since the end products contain both acetate and butyrate [19]. Besides, as yields increase the reaction becomes thermodynamically unstable. Another constraint of the process is the low conversion efficiencies of the substrate used.

### Hybrid fermentation technology

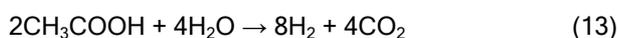
#### *Using dark fermentative and photofermentative bioreactors*

Hybrid fermentation technology might be one of the promising routes for the enhancement of  $H_2$  production yields. The synergy of the process lies in the maximum conversion of the substrate which otherwise fails to achieve a complete conversion due to thermodynamic limitations [21]. Thus, in this system the light independent bacteria and photosynthetic bacteria provide an integrated system for maximizing the  $H_2$  yield [22]. In such a system, the anaerobic fermentation of carbohydrate (or organic wastes) produces intermediates, such as low molecular weight organic acids, which are then converted into  $H_2$  by the photosynthetic bacteria in the second step in a photo-bioreactor. The overall reactions of the process can be represented as:

*i.* Stage I. Dark fermentation (facultative anaerobes):



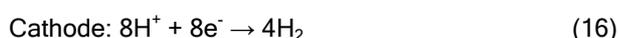
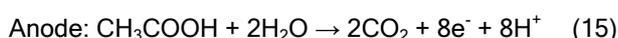
*ii.* Stage II. Photo-fermentation (photosynthetic bacteria):



So, theoretically it is evident that using glucose as the sole substrate in the dark anaerobic fermentation, where acetic acid is the predominant metabolite, a total of 12 mol H<sub>2</sub> could be expected in a combined process from one mol of glucose.

*Using bioelectrochemically assisted microbial bioreactors*

Microbial fuel cell (MFC) produces protons and electrons due to the oxidation of the organic matter by the bacteria [23,24]. Protons diffuse through the electrolyte towards the cathode, while the electrons travel around in a circuit to the cathode producing current. At the cathode oxygen reacts with the electrons and protons to form a reduced compound, as water. In a bioelectrochemically assisted microbial reactor (BEAMR), H<sub>2</sub> is evolved at the cathode [25] using any biodegradable material, including glucose, acetate, proteins, starch and cellulose.



At pH 7.0 the voltage that is required to produce H<sub>2</sub> is theoretically -0.61 V ( $V_{\text{cat}}$  versus Ag/AgCl) [26]. In practice, the minimum applied voltage to produce H<sub>2</sub> from the bioelectrolysis of acetate has been found to be more than 0.25 V due to ohmic resistance and electrode overpotentials [25] which is still substantially less than 1.8-2.0 V needed for H<sub>2</sub> production via water electrolysis (alkaline conditions) [27]. The BEAMR and MFC systems share many similar characteristics, and therefore many findings for improving the electricity generation in MFCs should be applicable for increasing H<sub>2</sub> production in the BEAMR system.

Each of the processes defined above have their own merits and demerits, however none of the technologies have achieved the level of perfection required for commercial purposes. On the other hand, the basic sciences on which biohydrogen technologies and process development must be based have greatly advanced over the past two decades, with an ever more intimate understanding of the genetics, biochemistry, and physiology of the microbial H<sub>2</sub> metabolism. This fundamental scientific information must now

be applied to the development of H<sub>2</sub> production processes that could deliver biohydrogen at acceptable costs. Conversely, we can also identify those approaches that are less promising to allow focusing the limited R&D resources only on the most promising alternatives.

## MAJOR CHALLENGES IN BIOLOGICAL HYDROGEN PRODUCTION PROCESSES

Major challenges need to be overcome for the smooth transition from the fossil fuel based economy to the H<sub>2</sub> energy based economy and may be outlined as follows:

- The yield of H<sub>2</sub> from any of the processes defined above is low for commercial application. The pathways of H<sub>2</sub> production have not been identified and the reaction remains energetically unfavorable.

- Processing of some biomass feed stock is too costly. There is a need to develop low cost methods for growing, harvesting, transporting and pre-treating energy crops and/or biomass waste products.

- There is no clear contender for a robust, industrially capable microorganism that can be metabolically engineered to produce more than 4 mol H<sub>2</sub>/mol of glucose.

- Several engineering issues need to be addressed which include the appropriate bioreactor design for H<sub>2</sub> production, difficult to sustain steady continuous H<sub>2</sub> production rate in the long term, scale-up, preventing interspecies H<sub>2</sub> transfer in non sterile conditions and separation/purification of H<sub>2</sub>.

- Sensitivity of hydrogenase to O<sub>2</sub> and H<sub>2</sub> partial pressure severely disrupts the efficiency of the processes and adds to the problems of lower yields.

- Insufficient knowledge on the metabolism of H<sub>2</sub> producing bacteria and the levels of H<sub>2</sub> concentration tolerance of these bacteria.

- A lack of understanding on the improvement of economics of the process by integration of H<sub>2</sub> production with other processes.

## SCIENTIFIC ADVANCEMENTS MADE AND TECHNICAL BREAKTHROUGHS REQUIRED

To overcome the above bottlenecks a number of scientific advances and technical breakthroughs are required.

### Genetic modification of the microorganism

The achievement of higher yields is a critical research objective for the sustenance of H<sub>2</sub> as the dream fuel of the future. Biohydrogen productivities of 605 mg

$\text{H}_2 \text{ h}^{-1} \text{ l}^{-1}$  by an undefined consortium is the highest productivity that has been reported so far [28]. But this process is still not commercially viable [29]. Various strategies have therefore to be applied both at the fermentative and genetic level to improve  $\text{H}_2$  production rates and yields.

Today, genetic engineering is the key area of focus for the improvement of strains for  $\text{H}_2$  production. In photosynthetic bacteria the presence of pigments hinders the intensity of the light in the photobioreactor. An approach for the improvement of  $\text{H}_2$  production by photosynthetic bacteria, therefore, would be to control the photosynthetic protein expression to allow efficient absorption of the light energy [30]. A purple bacterium *R. sphaeroides* is a strong  $\text{H}_2$  producer [31]. However, the light absorption by the bacterial cells reduces the penetration of light into the photobioreactor and suppresses the  $\text{H}_2$  production rate [32]. Towards this end, Miyake et al. [31] proposed a method for the enhancement of the bacterial light-dependent  $\text{H}_2$  production by altering the light harvesting (LH1/LH2) ratio of *Rhodobacter sphaeroides* RV. A model mutant strain, P3, was obtained by UV irradiation from the wild strain (RV). The amount of bacteriochlorophyll in LH1 was reduced to 35 % and that in LH2 was enhanced to 140 % in P3 compared to RV. This resulted in 1.4 times higher  $\text{H}_2$  production rates in P3 than RV [31].

Despite the access to new genetic engineering tools there is a practical limit to the number of heterologous genes that can be expressed and controlled by antibiotic resistance. To bypass the need for multiple plasmids Akhtar et al. demonstrated the convergence of all the *hyd* genes of *C. acetobutylicum* onto a single plasmid in the form of a synthetic *hydF-hydE-hydG-hydA* operon [33]. The significance and the applicative value of the development of the synthetic operon can be best understood in the broad scheme of metabolic engineering [34].

In another approach, it may be possible to harness the key metabolic network of the high  $\text{H}_2$  producing organisms, to alternate the model hosts like *E. coli* which grows much faster under the easily commercializable conditions so as to proceed from a laboratory model to a practically applicable strain.

At this moment, the acceptability of genetically modified microorganisms is a challenge because of the possibility of horizontal gene transfer [35]. However, this can be ruled out by chromosomal integration and the elimination of plasmids containing antibiotic markers with available molecular tools [36].

### Improvement of $\text{H}_2$ yield by using metabolically engineered organisms

Metabolic engineering involves genetically modified microorganisms to target and manipulate enzymatic, regulatory, or transport pathways that impact a particular microbial process such as  $\text{H}_2$  production. Recent success in genome sequencing and a gene expression analysis has enhanced the ability to engineer microorganisms for specific metabolic tasks.

The study of physiology of genetically modified photosynthetic microorganisms has shown that the electron flux can be redirected to the bidirectional [NiFe]-hydrogenase as demonstrated in a *ndhB* mutant (defective in the type I NADPH-dehydrogenase complex) of *Synechocystis* and that a change in carbon metabolism in mutants of *Rhodobacter capsulatus* can affect the flow of the reducing equivalent from organic substrates to nitrogenase [37]. Increasing the flux through an existing pathway or redirecting the enzyme-catalyzed reactions is an approach referred to as metabolic engineering.

It is well known that organic acids synthesized during microbial metabolism decrease the  $\text{H}_2$  producing efficiency of the microorganism since too low pH conditions force the microorganism to reduce the concentration of  $\text{H}^+$  thereby inhibiting the production of  $\text{H}_2$ . Kumar et al. have shown that the improvement in the yield of  $\text{H}_2$  can be significantly achieved through redirection of the metabolic pathways by blocking formation of alcohol and some organic acids in *Enterobacter cloacae* IIT-BT 08. The double mutant with defects in both alcohol and organic acid formation pathways was shown to give the yields of 3.8 mol/mol of glucose which is very close to the theoretical yield of 4 mol/mol glucose [38].

Several studies show that  $\text{H}_2$  production can be increased by directing the carbon flow into synthesis of formate. Yoshida et al. [39] have experimentally proved that faster induction of the enzyme formate  $\text{H}_2$  lyase (FHL) is possible by elimination of lactate and succinate formation. Increased yields from 1.08 mol/mol glucose to 1.82 mol/mol glucose in the *E. coli* SR15 strain lacking lactate and succinate production have been achieved [39].

Metabolic engineering may target the increase of the  $\text{H}_2$  yield by increasing the substrate utilization efficiency of the organisms. Cellulose is found in abundance on the earth and may prove to be an apt substrate for biohydrogen production. Recently, the cellulose-degrading capabilities of *C. cellulolyticum* was improved by the heterologous expression of pyruvate decarboxylase and alcohol dehydrogenase from *Zy-*

*momonas mobilis* [40]. This metabolic engineering approach lowered the excess pyruvate accumulation generated from cellulose break down and thereby effectively reduced the toxic effect of pyruvate build-up.

So, by understanding which metabolic pathways contribute and regulate the H<sub>2</sub>-production, elimination of hydrogen-consuming reactions may be targeted to sustain and regulate the H<sub>2</sub> production rates. Detailed studies can be conducted to use genetic tools to overcome the metabolic barrier by manipulating the electron flux in H<sub>2</sub> producing organisms. The development of microbes that ferment multiple sugars, or which can directly utilize the naturally abundant sugars cellulose/hemicellulose, can be targeted. Moreover, computed models can be constructed to guide metabolic engineering, for example, models to identify control points for manipulating the flow of electrons to hydrogenase or to predict how cellular activity may impact H<sub>2</sub> yields.

### Engineering of hydrogenases

Hydrogenase is the key enzyme of the biological H<sub>2</sub> production process. It can be classified into three types [NiFe]-hydrogenase, [Fe]-hydrogenase and metal free hydrogenase. Out of these [NiFe]-hydrogenase is an uptake hydrogenase found in both aerobic and anaerobic microorganisms while [Fe]-hydrogenases preferentially catalyze H<sub>2</sub> reduction in anaerobic microorganisms [41].

The presence of uptake hydrogenases appears to consume and reuse the H<sub>2</sub> gas, resulting in a decrease in net H<sub>2</sub> production. Since uptake hydrogenases seem to decrease the efficiency of the process, genetic engineering should target to eliminate or mutate the gene responsible for the production of uptake hydrogenase. This may be achieved by insertional inactivation of the gene [42] site directed mutations etc. Today, the development of [NiFe]-hydrogenase uptake negative mutant is one of the key research areas.

Some nitrogen-fixing cyanobacteria are potential candidates for H<sub>2</sub> production. H<sub>2</sub> production by nitrogenase is, however, an energy-consuming process due to hydrolysis of many ATP molecules. On the other hand, hydrogenase-dependent H<sub>2</sub> production by cyanobacteria and green algae is "economic" with respect to energy requirements (no ATP requirements). This mechanism of H<sub>2</sub> production is not however sustainable under light conditions due to the inactivation of hydrogenase by O<sub>2</sub>. During biophotolytic H<sub>2</sub> production, O<sub>2</sub> is released from the water-splitting reaction; thus, engineering hydrogenases with a sufficient activity and O<sub>2</sub> tolerance will be needed. Engineered hydrogenases then could be used under op-

timal conditions for H<sub>2</sub> production. In regard of this, Siebert et al. demonstrated that an in vivo mutagenesis and selection approach resulted in a 10-fold increase in O<sub>2</sub> tolerance for the *Chlamydomonas reinhardtii* hydrogenase [43].

Hydrogenases are the key enzymes of the process thus over-expression of the enzymes may lead to the increased yields of H<sub>2</sub>. In fact, a 1.7-folds increase in H<sub>2</sub> production over the wild type was achieved in *C. paraputrificum* M-21 strain by over-expression of the *hydA* gene encoding the [Fe]-hydrogenase [44].

For further improvements, advanced techniques for visualizing different stages of hydrogenase assembly or monitoring a hydrogenase activity in living cells, in vivo, will be critical for the building of predictive models that can be used to engineer hydrogenases.

### Development of improved bioreactor design

The performance of bioreactors needs to be monitored, especially under the conditions of industrial H<sub>2</sub> production for robust, reliable performance and high sustained H<sub>2</sub> yields. Problems that need to be addressed include: a combinatorial screening of enzymes, kinetic study of the enzymatic activity, and the development of synthetic analogs of enzymes. Different types of bioreactors have already been reported for different biological H<sub>2</sub> production processes, e.g. a multi-layer bioreactor, UASB, a rhomboidal reactor etc. (Table 2). However, the major problem lies in the gas-hold up, which decreases the working volume of the reactor to a great extent. This can be partially overcome by using a rhomboidal bioreactor [52]. A light distribution in the photobiological reaction can be improved by using a multi-layer photo-bioreactor [45].

### Innovative methods by studying system economics

Since the substrate conversion efficiency of the dark fermentation process is low, the process can be made more economical by combining it with other processes that can further utilize the spent medium. The research on various such integrated systems has already been initiated. For instance, the combination of photosynthetic and anaerobic bacteria can provide an integrated system for maximization of H<sub>2</sub> yields [43]. In such a system, anaerobic fermentation of carbohydrate (or organic wastes) which produces intermediates, such as low-molecular weight organic acids, can be converted to H<sub>2</sub> by photosynthetic bacteria in the second step by using a photobioreactor. Lee et al. have studied the combination of purple nonsulfur (PNS) photosynthetic bacteria and anaerobic bacteria for the efficient conversion of wastewater into H<sub>2</sub> [53]. In another study, Kim et al. combined dark fermentation with

photo-fermentation to improve H<sub>2</sub> productivity from the food processing wastewater and sewage sludge [54]. Similar studies have been reported by Nath et al. using glucose as a substrate in the dark fermentation process, and the spent medium from this process has been used as a substrate for anoxygenic phototrophic PNS bacteria, *Rhodospseudomonas palustris* for H<sub>2</sub> pro-

duction by photo-fermentation process [19]. The maximum yield reported for such two stage processes is 7.1 mol H<sub>2</sub>/mol of glucose against the theoretical maximum of 12 mol H<sub>2</sub>/mol of glucose. Thus, it has been observed that H<sub>2</sub>-production can be significantly improved by using two-stage processes (Table 3).

Table 2. Performances of modified bioreactor and solid matrices for the H<sub>2</sub> production

Microorganisms	Raw materials	Type of modification	Maximum rate of H <sub>2</sub> production, ml H <sub>2</sub> l <sup>-1</sup> h <sup>-1</sup>	Reference
<i>Rhodobacter sphaeroides</i> RV	Basal medium with lactate and glutamate	Multi-layered photobioreactor (MLPR)	2000*	45
<i>Rhodospseudomonas palustris</i> WP3-5	Acetate	Internal optical fiber illumination	44.0	46
Sewage sludge	Sucrose	Fixed bed bioreactor with activated carbon	1320	47
Activated sludge and digested sludge	Glucose	Anaerobic fluidized bed reactor	2360	48
Anaerobic sludge	Sucrose	Polymethy methacrylate(PMMA) immobilized cells	1800	49
Sludge from wastewater treatment plant	Sucrose	Carrier-induced granular sludge bed (CIGSB)	9310	50
Sludge from wastewater treatment plant	Sucrose	Fluisized bed reactor (FBR)	1321.6	51
<i>Enterobacter cloacae</i> IIT-BT 08	Sucrose	Rhomboidal bioreactor	1732	52

\*ml m<sup>-2</sup> h<sup>-1</sup>

Table 3. Comparison of different two-stage processes for hydrogen production

Microorganisms used	Raw materials	Maximum organic acid concentration, mg l <sup>-1</sup>	Maximum H <sub>2</sub> yield mol H <sub>2</sub> /mol of glucose	Rate of H <sub>2</sub> production ml H <sub>2</sub> l <sup>-1</sup> h <sup>-1</sup>	Reference
Activated sludge and <i>Rhodobacter sphaeroides</i> O.U.001	Olive mill wastewater	-	-	8.1	55
<i>Lactobacillus delbrueckii</i> and <i>Rhodobacter sphaeroides</i> RV	Glucose	16 mM acetic acid and 10 mM lactic acid	7.1	-	56
<i>Escherichia coli</i> HD701 and <i>Rhodobacter sphaeroides</i> O.U.001	Glucose	20 mM acetate, 15 mM lactate, 3 mM succinate	-	5.2	57
<i>Clostridium butyricum</i> NCIB 9576 and <i>Rhodobacter sphaeroides</i> E15-1	Makkoli wastewater	-	-	16	54
<i>Rhodobacter sphaeroides</i> O.U.001 and <i>Halobacterium salinarum</i>	Basal medium with lactate and glutamate	-	-	27	58
<i>Enterobacter cloacae</i> DM11 and <i>Rhodobacter sphaeroides</i> O.U.001	Glucose	2,500 mg/l of VFA	5.3	-	19
<i>Clostridium butyricum</i> & <i>Enterobacter aerogenes</i> HO-39 and <i>Rhodobacter</i> sp. M-19	Starch manufacturing wastes	-	7.2	-	59

In the second approach, H<sub>2</sub> and methane production can be linked through a two-stage process. In the first process, H<sub>2</sub> can be recovered during hydrolysis and fermentation of organic matter while in the second process the spent media may be utilized for the generation of methane [60].

In another approach, a microbial fuel cell technology may be economized. In this technology bacteria are used directly to generate electricity. Complete utilization of the carbohydrate content in the wastewater may thus be achieved by using the spent media obtained after H<sub>2</sub> production for the generation of electricity through a microbial fuel cell.

### Molecular profiling to provide an overall view of cellular activity during hydrogen production

Ameliorations in computational capabilities and large-scale molecular profiling techniques (transcriptomics, proteomics, metabolomics) is needed to obtain an overall view of microbial H<sub>2</sub> production. These computational analyses can guide experimental investigations by defining the gene regulatory networks involved in H<sub>2</sub> production. Hence, the pathways that get triggered or deactivated can be identified for multiple organisms under the varying condition to find the most optimal conditions and most suited organism for H<sub>2</sub> production.

### Use of cheaper raw material

Raw materials add to the cost of biohydrogen production processes. The main criteria for the selection of a substrate for H<sub>2</sub> production are its availability, cost, carbohydrate content and biodegradability [61]. Currently, the cost of H<sub>2</sub> generated from biological

processes is very high (Table 4). Strategies should therefore be developed to effectively lower the cost of H<sub>2</sub> production. This can be achieved by either using the low cost substrate or finding a means to effectively utilize the 67-85 % of the unused spent media [60]. Commercially produced food products, such as corn and sugar are not economical for H<sub>2</sub> production [7]. However, solid organic wastes from agricultural crops, industrial processes and domestic waste water represent a valuable resource for the energy production. Starch based wastewater has great potentiality for the H<sub>2</sub> production [66]. Disposal of these wastes is an economic load on the society. Therefore, its utilization in the generation of an energy carrier will greatly add to economize the process. Besides, the development of low cost techniques is also required for harvesting, pre-processing and pre-treatment of biomass. Recently, it has been observed that the sewage sludge in combination with cane molasses improves the H<sub>2</sub> yield of the process to a great extent [67].

Table 4. Comparison of the energy conversion efficiency and unit cost of various biological hydrogen production processes with that of conventional processes

Name of the process	Raw materials	Energy conversion efficiency	Unit cost of energy content of the fuel US \$/MBTU	Reference
Photobiological hydrogen	H <sub>2</sub> O and organic acids	10	10	60
Fermentative hydrogen	Molasses	28.34	30	63
Fast pyrolysis for hydrogen production	Coal, biomass	-	4	64
H <sub>2</sub> from advanced electrolysis	H <sub>2</sub> O	-	11	64
H <sub>2</sub> from thermal decomposition of steam	H <sub>2</sub> O	-	13	64
H <sub>2</sub> from photochemical	Organic acids	-	21	64
Fermentative ethanol	Molasses	-	31.5	65
Gasoline	Crude petroleum	-	6	64

### Pilot plant studies

Very little information is available on the pilot plant studies for the biological H<sub>2</sub> production. A pilot plant of 1.48 m<sup>3</sup> capacity was studied continuously for 200 days [68]. The H<sub>2</sub> bio-producing reactor (HBR) system was operated under the organic loading rates (OLR) of 3.11-85.57 kg of COD m<sup>-3</sup> d<sup>-1</sup> with molasses as the substrate. Both biogas and H<sub>2</sub> yields increased with OLR at the range of 3.11-68.21 kg of COD m<sup>-3</sup> d<sup>-1</sup>, but decreased at high OLR (68.21-85.57 kg of COD m<sup>-3</sup> d<sup>-1</sup>). The biogas is mainly comprised of CO<sub>2</sub> and H<sub>2</sub>. The biogas contains 40 to 52 % v/v. A maximum H<sub>2</sub> production rate of 5.57 m<sup>3</sup> of H<sub>2</sub> m<sup>-3</sup> d<sup>-1</sup>, with a specific H<sub>2</sub> production rate of 0.75 m<sup>3</sup> of H<sub>2</sub>/kg of MLVSS per day,

was obtained in the bioreactor. The H<sub>2</sub> yield was 26.13 mol/kg of COD removed within OLR range of 35-55 kg of COD m<sup>-3</sup> d<sup>-1</sup>. In addition, it is reported that the hydrogen yield is affected by the presence of ethanol and acetate in the liquid phase, and the maximum H<sub>2</sub> production rate occurred while the ratio of ethanol to acetate was close to one [68]. Ethanol-type fermentation was favorable for H<sub>2</sub> production [65]. One pilot plant of capacity 800 dm<sup>-3</sup> is in operation at the Indian Institute of Technology Kharagpur using immobilized whole cell (*E. cloacae* IIT-BT 08) bioreactor (Fig. 1). The rate of H<sub>2</sub> production of the bioreactor is comparable with that of the bench scale data (77.3 mmol H<sub>2</sub> l<sup>-1</sup> h<sup>-1</sup>) [52].



Fig. 1. Pilot plant studies ( $800 \text{ dm}^3$ ) at Indian Institute of Technology Kharagpur, India, using immobilized *Enterobacter cloacae* IIT-BT 08 for hydrogen production from cane molasses.

## CONCLUSION

Biological  $\text{H}_2$  production is the most challenging area of biotechnology with respect to environmental problems. A challenging problem in establishing biohydrogen as a source of energy is the renewable and environmentally friendly generation of large quantities of  $\text{H}_2$  gas. However, two major aspects need indispensable optimization, viz., a suitable renewable biomass/wastewater and ideal microbial consortia that can convert this biomass efficiently to  $\text{H}_2$ . Comparative studies on the available processes indicate that biohydrogen production requires greater improvement on the process mainly with respect to  $\text{H}_2$  yield from the cheaper raw materials. The future of biological  $\text{H}_2$  production depends not only on research advances, i.e., the improvement in efficiency through genetically engineered microorganisms and/or the development of bioreactors, but also on economic considerations (the cost of fossil fuels), social espousal and the development of  $\text{H}_2$  energy systems.

## FUTURE GOAL

Several research groups have already carried out significant progress on biological  $\text{H}_2$  production processes, but still the economy of the process is not attractive as compared to the conventional  $\text{H}_2$  production processes. In order to make the process more economically viable the following points require immediate attention:

- i.* the improvement of  $\text{H}_2$  yield of the processes using cheaper raw materials;
- ii.* the development of the mixed microbial consortium or the metagenomic approach may be used to develop the efficient microbial strain for the better

utilization of industrial wastewater, which has different carbon content;

*iii.* in two stage processes, the major bottleneck lies in the scaling up of the photo-fermentation process. The improvement of these processes will definitely improve overall  $\text{H}_2$  yields, as well as the economy of the process.

## Nomenclature

- ADP - Adenosine diphosphate;
- ATP - Adenosine triphosphate;
- BEAMR - Bioelectrochemically assisted microbial reactor;
- CEM - Cation exchange membrane;
- CoA - Coenzyme A ( $\beta$ -mercaptoethylamine + pantothenic acid + ADP with 3'-phosphate group);
- CSTR - Continuous stirred tank reactor;
- Fd(ox) - Ferredoxin (oxidised form);
- Fd(red) - Ferredoxin (reduced form);
- MFC - Microbial fuel cell;
- MLVSS - Mixed liquor volatile suspended solids;
- NADPH - Nicotinamide adenine dinucleotide phosphate (reduced form);
- OLR - Organic loading rate,  $\text{kg of COD m}^{-3} \text{ d}^{-1}$ ;
- PBR - Packed bed reactor;
- PNS - Purple non-sulfur;
- PSI - Photosystem-I;
- PSII - Photosystem-II;
- UASB - Upflow anaerobic sludge blanket.

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#### PROFESSOR DEBABRATA DAS

*Professor Debabrata Das* has pioneered the promising research and development of biohydrogen production process by applying fermentation technology. This is a major area of green technology whereby the future world will be rewarded with the boons of a dream fuel-hydrogen! He is actively involved in the research of hydrogen biotechnology for a period of last eleven years. His commendable contributions towards development of a commercially competitive and environmentally benign bioprocess began with the isolation and characterization of high-yielding hydrogen producing bacterial strain *Enterobacter cloacae* IIT-BT 08, which, as of today, is known to be the highest producer of hydrogen by fermentation. He has conducted basic scientific research on the standardization of physico chemical parameters in terms of maximum productivity of hydrogen by fermentation and made significant contribution towards enhancement of hydrogen yield by redirection of biochemical pathways. Prof. Das has also conducted modeling and simulation study of a continuous immobilized whole cell hydrogen production system using lignocellulosic materials as matrix. Presently, Prof Das is involved in Pilot Plant studies (800 L) for the commercial exploitation of the process using cane molasses, sewage sludge and industrial wastewater such as distillery effluent and cheese whey. The aim was to synchronize the bioremediation of wastewater with clean energy generation.

His another major contribution in the field of biohydrogen research was the molecular characterization of *hydrogenase* coded gene and the over expression of the same in a fast growing *E. coli*. Prof. Das has already established the presence of novel Fe-hydrogenase coded gene, which contains only the H-cluster (conserve region) but not the F-cluster (electron donor). Prof. Das worked on the development of a novel two-stage fermentation process (dark fermentation followed by photofermentation) with a view towards amelioration of hydrogen productivity. Prof. Das has authored/coauthored a good number of publications on different bioprocesses in various peer-reviewed journals of national and international repute. He has various sponsored projects on different aspects of hydrogen biotechnology under the aegis of DST (India), DBT (India), MNES (India), NSF (USA), DAAD (Germany), DTU (Denmark). He associated with international collaborative research work with University of Miami & Southern Illinois University, USA; Ruhr University & Aachen University, Germany; Technical University, Denmark; University of Berger, Norway. He is the member of the Editorial Board of the journals: *International Journal of Hydrogen Energy*, *Biotechnology for Biofuels* and *Indian Journal of Biotechnology*. Recently, he has been awarded *IAHE Akira Matsui Award 2008*.



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*Ms. Namita Khanna* has been doing Ph.D. work in the Department of Biotechnology, Indian Institute of Technology Kharagpur. Her present interest is to improve the biohydrogen production process by optimizing the operational parameters and microbial profiling.