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Article

Laccase Did It Again: A Scalable and Clean Regeneration System for NAD⁺ and Its Application in the Synthesis of 12-oxo-Hydroxysteroids

Fabio Tonin, Elisabet Martì, Isabel W. C. E. Arends [†] and Ulf Hanefeld * 

Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands; F.Tonin@tudelft.nl (F.T.); emartisolier@gmail.com (E.M.); I.W.C.E.Arends@tudelft.nl (I.W.C.E.A.)

* Correspondence: u.hanefeld@tudelft.nl; Tel.: +31-15-2789304

[†] Current address: Faculty of Science, Utrecht University, Budapestlaan 6, 3584 CD Utrecht, The Netherlands.

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Abstract: The specific oxidation of 12 α -OH group of hydroxysteroids is required for the preparation of cheno- and ursodeoxycholic acid (CDCA and UDCA, respectively). The C₁₂ oxidation of hydroxysteroids into their 12-oxo derivatives can selectively be performed by employing 12 α -hydroxysteroid dehydrogenases. These enzymes use NAD(P)⁺ as an electron acceptor, which has to be re-oxidized in a so-called “regeneration system”. Recently, the enzyme NAD(P)H oxidase (NOX) was applied for the regeneration of NAD⁺ in the enzymatic preparation of 12-oxo-CDCA from cholic acid (CA), which allows air to be used as an oxidant. However, the NOX system suffers from low activity and low stability. Moreover, the substrate loading is limited to 10 mM. In this study, the laccase/mediator system was investigated as a possible alternative to NOX, employing air as an oxidant. The laccase/mediator system shows higher productivity and scalability than the NOX system. This was proven with a preparative biotransformation of 20 g of CA into 12-oxo-CDCA (92% isolated yield) by employing a substrate loading of 120 mM (corresponding to 50 g/L). Additionally, the performance of the laccase/mediator system was compared with a classical ADH/acetone regeneration system and with other regeneration systems reported in literature.

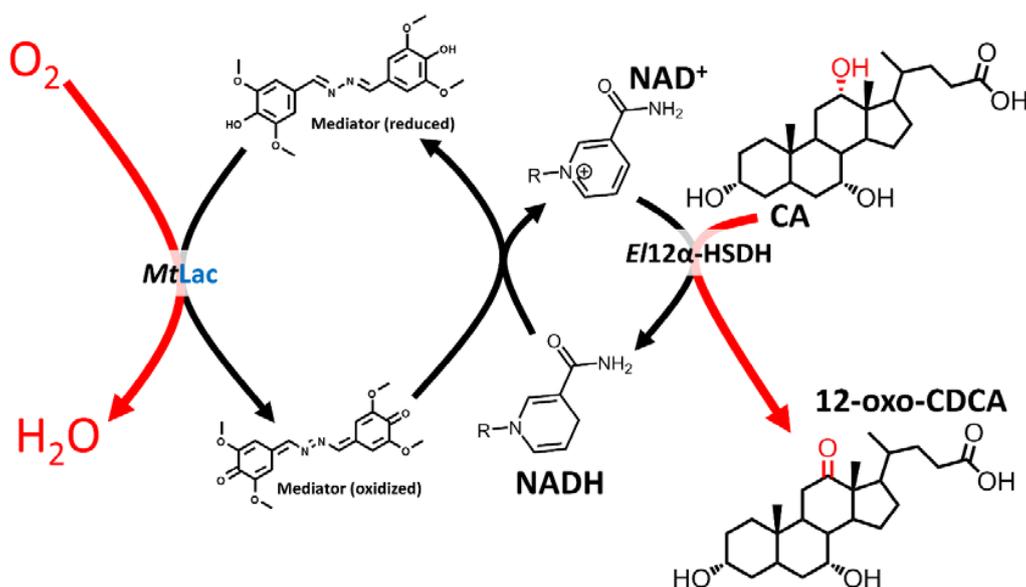
Keywords: hydroxysteroid dehydrogenase; laccase-mediator; NAD⁺ regeneration; biocatalysis; laccase

1. Introduction

Cheno- and ursodeoxycholic acid (CDCA and UDCA, respectively) are used for the treatment of cholestatic diseases by reducing the absorption of cholesterol and improving the digestion of fatty acids through stimulation of the liver functions [1–4]. CDCA can be produced via dehydroxylation of the readily available cholic acid (CA) at C₁₂, and subsequent epimerization at C₇ affords UDCA.

However, chemical synthesis routes for the conversion of CA to CDCA or UDCA employ toxic chemicals in stoichiometric amounts, suffer from low yields and lead to the formation of large amounts of waste. Despite these disadvantages, the chemical transformation of CA to CDCA or UDCA is still currently used, making a replacement with a benign approach urgent [5–7].

Alternatively, the dehydroxylation at C₁₂ can be performed in a two-step chemo-enzymatic reaction employing: (i) the selective oxidation of the 12-OH group to form 12-oxo-CDCA catalyzed by the enzyme 12 α -hydroxysteroid dehydrogenase (12 α -HSDH, Scheme 1); (ii) the Wolff-Kishner reduction of 12-oxo-CDCA or 12-oxo-UDCA to form CDCA or UDCA, respectively.



Scheme 1. Reaction scheme of the proposed enzymatic cascade for the synthesis of 12-oxo-CDCA.

12 α -HSDHs specifically oxidize the C₁₂ hydroxyl group to a ketone, with a concomitant reduction of NAD^+ [5]. To obtain an economical and sustainable process, a regeneration system for the nicotinamide cofactors is required. Previously, the enzyme NAD(P)H oxidase (NOX) was applied for the regeneration of NAD^+ in the enzymatic preparation of 12-oxo-CDCA [6]. NOX uses O_2 for the oxidation of NAD(P)H and only generates water as a side product. However, NOX regeneration systems suffer from low activity, low stability and high costs. Moreover, the substrate loading is limited to 10 mM.

Further $NAD(P)^+$ regeneration systems, such as glutamate dehydrogenase, *i*PrOH (alcohol) dehydrogenase and lactate dehydrogenase were recently reviewed for the enzymatic synthesis of mono- and diketo hydroxysteroids [7]. These methods involve the reduction of an organic sacrificial substrate (α -ketoglutarate, acetone or pyruvate, respectively) to the corresponding alcohol/amine. Other solutions for the regeneration of NAD^+ , employing molecular O_2 as final electron acceptor, were also identified. Profiting from the spontaneous auto-oxidation of FMN, a flavin reductase was employed to regenerate NAD^+ in the biocatalytic oxidation of CDCA to 7-oxo-LCA [8]. Laccase/mediator systems have been similarly applied in the same reaction [9,10]. Specifically, laccases catalyze the oxidation of several low molecular weight compounds, which function as mediators by spontaneously oxidizing $NADH$ without interfering with the enzymatic oxidation of CDCA [9,11–13].

To develop a feasible, preparative-scale oxidation of CA to 12-oxo-CDCA relying on oxygen as oxidant, the laccase/mediator system was coupled with the E112 α -HSDH enzyme (Scheme 1).

The influence of several factors on the enzymatic activity and stability of the system were tested (pH values, different mediator types and cosolvents, enzyme and mediator concentrations and substrate loading). Finally, a preparative scale reaction was performed under optimized conditions, employing a substrate loading of 120 mM CA (corresponding to 50 g/L) with as little as 150 μ M of syringaldazine as mediator.

The production of the enzyme E112 α -HSDH was improved, and the overall environmental impact was analyzed to ensure a functional process.

2. Results

2.1. *E112 α -HSDH* Expression Trials

Expression trials were initially carried out to optimize the heterologous production of *E112 α -HSDH* in *E. coli*. Several modifications of the commonly used Lysogeny Broth (LB) medium have been described with the aim of increasing cell yield, such as supplementing both fermentable and non-fermentable carbon sources and phosphate buffers. Based on this, different media compositions and the addition of NaCl at the moment of induction were investigated. The expression of *E112 α -HSDH* enzyme was investigated by measuring its activity in the crude extract.

The enzymatic activity varied considerably depending on the type of media used. A high expression of *E112 α -HSDH* was measured in YT (2928 U/L_{broth}) and TB (3020 U/L_{broth}) medium (Figure 1). The addition of NaCl to the medium subjects the cells to osmotic stress, and should have a positive effect on protein expression [14]. In this case, a negative effect was observed, lowering the expression of *E112 α -HSDH* in all the three different media. Overall, a five-fold increase in volumetric expression was obtained, in comparison with the previously reported expression in LB medium (638 U/L_{broth}) [6].

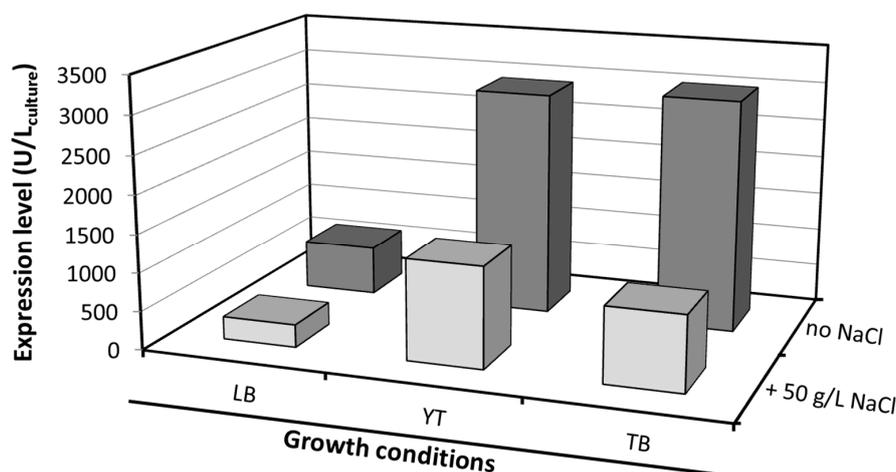


Figure 1. Expression trials of *E112 α -HSDH* in different media.

2.2. Laccase/Mediator System for the Regeneration of NAD⁺

To establish a suitable and efficient regeneration system for NAD⁺, several laccases from different origins were tested for their ability to oxidize NADH with three different mediators (acetosyringone, syringaldehyde and syringaldazine, Table S1). Laccase activity was assayed in the presence of 2 μ M mediator. The spectrophotometric assay was carried out at two different pH values (5 and 8) by following the consumption of NADH at 340 nm (Figure 2).

The laccase from *Myceliophthora thermophila* (*MtLac*) showed the highest activity at pH 8.0 (*E112 α -HSDH* is active and stable at this pH) [6], displaying 30-fold higher activity than the NOX system. *MtLac* is a commercially available enzyme, with a moderate-low price. Its safety in use, high activity and stability have been reported for other applications [15]. Therefore, *MtLac* was chosen for the subsequent studies towards the development of the proposed biocatalytic cascade.

In the next step, a wider selection of mediators was investigated with *MtLac* (Table S2). In this case, we chose to directly evaluate the bio-production of the desired 12-oxo-CDCA through incubation of 10 mM CA with *E112 α -HSDH*, in the presence of 0.5 mM of NAD⁺, laccase (0.34 mg/mL) and mediators (all at a concentration of 5 μ M) at pH 8.0. High conversions of CA to 12-oxo-CDCA were obtained within 4 h with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and syringaldazine as mediators, leading to 99% and 95% conversion, respectively. In contrast to previous studies, low reaction rates were observed with Meldola's Blue [9]. This difference can be attributed to the different

type of laccases used and to the lower concentration of mediator in the bioconversion (Table S2). To compare the different mediators, the specific activity of the different *MtLac*/mediator systems was evaluated by a spectrophotometric assay, following the oxidation of NADH at 340 nm (Figure 3). A high activity was observed when syringaldazine or ABTS were used as mediator, confirming the results obtained for the conversion of CA. Notably, syringaldazine is slowly oxidized by molecular oxygen and shows absorbance at 340 nm for different redox states. This results in an apparent background activity when syringaldazine is used as mediator [13]. Thus, ABTS and syringaldazine were selected for subsequent investigations.

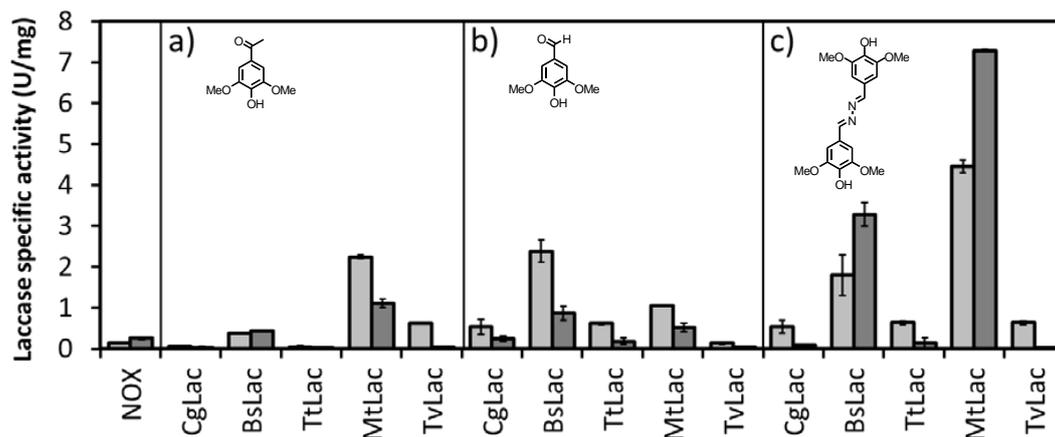


Figure 2. Screening of different laccases in combination with (a) acetosyringone, (b) syringaldehyde and (c) syringaldazine. The activities of the different laccase/mediator systems were spectrophotometrically assayed at pH 5.0 (light grey) and pH 8.0 (dark grey) following the decrease of absorbance at 340 nm (NADH oxidation). Activity of NOX in presence of 0.5 mM NADH at pH 5 and pH 8 is reported in the firsts two columns. Laccases from *Casuarina glauca* (CgLac), *Bacillus subtilis* (BsLac), *Trametes trogii* (TtLac), *Myceliophthora thermophila* (MtLac) and *Trametes versicolor* (TvLac) were used.

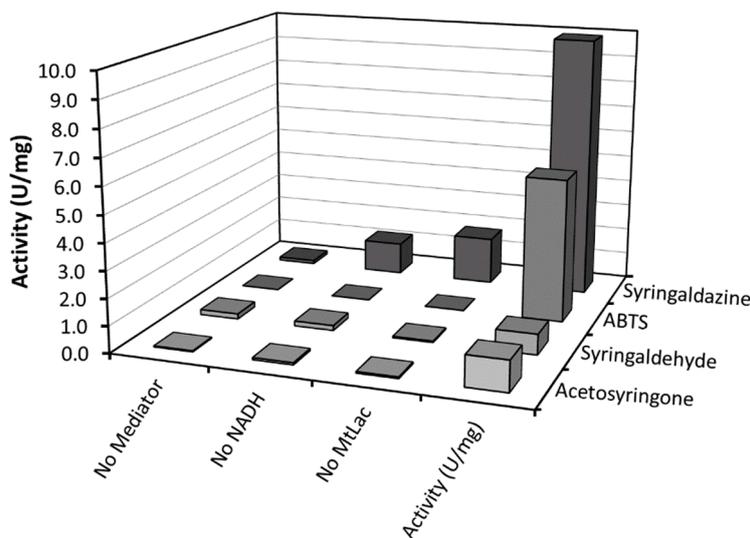


Figure 3. Activity of *MtLac* in presence of 5 μ M of different mediators, 0.5 mM NADH and 100 mM KPi buffer (pH 8.0) at 25 $^{\circ}$ C. The background activities of the controls in absence of NADH, mediator and laccases are reported.

The amount of mediator is another parameter that needs to be optimized for the bioconversion: ideally, mediators should be used at the lowest concentration possible, in order to obtain a clean reaction and minimize the generation of waste. Bioconversions of CA with three different concentrations

of ABTS and syringaldazine (0.01, 0.02 and 0.05 mM) were followed over time. Full conversion of 10 mM CA was obtained for all combinations within 6 h. While bioconversions with syringaldazine as mediator are faster and less dependent on the concentration of mediator used, bioconversions with ABTS are slower and the rate of the reaction depends on the concentration of mediator used (Figure 4). This can be attributed to the higher K_M of fungal laccases, such as *MtLac* for ABTS at basic pH values [16].

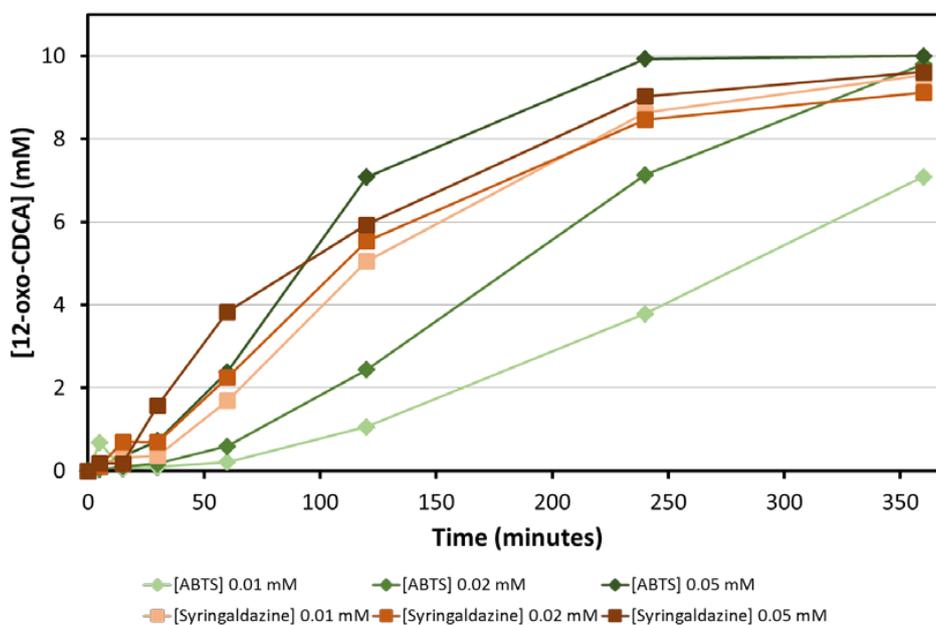


Figure 4. Bioconversion time courses employing *E12 α* -HSDH coupled with *MtLac* and varying amounts of syringaldazine (red lines) or ABTS (green lines). Reactions were performed in the presence of 10 mM CA, 0.5 mM NADH in 100 mM KPi buffer (pH 8.0) at 25 °C.

The pH optimum of *MtLac* depends on the respective substrate/mediator pair used and was previously reported by Berka et al. [17]. When ABTS is used as substrate, *MtLac* shows optimal activity in the acidic range (3–6), which is mismatched with the pH optimum of HSDHs (pH 7.0–9.0). In contrast, *MtLac* shows maximal activity around pH 7.0–7.5 when syringaldazine is used as mediator. Consequently, a pH value of 7.2 was chosen for further studies. Notably, CA is comparably soluble at pH 7.2 and 8.0 ($pK_a = 4.6$) [18,19].

2.3. Substrate Loading and Cosolvent

Preceding publications have highlighted the low solubility of hydroxysteroids in aqueous media as the main bottleneck for the biocatalytic preparation of 12-oxo-hydroxysteroids [5,20]. Having established the laccase/mediator system as a suitable way to regenerate NAD^+ , our focus changed toward increasing the substrate loading. Both monophasic and biphasic cosolvent systems have been shown to increase the substrate loading of hydroxysteroids in aqueous environments. However, the addition of organic solvents to the reaction mixture can influence the enzymatic activities [21].

Based on preliminary studies, MeOH and DMSO were selected as cosolvents and their effect on the activities of the enzymes involved in the biotransformation was investigated. Bioconversions with 20, 50, 100 and 250 mM of CA were set up in the presence of MeOH (10%, 20% and 40%) and DMSO (10%). Notably, 10% of DMSO was enough to fully solubilize 250 mM of CA, and 85.8 mM of 12-oxo-CDCA were obtained. For this reason, higher concentrations of DMSO were not investigated.

In contrast, >10% methanol is needed to increase the substrate concentration above 10 mM, resulting in a loss of activity and thus lower conversions (Table 1). Previous studies showed no decrease

of activity for *E112 α* -HSDH in the presence of 10% MeOH [6], while *MtLac* activity was reported to decrease over time when incubated for long periods with MeOH [22].

Table 1. Analytical scale bioconversions of CA to 12-oxo-CDCA when the *E112 α* -HSDH/*MtLac*/syringaldazine system is employed with different substrate loadings (10, 20, 50, 100 and 250 mM) and different cosolvents at pH 7.2. All reactions were carried out for 48 h at 25 °C. Conversions were determined by HPLC analysis. All bioconversions were carried out employing 0.34 mg/mL (1.8 U/mL) *MtLac* and 0.013 mg/mL (0.8 U/mL) *E112 α* -HSDH.

Entry	[CA] (mM)	[Cosolvent] (%)	[12-oxo-CDCA] (mM)	Conversion (%)	
1	10	MeOH	10%	9.6	96.1
2	20	MeOH	20%	14.5	72.7
3	50	MeOH	40%	1.5	3.0
4	100 ^a	MeOH	40%	0.7	0.7
5	250 ^a	MeOH	40%	n.c.	n.c.
6	10	DMSO	10%	9.3	93.3
7	20	DMSO	10%	19.7	98.3
8	50	DMSO	10%	49.7	99.5
9	100	DMSO	10%	85.8	85.8
10	250	DMSO	10%	43.9	17.6

^a Due to the substrate solubility limit, the reaction was carried out as slurry suspension of CA. n.c. no conversion was observed.

According to the GlaxoSmithKline (GSK) solvent sustainability guide [23,24], DMSO shows moderate complications when used at low temperatures. The main disadvantage consists in its difficult removal from the product mixture due to a high boiling point. However, since it enables a high enzyme activity as well as a better solubilization of CA in water, it was chosen as cosolvent for the preparative scale production of 12-oxo-CDCA.

2.4. 20 g Scale Preparation of 12-oxo-CDCA

To demonstrate the synthetic potential of the proposed system, a preparative scale bioconversion was carried out. 20 g of CA were dissolved in 100 mM KPi buffer, pH 7.2 in the presence of 10% DMSO, resulting in a final concentration of 50 g/L for CA (corresponding to 120 mM).

The solution was initially heated in order to completely dissolve the CA. Once the solution reached homogeneity, the mixture was cooled (25 °C) and supplemented with 0.5 mM NAD⁺, 50 μ M of syringaldazine, 0.013 mg/mL (0.8 U/mL) of *E112 α* -HSDH and 0.34 mg/mL (1.8 U/mL) of *MtLac*.

The reaction proceeded for 7 days, and samples were analyzed by HPLC at different times (Figure 5, blue line). The pH was checked every 24 h. When a small shift in pH was observed (0.1–0.2 pH points) the pH was adjusted with 1 M KOH.

After 85 h, a decrease of reaction rate was observed. This can be attributed to the degradation of the mediator, which was observed in previous experiments. The addition of 7 mg of syringaldazine (corresponding to a concentration of 50 μ M) resulted in the restart of the reaction. A second addition of syringaldazine after 115 h similarly restarted the reaction, driving it finally to completion (98%). A total of 21 mg of syringaldazine (final concentration of 150 μ M) were required to convert 20 g of CA.

The product (12-oxo-CDCA) was precipitated by acidification of the reaction mixture to pH 3 with 6 M HCl. It was subsequently extracted with diethyl ether (5 \times 200 mL). Notably, this volume could be reduced by removing the enzymes from the reaction mixture by ultrafiltration. The organic phase was dried over MgSO₄ and the ether was evaporated under reduced pressure. Finally, 18.4 g of product was obtained (92% isolated yield).

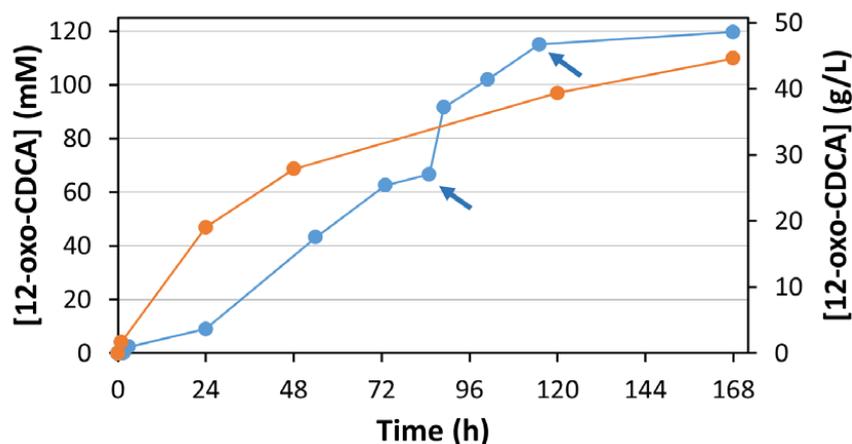


Figure 5. Bioconversion time-courses employing *El12 α* -HSDH (0.013 mg/mL–0.8 U/mL with CA as substrate) coupled with: (blue line) *MfLac* (0.34 mg/mL–1.8 U/mL), syringaldazine (50 μ M) and 10% DMSO; (orange line) ADH420 (0.05 mg/mL–0.06 U/mL) and 15% (v/v) acetone. All reactions were carried out in presence of 0.5 mM NAD⁺ in 100 mM KPi buffer, pH 7.2 at 25 °C. The addition of 7 mg of syringaldazine (corresponding to 50 μ M in the final mixture) is indicated with an arrow.

2.5. Cofactor Regeneration with a Classical Regeneration System

To compare the performance of the *MfLac*/syringaldazine system with a classical regeneration system (employing a ketone as stoichiometric oxidant by ADHs), 14 commercial NAD⁺-dependent enzymes from the EvoCatal (ADH Kit) were screened for their ability to oxidize *i*-PrOH with concomitant reduction of NAD⁺ to NADH (Table S3) under the conditions employed in our experiments. Acetone and *i*-PrOH are cheap sacrificial substrates that can ideally also be used as cosolvents.

The enzyme ADH420 (from unknown source) was selected for its ability to efficiently oxidize *i*-PrOH, showing a specific activity of 1.2 U/mg. Additionally, this enzyme does not accept CA as substrate, avoiding the formation of side-products.

In the next step, CA (2 g) was incubated with *El12a*-HSDH and ADH420. 15% acetone (corresponding to a final concentration of 2 M) was simultaneously used as cosolvent and sacrificial substrate. The reaction mixture was sampled at regular intervals and analyzed by HPLC (Figure 5, orange line). After 168 h of incubation, 12-oxo-CDCA was obtained in 92% isolated yield.

3. Discussion

To date, there is no cofactor recycling system that fully complies with the principles of Green Chemistry. Several enzymatic, chemical and electrochemical methods have been proposed in literature for the regeneration of NAD⁺, but it is still not possible to clearly state which one outperforms the others [25]. In the particular case of ADHs, biocatalytic reactions are driven by the addition of sacrificial substrates that generate waste compounds of no particular interest. In addition, due to the reversibility of the reactions involved, these sacrificial substrates have to be used in excess.

NOX has been identified as a possible candidate for the clean regeneration of NAD⁺. This enzyme catalyzes the oxidation of NAD(P)H using molecular oxygen as sacrificial substrate and generates water as the sole side product. Since this reaction is irreversible ($K_{eq} = 10^{80}$), oxygen is not needed in excess. Nonetheless, the poor solubility of molecular oxygen in water is frequently limiting its application as a stoichiometric reagent. However, different technologies can be used to overcome this limitation: for example, segmented-flow reactors have been shown to maximize mass transfer between the gas and the water phase, allowing for quicker reactions than the corresponding batch mode. Other types of reactor design (e.g., bubble-column reactors, sparger reactors, etc.) have been employed in biocatalytic processes [26,27].

In the particular case of hydroxysteroid oxidation, a previous study employing *E112* α -HSDH (for the C₁₂ oxidation of CA) and NOX (for the regeneration of NAD⁺) showed that the rate limiting reaction is the oxidation of NADH (produced by *E112* α -HSDH) by NOX. For this reason, we focused our investigation on the development of a laccase/mediator system for the regeneration of oxidized nicotinamide cofactors. In fact, this system catalyzes the same overall reaction as NOX, profiting additionally from the use of the highly active, stable and cheap enzyme *MtLac*. The application of the laccase/mediator system has already been shown by Monti et al. for the preparation of 7-oxo-CDCA, another important intermediate for the production of UDCA. By analogy, we focused on the identification of a laccase/mediator couple that can be used at higher pH value (7.0–8.0) compatible with the activity of ADHs (pH range 7.0–9.0). Previous studies showed high laccase activity towards phenolic compounds at pH > 7.0 [13,28,29]. Accordingly, a panel of phenolic mediators was selected and screened for their ability to oxidize NADH.

From the starting panel of selected laccases, the blue fungal laccase from *Mycelyophthora thermophilia* (*MtLac*) displayed high activity (7.2 U/mg) for the oxidation of NADH in combination with syringaldazine as mediator. Interestingly, its activity towards syringaldazine as substrate at pH 8.0 is even higher than at pH 5.0. In comparison to the NOX system (0.24 U/mg for NADH oxidation), the *MtLac*/syringaldazine couple showed a 30-fold higher activity. Furthermore, other mediators were tested for their ability to oxidize NADH. A detectable activity was observed with acetosyringone, syringaldehyde and ABTS. However, the maximal NADH oxidation rate was observed when syringaldazine was used as mediator. Several bioconversions with different amounts of mediator were set up in order to establish the minimal amount of mediator required for the reaction. Observing the time-course of the bioconversions, no particular differences were detected when 10, 20 and 50 μ M of syringaldazine were employed in the biotransformation. On the other hand, when ABTS was used, a dependence of the reaction rate on the amount of mediator was observed. This particular behavior can be related to a higher K_M of *MtLac* for ABTS at basic pH values. Full conversion of 10 mM of CA to the desired product (12-oxo-CDCA) was observed in all cases.

Having established *MtLac*/syringaldazine as a suitable system for the proposed biocatalytic process, the focus was shifted toward increasing the substrate loading to enable preparative and industrial scale applications.

DMSO and MeOH were evaluated as possible cosolvents for the reaction. At equal concentration, DMSO is a better cosolvent than MeOH; 10% (v/v) DMSO is enough to solubilize 120 mM of CA (in KPi buffer, pH 7.2). However, the use of this solvent can result in complications during downstream processing and in wastewater treatment. Notably, the activity of the enzymes involved in the biotransformation (*E112* α -HSDH and *MtLac*) are not compromised by the presence of this cosolvent.

Once the critical parameters for the biotransformation were identified and optimized, a preparative scale reaction starting from 20 g of CA was set up. A decrease in conversion rate was observed after 85 h of reaction. This effect is mainly due to slow degradation of syringaldazine under the reaction conditions. The addition of 7 mg of syringaldazine (50 μ M) enabled the restart of the reaction, yielding > 90% conversion within 120 h. A further addition of mediator was necessary to obtain a final conversion of 98%. The reaction was stopped after 168 h by the addition of HCl, leading to the inactivation of the enzyme and precipitation of the product. 18 g of 12-oxo-CDCA were isolated. Notably, the extraction procedure was not optimized, and a better extraction efficiency may well be obtained if the enzymes were to be removed before the acidification and extraction process. Nonetheless, the product was recovered with high yield and purity.

To compare this O₂ dependent enzymatic system with a classical regeneration system, *E112* α -HSDH was combined with ADH420 from the EvoCatal screening kit. This enzyme was identified for its ability to oxidize and reduce *i*-PrOH and acetone, respectively. It does not accept hydroxysteroids as substrate. A preparative scale reaction starting from 2 g of CA was performed. Interestingly, conversion values similar to the laccase mediator system were obtained: in both cases, >90% conversion was achieved after 7 days of reaction. The initial rate appears to be faster than that of the laccase/mediator system.

This effect can be related to gas-liquid mass transfer limitations of oxygen, which is required in the *E112* α -HSDH/*MtLac* system.

Despite the fact that both processes perform equally, several observations can be made (Table 2): (i) From the evaluation of green metrics (Table S4), the laccase mediator system is preferable, due to the absence of the sacrificial substrate (acetone) required by the classical system: this is apparent from a lower E-value (2.9 vs. 3.1) and a higher atom efficiency; (ii) the main difference between the two processes is the cosolvent employed in the biotransformation: DMSO (for the laccase/mediator NAD^+ regeneration system) and acetone (for the classical regeneration system). Notably, acetone also functions as final electron acceptor and is converted to *i*-PrOH during the reaction. Based on the GSK solvent sustainability guide, there are no particular health hazard related to the use of these solvents. Because of its low boiling point, acetone is easier to recycle than DMSO. In addition, it does not form an azeotrope with water, enabling a straightforward separation; (iii) the specific productivity of the ADH420/acetone regeneration system appears to be higher than the *MtLac*/syringaldazine system, while the other process metrics are similar for the two setups. However, for the *MtLac*/mediator system, difficulties could be encountered with larger reaction volumes, due to oxygen solubility and mass-transfer limitations.

Table 2. Comparison of the environmental process metrics calculated for the different NAD^+ regeneration systems employed in this study.

	<i>MtLac</i> Syringaldazine	<i>TpLac</i> Meldola's Blue	NOX	ADH420 Acetone	ADH Acetone
	(This Work)	(Ferrandi et al.) [9]	(Tonin et al.) [6]	(This Work)	(Fossati et al.) [30]
Oxidation position	12 α -OH	7 α -OH	12 α -OH	12 α -OH	12 α -OH
Product	12-oxo-CDCA	7-oxo-LCA	12-oxo-CDCA	12-oxo-CDCA	12-oxo-CDCA
Total Volume (L)	0.41	1	0.075	0.041	0.01
Substrate (mM)	120	50	10	120	98
NAD(P)^+ (mM)	0.5	0.5	0.5	0.5	0.1
Mediator (mM)	0.15	0.3	-	-	-
HSDH (U)	314	700	7.5	31.4	200
Regeneration enzyme (U)	750	280	7.4	2.5	160
Conversion (%)	>99%	>99%	>99%	95%	92%
STY (mM h^{-1})	0.7	2.1 (5.8) ^a	0.4	0.7	3.8
$\text{TN}_{\text{NAD(P)}^+}$ (h^{-1})	1.4	4.2 (11.6) ^a	0.04	1.4	38
SP_{HSDH} ($\text{mmol kU}^{-1} \text{h}^{-1}$)	1.0	3.0 (12.9) ^a	0.4	0.9	0.2
$\text{SP}_{\text{regeneration enzyme}}$ ($\text{mmol kU}^{-1} \text{h}^{-1}$)	0.4	7.4	4.2	11.7	0.3
$\text{TTN}_{\text{NAD(P)}^+}$	240	100	20	240	980
TTN_{HSDH}	1.04×10^6	4.2×10^5	8.7×10^5	9.9×10^5	6.1×10^4
E-value	2.9	n.c.	2.5	3.1	n.c.
Atom efficiency	95.8	n.c.	95.8	83.5	n.c.

^a Values from [9] calculated at 75% of conversion are indicated in the brackets. STY: Space-Time Yield; $\text{TN} = \text{STY}/[\text{cofactor}](\text{mM})$; $\text{TTN} = \text{mmol}_{\text{product}}/\text{mmol}_{\text{cofactor or HSDH}}$; $\text{E-value} = \text{kg}_{\text{waste}}/\text{kg}_{\text{product}}$; $\text{Atom efficiency} = \text{MW}_{\text{product}} \times 100/\text{MW}_{\text{reagents}}$.

The results obtained in this study were compared with the 7 α -HSDH/laccase/mediator system developed by Ferrandi et al. (for the oxidation of the 7 α -OH group of cholic acid) and with the 12 α -HSDH/acetone/ADH system developed by Fossati et al. (for the oxidation of the 12 α -OH group of cholic acid).

The here presented system (*MtLac*/syringaldazine) appears to be less productive than the Meldola's Blue/*Trametes pubescens* laccase system (specific productivity at full conversion for the regeneration systems are 0.4 vs. 7.4 $\text{mmol kU}^{-1} \text{h}^{-1}$, respectively). However, a sparger was used to increase the O_2 concentration in the preparative reaction performed by Ferrandi et al., while in our setup the reaction was performed in a simple round bottom flask in a shaker. The oxygen availability can certainly influence the rate of the laccase reaction and therefore the efficiency of the regeneration system. On the other hand, the ADH420/acetone system shows similar process metrics to the system developed by

Fossati et al. Particularly, the specific productivity values indicate that the use of the ADH420 for the regeneration of the NAD^+ is advantageous.

In conclusion, the *MtLac*/syringaldazine and ADH420/acetone systems are suitable for the regeneration of the NAD^+ in the preparation of 12-oxo-hydroxysteroids. In contrast to the NOX regeneration system, higher concentrations (120 vs. 10 mM) of CA can be successfully converted into 12-oxo-CDCA.

In addition, a productivity value of $6.4 \text{ g L}^{-1}\text{d}^{-1}$ was obtained for the *MtLac*/syringaldazine system: under these conditions, it will require a 500 L reaction run for 1 week to produce the amount of CDCA necessary to treat the 60 CTX Dutch patients for 1 year (roughly 16 kg). In our opinion, this is a feasible volume that is suitable for this biocatalytic process.

4. Materials and Methods

4.1. *El12 α -HSDH* Expression Trials

The NAD^+ -dependent HSDH from *Eggerthella lenta* (*El12 α -HSDH*; GenBank: WP_114518444.1) was expressed as reported previously [6]. Expression trials of *El12 α -HSDH* were performed in Erlenmeyer flasks with 500 mL of different media. LB media: 10 g/L Bacto-tryptone, 5 g/L Yeast extract, 10 g/L NaCl; TB media: 20 g/L Bacto-tryptone, 24 g/L Yeast extract, 0.231 g/L KH_2PO_4 , 1.254 g/L K_2HPO_4 , 4 mL/L Glycerol; YT media: 16 g/L Bacto-tryptone, 10 g/L Yeast extract, 5 g/L NaCl. The presence of NaCl as co-inducer was evaluated by adding 100 mL of NaCl solution (of a 250 g/L stock solution) at the moment of the induction (final concentration of 50 g/L).

E. coli cells were grown at 37 °C in a shaker incubator (200 rpm). When an $\text{OD}_{600\text{nm}}$ value of 1.0 was reached, the expression of the gene *El12 α -HSDH* was induced by the addition of 0.250 mM IPTG and the temperature lowered to 25 °C. After 24 h of incubation under these conditions, cells were separated from the broth by centrifugation. The crude extract was obtained by resuspension of the cells in lysis buffer, sonication and centrifugation at $50,000\times g$ (20 min, 4 °C).

Enzyme expression was evaluated measuring the *El12 α -HSDH* activity in the crude extract: 10 mL of the cultures were centrifuged at 7000 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in 2 mL of potassium phosphate buffer (100 mM, pH 8.0). Samples were then sonicated and subsequently centrifuged (7000 rpm, 10 min) to discard cell debris. Activity assay was carried out via spectroscopy, measuring the formation of NADH at the wavelength of 340 nm for 10 min. 25 μL of crude extract obtained by cells expressing the *El12 α -HSDH* enzyme grew in the different media were added to 200 μL of a stock solution consisting of 10 mM CA, 0.5 mM of NAD^+ and 50 mM potassium phosphate buffer (pH 8.0). Assays were carried out in triplicates.

4.2. Purification of *MtLac*

Laccase from *Myceliophthora thermophila* (*MtLac*) was purchased (see Laccases screening). The original sample consists in a centrifuged and concentrated broth sample of laccase with a brown color. Enzyme purification was performed by using a NGC apparatus (BioRad, Hercules, U.S.). The sample (17 mL, was diluted ten-times in 10 mM Tris-HCl, pH 7.2 and loaded onto a 70 mL Q-Sepharose column (GE Healthcare, Chicago, Illinois, U.S.) pre-equilibrated with the same buffer (accordingly to the procedure reported by Berka et al. [17]). *MtLac* was eluted with 20% of the same buffer containing 2 M NaCl (purification chromatogram is reported in Figure S1). *MtLac* has an intense blue color which corresponds to an absorbance peak at 600 nm, typical of multicopper oxidases. The blue fractions were collected, pooled together and concentrated by Amicon apparatus equipped with a membrane with a cut-off of 30 kDa.

4.3. Laccase Screening

Laccases from *Casuarina glauca* (*CgLac*), *Bacillus subtilis* (*BsLac*) and *Trametes trogii* (*TtLac*) were kindly donated by Vlada B. Urlacher (Heinrich-Heine-Universität, Düsseldorf, Germany). Laccase from

Myceliophthora thermophila (*MtLac*) was purchased from Novozymes (trade name Flavorstar). Laccase from *Trametes versicolor* (*TvLac*) was purchased from Sigma-Aldrich. NAD(P)H oxidase (PRO-NOX001) was purchased from Prozymix (Haltwhistle, UK). Enzyme concentrations were spectrophotometrically determined with Bradford assay, measuring the absorbance at 595 nm in a plate reader, using a calibration curve with BSA from 2 µg/mL to 50 µg/mL.

Laccase screening was carried out with 2 µM of mediators (syringaldazine, syringaldehyde and acetosyringone) in presence of 0.5 mM NADH and 100 mM potassium phosphate buffer (KPi), pH 8.0 or 100 mM sodium acetate buffer (NaAcO), pH 5.0. Reactions (200 µL) were started by the addition of 20 µL of 100-fold diluted enzyme stock solutions (final concentration in the assay: 5.9 µg/mL *CgLac*, 3.3 µg/mL *BsLac*, 5.5 µg/mL *TtLac*, 11.5 µg/mL *MtLac*, 90.9 µg/mL *TvLac*). Activity was spectrophotometrically assayed following the oxidation of NADH at 340 nm ($\epsilon_{340\text{nm}} = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$) (Figures 2 and 3). Assays were performed in 200 µL volume in a 96-well plate reader at 25 °C. Assays were carried out in triplicate.

4.4. Mediator Screening

All mediators used in this study are commercially available. Syringaldazine, acetosyringone, syringaldehyde, thionine, tartrazine, resazurin and Meldola's blue were purchased from Sigma-Aldrich (Germany). ABTS (2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) was purchased from Fluka (Germany).

Biocatalytic reactions (2 mL) were carried out in 15 mL tube in presence of different mediators. They were set up incubating 10 mM CA, 0.5 mM NAD⁺, 0.34 mg/mL *MtLac*, 0.013 mg/mL *E112α*-HSDH and 5 µM of different mediators. Reactions were carried out at 25 °C for 24 h. Samples were analyzed by HPLC chromatography (Table S2).

4.5. Enzymatic Activity Assays

The *E112α*-HSDH activity was determined spectrophotometrically following the production of NADH (ϵ_{NADH} at 340 nm = $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$) for ten minutes. Assay conditions: 1 mM CA, 1 mM NAD⁺, 50 mM KPi buffer pH 8.0, 25 °C. One unit of enzymatic activity was defined as the amount of enzyme that reduces 1 µmol of NAD⁺ per minute under the assay conditions. Specific activity of *E112α*-HSDH is 59.2 U mg⁻¹.

The *MtLac* activity was determined spectrophotometrically following the oxidation of ABTS (ϵ_{ABTS} at 420 nm = $36.0 \text{ mM}^{-1} \text{ cm}^{-1}$) for one minute. Assay conditions: 0.5 mM ABTS, 50 mM sodium acetate buffer pH 5.0, 25 °C. One unit of enzymatic activity was defined as the amount of enzyme that oxidizes 1 µmol of ABTS per minute under the assay conditions. Specific activity of *MtLac* is 5.4 U mg⁻¹.

The ADH420 activity was determined spectrophotometrically following the production of NADH (ϵ_{NADH} at 340 nm = $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$) for ten minutes. Assay conditions: 15% (v/v) isopropanol, 0.5 mM NAD⁺, 50 mM KPi buffer pH 8.0, 25 °C. One unit of enzymatic activity was defined as the amount of enzyme that reduces 1 µmol of NAD⁺ per minute under the assay conditions. Specific activity of ADH420 is 1.2 U mg⁻¹.

4.6. Optimization Studies

All bioconversions were carried out employing 0.34 mg/mL (1.8 U/mL) *MtLac* and 0.013 mg/mL (0.8 U/mL) *E112α*-HSDH. Analytical scale conversions were carried out in a 15 mL tube filled with 2 mL of reaction mixture and they were incubated at 25 °C on a rotator shaker (45 rpm) (IntelliMixer, Neolab Migge GmbH, Heidelberg, DE).

Effect of the mediator concentration: reaction mixtures containing different amounts of syringaldazine or ABTS (10, 20 and 50 µM), 10 mM CA, 0.5 mM NAD⁺ and 100 mM KPi buffer at pH 8.0 were prepared. The reaction was started by the addition of the enzymes and the reaction mixture was sampled at given times (Figure 4).

Effect of the cosolvent and substrate loading: reaction mixtures containing different amount of CA (from 10 to 250 mM), 50 μM of syringaldazine, 0.5 mM NAD^+ and 100 mM KPi buffer at pH 7.2 and different amount of MeOH (10–40% v/v) or DMSO (10%) were prepared (see Table 1). After the addition of the enzymes, the reaction mixture was sampled after 48 h.

4.7. Preparation of 12-oxo-CDCA (*MtLac* + Mediator Regeneration System)

In a 2 L round-bottom flask 20 g (49.2 mmol) of CA, 136 mg (0.205 mmol) of NAD^+ and 7 mg (0.019 mmol) of syringaldazine were dissolved in 100 mM of KPi buffer, pH 7.2 with 10% DMSO (final volume 410 mL). The reaction was initiated by adding 5.33 mg (314 U) of *E112 α -HSDH* and 139 mg (750 U) of *MtLac* (Flavostar, Novozyme). The reactions were gently stirred at 25 °C in an orbital shaker. Conversion was followed with HPLC. After 85 and 115 h, 7 mg (0.019 mmol) of syringaldazine was added to the reaction mixture. When the reaction was complete (168 h) the solution was acidified with HCl to pH 3.0 and extracted five times with diethyl ether (200 mL for each extraction). Solvent was dried with MgSO_4 and evaporated giving the final product (white powder). 12-oxo-CDCA: Isolated yield: 18.4 g (92%); ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$): δ 214.0 (C12=O), 174.8 (COOH), 70.0 (C3 α -OH), 66.0 (C7 α -OH), 56.3, 53.4, 46.1, 40.9, 37.4, 36.6, 35.5, 35.1, 35.1, 34.6, 31.1, 30.4, 30.3, 27.1, 23.3, 22.0, 18.5, 11.2. In accordance with literature data [30].

4.8. Preparation of 12-oxo-CDCA (*ADH420* + Acetone)

In a 50 mL falcon tube, 2 g (4.92 mmol) of CA and 13.6 mg (0.021 mmol) of NAD^+ were dissolved in 100 mM of KPi buffer, pH 7.2 with 15% acetone (83 mM) (final volume 41 mL). The reaction was initiated by addition of 0.53 mg (31.4 U) of *E112 α -HSDH* and 2.05 mg (2.5 U) of *ADH 420* (EvoCatal ADH screening kit). The reaction mixture was incubated at 25 °C on a rotatory shaker. Conversion was checked with HPLC. When the reaction was complete (168 h) the solution was acidified with HCl to pH 3.0 and extracted three times with diethyl ether (20 mL for each extraction). Solvent was dried with MgSO_4 and evaporated giving the final product (white powder). 12-oxo-CDCA: Isolated yield: 1.83 g (92%); ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$): δ 214.0 (C12=O), 174.8 (COOH), 70.0 (C3 α -OH), 66.0 (C7 α -OH), 56.3, 53.4, 46.1, 40.9, 37.4, 36.6, 35.5, 35.1, 35.1, 34.6, 31.1, 30.4, 30.3, 27.1, 23.3, 22.0, 18.5, 11.2. In accordance with literature data [30].

4.9. HPLC Analyses

The bioconversions were analyzed by HPLC: 50 μL of sample were diluted with water/ CH_3CN /TFA (70/30/0.1) solution to reach a final concentration of hydroxysteroid of 2 mM and centrifuged at 14,000 $\times g$ for 2 min. 10 μL of the obtained samples was analyzed by HPLC.

Reversed-phase, isocratic gradient HPLC analyses were performed on a Shimadzu (Kyoto, Japan) apparatus equipped with a LC20AT pump and an ELSD-LTII detector and fitted with a XTerra RP C18 column (Waters, Milford, Massachusetts, USA) (length/internal diameter 150/4.6 mm, pore size 3.5 μm) under the following conditions: eluent $\text{H}_2\text{O}/\text{CH}_3\text{CN}/\text{TFA}$ (70:30:0.1), flow 1.0 mL min^{-1} , temperature 35 °C.

5. Conclusions

The selective oxidation of CA to 12-oxo-CDCA is a fundamental reaction for the chemo/enzymatic synthesis of the pharmaceutical ingredient UDCA. This oxidation can be carried out by employing an alcohol dehydrogenase enzyme, namely 12 α -HSDH. Here, this reaction was achieved by employing *E112 α -HSDH* enzyme and the *MtLac*/syringaldazine mediator system for the oxygen dependent regeneration of the nicotinamide cofactor, required by HSDHs.

In comparison to our previous study, where NAD(P)H oxidase was used, the *MtLac*/syringaldazine system enables higher productivity while maintaining similar environmental metrics. This enzymatic system was employed in a preparative scale reaction, yielding 18 g of 12-oxo-CDCA in a simple batch mode.

6. Patents

There are no patents resulting from the work reported in this manuscript.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4344/10/6/677/s1>, Figure S1. NGC chromatogram of *MtLac* purification by ion-exchange chromatography on Q-Sepharose column; Table S1. List of laccases used in the study; Table S2. Bioconversion of 10 mM CA to 12-oxo-CDCA in presence of 0.34 mg/mL *MtLac*, 0.013 mg/mL *El12 α* -HSDH, 0.5 mM NAD⁺ and 5 μ M of different mediators; Table S3. Activity of EvoCatal ADH screening kit towards hydroxysteroids and isopropanol; Table S4. Calculation of E-values and atom efficiencies.

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References

1. Podda, M.; Ghezzi, C.; Battezzati, P.; Bertolini, E.; Crosignani, A.; Petroni, M.; Zuin, M. Effect of different doses of ursodeoxycholic acid in chronic liver disease. *Dig. Dis. Sci.* **1989**, *34*, S59–S65. [[CrossRef](#)] [[PubMed](#)]
2. Colombo, C.; Setchell, K.D.; Podda, M.; Crosignani, A.; Roda, A.; Curcio, L.; Ronchi, M.; Giunta, A. Effects of ursodeoxycholic acid therapy for liver disease associated with cystic fibrosis. *J. Pediatr.* **1990**, *117*, 482–489. [[CrossRef](#)]
3. Colombo, C.; Crosignani, A.; Assaisso, M.; Battezzati, P.M.; Podda, M.; Giunta, A.; Zimmer-Nechemias, L.; Setchell, K.D. Ursodeoxycholic acid therapy in cystic fibrosis—Associated liver disease: A dose-response study. *Hepatology* **1992**, *16*, 924–930. [[CrossRef](#)]
4. Crosignani, A.; Battezzati, P.M.; Setchell, K.D.; Invernizzi, P.; Covini, G.; Zuin, M.; Podda, M. Tauroursodeoxycholic acid for treatment of primary biliary cirrhosis. *Dig. Dis. Sci.* **1996**, *41*, 809–815. [[CrossRef](#)] [[PubMed](#)]
5. Tonin, F.; Arends, I.W.C.E. Latest development in the synthesis of ursodeoxycholic acid (UDCA): A critical review. *Beilstein J. Org. Chem.* **2018**, *14*, 470–483. [[CrossRef](#)]
6. Tonin, F.; Alvarenga, N.; Ye, J.Z.; Arends, I.W.C.E.; Hanefeld, U. Clean enzymatic oxidation of 12 α -hydroxysteroids to 12-oxo-derivatives catalyzed by hydroxysteroid dehydrogenase. *Adv. Synth. Catal.* **2019**, *361*, 2448–2455. [[CrossRef](#)]
7. Ferrandi, E.E.; Bertuletti, S.; Monti, D.; Riva, S. Hydroxysteroid dehydrogenases: An ongoing story. *Eur. J. Org. Chem.* **2020**. [[CrossRef](#)]
8. Chen, X.; Cui, Y.; Feng, J.; Wang, Y.; Liu, X.; Wu, Q.; Zhu, D.; Ma, Y. Flavin oxidoreductase-mediated regeneration of nicotinamide adenine dinucleotide with dioxygen and catalytic amount of flavin mononucleotide for one-pot multi-enzymatic preparation of ursodeoxycholic acid. *Adv. Synth. Catal.* **2019**, *361*, 2497–2504. [[CrossRef](#)]
9. Ferrandi, E.E.; Monti, D.; Patel, I.; Kittl, R.; Haltrich, D.; Riva, S.; Ludwig, R. Exploitation of a Laccase/Meldola's Blue System for NAD⁺ regeneration in preparative scale hydroxysteroid dehydrogenase-catalyzed oxidations. *Adv. Synth. Catal.* **2012**, *354*, 2821–2828. [[CrossRef](#)]
10. Aksu, S.; Arends, I.W.C.E.; Hollmann, F. A new regeneration system for oxidized nicotinamide cofactors. *Adv. Synth. Catal.* **2009**, *351*, 1211–1216. [[CrossRef](#)]
11. Könst, P.; Kara, S.; Kochius, S.; Holtmann, D.; Arends, I.W.C.E.; Ludwig, R.; Hollmann, F. Expanding the scope of laccase-mediator systems. *ChemCatChem* **2013**, *5*, 3027–3032. [[CrossRef](#)]
12. Hollmann, F.; Arends, I.W.C.E.; Buehler, K. Biocatalytic redox reactions for organic synthesis: Nonconventional regeneration methods. *ChemCatChem* **2010**, *2*, 762–782. [[CrossRef](#)]

13. Tetianec, L.; Chaleckaja, A.; Vidziunaite, R.; Kulys, J.; Bachmatova, I.; Marcinkeviciene, L.; Meskys, R. Development of a laccase/syringaldazine system for NAD (P) H oxidation. *J. Mol. Catal. B Enzym.* **2014**, *101*, 28–34. [[CrossRef](#)]
14. Tonin, F.; Rosini, E.; Piubelli, L.; Sanchez-Amat, A.; Pollegioni, L. Different recombinant forms of polyphenol oxidase A, a laccase from *Marinomonas mediterranea*. *Protein Expr. Purif.* **2016**, *123*, 60–69. [[CrossRef](#)]
15. Brinch, D.; Pedersen, P. Toxicological studies on laccase from *Myceliophthora thermophila* expressed in *Aspergillus oryzae*. *Regul. Toxicol. Pharm.* **2002**, *35*, 296–307. [[CrossRef](#)]
16. Tonin, F.; Melis, R.; Cordes, A.; Sanchez-Amat, A.; Pollegioni, L.; Rosini, E. Comparison of different microbial laccases as tools for industrial uses. *New Biotechnol.* **2016**, *33*, 387–398. [[CrossRef](#)]
17. Berka, R.M.; Schneider, P.; Golightly, E.J.; Brown, S.H.; Madden, M.; Brown, K.M.; Halkier, T.; Mondorf, K.; Xu, F. Characterization of the gene encoding an extracellular laccase of *Myceliophthora thermophila* and analysis of the recombinant enzyme expressed in *Aspergillus oryzae*. *Appl. Environ. Microbiol.* **1997**, *63*, 3151–3157. [[CrossRef](#)]
18. Cabral, D.J.; Hamilton, J.A.; Small, D.M. The ionization behavior of bile acids in different aqueous environments. *J. Lipid Res.* **1986**, *27*, 334–343.
19. Ekwall, P.; Rosendahl, T.; Sten, A. Studies on bile acid salt solutions II. The solubility of cholic acid in sodium cholate solutions and that of desoxycholic acid in sodium desoxycholate solutions. *Acta Chem. Scand.* **1958**, *12*, 1622–1633.
20. Tonin, F.; Otten, L.G.; Arends, I.W.C.E. NAD⁺-dependent enzymatic route for the epimerization of hydroxysteroids. *ChemSusChem* **2019**, *12*, 3192–3203. [[CrossRef](#)]
21. Gupta, M.N.; Batra, R.; Tyagi, R.; Sharma, A. Polarity index: The guiding solvent parameter for enzyme stability in aqueous-organic cosolvent mixtures. *Biotechnol. Prog.* **1997**, *13*, 284–288. [[CrossRef](#)]
22. López-Cruz, J.; Viniegra-Gonzalez, G.; Hernández-Arana, A. Thermostability of native and pegylated myceliophthora t hermophila laccase in aqueous and mixed solvents. *Bioconj. Chem.* **2006**, *17*, 1093–1098. [[CrossRef](#)]
23. Alder, C.M.; Hayler, J.D.; Henderson, R.K.; Redman, A.M.; Shukla, L.; Shuster, L.E.; Sneddon, H.F. Updating and further expanding GSK’s solvent sustainability guide. *Green Chem.* **2016**, *18*, 3879–3890. [[CrossRef](#)]
24. Henderson, R.K.; Jiménez-González, C.; Constable, D.J.; Alston, S.R.; Inglis, G.G.; Fisher, G.; Sherwood, J.; Binks, S.P.; Curzons, A.D. Expanding GSK’s solvent selection guide—Embedding sustainability into solvent selection starting at medicinal chemistry. *Green Chem.* **2011**, *13*, 854–862. [[CrossRef](#)]
25. Liu, W.; Wang, P. Cofactor regeneration for sustainable enzymatic biosynthesis. *Biotechnol. Adv.* **2007**, *25*, 369–384. [[CrossRef](#)] [[PubMed](#)]
26. Kantarci, N.; Borak, F.; Ulgen, K.O. Bubble column reactors. *Process Biochem.* **2005**, *40*, 2263–2283. [[CrossRef](#)]
27. Plutschack, M.B.; Pieber, B.U.; Gilmore, K.; Seeberger, P.H. The hitchhiker’s guide to flow chemistry. *Chem. Rev.* **2017**, *117*, 11796–11893. [[CrossRef](#)]
28. Niladevi, K.N.; Jacob, N.; Prema, P. Evidence for a halotolerant-alkaline laccase in *Streptomyces psammoticus*: Purification and characterization. *Process Biochem.* **2008**, *43*, 654–660. [[CrossRef](#)]
29. Ruijssenaars, H.; Hartmans, S. A cloned *Bacillus halodurans* multicopper oxidase exhibiting alkaline laccase activity. *Appl. Microbiol. Biotechnol.* **2004**, *65*, 177–182. [[CrossRef](#)]
30. Fossati, E.; Polentini, F.; Carrea, G.; Riva, S. Exploitation of the alcohol dehydrogenase-acetone NADP-regeneration system for the enzymatic preparative-scale production of 12-ketochenodeoxycholic acid. *Biotechnol. Bioeng.* **2006**, *93*, 1216–1220. [[CrossRef](#)]

