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Photobiocatalytic chemistry of oxidoreductases using water as the electron donor

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To date, water has been poorly studied as the sacrificial electron donor for biocatalytic redox reactions using isolated enzymes. Here we demonstrate that water can also be turned into a sacrificial electron donor to promote biocatalytic redox reactions. The thermodynamic driving force required for water oxidation is obtained from UV and visible light by means of simple titanium dioxide-based photocatalysts. The electrons liberated in this process are delivered to an oxidoreductase by simple flavin redox mediators. Overall, the feasibility of photobiocatalytic, water-driven bioredox reactions is demonstrated.

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Biocatalysts are gaining ground as competitive tools for organic chemistry^{1–8}. Their high selectivity enables shorter and more efficient synthetic routes and bears the promise for greener chemistry. Today, hydrolytic enzymes are well-established on industrial scale and oxidoreductases are ready to follow. This latter class of enzymes catalyses a broad range of synthetically useful transformations ranging from (enantio)selective reduction and oxidations to specific oxyfunctionalization or halogenation⁹.

Some oxidoreductases, such as dehydrogenases, are, however, cofactor-dependent enzymes, which represents a major drawback for practical applications. Usually, the required reductants (either for reduction of the substrate or reductive activation of molecular oxygen to perform oxyfunctionalisation chemistry) are delivered via natural electron transport chains. These electron transport chains often comprise several enzymes and cofactors to connect the oxidoreductases to the reduced nicotinamide cofactors NADH (β -nicotinamide adenine dinucleotide) or NADPH (β -nicotinamide adenine dinucleotide phosphate). The latter themselves are rather costly necessitating an enzymatic regeneration system to allow for their use in catalytic quantities. Today, a broad range of such regeneration systems are available, in principle solving this cofactor challenge^{10–13}. The resulting multi-enzyme cascades (Fig. 1) comprise up to two extra enzymes and cofactors in addition to the actual production enzyme.

Clearly, such complicated electron transport chains are difficult to optimize and lack robustness required for a chemical transformation at scale. This also gives an explanation why cofactor-dependent oxidoreductases (such as dehydrogenases or monooxygenases) are lagging behind ‘simple’ cofactor-independent enzymes (such as hydrolases, oxidases and even peroxidases) with respect to their implementation in preparative organic chemistry. A more elegant approach is to deliver the reducing equivalents directly from the cosubstrate to the oxidoreductases, thereby shortcutting the complicated natural electron transport chain. Ever since the pioneering work by Vilker and coworkers¹⁴, a range of direct regeneration approaches have been reported^{15–28}.

Another issue is the fact that the majority of cosubstrates are valuable compounds themselves as they are generally highly reduced compounds, which, when applied for cofactor

regeneration, are wasted as simple electron donors and generate additional (waste) by-products.

Indeed, water would be the most attractive sacrificial electron donor but its high stability and very low oxidation potential require additional energy input to activate water as electron donor. Nature’s answer to this challenge is photosynthesis where visible light is used as thermodynamic driving force to promote water oxidation leading to the formation of molecular oxygen and biogenic reducing equivalents in form of reduced nicotinamide cofactors (NAD(P)H). Natural photosynthesis, however, is challenging to apply *in vitro*, mainly due to stability issues^{29–31}. Nevertheless, photosynthesis has inspired chemists to search for robust (inorganic) photocatalysts capable of mimicking the natural process³². A great variety of potentially useful photocatalysts have resulted from the ongoing research on the conversion of solar energy into chemical energy^{33,34}. Light-driven water splitting is a particular focus of research, resulting in many elegant biomimetic photocatalytic systems, with titanium dioxide being the most widely used photocatalyst³⁵. TiO₂ doped with noble metals and non-metal elements, such as N, C and S, have also been extensively studied. However, to the best of our knowledge, utilization of these reducing equivalents to promote (bio)catalytic redox reactions has not been reported yet. It is however worth mentioning here that TiO₂-generated H₂O₂ has been used to promote peroxidase-catalysis in biosensor applications^{36–38}.

Here we use light-driven and TiO₂-promoted water oxidation to drive biocatalytic redox reactions. As a model transformation, we have chosen the stereospecific reduction of conjugated C=C-double bonds catalysed by old yellow enzymes. The feasibility of this photoenzymatic reaction scheme is demonstrated.

Results

Photobiocatalytic reduction reactions. To demonstrate the feasibility of the envisaged combination of photocatalytic water oxidation and enzymatic redox reactions, we chose the asymmetric reduction of conjugated C=C-double bonds as a model reaction (Fig. 2). As photocatalyst, a system formed by gold nanoparticles deposited on TiO₂, reported as an active catalyst

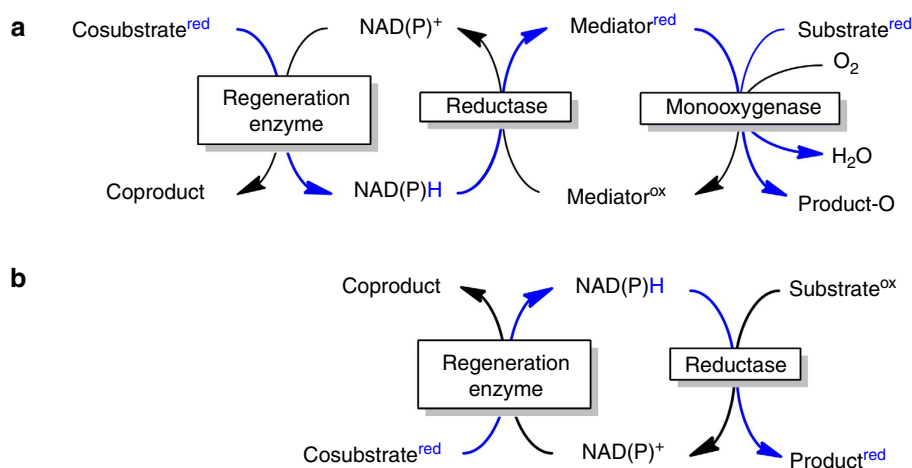


Figure 1 | Schematics of electron transport chains. Representation of the traditional electron transport chain to supply monooxygenases (a) and reductases (for example, alcohol dehydrogenases or old yellow enzymes) (b) with reducing equivalents. In both cases, a high-energy content cosubstrate is oxidized by a regeneration enzyme thereby regenerating the reduced nicotinamide cofactor (NADH or NADPH). The latter delivers two electrons either directly to the production enzyme (for example, alcohol dehydrogenases, old yellow enzymes but also some monooxygenases) or (as in case of many monooxygenases) indirectly via an electron mediator system (such as flavins or FeS-proteins). The flow of electrons is shown in blue. Typical examples for regeneration enzymes comprise formate dehydrogenase, alcohol dehydrogenase, glucose dehydrogenase and so on oxidizing the respective cosubstrates such as formic acid, ethanol/isopropanol or glucose.

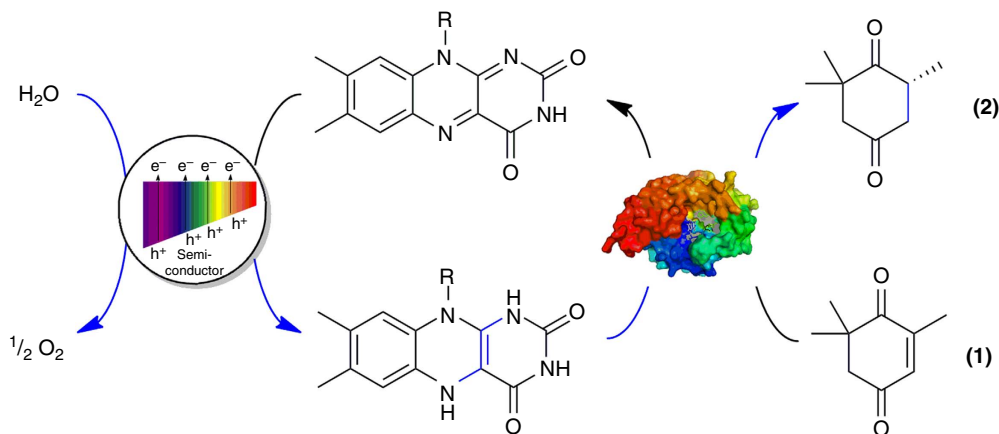


Figure 2 | The proposed photobiocatalytic reduction system. TiO₂-based photocatalysts mediate the light-driven oxidation of water yielding O₂ as sole by-product. The reducing equivalents liberated are transferred via flavin mediation to the active site of the oxidoreductase (here: the old yellow enzyme homologue from *Thermus scotoductus* SA-01).

for the photochemical water splitting reaction³⁹, was chosen. The model biocatalyst was the old yellow enzyme homologue from *Thermus scotoductus* SA-01 (*TsOYE*)^{40,41}, catalysing the stereoselective *trans*-hydrogenation of Ketoisophorone (**1**) to (*R*)-Levodione (**2**) (Fig. 2). Flavin mononucleotide (FMN) played a dual role as an enzyme prosthetic group and (photoexcitable) redox mediator delivering electrons from the photocatalyst to the enzyme.

Upon irradiating the catalytic components indicated in Fig. 2: Au-TiO₂ (10 gl⁻¹), FMN (200 μM) and *TsOYE* (15.2 μM) with UV light (Hg-doped Xe-lamp, 200 W) at 50 °C we were pleased to observe conversion of Ketoisophorone (**1**) (10 mM) into (*R*)-Levodione (**2**) (66% conversion, 86% ee after 6 h).

If the reaction was carried out in the dark and in the presence of H₂ only, 26% conversion and racemic product was obtained indicating that the cofactor is reduced as suggested in Fig. 2 and not (or only to a minor extent) by molecular H₂.

It is also worth mentioning that using stoichiometric amounts of NADPH (in the darkness under otherwise identical conditions) resulted in significantly lower yield (49% as compared with 66%). This may unambiguously be explained by the aerobic reaction conditions. Flavoproteins are known to be reoxidized in the presence of molecular oxygen. This competing uncoupling of the flavin reduction from substrate reduction was not compensated in the presence of stoichiometric amounts of NADPH, whereas the photocatalytic approach continuously provided *TsOYE* with reducing equivalents.

To prove that indeed water serves as sacrificial electron donor, qualitative experiments were carried out using labelled water (H₂¹⁸O) as reaction medium under standard reaction conditions. Gratifyingly, ¹⁸O₂ could be identified by GC/MS in the gas phase, thus strongly supporting our initial hypothesis.

The high enantiomeric purity of the product suggested a biocatalytic reduction reaction by *TsOYE* and FMN. However, also direct reduction of Ketoisophorone (**1**) at the photocatalyst surface (Au-TiO₂) and/or by free reduced FMN (FMNH₂) may occur. Indeed, performing the experiment described above in the absence of *TsOYE* and FMN gave essentially identical conversion but formed the racemic product.

Overall, these results suggest that Au-TiO₂-catalysed oxidation of water was the overall rate-limiting step in the photoenzymatic reduction of Ketoisophorone (**1**).

Kinetic studies. Aiming at further understanding of the catalytic mechanism, we performed a range of experiments systematically

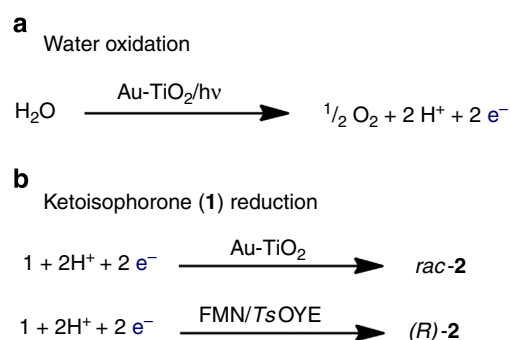


Figure 3 | Proposed elementary steps of the photoenzymatic reduction of ketoisophorone. (a) The photocatalyst (Au-TiO₂) oxidises water yielding O₂ and electrons in the valence band of the photocatalyst. (b) These electrons can be transferred to the substrate directly (non-enzymatic step, yielding racemic product) or via FMNH₂ to the enzyme (resulting in enantiomerically pure product).

varying the catalysts concentrations. Particularly, we aimed at understanding the apparent competition between *TsOYE*-catalysed and non-enzymatic reduction of Ketoisophorone (Fig. 3).

In a first set of experiments, we varied the concentration of photocatalyst under otherwise identical conditions. As shown in Fig. 4, the initial rate of the overall reaction directly depended on the photocatalyst concentration applied. The individual time courses are given in the Supplementary Fig. 1. The apparent saturation kinetics (leveling off of the curve at high photocatalyst concentrations) may be explained by the decreasing optical transparency of the reaction medium leading to suboptimal illumination of the TiO₂-particles. Alternatively, this saturation behaviour may indicate Michaelis–Menten-type saturation of the enzyme with FMNH₂ as ‘cosubstrate’ However, the formal specific activity of *TsOYE* determined at the highest TiO₂ concentration applied in Fig. 4 corresponds to 0.1 U mg⁻¹, which is somewhat lower than the reported activity (albeit under optimized reaction conditions)^{40,41}, indicating that the full catalytic potential of *TsOYE* is not fully exploited here (*vide infra*). Varying the biocatalyst concentration, however, had no significant influence on the initial rate of the reaction (Fig. 5). We like to interpret these observations as the photocatalytic water oxidation reaction being overall rate limiting. Still, the enzyme concentration had a marked influence on the optical purity of the Levodione (**2**) product. As shown in Fig. 5, racemic product was

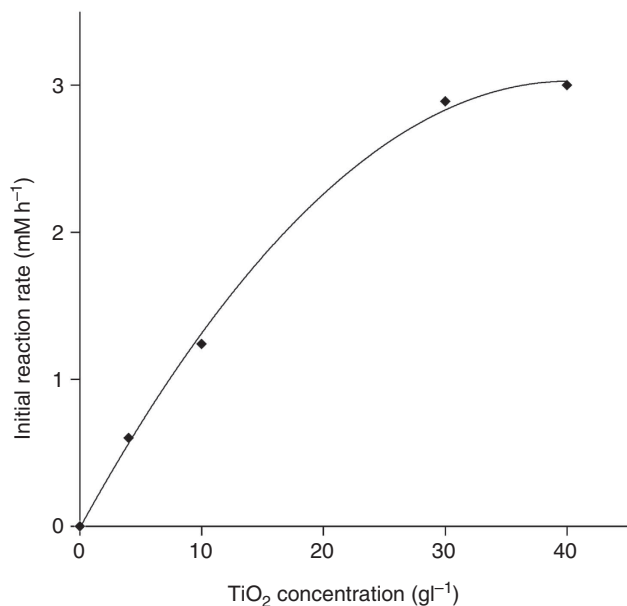


Figure 4 | Effects of photocatalyst concentration. Influence of the photocatalyst concentration on the initial rate of the photoenzymatic reduction of Ketoisophorone. Reaction conditions: 2.5 ml buffer (50 mM MOPS, pH 7.5, 10 mM CaCl₂) containing 10 mM Ketoisophorone; 200 μM FMN; 20 mg lyophilized TsOYE preparation (15.2 μM TsOYE); 323 K.

formed in the absence of TsOYE, whereas, already in the presence of relatively small concentrations of the enzyme, significant optical purities were observed. At saturation concentrations, the optical purities observed (94% ee) were essentially identical to those observed under natural conditions^{40,41}. Hence, we conclude that the reducing equivalents provided by the photocatalyst are more efficiently used by the biocatalyst (leading to highly enantiomerically pure product) than for spontaneous chemical reduction.

It is worth mentioning that the photocatalyst could be reused at least twice without appreciable activity reduction (Supplementary Fig. 2).

Towards visible light-driven reactions. As mentioned above, the Au-TiO₂-catalysed oxidation of water appeared to be the overall rate-limiting step. Therefore, we hypothesized that other dopants such as transition metal oxides easily changing their valence might be more efficient water oxidation catalysts. In this respect, V(V) oxide may be a suitable cocatalyst since V⁵⁺ can coordinate water to form hydroxyl radicals bonded to the vanadium and thereby catalyse water oxidation. In fact, substituting the previously used Au-TiO₂ by V-TiO₂ resulted in a significantly increased overall reaction rate (more than two times faster under otherwise identical conditions) and resulted in full conversion of the starting material into (*R*)-Levodione (**2**) at slightly decreased optical purity of the product (80% ee). Again, control experiments in the absence of the biocatalyst resulted in comparable yields albeit forming racemic product, while no product formation was observed in the dark (in the presence and absence of H₂). Representative progression curves for Au-TiO₂- and V-TiO₂-catalysed water oxidation to promote TsOYE-catalysed reduction reactions are shown in Fig. 6.

Even more interestingly about V-TiO₂ is its water oxidation activity in the visible-light range. Ideally, photoredox biocatalytic reactions would be performed under sunlight irradiation. Therefore, we also investigated V-TiO₂ as regeneration catalyst for TsOYE in the presence of visible light using polychromatic light

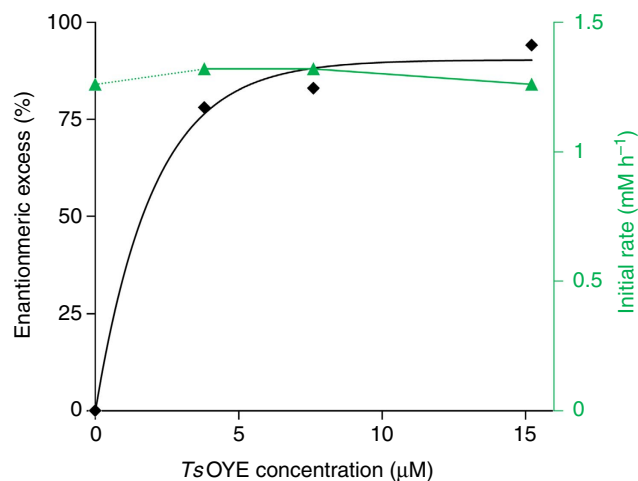


Figure 5 | Effects of biocatalyst concentration. Influence of the biocatalyst concentration on initial rate (green triangle) and enantioselectivity (black diamond) of the photoenzymatic reduction of Ketoisophorone. Reaction conditions: 2.5 ml buffer (50 mM MOPS, pH 7.5, 10 mM CaCl₂) containing 10 mM Ketoisophorone; 200 μM FMN lyophilized TsOYE preparation; 10 gl⁻¹ Au-TiO₂; 323 K. Reaction without enzyme: 2.5 ml buffer (50 mM MOPS, pH 7.5, 10 mM CaCl₂) containing 10 mM ketoisophorone.

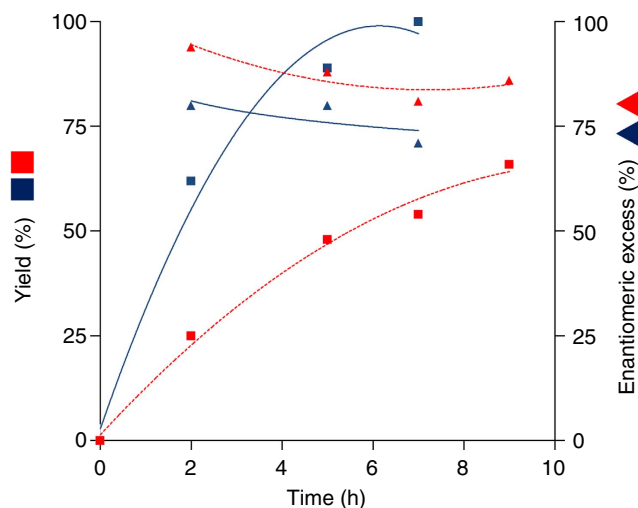


Figure 6 | Time courses of reactions. Comparison of the time courses of Au-TiO₂ (red symbols) and V-TiO₂ (blue symbols)-driven reductions of Ketoisophorone with respect to yield (squares) and optical purity of the product (triangles). Reaction conditions: 2.5 ml buffer (50 mM MOPS, pH 7.5, 10 mM CaCl₂) containing 10 mM Ketoisophorone; 200 μM FMN; 15.2 μM TsOYE; 10 gl⁻¹ photocatalyst; 323 K.

of wavelengths longer than 385 nm under otherwise identical conditions to the experiments reported before. Under these conditions, very promising results of more than 50% yield of Levodione (at optical purities greater than 85% ee) were obtained within 9 h reaction time.

Discussion

With this proof-of-concept study, we have demonstrated for the first time that catalytic water oxidation mediated by robust TiO₂ semiconductors can be productively coupled to oxidoreductases achieving photobiocatalytic redox reactions. Thereby, two

emerging technologies with great potential for the chemistry of the 21st century have been successfully combined to create a new, promising discipline of Green Chemistry. Admittedly, the technology is in its infancy, but further mechanistic studies will reveal optimal reaction conditions and expand this concept to other biocatalytic redox transformations. We are convinced that the concept introduced here will significantly have an impact on redox biocatalysis.

Methods

Materials. Ketoisophorone, FMN, HAuCl_4 , Calcium Chloride and MOPS were purchased from Sigma-Aldrich; Aerioxide TiO_2 P 25 was purchased from Degussa (Evonik).

Photocatalyst preparation. Au- TiO_2 (1% Au) and V- TiO_2 (1% V) were prepared according to literature procedures^{39,42}. An exemplary TEM picture of the photocatalyst preparation is shown in Supplementary Fig. 3.

Enzyme production. *TsOYE* was purified according to a previously published procedure⁴¹. *Escherichia coli* BL21 (DE3) harbouring a pET22b(+) plasmid containing the gene encoding for *TsOYE* was cultivated in minimal medium at 30 °C. Upon reaching an OD_{600} of 0.45, *TsOYE* expression was induced by the addition of 0.1 mM IPTG (isopropyl β -D-1-thiogalactopyranoside) followed by further cultivation for 12 h. The cell pellet (resulting from centrifugation and washing with 20 mM MOPS buffer pH 7.5 containing 5 mM CaCl_2) was suspended in fresh washing buffer. Cell disruption was achieved by two passages through the French press followed by heat treatment at 70 °C for 1.5 h. After centrifugation, to remove insoluble cell debris and denaturated proteins, a bright yellow solution of *TsOYE* (more than 95% pure as estimated by SDS-page analysis) was obtained. For storage, this solution was freeze dried and remained more than 90% of its original activity upon storage at 4 °C for at least several months.

Standard reaction. The photoreactor was a cylindrical quartz vessel (Vicilab[®]) (48 ml total volume). A typical reaction was performed as follows: 25 mg Au- TiO_2 (1% Au) or V- TiO_2 (1% V); 200 μM FMN and 10 mM CaCl_2 were added sequentially to 2 ml MOPS buffer pH 7. The whole system was purged with an argon flow for at least 30 min before irradiation to ensure the absence of oxygen in the system. Afterwards, ketoisophorone (10 mM final concentration) and 20 mg of lyophilized *TsOYE* dissolved in 500 μl MOPS buffer were added with a syringe to the reaction mixture. The system was heated until 323 K. Then, it was irradiated for certain time using 200 W Xenon-doped mercury lamp (Hamamatsu Lightningcure LC8, spot light source L9566-04; DB 4719 input 100–240 V 300 VA 50–60 Hz), without filter, 1 cm distance. For visible light experiment, the output of 200 W Xenon-doped mercury lamp was filtered through a cutoff filter ($\lambda > 380$ nm). GC analyses were performed with a HP-5 column (30 m \times 0.320 mm) from Agilent technologies. The amount of product was quantified from the peak areas using an external standard methodology.

Reduction reaction by H_2 . The reaction was carried out as follows: a mixture of 12.5 mg Au- TiO_2 (1% Au) or V- TiO_2 (1% V), Ketoisophorone (10 mM final concentration) were added sequentially to 1.25 ml MOPS buffer pH 7. The resultant suspension was bubbled with H_2 during 10 min, afterwards the autoclave was pressurized until 5 bars of H_2 . The system was heated at 323 K and kept it under vigorous stirring. GC analyses were performed with a HP-5 column (30 m \times 0.320 mm) from Agilent technologies. The amount of product was quantified from the peak areas using an external standard methodology.

H_2^{18}O experiment. The photoreactor was a cylindrical quartz vessel (Vicilab) (48 ml total volume) with an inlet and outlet with independent valves and equipped with a manometer to determine the pressure. The reaction was performed as follows: 12.5 mg V- TiO_2 (1% V); 200 μM FMN; 10 mM CaCl_2 and 10 mg of lyophilized *TsOYE* were added sequentially to 1 ml MOPS buffer pH 7 prepared with H_2^{18}O . The whole system was purged with an argon flow for at least 30 min before irradiation to ensure the absence of oxygen in the system. Afterwards, ketoisophorone (10 mM final concentration) was added with a syringe to the reaction mixture. The system was heated until 323 K. Then, it was irradiated for certain time using 200 W Xenon-doped mercury lamp (Hamamatsu Lightningcure LC8), 1 cm distance. $^{18}\text{O}_2$ evolved in course of the reaction was detected by GC/MS.

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Author contributions

M.M. designed and performed photobiocatalytic experiments, analysed the results and contributed to the preparation of the manuscript. S.G. prepared and characterized the biocatalyst used. S.I. and I.W.C.E.A. contributed to the analysis of the experiments and writing. A.C. and F.H. conceived the concept, designed the experiments, proposed the mechanism, supervised the work and mainly wrote the manuscript.

Additional information

Supplementary Information accompanies this paper at <http://www.nature.com/naturecommunications>

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