SI for: Photobiocatalytic alcohol oxidation using LED light sources

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Material and Methods

Materials

Unless stated otherwise all chemicals were purchased from Sigma-Aldrich (Steinheim, Germany), New England Biolabs (Ipswich, MA, USA) or Merck (Darmstadt, Germany) in the highest quality available and used without further purification.

LED light source

The brand of the LED light source is Paulmann, YourLED, Basic Set RGB 1.5m.

Bacterial strains and plasmids

Escherichia coli TOP10 and BL21 (DE3) were purchased from New England Biolabs (Beverly, MA, USA). The plasmid pET28a containing the gene encoding the alcohol dehydrogenase from *Equus caballus* (E-Isoenzyme, NCBI_Nucleotide accession-number: NM_001082528) bearing an additional N-terminal His_6-tag was kindly provided by Dr. Doerte Rother (Forschungszentrum Juelich, Juelich, Germany). The plasmid was transformed into the appropriate *E. coli* strain by the heat shock method.[1]

Cultivation conditions

Expression of HLADH was carried out by inoculation of 400 mL TB (terrific broth) medium supplied with the appropriate antibiotic (kanamycin) with an overnight culture to give an OD_{600} of 0.05. *E. coli* BL21 (DE3) cells were used as expression host. Cells were grown at 37°C in baffled shake flasks. HLADH expression was induced at an OD_{600} of 0.6-0.8 with 1.0 mM IPTG. Cultivation was continued at 25°C for 24 hours. Cells were harvested (centrifugation at 1344 *g* at 4°C for 15 min) and washed twice in Glycine-NaOH buffer (pH 9.0, 100 mM). The bacterial cell pellet was re-suspended in the same buffer to give a wet cell weight (WCW) of 100 g/L and disrupted with a French press at 2000 psi or directly frozen and freeze-dried afterwards.

Purification of HLADH

For purification, cell pellets obtained as described above were resuspended in 25 mL sodium phosphate buffer (100 mM, 300 mM NaCl, pH 7.5) containing 30 mM imidazole. For cell disruption, the cell suspension was passaged twice through a French pressure cell at 2000 psi. Cell debris was separated from the crude extract by centrifugation at 9000 *g* for 45 min.
Purification of HLADH was performed using the NGC-1 purifier (BioRad, Berkeley, USA). The filtrated supernatant was applied to a Nickel-NTA column (GE Healthcare, Munich, Germany). After washing the column with a triple volume of 100 mM sodium phosphate buffer containing 300 mM sodium

chloride at a flow rate of 1 ml min^{-1}, the protein was eluted with 100 mM sodium phosphate buffer containing 300 mM imidazole and 300 mM sodium chloride. The HLADH containing fractions were collected. The proteins were desalted by PD-10 columns against 100 mM Glycine-NaOH buffer (pH 9.0). Afterwards, the protein solution was concentrated using Centricons (10 kDa cut-off). To determine the protein content of the crude cell extract as well as of the purified and desalted fractions, the Bradford assay was used. Standard curves were made using BSA in a range of 0.02-2 mg/mL. Samples were measured in triplicates using suitable dilutions.

Determination of HLADH activity

The HLADH activity assay was established with benzaldehyde as standard substrate. The consumption of NADH during the enzymatic reaction was directly followed at 340 nm for 120 s. The benzaldehyde concentration in the assay was 0.25 mM. Additionally, the activity was determined against 3-methyl-1,5-pentane diol by following the increase of NADH at 340 nm for 120 s (Table S1).

Table S1: Activity of HLADH at different pH's and with different substrates.

against Benzaldehyde (0.5 mM final concentration)								
in Glycine-NaOH buffer (100 mM pH 9.0)								
Enzyme preparation	amount of enzyme [mL]	dilution factor	delta Abs/delta t	delta Abs/delta t	delta Abs/delta t	Average	**Volumetric activity [U/mL]**	**Specific activity [U/mg]**
HLADH1	0.015	0	0.075				0.793650794	1.248875022
	0.03	0	0.105				0.555555556	0.874212516
HLADH2	0.01	0.001	0.1882	0.2026	0.1979	0.196233333	31.14814815	3.990827356
HLADH3	0.01	0.001	0.2995				47.53968254	5.590832301
	0.005	0.0005	0.1846	0.1904		0.1875	59.52380952	7.000207389
against 3-methyl-1,5-pentane diol (0.5 mM final concentration)								
in Glycine-NaOH buffer (100 mM pH 9.0)								
	amount of enzyme [mL]	dilution factor	Substrate conc. [mM]	delta Abs/delta t	delta Abs/delta t	delta Abs/delta t	Average	**Volumetric activity [U/mL]**	**Specific activity [U/mg]**
HLADH2	0.01	0.001	0.5	0.1132				17.96825397	2.302165738
	0.01	0.001	1	0.1573				24.96825397	3.199034193
	0.01	0.001	2	0.1416				22.47619048	2.879740888
HLADH3	0.01	0.001	1	0.1699				26.96825397	3.171560628
	0.005	0.0005	1	0.1229	0.1202		0.12155	38.58730159	4.538001111

against 3-Methyl-1,5-pentane diol (0.5 mM final concentration)									
in Glycine-NaOH buffer (200 mM pH 9.5)									
	amount of enzyme [mL]	dilution factor	Substrate conc. [mM]	delta Abs/delta t	delta Abs/delta t	delta Abs/delta t	Average	**Volumetric activity [U/mL]**	**Specific activity [U/mg]**
HLADH2	0.01	0.001	1	0.1015	0.0948	0.0932	0.0965	15.31746032	1.96253528
HLADH3	0.005	0.0005	1	0.0971	0.0938	0.0895	0.093466667	29.67195767	3.489525603

Synthesis of racemic 4-methyltetrahydro-2H-pyran-2-one

Racemic 4-methyltetrahydro-2H-pyran-2-one was synthesized according to the procedure of Phillips and Graham (2008) in a slightly modified manner.[2] A mixture of *meso*-3-methyl-1,5-pentanediol (205 mmol, 1.215 g) and MnO_2 (17 equiv., 4.8 mol, 20.82 g) in $CHCl_3$ (50 mL) was stirred under reflux conditions at 55°C for 48 hours. After 48 hours the reacion mixture was aliquoted (30 mL each) into 50 mL Falcon tubes, centrifuged (10,000 rpm, 5 min), and the precipitate was washed with $CHCl_3$ (3 x 10 mL each). The solvent was removed under reduced pressure to give a yellowish oily compound (600 mg). ^{1}H-NMR analysis revealed also the presence of the lactole intermediate (4-methyltetrahydro-2Hpyran- 2-ol, 31%). Column chromatography was used for separation by slowly increasing the percentage volume of ethyl acetate in the mobile phase (heptane) from 1% to 50%. All collected fractions were analyzed with TLC. The product-containing fractions were analyzed with GC. The fractions containing either the lactole or the lactone were mixed together. The solvent was evaporated and both compounds were analyzed with ^{1}H NMR (Figure S1) and GC (Figure S2). Pure compounds (317 mg of the lactole with an isolated yield of 26% and 190 mg of the lactone with 16% isolated yield) could be obtained.

^{1}H-NMR: (400 MHz, $CDCl_3$) δ 1.06 (d, J = 6.3 Hz, 3H), 1.56–1.48 (m, 1H), 1.94–1.86 (m, 1H), 2.14–2.04 (m, 2H), 2.71–2.63 (m, 1H), 4.29–4.22 (m, 1H), 4.44– 4.39 (m, 1H).

The baseline separation of the peaks of enantiomers was established with GC analysis (Table S2). The following chromatograms (Figure S1) were obtained for the synthesized *rac*-4-methyltetrahydro-2H-pyran-2-one.

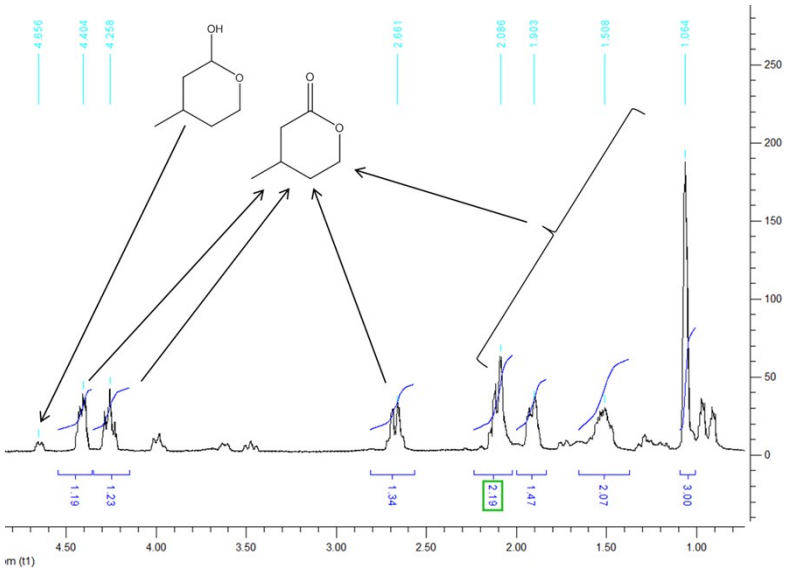


Figure S1. ^{1}H NMR in $CDCl_3$ analysis of the extracted product mixture. The peak at 4.6 ppm is a clear signal from the lactole intermediate.

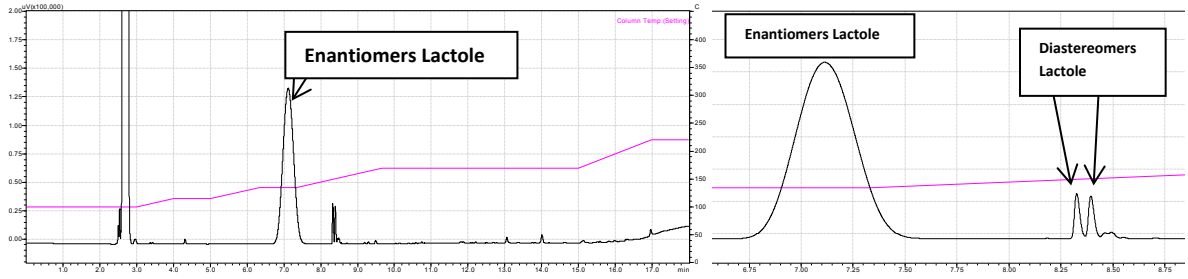


Figure S2. The GC chromatogram of the synthesized lactole intermediate 4-methyltetrahydro-2H-pyran-2-ol (Enantiomers t_R = 7.114 min). Right: Diastereomers of the Lactole (Diastereomer 1 t_R = 8.32 min, Diastereomer 2 t_R = 8.94 min).

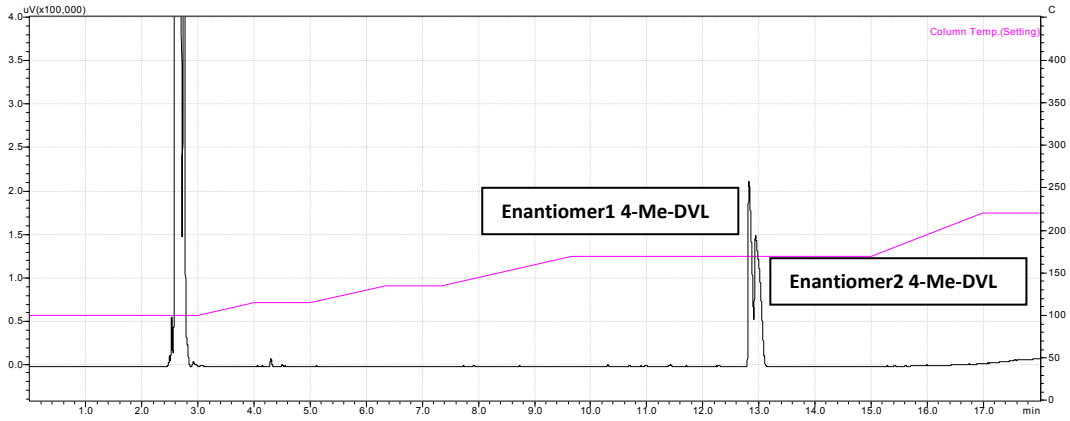


Figure S3. The GC chromatogram of the synthesized *rac*-4-methyltetrahydro-2H-pyran-2-one (Enantiomer 1 t_R = 12.825 min, Enantiomer 2 t_R = 12.943 min).

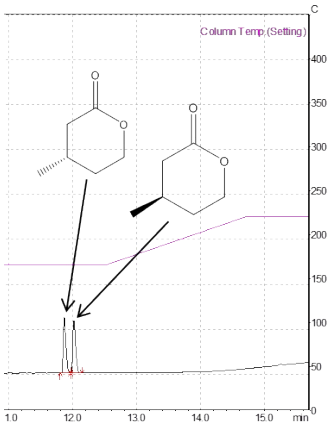


Figure S4. The GC chromatogram showing the baseline separation of both enantiomers of the synthesized *rac*-4-methyltetrahydro-2H-pyran-2-one (Enantiomer 1 t_R = 11.904 min, Enantiomer 2 t_R = 12.05 min).

Synthesis of (*S*)-4-methyltetrahydro-2H-pyran-2-one catalyzed by HLADH

The synthesis of (*S*)-4-methyltetrahydro-2H-pyran-2-one was performed as previously reported by Kara et al. (2013).[3] For this, a stock of *meso*-3-methyl-1,5-pentanediol (0.5 M), NAD^+ stock (25 mM), acetosyringone stock (2 mM), and HLADH stock (3 gL^{-1}) were freshly prepared in 50 mM Tris-HCl buffer at pH 8. The laccase was applied as delivered (0.2 mM solution). The mixture of *meso*-3-methyl-1,5-pentanediol stock (1 mL), acetosyringone stock (1 mL), NAD^+ stock (0.2 mL) and buffer (6.7 mL) was incubated at 30 °C for 5 min. Finally, laccase (0.1 mL) and HLADH solution (1 mL) were added. The starting concentrations were: 50 mM *meso*-3-methyl-1,5-pentanediol, 0.5 mM NAD^+, 200 µM acetosyringone, 0.3 gL^{-1} HLADH and 2 µM laccase. The reaction mixture (10 mL) was orbitaly shaken at 600 rpm in 50 mL Falcon tubes at 30 °C. Samples (50 µL) were taken at defined time intervals and mixed with 200 µL EtOAc (containing 5 mM acetophenone). The mixture was vortexed and dried over anhydrous MgSO4. A conversion of 72 % to the enantiopure (*S*)-4-methyltetrahydro-2H-pyran-2-one (ee > 99% according to GC analysis) was achieved after 16 hours. The reaction mixture (10 mL) was then saturated with NaCl and extracted with EtOAc (3 x 10 mL). After each extraction step the mixture was centrifuged (4000 rpm, 10 min). The collected clear organic phase was dried over anhydrous $MgSO_4$ and the solvent was removed under reduced pressure to give a yellowish oily compound (39 mg). Purification of the crude product was attempted by column chromatography (Pasteur pipette filled with Silica gel 60, 70-230 mesh particle size; solvent petroleum ether: ethyl acetate 9:1).

The following chiral-phase GC chromatogram (Figure S5) was obtained for the synthesized (*S*)-4-methyltetrahydro-2H-pyran-2-one.

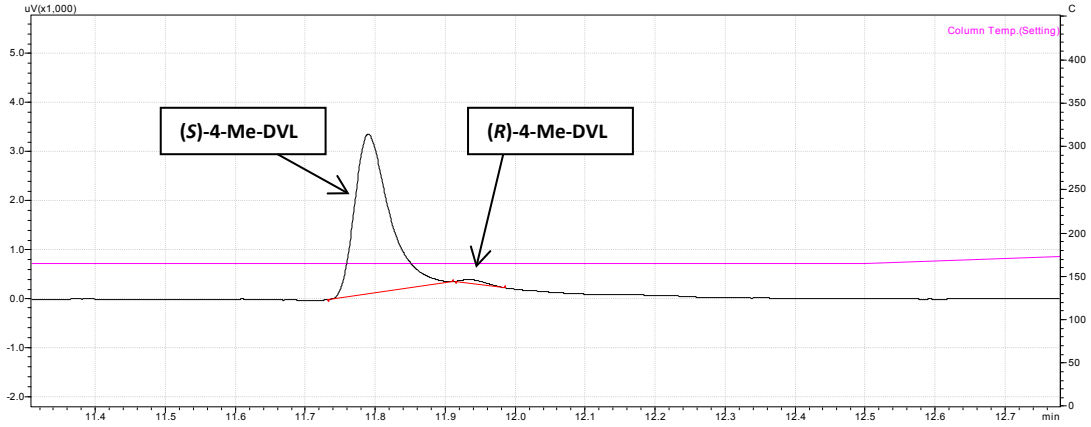


Figure S5. GC chromatogram of the synthesized (*S*)-4-methyltetrahydro-2H-pyran-2-one (t_R = 11.904 min).

(*S*)-4-methyltetrahydro-2H-pyran-2-one: ^{1}H-NMR: (400 MHz, $CDCl_3$) δ 1.07 (d, J = 6.3 Hz, 3H), 1.56–1.47 (m, 1H), 1.95–1.89 (m, 1H), 2.15–2.07 (m, 2H), 2.70–2.64 (m, 1H), 4.30–4.23 (m, 1H), 4.44–4.39 (m, 1H).

Isolated (*S*)-4-methyltetrahydro-2H-pyran-2-one contained 0.98% of the lactole intermediate (4-methyltetrahydro-2H-pyran-2-ol), proved by chiral-phase GC analysis.

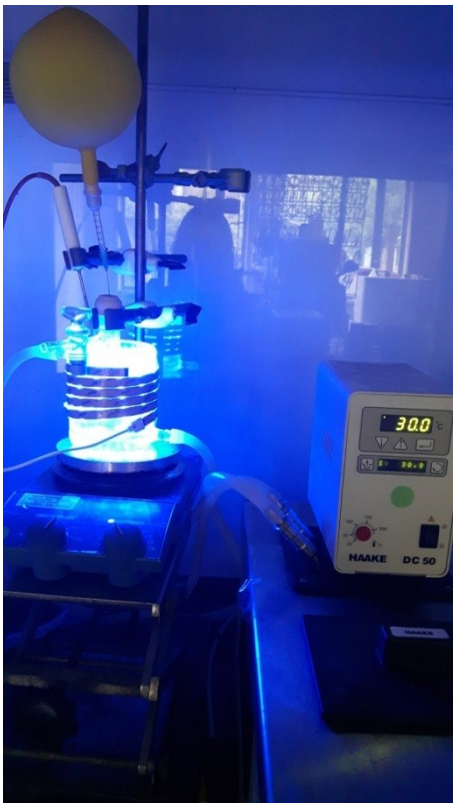


Figure S6. Picture of the reaction setup. Commercially available LED bands (3 colored) were wrapped around a thermostatted reaction vessel and used for illumination of the reaction mixture inside

(generally in a Schlenk vessel) a slight overpressure was achieved by an air-filled balloon to reduce O_2-transfer limitations to the reaction mixture.

Photochemical oxidation of NADH

For a total volume of 2 mL reaction, 1950 μL of 50 mM phosphate buffer (pH 7), 40 μL of NADH (10 mM) and 10 μL of FMN (0.4 mM) were added in a Schlenk flask. At intervals, 1 mL samples were withdrawn, analyzed spectrophotometrically (Agilent Technologies Cary 60 UV-Vis at 25 °C) and returned into the reaction mixture.

Biophotocatalytic oxidation *meso*-3-methyl-1,5-pentanediol

For a total volume of 3 mL reaction, 2100 μL of Glycine NaOH Buffer (pH 9, 100 mM), 150 μL of *meso*-3-methyl-1,5-pentanediol (200 mM), 300 μL of NADH (10 mM), 150 μL of purified HLADH (148 μM) and 5 drops of catalase were added in a Schlenk flask. A slight overpressure with ambient air was ensured by an air-filled balloon connected to the headspace.

For analysis, 50 μL samples of the reaction mixture were taken at intervals. The extraction of the substrate, the intermediate and the product was performed two times with 125 μL ethyl acetate (containing 5mM acetophenone as internal standard). The separation of the two phases was obtained *via* centrifugation (60 sec). The combined organic phases were dried over anhydrous $MgSO_4$ and transferred into GC vials for analysis.
All concentrations reported here are based on calibration curves obtained from authentic standards and treated in the same manner as described here.

Analytics

Gas chromatography (GC)

GC analysis - column

The evaluation of the synthesized product standards as well as the biocatalytic reactions for the chiral compounds was performed using a Shimadzu GC-14A equipped with a Lipodex E column (50 m x 0.25 mm, Macherey & Nagel, Düren, Germany) and flame-ionization detection (FID).

Quantification of conversion

Extraction procedure for GC analysis:

For analysis, samples of the reaction mixture were taken periodically (50 µl). The extraction of the substrate, the intermediate and the product was performed two times with 125 µL acetophenone (5 mM) in EtOAc. Acetophenone was used as internal standard. The separation of the two phases was obtained *via* centrifugation (60 sec).

The combined organic phases were dried over anhydrous $MgSO_4$ and transferred into GC vials for analysis.

GC analysis

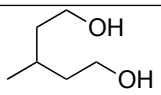
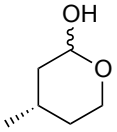
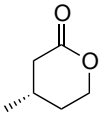
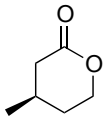
The analysis of the substrate *meso*-3-methyl-1,5-pentanediol, the lactole intermediate as well as the product (*S*)-or (*R*)-4-methyltetrahydro-2H-pyran-2-one was performed with the following GC method:

Rate	**Temperature [˚C]**	**Hold time [min]**
-	125	3.00
10.00	135	2.20
10.00	170	3.10
25.00	220	1.00

Split ratio: 10.00, Linear Velocity: 38 cm/sec, Total flow: 26 mL/min, Column Flow: 2.09 mL/min.

Using this method, it was possible to analyze all compounds in the reaction mixture and to separate the (*S*)- and (*R*)-enantiomers of the methyl-substituted lactone product (Table S2).

Table S2. Retention times of the reaction components.

Name	Compound	Retention time [min]
meso-3-methyl-1,5-pentanediol		9.674
4-methyltetrahydro-2H-pyran-2-ol		4.93
(*S*)-4-methyltetrahydro-2H-pyran-2-one		11.904
(*R*)-4-methyltetrahydro-2H-pyran-2-one		12.05

^{1}H-NMR spectroscopy

All measurements were recorded on a Bruker NMR unit (Bruker, Karlsruhe, Germany) at 400 (^{1}H) MHz. The ^{1}H NMR spectra were recorded in $CDCl_3$. The impurities observed in the spectra were identified according to Gottlieb et al.[4]

Supporting results

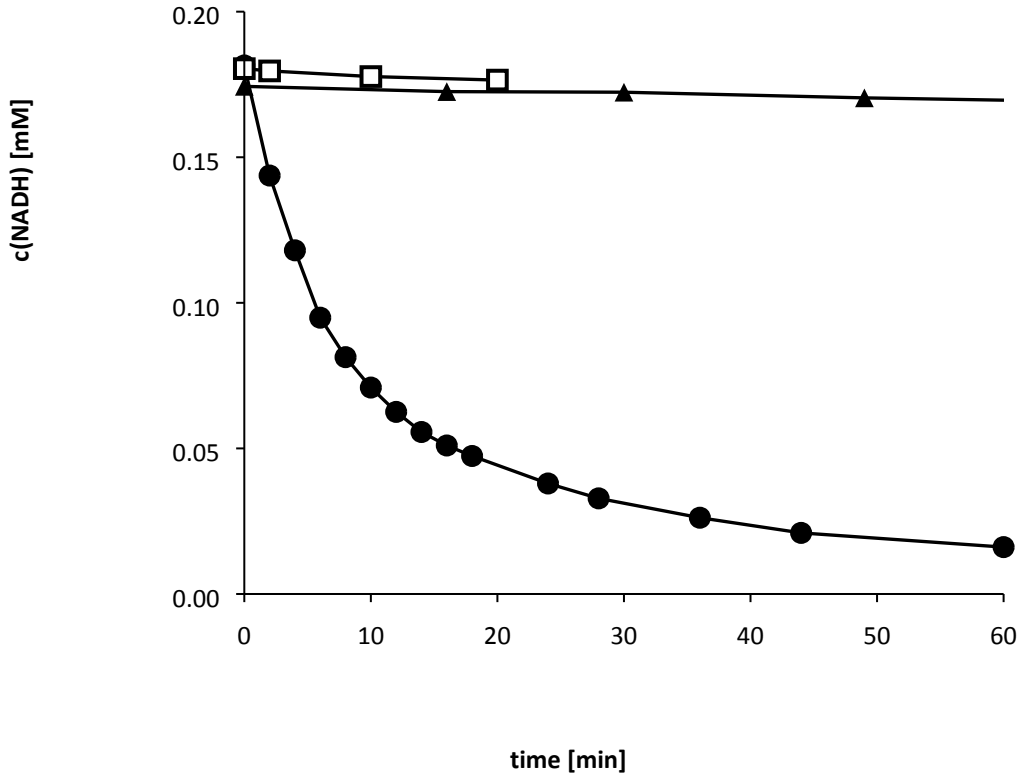


Figure S7. Photocatalytic oxidation of NADH using the entire system (●) or in the absence of either FMN (□) or light (■). Conditions: potassium phosphate buffer (pH 7, 50 mM), T = 30 °C, $[NADH]_0$ = 0.2 mM, $[FMN]_0$ = 2 μM.

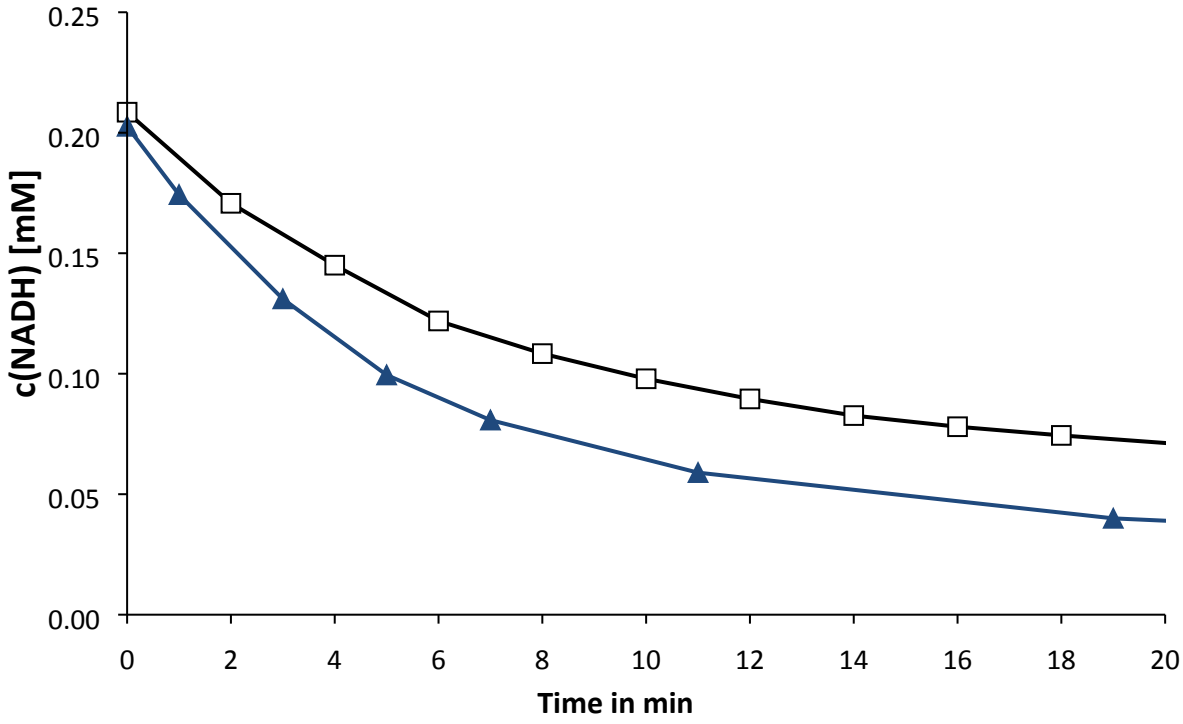


Figure S8. Photocatalytic oxidation of NADH using the traditional white light bulb (☐) or blue LED light (🟦). Conditions: 50 mM KPi buffer (pH 7), T = 30 °C, $[NADH]_0$ = 0.2 mM, [FMN] = 2 µM.

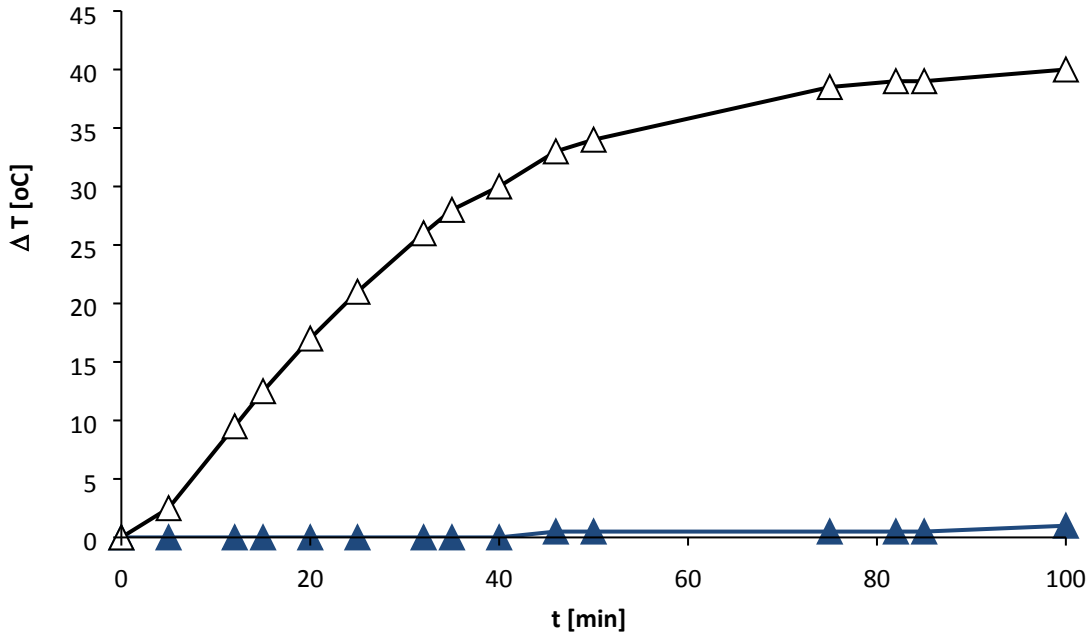


Figure S9. Effect of different light sources on the temperature of a non-thermostatted reaction vessel. LEDs (🟦) and white light bulb (❐).

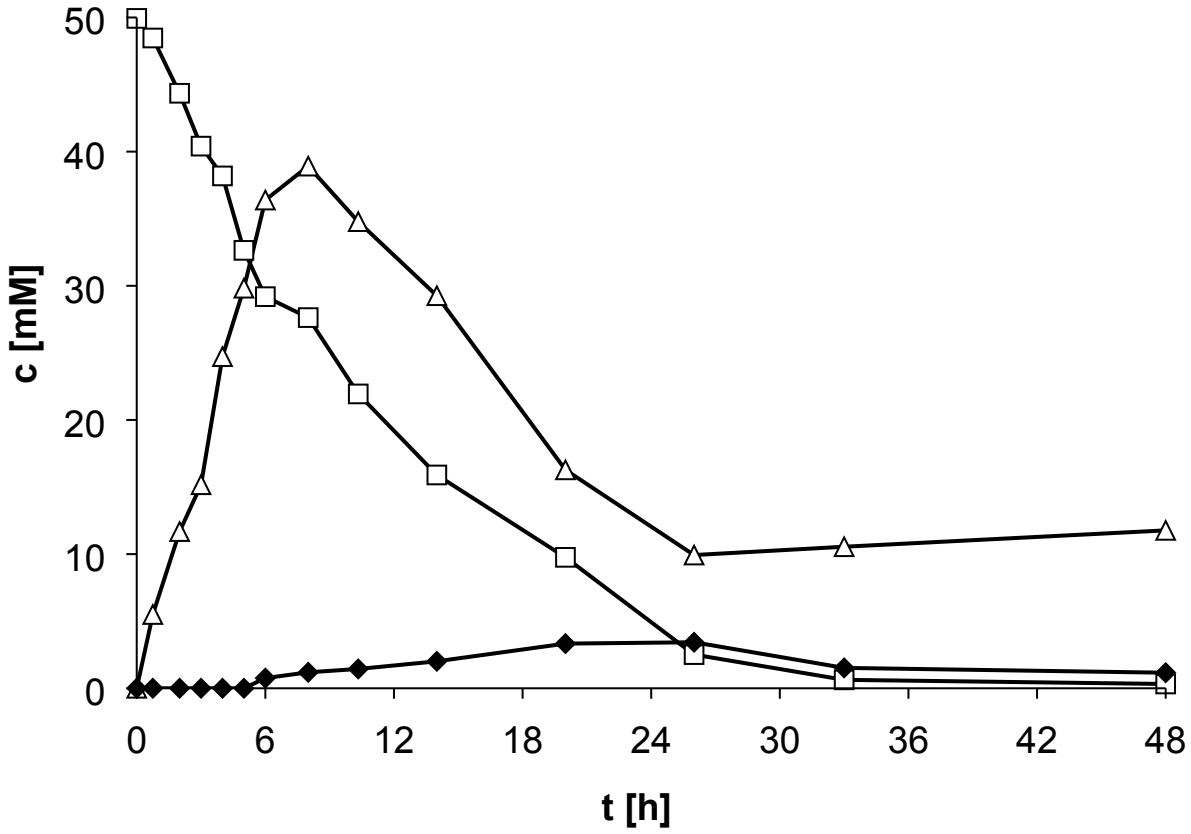


Figure S10. Time course of the photoenzymatic oxidation of *meso*-3-methyl-1,5-pentanediol (☐) to *(S)*-4-methyltetrahydro-2H-pyran-2-one (◆) via the intermediate lactol (❒). General conditions: 200 mM Glycine-NaOH buffer (pH 9.5), $[diol]_0$ = 50 mM, $[NADH]_0$ = 1 mM, [FMN] = 100 µM, [HLADH] = 7.4 µM, 5 drops of catalase, T = 30°C.

Estimation of the CO_2 emissions (E-factor on CO_2)

According to the European Energy Agency (www.eea.europa.eu; accessed on October 8th 2016) the average European CO_2 emission intensity (g CO_2/kWh) for public electricity in 2013 was 558 g CO_2/kWh. This value translates into 155 10^{-6} g(CO_2) per joule.

The power consumption of the white light bulb and the LEDs used in this study are 250 W and 24 W, respectively (manufacturers' data). These numbers translate into 139.5 and 13.4 g CO_2 h^{-1} emitted due to illumination using the white light bulb or the LED, respectively.

The volumetric productivity achieved in this study are approximately 10 mM h^{-1} (corresponding to 1.14 g $L^{-1}h^{-1}$). In absolute numbers (3 mL reaction volume) this corresponds to 3.42 * 10^{-3} g h^{-1}. The resulting E-factors (on CO_2 are summarized in Table S3).

Similarly, the CO_2 consumptions due to cooling can be estimated using the specific heat capacity of water (4185.5 $Jkg^{-1}K^{-1}$). From Figure S9, an average temperature increase of 71°C per hour was extrapolated using the white light bulb. Hence, roughly 297 kJ L^{-1} h^{-1} are consumed corresponding to 46 g L^{-1} h^{-1} CO_2 emission needed to maintain the reaction temperature.

Table S3. E-factor(CO_2) estimations.

E-factor [kg(CO_2)/kg(product)]	White light bulb	LED
illumination	40790	3920
cooling	40.4	-

References

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[3] S. Kara, D. Spickermann, J. H. Schrittwieser, A. Weckbecker, C. Leggewie, I. W. C. E. Arends, F. Hollmann **2013** *ACS Catalysis 3* (11), 2436-2439.

[4] H. E. Gottlieb, V. Kotlyar, A. Nudelman, *J. Org. Chem.* **1997**, *62*, 7512-7515.