“A hundred times a day I remind myself that my inner and outer lives are based on the labors of other people, living and dead, and that I must exert myself in order to give in the same measure as I have received and am still receiving”.

Albert Einstein (1879-1955)
3.1 Growth of *Chlamydomonas reinhardtii*, *Synechocystis PCC 6803* and *Chlorella vulgaris* at various illumination intensities

The influence of light intensity on growth of *Chlamydomonas reinhardtii*, *Synechocystis PCC 6803* and *Chlorella vulgaris* was assessed in the laboratory under optimum condition. All experiments were performed with low optical density cultures to avoid excessive growth. CO$_2$ and nitrogen (as nitrate) were provided in excess, in order to prevent growth limitation due to these nutrients and to reveal illumination effects only. Growth had a linear relationship with illumination when 12 hours of light and 12 hours of darkness were provided for growth (Fig. 1a). In 24 hours of continuous illumination, the growth was substantially hindered (Fig. 1b) whereas, in 24 hours of darkness (Fig 1c), the growth was less than the growth obtained in intermittent light cycle. This was expected as light may be limiting for growth but, if in excess, leads to oxidative stress resulting in cell death. However, since the light provided was almost double or maybe high, light usage fell by more than 50%. This indicated that when most external cells are exposed to intense illumination, they can cope with the resulting stress, but they use available energy with lower efficiency.
Photo-biological hydrogen production using algae and cyanobacteria species

Figure 1 a: Optical density at 750 nm depicting growth of algal cultures in 12 hours of light and 12 hours of darkness (optimal alternation of light and dark).

Figure 1 b: Optical Density at 750 nm depicting growth of algal cultures in 24 hours of illumination.

Figure 1 c: Optical Density at 750 nm depicting growth of algal cultures in 24 hours of darkness.
3.2 Pigment analysis at optimal alternation of light and dark

Pigment concentrations were determined spectro-photometrically. Under excess illumination, Chlorophyll a content decreases to reduce light-harvesting efficiency, and carotenoids, active in protecting against oxidative stress, are accumulated. Chlorophyll a increased with time showing linear relationship with illumination when given at intervals of 12 hours (Fig.2a). Carotenoid levels increased with time showing traces of oxidative stress due to the accumulation of light for several hours (Fig.3a).

Figure 2 a: Depicting Chlorophyll a content (mg/ml) in 12 hours of light and 12 hours of darkness (optimal alternation of light and dark)
Photo-biological hydrogen production using algae and cyanobacteria species

Figure 2 b: Depicting Chlorophyll a content (mg/ml) in 24 hours of darkness.

Figure 2 c: Depicting Chlorophyll a content (mg/ml) in 24 hours of illumination.
Photo-biological hydrogen production using algae and cyanobacteria species

Figure 3 a: Depicting Carotenoid content (mg/ml) in 12 hours of light and 12 hours of darkness (Optimal alternation of light and dark).

Figure 3 b: Depicting Carotenoid content (mg/ml) in 24 hours of darkness.
3.3 Effect of Illumination on Photosynthetic Apparatus measured by PAM Fluorometer

Fv/Fm (Fmax-Fmin/Fmax) (Fig.4a) was monitored in all cultures and cells grown at different levels of illumination. Fv/Fm is a useful parameter to evaluate photosynthetic efficiency in algae and mainly to highlight photo-inhibition due to excess illumination. PAM fluorometry results indicated that at optimal alteration of light and dark cycles the quantum yield is most (Fig.4b) whereas in continuous illumination (Fig.4c) or continuous darkness the quantum yield is low. In all cases along with the impaired growth, a reduction in Fv/Fm was also observed, indicating that the cells also underwent photoinhibition, although they were exposed to a low total amount of photons. The quenching analysis by PAM fluorometer gave the measure of photosynthetic efficiency in terms of quantum yield. The Fv/Fm series parallels the evolutionary sequence of these organisms and reflects several possible sources for improving the efficiency. An important measure of practical utility is
the photochemical quantum efficiency for PSII charge separation attainable at full solar flux. At total solar flux, Fv/Fm decreases relative to its dark-adapted level, owing to losses from increased probability of charge recombination in PSII as the proton/electron circuits back up.

Figure 4 a: Quenching Analysis of the three species in continuous darkness (24 hours); highest quenching was found in Chlamydomonas reinhardtii (Purple), in comparison to Chlorella vulgaris (Green) and minimum in Synechocystis PCC 6803 (Black). Y-axis depicts Fluorescence quenching and X-axis depicts time in seconds.
Figure 4 b: Quenching Analysis of the three species in optimal alteration of light and dark (12 hours light and 12 hours dark); highest quenching was found in *Chlamydomonas reinhardtii* (Pink), then in *Chlorella vulgaris* (Brown) and minimum in *Synechocystis PCC 6803* (Light Green).

Figure 4 c: Quenching Analysis of the three species in continuous illumination (24 hours); highest quenching was found in *Chlamydomonas reinhardtii* (Red), then in *Chlorella vulgaris* (Light Blue) and minimum in *Synechocystis PCC 6803* (Dark Blue).
3.4 Mapping intramolecular tunnels

The noteworthiness of tunnel structure of hydrogenases in their oxygen resistance is shown by multiple publications showing a high correlation between tunnel structure and oxygen resistance. Hydrogenases with low sensitivity to oxygen having constricted channels are an example. Moreover, oxygen-sensitive hydrogenases of sulfate-reducing bacterium became insensitive once amino acids surrounding the tunnel were replaced by amino acids with large radical. Additionally, there is no proof for the existence of 4Fe–3S clusters (which was shown to be responsible for the oxygen sensitivity) in the small subunits attributable to the shortage of extra cysteines needed for amalgamate this cluster (Figure 5). Also, there's no reason for the existence of selenocysteine residue within the active sites, which may well be a determinant of oxygen tolerance unless we are going to suppose a novel selenocysteine incorporation mechanism specific for these enzymes or the studied microorganism species. In previous attempts researchers could not identify large amino acid residues specific for hydrogen-sensing hydrogenases in the corresponding positions of studied enzymes. This is the first report of its kind in which mapping of the intramolecular tunnels in the four-hydrogenase enzymes disclosed potential variations between designed models and acknowledged structures.
Figure 5: Typical [NiFe] prosthetic group-The nickel atom (light blue) is bound by four cysteine thiolates. Two of these residues bridge to the iron atom (red). The iron atom carries two CN and as single CO ligand. Furthermore, it shares a free coordination site (X) with nickel. This bridging position is supposed to be the catalytic site. Nickel has another open coordination site (Y) responsible where external CO inhibits the enzyme.

Models of *C. reinhardtii* hydrogenase had two tunnels join into one (Figure 6 a, b), and models of *C. vulgaris* hydrogenase had one tunnel almost like two tunnels of *C. reinhardtii* (Figure 7 a, b), whereas hydrogenases from *A. vinosum* (Figure 8 a) and *D. vulgaris* (Figure 8 b) possessed six tunnels. Thus, there’s a definite likelihood of the existence of a “bottleneck” in hydrogenases of *C. reinhardtii* and *C. vulgaris* creating tunnel mapping and thus oxygen diffusion not possible.
Figure 6: (a, b): Models of *C. reinhardtii* hydrogenase tunnels: two tunnels joined into one. The green, cyan and white colored coils are showing the hydrogenase tunnels assumed to be connecting all the active sites of HydSL enzyme. The presence of tunnels is considered as the pathway that facilitates gas diffusion and substrate entry to the active site but can also promote hydrogenase inhibition, more the tunnels more the inhibition.
Figure 7 (a, b): Models of *C. vulgaris* hydrogenase tunnels; one tunnel almost like two tunnels. The cream and white colored coils are showing the hydrogenase tunnels assumed to be connecting all the active sites of HydSL enzyme.
Figure 8: (a) Model of *A. vinosum* hydrogenase tunnels consisting of six tunnels, (b) Model of *D. vulgaris* hydrogenase tunnels consisting of six tunnels. The green, cyan, cream and white colored coils are showing the hydrogenase tunnels assumed to be connecting all the active sites of HydSL enzyme.
It should be noted that energy minimization has pointed to the impossibility of tunnel mapping; it implies that most of the time these enzyme tunnels are in closed state in the solution. Besides that, the tunnel structure depended on chosen methodology for subunit imposition that evidences their sensitivity to the geometry of mutual position of the subunits. For additional precise mapping of potential pathways for entry of inhibitors to the site, long molecular dynamics simulations in a particular solvent in the presence of oxygen molecules are needed.

3.5 Mapping Electrostatic Potential

The static potential maps were calculated by PME technique in the AMBER03 field of force. The hydrogenase of *C. vulgaris* possesses regions of positive potential that is sort of immune to pH alterations, within the C-terminal region of tiny subunit and central part of the large one. Therefore, we are able to hypothesize that the optimum immobilization agent for this protein would be a negatively charged molecule (Figure 9 a, b, c).

As for the hydrogenase of *C. reinhardtii*, it's more sensitive to hydrogen ion concentration shifts. That’s why it looks that immobilization agents for this protein ought to be varied reckoning on pH. In acid or neutral conditions, negative molecules would be appropriate (Figure 10 a, b, c).
Photo-biological hydrogen production using algae and cyanobacteria species

Figure 9 a, b, c: HydSL from *Chlorella* Species, Maximal potential (corresponding to the brightest color) was set to 250 kcal/mol (1,046 kJ/mol). Positive potential areas are colored blue, negative–red, the neutral ones are gray. C-terminal parts of the small subunits are turned to the bottom. a) pH 5.0. b) pH 7.0. c) pH 9.0.
Figure 10 a, b, c: HydSL from Chlamydomonas Species, Maximal potential (corresponding to the brightest color) was set to 250 kcal/mol(1,046 kJ/mol). Positive potential areas are colored blue, negative–red, the neutral ones are gray. C-terminal parts of the small subunits are turned to the bottom.a) pH 5.0. b) pH 7.0.c) pH 9.0.
3.6 Super positioning of the HydSL proteins

The Root-mean-square deviation (RMSD) of backbone atoms between models and between models and templates and between templates was calculated using Super Pose Version 1.0 (Figures 11 and 12). The combined RMSD value for all the templates and models was 1.284. RMSD value of less than 2 Å is preferred as significant. RMSD < 2 Å from the empirical structure depicts the model as a “Highly successful homology model” (Figure 13).

Figure 11: Super Pose output for multiple chain superposition using WebMole applet; superimposition was done of 8 chains of 3MYR, 12 chains of 2FRV and 2 chains of 1H2A in backbone frame.
Figure 12: Super Pose output for multiple chain super-position using MolScript superposition applet showing atoms of the coordinating residues in a backbone frame and colored active sites supposedly connects all the tunnels.

Figure 13: Super Pose output for multiple chain super-position depicting RMSD score of 1.284, showing a successful homology of the models and templates.
3.7 Modeling HydSL hydrogenase from *Chlorella vulgaris*

The search for the best templates for homology modeling was carried out in BLAST program across Protein Data Bank database (PDB). The best template identified (i.e., sharing the largest identity with the target protein) was the 3D structure of HydSL hydrogenase from *A.vinosum* (PDB-entry assigned as 3MYR). It is a thermostable hydrogenase presented as a hetero-octameric crystal (consisting of four large and four small subunits).

This enzyme was obtained from purple sulfur bacteria; however, the initial coordinate file 3MYR.pdb from PDB was prepared for modeling in Yasara program. Then, each of the subunit of the hydrogenase was saved as a separate PDB file. Thus, the four corresponding subunits from 3MYR.pdb were used for modeling each of the subunit.

Modeling was performed using the MODELLER program (Sali and Blundell 1993) with ligand inclusion. For each of the subunit, 1,000 models were built and the estimation was done by following, these parameters: DOPE–discrete optimized protein energy (Shen and Sali 2006) and molpdf (objective function) a statistical potential based on density function of the probability of interatomic distances, angles between bonds and dihedrals. After modeling and choosing the four best models out of thousands, root-mean-square deviation (RMSD) of backbone atoms between models, between models and templates and between templates was calculated using Yasara Structure.
Then, small and large subunits were joined using Yasara Structure by superpositioning (Figure 14 a) with the template, after which intersubunit and intrasubunit hydrogen bonds were calculated.

Figure 14: HydSL Models (a) HydSL hydrogenase from *Chlamydomonas reinhardtii*: an Overview of HydSL hydrogenase model. The small subunit is colored yellow, the large one–gray. The atoms of coordinating residues are shown as sticks: green–sulfur, cyan–carbon, red–oxygen;(b) Overview of HydSL hydrogenase from *Chlorella variabilis*: the small subunit is colored yellow, the large one is gray; the small subunit fragment lacking homolog is colored magenta.
Photo-biological hydrogen production using algae and cyanobacteria species

Model validation was carried out using MolProbity online server (Chen et al., 2010); for clash score minimization (number of atom clashes more than 0.4 Å per 1,000 atoms), energy minimization was carried out using YASARA energy minimization program (Krieger et al., 2009), which lead to good clash scores (Table 1).

<table>
<thead>
<tr>
<th>Parameter model</th>
<th>Clashscore (a)</th>
<th>MolProbity score (a)</th>
<th>Improper bonds % (b)</th>
<th>Improper angles (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HydL</td>
<td>15.8/67 %</td>
<td>1.67/91%</td>
<td>0</td>
<td>0.62%</td>
</tr>
<tr>
<td>3MYR (chain B)</td>
<td>7.31/95 %</td>
<td>1.94/94%</td>
<td>0</td>
<td>0.00%</td>
</tr>
<tr>
<td>HydS (full-size)</td>
<td>10.3/77 %</td>
<td>2.06/90%</td>
<td>0</td>
<td>1.05%</td>
</tr>
<tr>
<td>HydSdelta18 (without C-terminal moiety)</td>
<td>9.78/82 %</td>
<td>1.97/90%</td>
<td>0</td>
<td>0.80%</td>
</tr>
<tr>
<td>3MYR (chain A)</td>
<td>5.51/97 %</td>
<td>2.12/72%</td>
<td>0.37</td>
<td>1.49%</td>
</tr>
<tr>
<td>HydSL</td>
<td>30.01/14 %</td>
<td>1.99/81%</td>
<td>0</td>
<td>0.91% (0.68%)c</td>
</tr>
<tr>
<td>(5.20/98 %)c</td>
<td>(1.71/99 %)c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HydSdelta18</td>
<td>25.74/24 %</td>
<td>1.93/36%</td>
<td>0</td>
<td>0.37% (0.02%)c</td>
</tr>
<tr>
<td>(0.44/100 %)c</td>
<td>(1.37/100 %)c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3MYRab</td>
<td>6.98/97 %</td>
<td>2.03/78%</td>
<td>0.12</td>
<td>0.48%</td>
</tr>
</tbody>
</table>

Table 1: Data of analysis of HydSL and moieties in Chlamydomonas reinhardtii.

x Clashscore and MolProbity score are present in absolute values and in percentiles among a group of PDB structures with 2.1 ±0.15 Å resolution, i.e., in the percentage of structures with a higher value of the corresponding parameter in PDB.

y Improper bonds and angles are present as the percentage of all bonds or angles in the structure.

z The values after energy minimization are shown in brackets.
3.8 Modeling HydSL hydrogenase from *Chlamydomonas reinhardtii*

The best template for homology modeling of HydSL hydrogenase was also the structure of HydSL hydrogenase obtained from *A. vinosum* (Figure 14 b).

3.9 Hydrogen bonds

Hydrogen bonds were calculated by the use of Yasara Structure program; the cutoff found for bond energy was 6.25 kJ/mol (Table 2).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HydSL <em>Chlamydomonas</em> sp.</th>
<th>HydSL <em>Chlorella</em> sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of intersubunit hydrogen bonds</td>
<td>24.6 ± 5.2</td>
<td>21.75 ± 4.8</td>
</tr>
<tr>
<td>Total energy of hydrogen bonds, kJ/mol</td>
<td>453 ± 82.0</td>
<td>371.8 ± 77.8</td>
</tr>
<tr>
<td>Number of hydrogen bonds in large subunit</td>
<td>412 ± 31.6</td>
<td>398 ± 26.14</td>
</tr>
<tr>
<td>Total energy of hydrogen bonds in large subunit, kJ/mol</td>
<td>7,731.3 ± 734.32</td>
<td>7,078.3 ± 699.26</td>
</tr>
<tr>
<td>Number of hydrogen bonds in small subunit</td>
<td>156 ± 8.02</td>
<td>149 ± 6.83</td>
</tr>
<tr>
<td>Total energy of hydrogen bonds in small subunit, kJ/mol</td>
<td>2,501 ± 200.3</td>
<td>2,606.66 ± 142.67</td>
</tr>
<tr>
<td>Number of intersubunit ion pairs</td>
<td>7.25 ± 1.60</td>
<td>11.75 ± 0.5</td>
</tr>
<tr>
<td>Number of ion pairs in large subunit</td>
<td>55.25 ± 3.30</td>
<td>55.75 ± 3.77</td>
</tr>
<tr>
<td>Number of ion pairs in small subunit</td>
<td>20 ± 2.16</td>
<td>16 ± 1.82</td>
</tr>
</tbody>
</table>

(Data are shown as average value for 4 structures ± standard deviation)

Table 2: Numbers and energies of intersubunit interactions in hydrogenases.

3.10 Mapping molecular surfaces (Catalytic Site Atlas database)

For mapping of the hydrophobic regions, the hydrophobic residues (Ala, Val, Ile, Leu, Met, Trp, and Phe) were denoted on molecular surfaces of the hydrogenases (Figure 15 a, b).
Figure 15: Hydrophobic residues (a) Hydrophobic residues on the surface of HydSL from *Chlorella vulgaris* (b) Hydrophobic residues on the surface of HydSL from *Chlamydomonas reinhardtii*.

Hence, we have built a quite realistic model of HydSL hydrogenase from *C. vulgaris* because we could get helpful information from existing models (already studied). We have also built a model of hydrogenase from *C. reinhardtii* with
ligands; the numbers of ionic pairs in this enzyme were shown to be less than HydSL from *C. vulgaris*, which emphasize on the role of other interactions (maybe other hydrophobic contacts or specific intrasubunit interactions), in its stabilization. Hydrophobic areas on hydrogenase molecular surfaces were mapped in this study, and it has shown that hydrogenases have hydrophobic regions, which could facilitate their incorporation into the membrane for interaction with photosynthetic electron-transport chain and could also be helping the coupling with hydrophobic parts of proteins from the electron-transport chain.

3.11 Insights into [FeFe]-Hydrogenase Structure, Mechanism, and Maturation

This part of the work represents the state of the field in terms of our understanding of the structure and mechanism of [FeFe]-hydrogenases and the exciting recent insights into H-cluster biosynthesis and [FeFe]-hydrogenase maturation (Figure 16).
Figure 16: Overview of [FeFe]-hydrogenase diversity, structure, and mechanism of cluster activation and final maturation: The cartoon depicts various ferredoxin-like Fe-S cluster-binding domains, gas-diffusion pathways, and a maturation channel for H-cluster insertion that completes formation of an active [FeFe]-hydrogenase (right, Cpl; PDB ID: 3C8Y). Elemental coloring scheme: C, gray; N, blue; O, red; Fe, rust; S, orange; unknown, magenta. All molecular figures were created in PyMOL (DeLano, 2002).

The simplest characterized [FeFe]-hydrogenases are observed in the chlorophycean algae, including C. reinhardtii, Chlorella fusca, and Scenedesmus obliquus, which express enzymes consisting of only the H-cluster without F-cluster domains (Florin et al., 2001; Happe and Kaminski, 2002; Forestier et al., 2003). These proteins have been exploited more recently for biochemical and spectroscopic characterization because they lack the additional FeS clusters observed in most native [FeFe]-hydrogenases that can complicate the direct examination of the H-cluster (Kamp et al., 2008; Mulder et al., 2009, 2010; Stripp et al., 2009).

When present, the accessory FeS cluster binding domains are predominantly comprised of different combinations of a: (1) two [4Fe-4S] bacterial ferredoxin-like
domain identified in sequence databases by a conserved arrangement of eight Cys residues that make up the thiolate ligands to the [4Fe-4S] clusters; (2) short domain containing a [4Fe-4S] cluster ligated by three Cys and one His; (3) [2Fe-2S]-containing plant-type ferredoxin-like domain; and/or (4) thioredoxin-like [2Fe-2S] cluster binding domain with homology to the NuoE subunit of Complex I of the respiratory chain that is found in the C terminus of some NAD(P)H-linked hydrogenases (Figure 17). A distinct uncharacterized domain with eight conserved Cys residues and a domain with homology to subunit NuoF of Complex I are present in a subset of [FeFe]-hydrogenases. The FeS cluster binding domains with homology to Complex I frequently exist as distinct subunits in many multimeric hydrogenases, but when present as a domain of a single polypeptide, are located at the C-terminal end of the H-cluster domain.

Various combinations of F-cluster domain modules are observed in different [FeFe]-hydrogenases, and Meyer (2007) proposed a nomenclature for classification based on F-cluster configurations (Figure 17). This phylogenetic analysis revealed eight distinct [FeFe]-hydrogenase groups that are unique according to domain architecture and/or subunit composition.

The structurally characterized DdH (D(M2) architecture) contains a H-cluster domain and a two [4Fe-4S] cluster domain (Nicolet et. al., 1999). The F-cluster domain in D(M2) is typically localized N-terminal to the H-cluster domain, and this domain organization has been proposed to be the likely ancestral hydrogenase
architecture (Meyer, 2007). CpI (M3 architecture), which has also been structurally characterized, has the H-cluster binding domain, the two [4Fe-4S] cluster-binding domain, as well as a short domain that binds a [4Fe-4S] cluster ligated by three Cys residues and a His residue, and an N-terminal [2Fe-2S] plant-type ferredoxin domain (Figure 17) (Peters et. al., 1998).

The specific biochemical and physiological role of this unique His-ligated cluster is not known, but it is possible that the ligand arrangement could contribute to defining the oxidation-reduction potential of the cluster (Peters et. al., 1998). The [NiFe]- hydrogenase from D. fructosovorans also contains a distal [4Fe-4S] cluster ligated by three Cys and a His. Site-directed mutagenesis of the His to a Cys or a Gly still yields assembly of the [4Fe-4S] cluster and complete protein folding; however, H2-oxidation rates measured by protein film voltammetry are significantly decreased in the engineered enzyme (Dementin et. al., 2006). These studies suggest that the unique His ligand of the [4Fe-4S] cluster may play a fundamental role in the intra and intermolecular electron transfer properties of the cluster (Dementin et. al., 2006).

The breadth of microbial genome sequencing is revealing novel sequences phylogenetically related to HydA that indicate increased diversity in accessory domain composition beyond the nomenclature articulated by Meyer (2007), and adaptations to the original nomenclature have been reported by Calusinska et. al., (2010). This updated analysis demonstrates that unique F-cluster module variants
have likely evolved in some enzymes and that further genome sequencing efforts
are likely to reveal new accessory domain architectures beyond the more frequently
observed configurations.

Figure 17: [FeFe]-hydrogenase Cpl and alignment of FeS cluster-binding motifs of
representative [FeFe]-Hydrogenases: The colored shapes depict different F-cluster
compliments from a variety of organisms (see legend in upper right). The sequence
motifs below allow for identification of F-cluster and H-cluster domains. The
unique histidine-ligated [4Fe-4S] cluster of Cpi (left, PDB ID: 3C8Y) is shown in
(A), along with structures of 23 [4Fe-4S] (B) (PDB ID: 1BLU) and [2Fe-2S]
ferredoxins (C) (PDB ID: 1AWD) that are structural analogs of Cpi F-cluster
domains. (D) depicts the thioredoxin-like [2Fe-2S] cluster-binding domain of
Complex I subunit NuoE (PDB ID: 3IAS).
Similar to [NiFe]-hydrogenases, [FeFe]-hydrogenases are reversibly inhibited by the addition of exogenous CO. EPR studies have demonstrated that inhibition by CO is reversible (Bennett et al., 2000), and additional EPR and FTIR studies have demonstrated that the inhibitory CO can be selectively photolyzed. In the oxidized state the H-cluster of CpI displays a characteristic rhombic EPR signal that is replaced by an axial signal after addition of CO (Bennett et al., 2000). An X-ray crystal structure of the CO-inhibited state of CpI (Hox-CO) clearly demonstrates that CO binding occurs at the same location as the H2O molecule in the Hox state, at the ligand exchangeable site (Figure 18). This observation, along with the structural differences observed for the enzyme in various oxidation states, indicates that H2 binding may occur at the distal Fe atom of the 2Fe subcluster. Recently, it was shown that formaldehyde also reversibly inhibits [FeFe]-hydrogenase, and it is proposed that formaldehyde binds at the distal Fe of the 2Fe subcluster, at the dithiolate ligand, or at one of the neighboring residues that interact with the cluster (Wait et al., 2011).
Figure 18. Structural models of H-cluster oxidation states representations were based upon the structural observations of the CO inhibited state and presumed oxidized state of CpI (Peters et al., 1998; Lemon and Peters, 1999) and the reduced state of DdH (Nicolet et al., 2001). The blue arrow indicates the ligand-exchangeable site located at the distal Fe. The magenta “X” indicates the dithiolate bridgehead group that is likely to be CH$_2$, NH/NH$_2^+$, or O.

The aforementioned observations begin to provide mechanistic clues as to how reversible H$_2$ oxidation takes place at the 2Fe subcluster, and a scheme can be proposed in which the ligand-exchangeable site is occupied by a water molecule in the oxidized state, but when the metal cluster is reduced or H$_2$ is available as a coordinating group, water is displaced by the formation of a hydride or by the binding of H$_2$. Heterolytic H$_2$ bond cleavage could take place at the terminal position of the distal Fe site. Interestingly, characterization of the [FeFe]-
hydrogenase (HydA1) from *C. reinhardtii* by FTIR spectro-electrochemistry revealed the bridging CO ligand in both the H\textsubscript{ox} and H\textsubscript{red} states, leading further support to the idea that H\textsubscript{2} binding takes place at the ligand-exchangeable site at the distal Fe (Silakov *et al.*, 2009). A wide variety of studies involving inorganic synthetic model complexes of the active site of [FeFe]-hydrogenases that probe the mechanism of the enzyme have also been reported, and it should be noted that hypotheses involving bridging hydrides have also been put forth (Tard and Pickett, 2009). Nevertheless, additional X-ray crystal structures of hydrogenases purposefully poised in different redox states could yield important insights into deciphering remaining details of the mechanism of hydrogenase catalysis by the unique active site cluster.

Although the 2Fe subcluster of the H-cluster is coordinated by only a single-shared Cys thiolate ligand from the protein, it does have a number of non-covalent interactions that both tune the H\textsubscript{2} activation properties of the cluster and stabilize it in the binding site (Figure 19). For CpI, amino acid residues within hydrogen bonding distance of CO and CN ligands include: (1) Ser232 (Og) with the terminal CN\textsubscript{terminal} ligand (2.8\textdegree A\textsubscript{o}) on the proximal Fe (Figures 19 a and 19 b); (2) Lys358 (Nz) with the terminal CN\textsuperscript{terminal} ligand (2.9 A\textdegree) on the distal Fe (Figures 19 b and 19 c); and (3) Met353 (S) with the bridging CO ligand (3.2 A\textdegree) (Figure 19b). Surprisingly, Ser232 is not conserved in the DdH structure in which the equivalent residue is Ala109; however, the peptide bond amide of this residue supplants the
role of the Ser hydroxyl in hydrogen bonding to the CN ligand (Nicolet et al., 2000). Cpl residues in the environment of the bridging sulfur atoms of the dithiolate ligand include the peptide carbonyl bonds of Phe417 and Cys299 at 3.4 and 3.6 Å away, respectively (Figure 19 c). Cpl residues in near proximity to the dithiolate ligand include Met497 and Cys299 sulfurs, which are 3.6 Å away from the bridgehead atom (Figures 19 a and 19 c). In addition, Cys299 is at a distance of 3.7 Å from the terminal H$_2$O molecule on the distal Fe atom of the oxidized H-cluster (Figure 19 c). It has been suggested that this residue could donate protons for H$_2$ formation during catalysis (Peters et al., 1998). Hydrophobic residues Ile268, Ala272, Pro324, Val423, and Phe416 create a pocket around the 2Fe subcluster protecting it from solvent.

Figure 19: Protein Environment of the H-cluster Dashes represents interactions of various residues (labeled) with the 2Fe subcluster and non-protein ligands. The coordinates are from PDB ID: 3C8Y. Elemental coloring scheme: C, gray; N, blue; O, red; Fe, rust; S, orange; unknown, magenta.
3.12 Hypothetical scheme for [FeFe]-hydrogenase (HydA) H-cluster activation by maturation enzymes

Preliminary characterization of reconstituted HydE and HydG from *T. maritima* demonstrated that HydE bound two [4Fe-4S] clusters, and HydG bound a single [4Fe-4S] cluster (although it was suggested from the presence of shoulders on the low and high-field EPR signal that an additional cluster was bound), and both enzymes could cleave SAM nonproductively (Rubach *et. al.*, 2005). Reconstituted HydF was found to bind a single [4Fe-4S] cluster and hydrolyze GTP to GDP (Brazzolotto *et. al.*, 2006).

These experimental observations helped to shape a hypothetical pathway for H-cluster biosynthesis, thereby providing a framework for additional investigation. HydE and HydG were proposed to be responsible for the synthesis of the bridging dithiolate ligand in the first step of the chemical modification of a [2Fe-2S] cluster. Subsequent generation of a glycyl radical by HydE and/or HydG and the interaction of this radical with an Fe(I)-thiolate site resulted in exothermic decomposition, yielding a CO, CN, and H₂O-coordinated Fe(I) center. HydF was proposed to serve as a scaffolding protein in this process, with cluster translocation from HydF to HydADEFG resulting in formation of holo [FeFe]-hydrogenase.

It was subsequently demonstrated that the component capable of activating HydADEFG resides on HydF and that this form of HydF could be purified (McGlynn *et. al.*, 2008). More detailed studies probing the specific actions of
HydE, HydF, and HydG on HydADEFG have provided additional evidence to support this H-cluster biosynthetic pathway (Figure 20).

Figure 20: Hypothetical Scheme for [FeFe]-Hydrogenase (HydA) H-Cluster Activation by Maturation Enzymes HydE, HydF, and HydG. In the molecular representations of 2Fe subcluster precursors and the H-cluster, the magenta “X” indicates the dithiolate bridgehead group that is likely to be CH₂, NH/NH₂⁺, or O.

The crystal structure of HydADEFG from *C. reinhardtii* heterologously expressed in *E. coli* provided important clues into the maturation process and cluster insertion (Figure 21a). The structural analysis (Mulder *et. al.*, 2010) together with detailed biochemical and spectroscopic characterization revealed that only the [4Fe-4S] cubane is present at the active site (Mulder *et. al.*, 2009), indicating that the [4Fe-4S] subcluster is synthesized by the *E. coli* housekeeping FeS cluster biosynthetic machinery before the synthesis and insertion of the 2Fe subcluster by the specialized hydencoded maturation machinery (Mulder *et. al.*, 2010; Shepard *et. al.*, 2010; Shepard *et. al.*, 2011).
Insertion of the 2Fe subcluster presumably occurs via a positively charged channel that closes following 2Fe subcluster incorporation by conformational changes in two conserved loop regions. This stepwise mechanism for cluster insertion involving protein structural rearrangement is not restricted to [FeFe]-hydrogenases, and similar mechanisms involving cluster and cofactor insertion have been proposed for nitrogenase (Schmid et. al., 2002) and for [Fe]-hydrogenase (Hiromoto et. al., 2009).
Figure 21: Structural insights and analysis of HydADEFG maturation (a) Structural representation of HydADEFG from *C. reinhardtii* (PDB ID: 3LX4) and zoom of the active site [4Fe-4S] cluster environment. The representation is colored according to secondary structure with the two-conserved loop regions colored violet. Residue numbering is according to NCBI GenBank accession number AAL23572.1. Elemental coloring scheme: C, green; N, blue; O, red; Fe, rust; S, orange. (b) Hypothetical visualization of 2Fe cluster incorporation by loop closure in HydADEFG. The loop linear interpolation (calculated with the Morph Server, [http://www.molmovdb.org/molmovdb/morph/](http://www.molmovdb.org/molmovdb/morph/)) begins at the open HydADEFG conformation (light violet) and ends at the closed homology of Cpl conformation (dark violet). (c) Potential salt bridges that may contribute toward stabilization of Cpl (PDB ID: 3C8Y). Elemental coloring scheme: C, green; N, blue; O, red; Fe, rust; S, orange; unknown, magenta.
Structural analysis of the channel formed by the loop rearrangement has given insight into the mechanism for insertion of the 2Fe sub cluster (Figure 22). The channel is solvent exposed, and many water molecules are observed to occupy the channel and the cavity adjacent to the [4Fe-4S] cubane in the crystal structure, suggesting that 2Fe subcluster insertion may be entropically driven. The surface of the channel is partially lined with positively charged residues, which could provide a favorable interaction for the presumably negatively charged 2Fe sub cluster during insertion.
Figure 22: Protein channel for 2Fe sub cluster insertion during [FeFe]-hydrogenase maturation (A) Transparent surface representation of HydADEFG (PDB ID: 3LX4) with channel-exposed residues represented as sticks. The [4Fe-4S] cluster (ball representation) present near the end of the channel is visible through the partial transparent surface rendering. (B) Superimposition of the two conserved loop regions showing structural rearrangement along with the 2Fe sub cluster from Cpl on HydADEFG (surface representation).
Figure 23: TLES Simulations of hH₂ Diffusion: Four 4 ns TLES simulations of 1000 copies of hH₂ diffusing out from the H cluster. Each independent simulation is shown in a different color, highlighting the fact that the space explored by hH₂ was consistent between simulations. Frames taken from every 100 ps of the trajectories of the hH₂ molecules located inside the protein are superimposed as a cloud. Shown in licorice are the iron sulfur clusters and H cluster, as well as the residues that line the two major exit pathways. Possible exits, based on the proximity of the external solution, are highlighted with arrows.
Figure 24: TLES Simulations of $O_2$ Diffusion: Three representative TLES simulations of 1000 copies of $O_2$ diffusing out from the H cluster or from the middle of a previously identified $H_2$ channel. Each independent simulation is shown in a different color, and one can see that, contrary to $H_2$ diffusion, the $O_2$ molecules move collectively through the same pathway for a given simulation, though they may employ different pathways for different independent simulations. Overall, the set of pathways explored by $O_2$ matches the dominant pathways explored by $hH_2$. Snapshots were taken every 50 ps. The representation of the protein is the same as in Figure 23.
O₂ and H₂ gas access was investigated by all-atom MD simulations of the diffusion of O₂ and H₂ molecules inside CpI, originating at the active site. We used a model for the gas molecules that did not include any partial charges, and for which O₂ and H₂ differed only in their Lennard-Jones parameters (H₂ = −0.1521 kcal/mol, Rmin/2 = 1.7682 Å; O₂ = −0.022 kcal/mol, Rmin/2 = 1.32 Å; according to the CHARMM form of the Lennard-Jones formula), bond spring constants and bond spring lengths (H₂: 0.72 Å; O₂: 1.16 Å).

As stated before, we used a heavy version of dihydrogen hH₂ instead of H₂, so that we could compare the diffusion properties of O₂ and H₂ based on their size differences alone.

Our model of hydrogenase was based on the X-ray structure of CpI [FeFe]-hydrogenase (PDB accession code 1feh). A series of atoms in the active site, or H cluster, were missing from the Protein Database structure and have been modeled here as a di (thiomethyl) amine bridge between the two H cluster sulfur atoms, as suggested by later studies (Fan and Hall, 2001). The partial charges for the rest of the H cluster were based on a density functional theory calculation on the 2-oxidation state (Torres et. al., 2003), and individual charges were adjusted by a maximum of ±0.02e to guarantee the system’s charge neutrality.

The structure was embedded in a water box, resulting in a 57,000-atom system consisting of 9,000 hydrogenase atoms, 16,000 water molecules, and 15 sodium ions to cancel the excess integer charge. The system was then equilibrated at a
constant temperature (310 K) and pressure (1 atm) for duration of 1 ns. The last frame of this equilibration was used as a starting point for all subsequent simulations. Aside from the initial equilibration, all simulations were performed at constant volume and temperature (310 K). In all cases, periodic boundary conditions were used. Temperature was regulated within the TLES approach by using Langevin dynamics with damping constants of 5 ps$^{-1}$ for unreplicated atoms and 10 ps$^{-1}$ for replicated gas atoms, respectively. Multiple time stepping was used, with integration time steps of 1 fs, 2 fs, and 4 fs, respectively, for bonded, nonbonded, and long-range electrostatic interactions (a nonbonded time step of 1 fs was used for the case of hH$_2$ TLES simulations because of energy stability issues when replicated hH$_2$ molecules enter the water solution environment) (Figure 23, 24).

Particle Mesh Ewald with a grid resolution of better than 1 Å was used for long-range electrostatics, and all other nonbonded interactions were calculated by using a cutoff of 12 Å. The CHARMM22 force field (MacKerell et. al., 1992, 1998) was employed for all protein interactions, and simulations were performed by using the NAMD (Kalé et. al., 1999) MD software, modified by the present authors to allow for TLES.
“Man needs his difficulties because they are necessary to enjoy success”.
A.P.J. Abdul Kalam (1931-2015)

CONCLUSION
• Biofuels from algae and cyanobacteria are highly attractive as renewable energy sources to replace, at least partially, fossil fuels.

• The genetics, biosynthesis, structure, function and $O_2$ sensitivity of hydrogenase have been focus of extensive research in recent years.

• Much focus has been placed on optimizing microorganisms and primarily microalgae, to efficiently produce compounds that can substitute for fossil fuels.

• However, the path to achieving economic feasibility is likely to require strain optimization through using available tools and technologies in the fields of Systems and Synthetic Biology.

Figure 1: Schematic drawing of the mechanism of the hydrogen production from hydrogenase enzyme.
• Photo biological production of H₂ gas, through split of water (e⁻ donor) into molecular H₂ and O₂ using sunlight, is a property of two types of photosynthetic microorganisms: green algae and cyanobacteria (Figure 1).

• These organisms use their photosynthetic apparatus to absorb sunlight and convert it into chemical energy. This study will focus on oxygenic organisms such as green algae and some cyanobacteria, which produce hydrogen directly from water, without an intermediary biomass accumulation stage.

• Interestingly, water splitting in each of these organisms is functionally linked to H₂ production by the activity of one of two major types of [FeFe] or [NiFe] hydrogenases (Figure 2).

![Figure 2: Schematic pictures of the hydrogen production and oxidation from FeFe hydrogenases.](image-url)
Both enzymes are phylogenetically distinct but perform the same catalytic reaction. This process is considered to have highest potential sunlight conversion efficiency to H₂, which could be on the order of 10 to 13 %.

\[
\begin{align*}
\text{Ni(III)} & \quad \text{[Fe–S]}^{2+} \\
& \quad \text{[Fe–S]}^{2+} \quad \text{Ni(II)} \\
& \quad \text{[Fe–S]}^{2+} \quad \text{[Fe–S]}^{2+} \quad \text{H}^+ \\
& \quad \text{Ni(I)–H}^+ \quad \text{Ni(II)–H}^+ \\
& \quad \text{[Fe–S]}^{2+} \quad \text{[Fe–S]}^{2+} \quad \text{H}^+ \\
& \quad \text{Ni(I)–H}^+ \quad \text{Ni(II)–H}^+ \\
\end{align*}
\]

Figure 3: Mechanism of NiFe hydrogenase functioning.

The influence of light intensity was checked with continuous illumination, darkness and alternation of light and dark cycles. Results show that these species can easily use even very intense light if dark cycles occur allowing re-oxidation of the electron transporters in photosynthetic cycle.

If the alteration of light and dark is not optimal, species undergo radiation damage, and photosynthetic productivity is significantly reduced.
Another regulation commonly observed in these organisms exposed to various light intensities is the alteration of chlorophyll content per cell.

Under excess illumination, Chlorophyll content decreases to reduce light-harvesting efficiency, and carotenoids, active in protecting against oxidative stress, are accumulated.

Fv/Fm (Fmax-Fmin/Fmax) was monitored in all cultures and cells grown at different levels of illumination. Fv/Fm is a useful parameter to evaluate photosynthetic efficiency in algae and mainly to highlight photo-inhibition due to excess illumination.

PAM fluorometry results indicated that at optimal alteration of light and dark cycles the quantum yield is most whereas in continuous illumination or continuous darkness the quantum yield is low.

In all cases along with the impaired growth, a reduction in Fv/Fm was also observed, indicating that the cells also underwent photo-inhibition, although they were exposed to a low total amount of photons.

The quenching analysis by PAM fluorometer gave the measure of photosynthetic efficiency in terms of quantum yield. The Fv/Fm series parallels the evolutionary sequence of these organisms and reflects several possible sources for improving the efficiency.

The possible determinant of oxygen stability of studied hydrogenases could be the presence of several intramolecular tunnels.
• The possible tunnels were traced out using MOLE 2 software, which showed several intramolecular pathways that may be connecting the active sites of the enzyme. The RMSD value showed a great deal of significance in the enzyme homology.

• This is the first report of its kind in which mapping of the intramolecular tunnels in the four-hydrogenase enzymes disclosed potential variations between designed models and acknowledged structures.

• The tunnel tracing study by Mole 2 indicated two tunnels joined into one in *C. reinhardtii* model whereas *C. vulgaris* model showed one tunnel almost like two tunnels. Templates of both the *A. vinosum* and *D. vulgaris* hydrogenase consisted of six tunnels.

• Electrostatic studies define electrostatic potential (ESP) that help shuttle protons to the active site. For HydSL from *Chlamydomonas* and *Chlorella Species*, the positive potential areas were marked.

• For HydSL from *Chlamydomonas* and *Chlorella Species* the maximal potential was set to 250 kcal/mol (1,046 kJ/mol) and the positive potential areas were recorded.

• Two contrasting modes of intra-protein transport for CO and O₂ were derived using VSAM, which finally predicted the greater active site affinity for CO. Hence, suggesting the suitability of CO as an effective non-destructive inhibitor providing protection against O₂ damage.