Supplementary Information

Boosting photobioredox-catalysis by morpholine electron donors under aerobic conditions

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CONTENTS

EXPERIMENTAL DETAILS

General considerations

All chemicals were obtained from commercial suppliers (highest purity available) and used without further purification unless otherwise stated.

Buffers / electron donor solutions

Photochemical experiments were carried out in one of the following solutions, which were prepared in dH_2O and the pH set to 7.5 with 1 M NaOH or 1 M HCl:

A) 100 mM MOPS buffer (3-(N-Morpholino)propanesulfonic acid, > 99.5%);

B) 100 mM Tris-HCl buffer (Tris base, > 99.0%);

C) 100 mM sodium phosphate buffer (Na_2HPO_4 / NaH_2PO_4, > 99.0%);

E) 100 mM MES buffer (2-(N-Morpholino)ethanesulfonic acid), > 99.0%);

F) 100 mM morpholine (tetrahydro-1,4-oxazine, > 99.5%).

G) 100 mM 4-methylmorpholine (> 99%)

H) 100 mM 4-(2-hydroxyethyl)morpholine (> 99%)

I) 100 mM triethanolamine (> 99%)

Growth of Bacterial Cells for Enzyme Expression and Isolation

XenB (enoate reductase from *Pseudomonas sp.*, GenBank: KF055345) was expressed in *E. coli* strain BL21(DE3) according to previously published procedures.[1, 2] The TsOYE gene from the thermophile *Thermus scotoductus* SA-01 (GenBank: CP001962) was ordered from GenScript and cloned into the pET28a(+) cloning vector utilizing the *Nde*I and *EcoR*I restriction sites[3, 4] and expressed in *E. coli* strain BL21(DE3). Lysogeny broth (LB) medium (6 mL) supplemented with ampicillin (100 μg mL^{-1}) was inoculated with pGASTON_XenB[5]. LB media supplemented with kanamycin (50 μg mL^{-1}) was inoculated with *E.coli* BL21(DE3) pET28a(+)_TsOYE. These were grown over-night at 37 ºC in an orbital shaker operated at 200 rpm. The cultures were transferred to 1 L baffled Erlenmeyer flask containing 250 mL LB/ampicillin or LB/kanamycin medium and shaken at 200 rpm and 37 ºC for approximately 2.5 h to a final optical density at 590 nm of approx. 0.6. L-rhamnose, 0.2%, was added for the induction of XenB, which was incubated for 18–22 h at 25 ºC. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 100 μM for the induction of TsOYE) and the flasks were incubated for 18–22 h at 30 ºC. Cells were harvested by centrifugation (4000 × g, 4 ºC, 15 min).

Enzyme Purification

Cell pellets were re-suspended in 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM FMN. Cells were placed on ice and sonicated using a Bandelin KE76 sonotrode connected to a Bandelin Sonoplus HD 3200 in 9 cycles (5 s pulse, 55 s break,

amplitude 50%). Cell debris were removed by centrifugation (15000 × g, 4 ºC, 45 min) and the clarified supernatants containing the polyhistidine-tagged XenB or TsOYE wild-type enzymes were loaded on a Ni^{2+}-Sepharose HP affinity column (5 mL, GE Healthcare bioscience) equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 0.5 M NaCl and 0.1 mM FMN. Enzymes were eluted in 4 column volumes within a linear gradient from 25 to 250 mM imidazole in 50 mM Tris-HCl buffer, pH 7.5, containing 0.5 M NaCl and 0.1 mM FMN. Fractions containing the enzymes were identified by SDS-PAGE analysis, pooled, desalted, washed with 50 mM Tris-HCl (pH 7.5) containing 0.1 mM FMN and concentrated by ultrafiltration by using ultra centrifugal tubes with a cut-off of 10 kDa. Protein concentrations were determined by the dye-binding method of Bradford using a pre-fabricated assay (Bio-Rad) and bovine serum albumin as the calibration standard. Buffer exchange was done using Amicon Ultra Centrifugal Filters (10 kDa, 0.5 mL, Millipore) for experiments performed in buffers other than 50 mM Tris HCl (pH 7.5). All the solutions used for the TsOYE isolation were supplemented with 10 mM $CaCl_2$.

Absorption measurements

Absorption spectra were recorded in a Shimadzu spectrophotometer (UV-1800) featuring a thermo-controlled 6-cell positioner (CPS-240A).

Light Source

For the daylight lamp experiments, the lamp was placed at 15 cm (XenB experiments) distance from the plate/vial. The light intensity on the plate level was measured with an Ocean Optics Spectrophotometer (USB2000+) at 450 nm for the daylight lamp (28 μW/cm^2). The region where the light intensity remained the same was determined and the experiments performed in the area where the amount of energy was constant.

Gas Chromatography (GC) Analysis

For GC analysis, 50 μL of the reaction mixture were added to 1.5 mL Eppendorf tubes containing 150 μL ethyl acetate supplemented with 1 mM methyl benzoate as internal standard. Samples were vortexed at maximum speed (IKA Vortex 4 basic) for 30 s and centrifuged for 1 min (VWR Silverstar bench top centrifuge). The organic layer was transferred into a new 1.5 mL Eppendorf tube and dried over Na_2SO_4. After centrifugation, the supernatant was transferred to a 1.5 mL GC glass vial equipped with a 0.1 mL micro-insert and subjected to GC analysis.

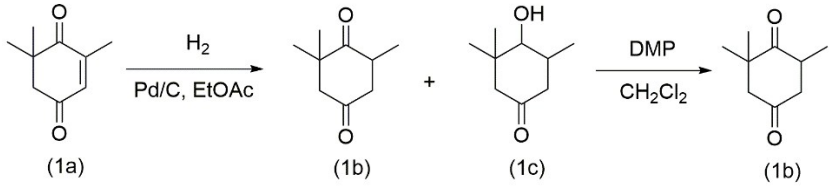
GC achiral analyses were performed with a BGB5 (30 m x 0.25 mm ID, 0.25 μm film) on a Thermo Finnigan Focus GC / DSQ II (Thermo Scientific) and GC chiral analysis, with a BGB175 column (30 m x 0.25 mm ID, 0.25 μm film) on a ThermoQuest Trace GC 2000 (Thermo Scientific). The amount of product was quantified from the peak areas using an internal standard (1 mM methyl benzoate) and corrected using

the response factor obtained from the linear regression adjust of the calibration curve, unless otherwise stated.

Determination of Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) was measured by the ABTS based assay. This assay quantifies the production of H_2O_2 in the presence of horseradish peroxidase (HRP), which uses the H_2O_2 to convert the colorless ABTS reactant to its oxidized turquoise form. The amount of H_2O_2 in the samples irradiated with the daylight lamp (300 W, Osram) containing FMN (100 µM) in 100 mM MOPS, in 100 mM Tris-HCl or in water, pH 7.5, as well as control samples without any flavin. For that, 50 µL aliquots were taken over time and added to 2 mM ABTS and 5.8 U/mL HRP to a final volume of 500 µL. The absorption spectrum was recorded for each sample and the amount of H_2O_2 quantified after calibration curve with commercial H_2O_2 at 734 nm (intercept = 0.067; slope = 0.02702 ± 0.0008, Adj-R^2 > 0.99).

Synthesis of 2,2,6-trimethyl-1,4-cyclohexanedione (levodione)



Ketoisophorone **(1a)** (500 mg, 3.3 mmol, 1 eq.) was placed in a 3-neck-round bottomed flask and ethyl acetate (5 mL) was added. 75 mg of Palladium on charcoal (75 mg, 10%$_{w/w}$ were added to the mixture and the mixture was evacuated and purged with argon three times. After the last purging step, the evacuated state was maintained and the Schlenk-line tube was exchanged with a hydrogen balloon. The apparatus was flushed with H_2 and the mixture was stirred overnight. Upon reaction completion (monitored by GC/MS analysis), the reaction mixture was filtered over Celite and the solvent was removed under reduced pressure (428 mg crude yield). The crude product (100 mg) was mixed with Dess-Martin-Periodinane (DMP, 300 mg, 0.7 mmol, 1.06 eq. *vs* **1c**) in CH_2Cl_2 and stirred for 15 minutes at room temperature. After completion (monitored by TLC), the reaction was extracted with a 10%$_{w/v}$ $Na_2S_2O_3$ solution followed by extraction with a saturated solution of NH_4Cl. The aqueous layers were re-extracted with CH_2Cl_2 and the resulting organic layer was dried over Na_2SO_4 and filtered through silica. The solvent was removed under reduced pressure resulting in a beige solid (**1b**, 42 mg).

^{1}H-NMR (400 MHz, $CDCl_3$): δ(ppm)= 1.14 (s, 3H), 1.17 (d, 3H, J^3= 6.5 Hz), 1.21 (s, 3H), 2.30- 2.42 (m, 1H), 2.54 (d, 1H, J^2= 15.5 Hz), 2.72- 2.83 (m, 1H), 2.96- 3.08 (m, 1H). Spectrum in accordance to the literature.[6]

^{13}C-NMR (100 MHz, $CDCl_3$): δ(ppm)= 14.6, 25.6, 26.5, 39.8, 44.2, 44.9, 52.8, 208.0, 214.1. Spectrum in accordance to the literature.[7]

SUPPLEMENTARY FIGURES

Stability of TsOYE and XenB in Tris-HCl at 30°C.

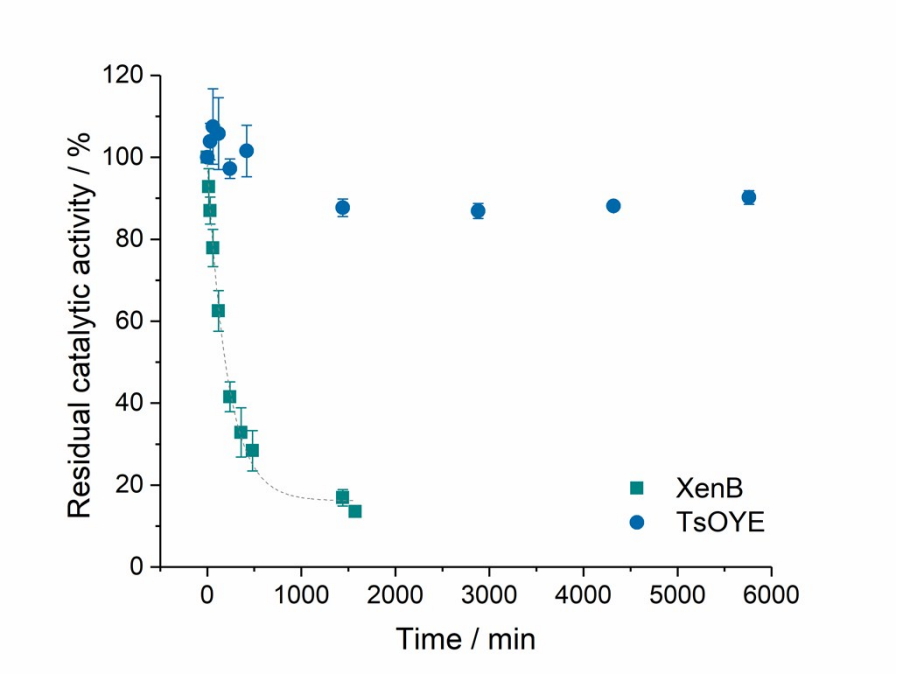


Fig S1. Stability of XenB (■, 10 μM) and TsOYE (●, 10 μM) in the presence of 100 μM FMN in 100 mM Tris-HCl buffer, pH 7.5, at 30 ºC. Enzyme activity was measured over time by the decrease in the NADPH absorption at 340 nm.

GC analysis of the photoenzymatic reduction of ketoisophorone (1a)

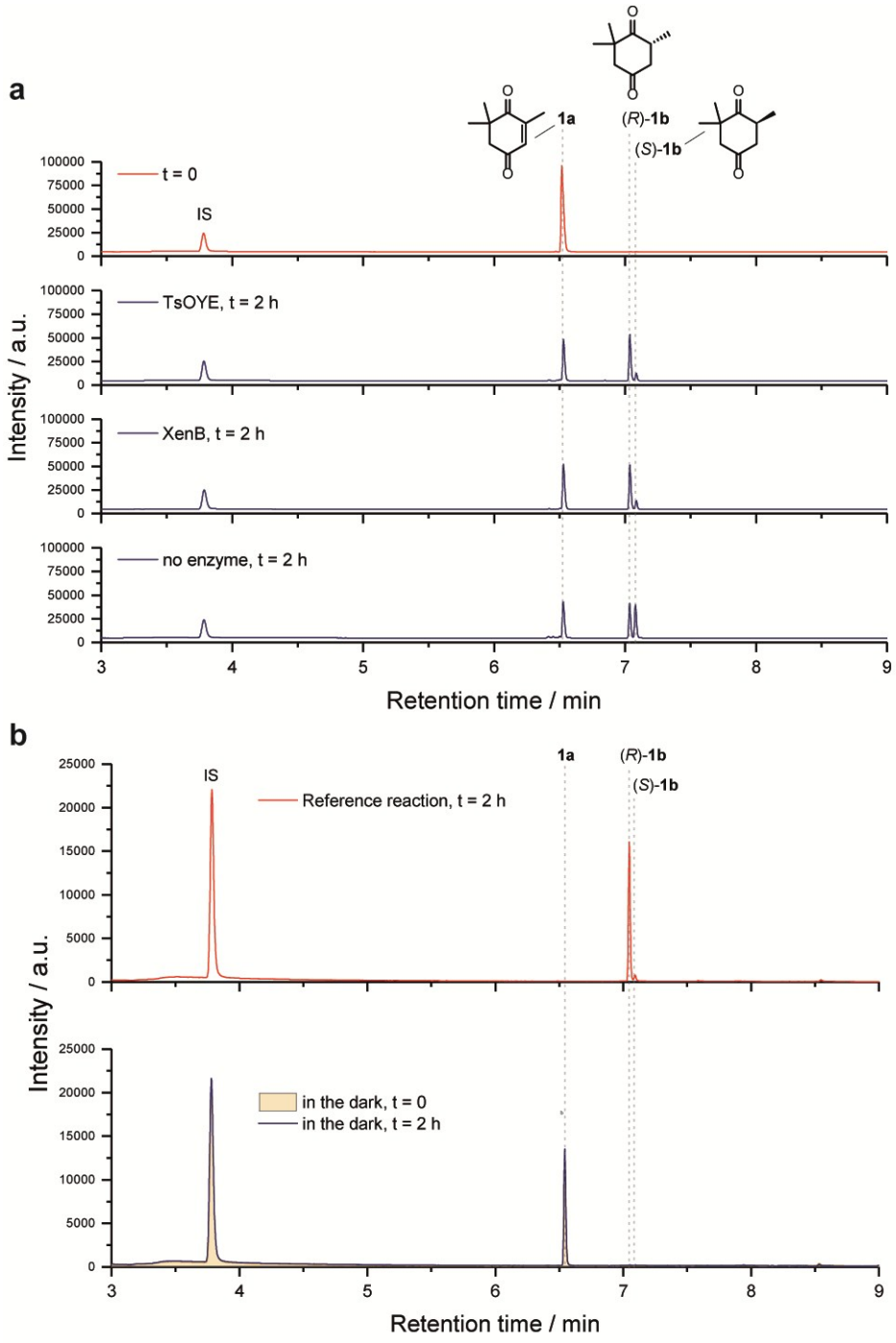


Fig S2. (a) GC analysis of the reaction mixture of the photoenzymatic reduction of ketoisophorone (**1a**, 10 mM) in the presence of TsOYE (15 µM) and XenB (15 µM) and FMN (200 µM) in 50 mM MOPS pH 7.5 after 2 h irradiation with the daylight lamp (300 W, Osram). (b) GC analysis of the reference reaction and the control experiment in the dark employing 1 mM ketoisophorone. IS: internal standard (1 mM methyl benzoate).

Effect of the concentration of XenB, FMN and $NADP^+$ on the product GC-yield and *ee*

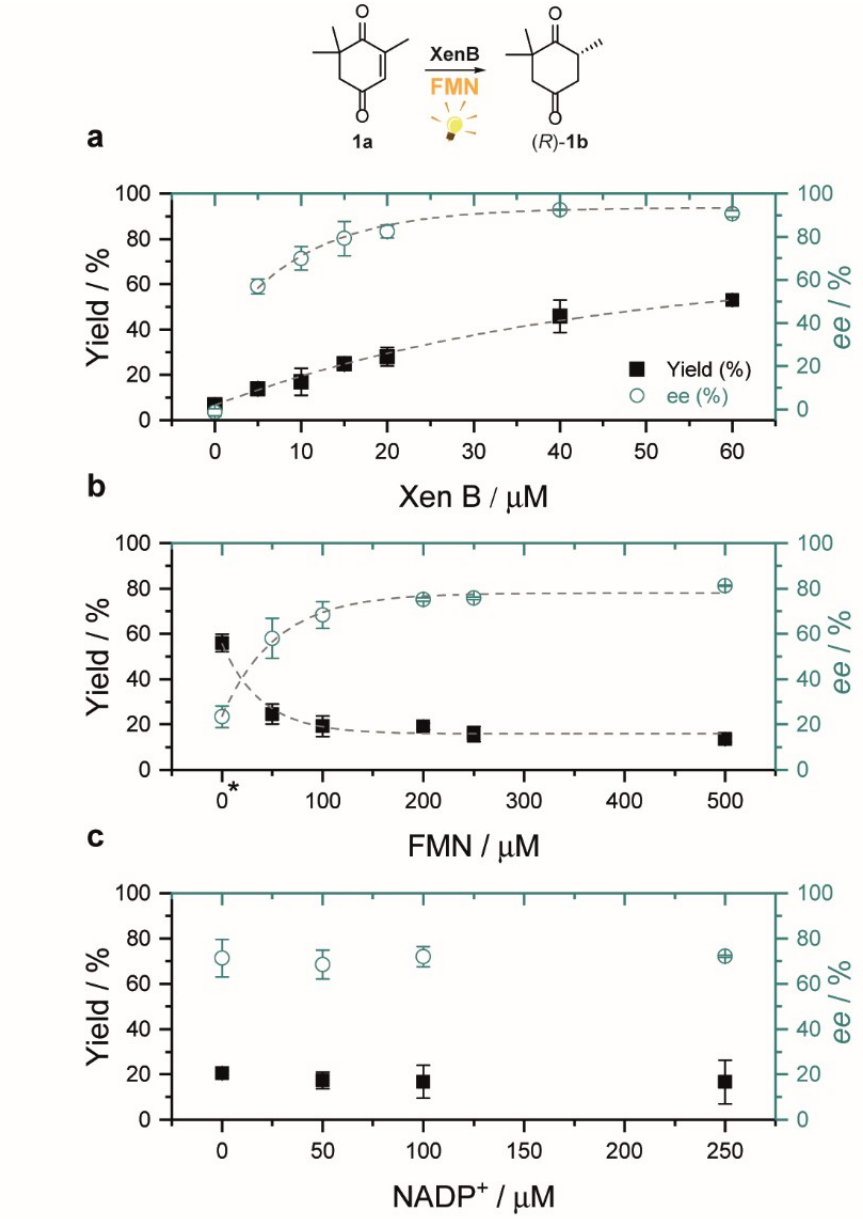


Fig S3. Photoinduced enzymatic reduction of ketoisophorone (1 mM) in 100 mM MOPS (pH 7.5). (a) Effect of the enzyme concentration, XenB (0 – 60 µM) in the presence of 100 µM FMN, (b) effect of the concentration of FMN (0^* – 500 µM) employing 10 µM XenB and (c) effect of the concentration of $NADP^+$ (0 – 250 µM) employing 10 µM XenB in the presence of 100 µM FMN. Irradiation time: 0.5 h with the daylight lamp (300 W, Osram). XenB was supplemented with FMN for storage to improve stability, therefore, it is not possible to determine the yield in FMN free condition and FMN = 0^* represents no addition of extra FMN. **Note:** Yield refers to GC-yield and is based on calibrated GC-data.

Effect of the concentration of XenB on the reaction performance

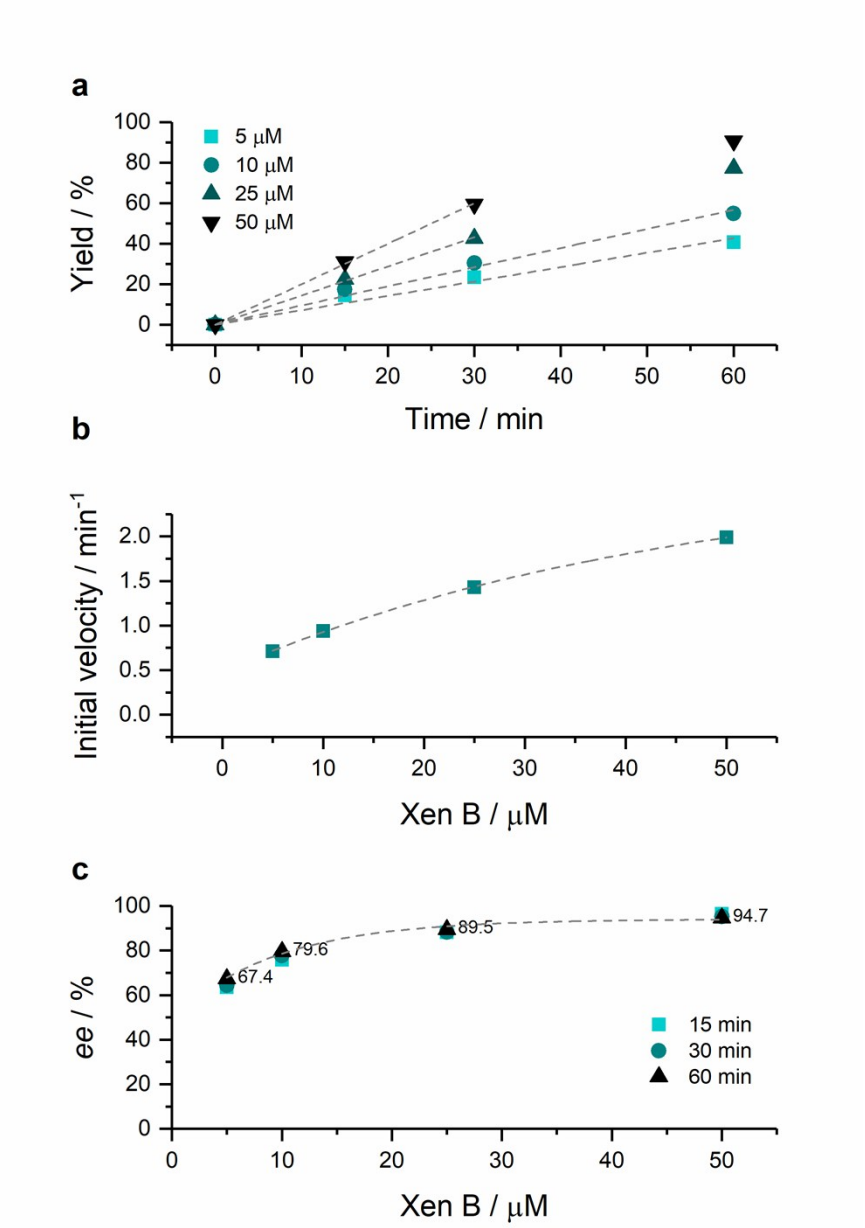


Fig S3. Photoinduced enzymatic reduction of ketoisophorone (**1a**, 1 mM) in 100 mM MOPS pH 7.5. (a) Effect of the enzyme concentration, XenB (0 – 50 µM), on the yield over time. (b) effect of the enzyme concentration on the initial velocity (min^{-1}). (c) Effect the enzyme concentration, XenB (0 – 50 µM), on the enantiomeric excess over time. Reactions in were performed in the presence of 100 µM FMN. Irradiation source: daylight lamp (300 W, Osram). **Note:** Yield refers to GC-yield and is based on calibrated GC-data.

Rate of the photoinduced enzymatic reduction of ketoisophorone (1a) by XenB

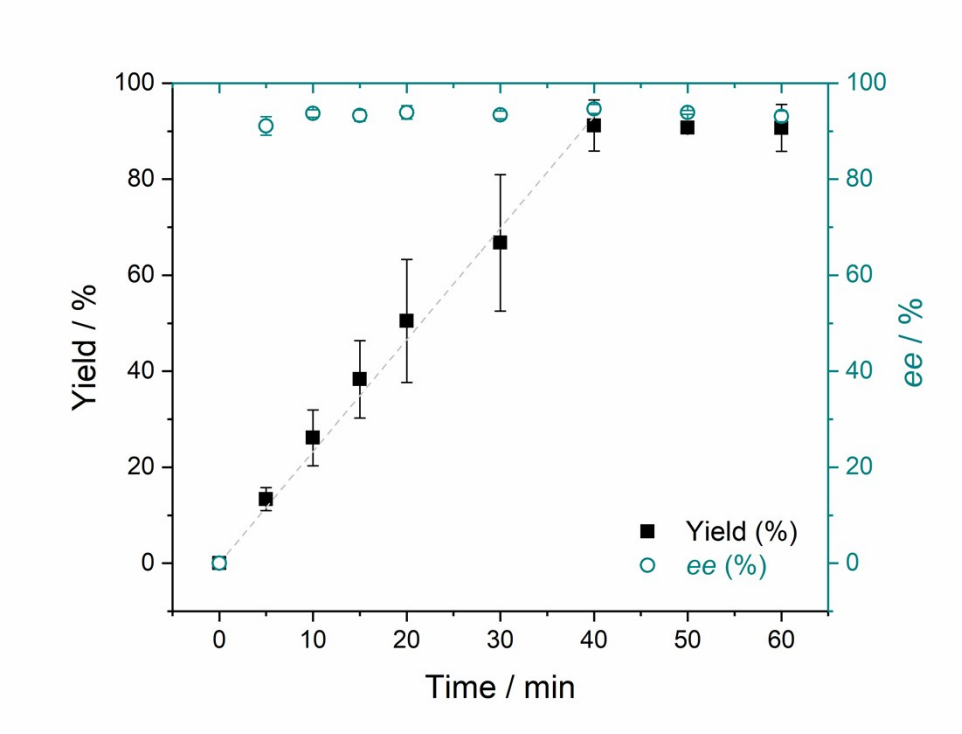


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Fig S5. Photoinduced enzymatic reduction of ketoisophorone (1 mM) employing XenB (40 µM) as biocatalyst and FMN (100 µM) as mediator. Product yield and *ee* over time of the reaction mixture irradiated with the daylight lamp (300 W, Osram) in 100 mM MOPS (pH 7.5). Linear regression slope (intercept = 0): 2.33 ± 0.06 (Adj-R^2 = 0.996).
Product formation rate: 1.84 ± 0.02 mM h^{-1}. Initial turnover frequency (TOF): 157.5 h^{-1}.
Note: Yield refers to GC-yield and is based on calibrated GC-data.

Formation of H_2O_2

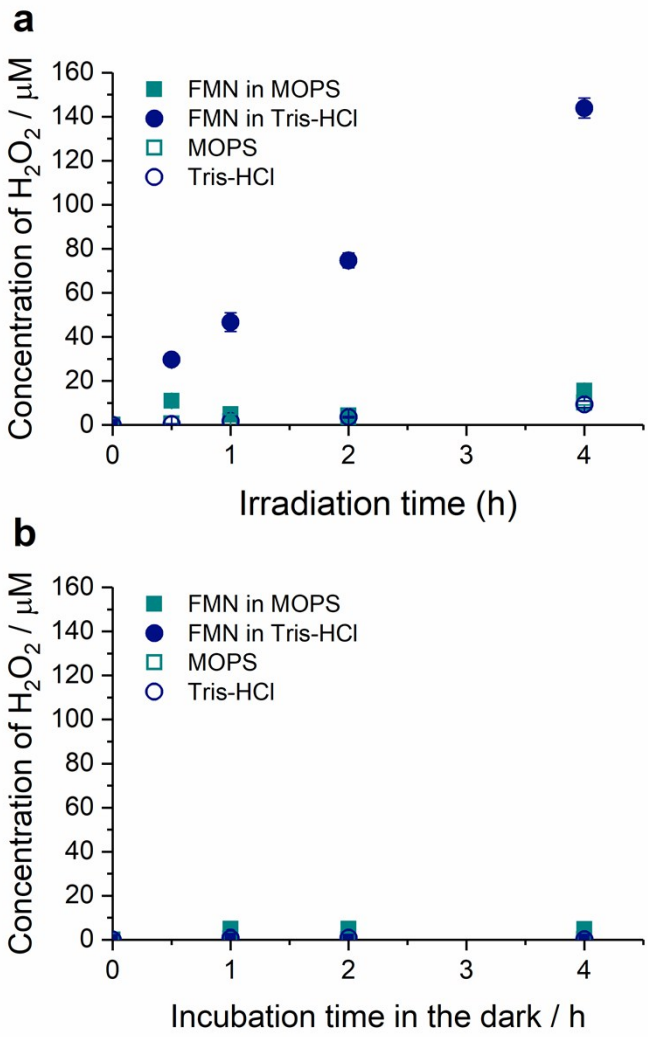

Fig S6. Analysis of the hydrogen peroxide (H_2O_2) concentration in samples containing FMN (100 µM) (a) under irradiation or (b) incubated in the dark. Light source: daylight lamp (300 W, Osram). The concentration was determined by the ABTS/HRP system (λ = 734 nm) using the calibration curve published in our previous work: slope = 0.02702 ± 0.0008 (intercept = 0.067), Adj-R^2 > 0.99.[8] Note: data between 50 – 100 µM show absorbance intensity above the Beer-Lambert law. Samples with larger response than permitted by the calibration curve were diluted for the measurement.

Effect of the electron donor on the absorption spectra of FMN

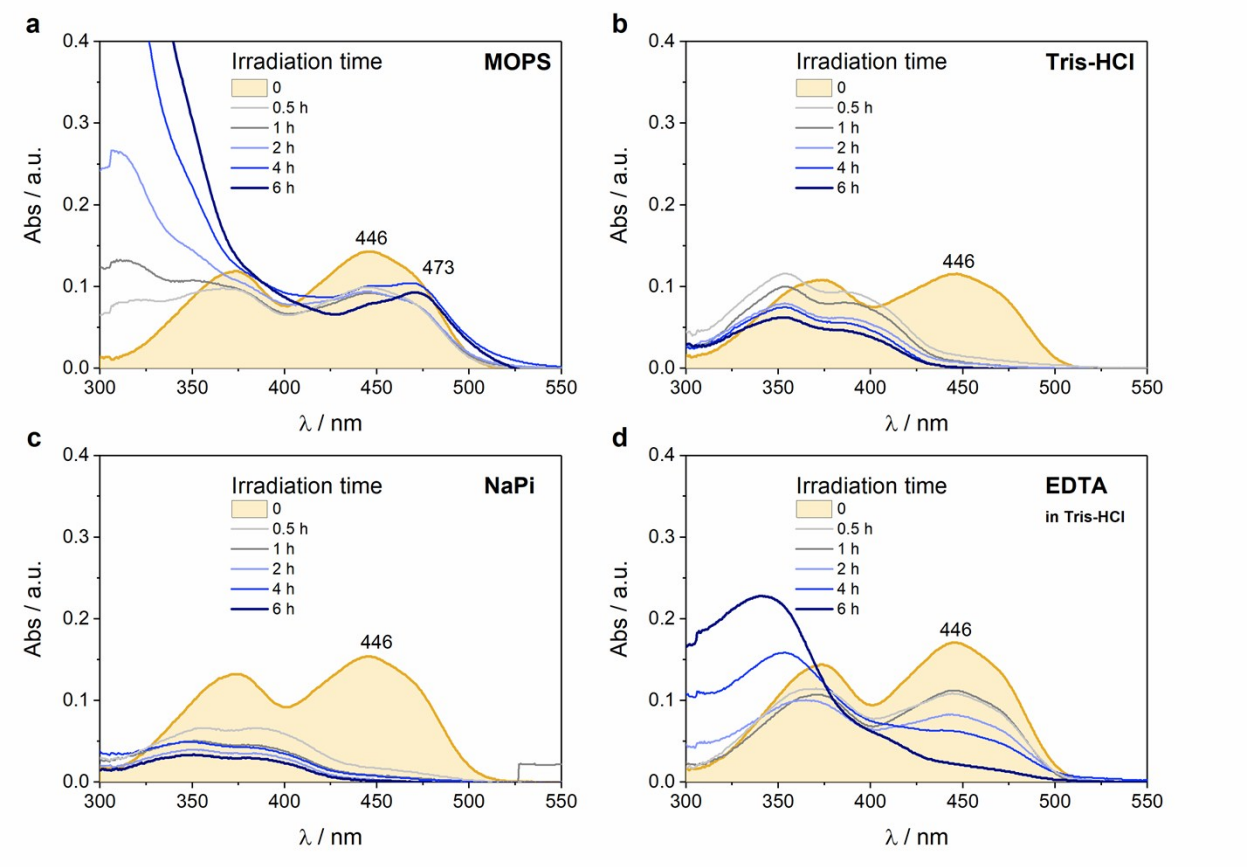

Fig. S7. Effect of the electron donor (ED) on the absorption spectra of 100 μM FMN upon light irradiation. The samples were irradiated over time with the Daylight lamp (300 W, Osram) in the following solutions (pH 7.5): (a) 100 mM MOPS buffer, (b) 100 mM Tris-HCl, (c) 100 mM phosphate buffer (NaPi) and (d) 25 mM EDTA in 100 mM Tris-HCl buffer.

Effect of the electron donor on the absorption spectra of FMN under turnover condition

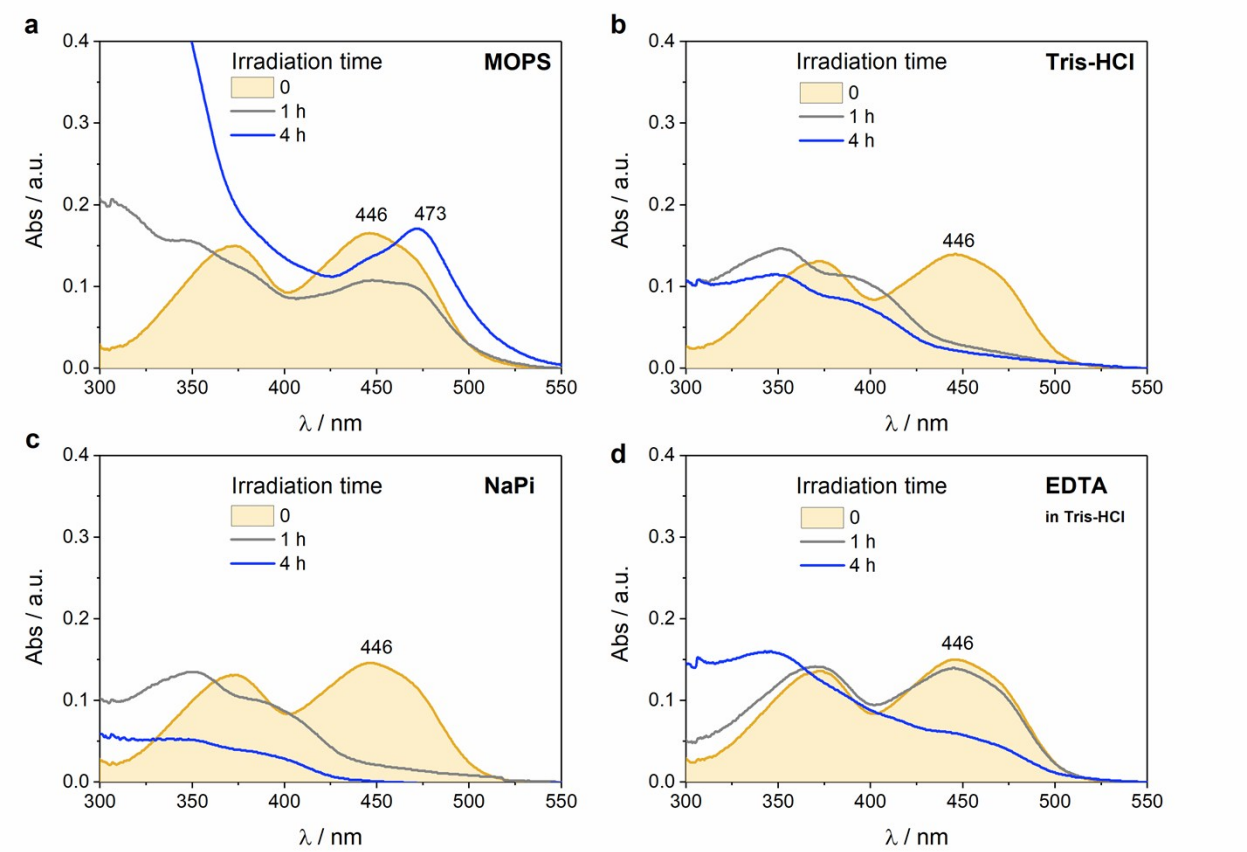

Fig. S8. Effect of the electron donor (ED) on the absorption spectra of 100 μM FMN during the photoinduced enzymatic reduction of ketoisophorone (**1a**, 1 mM) to levodione (**1b**) with XenB (10 μM). Samples were irradiated over time with the Daylight lamp (300 W, Osram) in the following (pH 7.5): (a) 100 mM MOPS buffer, (b) 100 mM Tris-HCl, (c) 100 mM phosphate buffer (NaPi) and (d) 25 mM EDTA in 100 mM Tris-HCl buffer.

Effect of the MOPS concentration on the absorption spectra of FMN

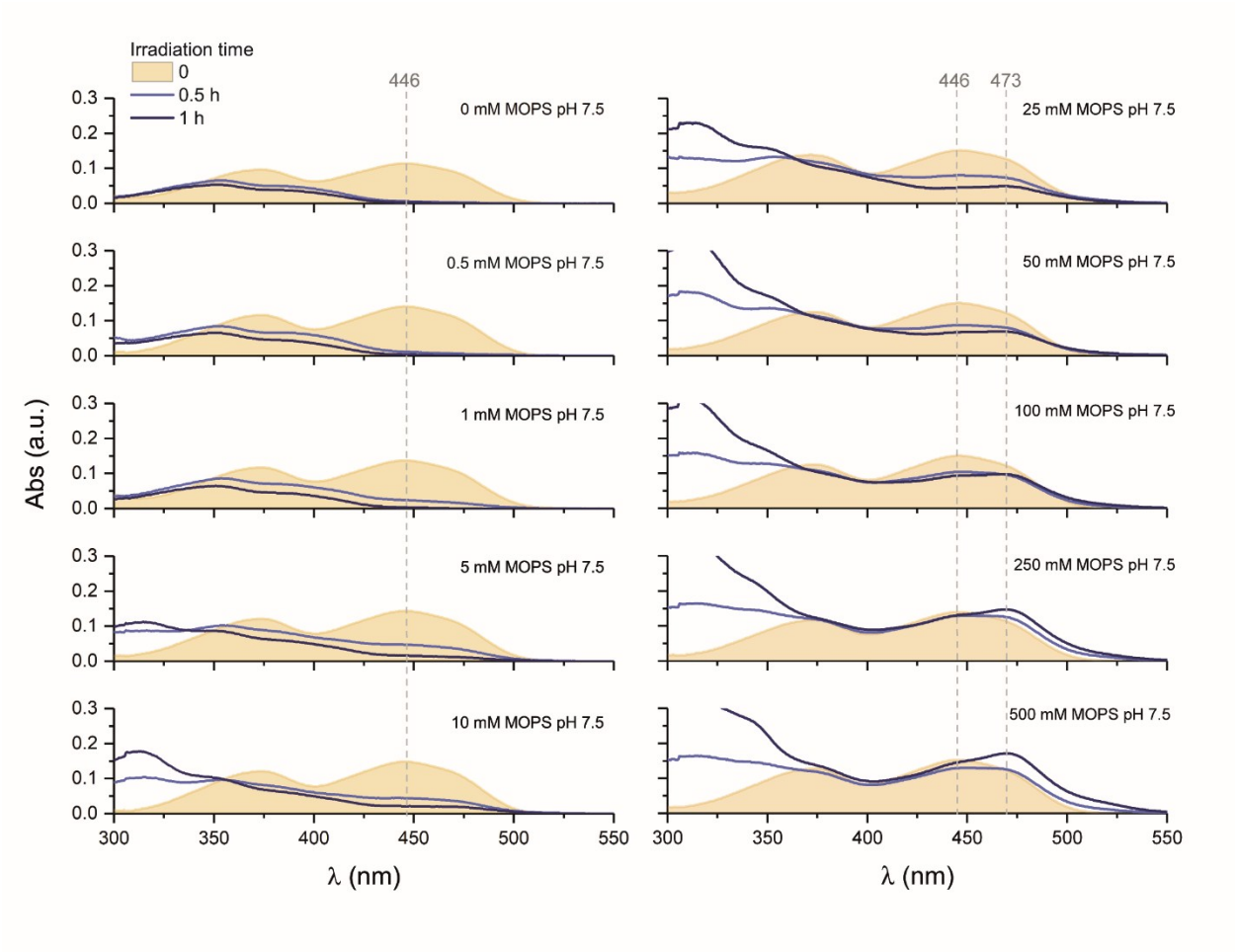

Fig. S9. Effect of the concentration of MOPS (0 – 500 μM, pH 7.5) on the absorption spectra of FMN (100 μM) at different irradiation time points (0 h, 0.5 h, and 1 h) with a Daylight Lamp (300 W, Osram).

Effect of the MOPS concentration on the absorption spectra of FMN under turnover condition

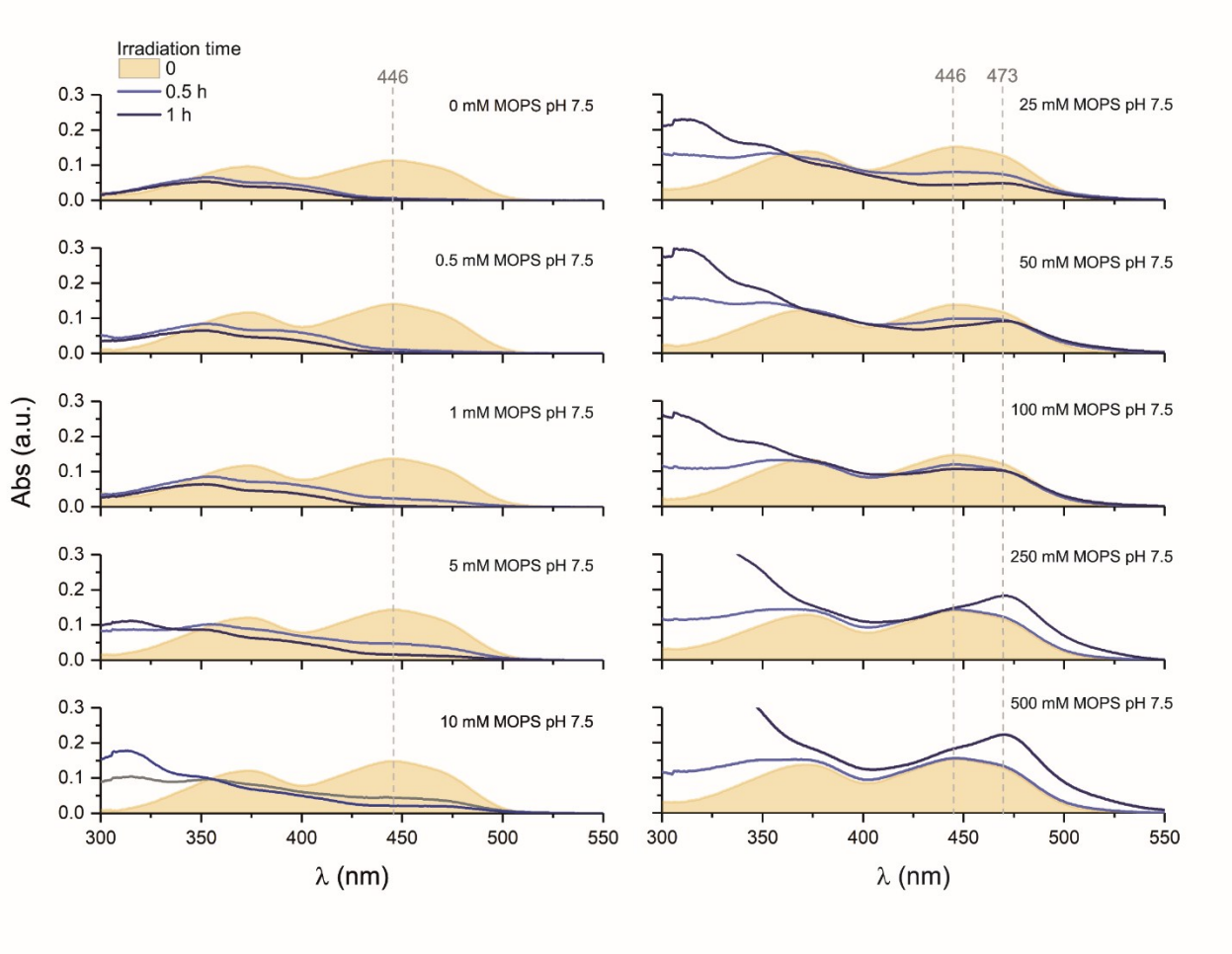

Fig S10. Effect of the concentration of MOPS (0 – 500 μM, pH 7.5) on the absorption spectra of FMN (100 μM) in the presence of XenB (10 μM) and ketoisophorone (1 mM) at different irradiation time points (0 h, 0.5 h, and 1 h) with a Daylight Lamp (300 W, Osram).

SUPPLEMENTARY TABLES

Table S1. Enzyme activity (U mg^{-1}) in 100 mM MOPS and 100 mM Tris-HCl (pH 7.5) at 30 ºC.

Enzyme	Substrate	Activity (U mg^{-1})		k_{cat} (s^{-1})	
		MOPS	Tris-HCl	MOPS	Tris-HCl
XenB	Ketoisophorone	7.9 ± 2.2	8.4 ± 1.8	5.0 ± 1.4	5.3 ± 1.1
TsOYE	Ketoisophorone	0.3 ± 0.1	0.5 ± 0.3	0.2 ± 0.1	0.3 ± 0.2

Data is reported as $\bar{x}$ ± 1SD (n=3).

Table S2. Effect of the temperature in the enzyme activity (U mg^{-1}) of TsOYE in 100 mM MOPS and 100 mM Tris-HCl (pH 7.5). Substrate: Ketoisophorone.

Temperature	Activity (U mg^{-1})	
	MOPS	Tris-HCl
30ºC	0.3 ± 0.1	0.5 ± 0.3
50ºC	0.6 ± 0.1[a]	0.7 ± 0.1[a]
65ºC	2.1 ± 0.1[a]	1.1 ± 0.3[a]

Data is reported as $\bar{x}$ ± 1SD (n=3).

Table S3. Rate constant of the substrate consumption and the product formation in the photo-induced enzymatic reduction of ketoisophorone (10 mM) in 50 mM MOPS (pH 7.5) by TsOYE and XenB.

	k (min^{-1})	
Enzyme	Substrate consumption	Product formation
TsOYE	1.13 ± 0.07	1.6 ± 0.16
XenB	1.00 ± 0.03	1.48 ± 0.38
No-enzyme	0.45 ± 0.20	0.45 ± 0.10

Data is reported as $\bar{x}$ ± 1SD (n=3), except for the data without enzyme (n = 1, error corresponds to the adjust).

Table S4. The percentage GC-yield and enantiomeric excess (*ee*) of levodione (**1b**) formation obtained in the presence of morpholines and controls.

Electron Donor	GC-yield (%)	*ee* (%)
MOPS	82.3 ± 8.3	95.3 ± 0.1
MES	86.5 ± 6.8	92.9 ± 1.4
HEMO	91.2 ± 4.1	95.8 ± 0.6
MMO	96.5 ± 0.1	93.5 ± 0.4
MO	18.3 ± 3.5	62.7 ± 6.1
EDTA-Tris/HCl	96.4 ± 0.6	94.1 ± 1.3
EDTA-NaPi	85.1 ± 2.6	71.2 ± 5.0
TEOA	95.1 ± 1.4	92.1 ± 2.6
Tris/HCl	33.1 ± 0.8	14.7 ± 1.0
NaPi	0.11 ± 0.1	n.d.

Reaction conditions: 10 μM XenB; 100 μM FMN and 1 mM **1a**; daylight irradiation for 1 h at pH 7.5. Data is reported as $\bar{x}$ ± 1SD (n=3). n.d.: not determined.

SUPPLEMENTARY REFERENCES

1. S. Milker, M. J. Fink, N. Oberleitner, A. K. Ressmann, U. T. Bornscheuer, M. D. Mihovilovic and F. Rudroff, *ChemCatChem*, 2017, **9**, 3420-3427.
2. N. Oberleitner, C. Peters, F. Rudroff, U. T. Bornscheuer and M. D. Mihovilovic, *J. Biotechnol.*, 2014, **192**, 393-399.
3. D. J. Opperman, L. A. Piater and E. van Heerden, *J. Bacteriol.*, 2008, **190**, 3076-3082.
4. D. J. Opperman, B. T. Sewell, D. Litthauer, M. N. Isupov, J. A. Littlechild and E. van Heerden, *Biochem. Biophys. Res. Commun.*, 2010, **393**, 426-431.
5. C. Peters, R. Kölzsch, M. Kadow, L. Skalden, F. Rudroff, M. D. Mihovilovic and U. T. Bornscheuer, *ChemCatChem*, 2014, **6**, 1021-1027.
6. X.-Q. Pei, M.-Y. Xu and Z.-L. Wu, *J. Mol. Catal. B-Enzym.*, 2016, **123**, 91-99.
7. A. Fryszkowska, H. Toogood, M. Sakuma, J. M. Gardiner, G. M. Stephens and N. S. Scrutton, *Adv. Synth. Catal.*, 2009, **351**, 2976-2990.
8. L. C. P. Gonçalves, H. R. Mansouri, E. L. Bastos, M. Abdellah, B. S. Fadiga, J. Sá, F. Rudroff and M. D. Mihovilovic, *Catal. Sci. Technol.*, 2019, **9**, 1365-1371.