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Hydrogenases – Types, Sources, Properties, and the Potential for Their Application

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Abstract

Hydrogenases are a group of versatile metalloenzymes that catalyse the reversible transformation between hydrogen gas and its constituent protons and electrons. These enzymes have gained attention more recently due to their ability to use molecular hydrogen as a substrate in the reactions they catalyse, as well as their potential to synthesise hydrogen, an important biofuel. Although the existence of hydrogenases has been known since the 1930s, current research has not yet made them viable on the industrial scale. They are explored mostly from the biochemical point of view, which is very important, but there is a lack of research on how to adapt these enzymes for real industrial-scale processes. Only a few studies address this gap. Therefore, in this brief literature review, we provide an overview of what is known about hydrogenases. We explore their background, classification, and phylogeny, highlighting their presence in many different sources in nature, such as bacteria, archaea, and certain eukaryotes. We also discuss key factors influencing their activity, along with their advantages and disadvantages. Furthermore, we summarise the methods available for determining their activity and emphasise the need for standardised units to ensure comparability of all data available in the literature. Finally, we discuss their potential applications, particularly in hydrogen production and synthetic reaction pathways for coenzyme regeneration, emphasizing the critical importance of these aspects in the investigation of hydrogenases.

Keywords

Hydrogenase, coenzyme regeneration, hydrogen production, hydrogenase classification, hydrogenase properties

1 Introduction

Hydrogenases (Fig. 1) are metalloenzymes that catalyse hydrogen consumption (uptake hydrogenases) and production (reversible hydrogenases).[1,2] *Stephenson and Stickland* first described hydrogenases as enzymes that activate H_2 to reduce electron acceptors such as sulphates, nitrates, fumarates, and oxygen.[3,4] Certain hydrogenases accelerate the reverse reaction of hydrogen production from an electron donor, such as methylviologen cation radical. They also catalyse the isotopic exchange between dihydrogen and water, as well as the conversion between para-H_2 and ortho-H_2.[1,2] Reactions of isotopic hydrogen exchange and para-ortho H_2 conversion played an important role in the development of mechanistic models for hydrogenase action.[2] With impressive catalytic abilities, hydrogenases play a key role in numerous ecological processes, *e.g.,* methanogenesis and nitrogen fixation. The performance of various synthesised bio-inspired molecular catalysts cannot repeat the success of hydrogenases.[5]

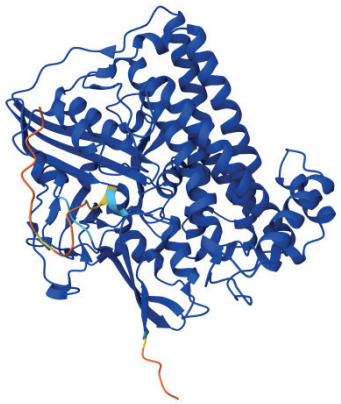

Fig. 1 – Structure of [NiFe] hydrogenase from *Desulfovibrio gigas* (Alphafold[6])

Slika 1 – Struktura [NiFe] hidrogenaze iz *Desulfovibrio gigas* (Alphafold[6])

1.1 Classification of hydrogenases

Hydrogenases can be classified based on their electron carrier specificity and the structure of the active site. In the following sections, we describe these classifications, and attempt to draw connections between the different groups. Namely, the scientific community employs both classification methods, though it is sometimes difficult to find a clear relationship between them. Here, we aim to clarify these connections and make them more apparent.

1.1.1 Classification of hydrogenases based on electron carrier specificity

Hydrogenases differ in electron carrier specificity, with ten types recognised by the IUBMB[7], as shown in Table 1. Depending on the origin, hydrogenases have different specificity to electron carriers. Generally, hydrogenases

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are named as enzymes catalysing H_2 uptake reaction to reduce their electron acceptors.[1] Each enzyme has a corresponding Enzyme Commission number or Enzyme Code (EC) consisting of four digits: the first digit distinguishes the enzyme class, the second and third digits describe the type of reaction catalysed, and the fourth digit differentiates between types of the same function based on the substrate used.[8] An example of this classification is provided in Fig. 2. Acceptors and a schematic representation of the catalysed reaction are presented in Table 1.[9] As shown in Table 1, in addition to the commonly used coenzymes such as NAD(P)$^+$, hydrogenases from different sources can use a variety of other acceptors.

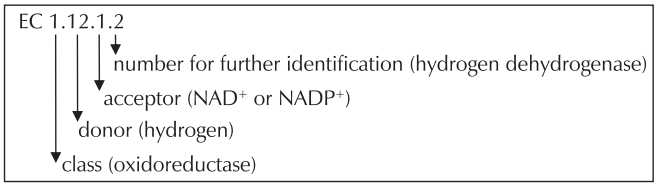

Fig. 2 – Classification of hydrogenases according to Enzyme Commission number
Slika 2 – Klasifikacija hidrogenaza prema broju Enzyme Commission

1.1.2 Classification of hydrogenases based on active site structure

Hydrogenases are divided into three phylogenetically unrelated classes based on the metal ions present in their active sites: [NiFe], [FeFe], and [Fe] hydrogenases.[10] Their catalytic activity depends on these metal cofactors embedded in the protein structure.[11] The hydrogenases listed in Table 1 are also classified according to these structural types, with no correlation between the two classifications.[1] The classification based on active site structure is shown in Table 2. Some EC numbers fall under multiple classes (marked ◆) according to this structural division, depending on the origin of the hydrogenase.[9] A short overview of the structural differences between hydrogenase families and their origin is presented in Table 3, with a more detailed description.[10,12]

The most extensively studied hydrogenases are [NiFe]-hydrogenases, categorised into five different phylogenetic groups: *Group 1* – H_2 uptake [NiFe]-hydrogenases, *Group 2* – cyanobacterial uptake [NiFe]-hydrogenases and sensory hydrogenases, *Group 3* – bidirectional heteromultimeric cytoplasmic [NiFe]-hydrogenases, *Group 4* – energy converting [NiFe]-hydrogenases, and *Group 5* – actinobacterial hydrogenases (AH).[10] The function and enzyme activity of these groups, although dependent on a heterobimetallic [NiFe] cofactor, vary greatly.[10] The first crystal structure of

Table 1 – List of hydrogenases registered by IUBMB and the corresponding catalysed reactions
Tablica 1 – Lista hidrogenaza registriranih u IUBMB-bazi i pripadajuće reakcijske jednadžbe

EC number	Enzyme name *(Names found in literature, but not accepted by IUBMB)* *Catalysed reaction*
EC 1.12.1.2	Hydrogen dehydrogenase *(NAD(P)$^+$-reducing hydrogenase)* $H_2 + NAD(P)^+ \rightleftharpoons H^+ + NAD(P)H$
EC 1.12.1.3	Hydrogen dehydrogenase (NADP$^+$) *(NADP$^+$-reducing hydrogenase)* $H_2 + NAD(P)^+ \rightleftharpoons H^+ + NAD(P)H$
EC 1.12.1.4	Hydrogen dehydrogenase (NAD$^+$, ferredoxin) *(Bifurcating hydrogenase)* $2H_2 + NAD(P)^+ + 2\ \text{oxidized ferredoxin} \rightleftharpoons 5H^+ + NAD(P)H + 2\ \text{reduced ferredoxin}$
EC 1.12.2.1	Cytochrome-c_3 hydrogenase $H_2 + 2\ \text{ferricytochrome c3} \rightleftharpoons H^+ + 2\ \text{ferricytochrome c3}$
EC 1.12.5.1	Hydrogen:quinone oxidoreductase *(Quinone-reducing hydrogenase)* $H_2 + \text{menaquinone} \rightleftharpoons \text{menaquinol}$
EC 1.12.7.2	Ferredoxin hydrogenase $H_2 + \text{oxidized ferredoxin}(\text{iron-sulfur protein}) \rightleftharpoons \text{reduced ferredoxin} + 2H^+$
EC 1.12.98.1	Coenzyme F_{420} hydrogenase *(F_{420}-reducing hydrogenase)* $H_2 + \text{oxidized coenzyme } F_{420} \rightleftharpoons \text{reduced coenzyme } F_{420}$
EC 1.12.98.2	5,10-methenyltetrahydromethanopterin hydrogenase *(H_2-forming 5,10-methylenetetrahydromethanopterin dehydrogenase)* $H + 5,10-\text{methenyltetrahydromethanopterin} \rightleftharpoons H + 5,10-\text{methenyltetrahydromethanopterin}$
EC 1.12.98.3	Methanosarcina-phenazine hydrogenase *(Methanophenazine hydrogenase)* $2H_2 + 2-(2,3-\text{dihydropentaprenyloxy})\text{phenazine} \rightleftharpoons 2-\text{dihydropentaprenyloxyphenazine}$
EC 1.12.99.6	Hydrogenase (acceptor) or Hydrogen: acceptor oxidoreductase $H_2 + \text{acceptor} \rightleftharpoons \text{reduced acceptor}$

Table 2 – Classification of IUBMB registered hydrogenases based on active site structure
Tablica 2 – Klasifikacija hidrogenaza registriranih u IUBMB-bazi temeljena na strukturi aktivnog mjesta

EC number	[NiFe]-hydrogenase	[NiFeSe]-hydrogenase	[FeFe]-hydrogenase	[Fe]-hydrogenase
EC 1.12.1.2	◆		◆	
EC 1.12.1.3	◆			
EC 1.12.1.4			◆	◆
EC 1.12.2.1	◆	◆	◆	◆
EC 1.12.5.1	◆			
EC 1.12.7.2	◆	◆	◆	◆
EC 1.12.98.1	◆			
EC 1.12.98.2				◆
EC 1.12.98.3	◆	◆		
EC 1.12.99.6	◆	◆	◆	◆

[NiFe]-hydrogenase from *Desulfovibrio gigas* was elucidated in 1995,[13] followed by numerous crystal structures from other microorganisms.[10] Alongside the [NiFe] active site located at the large protein subunit, these enzymes have a small subunit containing three FeS clusters (proximal, medial, and distal).[5] In the [NiFe] cofactor, the Ni ion is coordinated with four cysteine residues by S atoms and bound to the Fe ion by two bridging thiol ligands. The Fe ion is coordinated by one carbon monoxide and two cyanide ligands. Between Ni and Fe ions, a hydride bridging ligand is present. Substrates from the molecular surface pass through hydrophobic gas channels to the active site, while three FeS clusters serve as an electron transfer chain.[14]

[NiFeSe]-hydrogenases are a subclass that has a selenocysteine residue in place of one terminal cysteine residue in [NiFe]-hydrogenases. Structural studies have shown numerous structural differences between the two; namely, in the active site, the metal sites at the C-terminal region of the large subunit, and the medial FeS cluster. The active site difference results in an immediate activation upon reduction, as opposed to the slow activation of [NiFe]-hydrogenase.[10] In addition to a hydrophobic channel, found in [NiFe]-hydrogenases, [NiFeSe]-hydrogenases have a H_2 diffusion pathway that can accommodate higher H_2 concentrations, allowing them higher hydrogen production activity. In the [NiFeSe]-hydrogenase structure, three [4Fe-4S] clusters serve as an electron transfer chain.[12]

[NiFe]-hydrogenases tend to act as uptake hydrogenases, *i.e.*, they are biased toward hydrogen oxidation. Some [NiFe]-hydrogenases catalyse H_2 production, which is product (H_2) inhibited.[15] [NiFeSe]-hydrogenases, on the other hand, exhibit a high hydrogen evolution rate with low product inhibition.[12] The catalytic mechanism for [NiFe]-hydrogenase is known, and can be characterised in three catalytically active states: Ni-SI, Ni-C, and Ni-R.[10] Binding of H_2 to the [NiFe] active centre leads to the transition of the most oxidised active state Ni-SI to the most reduced Ni-R state. The Ni-C state is produced by oxidising the Ni-R state with one electron.[16] For [NiFeSe]-hydrogenase, only Ni-C and Ni-R active states have been observed, but as both [NiFeSe] and [NiFe]-hydrogenase follow a similar reaction mechanism, Ni-SI state is also proposed to exist.[12]

The most studied [FeFe]-hydrogenases originate from *Thermotoga maritima, Desulfovibrio desulfurican, Clostridium,* and green algae.[10] [FeFe]-hydrogenases display a highly modular genetic organisation, and their function cannot be predicted from the sequence alone. Studies have classified them as: *Group A* – prototypical and bifurcating, *Group B* – putative ancestral, *Group C* – putative sensory. The degree of [FeFe]-hydrogenases' characterisationvaries greatly, *e.g.*, by 2022, only three model enzymes belonging to *Group A* had been characterised experimentally in detail out of roughly 40 studied.[17] [FeFe]-hydrogenases are generally encoded in a polypeptide chain that can form homo-oligomers or be monomeric. Many are in a single subunit form, and consist of the catalytically active H-cluster as the only metal cluster buried deep in the centre. The H-cluster, a hexanuclear iron complex, consists of two sub-clusters: [2Fe-2S], which represents the active centre, and [4Fe-4S] responsible for electron transfer. In the [2Fe-2S] sub-cluster, each metal is coordinated by one carbon monoxide and one cyanide ligand.[10] [FeFe]-hydrogenases typically catalyse H_2 production, unlike [NiFe]-hydrogenases that are mostly uptake hydrogenases. Some [FeFe]-hydrogenases are also known as HDCR (hydrogen-dependent carbon dioxide reductase) for their ability to catalyse direct reduction of CO_2 using H_2.[17] Because of much shorter hydrophobic gas-access channels, they exhibit higher turnover frequency and lower oxygen tolerance than [NiFe]-hydro-

Table 3 – Structural differences between hydrogenase families
Tablica 3 – Strukturne razlike između različitih porodica hidrogenaza

Enzyme	Protein subunits	Clusters	Cofactors	Active site	Species
[NiFe]-hydrogenase	2 subunits	2 [4Fe-4S] and 1 [3Fe-4S]	[NiFe]-cluster	[NiFe]-cluster	*Desulfovibrio gigas*
[NiFeSe]-hydrogenase	2 subunits	3 [4Fe-4S]	[NiFeSe]-cluster	[NiFeSe]-cluster	*Desulfomicrobium baculatum*
[FeFe]-hydrogenase	1 subunit	3 [4Fe-4S] and 1 [2Fe-2S]	H-cluster	H-cluster	*Clostridium pasteurianum*
[Fe]-hydrogenase	homodimer	none	FeGP	Fe(II) site of FeGP	*Methanothermobacter marburgenis*

genases. Compared to the latter, [FeFe]-hydrogenases have more than 100 times faster specific hydrogen production rates (reaching up to 9000 molecules H_2 *per* second).[10,11] The catalytic mechanism of [FeFe]-hydrogenase has been elucidated.[10] The redox states involved in the catalytic cycle are H_{ox} (oxidised state), H_{red} (reduced state), and H_{sred} (superreduced state acquired by a second reduction).[18] The H_{red} state forms by one-electron reduction of H_{ox}, and a second one-electron reduction forms the H_{sred} state. The H_{hyd} state is formed by rearrangement of H_{sred}. Lastly, protonation of H_{hyd} regenerates active oxidised H_{ox} state and produces H_2.[19] Unlike [NiFe]- and [FeFe]-hydrogenases that contain a di-nuclear metal centre, [Fe]-hydrogenase contains a mononuclear metal centre. These enzymes are devoid of Fe-S clusters. For this reason, [Fe]-hydrogenases are known as iron-sulphur-cluster-free hydrogenases.[20] In the literature before (or around) 2002, when these mononuclear Fe centre enzymes were believed to be metal-free, [Fe]-hydrogenases meant today's [FeFe]-hydrogenases.[1] These iron-sulphur-cluster-free hydrogenases were first discovered in 1990 in methanogen *Methanothermobacter marburgenis*.[10] [Fe]-hydrogenase solely activates H_2 in the presence of substrate methenyltetrahydro-methanopterin. More specifically, it catalyses a stereospecific exchange of hydrogen from methylene-H_4MPT with protons from water. As opposed to bimetallic [FeFe]- and [NiFe]-hydrogenases, [Fe]-hydrogenases do not reduce artificial dyes, nor one-electron and two-electron acceptors.[21] Due to its ability to catalyse the reversible reduction of methenyl-H_4MPT^+, [Fe]-hydrogenase is also known as H_2-forming methylenetetrahydromethanopetrin (methylene-H_4MPT) dehydrogenase or Hmd. In these reactions, the methenyl-H_4MPT^+ substrate changes the structural conformation of the enzyme from open to closed.[10] The active site of [Fe]-hydrogenase, instead of the Fe-S cluster, is the iron guanylyl-pyridinol cofactor (FeGP), which is in charge of H_2 binding. A pyridinol nitrogen atom, an acyl carbon atom of the pyrdinol ring, two carbon monoxide ligands, one solvent molecule, and one cysteine sulphur atom ligate the Fe-centre.[21] Applicable for all hydrogenase classes, the large protein around the active site does not participate directly in catalysis, resulting in a limited number of active sites *per* square.[5]

2 Phylogeny

Hydrogenases are widely distributed in microbes. These enzymes serve different physiological functions in numerous metabolic pathways, *e.g.*, in methane formation, or in remediation of toxic heavy metals, parasites, and pathogenic bacteria.[10] *Greening et al.*[22] conducted a study on hydrogenase distribution in microbial H_2 metabolisms based on the 3286 protein sequences retrieved from publicly available databases. A comprehensive map of diversity and distribution showed that [NiFe]-hydrogenases are most widespread and diverse, whereas no [Fe]-hydrogenases were detected. Phylogenetic distribution was correlated with O_2 preference, where *obligate anaerobes* encode oxygen-sensitive [FeFe]- and [NiFe]-hydrogenases, *obligate aerobes* encode oxygen-tolerant [NiFe]-hydrogenases, and *facultative anaerobes* encode a diverse hydrogenase range.[22] The H_2 metabolism importance is evident as almost all anaerobic archaea and bacteria have more than one kind of [NiFe]-hydrogenase. For instance, three [NiFe]-hydrogenases were discovered in the hyperthermophilic archaea *Pyrococcus furiosus*. Groups of [NiFe]-hydrogenases discussed in Section 1.1.1 are mentioned here as a connection to the source of hydrogenases that are part of each group. Bacterial [NiFe]-hydrogenases mostly belong to *Groups 1* and *2*. *Group 5* hydrogenases are found in soil-living actinobacteria, while *Group 3* and most of *Group 4* are found in archaea. With the help of genome sequencing, the presence of bacterial hydrogenases was discovered in the two latter groups.[10] The most studied are oxygen-sensitive [NiFe]-hydrogenases from sulphate-reducing bacteria. They belong to *Group 1*, alongside the well characterised oxygen-tolerant [NiFe]-hydrogenases from *Ralstonia eutropha* and *Escherichia coli*.[14]

[FeFe]-hydrogenases are found in the genome of numerous microorganisms, anaerobic prokaryotes, and some anaerobic eukaryotes. As stated previously, the most studied [FeFe]-hydrogenases originate from *T. maritima, Clostridium*, *D. desulfurican,* and green algae.[10] Interestingly, the smallest identified [FeFe]-hydrogenase originates from *Chlorophyceae* green algae.[11] Within green algae, it is common to find two closely related [FeFe]-hydrogenase genes with *Clostridium acetobutylicum* as a good example of this feature. *Clostridium pasteruranium* has three [FeFe]-hydrogenases, alongside [NiFe]-hydrogenase (and nitrogenase). Furthermore, anaerobic *Thermoanaerobacterium saccharolyticum* has four [FeFe]-hydrogenase genes and a [NiFe]-hydrogenase gene. Different [FeFe]-hydrogenases in the same organism may exhibit different catalytic biases and functional roles.[17] Classification groups from Section 1.1.2. are referenced here for establishing the source of hydrogenases. *Group A* encompasses hydrogenases from *Clostridium*, as well as *T. maritima* and *D. desulfurican* hydrogenases. *Group B* [Fe]-hydrogenases remain uncharacterised. *Group C* hydrogenases also lack the functional information, but they are abundant in strictly anaerobic bacteria, such as *Thermotogae*, *e.g.*, the aforementioned *T. saccharolyticum*.[22] [FeFe]-hydrogenases cannot be found in archaea. Interestingly, proteins with sequences similar to [FeFe]-hydrogenase have been found in higher eukaryotes, including humans. In these cases, the H_2-linked functionality is lost, and other roles are assigned.[17] [Fe]-hydrogenases, on the other hand, are found only in methanogenic archaea, such as *Methanothermobacter thermoautotrophicum,* and *Methanocaldococcus junnaschii*, under dark conditions.[20,22]

In virtually all cases, organisms containing one or more nitrogenase, also contain uptake hydrogenase, both capable of H_2 metabolism.[23] Additionally, O_2-tolerant uptake hydrogenases are more distributed in nature than reported in the literature.[22] Moreover, in thermophilic microorganisms, hydrogenases capable of functioning at high temperatures can be found. Thermotolerant hydrogenases are for the most part [NiFe]-hydrogenases with activity in the temperature range from 50 °C to 125 °C.[24] Typically, [FeFe]-hydrogenases have a temperature optimum 50–60 °C.[25] Some of them can also withstand high pressures.[26] The discovery of thermotolerant hydrogenases has sparked discussions about their potential integration into various biotechno-

logical processes. These include coenzyme regeneration, bioremediation, biosensors, biosynthesis, and uses in the pharmaceutical industry.[24]

3 Factors affecting hydrogenase activity

Since the discovery of hydrogenases in 1931, their enzymatic activity has been studied using isotope exchange reactions and artificial electron carriers such as methylene blue, methyl viologen and benzyl viologen, with little attention initially given to natural electron carriers.[1] This section explores the factors that influence hydrogenase activity in more detail.

The catalytic activity of hydrogenases depends on pH.[27] Although these enzymes are active over a broad pH range,[1] H_2 reductions require acidic conditions, while oxidations require alkaline conditions.[28] Inhibitors, *i.e.*, molecules that bind to functional groups or active sites of the enzyme and reduce enzyme activity, can (reversibly or irreversibly) affect hydrogenase activity. Some known hydrogenase inhibitors are O_2, NO, CO, and CN^-.[29] Inactivation in the presence of O_2 is a significant limitation for using hydrogenases in hydrogen production.[30] In most cases, H_2 production can occur only in anaerobic conditions (less than 0.1 % of O_2).[31] While many [NiFe]-hydrogenases are reversibly inhibited by O_2 and can be reactivated upon its removal[30], [FeFe]-hydrogenases are for the most part irreversibly inactivated by traces of oxygen.[32] The recovery rates vary based on the inactivation conditions, duration of oxygen exposure, and redox potential.[30] [FeFe]-hydrogenases exhibit lower oxygen tolerance than [NiFe].[5] For instance, [FeFe]-hydrogenase from *T. maritima* exhibits 50 % inactivation after 10 s of oxygen exposure. The membrane-bound [NiFe]-hydrogenase from *P. furiosus* retains 50 % of its activity after four days, while the purified enzyme exhibits half of the initial activity after only 3 h.[24] Hydrogenase from *R. eutropha* exhibits the lowest sensitivity to oxygen. This hydrogenase can maintain H_2 oxidation activity in the presence of 20 % O_2 for more than 20 min.[33] However, it suffers from low hydrogen production activity under physiological conditions. This applies to many O_2-tolerant hydrogenases. Different rates of hydrogenase inactivation by oxygen are shown on a few examples in Table 4.[30] Several examples of oxygen-stable hydrogenases exist, although oxygen sensitivity is highly variable.[17] Oxygen binding occurs at an order of magnitude higher rate than the degradation (oxygen-bound state forms in a few hundred milliseconds, degradation in minutes). Enzyme activity loss can be represented by the 1st order rate law.[30] Oxygen tolerance has been improved by site-directed and random mutagenesis. Moreover, engineering strategies, such as hydrogel protection[34,35] and chemical treatment,[36] have shown success in O_2-driven damage protection.[30]

In the H_2 production reactions, the product inhibits hydrogenases as it diffuses from the solution through the gas channe l to the position close to the active site. Inhibition results from H_2 crowding in the gas-access channel of the hydrogenase structure.[15] *Fourmond et al.*[15] investigated product inhibition of [NiFe]-hydrogenase from *Desulfovibrio fructosovorans,* and [FeFe]-hydrogenase from *Chlamydomonas reinhardtii*. They developed a kinetic model of inhibition explaining the relationship between Michaelis (K_m) and inhibition constants (K_i) for hydrogenase activity from *C. reinhardtii*, and further tested it with the enzyme from *D. fructosovorans*. The kinetic model is based on what is known about H_2 transfer in hydrogenase. The effect of product inhibition of [FeFe]-hydrogenase is less pronounced, and the obtained K_i is 50-fold higher than that of the investigated [NiFe]-hydrogenase.[15]

Table 4 – Half-life of certain hydrogenases under exposure to oxygen

Tablica 4 – Vrijeme poluživota pojedinih hidrogenaza u prisustvu kisika

Type	Species	$p(O_2)$ / kPa	Half-life / min
[FeFe]	*Chlamydomonas reinhardtii*	1.0	38
[NiFe]	*Escherichia coli*	3.0	2.6
[FeFe]	*Clostridium acetobutylicum*	5.0	3.0
[FeFe]	*Clostridium pasteurianum*	5.0	7.7
[NiFe]	*Desulfomicrobium baculatum*	5.0	> 240
[FeFe]	*Desulfovibrio desulfuricans*	10.0	60
[NiFe]	*Klebsiella oxytoca*	10.0	≈ 720

The sensitivity of hydrogenases is visible also in the effect of UV-light. *Sensi et al.*[37] studied the photoinhibition of [FeFe]-hydrogenases from *C. reinhardtii* and *C. acetobutylicum*. The absorption of UV-B light (280–315 nm) irreversibly damaged the H-cluster, whereas visible light had no effect on its catalytic activity. The UV sensitivity stems from the presence of photolabile iron carbonyl bonds in the active site. Photoinactivation was somewhat slower when the enzyme was producing H_2 than when oxidising H_2.[37] Previously, photoinhibition of [FeFe]-hydrogenase from *D. desulfurican* was reported.[38,39,40]

Considering the potential of hydrogenases application in coenzyme regeneration reactions, it is of value to elucidate the effect of different solvents on enzyme activity and stability. *Serebryakova et al.*[41] determined the effect of methanol, ethanol, DMSO, THF, acetone, and acetonitrile in the H_2-dependent methyl viologen reduction by [NiFe]-hydrogenase from *Thiocapsa roseopersicina*. They found that significant enzyme activation can be obtained in the presence of methanol and ethanol in ratios 5–20 %. Still, at ratios above 40 %, the reaction rate decreased, and at 60 % the activity was inhibited. Similar findings were obtained for DMSO and THF, *i.e.*, activation at low concentrations followed by activity suppression, and complete inhibition at ratios above 50 % and 10–40 %, respectively. Acetone and acetonitrile caused no decrease in enzyme activity.[41] The residual activity of hydrogenase after incubation in buffer with organic solvents showed that all solvents decrease enzyme activity.[41]

4 Hydrogenase activity assays and hydrogenase activity

Comparative analysis of the activity of different hydrogenases is greatly hindered by the lack of a standard enzyme assay and unit of measurement.[24] The rate of hydrogenase activity is generally given in U mg^{-1} with 1 U defining the amount of enzyme needed for the conversion of 1 µmol of hydrogen *per* minute.[27] Enzyme activity has also been reported as: mol H_2 *per* mol substrate, litres of H_2 *per* hour *per* litre of culture, µmol H_2 *per* min *per* mg protein[24] and arbitrary units (AU) *per* ml.[42] Generally, [NiFe]-hydrogenases are 50–100 times less active than [FeFe]-hydrogenases. Activities of [FeFe]-hydrogenases range from $5 \cdot 10^{-3}$ to $1 \cdot 10^{-2}$ mol H_2 min^{-1} mg $protein^{-1}$ for H_2 production and $1 \cdot 10^{-2} – 5 \cdot 10^{-2}$ mol H_2 min^{-1} mg $protein^{-1}$ for H_2 uptake, with K_m for hydrogen ranging from $1 \cdot 10^{-4}$ to $1 \cdot 10^{-3}$ M at pH 8 and 30 °C. On the other hand, [NiFe]-hydrogenases have activity values $1 \cdot 10^{-4}$ to $1 \cdot 10^{-3}$ mol H_2 min^{-1} mg $protein^{-1}$ for both H_2 production and uptake, with K_m of two orders of magnitude lower than K_m for [FeFe]-hydrogenases. This corresponds to turnover frequencies of $6 \cdot 10^3 – 6 \cdot 10^4$ molecules H_2 s^{-1} *per* site for [FeFe]-hydrogenases and $1 \cdot 10^2 – 1 \cdot 10^3$ molecules H_2 s^{-1} *per* site for [NiFe]-hydrogenases. Nevertheless, the stated values highly depend on the applied activity assay.[43]

The catalytic activity of hydrogenase can be measured by following any reaction participant (H_2, e^- or H^+). When measuring gaseous H_2, gas chromatography, mass spectrometry or a hydrogen electrode can be used. Electrons can be tracked by protein film voltammetry, amperometry or UV-Vis spectroscopy.[44] Kinetic and thermodynamic measurements can also be conducted by cyclic voltammetry when measuring fairly active species and, for less active, more sensitive kinetic tests need to be developed. Utilising redox dyes is a common method for measuring hydrogenase activity assay.[43] If enzyme activity is determined by hydrogen-dependent reduction of artificial redox dyes, the accepted electron number should be considered, as different dyes can accept one (*e.g.*, methyl viologen), two (*e.g.*, methylene blue) or four (*e.g.*, nitoblue tetrazolium chloride) electrons per molecule.[27] Also, spectrophotometric activity assessment of soluble hydrogenase based on the hydrogen-dependent reduction of NAD^+ to NADH can be applied.[27] For non-buffered systems, enzyme activity can be determined by pH measurement following proton uptake or release.[44] By bidirectional measurement using the pH-stat method, activity can be measured without buffers, which may have a great influence on hydrogenase activity. Product formation can be measured using a pH-stat device, which allows for monitoring the reaction in both directions, which is not possible with methods like spectrophotometry.[45] H_2 formation can also be tracked using a Clark electrode, which consists of a Pt anode as the working electrode and an Ag/AgCl cathode, immersed in a 3 M KCl/10 M HCl electrolyte solution at a potential of +0.6 V. This electrode setup can continuously record the reaction kinetics of both H_2 reduction and oxidation, as the current is linearly dependent on the hydrogen concentration.[27]

5 Application of hydrogenases

Hydrogenases have potential applications in various fields. One of the most straightforward applications is in the photochemical production of H_2 that relies on electrochemically coupling photosensitizer compounds with hydrogen-producing catalytic centres.[11] These systems demonstrate the feasibility of water splitting catalysed by isolated hydrogenase. The first application of this type utilised [NiFeSe]-hydrogenase from *Desulfomicrobium baculatum* immobilised on dye-sensitized TiO_2 nanoparticles. This discovery enabled the construction of light-driven H_2 production systems with excellent photocatalytic activities, enzyme stability for several days, and high conversion rates.[12] *Gamache et al.*[46] studied a semi-artificial photocatalytic system using [FeFe] hydrogenase in whole cells for hydrogen gas production as well, focusing on the kinetics and photocatalytic performance.[46] Another application is in hydrogen fuel electrodes, which provide a clean energy source due to their high specificity for oxidising H_2 without producing environmentally harmful by-products.[47] The development of hydrogenase-based biofuel cells, however, is limited to O_2-tolerant hydrogenases. Early devices utilising hydrogenase anodes and oxygen-reducing cathodes were already comparable to platinum-based fuel cells, achieving open-circuit cell voltage values of up to 1.1 V (the thermodynamic limit is 1.23 V). Fuel supply fluctuations may result in high potentials under anode-limiting conditions. To prevent hydrogenase deactivation, cathode-limiting conditions are necessary. The immobilisation of hydrogenase in a redox hydrogel protects the enzyme from high potential deactivation and oxygen damage.[34] Hydrogenase electrodes prevent the issue of electrode poisoning with CO, since it has a reversible inhibition nature by CO. Unlike platinum, which can irreversibly lose 99 % of its initial activity (10 min under 0.1 % CO), hydrogenase electrodes maintain 90 % of their activity constantly (even under 1 % CO in H_2-CO mixture).[47] A critical factor for hydrogenase bioelectrocatalytic applications is the stability of the enzyme electrodes. Research has shown that immobilising hydrogenases on carbon filament material can significantly improve operational and storage stability.[48] Additionally, a graphite electrode coated with [NiFe]-hydrogenase exhibits activity that is two orders of magnitude greater than an electrode with a platinum atom on a metallic surface.[43] Immobilisation technology enhances the reusability, activity, stability, and electron-transfer efficiency of hydrogenases, making it a key technology for potentially replacing platinum as an electrocatalyst in the development of hydrogen biofuel cells.[10] Hydrogenases have recently been evaluated as enzymes that catalyse coenzyme regeneration, offering simplicity, and in some cases, independence from oxygen. For instance, the NAD^+ cofactor is required in many oxidative biocatalytic pathways as an electron acceptor. An effective regeneration system has been developed using oxygen-tolerant soluble hydrogenase from *R. eutropha*. Unlike standard NAD^+ regeneration systems that require additional oxidants and generate by-products, this hydrogenase-based system oxidises NADH with 100 % efficiency, producing only H_2 as a by-product.[49] Thermophilic hydrogenases are particularly promising for industrial applications in cofactor regeneration. Higher reaction temperatures can improve solubility, reaction rates, and re-

duce contamination risks, making hydrogenases that retain catalytic activity at elevated temperatures highly valuable.[24]

For example, *Assil-Companioni et al*.[50] achieved a successful whole-cell reduction of cyclic unsaturated ketones via hydrogen-driven cofactor regeneration, catalysed by ene-reductase in combination with a membrane-bound hydrogenase from *C. necator*.[50] Combining two or more enzyme-catalysed steps results in an enzyme cascade, which offers high substrate specificity, stereoselectivity, and regioselectivity, while avoiding the separation of intermediates and by-products.[51,52] *Ardao et al*.[42] demonstrated a cascade reaction for H_2 synthesis from cellobiose using a 13-enzyme synthetic *in vitro* metabolic pathway, including the hyperthermophilic *P. furiosus* hydrogenase. This multi-enzymatic system achieved a theoretical yield of 12 mol of H_2 *per* mol glucose, as opposed to 4 mol mol^{-1} yield from microbial fermentation, due to the unique combination of enzymes.[42] Multi-enzyme cascade systems are without a doubt more efficient than traditional step-by-step catalysis. Cascade reactions offer benefits such as handling of unstable intermediates, better control over unfavourable reaction equilibria, high yields, stereoselectivity, enantioselectivity, reduced waste, and lower costs.[52–54]

Despite the advantages, using multiple purified enzymes in industrial processes is costly. From an economic standpoint, industrial-scale application of H_2 production by hydrogenase from biomass is still unfeasible due to low volumetric productivity.[55] Many biocatalytic transformations require stoichiometric cofactor addition, necessitating recycling systems due to their high cost. An example of successful cofactor regeneration involves a multi-enzyme system where NAD^+ reductase and hydrogenase are adsorbed on graphite particles. The hydrogenase oxidises H_2, enabling direct electron transfer for NAD^+ reduction. The structure of both enzymes facilitates fast movement of electrons between the active centre and the conductive graphite particle. This approach has been used in the production of NADH cofactor for supplying NADH-dependent lactate dehydrogenase for the reduction of pyruvate to lactate.[56] *Reeve et al*.[56] tested this mechanism with NADH-dependent alcohol dehydrogenase for acetophenone reduction to phenylethanol. The availability of electrons from H_2 for NAD^+ reductase increases with the excess of hydrogenase on the particle. Therefore, with the increase in hydrogenase concentration, the rate of NADH production per mg of NAD^+ increases. Co-immobilisation of the NADH regeneration enzymes achieved higher reaction rates.[56] When applying cofactor regeneration systems in cascades, the effect that the cofactor concentration has on product formation must be considered. *Al-Shameri et al*.[49] studied a multi-enzyme cascade system for the oxidation of D-xylose to α-ketoglutarate by NADH dependent HsXyIDH2 xylose dehydrogenase. Soluble hydrogenase from *R. eutropha* was implemented in the reaction system for NAD^+ regeneration. By increasing NAD^+ concentration, significant product formation was achieved even at low enzyme concentration. As opposed to regeneration with NADH oxidase (NOX), the hydrogenase system offers higher conversion. The yield of the soluble hydrogenase system achieved after three hours was $2.8 \cdot 10^{-2}$ mol l^{-1} h^{-1} and $1.6 \cdot 10^{-2}$ mol l^{-1} h^{-1} after 16 h, exceeding those reported for NOX systems.[49]

6 Conclusion

Hydrogenases are versatile metalloenzymes found across all domains of life. However, our understanding of these enzymes is limited due to the relatively small number of characterised hydrogenases. Alongside low volumetric hydrogen productivity, oxygen sensitivity is the main obstacle in applying hydrogenases to ecologically friendly H_2 production. Ongoing research focuses on developing methods to block O_2 diffusion to the active site without compromising H_2 production activity. Despite these challenges, the main advantage of these enzymes is their high catalytic conversion rate, making them the most efficient hydrogen production catalysts. In industrial applications, hydrogenases are particularly promising for cofactor regeneration (*e.g.*, NADH). Integrating hydrogenases into cascade systems for supplying NADH-dependent enzymes can enhance reaction rates and yields. Although more research is required for successful scale-up and industrial application, recent studies have paved the way for the use of hydrogenases in small-scale biocatalytic reactions for H_2 biosynthesis.

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SAŽETAK

Hidrogenaze – tipovi, izvori, svojstva i potencijal njihove primjene

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Hidrogenaze su skupina svestranih metaloenzima koji kataliziraju reverzibilnu transformaciju između plinovitog vodika i njegovih sastavnih protona i elektrona. Ti su enzimi nedavno privukli pažnju zbog svoje sposobnosti primjene molekularnog vodika kao supstrata u reakcijama koje kataliziraju, kao i sposobnosti sintetiziranja vodika, danas vrlo važnog biogoriva. Iako je poznato da hidrogenaze postoje još od 1930-ih, dosadašnja istraživanja nisu dovoljna za njihovu primjenu na industrijskoj razini. Istražuju se uglavnom s biokemijskog gledišta, što je vrlo važno, ali nedostaju istraživanja koja bi te enzime učinila primjenjivijima u stvarnim industrijskim procesima. Postoji samo nekoliko studija u tom području. Stoga ovdje predstavljamo kratak pregled literature o onome što je poznato o hidrogenazama. Pokušali smo objasniti njihovu pozadinu, klasifikaciju i filogeniju, budući da se mogu pronaći u mnogim različitim izvorima, poput bakterija, arheja i u nekim eukariotima. Pobrojali smo važne čimbenike koji utječu na njihovu aktivnost te dali naglasak na njihovim prednostima i nedostacima. Uz to, predstavili smo dostupne metode za određivanje njihove aktivnosti i naglasili važnost postojanja jedinstvenih jedinica za njihovo izražavanje, jer je trenutačno teško usporediti sve dostupne podatke. Na kraju smo dali prikaz njihove potencijalne primjene kao ključne teme u istraživanjima, a to je u proizvodnji vodika te u sintetskim reakcijskim putevima za regeneraciju koenzima.

Ključne riječi
Hidrogenaza, regeneracija koenzima, proizvodnja vodika, klasifikacija hidrogenaza, svojstva hidrogenaze

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