Supporting Information

Expanding the Spectrum of Light-Driven Peroxygenase Reactions

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1. Materials

Ethylbenzene, (*R*)-1-phenylethanol, (*S*)-1-phenylethanol, acetopheone, β-NAD^+ were purchased from Sigma–Aldrich in the highest purity available and used without further purification. Formate dehydrogenase from *Candida boidinii* and the peroxygenase form *Agrocybe aegerita* were produced and purified according to previously published procedures. [1-2]

2. Reaction conditions

Unless mentioned otherwise, reactions were performed at 30℃ in 50 mM KPi buffer pH 7.0, 300 rpm, 200 mM methanol, 400 µL reaction volume in 4 mL glass vials.

The reactions were performed in a jacketed beaker with commercial LEDs (24 W) winded around (Figure S1 and Figure S2). The reactions vessels were placed in a homemade holder to ensure an equidistance between the reaction vessels and the light source (Figure S1).

For the reactions using a broad spectrum light source, a LIGHTINGCURE LC8 L9566 (Hamamatsu) was used. The optic fiber of the light source was placed on top of a water bath.

Anaerobic reactions were performed in a glovebox with an atmosphere consisting in 98 % N_2 and 2% H_2.

At different time intervals, aliquots were extracted with ethyl acetate (containing 5 mM 1-octanol as an internal standard). The organic phase was dried with magnesium sulfate, centrifuged and analyzed by GC.

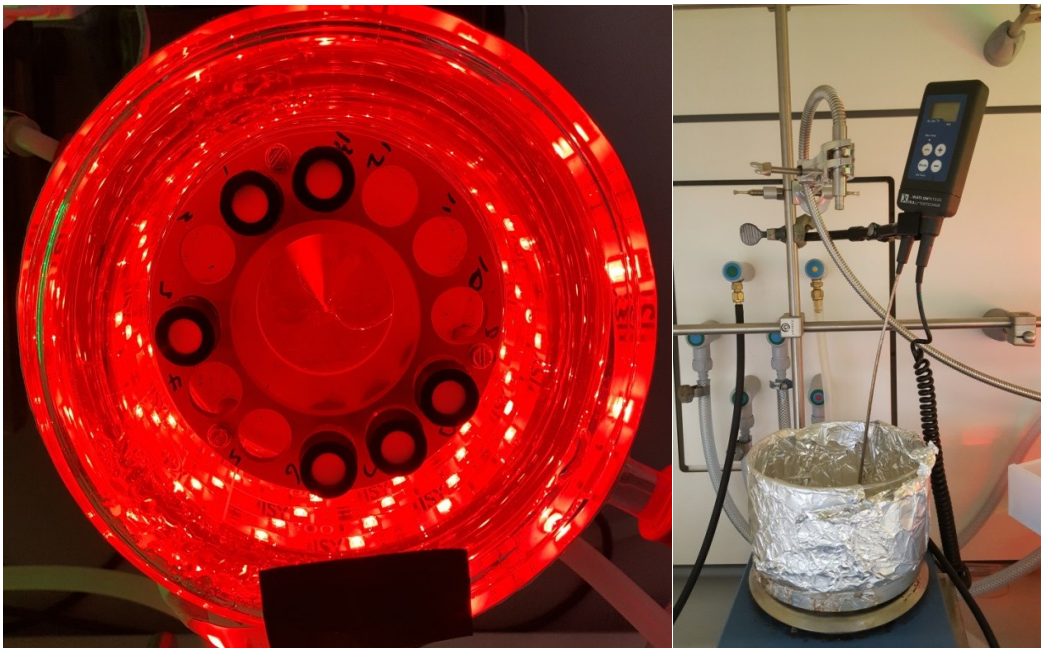


Figure S1. In-house light set up consisting in a jacketed beaker with LEDs winded around (left) and white light set-up consisting on a water bath light beam coming from the top via an optic fiber (right). Photograph taken by S.J.-P. Willot.

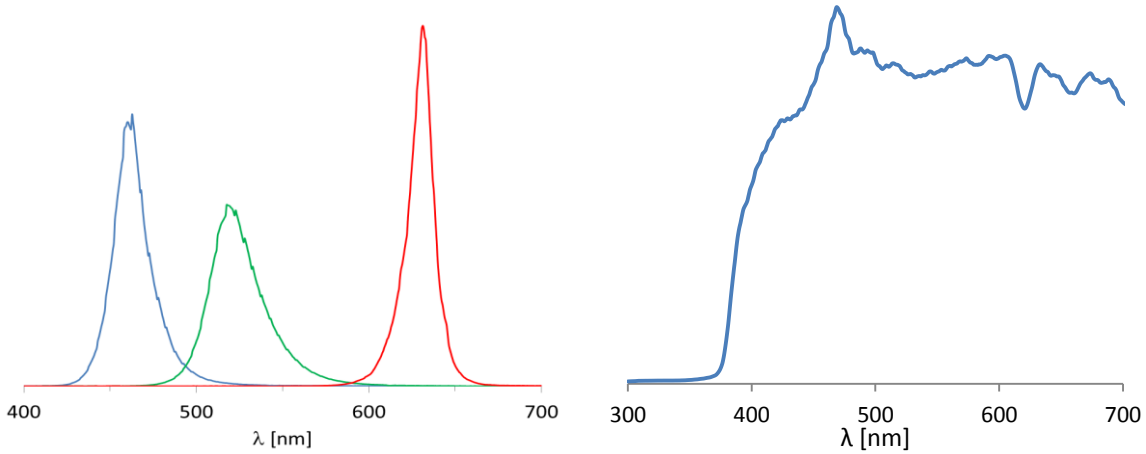


Figure S2. Emission spectra of the LEDs(left) and of the Hamamatsu LIGHTINGCURE LC8 L9566 (right).

3. Analytical procedures

3.1. Activity assays

Activity measurements were performed spectrophotometrically using the Agilent Technologies Cary 60 UV-Vis spectrophotometer (equipped with a single cell Peltier accessory) by monitoring the NADH consumption or generation, for FDH, respectively, at 340 nm in 1 mL cuvettes.

The activity of FDH was measured in potassium phosphate buffer (50 mM) at 30˚C using NAD^+ (0.5 mM) and sodium formate (150 mM). The volumetric activities were calculated using an extinction coefficient of ε=6.3 $mM^{-1}cm^{-1}$.

3.2. GC measurements

GC measurements were performed using a GC-214 Gas Chromatograph (Shimadzu) equipped with a FID-Detector and a AOC-20i auto injector. Quantification of ethylbenzene, 1-phenylethanol and acetophenone were performed using a CP Wax 52CB column from Agilent (25m x 0.25mm x 1.2 µm); temp. program 150 °C hold 2.2 min; 25 °C/min to 210 °C hold 4.2 min, 30 °C/min to 250 °C hold 1 min. Retention times: ethylbenzene 1.60 min; 1-phenylethanol 7.15 min; acetophenone 5.90 min; 1-octanol (internal standard) 4.65 min (Figure S3). The enantiomeric excess for 1-phenylethanol was measured on a CP Chirasil Dex CB column from Agilent (25 m x 0.32 mm x 0.25 µm); temp. program 120 °C hold 2.6 min; 15 °C/min to 135 °C hold 3.3 min, 25 °C/min to 225 °C hold 1 min. Retention times: ethylbenzene 2.40 min; (*R*)-1-phenylethanol 6.20 min, (*S*)-1-phenylethanol 6.47 min; acetophenone 4.20 min (Figure S4).

All measurements have been performed at least as duplicates. Numbers shown are always based on calibration curves with authentic standards of the reagents (using internal standards). Error bars shown represent the standard deviation.

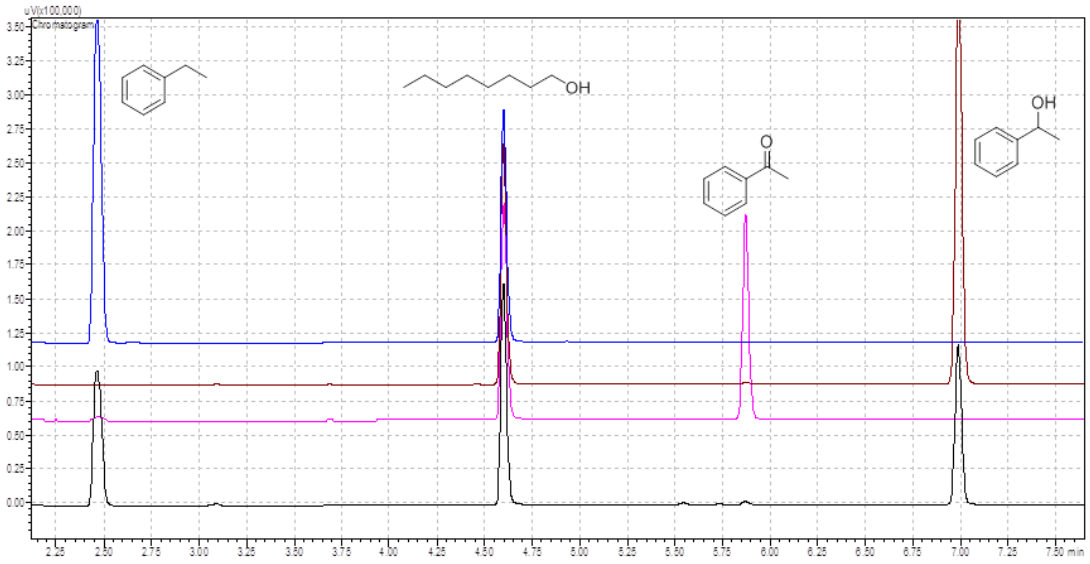


Figure S3. Example of chromatograms to quantify product formation. blue: ethylbenzene standard with octanol, brown: phenylethanol standard with octanol, pink: Acetophenone standard with octanol, black: example of reaction

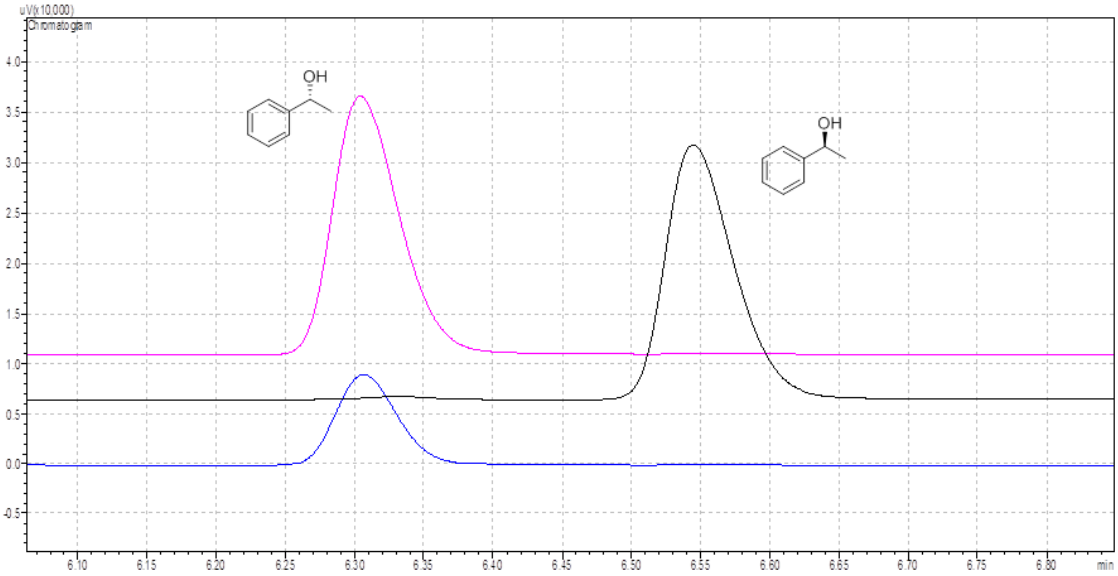


Figure S4. Exemplary chiral GC chromatograms of authentic (*R*)-1-phenylethanol and (*S*)-1-phenylethanol as well as one exemplary reaction product.

3.3. H_2O_2 concentration measurement

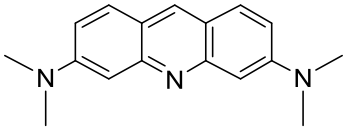
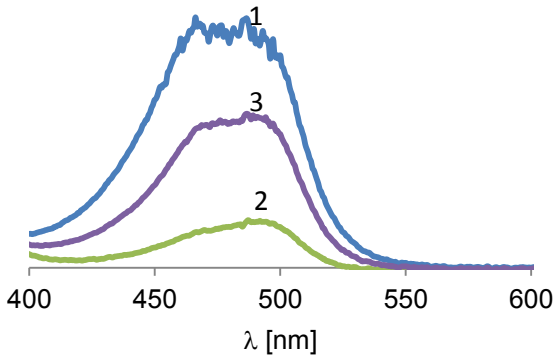
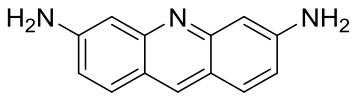
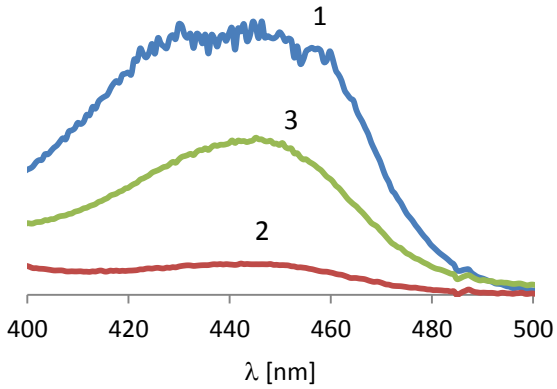
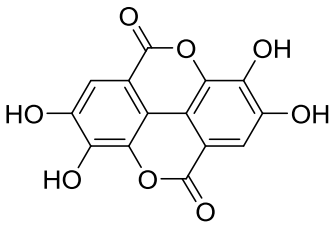
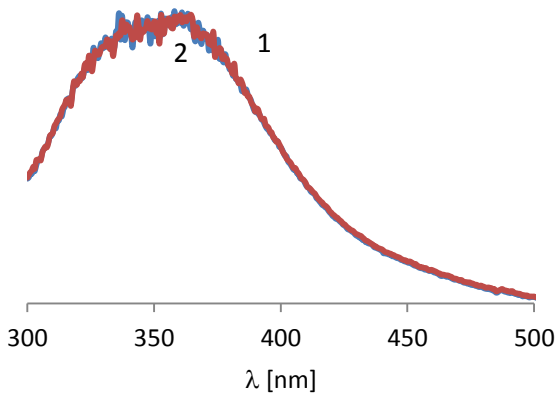
H_2O_2 concentration measurements were performed using an ABTS assay. Aliquots were mixed with 5 U (µmol. min^{-1}) of horseradish peroxidase and 1 mM 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) in 50 mM phosphate buffer pH 7.0 at room temperature. The corresponding reaction will lead to the formation of an absorbance peak at 420 nm which can be connected to the concentration of H_2O_2 with a calibration curve.

4. Additional Data

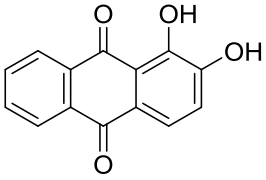
4.1. Photocatalyst screening

Several commercially available dyes have been screened. First, a reaction mixture of the photocatalyst and NADH were illuminated with the best overlapping LEDs light mode (blue, red or green) for 5 or 10 minutes. Then, if a significant change in the UV-Visible spectrum could be recorded, O_2 is introduced in the reaction mixture by bulling pure oxygen through it. The reaction is then placed in the dark and the UV-Visible spectrum is recorded again later on. The photocatalysts were successful if the initial spectrum could be partially recovered within a few minutes.

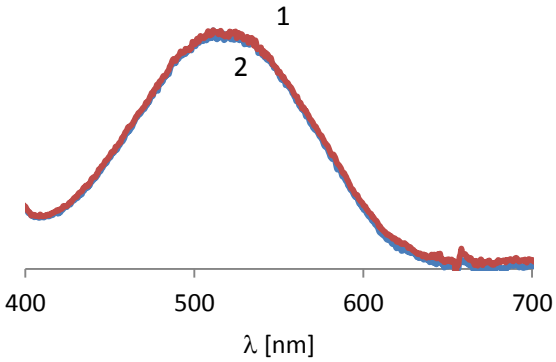
Table S1. UV-Visible spectra and conditions of the screened compounds.

Reaction steps	UV-Visible spectrum recorded
Acridine Orange  1. Initial spectrum 2. 5 minutes blue light illumination 3. 5 minutes in the dark with O_2	
Proflavin  1. Initial spectrum 2. 5 minutes blue light illumination 3. 5 minutes in the dark with O_2	
Alizarin yellow  1. Initial spectrum 2. 5 minutes blue light illumination	

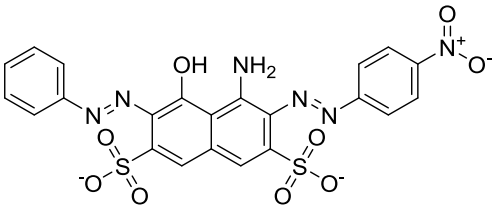
Alizarin red



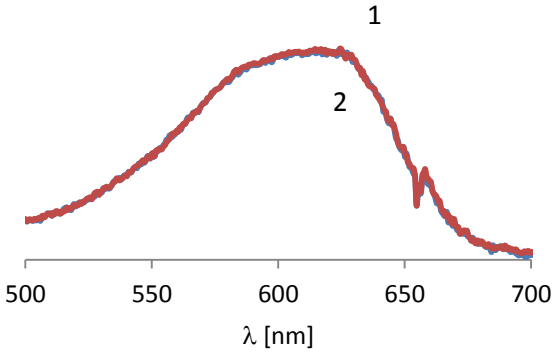
1. Initial spectrum
2. 5 minutes green light illumination



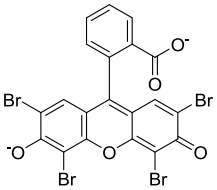
Amido Black



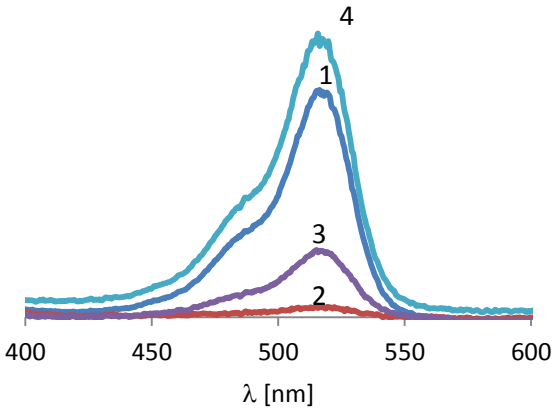
1. Initial spectrum
2. 5 min red light illumination



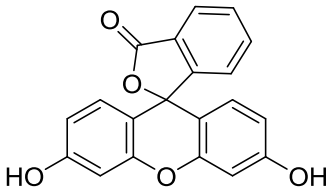
Eosin Y



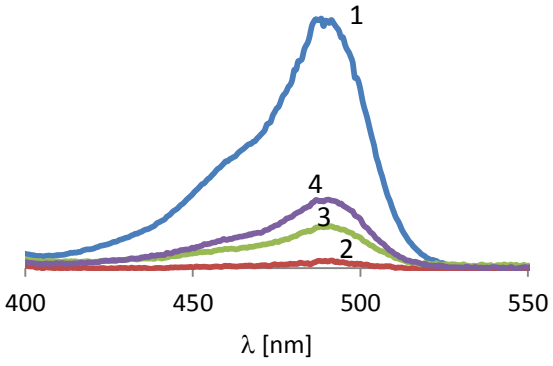
1. Initial spectrum
2. 5 min green light illumination
3. 30 min in the dark with O_2
4. 60 min in the dark with O_2



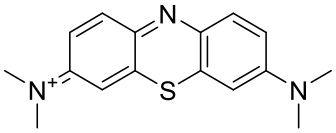
Fluorescein



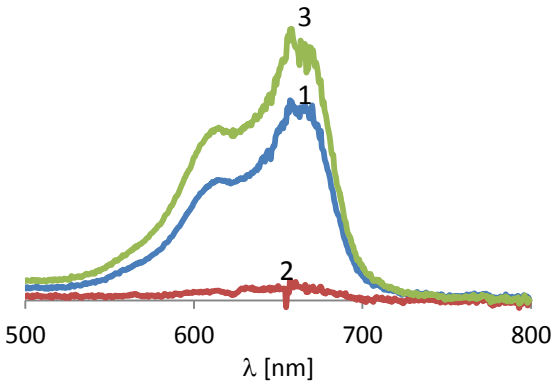
1. Initial spectrum
2. 10 min blue light illumination
3. 60 min in the dark with O_2
4. 180 min in the dark with O_2



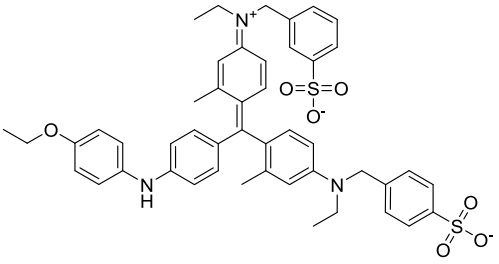
Methylene blue



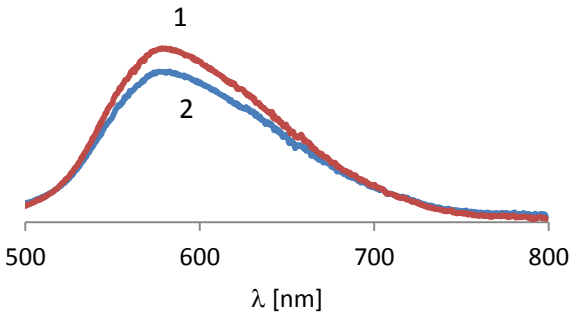
1. Initial spectrum
2. 5 min red light illumination
3. 5 min in the dark with O_2



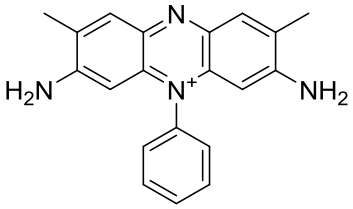
Serva blue G



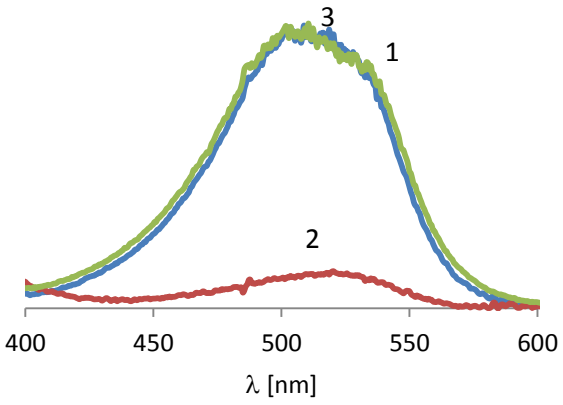
1. Initial spectrum
2. 5 min red light illumination



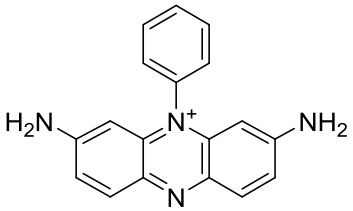
Safranin O



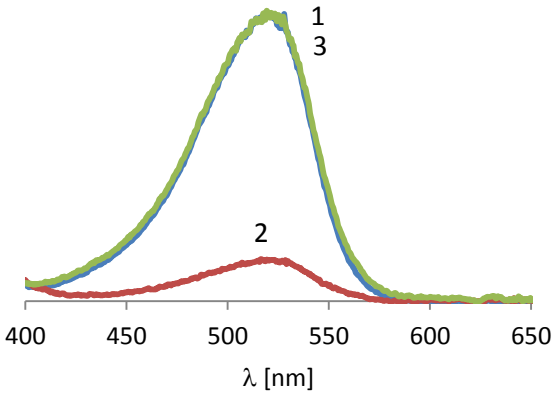
1. Initial spectrum
2. 5 min green light illumination
3. 5 min in the dark with O_2



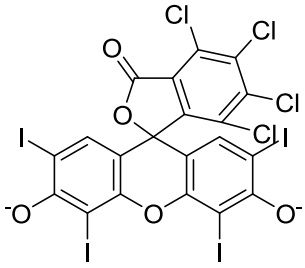
Phenosafranin



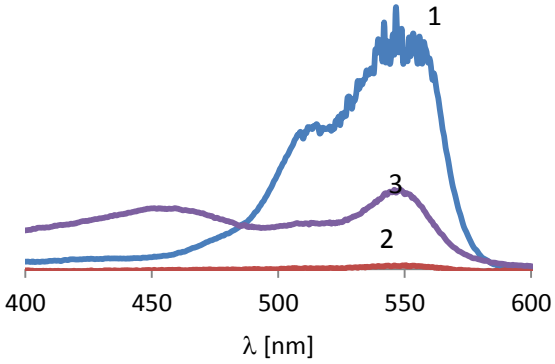
1. Initial spectrum
2. 5 min green light illumination
3. 5 min in the dark with O_2



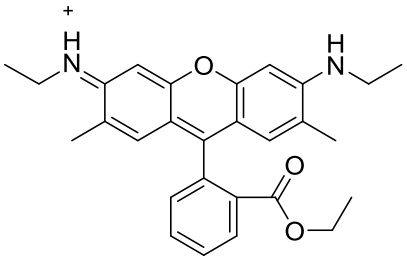
Rose Bengal



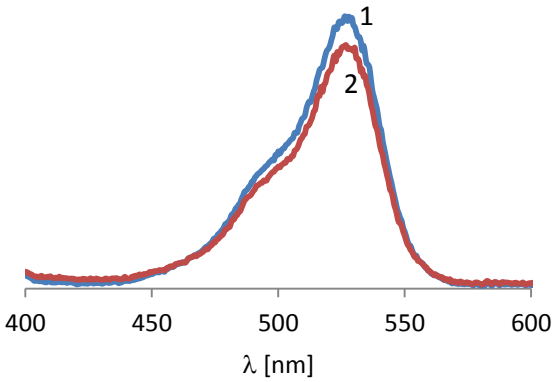
1. Initial spectrum
2. 5 min green light illumination
3. 24 h in the dark with O_2



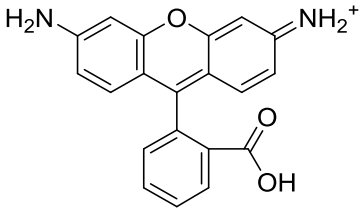
Rhodamine 6G



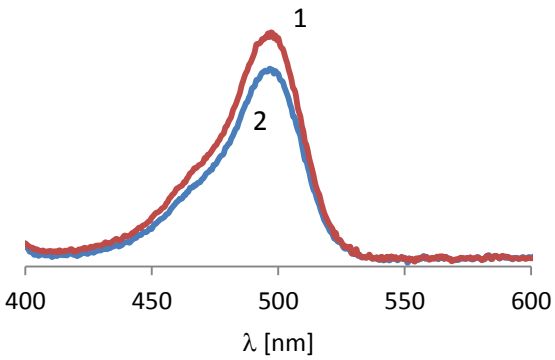
1. Initial spectrum
2. 10 min green light illumination



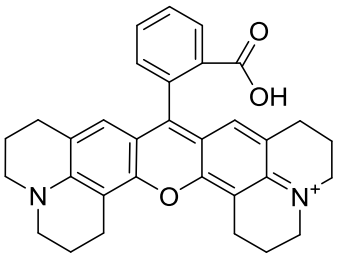
Rhodamine 110



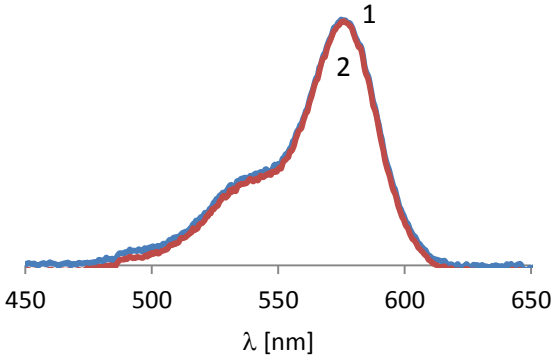
1. Initial spectrum
2. 10 min green light illumination



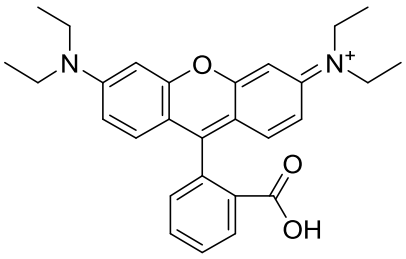
Rhodamine 101



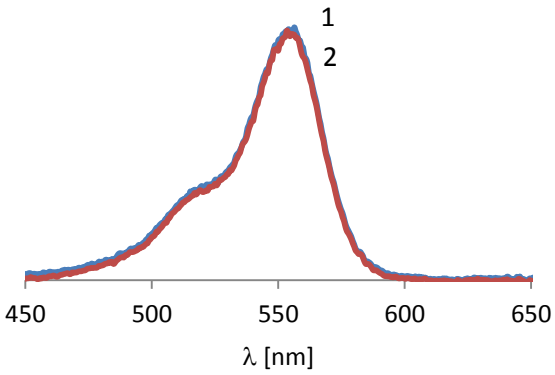
1. Initial spectrum
2. 10 min green light illumination



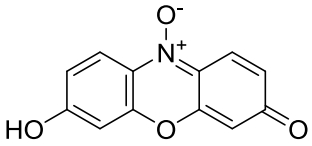
Rhodamine B



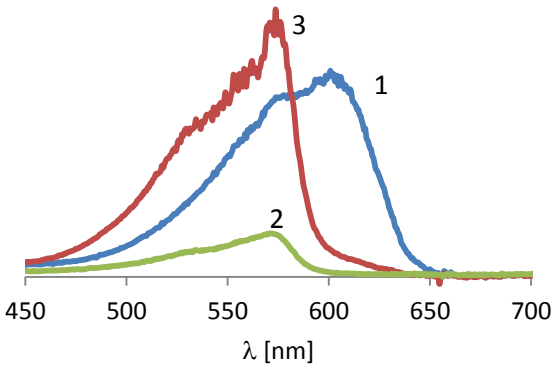
1. Initial spectrum
2. 10 min green light illumination



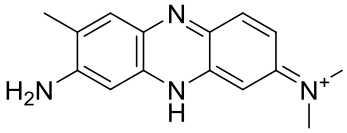
Rezasurin



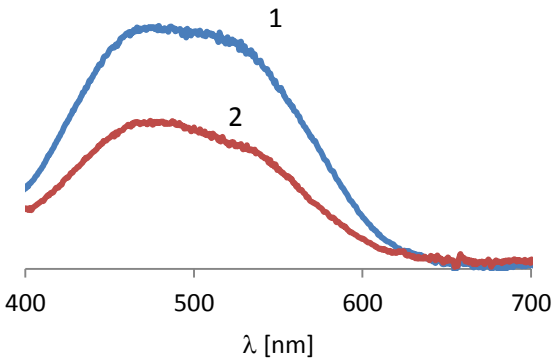
1. Initial spectrum
2. 10 min green light illumination
3. 24 h in the dark with O_2



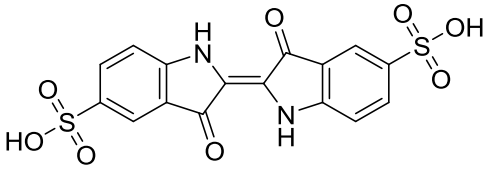
Neutral Red



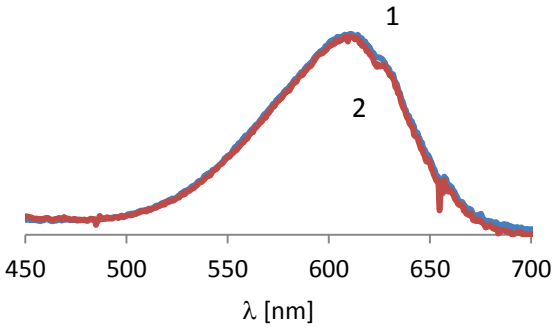
1. Initial spectrum
2. 5min red light illumination

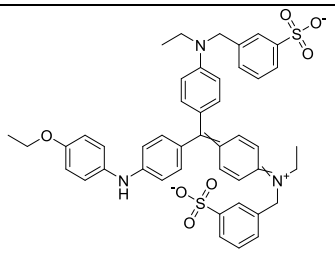


Indigo Carmin

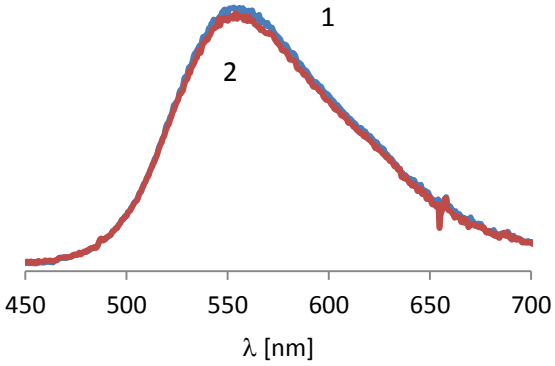


1. Initial spectrum
2. 5min red light illumination

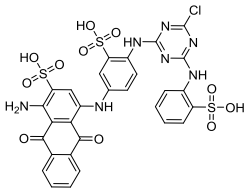




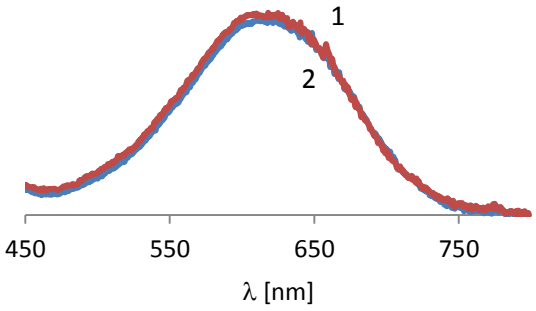
Coomassie blue
1. Initial spectrum
2. 5min red light illumination



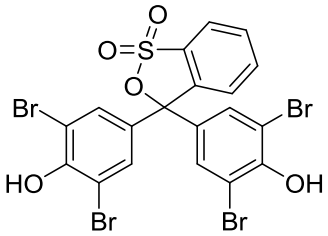
Cibracon 3G-A



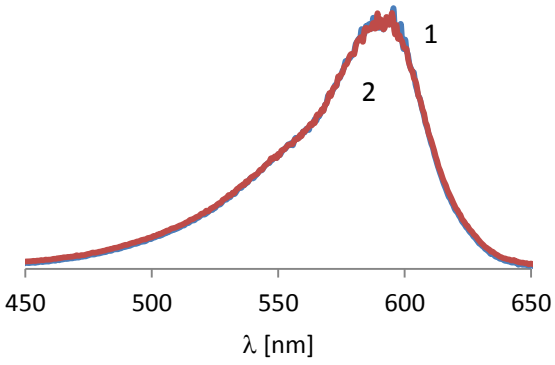
1. Initial spectrum
2. 5min red light illumination



Bromophenol blue



1. Initial spectrum
2. 5min red light illumination



General conditions: NADH > 300 µm, 50 mM phosphate buffer pH 7.0, 30 °C, anaerobic conditions (100% N_2), 300 rpm.

4.2. Reduction of photocatalysts

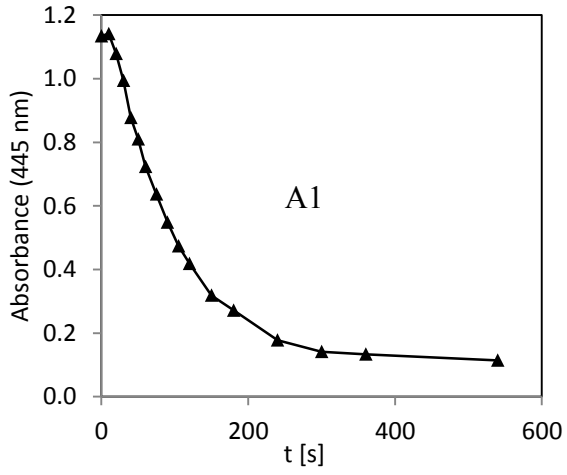
The compounds complying with our criteria were then studied when other electron donors are applied. EDTA, tris(hydroxymethyl)aminomethane (Tris), MOPS and ascorbic acid were tested (Table S2 and S3).

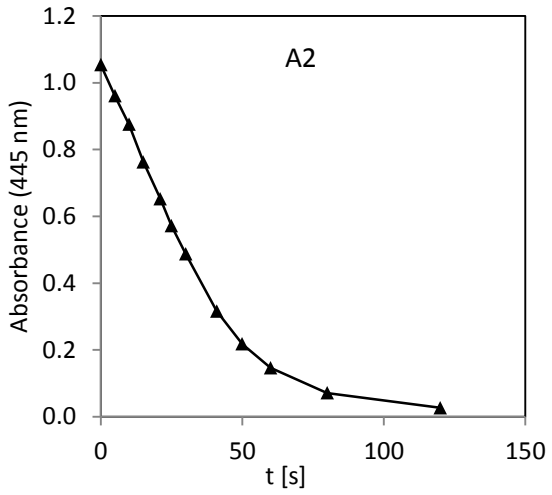
Table S2. Reduction kinetics of the photocatalysts depending on the electron donor. Conditions: room temperature (22-24°C),50 mM phosphate buffer pH 7, 1 mM reducing agent, anaerobic conditions, blue LEDs (proflavin, acridine orange) or green LEDs (safranin O and phenosafranin), red LEDs (methylene blue).

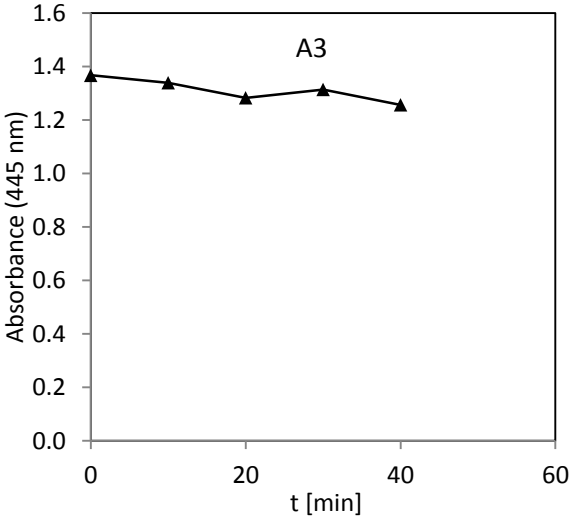
	$k_{Proflavin}$ (h^{-1})	$k_{Safranin\ O}$ (h^{-1})	$k_{Phenosafranin}$(h^{-1})	$k_{Methylene\ blue}$(h^{-1})	$k_{acridine\ orange}$(h^{-1})
EDTA	30 ± 3	52.2 ± 0.6	54 ± 4	80 ± 4	n.d.
NADH	109 ± 4	55 ± 2	51 ± 3	348 ± 24	5.9 ± 0.6
MOPS	0.12 ± 0.00	0.05 ± 0.00	0.300 ± 006	2.10 ± 0.006	n.d.
Ascorbic Acid	5.52 ± 0.03	n.d.	n.d.	3.8 ± 0.4	not determined
Tris	n.d.	n.d.	n.d.	0.24 ± 0.00	n.d.

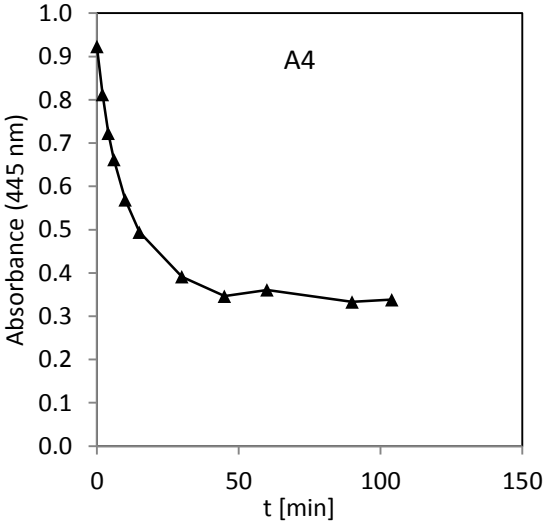
Table S3. Example of reduction time-courses.

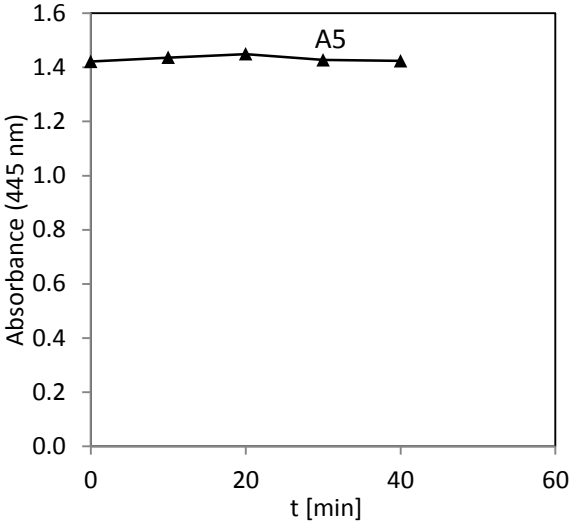
	proflavin	Safranin O	phenosafranin	Methylene blue	Acridine orange
EDTA	A1	B1	C1	D1	E1
NADH	A2	B2	C2	D2	E2
MOPS	A3	B3	C3	D3	E3
Ascorbic Acid	A4	B4	C4	D4	E4
Tris	A5	B5	C5	D5	E5

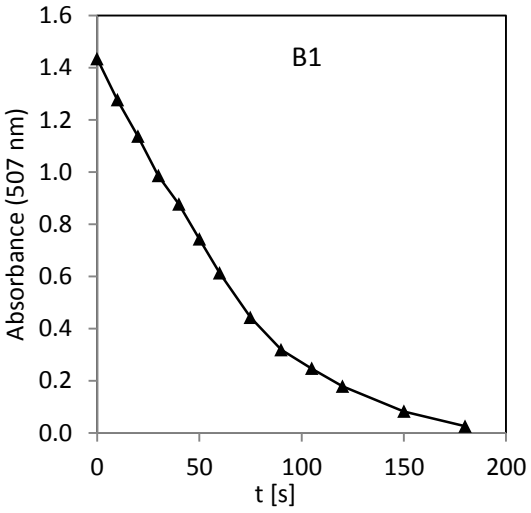


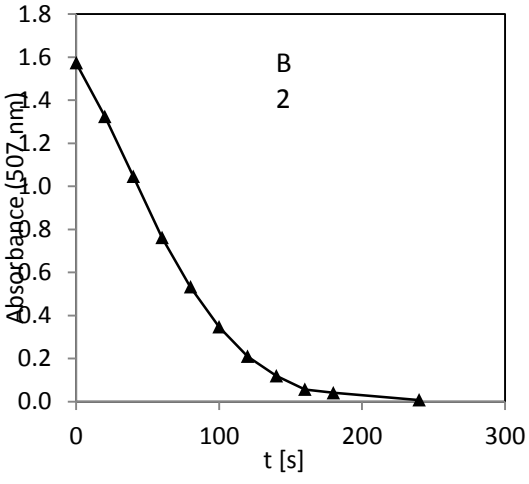


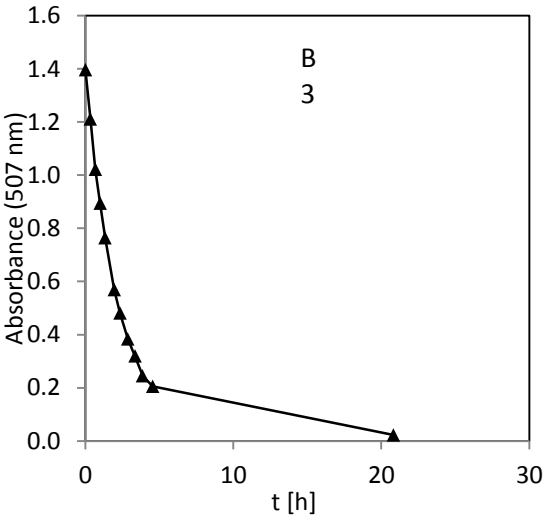

A3
Absorbance (445 nm)
t [min]

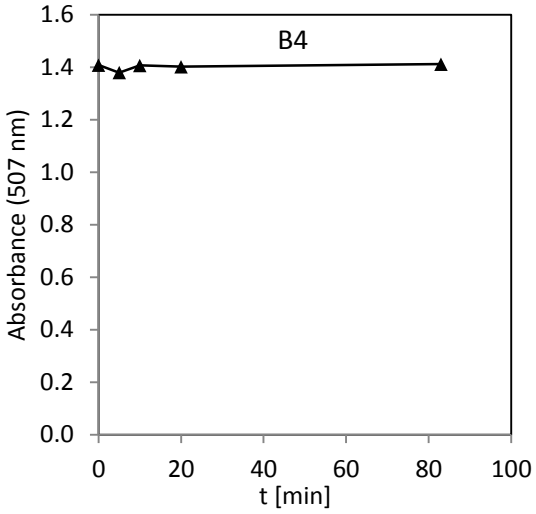

A4
Absorbance (445 nm)
t [min]

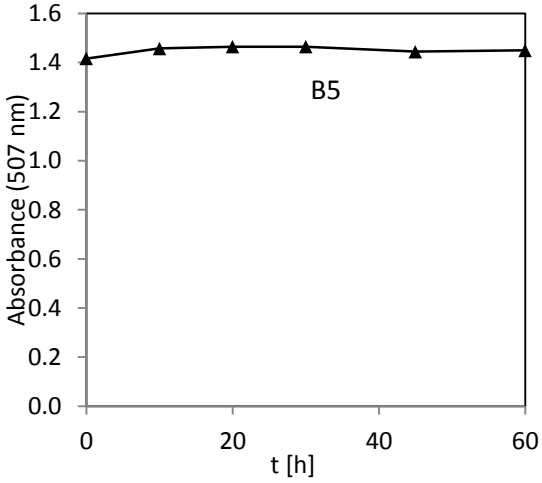

A5
Absorbance (445 nm)
t [min]

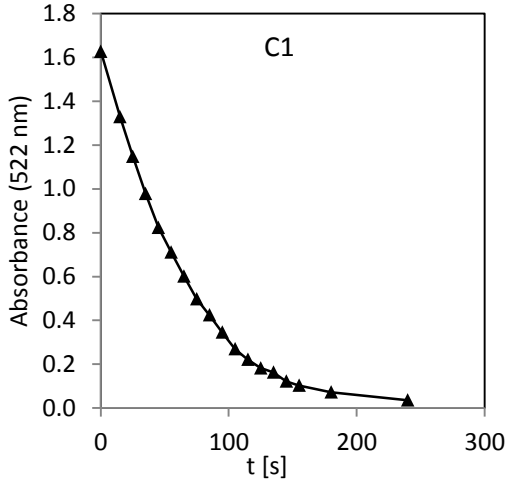

B1
Absorbance (507 nm)
t [s]

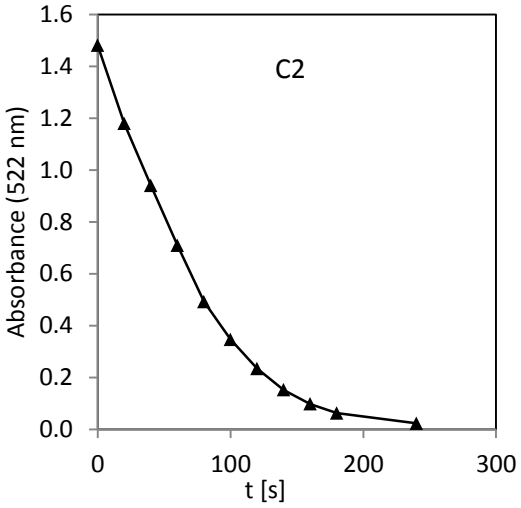

B
2
Absorbance (507 nm)
t [s]

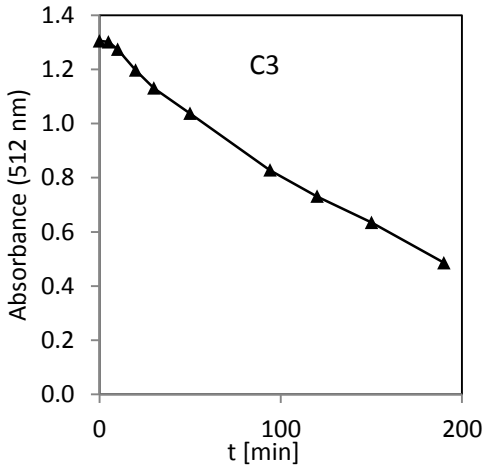

B
3
Absorbance (507 nm)
t [h]


B4
Absorbance (507 nm)
t [min]


B5
Absorbance (507 nm)
t [h]


C1
Absorbance (522 nm)
t [s]


C2
Absorbance (522 nm)
t [s]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Absorbance (512 nm)
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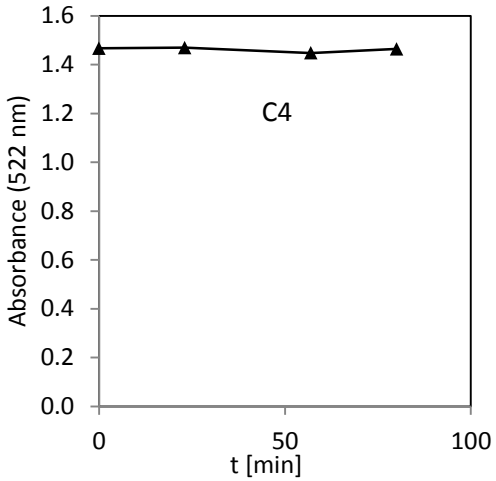
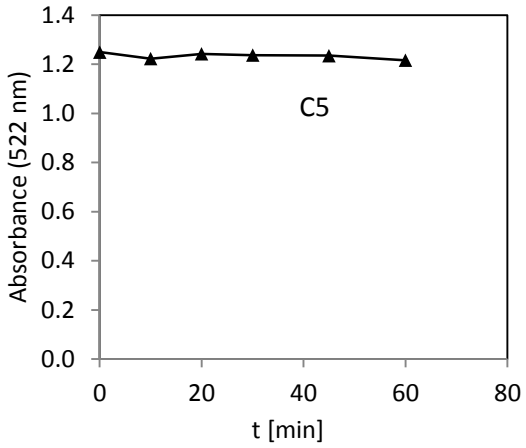
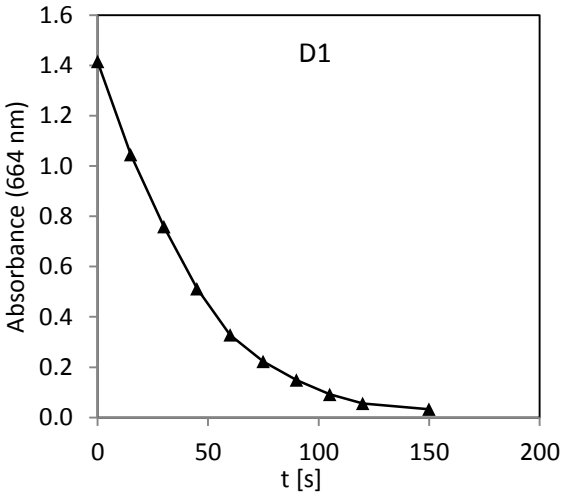
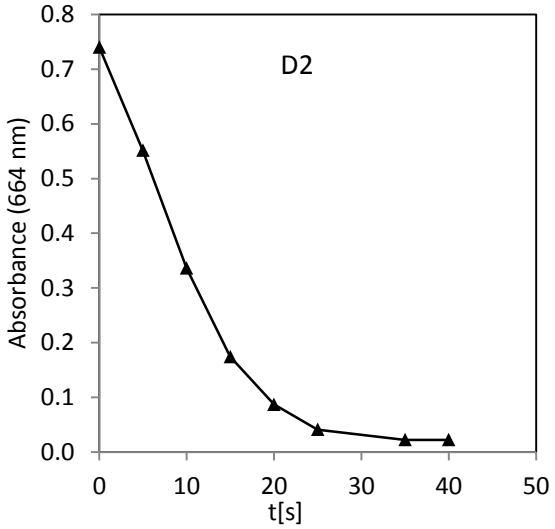
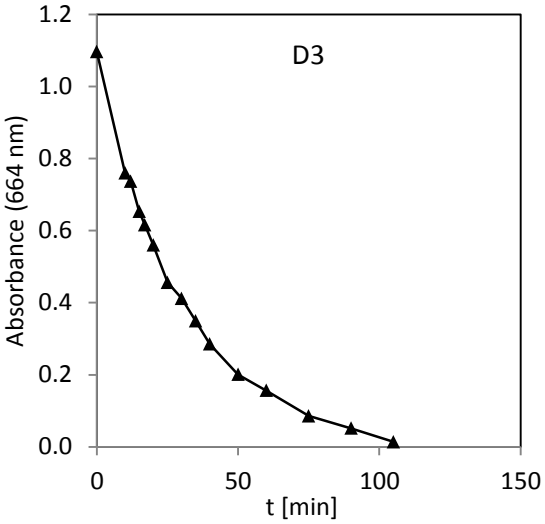

C4
Absorbance (522 nm)
t [min]


C5
Absorbance (522 nm)
t [min]


D1
Absorbance (664 nm)
t [s]


D2
Absorbance (664 nm)
t[s]


D3
Absorbance (664 nm)
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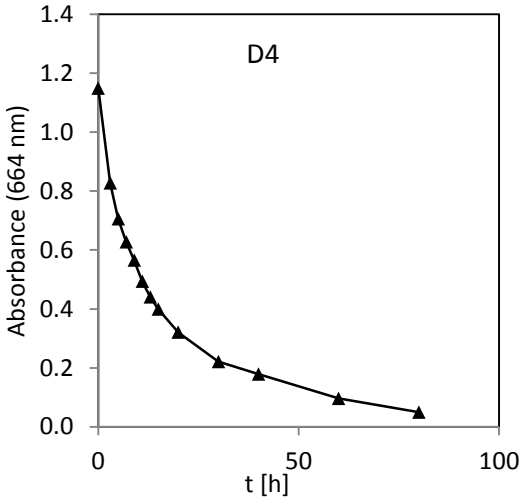
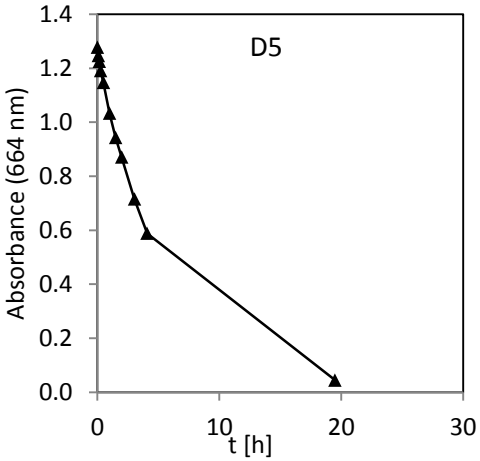
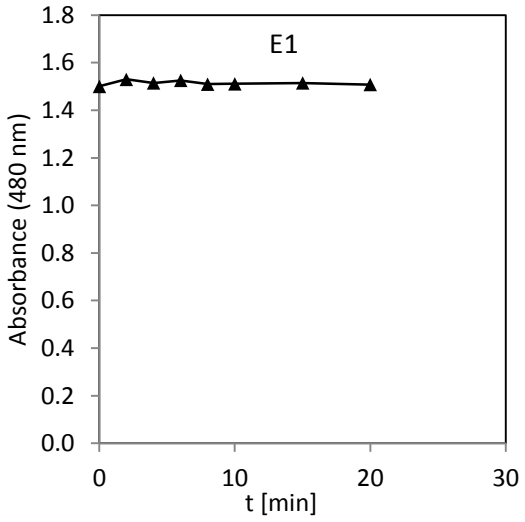
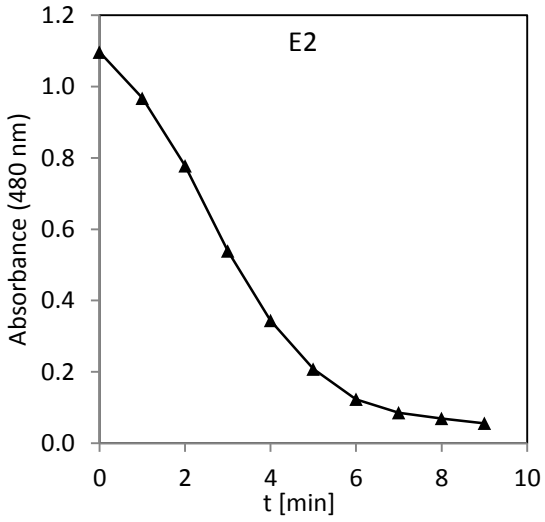

D4
Absorbance (664 nm)
t [h]


D5
Absorbance (664 nm)
t [h]


E1
Absorbance (480 nm)
t [min]
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
0
10
20
30


E2
Absorbance (480 nm)
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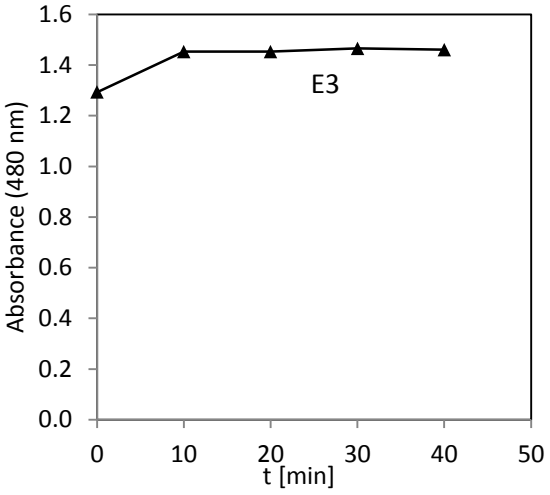
0.6
0.8
1.0
1.2
0
2
4
6
8
10


E3
Absorbance (480 nm)
t [min]
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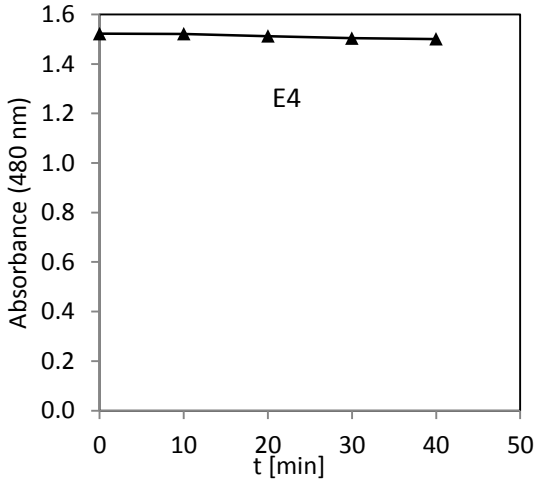
0.6
0.8
1.0
1.2
1.4
1.6
0
10
20
30
40
50


E4
Absorbance (480 nm)
t [min]
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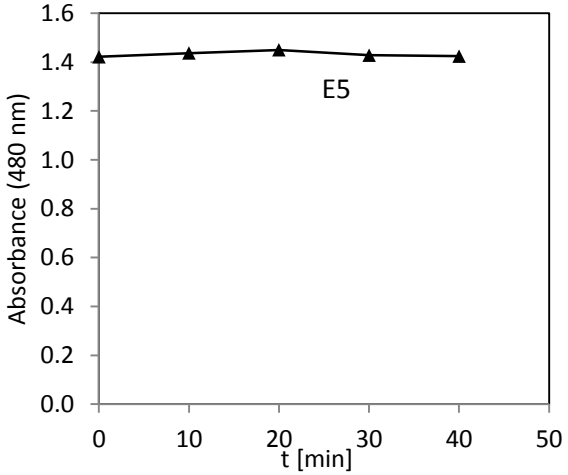
0.6
0.8
1.0
1.2
1.4
1.6
0
10
20
30
40
50


E5
Absorbance (480 nm)
t [min]
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0
10
20
30
40
50

4.3. H_2O_2 production by selected photocatalyst

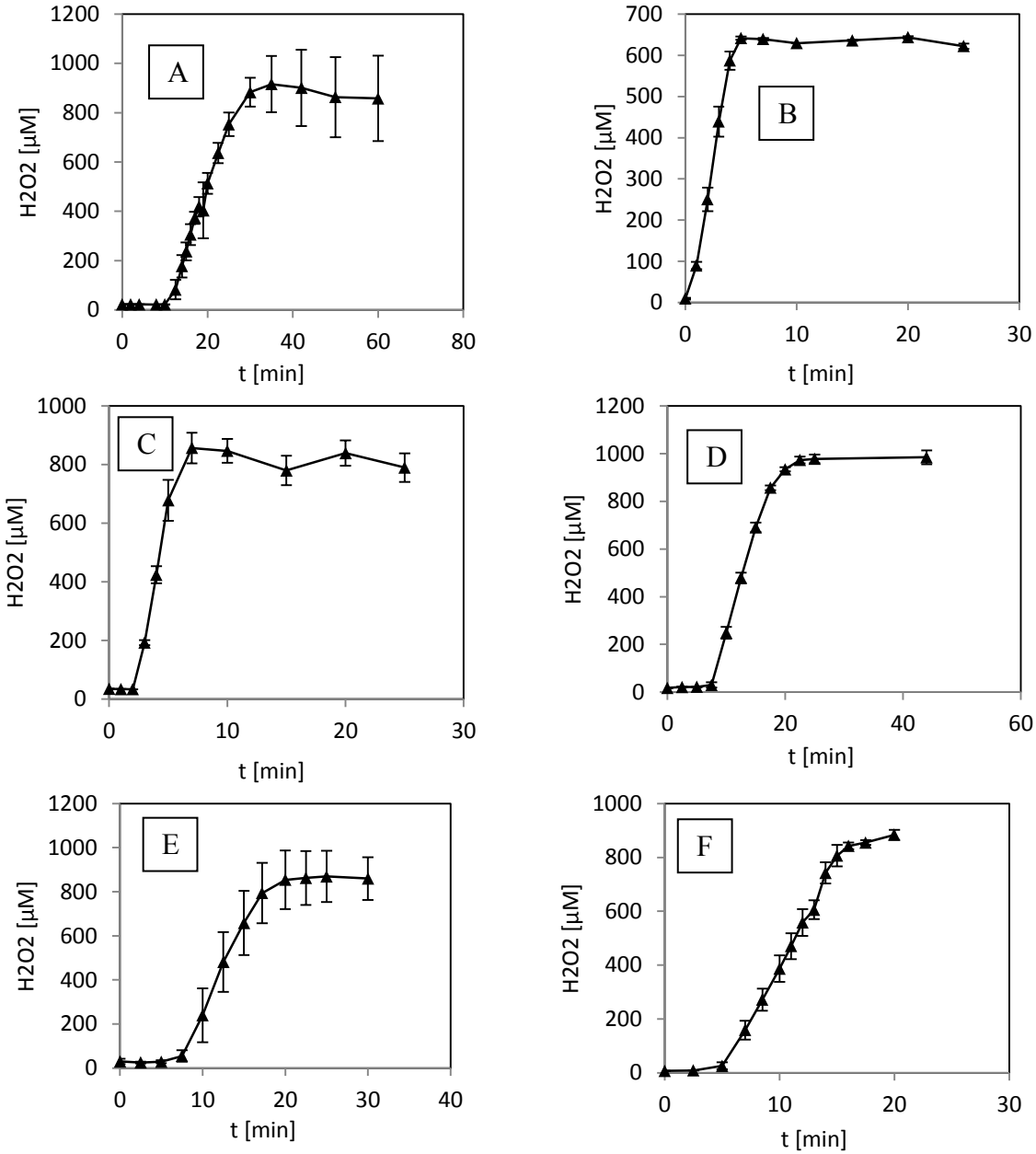


Figure S5. H_2O_2 production from 1 mM NADH with 50 µM (A) acridine orange, (B) FMN, (C) proflavin, (D) phenosafranin, (E) safranin O, (D) methylene blue. Conditions: 50 mM photocatalysts, 1 mM NADH, 50 mM phosphate buffer pH 7.0, aerobic, 30°C, (proflavin, acridine orange) or green LEDs (safranin O and phenosafranin), red LEDs (methylene blue).

4.4. Methylene blue pre-illumination with red light

A lag phase could be noticed when methylene blue was applied in little amount. Thus, we decided to incubated methylene blue in light prior to the reaction to verify if light was the cause of this activation.

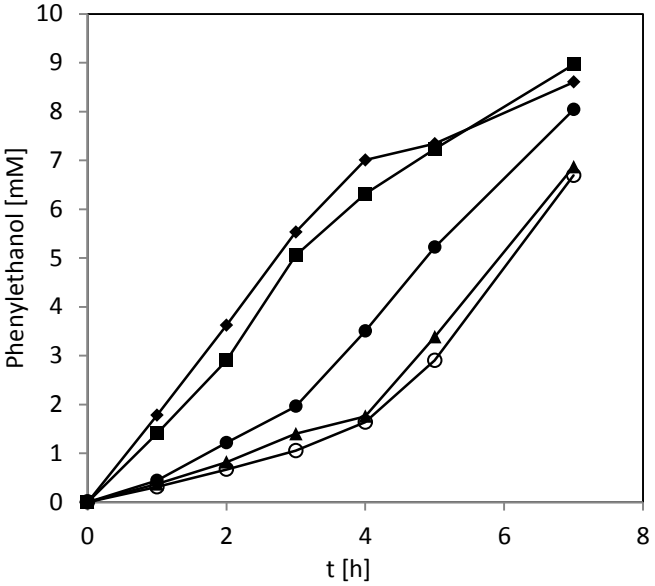


Figure S6. Phenylethanol production by the photoenzymatic cascade using methylene blue as photocatalyst. Prior the actual reaction methylene blue was illuminated with red light for (●) 20 min red light, (■) 60 min red light, (♦)120 min. For comparison: illuminations with blue light for (▲) 0 min or (○) 60 min.
Conditions: 75 mM formate, 0.4 mM NAD^+, 4.8 µM *Cb*FDH, 0.8 %(v/v) Methanol, 100 nM r*Aae*UPO, 50 mM Kpi pH 7.0, 30˚C.

4.5. Stability of FDH

*Cb*FDH was incubated in the presence of some photocatalysts and illuminated at their respective photoexcitation wavelength. At intervals the residual activity of *Cb*FDH was determined.

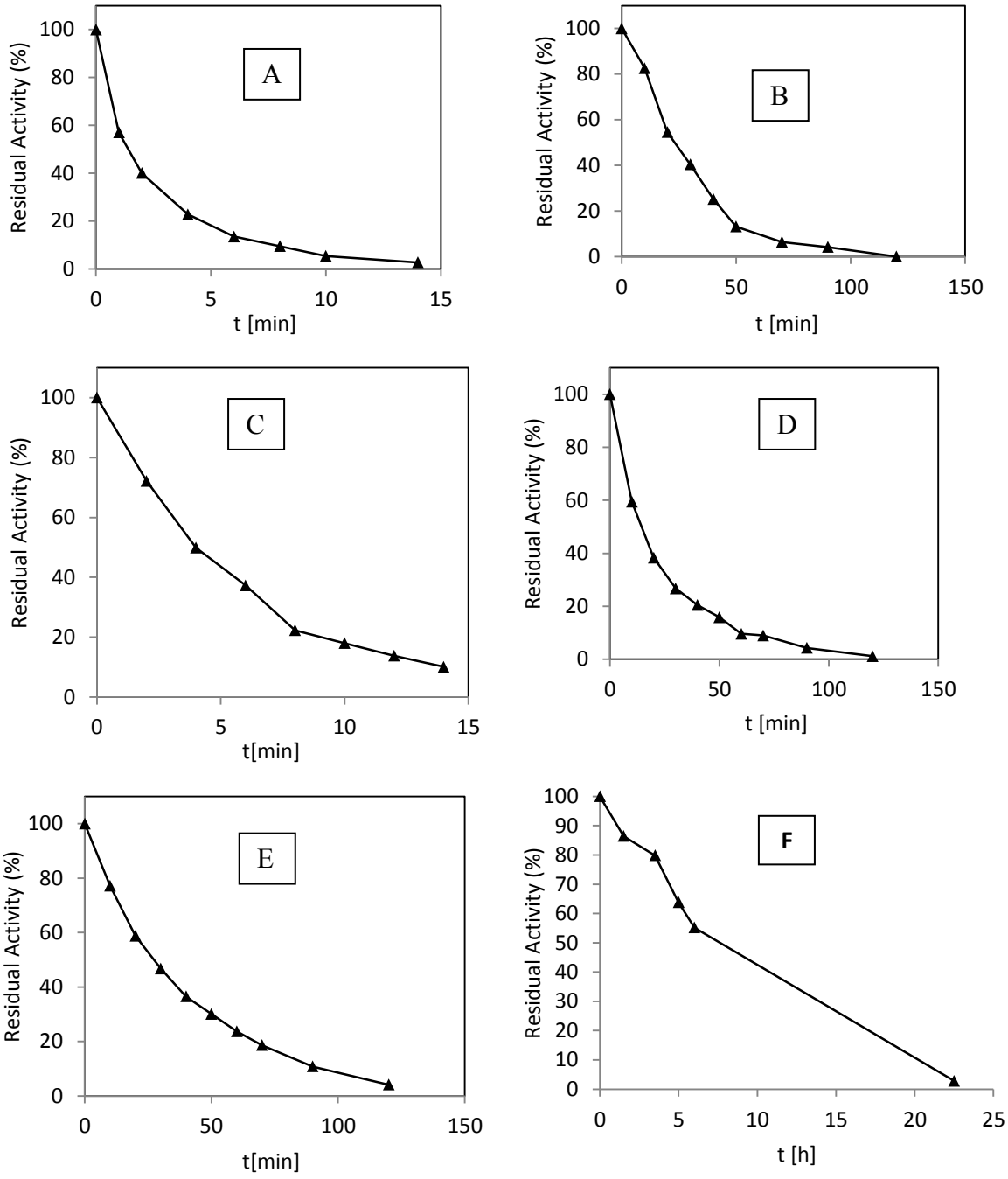


Figure S7. Detailed time course of enzyme activity when exposed to photoexcited catalysts. A: FMN, B: acridine orange, C: proflavine, D: phenosafranin, D safranin O, E: Methylene blue, F: no photocatalyst ('positive control'); Conditions: 2.4 µM FDH, 50 µM photocatalysts, 50 mM phosphate buffer pH 7.0, aerobic atmosphere, 30°C, 300 rpm, LED.

4.6. Stability of the photocatalysts

The photostability of the photocatalysts used was examined by illuminating them and following the intensity of the most prominent absorption peak.

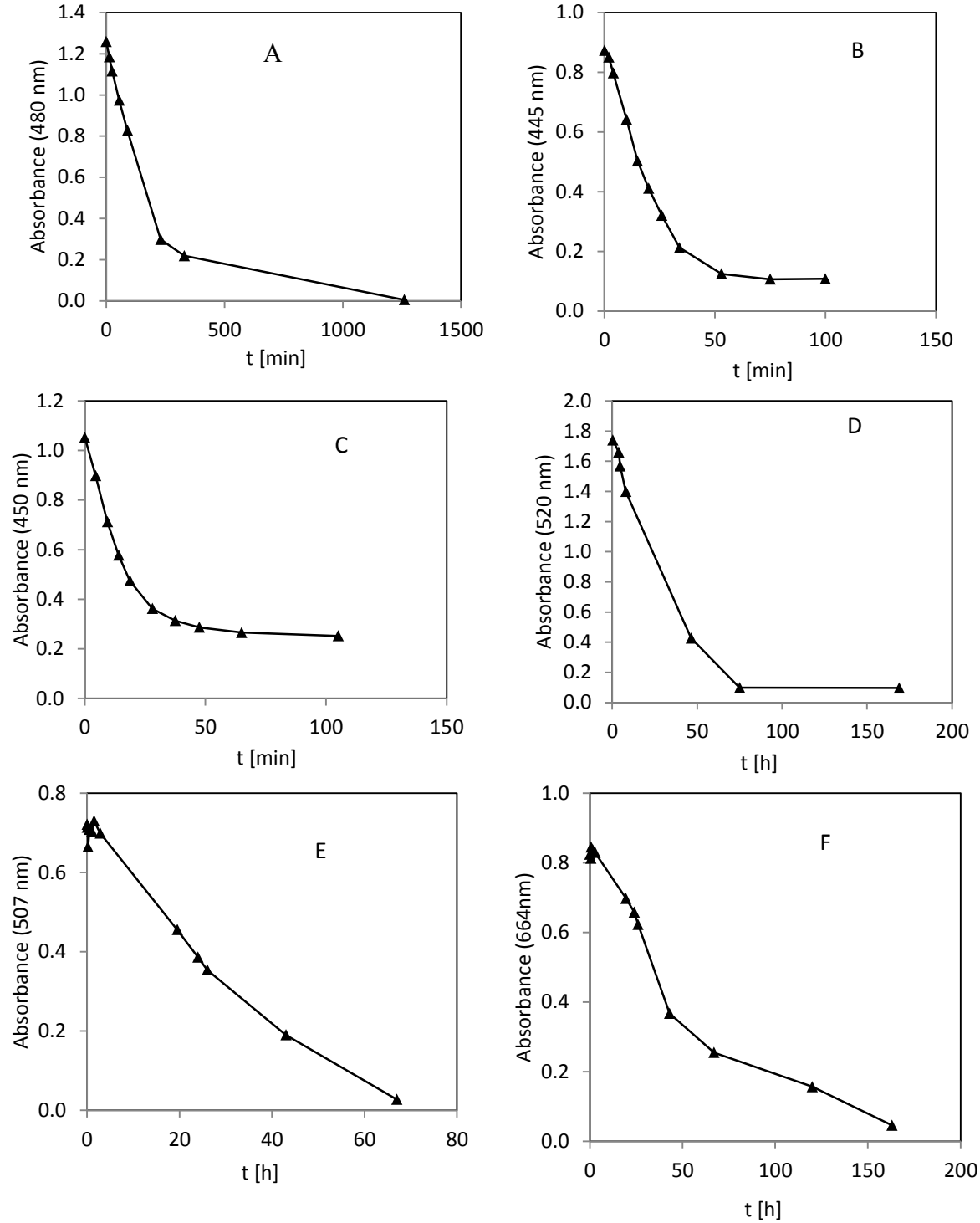


Figure S8. Stability of the photocatalysts against light. A: acridine orange, B: proflavin , C: FMN, D: phenosafranin, E: safranin O, E: methylene blue. Conditions: 100 μM photocatalyst, 50 mM phosphate buffer pH 7.0, aerobic atmosphere, 30°C, 300 rpm, LED.

4.7. Experiments using a polychromatic (sun light-simulating) light source

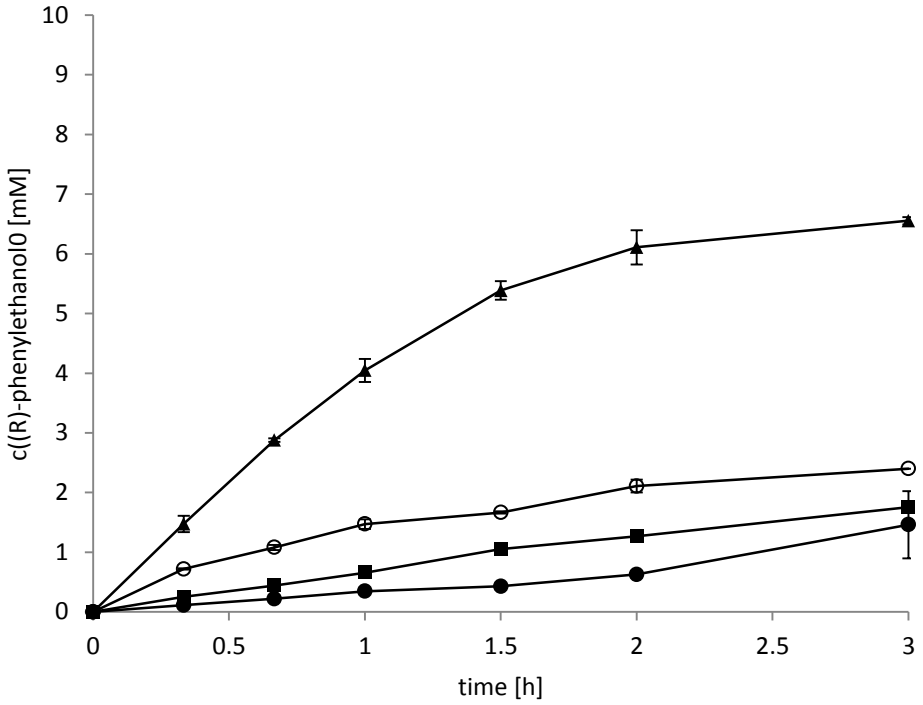
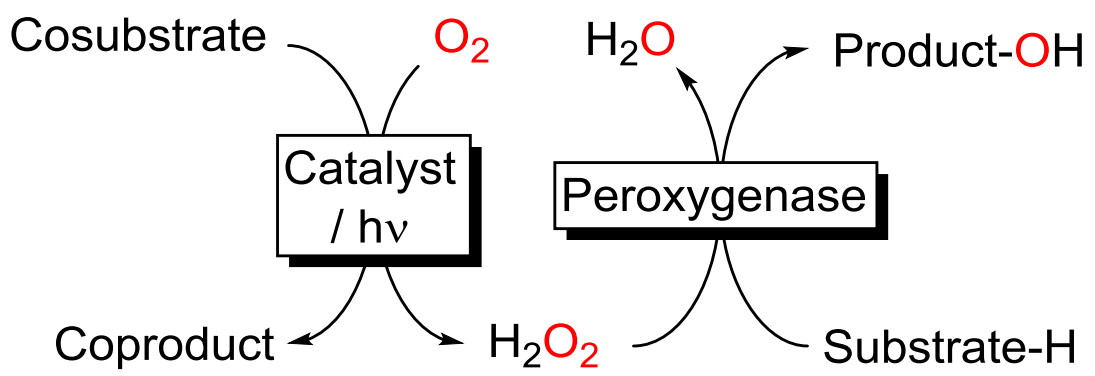


Figure S9. Time courses of some photoenzymatic transformations using a polychromatic light source((○) ethylbenzene, (▲) phenylethanol). (▲): 5 µM phenosafranin + 10 µM methylene blue + 5 µM FMN, (■) 5 µM phenosafranin, (●) 10 µM methylene blue, (○) 5 µM FMN. Conditions: 75 mM formate, 0.4 mM NAD^+, 4.8 µM FDH, 0.8 %(v/v) Methanol, 100 nM UPO, 50 mM Kpi pH 7.0, 30˚C

Table S4. Comparison of some *in situ* H_2O_2 generation systems to promote peroxygenase-catalyzed oxyfunctionalisation reactions.



Cosubstrate	Coproduct	Photocatalyst / wavelengths	Co-catalyst(s)	Waste [$g_{Coproduct}$ $mol^{-1}_{product}$]	ref
Glucose	glucuronic acid	-	GOx	196	[3]
MeOH	CO_2	-	AOx / FDM / FDH / 3HB6H/ NAD	15	[4]
HCO_2H	CO_2	-	FDH / NAD / YqjM	44	[5]
H_2O	-	Au-TiO_2 / >300 nm	-	-	[6]
MeOH	CO_2	Au-TiO_2 / >300 nm		15	[7]
Cathode	-	Flavin-SWCNT / 450 nm	-	-	[8]
EDTA	EDTriA / CH_2O / CO_2	FMN / 450 nm	-	308	[9-11]

GOx: glucose oxidase; AOx: alcohol oxidase; FDM: formaldehyde dismutase; FDH: formate dehydrogenase; 3HB6H: 3-Hydroxybenzoate-6-hydroxylase; NAD: nicotinamide cofactor; YqjM: ene-reductase from *Bacillus subtilis*; Flavin-SWCNT: flavin-modified carbon nanotube-functionalized cathodes; Ethylene diamine tetraacetate; EDTriA: ethylene diamine triacetate; FMN: flavin mononucleotide.

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