CHAPTER 1

Introduction

1.1 ENERGY CRISIS

The Intergovernmental Panel on Climate Change (IPCC) consisting of 2,500 leading scientists has warned that the phenomenon of global warming would raise the earth's temperature by as much as 3-5 °C over the next hundred years causing a world wide disaster (The Hindustan Times, 1996). Human activities are the likely cause of the planet heating up. The report says that global warming will have potentially irreversible effects, including loss of habitat, increase in human diseases, and loss of life.

Most of the gases are given off by burning fossil fuels resulting into the greenhouse effect and thinning of ozone layers (Selvam, 1991). Ultimately the world must move away from the use of traditional fuels for most of its energy needs. The quest for an alternate fuel source has gathered momentum ever since the world was exposed to the Gulf War. The realisation of short-lived oil supplies has been the final signal to make the search for a petrol substitute to go into hyperdrive.

Fossil fuels, particularly oil and gas, which presently provide most of our energy needs, are rapidly being depleted (Veziroglu, 1983). Scientists predict planet earth may have a century of oil reserves left and after that nobody knows what will happen. Coal has been less rapidly utilised because it is less convenient and creates more environment problems.
1.2 ALTERNATIVE SOURCES

Alternate sources of energy are available but are relatively undeveloped technologically or not utilised fully (Veziroglu, 1983). Some of these are renewable, such as solar heat, solar electricity, hydro, wind, ocean thermal and salinity gradient energy.

Others are depletable but relatively untapped, such as geothermal heat or synthetic fuels from coal or wastes. Nuclear fusion is still a hope for the future. However, biofuels -- by providing domestic resources to meet part of this demand -- can play a major role in stabilizing energy prices, improving national energy security and ensuring rural and regional economical development (Overend, 1993).

This is an effort to find a solution to the important and growing problem of our times: ENERGY.

Of all the known non-fossil energy sources, only a few are far enough along in their development to be counted on: solar and hydrogen (in particular, solar produced hydrogen), neither of which produces any greenhouse gases at all. They are very attractive because they produce no waste and they are inexhaustible. It is hoped to see vast tracts of photovoltaic collectors producing cheap electricity that can be transmitted over long distances. Alternately, the electricity could be used to produce hydrogen from water (biophotolysis).

Scientists have put forward a realistic argument for an energy-efficient, hydrogen-based future (Dostrovsky, 1991). Considering that nearly 80 % of the earth's surface is made up of water in various forms, the theory seems to be eminently plausible.

Hydrogen is as close to an ideal fuel as man can ever hope for (Gupta & Narsimharao, 1987). When burnt it releases no carbon dioxide, no sulphur, no carbon particles, no sulphur, no hydrocarbons. All it emits is steam that condenses
FIG. 1.1 : PROPOSED BIO-SOLAR HYDROGEN PRODUCTION PLAN.
into water thus making it environment-friendly. This theory is now being put to test, successfully.

Experiments have revealed that hydrogen indeed has immense commercial potential and the world may be very close to being presented with a viable hydrogen-based engine (Fig. 1). There are at least four multinational automakers -- Mercedes, BMW, Mazda and Honda, who have developed prototypes based on hydrogen (Ogden & Williams, 1989).

Scientists suggest that in the next century there is no option but to have a hydrogen-based economy (Ogden & Williams, 1989). This model suggests vast endless arrays of highly efficient solar cells that electrolyze water from underground aquifers and the gas thus produced will be pumped across the country. The hydrogen generated will in turn be used to produce energy in vehicles, factories and homes. Interestingly, this hydrogen will be burnt in power plants to produce electricity.

1.3 LIQUID MEMBRANE BILAYERS AND PHOTO-OSMOSIS

As of today, the only way, hydrogen can become a cheap power source is through increased efficiency of solar cells and proper harnessing of solar energy. To harness the inexhaustible flow of solar energy, presently, expensive solid-state materials, such as crystalline silicon is used as receptors of light energy (Lehninger, 1982).

Nature has put itself the problem of how to catch light streaming to the earth and to store the most elusive of all powers in a rigid form (Stryer, 1995). This power is trapped by photoreceptors like chlorophyll a, a substituted tetrapyrrole. Plants take in one form of power, light; and produce another power, chemical energy.
Four taxonomic groups of classical prokaryotes and one group of archaebacteria can convert light energy into chemical bond energy (Woese et al., 1978). They belong to three functional categories in terms of the photochemical mechanisms involved (Table 1).

**TABLE 1.1**

<table>
<thead>
<tr>
<th>Metabolic process</th>
<th>Taxonomic group</th>
<th>Pigment associated with energy conversion</th>
<th>Primary product(s) of energy conversion</th>
<th>Carbon source(s)</th>
<th>Photosynthetic electron donors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anoxygenic-photosynthesis</td>
<td>Purple bacteria</td>
<td>Bacteriochlorophyll a/b</td>
<td>ATP</td>
<td>Organic and/or</td>
<td>H₂, H₂S, S</td>
</tr>
<tr>
<td></td>
<td>Green bacteria</td>
<td>Bacteriochlorophyll a</td>
<td>ATP</td>
<td>CO₂</td>
<td>H₂, H₂S, S</td>
</tr>
<tr>
<td>Oxygenic-photosynthesis</td>
<td>Cyanobacteria*</td>
<td>Chlorophyll a</td>
<td>ATP + NADPH</td>
<td>CO₂</td>
<td>H₂O</td>
</tr>
<tr>
<td></td>
<td>Prochlorophytes</td>
<td>Chlorophyll a</td>
<td>ATP + NADPH</td>
<td>CO₂</td>
<td>H₂O</td>
</tr>
<tr>
<td>Bacteriorhodopsin*</td>
<td><em>Halobacterium</em></td>
<td>Bacteriorhodopsin</td>
<td>ATP</td>
<td>Organic</td>
<td>None</td>
</tr>
</tbody>
</table>

a Some cyanobacteria can perform facultative anoxygenic photosynthesis, using H₂S as a reductant for CO₂  
b An archaebacterium (Woese, Magrum, and Fox, 1978)


In all phototrophic prokaryotes, as in green plants, energy conversion is a chlorophyll-based photosynthetic process. Purple and green bacteria perform anoxygenic photosynthesis, so named because it is unaccompanied by a photochemical cleavage of water and therefore does not lead to the formation of oxygen. The cyanobacteria — by far the largest group of phototrophic prokaryotes — perform oxygenic photosynthesis, quite identical with eukaryotic, chloroplast-mediated photosynthesis. The prochlorophytes, a small group of phototrophic prokaryotes, that were recently discovered as endosymbionts of ascidians, also perform oxygenic photosynthesis (Lewin, 1976) (Table 2).
### TABLE 1.2

**COMPARITIVE CHEMISTRY OF THE PHOTOSYNTHETIC APPARATUS OF THE PROKARYOTES**

<table>
<thead>
<tr>
<th>Light harvesting pigments</th>
<th>Principal electron carriers</th>
<th>Membrane glycolipids (diglycerides)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purple bacteria</td>
<td>Bacteriochlorophyls:</td>
<td>&quot;Bacterial&quot; ferredoxins;</td>
</tr>
<tr>
<td>Green bacteria</td>
<td>group-specific aliphatic</td>
<td>group-specific quinones;</td>
</tr>
<tr>
<td></td>
<td>or monocyclic carotenoids</td>
<td>and cytochromes</td>
</tr>
<tr>
<td>Cyanobacteria and rhodophyta chloroplast</td>
<td>Chlorophyll a: phycobiliproteins</td>
<td>&quot;Plant&quot; ferredoxin: plastocyanin;</td>
</tr>
<tr>
<td>Prochlorophytes and chlorophyta chloroplast</td>
<td>Chlorophylls a and b: β-carotene</td>
<td>plastocyanin; cytochrome f</td>
</tr>
</tbody>
</table>


The comparison of prokaryote chlorophyll absorption maxima in vivo and in vitro is as listed in Table 3.

### TABLE 1.3

**COMPARISON OF PROKARYOTE CHLOROPHYLL ABSORPTION MAXIMA IN VIVO AND IN VITRO**

<table>
<thead>
<tr>
<th>Biological group</th>
<th>Chlorophyll species</th>
<th>Wavelength maxima (nm)</th>
<th>Red shift (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ether</td>
<td>Intact cells</td>
</tr>
<tr>
<td>Cynobacteria</td>
<td>a</td>
<td>665</td>
<td>690-693</td>
</tr>
<tr>
<td>Green bacteria</td>
<td>Bacterio-c</td>
<td>660</td>
<td>745</td>
</tr>
<tr>
<td>Green bacteria</td>
<td>Bacterio-d</td>
<td>650</td>
<td>725</td>
</tr>
<tr>
<td>Purple bacteria</td>
<td>Bacterio-a</td>
<td>775</td>
<td>850-910</td>
</tr>
<tr>
<td>Purple bacteria</td>
<td>Bacterio-b</td>
<td>790</td>
<td>1,020-1,035</td>
</tr>
</tbody>
</table>

The primitive archaebacterial group *Halobacterium* cannot, strictly speaking, be construed as phototrophic organisms, since their growth does not occur at the sole expense of light. Chemoorganotrophy is the dominant nutritional mode in these organisms, and oxygen-linked respiration is the normal mechanism of ATP synthesis (Stanier et al., 1986).

However, when placed under conditions of oxygen limitation, the halobacteria become derepressed for the synthesis of a chromoprotein, bacteriorhodopsin, which is incorporated into discrete patches (‘purple membrane’) in the cell membrane. The name bacteriorhodopsin was coined by analogy with the vertebrate visual pigment rhodopsin (Oesterhelt & Stoeckenius, 1973). In both of these pigments, the \( C_{20} \) carotenoid retinal serves as chromophore; and both are light sensitive, undergoing a complex series of photochemical transformations (Kung et al., 1975).

Illumination of cells of *Halobacterium* that contain purple membrane results in bleaching of the chromoprotein, accompanied by a release of protons into the extracellular milieu. The reaction is reversible (Oesterhelt & Stoeckenius, 1973). The establishment of a transmembrane proton gradient permits ATP synthesis, maintained as long as illumination of the cells continues.

By virtue of their extreme and obligate halophily, the aerobic halobacteria are largely confined to surface layers of hypersaline aquatic environments, where they are exposed to intense solar irradiance. Since the solubility of oxygen in concentrated salt solutions is considerably less than in water, the concentration of dissolved oxygen in such environments can easily become growth-limiting for an aerobic organism. The derepression of bacteriorhodopsin synthesis specifically triggered by oxygen depletion, thus has an evident adaptive value: it brings into operation an ancillary mechanism of ATP synthesis that is light dependent, and not oxygen dependent. This mechanism of energy conversion is not known to exist in any other biological group (Stanier et al., 1986).

By contrast, light is often a limiting factor for cyanobacteria (blue-green algae) and red algae. They possess accessory light-harvesting pigments that enable them
to trap light that is not absorbed by the chlorophylls of photosynthetic organisms lying above them (Stryer, 1995). Phycobilisomes constitute the light-harvesting antennae of cyanobacteria (Lehninger, 1993). Biochemical analyses have demonstrated that proteins are the only components of the phycobilisomes (Capuano et al, 1990). The phycobiliproteins belong to the antennal complex of photosystem II (PS-II) of cyanobacteria and red algae (Bekasova, 1993). They attach to the cytoplasmic surface of thylakoid membranes forming in the cells, highly ordered rows. The number of phycobiliproteins per unit area (μm²) varies from 400 to 1200. The size of the phycobilisomes ranges within the limits (Å²): 320-700 (diameters), 250-450 (height) and 120-400 (thickness).

The cyanobacteria are richly coloured, unicellular or filamentous oligocellular micro-organisms (Papageorgiou, 1996). Three classes of protein-associated, visible light absorbing pigments are present in them

1. phycobilins
2. carotenoids (including xanthophylls),
3. Chl a

Phycobilins and Chl a emit characteristic fluorescence, whereas carotenoids do not.

1.4 THE PHOTOSYNTHETIC MACHINERY OF CYANOBACTERIA

According to some interpretations, Nature invented photosynthesis twice: once in the case of halophilic bacteria (archaeobacteria) and the second time in cyanobacteria (eubacteria) (Papageorgiou, 1996). Other interpretations, however, suggest that cyanobacteria and halobacteria may have descended from a common progenitor, the photocyte, in which case Nature had to invent photosynthesis only once. Once or twice invented, however, the information passed from cyanobacteria
to ancestral eukaryotic hosts, giving rise to present day chloroplasts. The photosynthetic machinery, particularly its membrane-embedded hydrophobic components, remained remarkably conserved. This hypothesis is quite firmly rooted in the many structural and functional similarities of homologous gene clusters and gene products that play important roles in photon harvesting, electronic excitation transduction, and photosynthetic electron transport in higher green plants and cyanobacteria.

1.5 PHYCOBILISOMES: THE EXTRINSIC LIGHT-HARVESTING ORGANELLES OF CYANOBACTERIA

Haxo and Blinks were the first to note the importance of phycobiliproteins (Papageorgiou, 1996). They found that light absorbed by them is photosynthetically more active than light absorbed by chlorophylls. This observation posed the question as to how efficiently phycobiliproteins transfer electronic excitation to chlorophylls. The answer was sought by means of sensitized Chl a fluorescence, namely the indirect generation of Chl a fluorescence, by phycobiliprotein excitation. The physical basis for this approach is the spectral overlap of phycobiliprotein fluorescence with Chl a absorption.

There are four main kinds of phycobiliproteins, characterized by their phycobilin chromophores:

- Phycoerythrocyanins (PEC; $A_{\text{max}}: 568$ nm)
- Phycoerythrins (PE; $A_{\text{max}}: 565$ nm)
- Phycocyanins (PC; $A_{\text{max}}: 620-628$ nm)
- Allophycocyanins (APC; $A_{\text{max}}: 650$ nm)

These proteins form large multisubunit organelles, the phycobilisomes (PBS), which attach non-covalently to the cytoplasmic side of the thylakoid membrane. A typical PBS is hemidiscoidal or ellipsoidal and comprises a core of three cylinders, stacked
along side, and six more cylinders, or rods, that converge on the curved surface of the core. Rods comprise face-to-face stacks of phycobiliprotein hexamers occupy the peripheral end of the rod, the phycocyanin hexamers the end that makes contact with the core. Core cylinders are allophycocyanin trimers that also include the special monomers allophycocyanin B (APC-B; $A_{\text{max}} : 670$ nm) and LCM ($A_{\text{max}} : 670$ nm). The latter protein links the PBS to intrinsic Chl a -- proteins of the thylakoid membrane. Other linker proteins join rod hexamers ($L_R$) and core trimers ($L_C$) together as well as rods to the core ($L_{\text{RC}}$).

The pigments in the phycobilisomes are joined by low molecular weight protein bonds of at least three types. The structure of the phycobilisome is maintained by hydrophobic and ionic interactions. The phycobilisomes are stable only in the presence of high salt concentrations. The isolated phycobilisomes retain functional integrity, as is confirmed by the agreement of the kinetics of energy transfer between the phycobiliprotein in purified phycobilisome and intact cells. The phycobilisomes isolated from the different species of cyanobacteria differ in the number of pigments incorporated into them -- from two in *Synechococcus* sp. to eight in *Nostoc muscorum*.

Allophycocyanin and phycocyanin are usually present in blue-green algae. Phycoerythrin, a red phycobiliprotein with a bile pigment chromophore different from the one common to phycocyanin and allophycocyanin, is present in a number of blue-green algae. The polypeptides of phycoerythrin, phycocyanin, and allophycocyanin are all different. The pigment composition of some algae depends upon the colour of light in which the organisms are growing. Adaptation to light is complementary. Algae growing in red light do not synthesize phycoerythrin and tend to accumulate more phycocyanin and allophycocyanin; those growing in green light accumulate phycoerythrin in addition to phycocyanin and allophycocyanin (Stryer, 1995).

Relatively little is known about the composition and properties of allophycocyanin (Brejc et al., 1995). This biliprotein was originally thought to be a
breakdown product of phycocyanin but report of its wide distribution and successful extraction and purification support its position as a unique biliprotein. Although allophycocyanin and phycocyanin are normally distinguished from one another by their characteristic absorption bands at 650 and 620 nm, respectively, phycocyanobilin is the only chromophore in both biliproteins. The number of phycocyanobilin residues attached to the subunits in allophycocyanin is uncertain. Since allophycocyanin's thought to mediate energy transfer between other biliproteins and chlorophyll a, it maybe the evolutionary antecedent of phycocyanin and phycoerythrin.

Phycobiliproteins are water soluble, coloured, highly fluorescent compounds consisting of prosthetic groups (the bilin chromophore) covalently attached to protein (Patterson, 1996). They exist naturally in cryptomonads, red algae, and cyanobacteria, where they can make up as much as 40% of the protein of the cell.

Phycofluors (primarily phycoerythrin and allophycocyanin) are used in fluorescence-activated cell sorting, flow cytometric analysis, and histochemistry (Patterson, 1996). They can be easily coupled to proteins (monoclonal antibodies, avidin, and streptavidin) or to small molecules (biotin and digoxigenin) with little alteration in the spectroscopic properties of the chromophore. Novel phycobiliproteins, with improved fluorescence characteristics, have been discovered and characterized. Screening of additional cyanobacteria for the presence of biliproteins with desirable characteristics likely will result in additional discoveries of this nature.

It has been possible to develop systems of pigments for solar energy conversion into electrical power (Srivastava et al, 1983). Such systems containing chlorophyll-like pigment and bacteriorhodopsin have gained world-wide acclaim. Recent studies on photo-osmosis through liquid membrane bilayers report that substances like chloroplast extract, haemoglobin, protoporphyrin and cyanocobalamin (Srivastava et al, 1984), cytochrome-C (Srivastava et al, 1985); Lecithin, cholesterol, and lecithin-cholesterol mixtures (Srivastava & Jakhar, 1982); mixture of bacteriorhodopsin and cyanocobalamin (Madamwar & Jain, 1992);
phycoerythrin and bacteriorhodopsin (Patel & Madamwar, 1993); phycocyanin and bacteriorhodopsin (Madamwar & Jain, 1993); and bacteriorhodopsin with cytochrome-C, myoglobin, or haemoglobin show the phenomenon of photo-osmosis (Garg & Madamwar, 1995). Trends observed in the data are consistent with those reported on bilayer lipid membranes (BLMs) (Tien, 1974). In view of these observations it is logical to expect that the liquid membrane bilayers generated by bacteriorhodopsin and allophycocyanin should also show the photo-osmotic effect, and hence the investigation.

Therefore, experiments were carried out with a view to studying the phenomenon of photoosmosis through liquid membrane bilayers generated by a mixture of bacteriorhodopsin with allophycocyanin, which is known for electron generation, so that H⁺ ion liberation could be enhanced. This is to support the view that when bacteriorhodopsin is combined with substances that liberate electrons, the rate of photo-osmotic velocity is much greater than that generated by the individual pigments. Thus, to provide a concept and experimental initiative for establishing a viable system for conversion of solar energy into electrical power, our study possesses importance.

Fundamental biochemical and biophysical research on energy transducing membranes of photosynthetic organisms is therefore not only important for an understanding of nature but may also have long range practical implications in energy production.

1.6 PHOTOHYDROGEN

Hydrogen is the most energetic fuel (Ali & Basit, 1993). But the greatest stumbling block facing experts is "how to produce and distribute hydrogen". At present, the costs of production are prohibitive. The only simple method to produce hydrogen is by electrolysis of water — by passing electric current through electrodes immersed in water. This process needs great charges of electricity which at present is generated
by coal or oil, the conventional sources of energy. Thus, apart from high costs, the whole process of using hydrogen as fuel is lost.

Suggestions have been made that one can use solar energy to generate electricity and in turn produce hydrogen. But this ideology also has its limitations. For example, even the best of photovoltaic cells can utilise only 18-20% of the sun rays that fall on them. Therefore, efforts are ongoing to evaluate and develop the potential for biomass as a renewable energy resource for power generation. For the longer term, biomass-fuelled generating systems promise an effective and economically feasible approach to reducing the contribution of fossil-fuel-based generation to emissions of the greenhouse gas carbon dioxide.

Biologically assisted hydrogen production from various sources has been suggested as one route for obtaining hydrogen for the putative hydrogen economy (Williams, 1983). The production of hydrogen from water by using a biological catalyst and sunlight as an energy source (biophotolysis) could substitute for natural gas (Jeffries et al., 1976). Biological hydrogen production especially by microorganisms has attracted scientists all over the world. This area still remains an academic exercise but has foreseen to be a potential commercial possibility. Before the commercial scaling up could be reached, it is essential to develop suitable organisms for efficient as well as sustained production of hydrogen.

Hydrogen photoproduction by photosynthetic micro-organisms has been studied as one of the ways to produce clean, renewable energy source (Mitsui & Kumazawa, 1977; Mitsui, 1992). The process of hydrogen production in photosynthetic bacteria was first observed by Gest and Kaman (1949) in *Rhodospirillum rubrum* (Meyer et al., 1978). After this, hydrogen production by photosynthetic bacteria became the focus of interest and a number of laboratories worldwide are involved in this. Although, photosynthetic bacteria produce larger amounts of hydrogen than other micro-organisms, they require organic acids as electron donors and hence are not suitable for commercial hydrogen production (Kumazawa & Mitsui, 1980). Among photosynthetic micro-organisms, nitrogen-fixing cyanobacteria, which carry out both oxygenic photosynthesis and
anoxygenic nitrogenase-catalyzed hydrogen production, seem to be suitable for the study on hydrogen production. However, concurrent hydrogen oxidation was shown to substantially reduce the net amount of hydrogen produced (Kumazawa & Mitsui, 1994).

1.7 USE OF CYANOBACTERIA IN PROJECTS OF SOLAR ENERGY CONVERSION TO HYDROGEN

In biological systems, hydrogen can be produced by fermentative processes or by light dependent reactions (Bothe et al., 1978). As regards to fermentation, it was recently pointed out that at most 33 per cent of the combustible energy of organic matter can theoretically be conserved in hydrogen which is not so favourable as the formation of methane where maximally 85 per cent is saved. Independent of external organic substrates, the green and the blue-green algae are able to evolve hydrogen photosynthetically. Photoproduction of the gas by green algae is experimentally critical. It requires an incubation under anaerobic conditions for several hours to commence and it is severely inhibited even by low concentrations of oxygen. Therefore, such photoproduction experiments can only be performed at low light intensities in order to prevent a photosynthetic production of oxygen in quantities which are deleterious to hydrogenase. These features make green algae doubtful candidates for practical biophotolysis systems.

Contrary to this, the hydrogen-formation by cyanobacteria does not require an adaptation to anaerobic conditions, is insensitive to air (heterocystous cyanobacteria) and also to high light intensities. The evolution of hydrogen in these organisms is catalysed by nitrogenase which requires the expenditure of energy. This is, however, not a serious obstacle -- as was argued -- since cyanobacteria have a very active cyclic photophosphorylation. Thus, unless model-systems are preferred, cyanobacteria have a certain promise in programmes of solar energy conversion to hydrogen.
Among photosynthetic micro-organisms, nitrogen-fixing cyanobacteria, which carry out both oxygenic photosynthesis and anoxygenic nitrogenase catalysed hydrogen production, seem to be suitable for the study on hydrogen production (Kumazawa & Mitsui, 1994). Cyanobacteria are a group of extraordinarily diverse prokaryotes that range from unicellular to multicellular, coccoid to branched filaments, nearly colourless to intensely pigmented, heterotrophic to autotrophic, psychrophilic to thermophilic, marine to freshwater (Allnut, 1996). They are found in the Antarctic in near permanent deep freeze and in thermal springs at near boiling temperatures. They are found both free living and as endosymbionts. Although primarily photoautotrophic, some are capable of heterotrophic and / or mixotrophic growth. They are also distinct from other algae (being eukaryotic) as well as from archaeobacteria and are classed as eubacteria. They comprise a unique group of organisms that could provide clues to basic scientific questions (for example photosynthesis, which has been the main focus of cyanobacterial research) as well as provide unique characteristics or products that could make them indispensable as a source for new drugs, chemicals or services. The genetic diversity they represent is immense and needs to be productively tapped in the future.

Cyanobacteria are photosynthetic prokaryotes that carry out water-splitting photosynthesis through the operation of two photosystems very similar to those present in higher plants (Luque et al, 1994). The organisms appear to have played an important role in the evolution of life on Earth, both in the genesis of an aerobic atmosphere and as precursors of higher plant plastids.

Cyanobacterial hydrogen production was first demonstrated with nitrogenase enzyme of Anabaena cylindrica under in vitro condition (Subramanian & Prabaharan, 1994). Later, hydrogen production was observed in intact filaments of A. cylindrica. When the cyanobacteria are incubated under Ar-CO₂ atmosphere the organisms produce hydrogen gas (Subramanian & Prabaharan, 1994). The enzyme nitrogenase is responsible for hydrogen production when protons are reduced under suitable conditions.
Though a number of micro-organisms produce hydrogen, cyanobacteria seem to be best suited since these unique photosynthetic prokaryotes have a number of advantages over other bacteria and green algae, in view of their trophic independence for nitrogen as well as carbon, making the inputs cheaper.

1.8 ENZYMES INVOLVED IN HYDROGEN METABOLISM

In cyanobacteria three distinguishable enzymes are involved in hydrogen metabolism (Subramanian & Prabaharan, 1994) as follows:

1. **Nitrogenase**, which catalyses an ATP dependent hydrogen production
2. **Uptake Hydrogenase**, that catalyses the oxidation of molecular hydrogen, providing reductants to the photosynthetic and respiratory electron transport chains
3. **Reversible Hydrogenase** that catalyses both the production and uptake of molecular hydrogen

These three enzymes occur in several different combinations depending on the organism and the growth conditions.

NITROGENASE:

All cyanobacterial nitrogenases, studied so far, consist of two oxygen-sensitive proteins - neither of which can function alone; one (molybdoferredoxin, azofermo) contains iron, labile sulphide and molybdenum, while other (azoferredoxin, azofer) contains iron and labile sulphide only.

The sequence of electron transfer in the nitrogenase system was established primarily by observations of the characteristic EPR signals of dinitrogenase and dinitrogenase reductase at low temperature (Burris, 1991). Dinitrogenase reductase is reduced by ferredoxin or flavodoxin (or by Na₂S₂O₄ in reconstructed systems). It binds MgATP, and this lowers its potential by about
100 mV to around -400 mV. At that potential it can transfer electrons to dinitrogenase. The transfer is accompanied by the hydrolysis of MgATP to MgADP and Pi. As the ADP (not ATP) is inhibitory to the reaction, it must be converted back to ATP. One electron is transferred for each 2MgATP hydrolyzed. As a single electron is inadequate to reduce N₂, the cycle must be repeated until the dinitrogenase has accumulated adequate electrons to reduce N₂.

As the reduction of N₂ is accompanied by an obligatory reduction of 2H⁺ → H₂, the overall reaction becomes as follows:

N₂ + 8H⁺ + 8e⁻ → 2NH₃ + H₂

As 2MgATP are required for each electron transferred, the reaction requires a minimum of 16MgATP under ideal conditions. However, under normal physiological conditions the requirement is closer to 20-30 MgATP.

In all normal hydrogen producing systems, nitrogenase is deprived of nitrogen and is made to reduce protons to hydrogen resulting in a much larger production of hydrogen than what would be obtained during nitrogen fixation (Subramanian & Prabaharan, 1994).

Nitrogenase, the enzymatic system for nitrogen fixation, is irreversibly inactivated by oxygen when the enzyme is extracted from any organism so far tested (Flores & Herrera, 1994). In nitrogen-fixing cyanobacteria this inactivation is mainly prevented in two ways i.e., by a spatial separation in heterocystous forms and a temporal separation in non-heterocystous (unicellular and filamentous) forms (Apte, 1992). A “separation in space” of nitrogenase into heterocysts, and oxygenic photosynthesis occurs in the unicellular and filamentous, whereas in non-heterocystous cyanobacteria the nitrogenase activity is restricted to the dark phase only. Assay of nitrogenase activity in such forms usually needs creation of anaerobiosis by argon-sparging or addition of DCMU.

It now is accepted that there probably are four distinct nitrogenases, the MoFe nitrogenase, a vanadium nitrogenase, tungsten nitrogenase and an iron nitrogenase.
that apparently is devoid of molybdenum, tungsten and vanadium (Burris, 1991). So these alternative nitrogenases currently are attracting considerable research attention, and the genetics of the systems are being explored (Takahashi, 1994). In general they are less active than the MoFe nitrogenases, and they differ in substrate specificity.

The nucleotide sequence for dinitrogenase reductase has been established for each of the structural genes, \( \text{nif} \ H, \ \text{vnf} \ H \) and \( \text{anf} \ H \). A major difference is that both the alternative vanadium and iron nitrogenases carry a third subunit, \( \delta \), that is coded for by \( \text{vnf} \ G \) and \( \text{anf} \ G \), rather than only two subunits characteristic of other dinitrogenases.

A most important finding with regard to the catalysis by nitrogenase was the discovery by Bulen, Burns and Le Comte (Mortenson, 1978) that hydrogen is evolved by nitrogenase in the absence of nitrogen. The hydrogen production required reductant and \( \text{MgATP}^2 \) just like \( \text{N}_2 \) reduction. The following reactions show two ways by which hydrogen might be produced by nitrogenase:

\[
\begin{align*}
\text{(1) } & \quad E + 2e^- \longrightarrow E^* : 2H \ [\rightarrow E + H_2] \\
\text{(2) } & \quad E^* : 2H + N, E^*.N_2 + H_2 \ [\text{+uptake hydrogenase carrier} \rightarrow \rightarrow \text{carrier} . 2e^-]
\end{align*}
\]

Reactions (1) and (2) show two ways by which hydrogen might be produced by nitrogenase. Reaction (1) would occur only in the absence of nitrogen whereas reaction (2) would occur only in the presence of nitrogen. When nitrogen is limiting, then additional hydrogen is produced, perhaps by a combination of reactions (1) and (2).

Nitrogenase reduces a variety of substrates and not just \( \text{N}_2 \). There is an absolute requirement of \( \text{Mg-ATP} \) to effect reduction of the 'Nitrogenase substrates'. The specific inhibitors of nitrogen fixation can be classified as those which are alternative substrates and those which are not. The first group includes \( \text{N}_2\text{O}, \ \text{NaN}_3, \)
C₂H₂, HCN, CH₃CN, and the second group includes H₂, CO, NO and analogs of these compounds.

In a recent study, Mo, V and Fe nitrogenases were induced in Anabaena variabilis (Tsygankov et al, 1997) The influence of neutral and alkaline pH's on nitrogenase activity and rates of hydrogen photoproduction was investigated to establish the optimum activity for hydrogen evolution under varying growth and hydrogen production conditions. Growth rates in batch cultures at pH 7 with Mo or V nitrogenases were three times higher than with Fe-nitrogenase cultures. The Mo-nitrogenase activity decreased from pH's 7 to 9, and at pH 10 the culture was unable to grow. Fe-nitrogenase cells (no Mo or V) showed similar behaviour over pH 7-8. Cultures expressing V-nitrogenase exhibited the highest resistance to alkaline pHs and grew even at pH 10; hydrogen evolution was practically independent of the culture at pH 7-9. With cells grown under more alkaline conditions, the pH optimum for the hydrogen production was more alkaline. The maximal rates of hydrogen photoproduction were observed with V-nitrogenase containing cells (Tsygankov et al, 1997).

HYDROGENASES

Unlike nitrogenase, hydrogenases are very diverse in their relative molecular mass, co-factor composition and spectroscopic properties (Subramanian & Prabaharan, 1994). The term ‘Hydrogenase’ refers not to a single enzyme but a class of enzymes. Hydrogenases are found in many species of bacteria, both aerobic and anaerobic and in prokaryotic as well as eukaryotic algae.

Most of the work regarding structure and function of hydrogenases was done only in bacteria. In cyanobacteria, uptake hydrogenase and reversible hydrogenase are bound but apparently to different membranes in cyanobacteria.

Uptake hydrogenase has been found in all heterocystous cyanobacteria so far examined, but is yet to be widely reported in non-heterocystous
cyanobacteria (Subramanian & Prabaharan, 1994). Uptake dehydrogenase, which normally co-occurs with nitrogenase, traps and reoxidizes any hydrogen produced (Patterson, 1996). The function of soluble dehydrogenase (also known as 'reversible' dehydrogenase) is not well understood in cyanobacteria; since this enzyme is apparently most active under anaerobic conditions it is unlikely to be involved in respiration.

The efficient hydrogen production depends on maximizing the rate of production while simultaneously minimizing the rate of concurrent oxidation in order to obtain the maximal net productivity. In this regard, some groups have concentrated on manipulation of the activities of the respective enzymes (Patterson, 1996). Likewise, cloning and genetic manipulation of the hydrogenase enzymes could lead to development of a hydrogenase-deficient strain. Genes coding for subunits of the soluble dehydrogenase of *Anabaena cylindrica* and *Synechococcus PCC 6714* have been cloned and sequenced. So far, however, the membrane-bound uptake dehydrogenase has not been purified. Another approach that offers significant promise is that of Mitsui and co-workers (Patterson, 1996). For many years, this group has screened numerous marine cyanobacteria, searching for strains with high rates of hydrogen photoproduction. One strain, designated *Synechococcus* sp. Miami BG043511, has little hydrogen oxidation activity. This strain is capable of high rates of hydrogen evolution [250 \( \mu \text{mol (mg chlorophyll)}^{-1} \text{h}^{-1} \)], and can sustain hydrogen photoproduction in closed vessels at high cell density (Kumazawa & Mitsui, 1994).

1.9 CHARACTERIZATION OF *PHORMIDIUM VALDERIANUM*:

The majority of cyanobacteria behave as photoautotrophic organisms, although some of their representatives are also able to use several saccharides as carbon sources, in either the dark or light (Luque *et al*, 1994). With regard to the utilization of nitrogen, these organisms preferentially use inorganic nitrogen for growth. Nitrate
and ammonium are in general, excellent nitrogen sources for cyanobacteria; they can also grow on nitrite and many of their representatives are able to perform nitrogen fixation. In these organisms, there are permeases that facilitate the uptake of nitrate, nitrite and ammonium (Luque et al, 1994).

The marine cyanobacterium *Phormidium valderianum* was isolated and purified from the southern east coast of India (Prabaharan & Subramanian, 1990). This organism was characterized morphologically and physiologically to a certain extent with a view to exploiting it for one or more biotechnological purposes. *P. valderianum* was found to grow very well in sea water supplemented with both urea and superphosphate compared to other sources of nitrogen and phosphorus either in combination or individually. It was also found to grow well, when common salt was used to make sea water. This organism showed a fairly direct correlation with increase in pH 6 to 10 by regulating the pH in all cases to 9 within about 10 days (Prabaharan & Subramanian, 1990). However, it could not grow well at very low pH. Similarly, this organism was able to tolerate and grow well in a wide range of salinity level (0-99 ppt). Maximum yield of biomass of this organism was obtained with aerated cultures compared to shake and still cultures. This partial characterization of *P. valderianum* has opened up several lines of investigation, to be followed in future.

1.10 ACETYLENE REDUCTION AND HYDROGEN PHOTOPRODUCTION:

There are several reports on acetylene reduction activity and hydrogen photoproduction capacity in non-heterocystous filamentous cyanobacterium under anaerobic, microaerobic and also in aerobic conditions (Stewart & Lex, 1970; Carpenter & Price, 1976; Rippka & Waterbury, 1977; Rippka et al, 1979; Pearson *et al*, 1979; Kumazawa & Mitsui, 1981). *P. valderianum* was screened for acetylene reduction activity in ASN III medium under different conditions such as anaerobic and microaerobic in a nitrogen atmosphere and also in aerobic
atmosphere. In addition, the effect of various nitrogen sources like nitrate nitrogen, ammonium nitrogen, organic nitrogen (amino acids) was also tested. Of all these different conditions *P. valderianum* showed acetylene reduction activity and photohydrogen production capability only in an anaerobic atmosphere free of combined nitrogen.

This shows that *P. valderianum* is one of the promising organisms for developing a biophotolysis system.

### 1.11 COMBINED SYSTEM FOR PHOTOHYDROGEN PRODUCTION

In addition to cyanobacteria, other systems have been considered, including two-stage and electrode systems. Two types of two-stage systems have been envisaged. The first has been suggested for non-heterocystous filamentous cyanobacteria, such as *Plectonema*, which show a temporal separation of photosynthetic oxygen evolution and nitrogen fixation since these activities are inherently incompatible (Weare & Benemann, 1974). It might be possible to obtain oxygen formation during the day and hydrogen formation with stored reductants at night. The second employs two different organisms not normally found together.

Weaver and associates (1980) have reviewed work using *Anacystis nidulans* and *Rhodopseudomonas capsulata* with an NADP+ / NADPH and oxaloacetate / malate couple to form hydrogen. One suggestion is that cyanobacteria could be modified to produce formate which can be converted to hydrogen and carbon dioxide in the dark by *Escherichia coli* (Weaver *et al*, 1980). Another possibility might be to modify cyanobacteria, to photoproduce sugars or organic acids which could be then be used by photosynthetic bacteria to produce hydrogen at high efficiency (Macler *et al*, 1980). A major question regarding such two-stage systems must be the overall efficiency compared to that obtained with cyanobacteria alone.
In our laboratory an effort was made to couple the reducing potential of the electrons with protons, released from bacteriorhodopsin, in presence of hydrogenase / nitrogenase enzyme such that molecular hydrogen is evolved (Patel & Madamwar, 1994). The nitrogen-starved culture of non-heterocystous marine cyanobacterium *Phormidium valderianum* produces hydrogen under an argon atmosphere. In combination with *Halobacterium halobium* and *Escherichia coli*, the hydrogen production goes up by several folds due to electron generation ability of its photosynthetic pigments alongwith protons generated by the bacteriorhodopsin of *H. halobium* (Patel & Madamwar, 1994). The process of coupling protons with electrons is mediated by *E. coli* hydrogenase.

However, hydrogen production goes down within six days. To establish a technically viable system the biological catalyst must be synthesized inexpensively, operate efficiently, and be stable for at least several weeks. Moreover, the simplest scale-up of hydrogen producing system is one in which the analytical minireactor is scaled up to a level where gas production could be observed by collection. This coupled system is functionally viable and therefore we attempted to stabilize this system for continuous production by immobilization (Patel & Madamwar, 1995).

### 1.12 CONTINUOUS PRODUCTION OF PHOTOHYDROGEN

The use of immobilized microbial and plant cell components for the production of fuels and biochemicals has become a rapidly advancing field of biotechnology (Markov et al, 1993). In comparison with batch or continuous culture fermentations where free cells are used, immobilized cells may offer certain specific advantages such as:

1. accelerated reaction rates due to increased cell density per unit volume
2. increased cell metabolism and cell wall permeability
3. no wash-out of cells
4. high operational stability and better control of the catalytic processes
5. separation and reuse of catalyst.
Many methods are available for the immobilization of cells. Active entrapment has been achieved using a variety of natural polymers such as agar, alginate, carrageenan etc. The use of these natural polymers did not seem to affect the viability of cells. However, active entrapment using these polymers serve limited purposes and the best of these, namely calcium alginate poses problems of disruption due to the presence of phosphate or citrate as components of the growth medium. Stabilizing the beads by lowering the pH to 5.5 proves deleterious to cyanobacteria since most of them prefer alkaline pH for growth and activity. Synthetic polymers such as polyacrylamide, serum albumin, glutaraldehyde and polyurethane have also been used for entrapment and have not been found very useful.

Immobilization techniques have been developed for cell stabilization and ease of operation. Immobilization of photosynthetic bacteria using porous glass for hydrogen photoproduction has also been attempted (Tsygankov et al., 1994). Several kinds of polymeric materials are also being used as carriers for immobilization since they have different functional groups and therefore can be modified chemically (Kuu & Wisecarver, 1992).

For possible commercial application, when compared to free living cultures, organisms immobilized in some matrix would be ideal, because the immobilized systems offer several advantages including an enhanced rate of hydrogen production (Kuwada & Ohta, 1987). Considerable work on immobilization using a variety of matrices such as glass, alginate, agar, carrageenan, foam etc. for hydrogen production has been carried out. The results were quite encouraging (Phlips & Mitsui, 1986). Immobilization of Oscillatoria sp. Miami BG 7 cells in 1.5% agar matrix significantly enhanced the rate, yield and stability of hydrogen production, compared to free cell suspension (Phlips & Mitsui, 1986). Brouers and Hall (1986) have shown that the hydrogen produced under argon by polyvinyl foam immobilized with A. azollae was twice that produced by free living cells. This could be either due to the stabilization of the enzymes on immobilization or to a delay in the development of the uptake hydrogenase activity. Markov and his co-
workers (1992) reported photoproduction of hydrogen by the nitrogen fixing cyanobacteria *A. variabilis* and *Nostoc muscorum* (Markov *et al.*, 1993). They observed continuous hydrogen production in a hollow-fibre photobioreactor over a period of 5 months using the organism *A. variabilis* under partial vacuum, thereby reducing the need for inert atmospheres. Such systems are promising for speciality cultures providing fundamental problems can be overcome, such as cell adhesion within the hollow fibres.

As carriers for immobilization, polymeric materials have been used extensively since they can have various functional groups and be easily modified chemically (Kuu & Wisecarver, 1992). Recently the use of polyvinyl alcohol (PVA) for cell immobilization has been investigated. Ariga *et al.* used the technique of iterative freezing and thawing of PVA to form a gel suitable for cell immobilization. They found that this technique produced a low-cost material with a rubber-like elasticity and high strength. Hashimoto and Furukawa crosslinked the PVA using a boric acid solution, producing a monodiol type PVA-boric acid gel lattice. The agglomeration problem of the PVA-boric acid method was eliminated after the addition of a small amount of calcium alginate by Kuu and Wisecarver (Kuu & Wisecarver, 1992).

We attempted to produce continuous and stable hydrogen production by immobilizing the combined system of *Phormidium valderianum*, *Halobacterium halobium* and *Escherichia coli* in a PVA-alginate matrix. PVA was cross-linked with glutaraldehyde, with the addition of a small amount of sodium alginate. The PVA film produced possessed high elasticity and was durable. The PVA pieces were packed in a downflow packed bed reactor and hydrogen produced continuously for eight weeks with no sign of breakage or disintegration of the PVA pieces. Our objective in the present study was to determine optimal nutritional and physiological conditions necessary for continuous and stable hydrogen production by manipulating light intensity, temperature and pH. The concentration of sodium chloride, sugar and electron donor was also optimized. The intermittent supply of nitrogen was found to be essential to retain cellular metabolic activities which in turn showed
prolonged production of hydrogen. The effect of combined nitrogen as a regulatory agent was also optimized and hydrogen production continued for more than four months.

1.13 HALOPHILIC α-AMYLASE

The extreme halophile *Halobacterium halobium* has attracted attention in recent years from the point of view of solar energy conversion and bioenergetics. It was procured and used in our laboratory for the sole purpose of energy generation. During the characterization of this bacterium it was observed that it produces α-amylase. Though the amount produced is meagre in comparison to the commercially use bacteria, it is interesting since it is halophilic in nature.

Bacteria growing best at salt concentrations between 20% (w/v) and saturation about 30% (w/v) are often referred to as "extremely halophilic bacteria" or "extreme halophiles" (Larsen, 1986). Until recently, only two types of bacteria were recognised as extreme halophiles, and in the eighth edition of Bergey's Manual of Determinative Bacteriology, they constitute the family Halobacteriaceae, comprised of the two genera *Halobacterium* and *Halococcus*. Besides their extreme requirement for NaCl, the representatives of this family have other features in common, such as a red-to-orange pigmentation, and other biochemical qualities. Morphologically and with respect to the chemical composition of their cell envelopes, the two genera are quite different, although both belong to the archaebacteria (Buchanan & Gibbons, 1974).

Considerable interest has been taken in the Halobacteriaceae, in part because of the intriguing problems presented by their preference for such an extreme environment, and in part because these organisms display biochemical qualities that make them interesting models for the study of intriguing problems of a more general kind in biochemistry and molecular biology (Larsen, 1980).
Besides the Halobacteriaceae, the only organisms known at present to be extremely halophilic are two representatives of the genus *Ectothiorhodospira*, namely *E. halophila* (Raymond & Sistrom, 1969) and *E. halochloris* (Imhoff & Truper, 1977). These are phototrophic organisms. A variety of other bacterial types have been described as halophilic, but their requirement for salt is modest, and thus they are referred to as "moderate halophiles" or "slight halophiles". Bacteria indigenous to the marine environment could be grouped with the slight halophiles.

As a general rule, the Halobacteriaceae are found in nature in extremely saline natural ponds and lakes, and in marine salterns where salt is commercially produced from sea water by evaporation of the water by the sun. There are reports on the occurrence of these organisms, often in very high numbers, in such localities from most parts of the world. The ecological aspects have been reviewed by Larsen (Larsen, 1986).

The Halobacteriaceae are chemoorganotrophic organisms and thus dependent on a supply of organic material for their growth and development. Most known isolates utilize proteins and amino acids rather than carbohydrates as a source of carbon. They are obligate aerobes (or respiratory, some strains develop anaerobically at the expense of nitrate) and thus are confined to localities which are exposed to air (Larsen, 1986).

Halophilic organisms can grow in very high salt concentrations without denaturation of proteins, making them biotechnologically important (Eisenberg & Wachtel, 1987; Danson, 1988). Enzymes from normal organisms are often inactive in these situations but clearly those from extremely halophilic archaebacteria maybe of use (Hough & Danson, 1989). The halobacteria have adapted to such an environment by accumulating salt intracellularly to at least the exterior concentration (Lanyi, 1978). Growth is optimal between 3.5 and 5 M salt, which must be NaCl, although KCl is also required. The potassium is concentrated by the cells and constitutes the major intracellular cation. These organisms have overcome the osmotic barrier by maintaining a high intracellular KCl concentration.
of about 4M (D’Souza et al, 1992). As a consequence, the halobacterial biochemical machinery is geared to function in high concentrations of salt. A number of reports have appeared on the various metabolic and enzymological aspects of these organisms (D’Souza et al, 1992). Thus enzymes from halophilic organisms have been shown to require a high concentration of KCl / NaCl for their activity and stability. Most of these studies, however have been carried out using cell-free enzymes either in a crude or purified form.

Amylases hydrolyze starch molecules to give a range of dextrins and smaller oligosaccharides (Denault & Underkofler, 1963). There is considerable understanding of amylases and amyloglucosidases especially for their commercial applications (Cornelis, 1987; Sivandane et al, 1983). But scanty information is available regarding the production of α-amylase by *H. halobium* (Good & Hartman, 1970; Patel & Madamwar, 1993). Therefore, the present investigation is an attempt made towards the stabilization of halophilic α-amylase by immobilization. Such an approach had previously been successful in an investigation of the thermostability of immobilized enzymes (Koch-Schmidt et al, 1979).

The use of alginate gel beads stands out as the most promising and versatile method yet (Smidsrod & Skjak-Braek, 1990). Since immobilization can impart stability to enzymes (Klibanov, 1983), the alginate immobilized halophilic α-amylase retained its activity for a longer period in a laboratory scale upflow packed bed reactor (Patel et al, 1996).

The use of immobilized cells provides several advantages over free cells (Corcoran, 1985). Recently, the use of polyvinyl alcohol (PVA) for cell immobilization has been investigated (Kuu & Wisecarver, 1992). *H. halobium* cells were also characterised in terms of temperature and pH in PVA-alginate immobilized condition for α-amylase production (Bagai & Madamwar, 1997).
The multiplicity of regulatory phenomena revealed in recent years in the course of in vitro studies on enzyme behaviour has given rise to some concern over the validity of extrapolation to the in vivo situations. In view of this there is currently a great deal of interest in studying enzyme activities in the presence of cellular structural components and in a natural atmosphere high in proteins (D'Souza et al., 1992). Permeabilized whole cells provide a simple system simulating in vivo conditions for studies on catalytic and regulatory behaviour of enzymes. The regulatory and kinetic properties of enzymes have often been found to be different in situ as compared to in vitro studies (D'Souza et al., 1992).

To date, however such in situ studies on enzymes have not been carried out using halophilic organisms. This is mainly due to their osmotic instability, especially when used in a medium of low salt concentrations. This prevents the study of in situ characteristics under varied conditions due to lysis of cells.

Also, to date, to the best of our knowledge, methods have not been developed for obtaining permeabilized halobacterial cells. Osmotically stabilized and permeabilized cells of halobacteria can serve as a tool for studying the in situ characteristics of halophilic enzymes and other macromolecules. The work with the halobacterial system in our laboratory aims at understanding the molecular mechanisms of adaptation to extreme environments. Such studies may also lead to a better general understanding of solvent effects on protein folding.

Even though potentials of halobacteria to catalyze reactions under extreme conditions of salt are known, hitherto no reports are available on their application in an immobilized form. This is mainly because these cells are very mechanically fragile and lyse very easily. Thus, we have attempted to develop a technique for continuous production of α-amylase which would obviate these problems and may also open up new avenues for utilisation of halobacterial cells for various biotechnological applications.
Thus, the halophilic α-amylases provide an area of study where basic and applied information both contribute to an understanding of microbiological and biochemical processes (Ingle & Erickson, 1978). The halobacterial cells constitute a unique system for inactivation-reactivation studies, which can shed light on the type of interactions involved in the maintenance of the integrity of native structures (Mevarech et al., 1976). These micro-organisms growing in extreme environments are reservoirs of enzymes that could change the face of biocatalysis (Adams & Kelly, 1995).

1.14 PROBLEM DELINEATED

The basic source for all the energy stored in conventional fuels such as oil, gas, coal and wood can be traced back to the sun. Photosynthesis in the past has provided the Earth with a very rich deposit of fossil fuels. The enormous demand for energy in the past two decades combined with decreasing land available for natural photosynthesis (agriculture and forestry) has created a serious and continuous energy problem. Alternate sources of energy are being explored and one of the solutions proposed is to find additional ways of harnessing the solar radiation falling on the earth to supplement the energy fixed through natural photosynthesis by plants and algae. If we can develop a system which does not have the physiological limitations of plants and does not use fertile land but just mimics the basic process of photosynthesis we may have a good way of harnessing solar energy in the future. The use of sunlight to split water to hydrogen and oxygen is an attractive concept since the energy source (sun) and the substrate (water) are abundant and the product (hydrogen) is a storable non-polluting fuel. Hydrogen has been suggested as a fuel of the future since it can readily be stored and handled and can be converted to other types of fuels, chemicals and electricity.

The splitting of a molecule of water to hydrogen and oxygen requires 237 KJ of energy. Though one Einstein of blue or violet light can supply this amount of
energy, since water molecules are transparent to visible solar radiation the direct decomposition of water by absorption of solar radiation is not possible. Direct photolysis of water can be achieved by the use of photosensitizers and light but the yields are generally poor. The plants have solved this problem billion of years ago and have developed a highly organized membrane system capable of splitting water.

Keeping these possibilities in mind, the research problem was framed so that the knowledge gained from these studies would help us to construct a light driven-water-splitting apparatus using biological and/or synthetic components which would continuously generate hydrogen (and oxygen) from water. Therefore, the research problem was outlined as:

* Characterization of bacteriorhodopsin combined with allophycocyanin to detect a system which could show enhanced photo-osmotic velocity

* Preparation of an immobilized combined system of Phormidium valderianum, Halobacterium halobium, and Escherichia coli and optimization of the reaction conditions used in the analytical photobioreactor

* Determination and optimisation of parameters for the long term stability of the combined system of P. valderianum, H. halobium, and E. coli toward prolonged production of hydrogen

* Immobilization of H. halobium for continuous production of α-amylase

The purple membrane of H. halobium has been considered as a potential candidate for solar energy conversion and therefore these studies appear relevant. Moreover, this is an attempt to develop a system of bacteriorhopsin in combination with allophycocyanin to provide a concept for experimental initiatives for a viable and potential energy producing system.
The simplest scale-up of hydrogen producing system is one in which the analytical minireactor is scaled up to a level where gas production can be observed by collection. Therefore, we optimised the different nutritional and physiological conditions necessary for the combined system of *P. valderianum*, *H. halobium*, and *E. coli* toward stable and continuous hydrogen production.

Biologically assisted hydrogen production from various sources has been suggested as one route for obtaining hydrogen for the putative hydrogen economy. This is a small step towards achieving that goal.

In addition we tried to exploit *H. halobium* for α-amylase production. Our aim has been to develop a technique for continuous α-amylase production which would obviate the problems faced and open up new avenues for utilisation of halobacterial cells for various biotechnological applications.
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