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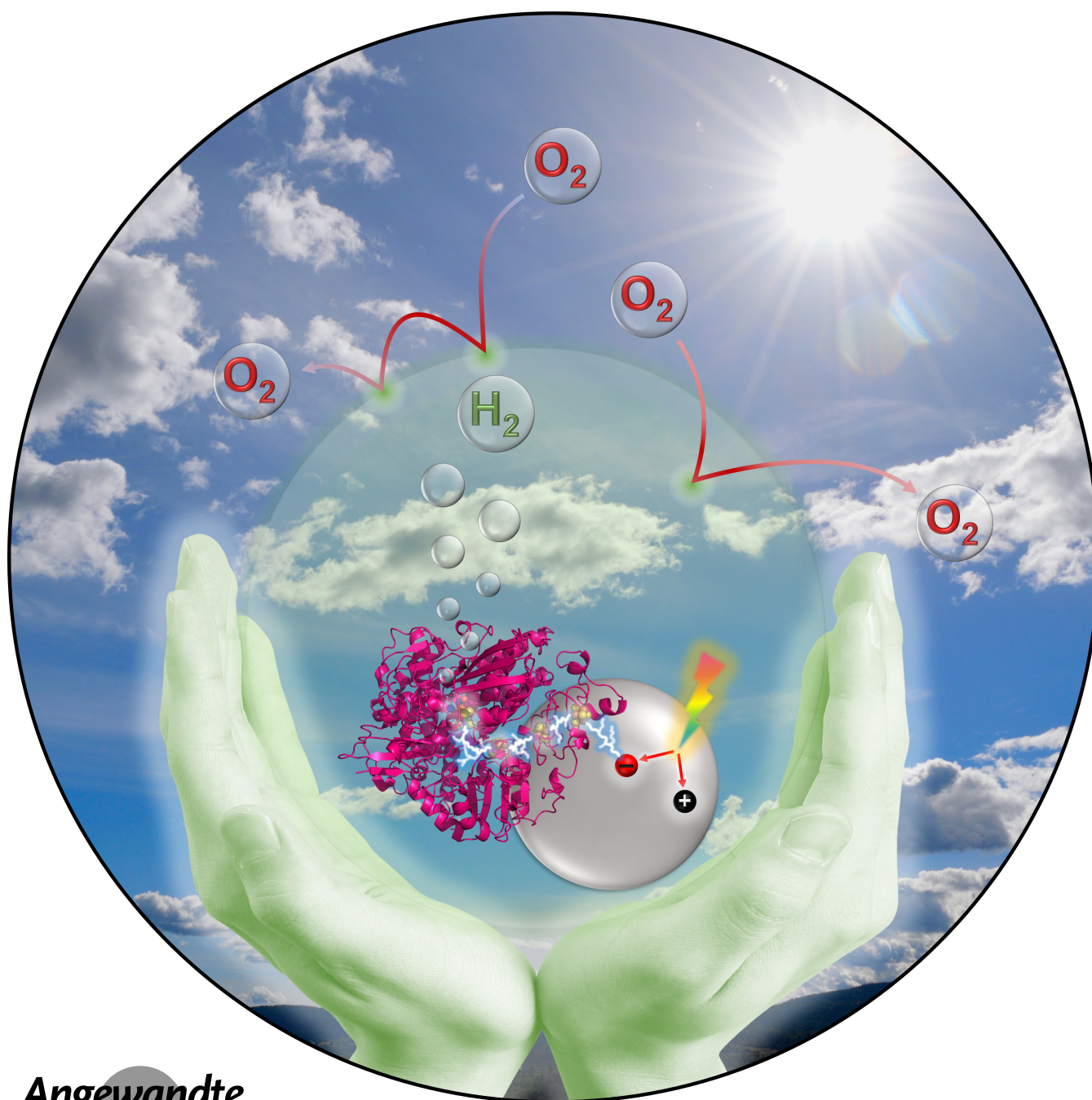
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Augmenting the Performance of Hydrogenase for Aerobic Photocatalytic Hydrogen Evolution via Solvent Tuning*Michael G. Allan, Thomas Pichon, Jade A. McCune, Christine Cavazza, Alan Le Goff, and Moritz F. Kühnel**

Abstract: This work showcases the performance of [NiFeSe] hydrogenase from *Desulfomicrobium baculatum* for solar-driven hydrogen generation in a variety of organic-based deep eutectic solvents. Despite its well-known sensitivity towards air and organic solvents, the hydrogenase shows remarkable performance under an aerobic atmosphere in these solvents when paired with a TiO₂ photocatalyst. Tuning the water content further increases hydrogen evolution activity to a TOF of 60 ± 3 s⁻¹ and quantum yield to 2.3 ± 0.4 % under aerobic conditions, compared to a TOF of 4 s⁻¹ in a purely aqueous solvent. Contrary to common belief, this work therefore demonstrates that placing natural hydrogenases into non-natural environments can enhance their intrinsic activity beyond their natural performance, paving the way for full water splitting using hydrogenases.

Photocatalytic water splitting is viewed as a favourable method of producing green H₂ to combat global energy challenges without requiring large investments into electrolyzers and power grids.^[1] Suitable systems for efficient solar H₂ production should focus on materials which are robust, cheap, and readily available.^[2] A research-intensive area in this field is the development of non-precious co-catalysts for the hydrogen evolution half-reaction (HER) and its reverse hydrogen oxidation reaction (HOR).^[3] [NiFe], [FeFe] and [NiFeSe] hydrogenases (H₂ases) are biological catalysts which can reversibly convert protons and electrons into H₂ at low overpotentials without being based on precious metals.^[4] Solar-driven H₂ generation has been demonstrated using a range of H₂ase-photocatalyst combinations^[5,6] with [NiFeSe] H₂ases shown to be particularly active.^[7] An

interesting aspect of [NiFe] and [NiFeSe] H₂ases is their O₂ tolerance,^[7g,8] whereby they show only a partial and reversible decrease in catalytic activity under aerobic conditions. Photocatalytic H₂ evolution in high levels of O₂ is an important property as catalytic components in photoreactors may be exposed to O₂ via in situ O₂ formation resulting from water oxidation or through leakage. Despite this, O₂-tolerant H₂ases show performances and lifetimes for H₂ evolution much lower in aerobic environments versus an inert environment.^[7d] Solving the O₂ sensitivity is considered a key step towards industrial application of hydrogenases.^[9]

We recently reported on a novel approach to enabling O₂-tolerant H₂ evolution through solvent design.^[10] Efficient photocatalytic H₂ evolution under aerobic conditions was achieved using a Pt/carbon nitride photocatalyst employing deep eutectic solvents (DESSs) with a low O₂ solubility and diffusivity as a reaction medium. DESSs have attracted attention in recent years as an alternative class of ionic liquids, as they possess low toxicities and can be prepared from cheap and readily available precursors.^[11] However, H₂ases have so far not been employed outside of conventional aqueous solvents for solar H₂ evolution due to incompatibility with different environments, particularly organic solvents.^[12] In the past, chemical modifications to H₂ases have been investigated to allow them to function in organic solvents.^[13] Protection of H₂ase from O₂ has been achieved by integration with hydrogels^[14] and redox-active films.^[15] In this work, we showcase the applicability of solvent engineering to the photocatalytic H₂ evolution at a [NiFeSe] H₂ase with TiO₂ as a light-absorber (Figure 1). We highlight for the first time not only the stability and catalytic activity of [NiFeSe] H₂ase in organic-based solvents for photocatalytic H₂ production, but also a remarkable enhancement in O₂ tolerance induced in these non-natural solvents without any enzyme modification or catalyst redesign.

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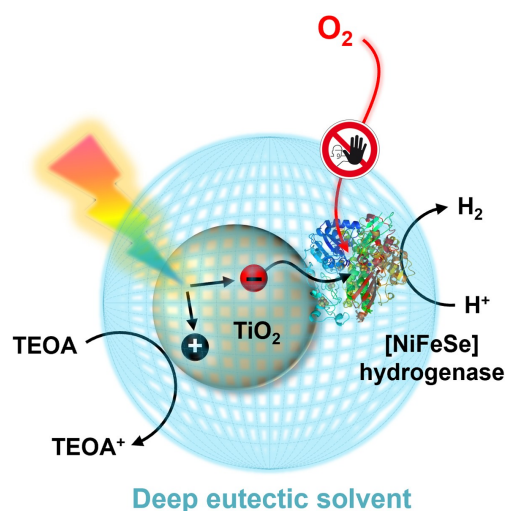


Figure 1. Schematic representation of the work presented here. Deep eutectic solvents induce oxygen tolerance to a TiO₂-hydrogenase based photocatalyst for solar-driven hydrogen evolution under aerobic conditions.

First, we assessed the general effect of DESs on the activity of H₂ases to investigate their potential for biophotocatalytic hydrogen evolution in non-conventional solvents. Previous work has shown that DESs can stabilise air-sensitive species^[16] and possess a high degree of biocompatibility^[17] with an ability to stabilise proteins.^[18] We chose [NiFeSe] H₂ase from *Desulfomicrobium baculatum* (*Db*) as a HER catalyst for this work, due to its previously reported suitability for use as a co-catalyst with various photocatalysts.^[7] The photocatalytic performance for H₂ generation was investigated in a heterogeneous TiO₂-[NiFeSe] catalyst system comprised of TiO₂ (2.5 mg mL⁻¹) and *Db*[NiFeSe] H₂ase (21 pmol) in a variety of DES-water mixtures. Aqueous TEOA was used as an electron donor, with the pH adjusted to 7.0 prior to mixing with the DES (Table S1). Samples were irradiated with simulated solar light (AM 1.5G) at 40 °C under a continuous purge of N₂, and H₂ evolution was quantified by gas chromatography (see Supporting Information for full details).

In all solvents tested, we clearly observed photocatalytic H₂ evolution, thus proving that *Db*[NiFeSe] H₂ase retains its catalytic HER activity in DES-based solvents. H₂ generation by the TiO₂-[NiFeSe] photocatalyst system was sustained for >24 h in all solutions containing the DES glyceline (choline chloride:glycerol 1:2). The reaction rate increased upon increasing the water content in the solvents (Figure 2). In an 80 % vol. aq. glyceline solution, TiO₂-[NiFeSe] generated 24.10 ± 0.55 μmol_{H₂} (TON > 1080000 ± 100000) after 24.9 h irradiation whereas the same photocatalyst in a 20 % vol. aq. glyceline solution showed a TON > 4350000 ± 500000 (91.44 ± 11.96 μmol_{H₂}) with an apparent quantum yield (AQE) of 2.3 ± 0.2 % (Table S2). Comparable performance was also observed in other DESs such as ethaline (choline chloride:ethylene glycol, Figure S1). This compares favourably with an aqueous solvent, in which TiO₂-[NiFeSe] exhibited a TON > 3500000 ± 31000 after 24.1 h under otherwise identical conditions. Remarkably, the activity in 20 % vol. aq. glyceline exceeds that observed in a purely aqueous environment by approx. 19% under otherwise identical conditions. Negligible amounts of H₂ were produced when TiO₂ was irradiated in 60% vol. aq. glyceline

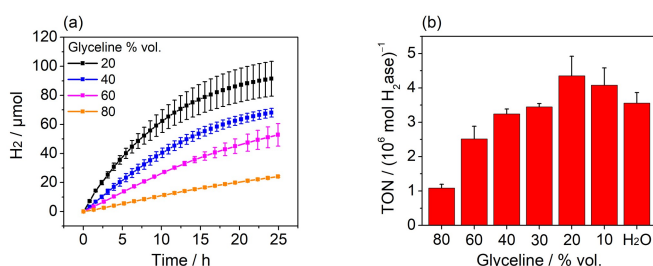


Figure 2. Photocatalytic H₂ generation using a photocatalyst system based on TiO₂ and *Db*[NiFeSe] H₂ase in various glyceline-water mixtures. (a) H₂ generation over time and (b) turnover number after 24 h irradiation in solvents of varying glyceline content under inert conditions. Conditions: TiO₂ (5.0 mg), *Db*[NiFeSe] H₂ase (21 pmol), 2.0 mL solvent, TEOA (0.4 M), AM 1.5G, 1 sun, 40 °C, constant N₂ purge.

without added *Db*[NiFeSe] H₂ase (Figure S2). To the best of our knowledge, this is the first report of a natural H₂ase functioning in an organic solvent for solar H₂ production—and even slightly better than in purely aqueous conditions in which it evolved. It also exceeds most previous reports on photocatalytic H₂ evolution using a [NiFeSe] H₂ase (Table S3).

Further insight into the origin of the varying H₂ase activity depending on the DES content of the medium was sought from electrochemical measurements of *Db*[NiFeSe] H₂ase adsorbed onto an adamantane-modified multi-walled carbon nanotube electrode (MWCNT)^[19] in varying concentrations of glyceline. Protein film electrochemistry^[20] shows that the enzyme retains its reversible activity towards both HER and HOR with near zero-overpotential in the DES (Figure 3a). Upon increasing the glyceline content in the solvent, the HER current increases before gradually decreasing (Figure 3b). The presence of an HOR current arises from the production of H₂ in the MWCNT layer upon proton reduction. Its decrease upon increasing the glyceline content might not only be caused by a decrease in HER activity but may be amplified by a decrease in H₂ solubility with higher DES concentrations, caused by the “salting out” effect of solutions with high ionic strengths.^[21] A lowered H₂ solubility in turn would mitigate the well-documented inhibition of H₂ase by H₂,^[22] thus allowing for a higher HER activity in DESs. The initial increase in HER current is consistent with the observed increase in photocatalytic H₂ generation reflecting the solvent effect on increasing the activity of the enzyme for proton reduction.

To exploit the impact of the DES content in the solvents on the stability of *Db*[NiFeSe] H₂ase under demanding conditions, the TiO₂-[NiFeSe] photocatalyst system was tested in the same solvents under a constant purge of air (21 % O₂). In an aqueous aerobic sample, the photocatalyst produced 2.73 ± 1.3 μmol_{H₂} after 24.1 h irradiation (TON = 130000 ± 60000) corresponding to only 3.7 ± 1.7 % of the amount produced under inert but otherwise identical conditions (Figure 4), owing to the known O₂ inhibition of H₂ase. In the DES-based solutions however, a high level of oxygen tolerance is exhibited without making changes to the photocatalyst or enzyme, particularly at higher percentages

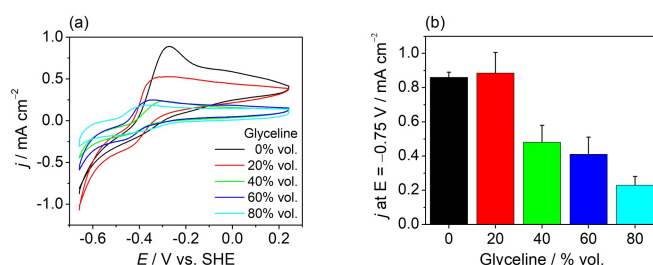


Figure 3. Protein film electrochemistry of *Db*[NiFeSe] H₂ase adsorbed on a MWCNT electrode in glyceline-water mixtures of varying composition. (a) Cyclic voltammograms and (b) observed current density at -0.75 V vs. SHE. Conditions: *Db*[NiFeSe] H₂ase-MWCNT working electrode, TEOA (0.4 M), pH 7.0, 25 °C, 0.01 V s⁻¹ scan rate, de-aerated solution.

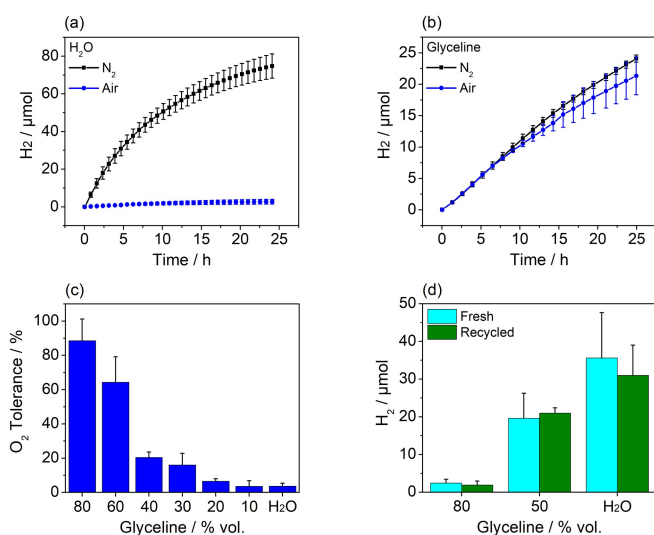


Figure 4. Oxygen tolerance of the photocatalytic H₂ generation using a photocatalyst system based on TiO₂ and Db[NiFeSe] H₂ase. (a) Photocatalytic H₂ generation in water and (b) in 80% vol. aq. glyceline under inert conditions and atmospheric levels of O₂. (c) Oxygen tolerance of TiO₂-[NiFeSe] determined from total H₂ produced in inert and aerobic conditions after >24 h irradiation in solvents of varying concentrations of glyceline in water. (d) H₂ produced by TiO₂-[NiFeSe] in fresh and resuspended solutions containing varying concentrations of glyceline after 9.3 h irradiation under N₂. Conditions: TiO₂ (5.0 mg), Db[NiFeSe] H₂ase (21 pmol), 2.0 mL solvent, TEOA (0.4 M), AM 1.5G, 1 sun, 40 °C, constant N₂ or air purge.

of DES. In an 80% vol. aq. glyceline solution, the O₂ tolerance was near 90% within experimental error with a total H₂ production of $21.34 \pm 3.03 \mu\text{mol}_{\text{H}_2}$ (TON = 1020000 ± 144000) after >24 h. Similarly, the AQE of H₂ evolution in an aerobic 60% vol. aq. glyceline solution was determined to be $2.3 \pm 0.4\%$ after 1 h, identical to the value determined under anaerobic conditions. After 5.2 h irradiation in air, the AQE was still at $1.4 \pm 0.3\%$, more than 70% of the activity in inert conditions (Table S2). While higher water contents in the solvent increased the photocatalytic activity under inert conditions (vide supra), the associated decrease in DES content lowers the O₂ tolerance. This decrease in O₂ tolerance is also observed when other DESs are used as the reaction medium (Figure S3). The optimum compromise is observed at 60% vol. aq. glyceline whereby TiO₂-[NiFeSe] continuously generates H₂ during 72 h irradiation at a remarkable overall TOF of $60 \pm 3 \text{ s}^{-1}$ in air, and a total TON of $>1800000 \pm 180000$ after the 72-h period (Figure S4). This retention of photocatalytic activity represents a major improvement over previous work showing that even O₂-tolerant H₂ases in water undergo considerable inactivation during photocatalytic H₂ evolution in the presence of air (Table S4). A [NiFe] H₂ase from *D. vulgaris* shows 65% retention of activity in air vs. N₂ when embedded in a nanoporous glass plate.^[23] In addition, an engineered [NiFe] H₂ase from *E. Coli* irradiated with a carbon nitride-TiO₂ photocatalyst system retains 20% of its activity in air.^[24] [NiFe] H₂ase from *T. Roseopersicina* covalently bound to a Ru photosensitiser was reported to

maintain 11% of its initial rate in the presence of air.^[5c] The Db[NiFeSe] H₂ase used here has also been shown to exhibit photocatalytic performance in air when used with dye-sensitised TiO₂ as the photoabsorber in water, with a lowered H₂ production rate even after just 30 minutes of exposure to air.^[7e]

To rationalise the dependence of O₂ tolerance on the solvent composition, we determined the O₂ solubilities $c(\text{O}_2)$ and diffusion coefficients $D(\text{O}_2)$ in glyceline and water using stepped-potential microwave chronoamperometry (Figure S5).^[25] Using the Krichevsky^[26] and Wilke-Chang^[27] equations, we estimated $c(\text{O}_2) \times D(\text{O}_2)$ for the different DES/water mixtures. Table S5 shows that as the water content increases from 0 to 100%, $D(\text{O}_2)$ increases approx. 800-fold, while $c(\text{O}_2)$ decreases only marginally. In line with our previously reported model for the O₂ tolerance in which O₂ intolerance is treated as diffusion-controlled O₂ reduction at a spherical photocatalyst particle,^[10a] we observe a good correlation between $c(\text{O}_2) \times D(\text{O}_2)$ and the O₂ tolerance when $c(\text{O}_2) \times D(\text{O}_2)$ is small, i.e. when the local concentration of O₂ at the photocatalyst is mass transport limited and it competes with the much faster proton diffusion (Figure S6).

In addition to TiO₂, photocatalytic H₂ evolution at Db[NiFeSe] H₂ase was further investigated using Eosin Y (EY) as a photoabsorber in aerobic and inert conditions to test the suitability of solvent tuning on a homogeneous photocatalytic system and thus the generality of this approach. In a previous report, it has been shown that EY-Db[NiFeSe] is active for H₂ evolution under visible light irradiation in the presence of atmospheric levels of O₂, however the photoreactor was not subject to resupply of O₂ and the activity retained in air was only 11% relative to an inert atmosphere.^[7d] We observed in glyceline-based solvents that increasing the DES content led to an increase in O₂ tolerance of EY-Db[NiFeSe] H₂ase (Figure S7) similar to the aforementioned heterogeneous system, however the overall activity of the photocatalyst system was low. The intrinsically low activity in DESs is in line with a previous report, where photocatalytic H₂ production at EY with synthetic HER catalysts was lower in DESs versus water but showed increased relative activity in air.^[10a]

To investigate the origin of decreased activity at higher glyceline concentrations, solutions containing TiO₂ and Db[NiFeSe] H₂ase were subject to centrifugation, with the supernatant subsequently decanted (see Supporting Information for details). The thus obtained TiO₂-[NiFeSe] pellet was resuspended in a fresh solution of the solvent in question without added H₂ase and then irradiated, and the H₂ production was compared to a TiO₂-[NiFeSe] pellet resuspended in the originally decanted supernatant. H₂ production performance of the TiO₂-[NiFeSe] in the recycled supernatant was similar to the performance in a fresh solution (Figure 4d). This indicates that the solvent does not hinder the adsorption of the hydrogenase enzyme to the TiO₂ surface, allowing for efficient charge transfer from the photocatalyst to H₂ase.

In summary, we have shown that DESs can act as alternative reaction media to water for the photocatalytic

hydrogen evolution using natural hydrogenase enzymes. By tuning the water content in the DESs, both activity and stability of the H₂ evolution activity can be increased to match and even outcompete pure water as a solvent for H₂ evolution. The H₂ evolution activity of TiO₂[NiFeSe] in aerobic conditions is drastically improved in DESs, with nearly 90 % activity retained in air, whereas H₂ evolution in water is almost completely quenched in air. This work shows the first instance of Db[NiFeSe] H₂ase employed in organic solvents for the H₂ evolution reaction and thus highlights the potential of solvent engineering as a novel, highly effective approach to improve natural enzyme performance. Further studies into the influence of temperature and pH on H₂ase activity and O₂ tolerance as well as their correlation with H₂ and O₂ solubilities in the respective solvent mixtures will provide key factors for designing tailored solvents in the future that can achieve a high O₂ tolerance without lowering the H₂ase activity.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article. Additional raw data is freely available from the Zenodo repository at <https://doi.org/10.5281/zenodo.7573371>.

Keywords: Deep Eutectic Solvents · Hydrogen · Hydrogenase · Oxygen Tolerance · Photocatalysis

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