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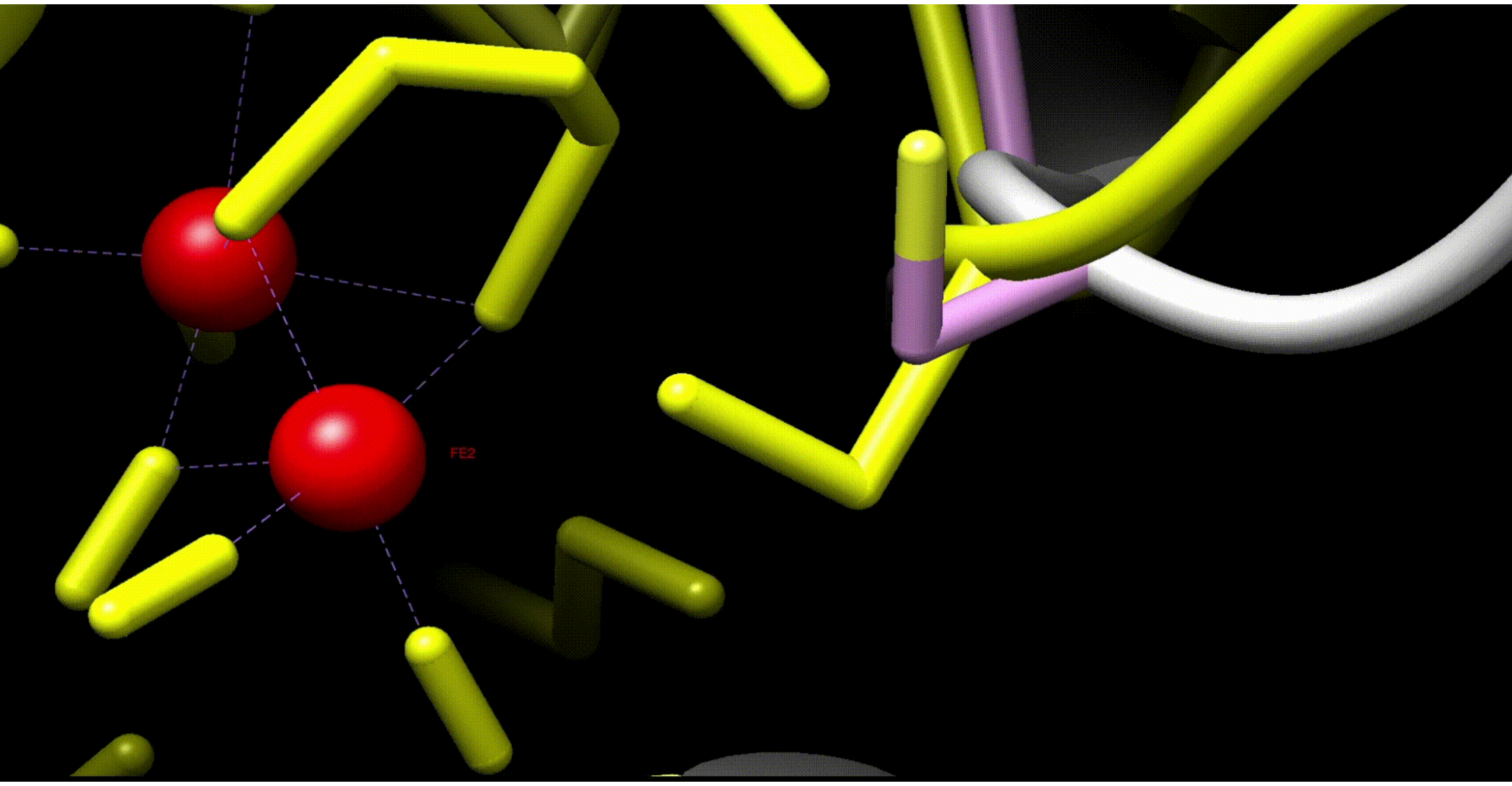
Special enzymes, like “Easter Eggs” with wonderful functions for biotechnology



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SCUOLA

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We guess you know very well what an Easter Egg is in a video game: a special surprise that was hidden in a secret area or a bonus object.

We discovered [Morra *et al.,* 2016a, check Bibliography for info and link to this original scientific paper and to other papers cited] a similar "Easter Egg" in a protein that functions in a very useful bacterium, and this suggested us how proteins and enzymes can contribute to a bio-sustainable future. But first let us introduce you the scientific background and the context.

THE BACKGROUND

As you know proteins are responsible of the cell structure from bacteria to animals, plants and humans but they are also in charge of making the organisms function: special proteins called enzymes can promote the chemical reactions in cells, the transport of substances and very complex machineries such as the muscle contraction in animals or the process of photosynthesis in plants.

The enzymes, as such, are very finely designed nano-machines: **nano** because they are in the dimensional range of nanometers (10^{-9} meters), **machines** because they exploit their chemical features and flexible structure to perform very specialized biotechnological functions. The design is of course directed by evolution, which selects the structure and functions that works better for the survival of the organisms.

THE CONTEXT

In our case the enzyme we were studying is called hydrogenase. In a bacterium called *Clostridium beijerinckii*, that lives in soils and grows in absence of oxygen, this enzyme allows the bacterium to exploit the waste organic material for gaining energy. As a product, not used by the bacterium, it produces hydrogen gas, which for the bacterium is a way to eliminate excess electrons. Hydrogen is nowadays gaining interest since it is a fuel that can be used without producing CO_2. Hydrogen-fuelled trains, car, ships and even planes are on the go, which will have a zero impact on the greenhouse gas emissions.

In natural habitats other organisms, such as **Archea**[1] that produce methane, can use the hydrogen gas, but now, in our society, we can also use our *Clostridium beijerinckii* bacterium to recover hydrogen gas from the huge amount of waste that we produce in our cities, from the organic waste, or from the agricultural waste and collect this precious fuel. The technology is already available: we can build **anaerobic digestion plants**[2] in which the waste is treated in presence of many bacteria and the hydrogen gas flow is recovered [Arizzi *et al*., 2016 and 2021, Morra *et al.* 2014] and stored in special materials, for us to exploit as a fuel. The treated waste can be further digested to produce methane, is then recovered (it is then called digestate) and can be processed to produce bio-fertilisers for a more sustainable agriculture fuel [Fuldauer *et al*., 2018].

OUR DISCOVERY

But let's go back to our studies: why is the hydrogenase of *Clostridium*

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[1] **Archea:** *microorganisms which are similar to bacteria in size and simplicity of structure but radically different in molecular organization. They are now believed to constitute a separate kingdom which is intermediate between the bacteria and eukaryotes*

[2] **Anaerobic digestion plants:** *structures in which the waste is converted in smaller molecule in absence of oxygen. We can recover hydrogen gas or, pushing the reaction further -but always via hydrogen- methane (both gases are mixed with CO2 but they can be purified).*

beijerinckii so special? It resists oxygen damage! Normally oxygen is toxic to the hydrogenase enzymes present in this organism. The organism itself can protect from oxygen by forming a spore (a very small and robust version of the bacterial cell that can stay quiet and regrow when condition gets better) but the enzyme gets irreversibly damaged by oxygen, so the bacterium has to recreate the enzyme from scratch at the cost of some energy. But for the enzyme we have discovered, the behaviour is different: the enzyme, although very similar to other sensitive enzymes, is not damaged in the presence of oxygen, it simply becomes temporarily inactive and then starts working again, operating this protective mechanism whenever oxygen occurs. How is this possible?

In our studies we observed that a change in the structure of the protein, (probably by chance, due to mutations in DNA and therefore in the amino acids composing the protein) made the enzyme slightly different. A part of the protein is more flexible [Winkler *et al.,* 2021] and can swing to block the part of the protein that is attached by oxygen (Figure 1). So, the protein has an intrinsic locking mechanism auto-protecting the hydrogenase from oxygen (in few words a real “Easter Egg”).

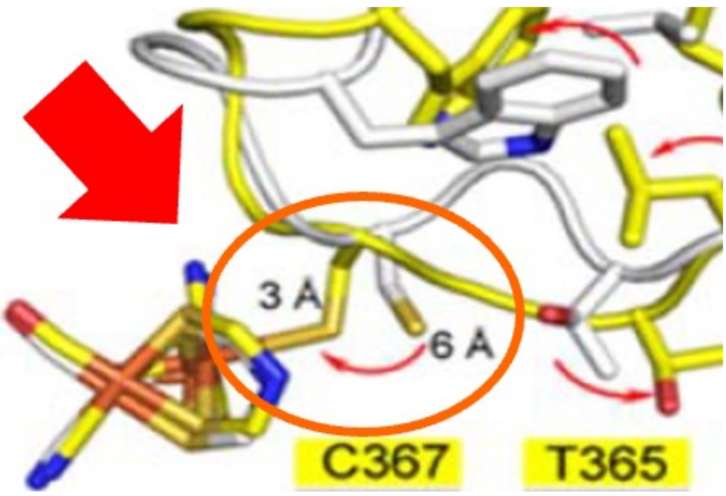

Figure 1 The structure of the locking mechanism: the protein loop swings from the position in white to the position in yellow and locks the oxygen-

reactive site (from *Winkler et al, 2021. Creative Commons Attribution 4.0 License https://creativecommons.org/licenses/by/4.0/)*

This mechanism works as a sort of reversible shield, stopping the oxygen reaching the enzyme metal-containing site, which in turn will produce reactive oxygen species[3] (**ROS, check glossary**) that would normally damage the enzyme itself. As soon as the oxygen is gone the enzyme starts to function again. The bacterium spares energy and can restart growing quickly, with advantages over other bacteria that cannot use their proteins which have been damaged by oxygen. This makes *Clostridium beijerinckii* a fast-growing bacterium and a highly efficient hydrogen producer.

FURTHER APPLICATIVE ADVANTAGES

But this is not all: the "super-powered" hydrogenase that can auto-protect from oxygen is very interesting for scientists, since it is a biocatalyst with a special feature that can be copied: Nature showed us how this mechanism of auto-protection can be modelled in an enzyme, and we can design hydrogen producing enzymes, equally protected from oxygen, that can be used outside the cell.

Isolated enzymes produced by bacteria [Morra 2022, Morra *et al*., 2016b] can be used to perform a specific reaction: in this case, only to produce or

[3] **ROS:** *acronym of* ***R****eactive* ***O****xygen* ***S****pecies, unstable molecules that contain oxygen and that easily react with other molecules in a cell. May cause damage to DNA, RNA, and proteins, and may cause cell death. Reactive oxygen species are usually formed when molecular oxygen* O_2 *reacts with some metals such as iron. They include free radicals (with reactive free electrons) such as superoxide and hydroxyl radical and non-radical species such as hydrogen peroxide (*H_2O_2*).*

consume hydrogen in technological systems (**electrolysers**[4] or **fuel-cells**[5],) that are essential for using hydrogen as a fuel or as energy storage (a general scheme in Figure 2). So, protein engineers, researchers like us who study how an enzyme structure conveys function and special features, can learn from Nature and redesign, even further, special enzymes that we can use.

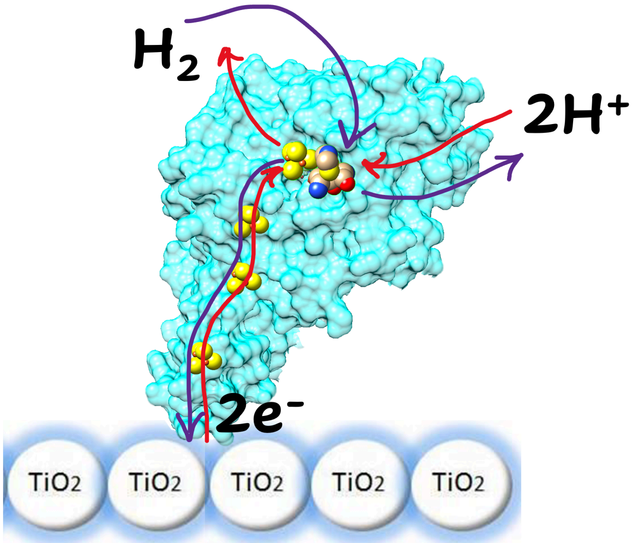

Figure 2 a scheme of an hydrogenase enzyme on an electrode made of a semiconductor material (Titanium dioxide or TiO_2), able to reversibly exchange electrons to generate hydrogen gas from electricity and protons of water (red trace) and vice versa (blue trace).

The general advantage of enzymes over normal metal **catalysts**[6] (such as

[4] **Electrolyser:** a system or device that uses electricity to split water molecules into hydrogen and oxygen, thereby producing hydrogen gas as a sustainable source of clean energy

[5] **Fuel-cell:** an electrochemical cell that converts the chemical energy of a fuel (often hydrogen) and an oxidizing agent (often oxygen) into electricity

[6] **Catalyst:** a substance that increases the rate of a chemical reaction without itself undergoing any permanent chemical change. Enzymes are the biological catalysts inside the cells.

platinum used in devices producing or exploiting hydrogen as a fuel) is the low cost (including also the independence from rare metals) the mild conditions of use, by definition “bio-compatible”, and the sustainable production and degradation.

In hydrogen technologies, in particular, the production and exploitation of hydrogen often involves the **inter-conversion of electricity into hydrogen**[7] and vice versa. Compared with non-biological catalysts, hydrogenases are capable of performing these reactions with higher efficiencies (10000 reactions/second) and what is called **low over-potential**[8] [Armstrong and Hirst, 2011]**.** The enzyme we discovered could be used instead of platinum and other rare and expensive metals to convert intermittent electrical energy, such as the current created in photovoltaic solar panel systems or by wind turbines, into hydrogen and vice versa, allowing it to be stored when in excess and reused when needed.

Research and development are still needed to make it possible to replace traditional catalysts with biological ones such as hydrogenases, but we are working on it!

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[7] **Inter-conversion of electricity into hydrogen:** Hydrogen gas and electricity are complementary energy carriers, interconverted by electrolysers and fuel cells, that have distinct characteristics. In general hydrogen gas can be a way of storing non-continuous electricity sources such as photovoltaics, wind, tidal waves and hydroelectric.

[8] **Low over-potential:** for a reduction or an oxidation reaction (exchange of electrons) to occur the potential (voltage) must be set at a certain value, according to the energy required. Often the reaction starts only if an excess energy is provided: this is called over-potential. Biological systems work at very low (close to zero) overpotential, thus minimising the waste of energy.

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