Chapter 10

Producing hydrogen as a fuel

K. Krishna Rao and Richard Cammack

With contributions from *Catherine E. Gregoire Padró, Bärbel Hüsing, Kornél L. Kovács, Peter Lindblad, Paula Tamagnini, Paola M. Pedroni* and *Paulette M. Vignais*

Over the last thirty years, many approaches for H_2 production by whole-cell and cell-free systems have been explored, and a number of pilot-scale feasibility studies have been performed. These have been reviewed in recent books (Zaborsky 1998; Miyake *et al*. 2000). In this chapter we review the ways in which our knowledge of hydrogenases in Nature can guide our future research on hydrogen energy, focusing on biotechnological and biomimetic approaches.

10.1. Introduction

10.1.1. The hydrogen economy

Periodic crises in the supply and price of fossil fuels have drawn attention to the fact that renewable energy sources are the only long-term solution to the energy requirements of the world's population. Molecular hydrogen is a future energy source/carrier that is being actively investigated as an alternative to fossil fuels. It reacts with oxygen, forming only water; hence, it is a clean renewable energy source. It has a high calorific value, and can be transported for domestic consumption through conventional pipelines. Contrary to a widely held belief (the 'Hindenburg disaster syndrome'), hydrogen gas is safer to handle than domestic natural gas.

When fossil fuels are no longer abundant, or their use is curtailed because of concerns over changes in the atmosphere, the way in which we use energy will be fundamentally changed. For example, the present methods of generating electricity are a compromise between the efficiency of large power stations and the losses in transmission over long distances. It is more efficient to transmit H_2 gas through pipelines, than electricity through power lines. Electricity could be produced locally, even domestically, from H_2 and air, in fuel cells. The risks of using H_2 are offset by the use of lower electric voltages. The switch to a hydrogen economy could be a gradual transition. Hydrogen can be mixed with methane in domestic gas supplies with minimal change to the equipment. But the greatest benefits for H_2 will come when exploiting the thermodynamic advantage of fuel cells, converting chemical energy directly to electricity.

A great deal of research is being applied to the use of hydrogen as a fuel in transportation, for cars and aeroplanes. The goal here is the promise of near-zero emissions. H_2 is also being considered as an alternative to batteries for electronic equipment.

For transport, the difficulties are concerned with finding a compact storage for the low-density fuel; and the expense of the catalysts. Various approaches are being used for storage. One is to store it as a higher-density liquid such as methanol, and reform it to H_2 as required. This is somewhat analogous to the biological approach (Chapter 1), though it leads to the release of CO_2. Other options are to compress the H_2, store it as liquid hydrogen at very low temperatures, or combine it with metals to form hydrides from which the gas can be released at will. Another method, which again resembles the biological solution (Section 8.2.3) is to store the H_2 in carbon nanotubes, which offer high-density and lightweight storage.

At present most of the H_2 is produced industrially by conversion of fossil fuels, either directly or indirectly. This leads inevitably to the net production of the greenhouse gas CO_2. New methods will have to involve recycling of organic matter, or direct production of H_2 from water using energy sources such as sunlight. This can be achieved either directly in photochemical fuel cells, or by using photovoltaic cells, which use solar radiation to electric current for electrolysis of water into H_2 and O_2. A great deal of effort has gone into the development of silicon solar cells (photovoltaics) for production of energy from sunlight, which have become less expensive and improved in efficiency. The costs of production of H_2 from the electricity produced are decreasing steadily, but they still involve noble-metal catalysts.

10.1.2. Biological hydrogen production

The most challenging option is the production of hydrogen by photosynthetic microorganisms. This biological approach is environmentally friendly, as it uses renewable materials, produces no toxic waste products, CO_2 or NO_x, and avoids the need for precious metals. It is not a new idea. It was known that algae and cyanobacteria could evolve small amounts of H_2 under certain growth conditions (Gaffron and Rubin 1942). Microbes produce H_2 for two principal reasons. The first is to dispose of excess reducing equivalents during fermentative metabolism either carried out in a dark anaerobic process or associated with the anoxic photosynthetic activity. An example is the production of hydrogen during the phosphoroclastic reaction of pyruvate and other 2-oxoacids (Fig. 10.1). This reaction often uses an [Fe] hydrogenase, although bidirectional [NiFe] hydrogenases are also used. A cell may produce H_2 in some stages of growth, then consume it in others (Belaich 1991). Second, H_2 is a by-product of the action of nitrogenase (Fig. 10.2), the enzyme that is needed for the fixation of atmospheric N_2. This is an extremely complex reaction; the enzyme is slow, and produces at least one H_2 for every N_2 fixed. The H_2 released by nitrogenase is often recovered within the nitrogen-fixing cells, by means of the membrane-bound uptake [NiFe] hydrogenase, and so less than proportional net H_2 production is observed. The membrane-bound respiratory chain, using H_2 as a fuel, produces ATP, which partly compensates for the energy consumed in the nitrogenase reaction.

Photoheterotrophic bacteria conduct a simple type of photosynthesis, in which there is only one photosystem, and reduced inorganic or organic compounds instead of water act as electron donors. Photosynthesis by these organisms can be used to produce hydrogen, with various forms of organic compounds as electron donors. They can be used, in principle, to produce H_2 from dairy and agricultural waste matter using light energy.

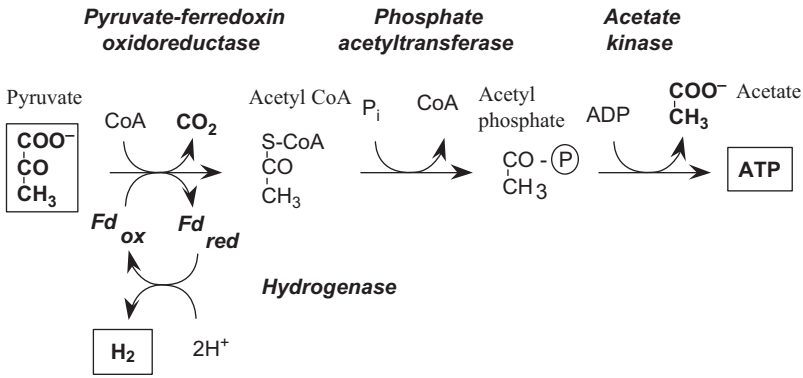

Figure 10.1 The phosphoroclastic reaction of pyruvate.

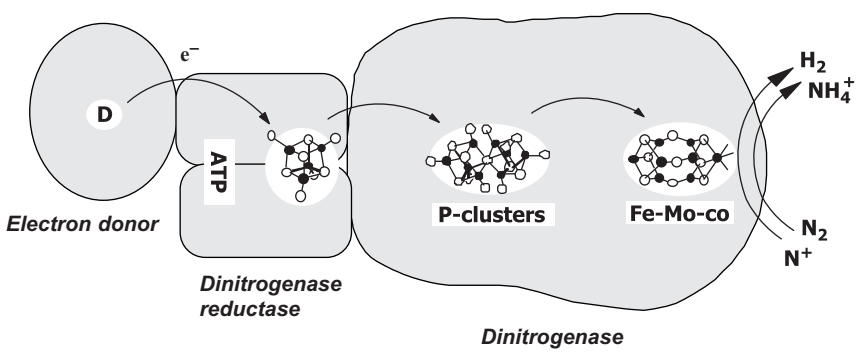

Figure 10.2 Cartoon of the electron transfer pathway in nitrogenase.

Artificial cell-free systems have been investigated, to test models of photosynthetic production of H_2. Benemann *et al*. (1973) demonstrated that it was possible to produce H_2 and O_2 by combining chloroplasts from green plants and bacterial hydrogenase, with ferredoxin as the intermediate electron carrier:

$$2\ H_2O \rightarrow 2\ H_2 + O_2.$$

In this system, oxygen is produced by photosystem II, as in green plants and cyanobacteria. The photosynthetic electron transfer, via photosystem I, is linked by low-potential electron carriers to hydrogenase, which produces H_2 (Fig. 10.3). Benemann and Weare (1974) then went on to investigate H_2 evolution by N_2-fixing cyanobacterial cultures as a whole-cell source of hydrogen energy.

An alternative approach, using semiconductors as light-driven electron donors, has been demonstrated in model systems (Grätzel 1982; Nikhandrov *et al*. 1988). These are more stable than the photosystems, but show lower photochemical conversion efficiencies owing to short-circuiting of reducing equivalents. The presently used

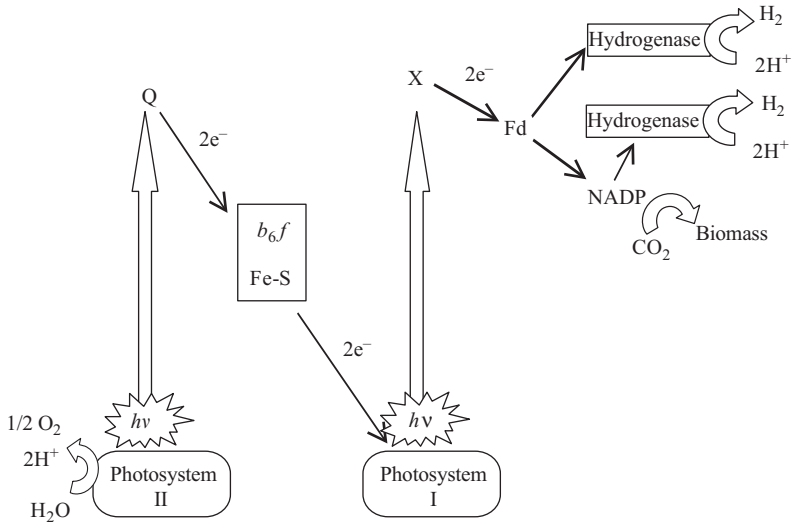

Figure 10.3 'Z-scheme' of oxygenic photosynthesis in green algae and cyanobacteria, showing links to hydrogenase. Q (plastoquinone) and X (an iron–sulfur cluster) are electron acceptors from photosystems II and I, respectively. The two hydrogenases shown are the NADP-dependent bidirectional hydrogenase and a ferredoxin-dependent enzyme.

semiconductors such as titanium dioxide (TiO_2) use only the blue/ultraviolet end of the whole energy spectrum.

10.2. Technologies to produce hydrogen by biological systems

Hydrogen can be produced from water using a variety of energy sources such as nuclear, solar, electrical, biological and chemical. Since both water and solar radiation are abundant, ubiquitous and 'free', photolysis of water (either direct or via additional components) is a preferred route to generate hydrogen from water. Other sources for hydrogen that are currently used, such as the reforming of hydrocarbons, are not neutral in terms of net CO_2 production.

The use of solar energy to split water to hydrogen and oxygen is an attractive means to harvest the Sun's energy falling on the Earth, and convert it to storable chemical energy. Photochemical, photoelectrochemical and photobiological technologies are being investigated for the development of sustainable, economically viable hydrogen production systems. Photosynthetic microorganisms (phototrophs) containing hydrogenases, as well as isolated hydrogenases, are used in these studies.

A major drawback in obtaining sustainable hydrogen production is the instability of the enzyme during continued operation. One approach is to use living cells, which have the ability to repair, maintain and reproduce themselves. The alternative would be to create a stable and inexpensive synthetic catalyst, which would mimic the properties of the natural enzyme, which is the rapid and reversible activation of H_2 at water temperature (below 100°C) and near-neutral pH. Such a catalyst would be most welcome in

processes like the production of hydrogen by photobiological/photochemical methods and in the generation of hydrogen for fuel cells. In short, the 'cheap' synthetic hydrogenase would replace the expensive platinum as a hydrogenation catalyst. The development of such catalysts is at an early stage, as described in Chapter 8 (Section 8.6).

In principle, the conversion of energy to H_2 production by biological systems could be quite efficient. The light reactions of photosynthesis convert light to chemical energy with very high efficiency, and hydrogenases, as already described in this book, are highly efficient enzymes. However, the potential has not been realized in the small-scale studies done so far, where conversion efficiency of light energy is typically less than 1 per cent. It is generally considered that efficiencies of 10 per cent or more would be needed to support our energy needs. It has become apparent that the development of viable large-scale systems would need a very significant commitment to applied and fundamental research.

The principal problems that were encountered were:

1 The oxygen-evolving photosynthetic systems are exposed to the damaging effects of light, and without the repair machinery present in the plant, soon lose activity. Plants have mechanisms to minimize the damage, but these reduce the conversion efficiency.
2 The hydrogenase and nitrogenase, which produce H_2, are inhibited or irreversibly damaged by the oxygen produced.
3 The product is a mixture of hydrogen and oxygen in solution, which have to be transferred to the gas phase (requiring a large surface area) and separated from each other before storage.
4 Sunlight as an energy source, although free, is intermittent and diffuse.
5 Hydrogen is the lowest-density gas with a very low boiling point, and storing it requires either large volumes, very low temperatures, or high pressures.
6 Wild-type microorganisms such as hydrogen-oxidizing and methanogenic bacteria would be competitors, growing like weeds, in systems for H_2 production, and have to be excluded.

Over the last thirty years, investigators around the world have explored methods of overcoming these obstacles. The yields can be increased by genetic manipulation of the enzymes and the machinery for producing them. For example, the yield of H_2 production by nitrogenase can be enhanced by mutations, and expression of the enzyme can be increased by modifying the way in which it is repressed by fixed nitrogen. Further development is needed, to improve the resistance of hydrogenase to oxygen and carbon monoxide.

A fundamental difficulty is that the main purpose of living organisms is the efficient production of their own cell material. H_2 is a by-product of their metabolism. Ultimately, in order to progress towards large-scale H_2 production, it may be necessary to engineer, by molecular biology techniques, a new type of organism or consortium of organisms, in which the photosynthetic production of H_2 is the major metabolic activity. This is an ambitious aim, but there are historical precedents in the breeding of animals and agricultural plants for improved agricultural productivity. The new breed of H_2-producing organisms would have to be grown in an environment from which other microorganisms are excluded.

New directed evolution techniques make it possible to modify the genetic makeup of an organism in a wide variety of ways. They make it possible to speed up the natural process of evolution from millions of years to a few months. Many possibilities for improving the metabolism and enzymology of organism can be explored. What is required is an effective way of applying the right selection pressure to ensure that the most effective systems emerge.

If solar energy were to supply the energy needs of the world's population, it would require large areas to be dedicated to it. These could be in sunny areas of low population such as deserts, or the oceans. It should be remembered that salt water is no barrier to the growth of many of these organisms. However, technological advances on this scale are obviously long term.

We now present some reports on the state of research at present, and some forecasts of the way in which biological H_2 production might develop in the future.

10.3. Hydrogen production by cyanobacteria and algae

K. Krishna Rao, Paula Tamagnini and Peter Lindblad

Cyanobacteria (prokaryotes) and unicellular green algae (eukaryotes) have been considered as promising phototrophs for the development of environment-friendly photobiological H_2 production systems. They are ubiquitous, and relatively inexpensive to grow. For growth, they need only water, air and simple minerals, and light as energy source.

10.3.1. Photosystems

Biophotolysis of water involves the use of the photosynthetic apparatus to generate hydrogen from water and sunlight. Solar energy (photons) captured by the antenna pigment–protein complexes excites the reaction centre chlorophylls to produce strong reducing potentials (Fig. 10.3). Photosystem II of cyanobacteria and plants takes electrons from water via the manganese-containing oxygen-evolving complex, and donates electrons to quinone. The electrons are passed through the cytochrome b_6f complex, to Photosystem I, which provides a strong reductant for ferredoxin. Ferredoxin:NADP reductase provides NADPH, for fixation of CO_2. It could also be used to reduce the bidirectional hydrogenase. Photosynthetic electron transport also creates a proton gradient (proton motive force) across the photosynthetic membrane, which promotes the synthesis of ATP. ATP and reduced ferredoxin are the important molecules involved in H_2 photoproduction from nitrogenase. (Hall and Rao, 1999).

10.3.2. Enzymes for photosynthetic hydrogen production

Cyanobacteria and green algae are capable of H_2 photoproduction from water, although the enzymology of H_2 production is different in the two groups. H_2 production in cyanobacteria is observed principally in diazotrophic strains under nitrogen-fixing conditions, and is catalysed by nitrogenase. Green algae are eukaryotes and not capable of N_2 fixation; H_2 evolution is catalysed by hydrogenase. The hydrogenase of *S. obliquus* has recently been characterized, and shown to have a unique structure with a particularly short polypeptide sequence (Florin *et al.* 2001). Nitrogenases and

the algal hydrogenases are all extremely O_2 sensitive and so are deactivated by photosynthetically generated O_2 (Rao and Hall 1996; Hansel and Lindblad 1998; Appel and Schulz 1998). Research efforts have concentrated on enhancing these activities, and on strategies for avoiding interference by O_2.

Nitrogenase

Nitrogenase, the enzyme that catalyses N_2 fixation, is essential for the maintenance of the nitrogen cycle on Earth, since fixed nitrogen is often limiting for the growth of living organisms (Postgate 1987; Burris 1991; Gallon 1992). The ability to fix N_2 is restricted to prokaryotic organisms. However, a representative number of eukaryotes, notably green plants, can establish symbiosis with N_2-fixing bacteria. A broad range of microorganisms, including both archaea and eubacteria, has been found to have the capacity to fix N_2. This diversity of organisms contrasts with the remarkable conservation of nitrogenase itself (Flores and Herrero 1994; Haselkorn and Buikema 1992; Smith 1999).

The most common form of nitrogenase is the molybdenum-containing enzyme. It consists of two proteins: the dinitrogenase (MoFe protein or protein I) and the dinitrogenase reductase (Fe protein or protein II). The dinitrogenase is an $\alpha_2\beta_2$ heterotetramer of about 220–240 kDa, which binds four 4Fe4S clusters, organized into two 'P clusters', and two FeMo cofactors (Flores and Herrero 1994). It is generally accepted that the FeMo-cofactors constitute the active site at which N_2 is reduced to ammonia. The α and the β subunits are encoded by the genes nifD and nifK, respectively. The Fe protein, encoded by the gene nifH, is a homodimer of about 60–70 kDa and has the specific role of mediating the transfer of electrons from the external electron donors (a ferredoxin or a flavodoxin) to the P clusters of dinitrogenase. Together, the subunits bind one intersubunit 4Fe4S cluster. In addition to the three structural genes mentioned above, many other genes are involved in the nitrogen-fixation process and its regulation (for a recent review, see Böhme 1998).

The reduction of nitrogen to ammonium, catalysed by nitrogenase, is a highly endergonic reaction requiring metabolic energy in the form of ATP. First, the dinitrogenase reductase is reduced by a ferredoxin or a flavodoxin and binds ATP, which lowers its potential. At this lower potential (around -400 mV), the dinitrogenase reductase transfers electrons to dinitrogenase. The transfer is accompanied by the hydrolysis of ATP to ADP + P_i. This reaction is also accompanied by an obligatory reduction of protons to H_2. At infinite pressure of N_2, 75 per cent of the electrons would be allocated for N_2 reduction and 25 per cent for H^+ reduction. As two ATP are required for each electron transferred from dinitrogenase reductase to dinitrogenase, the reaction requires a minimum of sixteen ATP until the dinitrogenase has accumulated enough electrons to reduce N_2 to NH_3. The overall reaction can be written as follows:

$$N_2 + 8e^- + 10H^+ + 16ATP \rightarrow 2NH_4^+ + H_2 + 16ADP + 16P_i$$

although it should be noted that this represents an ideal efficiency under optimal conditions. In practice, nitrogenase tends to produce more H_2 than this equation would suggest.

The 'alternative' nitrogenases contain vanadium or iron instead of molybdenum. They are encoded by the *vnf* and *anf* gene clusters, respectively. Structurally they are similar to the molybdenum-containing nitrogenases, but have catalytic clusters containing vanadium (FeVa cofactor) or iron only (FeFe cofactor) respectively. Nitrogenase is usually detected in cells by its ability to reduce acetylene (ethyne) to ethylene (ethene), which can readily be detected by gas chromatography. The alternative nitrogenases are less active in this reaction, and also tend to continue the reduction as far as ethane. They are repressed by molybdenum and for this reason they were isolated and characterized much later than the molybdenum enzyme. Significantly for bio-hydrogen production, they allocate a higher proportion of electrons to the reduction of H^+ to H_2 when compared to the conventional Mo-enzyme complex. For the Fe nitrogenase a minimum ratio $H_2 : N_2$ of 7.5 : 1 was estimated (Schneider *et al.* 1997).

In the heterocystous cyanobacterium *Anabaena variabilis*, four different nitrogenases have been identified and characterized. Two are Mo-dependent enzymes. While one (the so-called conventional nitrogenase, encoded by the nif1 gene cluster) functions only in the heterocysts under both aerobic or anaerobic growth conditions, the other (encoded by the nif2 gene cluster) functions strictly under anaerobic conditions in both the vegetative cells and the heterocysts. Furthermore the differences between the two nif clusters suggest that the conventional nitrogenase is developmentally regulated, while the other is regulated by environmental factors. The occurrence of a V-containing nitrogenase was first reported by Kentemich *et al.* (1988) and, subsequently, confirmed by Thiel (1993). This enzyme is encoded by the vnfDGK gene cluster, that is transcribed in the absence of molybdenum, and in which vnfDG are fused in a single ORF. Physiological evidence indicates that the fourth nitrogenase is an Fe enzyme similar to the one encoded by the anf gene cluster in *Azotobacter vinelandii* (Bishop and Premakumar 1992; may also occur in *A. variabilis,* although the gene characterization is still lacking, only in the Anabaena strain isolated from the fern Azolla, Kentemich *et al.* 1991).

The biosynthesis of nitrogenase is under strict regulation and it is suppressed by many factors, including the availability of other sources of fixed nitrogen, and the presence of oxygen. The optimal yields of nitrogenase are obtained by working under argon, when nitrogenase has no substrate to work on, but this is unlikely to be feasible on the large scale. It should be possible to engineer the regulatory mechanisms to increase the yield of the enzyme. Modified enzymes would be able to change the ratio of $H_2 : N_2$ produced. The disadvantages of nitrogenases are their low turnover numbers, high demand for ATP and oxygen sensitivity.

Uptake hydrogenase

All nitrogen-fixing unicellular and filamentous cyanobacteria examined so far have been found to possess an uptake hydrogenase. The enzyme seems to be membrane bound and, in some filamentous strains, is particularly expressed in the N_2-fixing heterocysts with no or minor activity in the photosynthetic vegetative cells. It is expressed under the same conditions as nitrogenase, with the evident function of catalysing the uptake of H_2 produced by nitrogenase. As a consequence release of H_2 is not normally observed. The uptake hydrogenase, like the membrane-bound hydrogenase of hydrogen bacteria such as *Ralstonia eutropha*, is linked to the respiratory

chain and in cyanobacteria also to photosystem I, ultimately to cytochrome oxidase. The ATP generated is used for biosynthetic reactions, including further N_2 fixation.

Syntheses of uptake hydrogenases of cyanobacteria, as well as uptake hydrogenases of other bacteria, have been shown to be dependent on the availability of Ni^{2+} in the growth medium. The enzymes are insensitive to O_2 in whole-cell preparations, but become sensitive after cell extraction (Houchins and Burris 1981b).

Bidirectional hydrogenase

Cyanobacteria also contain a reversible/bidirectional hydrogenase, which is soluble and has the capacity to both take up and produce H_2 (Bothe *et al.* 1991; Flores and Herrero 1994; Appel and Schulz 1998; Hansel and Lindblad 1998). The latter enzyme was called 'bidirectional hydrogenase' until the respective structural genes were sequenced and characterized by Schmitz *et al.* (1995). It has been the subject of some interest as another possible means to produce H_2 photosynthetically.

In *Anabaena* sp. strain PCC7120, the uptake hydrogenase is less sensitive to carbon monoxide inhibition than the bidirectional enzyme. Both enzymes have low K_m for H_2 but only the uptake hydrogenase activity was elicited by addition of H_2 to the gas phase.

In contrast with the uptake hydrogenase, the soluble, or loosely membrane-associated bidirectional hydrogenase was believed to be a constitutive enzyme (Kentemich *et al.* 1989, 1991; Serebriakova *et al.* 1994) widely distributed among N_2-fixing and non-N_2-fixing cyanobacteria. The activity of this enzyme, in heterocystous strains, increases considerably under anaerobic or microaerobic conditions, whereas in the unicellular non-N_2-fixing *Gloeocapsa alpicola* the partial pressure of oxygen does not seem to have any significant influence (Serebryakova *et al.* 1998). The hydrogenase activity in vegetative cells of *A. variabilis* has been suggested to be under a form of 'redox control' whereby the enzyme is only activated upon removal of light and oxygen. This mechanism may involve a thioredoxin (Spiller *et al.* 1983).

Schmitz *et al.* (1995) sequenced a set of structural genes (hox genes) encoding a bidirectional hydrogenase in the filamentous heterocystous *A. variabilis*, and proposed that the bidirectional enzyme is a heterotetrameric enzyme consisting of a hydrogenase part (encoded by hoxYH) and a diaphorase part. Nucleotide sequence comparisons showed that there is a high degree of homology between the hox genes of cyanobacteria and genes encoding the NAD^+-reducing hydrogenase from the chemolitotrophic H_2-metabolizing bacterium *R. eutropha* as well as methyl viologen-reducing hydrogenases from species of the methanogens (Chapter 2). The bidirectional hydrogenase of *A. variabilis* has been partially purified and characterized (Serebryakova *et al.* 1996).

10.3.3. Oxygen sensitivity and how to avoid it

The nitrogenases are deactivated immediately on exposure to air or O_2; the process is almost irreversible. All diazotrophs must protect the enzymatic complex from the deleterious effects of O_2. Cyanobacteria perform oxygenic photosynthesis, and must, therefore, protect their N_2-fixing machinery not only from atmospheric O_2 but also from the intracellularly generated O_2. Diverse strategies have evolved for separating the production of H_2 and O_2, either spatially or temporally. Some filamentous nitrogen-fixing

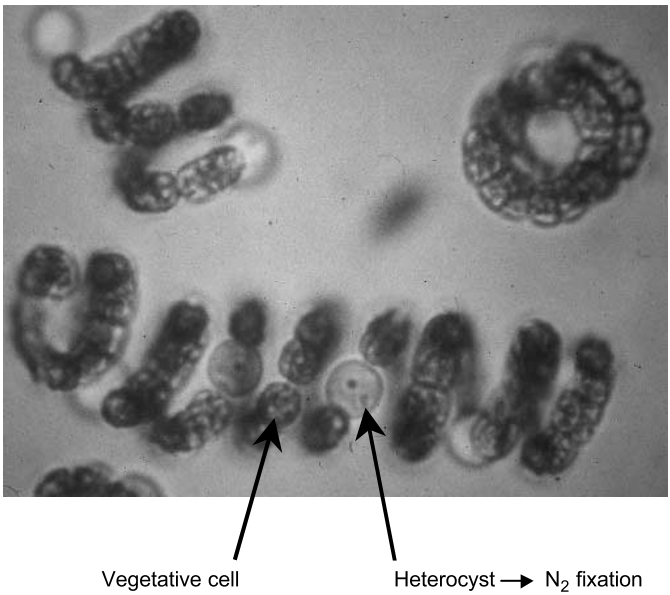

Figure 10.4 Cyanospira rippkae (courtesy of Prof. H. Bothe).

cyanobacteria have specially adapted cells, called heterocysts, which contain nitrogenase and do not evolve O_2 (Fig. 10.4).

The non-heterocystous cyanobacteria have attracted attention because of the apparent paradox of being able both to fix N_2 and to evolve O_2. Temporal separation between photosynthetic O_2 evolution and N_2 fixation seems to be the most common strategy adopted. A very restricted number of strains are able to perform N_2 fixation under aerobic conditions, and there is no evidence that the two processes, oxygenic photosynthesis and N_2 fixation, may occur simultaneously within a single cell (Bergman *et al*. 1997). However, not all strains fix N_2 exclusively during the dark phase of light/darkness cycle. N_2 fixation requires ATP and reductant, and the fermentation of stored carbohydrates may not be sufficient to cover the energy demand of nitrogenase activity. In natural populations of *Oscillatoria limosa*, the nitrogenase activity coincides with the transitions from dark to light and light to dark with a maximum at sunrise. The last event could be explained by light energy driven N_2 fixation in an initially low O_2 environment. The situation in the marine filamentous non-heterocystous *Trichodesmium* is far more complex. A spatial separation between the N_2 fixation and the photosynthesis probably occurs, without any obvious cellular differentiation. In contrast with the permanent changes occurring during heterocyst differentiation, those occurring in non-heterocystous cyanobacteria can be reversed.

When filamentous cyanobacteria such as *Nostoc* or *Anabaena* are grown under limited combined N_2, 5–10 per cent of their vegetative cells differentiate to form heterocysts. Heterocyst formation does not take place randomly within the filament. Most probably it is a dynamic selection in response to nitrogen deprivation but neither the existence of predetermination nor a combination of both events can be ruled out (Wolk 1996).

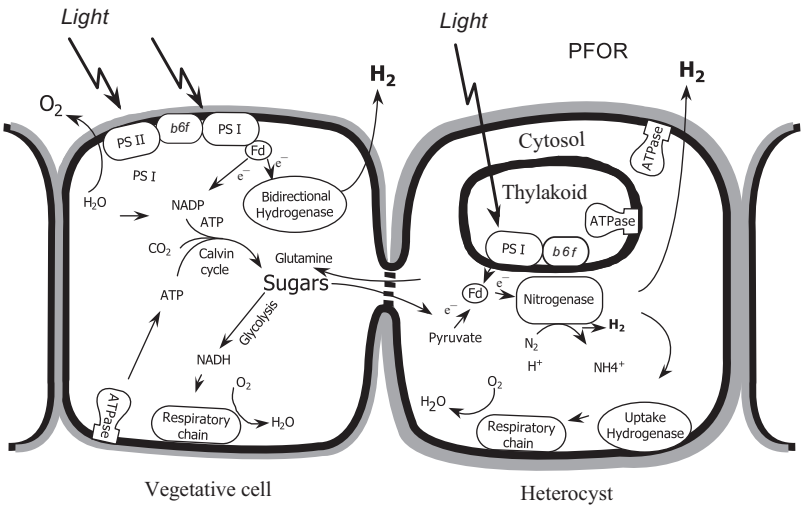

Figure 10.5 Scheme of electron transfer in a filamentous heterocystous cyanobacterium.

The heterocyst provides a virtually anaerobic environment suitable for the functioning of nitrogenase since: it lacks photosystem II activity, and therefore does not produce O_2 as a by-product of photosynthesis. The glycolipids on the thick outer envelope of the heterocysts restrict the diffusion of O_2 into the cells, which have a higher rate of respiratory O_2 consumption (Fay 1992; Wolk *et al.* 1996). The connection between the vegetative cell and the heterocyst is narrow and occurs via microplasmodesmata. Heterocysts import carbohydrates and in return export glutamine to the vegetative cells (Fig. 10.5). ATP formation in heterocysts is not completely understood. ATP can be produced either by cyclic photophosphorylation (PSI) in the light, or by oxidative phosphorylation; the latter process consumes oxygen and uses pyridine nucleotides or hydrogen as electron sources. It has been suggested that NADPH provides electrons for nitrogenase via a heterocyst-specific ferredoxin (FdxH) and ferredoxin:$NADP^+$ oxidoreductase (Böhme 1998).

An interesting feature of heterocyst differentiation in cyanobacteria is the occurrence of developmentally regulated genome rearrangements (Carrasco *et al.* 1995; Carrasco and Golden 1995; Golden *et al.* 1985, 1987, 1988; Matveyev *et al.* 1994). These rearrangements occur late during heterocyst differentiation at about the same time as the nitrogen-fixation genes are transcribed. Inserted elements in the genes for nitrogenase, uptake hydrogenase and ferredoxin are excised, during heterocyst differentiation, by site-specific recombinases.

10.3.4. Cyanobacterial hydrogen production: Present status and future potential

Filamentous cyanobacteria have been used in bioreactors for the photobiological conversion of water to hydrogen. However, the conversion efficiencies achieved are low because of the competing processes of hydrogen production and consumption within

the cells. Fixation of N_2 is accompanied by H_2 production which is an inevitable side reaction of nitrogenase, but often the H_2 is quickly reabsorbed by a unidirectional uptake hydrogenase. Cyanobacteria contain, in addition, a bidirectional (reversible) enzyme, which under some growth conditions oxidizes the H_2, and in others evolves it.

Investigations are continuing on ways of improving the efficiency of photobiological hydrogen production by cyanobacteria. Screening of natural strains and mutants with high nitrogenase activity, and improved aerobic stability is being pursued vigorously. In laboratory-scale experiments the H_2-evolving activity of nitrogenase is enhanced by substituting molybdenum in the standard culture medium with vanadium. Improvements of the conversion efficiencies have been achieved through the optimization of the conditions for H_2 evolution by nitrogenase, through the production of mutants deficient in H_2 uptake activity. *A. variabilis* PK84, a mutant of *A. variabilis* ATCC29413, impaired in uptake hydrogenase activity, was used by Borodin *et al*. (2000) for the continuous production of H_2 in air and CO_2, in an outdoor photobioreactor (Fig. 10.6). Alternatively, increased H_2 evolution has been obtained by the bidirectional enzyme. Symbiotic cells are of fundamental interest since they function as 'bioreactors': high metabolism, transfer of metabolite(s) from symbiont to host but almost no growth.

Combining all the physiological and immunological works with the recent molecular data, notably with the presence/absence of the rearrangement within the gene

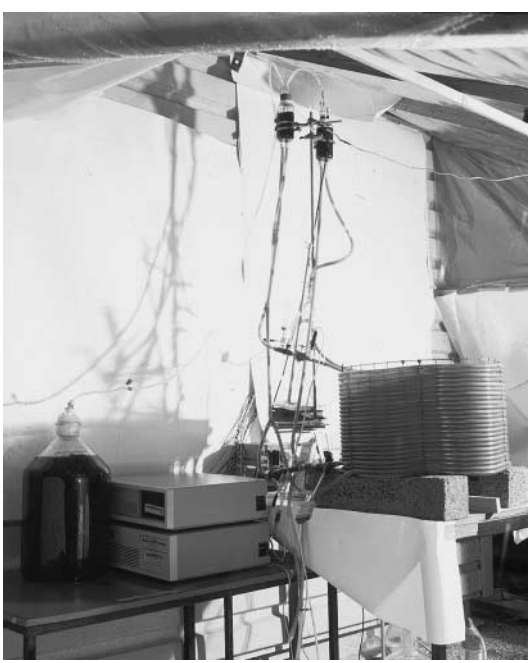

Figure 10.6 Helical photobioreactor for testing the effects of growth parameters during continuous H_2 production. The test reactor was constructed from 50 m of PVC tubing, wound round a wire former, and contained 3 l of a culture of *A. variabilis*. An airlift flushed the culture with air + 2 per cent CO_2, and circulated it through a degasser and computer-controlled monitoring equipment (Borodin *et al.* 2000).

hupL in the filamentous strains, it is impossible to establish one single pattern of cellular/subcellular localization and regulation of the uptake hydrogenase, even for closely related strains.

10.4. H_2 production by eukaryotic algae

Eukaryotic green microalgae such as *Chlamydomonas reinhardtii* and *S. obliquus* have been considered as sources of H_2 (Ghirardi *et al.* 2000). They have a more complex cellular organization than the cyanobacteria, and can live under a wide range of conditions. They do not have nitrogenase, but they contain inducible hydrogenases of the [NiFe] types and [Fe] types, and can produce hydrogen using hydrogenase under certain growth conditions. Dark hydrogen production, like the hydrogen-producing protozoa and anaerobic bacteria such as clostridia, occurs under anoxic conditions and is probably by the action of pyruvate:ferredoxin oxidoreductase, ferredoxin and hydrogenase. There is also evidence that hydrogenase can be coupled to photosynthetic electron transport, where it provides an overspill mechanism for excess reducing equivalents (Appel *et al.* 2000).

Melis *et al.* (2000) have described a novel approach for sustained photobiological production of H_2 gas via the reversible hydrogenase pathway in the green alga *C. reinhardtii*. Inhibition of the reversible hydrogenase by photosynthetic O_2 evolution was prevented by a two-stage procedure in which the photosynthetic phase was alternated with H_2 production. A transition from one stage to the other was by sulfur deprivation of the culture, which reversibly inactivated photosystem II (PSII) and O_2 evolution. Under these conditions, oxidative respiration by the cells in the light depleted O_2 and caused anaerobiosis in the culture, which was necessary and sufficient for the induction of the reversible hydrogenase. H_2 gas production was supported by photosynthetic electron transport through Photosystem I, from endogenous substrate to the ferredoxin and reversible hydrogenase (compare Fig. 10.5).

10.5. Hydrogen production by photoheterotrophic and heterotrophic bacteria

Paola M. Pedroni, Paulette M. Vignais, Kornél L. Kovács

10.5.1. Photoheterotrophic bacteria

Research at Enitechnologie (Italy) has been particularly addressed to develop technologies that combine the bioproduction of gas to the disposal of waste compounds exploiting sunlight as an energy source. This strategy is expected to reduce costs of process realization and management, and represents the basic concept of both *in vivo* and *in vitro* hydrogen bioproduction technologies developed (Fig. 10.7). In particular, the *in vivo* system is based on the cultivation of the photosynthetic bacterium *Rhodobacter sphaeroides*, which is able to evolve hydrogen utilizing organic wastes as growth substrates and harvesting sunlight as an energy source. Suitable photobioreactors have been designed to optimize bacterial cultivation with regard to solar irradiation.

Less investigated as potential methods for hydrogen bioproduction, *in vitro* systems make exclusively use of the enzymatic component responsible for hydrogen evolution

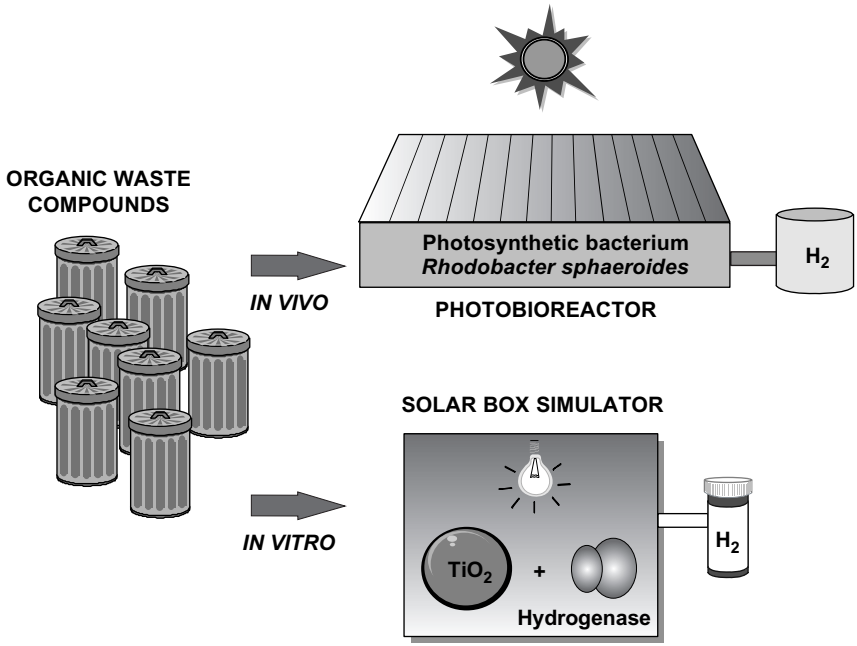

Figure 10.7 *In vivo* and *in vitro* hydrogen production technologies developed to combine the bioproduction of gas to the disposal of waste compounds exploiting the sunlight as an energy source (courtesy of Enitecnologie, Milano).

instead of the whole bacterial cell. In particular, in an *in vitro* system the gas is photocatalytically produced by coupling the hydrogenase enzyme with the inorganic semiconductor titanium dioxide (TiO_2). The reaction takes places in a solar simulator apparatus, which is used as a source of light. Organic waste compounds will be tested as renewable source of electrons.

The goal of research activities concerning *in vivo* hydrogen bioproduction is to improve the hydrogen evolution performance of *R. sphaeroides* wild-type strain by genetic engineering techniques. In this photosynthetic bacterium, at least two different enzymes are involved in hydrogen metabolism: the nitrogenase complex, responsible for hydrogen production, and a membrane-bound uptake hydrogenase which mediates hydrogen consumption (Fig. 10.8A). Since the physiological function of the latter enzyme is to recycle the hydrogen evolved by the nitrogenase, its activity reduces the net amount of this gas photoproduced by the bacterium. To isolate a hydrogen-overproducing strain starting from the wild type, an Hup^- mutant has been constructed in which the hydrogen recycling was eliminated by abolishing the uptake hydrogenase activity. This was achieved by interposon mutagenesis of the gene encoding the catalytic large subunit of the uptake hydrogenase heterodimer, since this disruption was considered as the most decisive at the functional level. Interposon mutagenesis technique led to the replacement of the functional gene copy (*hupL*) on the bacterial genome, with a copy of the same gene inactivated by the insertion in its reading frame of heterologous DNA. The hydrogen photoproduction performance of

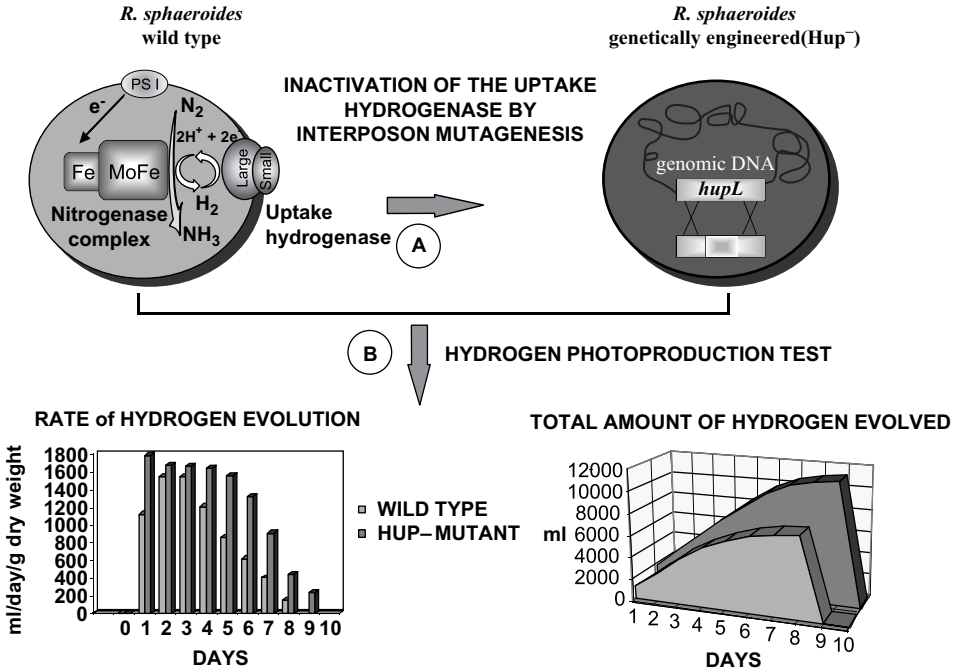

Figure 10.8 Improvement of the hydrogen production capacity of *R. sphaeroides* wild type. Construction of the Hup⁻ mutant strain by interposon mutagenesis (A) and evaluation test of its hydrogen photoproduction performace in comparison to the wild type (B) (courtesy of Enitecnologie, Milano).

the Hup⁻ mutant strain was then assayed in comparison to that of the wild type using an indoor 1 L-scale photobioreactor as an initial evaluation test (Fig. 10.8B). The hydrogen photoproduction rate of the mutant strain, measured as milliliters of gas evolved per day per gram of dry weight, was significantly higher than that of the wild type during all the fermentation period, thereby determining an increase of about 50 per cent in terms of total amount of hydrogen photoproduced.

In another approach, inactivation of hydrogen uptake activity in the mutant deficient in pleiotropic HypF accessory gene function, which is necessary to the assembly of active [NiFe] hydrogenases (Chapter 4), resulted in dramatic increase in the hydrogen evolution capacity of *Thiocapsa roseopersicina* under nitrogen-fixing conditions. Remarkably the *hypF* minus mutant strains showed a more than sixty-fold increase in H_2 evolution under nitrogen-fixing conditions when compared to the wild-type controls (Fig. 10.9). Firstly, this observation indicated that a substantial amount – probably all – hydrogen uptake activity has been inactivated in the *hypF*⁻ cells. Secondly, *hypF* mutation clearly offers an important avenue for the development of efficient biological hydrogen production systems using this organism. Growing under phototrophic and nitrogen fixing conditions, *T. roseopersicina* can evolve hydrogen at a practically significant level (B. Fodor, G. Rákhely, Á. T. Kovács and K. L. Kovács, University of Szeged, Hungary, in press).

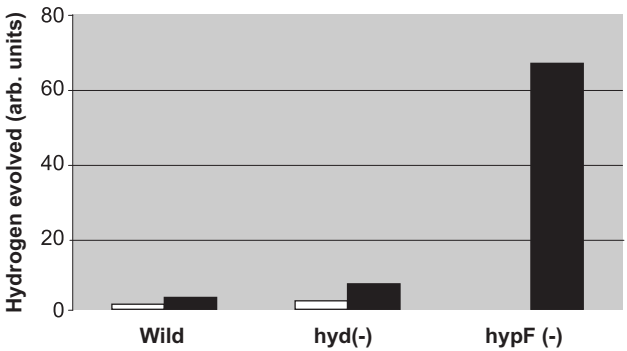

Figure 10.9 Relative hydrogen production of the wild type and the *hypF* defective (M539) *T. roseopersicina* strains *in vivo* under nitrogenase repressed (white columns) and derepressed (black columns) conditions. Samples were measured after cultivation for three days. The amount of H_2 evolved by the wild-type strain under non-nitrogen-fixing condition was chosen as 1.

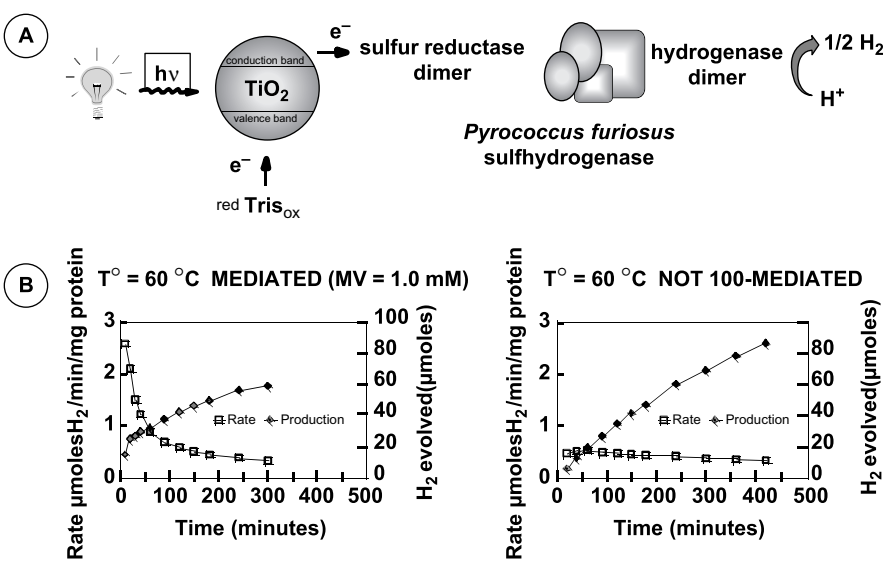

Figure 10.10 Optimization of the *in vitro* hydrogen bioproduction technology. Schematic representation of the electron transfer pathway among reaction components in un-mediated condition (A) and performance of the simplified system at 60°C compared to that of the previously developed version also including MV (B) (courtesy of Enitecnologie, Milano).

Figure 10.10A shows the reaction scheme of the system outlining the electron transfer pathway among the different components, namely the electron donor Tris, the semiconductor TiO_2 and the sulfhydrogenase of the hyperthermophilic archaeon *P. furiosus*, a bifunctional enzyme catalysing either proton or sulfur species reduction.

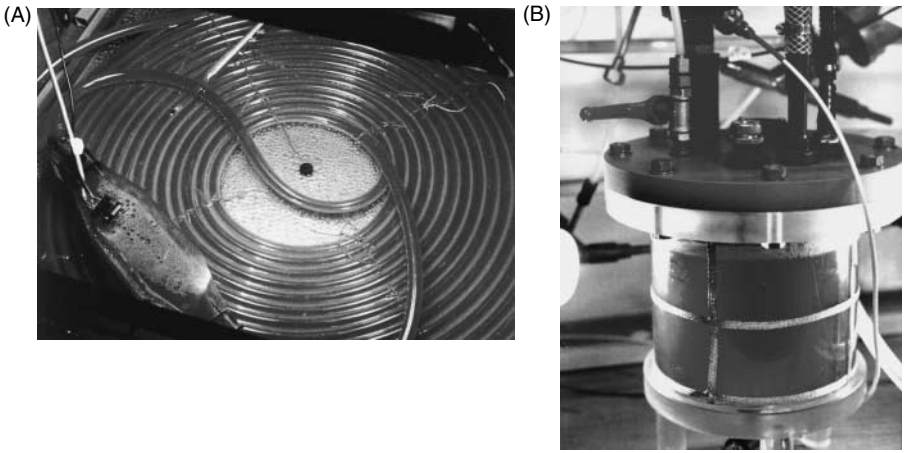

Figure 10.11 (A) Spiral-coiled plane photobioreactor filled with a culture of the photosynthetic bacterium *R. capsulatus*. A semiconductor temperature sensor, placed in contact with the culture in the center of the bioreactor is shown. (B) Degassing chamber (4.6 l) equipped with two level sensors.

This enzyme has been selected to enhance the stability of the biological catalyst, one of the most relevant problems connected with *in vitro* hydrogen photoproduction systems. Thermotolerant enzymes, in fact, are endowed with biochemical and structural features, such as high catalytic efficiency and resistance to chemical and denaturing agents, particularly suitable for practical purposes. Under light irradiation, the TiO_2 particle generates electron/hole couples. The hole is a relatively high-energy oxidative site and can accept electrons from Tris, the donor compound. As a consequence, an excess of negative charge is accumulated on the TiO_2 particle and electrons are then directly transferred from the light-excited semiconductor to the *P. furiosus* sulfhydrogenase which in turn reduces protons to hydrogen. This un-mediated reaction scheme represents a simplified and improved version of the *in vitro* system we previously set up. This latter included an additional component, the artificial redox mediator methyl viologen (MV) which mediated electron transfer from the irradiated TiO_2 particle to the biological catalyst. Its presence affected the life-time of the reaction, undergoing an irreversible degradation and therefore represented a limit to the total amount of hydrogen photoproduced. Besides optimizing the system with regard to reaction components, we also improved its performance as a function of temperature. In fact, lowering the reaction temperature from 80 to 60°C enhances the thermochemical stability of the reaction components and therefore had a positive effect on the life-time of the hydrogen photoproduction.

Figure 10.10B shows the results obtained at 60°C comparing the performance of the simplified un-mediated version to that of the mediated system in terms of rate and amount of hydrogen evolved. In the absence of the the artificial redox mediator MV, the initial rate of hydrogen production is lower, but its trend is more constant and the reaction life-time can be extended from 5 to 8 h, giving rise to a significantly higher amount of H_2 evolved.

A 10 l photobioreactor has been developed at CEA/GRENOBLE (France), which consists of transparent polyvinyl chloride (PVC) tubing, 30 m long, 15 mm internal diameter, volume 5.3 l), spiral-coiled so as to form a plane light captor of 1 m^2, was used to study the degradation of lactate by the photosynthetic bacterium *R. capsulatus*, strain B10 (Delachapelle *et al.* 1991) (Fig. 10.10). The bacterial culture was continuously circulated in the reactor so as to maintain a homogeneous suspension to optimize illumination of the cells, avoid bacterial self-shading, allow regeneration of ATP by photophosphorylation and make a well-mixed reactor for optimal nutrient transfers and for degassing of the medium. The bacteria were cultivated anaerobically under photoheterotrophic and N-limited conditions to allow the synthesis of the nitrogenase enzyme, the catalyst, which produces H_2.

To run it under automated conditions, the bioreactor was equipped with two temperature sensors, two pH electrodes, a water level detector, a manometer and a computer-controlled electric valve. Control of key parameters (pH, temperature, dilution rates) has allowed to define the culture conditions producing maximal amounts of molecular hydrogen.

The production of H_2 accompanying lactate degradation was maximal in diluted nitrogen-limited continuous cultures. It was observed at a dilution rate of 0.04 h^{-1} with 5 mM glutamate in the influent medium, the optical density of the culture being 2.1 at 660 nm. Under these conditions an average H_2 production of 85 ml h^{-1} l^{-1} (0.85 l h^{-1} for the whole bioreactor) was observed over a 200 h period and up to 90 per cent of the added lactate (initial concentration 30 mM) was degraded. The concentration of degradation products (formate, acetate) remained below 2 mM. At higher bacterial concentrations, the limitation of light energy resulted in a decrease in nitrogenase activity and therefore in a drop in H_2 production.

10.5.2. Heterotrophic microorganisms

Fermentative bacterial hydrogen production has been studied extensively as H_2 is a major product of anaerobic fermentation and nitrogen fixation. The stoichiometric fermentation of glucose can, in principle, yield 12 mol of H_2/mol glucose. However, if all chemical energy stored in the sugar molecule is converted to H_2, the microorganisms cannot generate ATP. This is not preferred because generation of ATP, the general chemical energy carrier and storage molecule in any living cell, is the ultimate driving force to carry our metabolic conversion of glucose. In order to maintain energetic balance, it has been suggested that the conversion of only about one-third of the glucose energy to H_2 is feasible for microorganisms carrying out anaerobic dark fermentation. This is in line with observations in growing bacterial cultures, which produce only 1–2 mol of H_2/mol glucose. This compares poorly with other potentially useful products of intermediate metabolism, e.g., about 80–90 per cent of the chemical energy of glucose can be converted to either ethanol or methane. However, this line of argument only applies to vigorously growing bacterial cultures needing plenty of energy in the form of ATP. Under real life conditions, and under conditions existing in large fermenters, cell growth is very often limited by the availability of nutrients or by unfavourable environmental stresses (Keasling *et al.* 1998).

Heterotrophic fermentation leading to H_2 production can be carried out by a wide variety of mesophilic and thermophilic microorganisms under anoxic conditions.

Under mesophilic conditions (e.g. in *Escherichia coli* or *Enterobacter aerogenes*) maximum conversion yields of 4 mol of H_2/mol glucose have been reported (Kengen *et al*. 1996). Twice this yield would be sufficient for economic H_2 fermentation (Keasling *et al*. 1998). This is valid if the fermentation costs are similar to those currently used in ethanol or methanol fermentations. The remaining chemical energy of glucose appears in the form of various by-products, such as acetate and lactate. These compounds are difficult to convert to H_2 under regular dark anaerobic fermentation conditions. However, phototrophs can utilize acetate and/or lactate and produce H_2 (Section 10.2.1). In general, factors determining optimal H_2 yield (e.g. pH, temperature, light intensity and quality, cell density and nutritional status) differ from optimal growth conditions (Sasikala *et al*. 1993). This further emphasizes the need to separate cultivation from H_2 production.

Alternatively, the biological system also favours H_2 production at elevated temperature, because in hyperthermophiles the affinity for H_2 decreases and the thermodynamic equilibrium of H_2 formation from acetate is favoured (Adams 1990). For heterotrophic hyperthermophiles, such as the species *Pyrococcus furiosus* (Pedroni *et al*. 1995) and *Thermococcus litoralis* (Rakhely *et al*. 1999), energy for growth is derived from the fermentative metabolism of peptides and sugars. In these cases, excess reductant, formed during the redox metabolic processes, is disposed of by a tetrameric hydrogenase producing H_2. An additional feature of interest is that these H_2-producing NiFe hydrogenases contain nucleotide cofactors, in addition to the regular NiFe and Fe clusters (see Section 2.3.3). These cofactors are known to be very sensitive to heat and fall apart at hyperthermophilic temperatures (above 80°C) very quickly. One of the puzzles surrounding hyperthermophilic hydrogenases is how they manage to stabilize their nucleotide cofactors.

It has been recognized that dark, anaerobic fermentative H_2 production, similarly to H_2 photoproduction by photosynthetic bacteria, only takes place to a limited degree in Nature. In mesophilic dark fermentation, the reason is thermodynamic preference of other metabolic products by the bacteria. However, metabolic engineering, coupled with the beneficial effect of high temperature, may bring about dramatic changes as demonstrated recently (Woodward *et al*. 2000). In an *in vitro* attempt, mixing enzymes of the so-called pentose phosphate cycle and hydrogenase from *P. furiosus*, 11.6 mol H_2 were obtained from 1 mol of glucose-6-phosphate. This experiment proved that 97 per cent of the maximum stoichiometric yield is indeed attainable, although sustained H_2 production in such artificial systems can only be maintained for a short period because of the inactivation of the participating enzymes. At any rate, hydrogen fermentations rank highest in their potential for both the likely economics of biological hydrogen production systems and environmental sustainability. Both in photoheterotrophic and dark heterotrophic fermentations there is plenty of room for improvement through genetic modification of metabolic balances. Besides the production of hydrogen in high yield, the use of hyperthermophiles exploits the advantage of an automatic selection pressure for hyperthermophilic hydrogen producers, preventing other processes which compete for the substrate(s) or consume the evolved H_2. Furthermore, some hyperthermophiles excrete a variety of enzymes enabling them to utilize biopolymers, such as starch and cellulose, thus facilitating the use of cheap biomass.

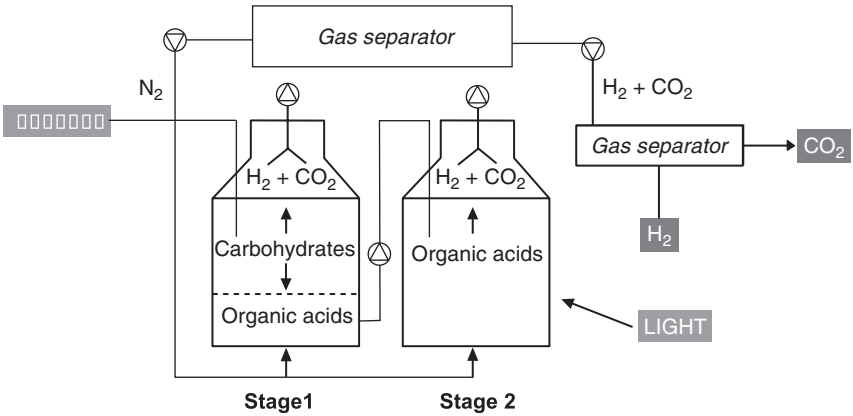

Figure 10.12 Scheme of the two stage biohydrogen fermentation system.

An interesting combination of photoheterotrophic and hyperthermophilic hydrogen fermentation was tested in a European Union 5th Framework Project, coordinated by the Wageningen University, The Netherlands. The main objective of this project was the development of an integrated process in which biomass is utilized for the biohydrogen fermentation. The biological fermentation process takes place in two steps (Claasen *et al*. 1999). In the first stage, the biomass is fermented to acetate, CO_2 and H_2 in a thermophilic dark fermentation. In a separate photobiological anaerobic fermenter (Fig. 10.12), acetate is converted to H_2 and CO_2. The ambitious aim is to get as close to the theoretical maximum of production 12 moles of H_2/mol glucose equivalent as possible. At present, the major environmental disadvantage of the utilization of hydrogen as a fuel is the vast amount of fossil carbon dioxide, which is released into the environment during the production of hydrogen from natural gas. In the proposed bioprocess, the ratio of carbon dioxide released to hydrogen produced will be the same. Moreover, biohydrogen fermentation of sugar or sugar containing agricultural waste can still be considered as 'green' technology and not a potential contribution to global warming because the released CO_2 originates from photosynthetic CO_2 fixation; therefore net CO_2 production does not take place.

10.6. The road to the hydrogen future: R&D in the US Hydrogen Program

Catherine E. Gregoire Padró NREL

The US Department of Energy (DOE) conducts R&D for the development of safe, cost-effective hydrogen energy technologies that support and foster the transition to a hydrogen economy. Of particular interest is the innovative research supported by the DOE's Hydrogen Program, focused primarily on exploration of long-term, high-risk concepts that have the potential to address large-scale energy needs.

Hydrogen can be produced directly from sunlight and water by biological organisms and using semiconductor-based systems similar to photovoltaics, or indirectly,

via thermal processing of biomass. These production technologies have the potential to produce essentially unlimited quantities of hydrogen in a sustainable manner.

Storage of hydrogen is an important area for research, particularly when considering transportation as a major user, and the need for efficient energy storage for intermittent renewable power systems. Although compressed gas and liquid hydrogen storage systems have been used in vehicle demonstrations worldwide, the issues of safety, capacity and energy consumption have resulted in a broadening of the storage possibilities to include metal hydrides and carbon nanostructures. Stationary storage systems that are high efficiency with quick response times will be important for incorporating large amounts of intermittent photovoltaic and wind into the grid as base load power.

In addition to the extensive fuel cell development programs in other offices within DOE, the Hydrogen Program conducts fuel cell research focused on development of inexpensive, membrane electrode assemblies and the development of reversible fuel cells for stationary applications. The Program also supports research in the development of hydrogen/methane blends and hydrogen-fuelled internal combustion engines and generator sets.

A large hurdle to expanded use of hydrogen is public perception. Widespread hydrogen use represents an extraordinary educational challenge, as well as the absolute requirement that safety be intrinsic to all processes and systems. The development of reliable, low-cost hydrogen sensors is an important aspect of the Program, as is the development of codes and standards for the safe use of hydrogen.

The use of solar energy to split water into oxygen and hydrogen is an attractive means to directly convert solar energy to chemical energy. Biological, chemical and electrochemical systems are being investigated within DOE as long-term (more than ten years), high-risk, high-payoff technologies for the sustainable production of hydrogen.

10.6.1. Biological systems

In Nature, algae absorb light and utilize water and CO_2 to produce cell mass and oxygen. A complex model referred to as the 'Z-scheme' has been identified to describe the charge separation and electron transfer steps associated with this process that ultimately drives photosynthesis. A number of enzymatic side pathways that can also accept electrons have been identified. Of interest is a class of enzymes known as hydrogenases that can combine protons and electrons obtained from the water oxidation process to release molecular hydrogen. These algal hydrogenases are quickly deactivated by oxygen. Researchers have identified mutant algal strains that evolve hydrogen at a rate that is four times that of the wild type, and are three to four times more oxygen tolerant (Ghiradi *et al*. 1997).

Photosynthetic organisms also contain light harvesting, chlorophyll–protein complexes that effectively concentrate light and funnel energy for photosynthesis. These antenna complexes also dissipate excess incident sunlight as a protective mechanism. The amount of chlorophyll antennae in each cell is directly related to the amount of 'shading' experienced by subsequent layers of microorganisms in a mass culture. In a recent set of experiments, researchers have observed that green alga grown under high light intensities exhibit lower pigment content and a highly truncated chlorophyll antennae size. These cells showed photosynthetic productivity (on a per chlorophyll basis) that was six to seven times greater than the normally pigmented cells, a

phenomenon that could lead to significant improvements in the efficiency of hydrogen production on a surface-area basis.

These technical challenges are being addressed by a team of scientists from Oak Ridge National Laboratory (ORNL), the University of California Berkeley, and the National Renewable Energy Laboratory (NREL). Various reactor designs are under development for photobiological hydrogen production processes (single-stage vs two-stage, single organism vs dual organism). At the University of Hawaii's Natural Energy Institute (HNEI), a new, potentially low cost, outdoor tubular photobioreactor is under development to test a sustainable system for the production of hydrogen.

In addition to the photosynthetic production of hydrogen from water, the Program supports the development of systems to convert CO (found in synthesis gas) to hydrogen via the so-called water-gas shift reaction ($CO + H_2O = CO_2 + H_2$). This reaction is essential to the widely used commercial steam methane reforming process for the production of hydrogen. In the industrial process in use today, high-temperature (450°C) and low-temperature (230°C) shift reactors are required to increase the overall hydrogen production efficiency and to reduce the CO content to acceptable levels. In this project, microorganisms isolated from Nature are used to reduce the level of CO to below detectable levels (0.1 ppm) at temperatures of around 25–50°C in a single reactor. This process, under development at NREL, has significant potential to improve the economics of hydrogen production when combined with the thermal processing of biomass or other carbon-containing feeds.

10.6.2. Photochemical systems

Among the technologies that have been investigated, photocatalytic water splitting systems using relatively inexpensive, durable and nontoxic semiconductor photocatalysts show promise. Supported catalysts such as Pt-RuO_2/TiO_2 have sufficient band gaps for water splitting, although the current rate of hydrogen production from these systems is too low for commercial processes. Modifications to the system are required to address issues such as the narrow range of solar wavelengths absorbed by TiO_2, the efficiency of subsequent catalytic steps for formation of hydrogen and oxygen, and the need for high surface areas. Binding of catalyst complexes that absorb light in the visible range to the TiO_2 should improve the absorption characteristics. Aerogels of TiO_2 as a semiconductor support for the photocatalysts have potential for addressing reaction efficiency and surface area issues. The University of Oklahoma is investigating these systems.

The Florida Solar Energy Centre (FSEC), in conjunction with the University of Geneva, is investigating tandem/dual bed photosystems using sol/gel-deposited WO_3 films as the oxygen-evolving photocatalyst, rather than TiO_2. In this configuration, the dispersion containing the wider band gap photocatalyst must have minimal light scattering losses so that the lower band gap photocatalyst behind it can also be illuminated.

10.6.3. Photoelectrochemical systems

Multijunction cell technology developed by the photovoltaic industry is being used to develop photo-electrochemical light harvesting systems that generate sufficient

voltage to split water and are stable in a water/electrolyte environment. The cascade structure of these devices results in greater utilization of the solar spectrum, resulting in the highest theoretical efficiency for any photoconversion device. In order to develop cost-effective systems, a number of technical challenges must be overcome. These include identification and characterization of semiconductors with appropriate band gaps; development of techniques for preparation and application of transparent catalytic coatings; evaluation of effects of pH, ionic strength, and solution composition on semiconductor energetics and stability, and on catalyst properties; and development of novel photovoltaic/photo-electrochemical system designs. NREL's approach to solving these challenges is to use the most efficient semiconductor materials available, consistent with the energy requirements for a water splitting system that is stable in an aqueous environment. To date, a photovoltaic/photo-electrochemical water splitting system with a solar-to-hydrogen efficiency of 12.4 per cent lower heating value (LHV) using concentrated light, has operated for over 20 h (Khaselev and Turner, 1998). HNEI is pursuing a low-cost amorphous silicon-based tandem cell design with appropriate stability and performance, and is developing protective coatings and effective catalysts. An outdoor test of the an Si cells resulted in a solar-to-hydrogen efficiency of 7.8 per cent LHV under natural sunlight.

10.6.4. Indirect hydrogen production technologies

These systems offer the opportunity to produce hydrogen from renewable resources in the mid-term (five to ten years). Using agricultural residues and wastes, or biomass specifically grown for energy uses, hydrogen can be produced using a variety of processes.

Biomass pyrolysis produces a bio-oil that, like petroleum, contains a wide spectrum of components. Unlike petroleum, bio-oil contains a significant number of highly reactive oxygenated components derived mainly from constitutive carbohydrates and lignin. These components can be transformed into hydrogen via catalytic steam reforming using Ni-based catalysts. By using high heat transfer rates and appropriate reactor configurations that facilitate contact with the catalyst, the formation of carbonaceous deposits (char) can be minimized. The resulting products from the thermal cracking of the bio-oils are steam reformed at temperatures ranging from 750–850°C. At these conditions, any char formed will also be gasified. At NREL and the Jet Propulsion Laboratory, research and modelling are underway to develop processing technologies that take advantage of the wide spectrum of components in the bio-oil, and address reactivity and reactor design issues (Miller and Bellan, 1997). Evaluation of co-product strategies indicates that high value chemicals, such as phenolic resins, can be economically produced in conjunction with hydrogen.

Biomass is typically 50 weight per cent (wt per cent) moisture (as received); biomass gasification and pyrolysis processes require drying of the feed to about 15 wt per cent moisture for efficient and sustained operation, in addition to requiring size reduction (particle size of ~1 cm). In supercritical gasification processes, feed drying is not required, although particle size reduction requirements are more severe. A slurry containing approximately 15 wt per cent biomass (required size reduction ~1 mm) is pumped at high pressure (>22 MPa, the critical pressure of water) into a reactor, where hydrothermolysis occurs, leading to extensive solubilization of the lignocellulosics at

just above the supercritical conditions. If heat transfer rates to the slurry are sufficiently high, little char is formed, and the constituents of biomass are hydrolysed and solubilized in the supercritical medium. Increasing the temperature to ~700°C in the presence of catalysts results in the reforming of the hydrolysis products. Catalysts have been identified that are suitable for the steam reforming operation (Matsunaga *et al.* 1997). HNEI, Combustion Systems Inc., and General Atomics are investigating appropriate slurry compositions, reactor configurations, and operating parameters for supercritical water gasification of wet biomass.

10.6.5. Hydrogen storage, transport and delivery

The storage, transport and delivery of hydrogen are important elements in a hydrogen energy system. With keen interest in mobile applications of hydrogen systems, and as intermittent renewables penetration of the electric grid increases, storage becomes essential to a sustainable energy economy. Light weight and high energy density storage will enable the use of hydrogen as a transportation fuel. Efficient and cost effective stationary hydrogen storage will permit photovoltaic and wind to serve as base load power systems.

Compressed gas storage tanks

Currently, compressed gas is the only commercially available method for ambient-temperature hydrogen storage on a vehicle. Compressed hydrogen stored at 24.8 MPa in a conventional fibreglass-wrapped aluminum cylinder results in a volumetric storage density of 12 kg of hydrogen per m^3 of storage volume and a gravimetric density of 2 wt per cent (grams of hydrogen per gram of system weight). Carbon fibre-wrapped polymer cylinders achieve higher densities (15 kg/m^3 and 5 wt per cent), but are significantly below target values required for hydrogen to make major inroads in the transportation sector (62 kg/m^3 and 6.5 wt per cent). Advanced lightweight pressure vessels have been designed and fabricated by Lawrence Livermore National Laboratory (Mitlitsky *et al.* 1998). These vessels use lightweight bladder liners that act as inflatable mandrels for composite overwrap and as permeation barriers for gas storage. These tank systems are expected to exceed 12 wt per cent hydrogen storage (at 33.8 MPa) when fully developed.

Carbon-based storage systems

Carbon-based hydrogen storage materials that can store significant amounts of hydrogen at room temperature are under investigation. Carbon nanostructures could provide the needed technological breakthrough that makes hydrogen powered vehicles practical. Two carbon nanostructures are of interest S single-walled nanotubes and graphite nanofibres. Single-walled carbon nanotubes, elongated pores with diameters of molecular dimensions (twelve-diamensional), adsorb hydrogen by capillary action at non-cryogenic temperatures. Single-walled nanotubes have recently been produced and tested at NREL in high yields using a number of production techniques, and have demonstrated hydrogen uptake at 5–10 wt per cent at room temperature (Dillon *et al.* 1997). Graphite nanofibres are a set of materials that are generated from the metal catalysed decomposition of hydrocarbon-containing mixtures. The

structure of the nanofibres is controlled by the selection of catalytic species, reactant composition and temperature. The solid consists of an ordered stack of nanocrystals that are evenly spaced at 0.34–0.37 nanometers (depending on preparation conditions). These are bonded together by van der Waals forces to form a 'flexible wall' nanopore structure.

Metal hydride storage and delivery systems

Conventional high capacity metal hydrides require high temperatures (300–350°C) to liberate hydrogen, but sufficient heat is not generally available in fuel cell transportation applications. Low temperature hydrides, however, suffer from low gravimetric energy densities and require too much space on board or add significant weight to the vehicle. Sandia National Laboratories (SNL) and Energy Conversion Devices (ECD) are developing low-temperature metal hydride systems that can store 3–5 wt per cent hydrogen. Alloying techniques have been developed by ECD that result in high-capacity, multicomponent alloys with excellent kinetics, albeit at high temperatures. Additional research is required to identify alloys with appropriate kinetics at low temperatures.

A new approach for the production, transmission and storage of hydrogen using a chemical hydride slurry as the hydrogen carrier and storage medium is under investigation by Thermo Power Corporation. The slurry protects the hydride from unanticipated contact with moisture and makes the hydride pumpable. At the point of storage and use, a chemical hydride/water reaction is used to produce high purity hydrogen. An essential feature of the process is recovery and reuse of spent hydride at a centralized processing plant. Research issues include the identification of safe, stable and pumpable slurries and the design of an appropriate high temperature reactor for regeneration of spent slurry.

10.6.6. End use technologies

Proton exchange membrane (PEM) fuel cells could provide low-cost, high-efficiency electric power, and be operated 'in reverse' as electrolysers to generate hydrogen. There has been a significant increase in industry activity for the development of PEM fuel cells for vehicular applications, with a number of active demonstration projects. Improvements in catalyst loading requirements, water management and temperature control have helped move these power units from mere curiosities to legitimate market successes. In order to increase the market penetration in both the transportation and utility sectors, additional improvements are required. Los Alamos National Laboratory is developing non-machined stainless steel hardware and membrane electrode assemblies with low catalyst loadings to achieve cost reductions and efficiency improvements (Cleghorn *et al*. 1997). The most important barriers to implementation of low-cost PEM fuel cells are susceptibility of the metal or alloy to corrosion, water management using metal screens as flow fields, and effective stack sealing. Operating the PEM fuel cell 'in reverse' as an electrolyser is possible, but optimum operating conditions for the power production mode and for the hydrogen production mode are significantly different. Design issues for the reversible fuel cell system include thermal management, humidification, and catalyst type and loading.

In an effort to promote near-term use of hydrogen as a transportation fuel, the Program is investigating the development of cost-effective, highly efficient, and ultra-low

emission internal combustion engines (ICE) operating on pure hydrogen and hydrogen-blended fuels. Research at SNL is focused on the development of a hydrogen fueled ICE/generator set with an overall efficiency of >40 per cent while maintaining near zero NO_x emissions (van Blarigan, 1998).

10.6.7. Safety

Hydrogen leak detection is an essential element of safe systems. The development of low-cost fibre optic and thick film sensors by NREL and ORNL, respectively, will provide affordable and reliable options for hydrogen safety systems. NREL is using optical fibres with a thin film coating on the end that changes optic properties upon reversible reaction with hydrogen. Change in the reflected light signal is an indication of the presence of hydrogen. Sensitivity and selectivity are important research issues. ORNL is focused on the development of monolithic, resistive thick film sensors that are inherently robust, selective to hydrogen, and easy to manufacture. Research issues include developing appropriate techniques for active (versus traditional passive) thick film applications.

Recognizing the importance of safe use of hydrogen, the DOE, in conjunction with Natural Resources Canada, has compiled a comprehensive document of prevailing practices and applicable codes, standards, guidelines, and regulations for the safe use of hydrogen. The Sourcebook for Hydrogen Applications is intended to be a 'living document' that can be updated to reflect the current state of knowledge about, and experience with, safely using hydrogen in emerging applications. DOE also supports the development of codes and standards under the auspices of the International Standards Organization.

In conclusion, the DOE Hydrogen Program conducts R&D in the areas of production, storage, and utilization, for the purpose of making hydrogen a cost-effective energy carrier for utility, buildings, and transportation applications. Research is focused on the introduction of renewable-based options to produce hydrogen; development of hydrogen-based electricity storage and generation systems that enhance the use of distributed renewable-based utility systems; development of low-cost technologies that produce hydrogen directly from sunlight and water; and support of the introduction of safe and dependable hydrogen systems including the development of codes and standards for hydrogen technologies.

10.7. Japan: International cooperations/networks

Japan has been active in supporting the exploitation of bio-solar technology for H_2 production. The international project RITE (Research Innovative Technologies of the Earth), under the National Organization NEDO (New Energy Development Organization) was supported by MITI (Ministry of International Trade and Industry). A major initiative was the International program 'IEA Agreement of the Production and Utilization of Hydrogen' (Annex 10 'Photoproduction of hydrogen'; Annex 15 'Photobiological Hydrogen Production'). The main objectives were to investigate and to develop processes and equipment for the production of hydrogen by direct conversion of solar energy to molecular hydrogen using biological systems.

Many of the outcomes of this work, together with contributions from around the world, have been included in the recent book Biohydrogen II, edited by J. Miyake *et al.* (2000).

10.8. Future challenges

Bärbel Hüsing

The major rationale for bio-hydrogen R&D is the possible use of bio-hydrogen as an energy source and raw material as a component within a future solar energy system. This is, however, a very long-term option. Its realization is not likely before the year 2030–2040, and only if drastic reductions in CO_2 emissions must be reached (Hüsing and Reiß 1996). From today's knowledge, for thermodynamic and logistic reasons the technological option of bio-hydrogen production which seems to be the best for the above mentioned purpose is biophotolysis, i.e. splitting water into oxygen and hydrogen by absorption of sunlight. This process is catalysed by photosystems and hydrogenase.

Bio-hydrogen is part of the broader concept of developing environmentally friendly technologies, especially zero-emission technologies. The technological options of bio-hydrogen production that mainly contribute to this concept are photoproduction from biomass and production from biomass by anaerobic fermentation. Both processes depend on the supply of organic substrates and are therefore ideally suited for coupling with treatment of biomass, waste and waste water. H_2 production by these metabolic routes is catalysed by nitrogenase and/or hydrogenase, and it is much closer to practical application than bio-hydrogen production by biophotolysis.

However, hydrogenases should not only be considered in the context of large-scale hydrogen production. There is considerable progress in microsystems engineering and nanotechnology, and biotechnology is beginning to play a role in this field. It would be an interesting option for future hydrogenase research and development (R&D), to exploit the potential of hydrogenases also as an energy source in 'small-scale applications', e.g. in biomolecular devices in microsystems engineering or as power supply for microsurgery robots or artificial organs which operate inside the human body. First attempts in this direction are already underway (Sasaki und Karube 1999). Other application potentials are hydrogenases in enzymatic analytical test systems, in biosensors, and in electrochemical regeneration of redox enzymes used in chemical synthesis (Somers *et al.* 1997).

If these three goals in bio-hydrogen R&D are to be pursued, progress in the following fields of bio-hydrogen R&D is required.

10.8.1. Improving the performance of the production organism

Optimizing the performance of the bio-hydrogen production organism comprises two strategies which complement each other: first, isolation and selection of natural H_2-producing strains by conventional screening methods; and second, improvement of the H_2-producing organisms by genetic engineering.

In the past, screening for excellent natural H_2-producing organisms has yielded very promising strains, but was time consuming and labour intensive. New approaches and

selection methods must be developed in order to speed up the screening process and to increase the likelihood of discovering excellent H_2 producers. This can be achieved by:

- screening the biodiversity of still untapped resources (e.g. extreme environments);
- applying automated, computer-controlled, miniaturized screening procedures;
- developing rapid identification tests based on information on the metabolic pathways involved in H_2 metabolism;
- developing rapid and miniaturized tests for the H_2-production ability of large numbers of organisms or mutants (ideally with positive selection); and
- screening for organisms with properties that are important for biotechnological exploitation, such as tolerance towards oxygen, pH, light, temperature, waste components, lack of adhesion to surfaces of culture vessels, and easy separation from the culture medium.

Once isolated, the natural H_2 producers can be optimized by conventional mutagenesis, and they should be studied so that we can understand those features that make them the best H_2 producers. This characterization would involve the analysis of metabolic fluxes (Stephanopoulos and Sinskey 1993; Schuster *et al*. 1999) and molecular genetics. It would result in new, previously unknown adaptations necessary for improved H_2 production, and could provide information on the most important mutations that are required to obtain excellent H_2 producers. Information obtained from these experiments should be used in genetic engineering approaches for optimizing H_2 producers. Moreover, excellent H_2 producers should be used in bioengineering approaches.

Parallel to the characterization of natural excellent H_2 producers is the rational optimization of H_2 producers by metabolic engineering, using genetic engineering approaches. This type of research can be performed in laboratory strains, which are relatively easy to handle. If certain approaches prove to be successful with respect to H_2 production they may also be transferred to the excellent natural H_2 producers. However, at present, there are hardly any model organisms or model hydrogenases that are well-characterized biochemically, chemically, spectroscopically, physiologically and by molecular genetics. If efforts were concentrated on a few model systems by combining different disciplines and approaches for a thorough investigation, synergies between the different methodological approaches could be exploited. This interdisciplinary approach could yield results that could not be obtained otherwise.

10.8.2. Improving the performance of hydrogenases in technical systems

At present, deepening our understanding for structure-function relationships in hydrogenases has mainly remained basic research. It is important for applications of hydrogenases in both 'large and small scale applications' to exploit this knowledge for the rational optimisation of hydrogenases with properties of biotechnological interest. This could be hydrogenases with e.g. enhanced stability and activity or biomimetic catalysts with these desired properties. Moreover, the 'irrational' design of hydrogenase properties could also be taken into consideration (Kuchner and Arnold 1997).

10.8.3. Bioprocess engineering, scaleup to a functional prototype of technical scale

The bioprocess engineering approach comprises the identification of factors influencing H_2 metabolism and growth, the optimization of culture conditions for H_2 producers, as well as bioreactor design, construction and operation. In the past, progress in this field has been hindered by the fact that research groups worked more or less isolated with their own system, so that comparisons and knowledge transfer between different systems were difficult to perform and therefore, possible synergies were not exploited. In order to provide data which allow the scaleup of bio-hydrogen production to a functional prototype of technical scale, future challenges of bio-hydrogen R&D are:

- to define standard culture conditions (e.g. light) and a standard report format for values (e.g. specific H_2-production rate, conversion efficiency, light conditions) to make comparisons between different strains and different R&D groups possible (especially for the evaluation of new screening isolates);
- to use the same strain wherever possible;
- to perform comparisons and 'benchmarking' of results wherever possible; and
- to systematically vary strains, culture conditions or bioreactor design while all other parameters are kept constant.

Up to now, there are hardly any photobioreactors which allow a cost-efficient production even of high-value products from phototrophic organisms. The development of such photobioreactors is an attractive goal. They could – as a 'spin-off' – prove useful for other production processes using phototrophic organisms. Another goal could be the design of a production process with minimal requirements of energy, water, cooling/heating, etc., minimal output of waste, and maximum product recovery. Technologies to achieve this goal (including e.g. membranes, electrodialysis) could also be applicable to other biotechnical production processes and would contribute to the implementation of the zero-emission concept.

10.8.4. Economic and ecological assessments parallel to experimental R&D

At the present state of bio-hydrogen R&D there are still many different options as to how a future bio-hydrogen production process might look; definite decisions about successful solutions cannot yet be made. Moreover, practical bio-hydrogen production will have to compete with alternative, often established technologies, and must therefore show clear comparative, specific advantages over these competing technologies. In addition, the need for bio-hydrogen is substantially influenced by priority-setting in environmental and energy policy.

Therefore, it is very important to periodically adjust bio-hydrogen R&D to new developments in the relevant frame conditions. For this purpose, relevant developments in bio-hydrogen R&D and related scientific fields, in environmental and energy policy, and in competing technologies should be monitored. Life-cycle assessments can contribute to defining the role of bio-hydrogen in relation to competing technologies,

to identifying weak spots in technical processes and to improving process performance from the environmental perspective. Economic analysis can help to define the cost targets for bio-hydrogen in order to become economically competitive, and market studies can help in the identification of markets and niches for bio-hydrogen and its by-products (e.g. products from excess biomass). Information from such activities should support strategic decision-making within future bio-hydrogen R&D and its fine-tuning.

Finally, progress in the field of bio-hydrogen R&D will significantly depend on how well close interaction and feedback between these different fields of R&D can be achieved in order to obtain intensive knowledge transfer, make tacit knowledge available, and to exploit synergies.

10.9. Concluding remarks

K. Krishna Rao and Richard Cammack

It is clear that in the twenty-first century, much more effort will have to be directed at alternative energy sources, of which hydrogen is one of the most promising. This book has focused on the science that underpins the biological approach to the production of hydrogen energy.

Photosynthetic organisms have evolved for maximum efficiency at what they do, and are capable of light conversion efficiencies of over 10 per cent. This is comparable with the best solar cell systems for generating electricity. The practical pilot systems are only approaching 1 per cent. In order to bridge that gap, an enormous investment in the evolution of artificial organisms might be required. We should not forget that there has been an enormous investment of resources, time, effort and ingenuity into perfecting the way we currently harness fossil fuels as energy sources. The effort that will be needed to bring newer energy technologies into use is no less formidable.