Article

Supplementary Material

Laccase did it again: a scalable and clean regeneration system for NAD+ and its application in the synthesis of 12-oxo-hydroxysteroids

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Contents

Figure S1. NGC chromatogram of *Mt*Lac purification by ion-exchange chromatography on Q-Sepharose column. Absorbance at 280 nm and concentration of elution buffer are depicted with blue and red lines, respectively. Fractions containing *Mt*Lac are highlighted in the grey box.

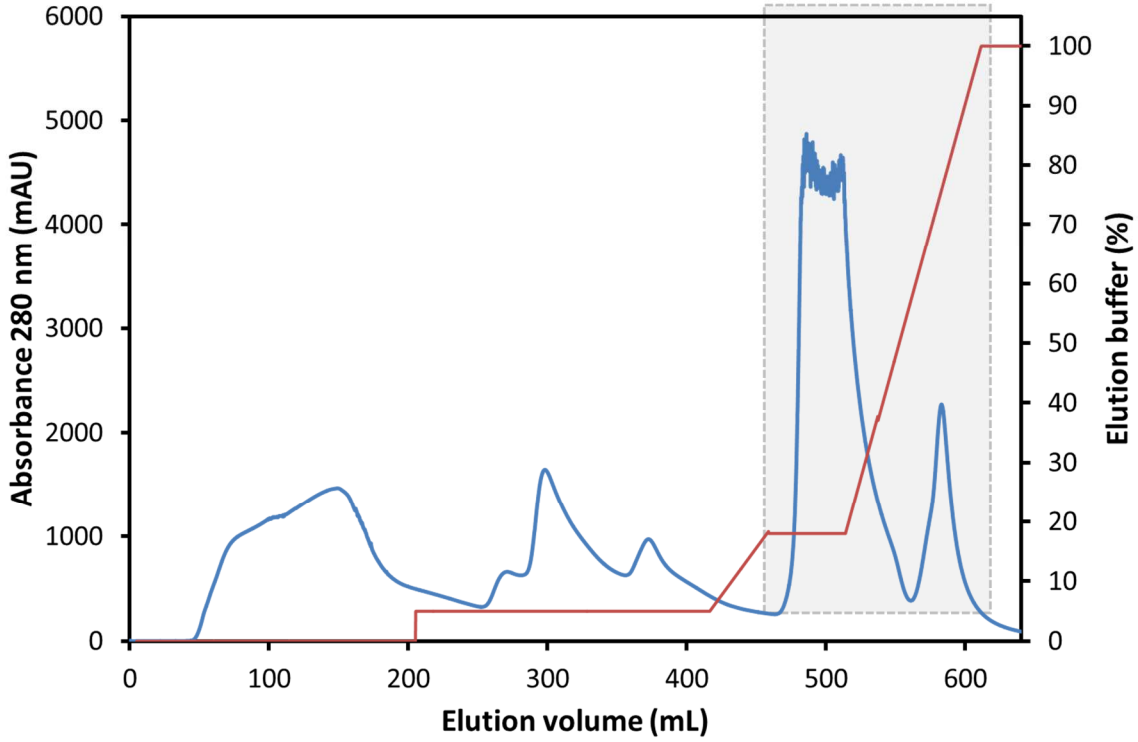
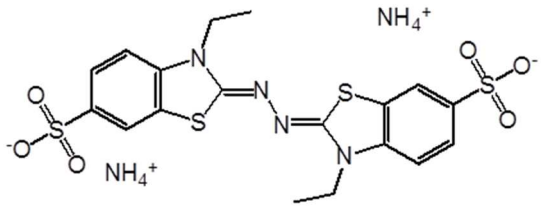
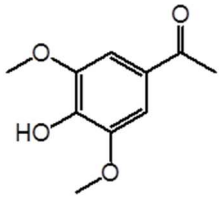
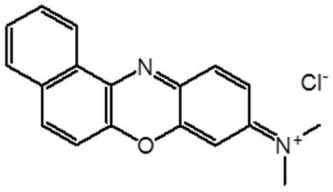
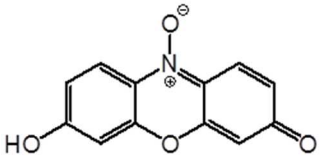
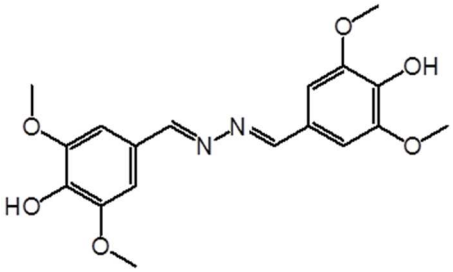
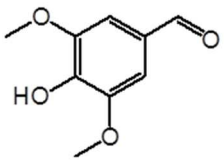


Table S1. List of laccases used in the study. The enzyme concentration in stock solutions is reported.

Organism	Abbreviation	[Enzyme] (mg/mL)
Bacillus subtilis	*Bs*Lac	3.6
Casuarina glauca	*Cg*Lac	6.5
Myceliophthora thermophila	*Mt*Lac	12.7
Trametes trogii	*Tt*Lac	6.0
Trametes versicolor	*Tv*Lac	1.0

Table S2. Bioconversion of 10 mM CA to 12-oxo-CDCA in presence of 0.34 mg/mL *Mt*Lac, 0.013 mg/mL *El*12α-HSDH, 0.5 mM NAD^+ and 5 μM of different mediators. Reactions were carried out in presence of 100 mM KPi buffer pH 8.0 at 25 °C for 24 h. Samples were analyzed by HPLC chromatography. In all the cases we observe a reaction selectivity of 100%.

Mediator		Conversion (%)
ABTS		**99.7%**
Acetosyringone		2.9%
Meldola's blue		0.7%
Resazurine		0.0%
Syringaldazine		**95.6%**
Syringaldehyde		3.8%

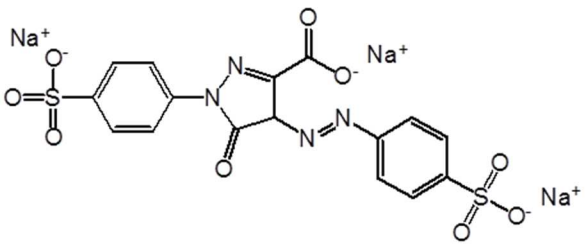
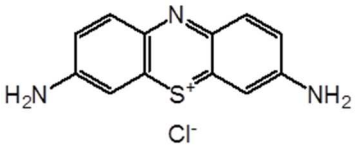
Tartazine		0.0%
Thionine		0.0%

Table S3. Activity of EvoCatal ADH screening kit towards hydroxysteroids and isopropanol. EvoCatal ADH030, ADH200 and ADH420 were compared in terms of efficiency in regenerating the cofactor NAD^+ to facilitate a double-enzyme-coupled system.

	Activity (U/g)[a]				Conversion[b]
	CDCA	DCA	UDCA	isopropanol	(%)
ADH010	5	4	0	74	
ADH020	10	3	1	35	
ADH030	**11**	**3**	**1**	**1411**	**12.6%**
ADH040	16	4	1	4	
ADH130	4	1	1	n.d.	
ADH140	5	1	1	n.d.	
ADH200	**17**	**5**	**4**	**366**	**4.5%**
ADH210	7	2	1	11	
ADH280	9	2	1	6	
ADH380	10	3	1	141	
ADH420	**6**	**2**	**0**	**1225**	**57.2%**
ADH430	4	0	1	28	
ADH441	2	2	1	287	
ADH442	8	1	0	11	

[a]The activity of the different ADHs was spectrophotometrically assayed following the NAD^+ reduction at 340 nm (ε_{340nm} = 6.2 mM^{-1} cm^{-1}). The activity towards hydroxysteroids was determined in presence of 10 mM substrate (CDCA, DCA and UDCA), 0.5mM NAD^+, 50 mM KPi buffer pH 8.0. The activity towards isopropanol was determined in presence of 15% (v/V) isopropanol, 0.5 mM NAD^+, 50 mM KPi buffer pH 8.0. All the reactions were carried out at 25 °C for 10 minutes. [b]Enzymatic oxidation of the 12α-OH group of CA to 12-oxo-CDCA was performed. Bioconversion were set up in presence of 120 mM of CA, 0.5 mM NAD^+, 100 mM KPi pH 7.2, 15% acetone, 0.013 mg/mL of *El*12α-HSDH and 0.05 mg/mL of commercial ADH. Reactions were carried out at 25 °C for 24 h. Samples were analyzed by HPLC chromatography. In all the cases we observe a reaction selectivity of 100%.

Table S4. Calculation of E-values and atom efficiencies. E-values were calculated as $Weight_{waste}$ / $Weight_{product}$. Atom efficiencies were calculated as $MW_{product}$ x 100 / $MW_{reactants}$.

a) *El*12α-HSDH + *Mt*Lac + Syringaldazine (this study).

$$CA + ½\ O_2 \rightarrow 12\text{-oxo-CDCA} + H_2O$$

	Compound	Weight (mg)
Product	12-oxo-CDCA	18377
Waste	CA (wasted)	1975
	NAD^+	136
	Syringaldazine	21
	DMSO	45100
	KPi pH 7.2	6700
E-Value	**2.9**	

	Compound	Equivalent	MW
Product	12-oxo-CDCA	1	406
Reagents	CA	1	408
	O_2	0.5	32
Atom efficiency	**95.8**		

b) *El*12α-HSDH + ADH420 + Acetone (this study).

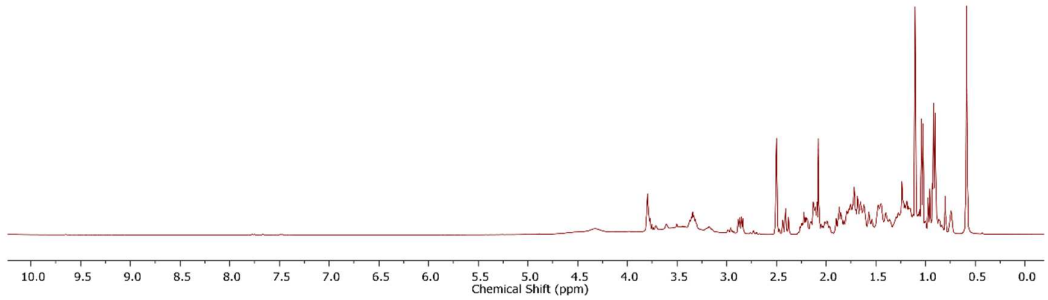
$$CA + Acetone \rightarrow 12\text{-oxo-CDCA} + i\text{-PrOH}$$

	Compound	Weight (mg)
Product	12-oxo-CDCA	1829
Waste	CA (wasted)	178
	NAD^+	14
	Acetone	4822
	KPi pH 7.2	670
E-value	**3.1**	

	Compound	Equivalent	MW
Product	12-oxo-CDCA	1	406
Reagents	CA	1	408
	Acetone	1	78
Atom efficiency	**83.5**		

Figure S2. (a) ^{1}H-NMR (400 MHz, DMSO-*d6*) and (b) ^{13}C-NMR (100 MHz, DMSO-*d6*) spectra of the obtained 12-oxo-chenodeoxycholic acid.

a)



b)