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Engineering an enzymatic regeneration system for NAD(P)H oxidation

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ABSTRACT

A recently proposed coenzyme regeneration system employing laccase and a number of various redox mediators for the oxidation of NAD(P)H was studied in detail by kinetic characterization of individual reaction steps. Reaction engineering by modeling was used to optimize the employed enzyme, coenzyme as well as redox mediator concentrations. Glucose dehydrogenase from *Bacillus* sp. served as a convenient model of synthetic enzymes that depend either on NAD$^+$ or NADP$^+$. The suitability of laccase from *Trametes pubescens* in combination with acetosyringone or syringaldazine as redox mediator was tested for the regeneration (oxidation) of both coenzymes. In a first step, pH profiles and catalytic constants of laccase for the redox mediators were determined. Then, second-order rate constants for the oxidation of NAD(P)H by the redox mediators were measured. In a third step, the rate equation for the entire enzymatic process was derived and used to build a MATLAB model. After verifying the agreement of predicted vs. experimental data, the model was used to calculate different scenarios employing varying concentrations of regeneration system components. The modeled processes were experimentally tested and the results compared to the predictions. It was found that the regeneration of NADH to its oxidized form was performed very efficiently, but that an excess of laccase activity leads to a high concentration of the oxidized form of the redox mediator – a phenoxy radical – which initiates coupling (dimerization or polymerization) and enzyme deactivation.

1. Introduction

During the last years laccase/mediator systems have been proposed for the regeneration of NAD(P)$^+$-dependent enzymatic processes in synthetic applications [1,2]. These initial studies showed a high potential for up-scaling, but more detailed investigations are necessary to understand the strengths and weaknesses of these regeneration systems. In this work we studied the underlying principles for an efficient and stable enzymatic regeneration process of NAD(P)$^+$, which does not show the restrictions of alternative systems, e.g. electrochemical methods [3]. Coenzymes are costly [4], which makes them too expensive to employ more than the minimal amount that still guarantees fast conversion of the synthetic enzyme [5]. High costs have been an obstacle in the wider application of coenzyme-dependent oxidreductases, but this is also the strongest argument for applying efficient and economical coenzyme regeneration systems. Various methods such as chemical, biological, photochemical, electrochemical or enzymatic approaches have been suggested and reviewed for this purpose [6,7]. Among them, the enzymatic methods seem to be the most convenient and useful. Such in situ regeneration reactions have been used in a number of oxidoreductase-catalyzed reactions, and some of them have been up-scaled to large-scale syntheses [1].

As suggested by Chenault and Whitesides [8] an ideal enzymatic regeneration system should meet the following criteria: (i) the enzymes should be inexpensive and stable, (ii) the enzymes should have high specific activity, (iii) simple and inexpensive reagents that do not interfere with the isolation of the product of interest or with enzyme stability should be employed, (iv) high turnover numbers should be obtained, (v) the total turnover number of the coenzyme should be at least between 10^2 and 10^4, and (vi) an overall equilibrium for the coupled enzyme system favorable to product formation should be reached. These criteria have been already partially met for NAD$^+$-reducing enzymes

Abbreviations: ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); GLC, D-glucose; GL, D-glucono-1,5-lactone; GA, gluconic acid; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); O_2, molecular oxygen; SHE, standard hydrogen electrode; STY, space-time yield (mM h^{-1}).

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Nomenclature

ε	molar absorption coefficient ($M^{-1}\,cm^{-1}$)
eff	efficiency
k_2	second-order rate constant measured for coenzymes and redox mediators ($M^{-1}\,s^{-1}$)
K_I	inhibition constant (μM)
K_M	Michaelis–Menten constant (μM)
r_1	rate of the regenerating (laccase) reaction ($M\,s^{-1}$)
r_2	second order rate constant ($M^{-1}\,s^{-1}$)
r_3	rate of the synthetic (GDH) reaction ($M\,s^{-1}$)
r_{hyd}	rate of D-glucono-1,5-lactone hydrolysis (s^{-1})
RM	redox mediator
t	time (h)
V_{max}	maximum enzymatic turnover rate at infinite substrate concentration ($M\,s^{-1}$, $U\,L^{-1}$)

such as alcohol dehydrogenase, lactate dehydrogenase and glutamate dehydrogenase [7,9,10]. However, the enzymatic oxidation of NAD(P)H is not satisfactorily developed to date. The use of laccase for NAD(P)H oxidation seems to fulfill most of the postulated criteria: (i) Laccases are technical enzymes employed for decolorization or delignification processes, which can be produced recombinantly and inexpensively. (ii) Laccase, a member of the blue multicopper oxidase family, has a high specific activity for various substrates, which can reach up to several hundred per second. (iii) Most of the investigated redox mediators, which typically are used in low concentrations, are inexpensive, but more work needs to be done on their removal from the product. Oxygen, the second substrate of laccase, can be easily provided to a biocatalytic process, and since water is produced by its reaction no purification of a by-product is required. (iv) It should be possible to obtain high turnover numbers for the coenzyme in a biocatalytic process when considering both the reported high stability and high specific activity of laccases, and (v) based on this high stability/high activity high total turnover numbers for the enzyme (laccase) should be achievable as well. (vi) The high redox potential of laccase of up to 800 mV vs. SHE allows to oxidize even redox mediators with high potentials [11,12]. The high thermodynamic driving force of oxygen reduction makes processes irreversible and drives coenzyme-dependent reactions toward completion [2]. The ideal mediator in these reactions should be non-toxic, cheap and efficient, with stable oxidized and reduced forms that do not inhibit the enzymatic reaction [13].

Laccase/mediator systems have been reported to be applicable for NAD^+ regeneration [2,14,15]. The main advantages of such systems are high process stability, low co-substrate costs and tolerance toward co-solvents. Laccase substrates such as ABTS, Meldola's blue, acetosyringone, syringaldehyde, caffeic acid, *p*-coumaric acid, vanillin, acetovanillone, 3-hydroxyanthranilic acid, 4-hydroxybenzoic acid, hydroquinone, phenolsulfonphthalein [16] have been used as mediators. Among these, acetosyringone and syringaldehyde are fastly oxidized by laccase and also exhibit high NAD(P)H oxidation rates.

In this work a laccase from *Trametes pubescens* and acetosyringone are used as an enzyme/mediator system to regenerate the oxidized coenzyme $NAD(P)^+$ from NAD(P)H. Glucose dehydrogenase (GDH) from *Bacillus* sp. is here employed as the model synthesizing enzyme that uses the oxidized coenzyme $NAD(P)^+$, which is reduced to NAD(P)H, for the oxidation of D-glucose to D-glucono-1,5-lactone. The latter spontaneously hydrolyses to D-gluconic acid (Fig. 1). Glucose oxidation catalyzed by GDH is a popular model system, since it can use both NAD^+ and the phosphorylated form $NADP^+$ [17,18]. The full rate equation of *Bacillus sp.* GDH, which is often applied for the regeneration of both NADPH and NADH, was recently elucidated [19]. Furthermore, modeling provides guidance in converting batch to continuous conversions as recently demonstrated for lactobionic acid production [20]. Here we used modeling together with experimental approaches to obtain knowledge on enzyme and redox mediator stability under reaction conditions, as well as on the necessary activities of enzymes and minimum concentrations of redox mediator and coenzyme to design an efficient enzymatic process. Overall, we obtained detailed information on the strengths and possible limitations of the laccase/redox mediator regeneration system.

2. Materials and methods

2.1. Materials

Acetosyringone (4′-hydroxy-3′,5′-dimethoxyacetophenone), syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde), NAD^+, $NADP^+$, NADH, NADPH, D-glucose and D-glucono-1,5-lactone were purchased from Sigma–Aldrich (Steinheim, Germany). 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was purchased from Amresco (Ohio, USA). Stock solutions (200 mM) of acetosyringone and syringaldehyde were prepared in ethanol. All buffer reagents and other chemicals were of analytical grade. Water was purified by reversed osmosis and scavenger resins to a resistivity of >18 MΩ cm. Laccase (EC 1.10.3.2) from *T. pubescens* CBS 696.94 with a specific activity of 594 U/mg at pH 5.0 was produced by cultivation of the fungus in a bioreactor under copper induction following published procedures [21,22]. Glucose dehydrogenase (GDH, EC 1.1.1.47) from *Bacillus* sp. with a specific activity of 18.5 U/mg at pH 5.0 was a gracious gift from Amano Enzyme Inc. (Nagoya, Japan).

2.2. Enzyme activity assays

Laccase activity was determined with ABTS as colorimetric substrate. The assay mixture contained 1 mM ABTS in air-saturated, 100 mM sodium-citrate buffer, pH 5.0, incubated for 15 min at 30 °C before the measurement. After addition of a suitable amount of laccase, the oxidation of ABTS was monitored by following the increase of absorbance at 420 nm (ε_{420} = 36.0 $mM^{-1}\,cm^{-1}$) for 180 s.

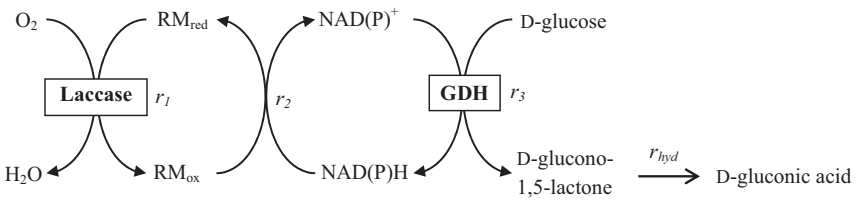

Fig. 1. Reaction scheme for the bi-enzymatic system employing laccase as regenerating enzyme and glucose dehydrogenase (GDH) as synthetic enzyme. The redox mediator in its reduced form RM_{red} is oxidized by laccase to RM_{ox} with the rate r_1. The bimolecular rate observed for the reaction between RM_{ox} and the reduced form of the coenzyme NAD(P)H is given as r_2. NAD(P)H is reduced by GDH with the rate r_3. The concomitantly formed product is gluconolactone, an inhibitor of GDH. Its hydrolyzation rate to the non-inhibiting final product is r_{hyd}.

One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per min.

GDH activity was followed by the increase in NAD(P)H absorbance at 340 nm (ε_{340} = 6.22 mM^{-1} cm^{-1}) for 180 s at 30 °C. The assay contained glucose (100 mM), the respective coenzyme (NAD^+ or $NADP^+$, 0.5 mM), 100 mM sodium-citrate buffer, pH 5.0 and a suitable amount of enzyme. One unit of GDH activity was defined as the amount of enzyme required to reduce 1 μmol of $NAD(P)^+$ per min. Measurements of enzymatic activities in samples taken from conversion experiments were affected by a small error introduced by the regenerating enzyme, which was corrected by performing reference measurements.

2.3. Determination of pH profiles and catalytic constants

The pH profile of laccase activity with the redox mediators was determined in air-saturated, 100 mM phosphate-citrate buffer solution at various pH values (3.0–6.5). The assays were carried out at 30 °C using 1 mM of acetosyringone (ε_{400} = 1.7 mM^{-1} cm^{-1}) or syringaldehyde (ε_{380} = 1.6 mM^{-1} cm^{-1}). The molar absorption coefficients of the two redox mediators were determined from standard curves (Supplemental Fig. S1). Catalytic constants of laccase for the redox mediators were calculated by non-linear least-squares regression, fitting the observed data to the Henri–Michaelis–Menten equation using SigmaPlot v.12 (Systat Software Inc, CA, USA). The K_M value of laccase for oxygen (0.41 mM) was taken from [21]. The second-order rate constants of the oxidation reaction of NAD(P)H by redox mediators were measured at 30 °C in 100 mM phosphate-citrate buffer, pH 5.0 by following the reduction of the NAD(P)H absorption band at 340 nm. The fast reaction of the redox mediator and NAD(P)H (both at 150 mM final concentration) was recorded with an Applied Photophysics SX20 stopped-flow spectrophotometer. Results are averaged from four independent measurements and standard errors are given.

2.4. Batch conversion experiments

All batch conversion experiments were performed in a parallel 0.5-L bioreactor system (Sixfors, INFORS HT, Bottmingen, Switzerland) with a working volume of 0.3 L. The reactions contained 200 mM D-glucose, 380–900 U L^{-1} laccase, 220–500 U L^{-1} GDH, 100–500 μM NAD^+ and 100–500 μM acetosyringone as redox mediator in 100 mM sodium citrate buffer, pH 5.0. Four batch conversion experiments (A, B, C and D) employing different enzymatic activities, redox mediator and coenzyme concentrations were run. The reaction solution was prepared as follows: first glucose was dissolved in an appropriate amount of buffer. Then, the redox mediator, coenzyme and laccase were added. The redox mediator was dissolved in 1 mL ethanol (96%) before adding to the reactor. Finally, the reaction was started by the addition of GDH. The pH was regulated by automatic titration with an aqueous sodium carbonate solution (500 mM). The reaction solutions were thermostatted to 30 °C, continuously stirred at 250 rpm and oxygenated using pure oxygen which was bubbled through a sparger. The dissolved oxygen concentration was measured by using an oxygen electrode (OxyFerm, Bonaduz, Switzerland) and the oxygen saturation was set to 21%, equal to air saturation. Samples were taken periodically and used immediately for enzymatic activity measurements or heated at 99 °C for 5 min and then frozen at −18 °C until HPLC analysis of glucose and gluconic acid.

2.5. HPLC analysis

The conversion of glucose was monitored from reaction samples by HPLC (Dionex Summit and Chromeleon software, Sunnyvale, CA, USA) using an Aminex HPX-87 H-column (BioRad Laboratories, CA, USA) at 60 °C and 5 mM H_2SO_4 as eluent with a flow rate of 0.5 mL min^{-1}. The components were detected by UV at a wavelength of 210 nm. Calibration was performed with authentic samples of glucose and gluconic acid in a concentration range from 5 to 20 mg L^{-1}. Because of the heat treatment of the sample the formed intermediate glucono-1,5-lactone was fully converted to gluconic acid and could not be determined.

2.6. Rate equation and MATLAB model

For the bi-enzymatic process employing GDH and laccase (Fig. 1) a set of non-linear, differential equations was derived from mass balances and the mass conservation law. Laccase activity is modeled by a ping-pong *bi-bi* reaction mechanism [23] although this is only an approximation of the more complicated reaction mechanism for which no kinetic model is published. GDH activity was modeled by sequential ordered *bi-bi* mechanism kinetics with the coenzyme binding first [24]. The product of the laccase reaction, the oxidized redox mediator, reacts with one of the products of the GDH reaction, the reduced nicotinamide coenzyme, in a second-order reaction [5] (this work). The reaction rates for laccase (r_1), the redox mediator/NAD(P)H (r_2) and GDH (r_3) are given by the Eqs. (1)–(3):

$$r_1 = V_{max,LAC} \times \frac{[O_2] \times [RM_{red}]}{K_{M,RM} \times [O_2] + K_{M,O_2} \times [RM_{red}] + [O_2] \times [RM_{red}]} \quad (1)$$

$$r_2 = k_2 \times [RM_{ox}] \times [NADH] \quad (2)$$

$$r_3 = V_{max,GDH} \times \frac{[NAD] \times [GLC]}{K_{I,NAD} \times K_{M,GLC} \times \left(1 + \frac{[NADH]}{K_{I,NADH}} + [GL] \times \frac{K_{M,NADH}}{K_{I,NADH} \times K_{M,GL}}\right) + K_{M,GLC} \times [NAD] \times \left(1 + [GL] \times \frac{K_{M,NADH}}{K_{I,NADH} \times K_{M,GL}}\right) + K_{M,NAD} \times [GLC] \times \left(1 + \frac{[NADH]}{K_{I,NADH}}\right) + [NAD] \times [GLC] \times \left(1 + \frac{[GL]}{K_{I,GL}}\right)} \quad (3)$$

The concentration of the redox mediator RM in the oxidized and reduced states, $NAD(P)^+$, NAD(P)H, D-glucose (GLC), D-glucono-1,5 lactone (GL) and gluconic acid (GA) can be described by the set of differential equations (4)–(8):

$$\frac{d[GLC]}{dt} = -r_3 \quad (4)$$

$$\frac{d[NAD]}{dt} = -\frac{d[NADH]}{dt} = r_2 - r_3 \quad (5)$$

$$\frac{d[RM_{red}]}{dt} = -\frac{d[RM_{ox}]}{dt} = r_2 - r_1 \quad (6)$$

$$\frac{d[GL]}{dt} = -\frac{d[GLC]}{dt} - \frac{d[GA]}{dt} = r_3 - r_{hyd} \quad (7)$$

$$\frac{d[GA]}{dt} = k_{hyd} \times [GL] \quad (8)$$

The hydrolysation rate $k_{hyd} = 2.5 \times 10^{-4}\ s^{-1}$ at pH 5.0 was interpolated from data reported by Pocker and Green [25]. The space-time yield (STY) for the sum of products (glucono-1,5-lactone plus gluconic acid) was calculated for the initial reaction to exclude enzyme deactivation and redox mediator degradation and after 10 h (STY_{10}) when the reactions were terminated (9):

$$STY\left[mol\,(GL + GA)\ L^{-1}\,h^{-1}\right] = \frac{C^0_{GLC} - C^t_{GLC}}{t} \quad (9)$$

To solve the set of ordinary differential equations the ODE15s subroutine in the MATLAB R2009a software (The MathWorks, Natick, MA, USA) was used, which is designed specifically to deal with stiff differential systems of equations. For better comparison with the experimental results the predicted glucono-1,5-lactone and gluconic acid concentration are summed up and displayed in Fig. 3 in only one curve for the end product (gluconic acid).

3. Results

3.1. Determination of catalytic constants and reaction rates

Laccase catalyzes the oxidation of acetosyringone and syringaldehyde to the corresponding phenoxy radicals at the expense of molecular oxygen, which is reduced to water, with a stoichiometry of 4:1. The pH optima of both reactions were determined photometrically using molar absorption coefficients determined at pH 5.0 (Fig. S1). Laccase from *T. pubescens* exhibited a pH optimum at 3.5 for both redox mediators (Fig. 2), with the activity toward acetosyringone being higher at less acidic pH values than syringaldehyde. At pH 5.0 the specific activity for acetosyringone was 243 U mg^{-1}, which is 1.9-fold higher than that for syringaldehyde (126 U mg^{-1}). To calculate r_1 in modeled processes, the catalytic constants of laccase were measured for both redox mediators. For measurements and batch reactions pH 5.0 was chosen as a compromise between the pH optima of laccase and GDH. The k_{cat} of laccase for acetosyringone is 2.6-fold higher and the K_M is 1.9-fold higher compared to syringaldehyde, which results in a 1.3-fold increased catalytic efficiency for acetosyringone at pH 5.0 (Table 1). The catalytic constants of GDH for glucose and NAD^+ and the inhibition constants for gluconolactone and NADH at pH 5.0 ($K_{I,GL} = 413 \pm 12$ mM, $K_{I,NADH} = 0.801 \pm 0.21$ mM) were also determined to model r_3 in batch conversion experiments. The last missing piece of kinetic information (to model r_2) was obtained by determining the second order rate constants (k_2) for the redox mediator/coenzyme couples. The second-order reactions give the following rate constants: $k_{2\ NADH/acetosyringone} = 294 \pm 5\ M^{-1}\ s^{-1}$, $k_{2\ NADPH/acetosyringone} = 152 \pm 11\ M^{-1}\ s^{-1}$, $k_{2\ NADH/syringaldehyde} = 136 \pm 5\ M^{-1}\ s^{-1}$ and $k_{2\ NADPH/syringaldehyde} = 103 \pm 8\ M^{-1}\ s^{-1}$.

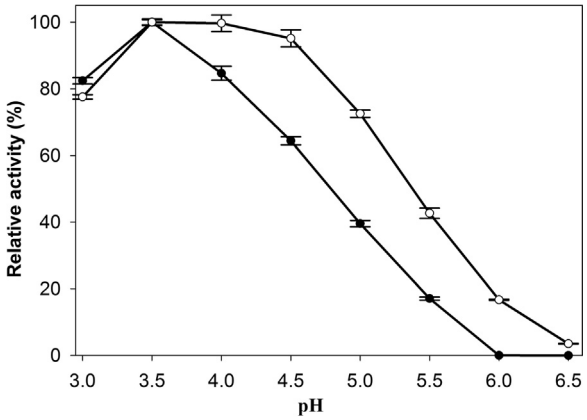

Fig. 2. pH profile of laccase activity with either acetosyringone (○) or syringaldehyde as substrate (●). Relative activities are shown for better comparison of the shape of the profile. At pH 3.5 the specific activitiy of laccase for acetosyringone and syringaldehyde were 334 and 318 U/mg, respectively.

3.2. Experimental evaluation of the MATLAB model

The first conversion experiment, Batch A, was performed with a 4.1-fold higher laccase activity than GDH activity (Table 2). A higher volumetric activity of the regenerating enzyme together with high concentrations of both the redox mediator acetosyringone and the coenzyme NAD^+ (500 μM each) was chosen to ensure efficient NAD^+ regeneration. Because of experimental reasons (sampling, enzyme degradation), only the first 10 h of the process were followed by sampling. In these ten hours 30.2% of glucose were converted, which correlates well with the predicted degree of conversion of 35.7% when considering enzyme deactivation. The measured and predicted data for Batch A are shown in Fig. 3A. The averaged modeled NAD^+ concentration during this steady-state phase is ~485 μM (Table S1) and the acetosyringone concentration is ~33 μM (Table S2). The measured laccase activity decreased by 20.8% and the GDH activity by 26.7% during these 10 h of reaction, which led to a too big deviation from the initial settings to continue the experiment. At this point the total turnover number of laccase was 6.65×10^6 and of GDH 2.03×10^6. The averaged enzyme consumption numbers for laccase and GDH are 2.81 and 0.88 U mmol^{-1} product, respectively.

Table 1
Catalytic constants of laccase and GDH for their substrates and co-substrates. These constants were measured at 30 °C in air-saturated, 100 mM sodium-citrate buffer, pH 5.0.

Enzyme/substrate	Wavelength monitored (nm)	Extinction coefficient ($mM^{-1}\ cm^{-1}$)	V_{max} (U mg^{-1})	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}\ s^{-1}$)
Laccase						
Acetosyringone	400	1.7	289 ± 5	161 ± 10	289 ± 5	1.78×10^{-6}
Syringaldehyde	380	1.6	113 ± 2	85 ± 5	113 ± 2	1.34×10^{-6}
Oxygen[a]	420	36	2900		2900	7.0×10^6
Enzyme/substrate	**Wavelength monitored (nm)**	**Extinction coefficient ($mM^{-1}\ cm^{-1}$)**	**V_{max} (U mg^{-1})**	**K_M (mM)**	**k_{cat} (s^{-1})**	**k_{cat}/K_M ($M^{-1}\ s^{-1}$)**
GDH						
Glucose	340	6.22	21.6 ± 0.2	16.7 ± 0.6	37.7 ± 0.3	2.26×10^{-3}
NAD^+	340	6.22	28.9 ± 0.4	0.26 ± 0.01	50.5 ± 0.8	194×10^{-3}

[a] From [21], were determined at 25 °C using 1 mM ABTS as the electron donor.

Table 2
Batch conversion processes employing the system laccase/GDH/NAD(H)/acetosyringone. The reactions were performed with a working volume of 300 mL and an initial glucose concentration of 200 mM in 100 mM sodium-citrate buffer, pH 5.0. The measured and modeled data are taken after 10 h of reaction. Space-time yield (STY) and specific productivity are calculated for 10 h of reaction. Turnover numbers of enzymes, redox mediator and coenzyme were calculated by dividing the STY_{10} by the respective molar concentration.

Batch	A	B	C	D
Initial values				
Acetosyringone (μM)	500	500	500	100
NAD^+ (μM)	500	500	100	500
Laccase ($U L^{-1}$)	900	380	900	900
GDH ($U L^{-1}$)	220	480	300	500
Measured and modeled data				
Time to reach a conversion of 99% (h)				
Modeled	37.5	28.1	66.5	23.4
Conversion after 10 h (%)				
Experiment	30.2	37.7	17.7	13.0
Modeled	35.7	37.7	19.9	49.9
STY_{10} ($mM h^{-1}$)				
Experiment	6.04	7.69	3.61	2.65
Modeled	7.14	7.69	4.06	10.2
Specific $productivity_{LAC}$ ($mmol kU^{-1} h^{-1}$)				
Experiment	6.76	20.3	4.04	2.97
Modeled	7.98	20.2	4.54	11.4
Specific $productivity_{GDH}$ ($mmol kU^{-1} h^{-1}$)				
Experiment	27.3	16.0	11.9	5.29
Modeled	32.3	16.0	13.4	20.3
TN_{LAC} (h^{-1})				
Experiment	6.65×10^5	1.99×10^6	3.97×10^5	2.92×10^5
Modeled	7.85×10^5	1.99×10^6	4.47×10^5	1.12×10^6
TN_{GDH} (h^{-1})				
Experiment	2.03×10^5	1.19×10^5	8.86×10^4	3.93×10^4
Modeled	2.40×10^5	1.19×10^5	9.96×10^4	1.50×10^5
TN_{NAD^+} (h^{-1})				
Experiment	12.1	15.4	36.1	5.31
Modeled	14.3	15.4	40.6	20.3
TN_{RM} (h^{-1})				
Experiment	12.1	15.4	7.22	26.5
Modeled	14.3	15.4	8.12	102

3.3. Process modeling and engineering

To study the influence of different volumetric activities of laccase and GDH on the conversion rate in combination with three preselected, initial concentrations of redox mediator and coenzyme, productivity charts were generated from 1681 calculations per plot (Fig. 4A–C). The availability (the concentration) of redox mediator and coenzyme is shown to be an important factor influencing STY. Three laccase/GDH activity ratios for Batches B, C and D were selected to test for the rate limiting step in the regeneration system, which are indicated in the plots. To investigate the influence of enzyme activity on the whole process in more detail, specific productivities for each enzyme were modeled for different combinations of the other enzyme's activity, redox mediator and coenzyme concentration. Isoproductivity plots for GDH and laccase gave a critical activity, below which the STY drops, and are given for Batches A and B (Fig. 5A and B) and Batches C and D (Fig. S2A–D). Finally the effect of the initial redox mediator and coenzyme concentration on the STY and turnover numbers for coenzyme and redox mediator was investigated. The simulations shown in Fig. 6 illustrate the effect for the laccase/GDH activity ratio used in Batch A (900 and 220 $U L^{-1}$, respectively). Simulations for the conditions employed in Batches B, C and D are given in the Supplemental Information (Fig. S3A–F).

3.4. Limiting activity of the regenerating enzyme

The settings for Batch B were selected to test the model of the regeneration system (laccase and redox mediator) and investigate the effect of a decreased efficiency of the regeneration system by making the laccase reaction rate limiting. The ratio of laccase:GDH volumetric activities was 0.8 while all other parameters were kept constant (Table 2). Glucose conversion was 37.7% during 10 h of reaction, which correlated very well with the predicted degree of conversion. The measured and predicted data for Batch B are shown in Fig. 3B. The modeled, averaged NAD^+ concentration during time is ~170 μM (Table S1) and acetosyringone concentration is ~478 μM (Table S2). The measured activity decreased by 15% for laccase and 17% for GDH during these initial 10 h of reaction. The total turnover number after 10 h was 1.99×10^7 for laccase and 1.19×10^6 for GDH. The averaged enzyme consumption numbers for laccase and GDH were 0.76 and 1.08 U $mmol^{-1}$ product, respectively.

3.5. Limiting coenzyme concentration

Batch C was conducted to evaluate the effect of a decreased coenzyme concentration on coenzyme regeneration and especially the synthetic reaction. In this experiment, the coenzyme concentration was 5-fold lower compared to Batches A and B, while keeping the laccase:GDH activity ratio high (3:1) (Table 2). The observed glucose conversion was 17.7% within 10 h. The correlation with the predicted degree of conversion of 19.9% is good, further measured and predicted data for Batch C are shown in Fig. 3C. The modeled, averaged NAD^+ concentration during the first ten hours is very constant with ~92 μM (Table S1) and the acetosyringone concentration is ~15 μM (Table S2). During the conversion the measured laccase activity decreased by 15.6% and GDH activity by 54.8%. The total turnover number after 10 h was 3.87×10^6 for laccase and 8.86×10^5 for GDH. Both enzymatic activities decreased linearly. The averaged enzyme consumption numbers for laccase and GDH were 3.97 and 4.64 U $mmol^{-1}$, respectively.

3.6. Limiting the redox mediator concentration

In the fourth experiment, Batch D, the initial redox mediator concentration was decreased 5-fold from standard conditions to evaluate the effect of a decreased redox mediator concentration on both enzymatic cycles. The laccase:GDH activity ratio was 1.8:1 (Table 2). Glucose conversion was only 13% in 10 h, which was a result of an almost complete stop of the reaction after 4 h. The measured conversion after 1 h was 7.40%, which correlates roughly with the predicted value of 5.29% when considering the difference of actually added GDH activity and V_{max}. However, for later time points a much lower conversion is measured than predicted by the model. Data for Batch D are shown in Fig. 3D. The modeled, averaged NAD^+ concentration during the first hour is ~240 μM (Table S1) and acetosyringone concentration is ~60 μM (Table S2). The measured specific productivity of GDH for the first hour is 29.5 $mmol kU^{-1} h^{-1}$, which is the second highest after Batch A. However, after 1 h a dramatic drop in the specific productivity of GDH and laccase is observed. During the first 2 h the measured laccase activity decreases faster than thereafter, but the total loss of activity after 10 h is only 20.1%. Little GDH deactivation is observed in this reaction (~5%). The total turnover number after 10 h was 2.92×10^6 for laccase and 3.93×10^5 for GDH. The averaged enzyme consumption numbers for laccase and GDH were 6.96 and 0.96 U $mmol^{-1}$, respectively. The model was used to calculate the theoretical, residual amount of redox mediator present in Batch D, based on the deviation of predicted and measured reaction rate for each hour. The reduction of the acetosyringone concentration was calculated to be 54, 15.6 and 7.3 μM after 2, 3 and 4 h, respectively.

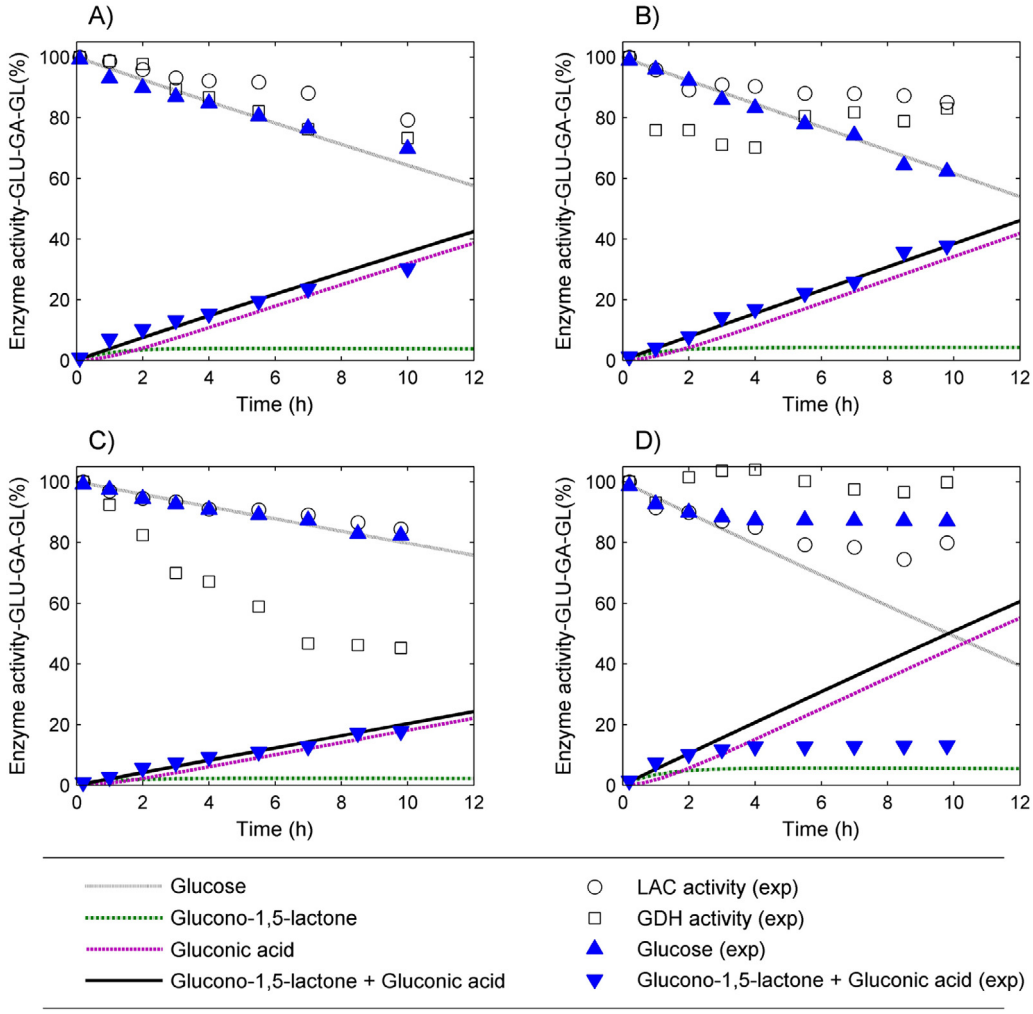

Fig. 3. Batch conversions A–D. Measurements were stopped after 10 h of conversion. Measured values are indicated by data points, calculated values are indicated by lines. The *Y*-axis gives concentrations and activities as a percentage of their initial values to avoid multiple axes. Initial concentrations, activities and calculated performance numbers are given in Table 2. The reaction product glucono-1,5-lactone hydrolyzes to gluconic acid during HPLC sample preparation and is therefore lumped with the already present gluconic acid (triangles down).

4. Discussion

To find the best pH for coupling acidic *T. pubescens* laccase and GDH, the pH optima of laccase activity for the two previously published, NADH-oxidizing redox mediators acetosyringone and syringaldehyde were determined [15]. The rate constants with the laccase at pH 5.0, were high enough to use the selected laccase together with GDH. The catalytic performance of GDH at pH 5.0 compared to pH 8.0 [24] is still good enough to allow its application. One potential problem arising from employing GDH at low pH is the low hydrolysis rate of the reaction product gluconolactone, which results in product inhibition. Another critical factor is the

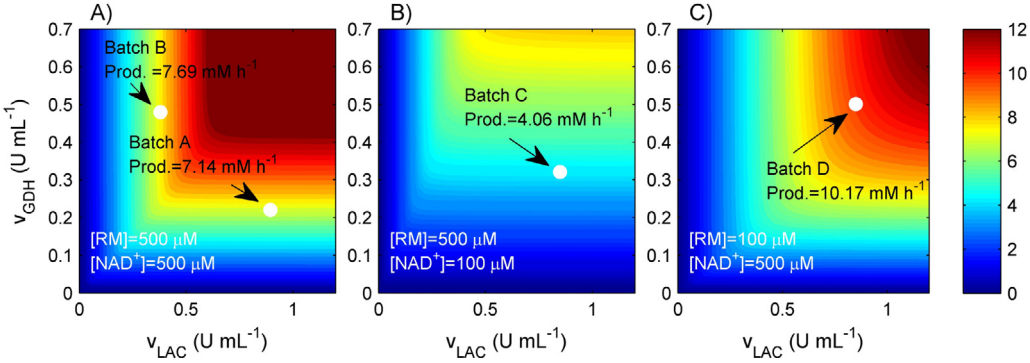

Fig. 4. Isoproductivity charts calculated from space-time yields after 10 h of reaction. The simulation was based on the following initial concentrations: 200 mM glucose and (A) 500 μM redox mediator and 500 μM coenzyme (NAD^+), (B) 500 μM redox mediator and 100 μM coenzyme (NAD^+), (C) 100 μM redox mediator and 500 μM coenzyme (NAD^+).

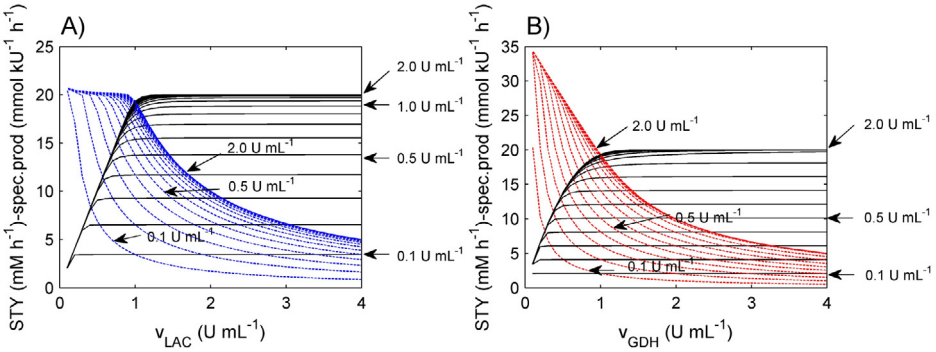

Fig. 5. Isoproductivity plots based on the specific productivity after 10 h of reaction in regard to the laccase activity (A) and GDH (B) activity, simulated for the conditions in Batch A and B (500 μM redox mediator and 500 μM coenzyme). The activities indicated in the diagrams are the opposite enzyme as given on the *X*-axis. STY (straight lines), specific productivity (dashed lines).

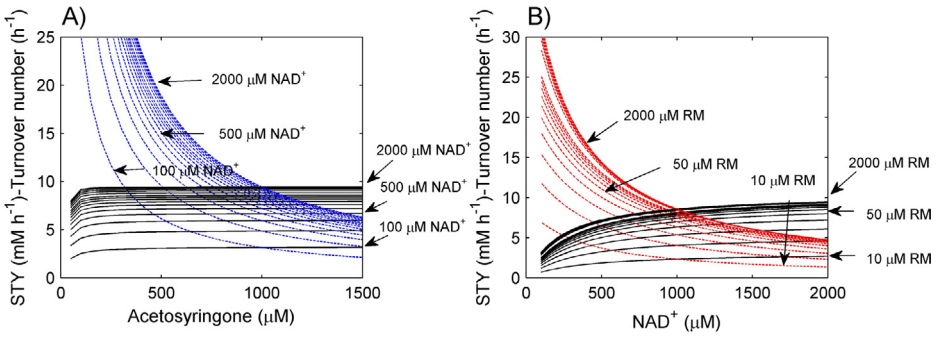

Fig. 6. Effect of the initial redox mediator concentration (A) and coenzyme concentration (B) on STY and turnover numbers for the coenzyme and redox mediator after 10 h of reaction. The simulated laccase:GDH activity ratio corresponds to that used in Batch A (900 U L^{-1} laccase and 220 U L^{-1} GDH). STY (straight lines), turnover numbers (dashed lines), NAD^+ stands for the total of applied coenzyme which was in the oxidized form.

supply of oxygen, which governs the regenerating laccase reaction. Therefore, the employed enzymatic activities were selected sufficiently low to avoid critical gluconolacone build-up or a decrease of the oxygen concentration in the reactor. Laccase from *T. pubescens* exhibits more favorable catalytic constants for acetosyringone than syringaldehyde at pH 5.0, which was one reason to study acetosyringone as redox mediator in this work. The other reason to employ acetosyringone was its higher rate constants with the coenzymes NADH and NADPH. The rate constants obtained for the reaction of syringaldehyde or acetosyringone with NADH were 1.3 to 2-fold higher than that for NADPH. The phosphorylation of the coenzyme reduces the re-oxidation rate. Considering the similar redox potential of both coenzyme forms (−320 mV vs. SHE) this is an unecpected result. The reason might be the additional negatively charged phosphate group in NADPH which may lead to increased electrostatic repulsion with the deprotonated phenolate in acetosyringone.

The experimental evaluation of the MATLAB model for the GDH reaction was based on Batch A, in which a high laccase activity was employed to ensure a high NAD^+ concentration. According to the model ~460 μM NAD^+ were present during the reaction. This allows the verification of the model for the synthetic GDH reaction without limitation from the laccase regeneration system. The difference of the observed and predicted substrate conversion during in first two hours shows that the activity obtained with the used assay does not reliably estimates the V_{max} of GDH, but underestimates it by a factor of ~1.6, because of the limited glucose concentration (K_M = 16.7 mM, S = 100 mM), but especially the limited coenzyme concentration (K_M = 260 μM, S = 500 μM) in the assay. GDH deactivation during the process leads to a convergence of the actual and predicted glucose conversion.

The experimental evaluation of the MATLAB model for the laccase reaction was based on Batch B, which employed a low laccase activity to keep the concentration of the reduced form of acetosyringone high. The model predicted a concentration of 478 μM acetosyringone. The volumetric activity of laccase was well estimated by the enzymatic assay and its conversion from ABTS to acetosyringone activity by the estimated factor of 1:0.54. Since the oxygen concentration was kept constant, no deviation between the volumetric activity and V_{max} was found.

Process modeling and engineering was based on the verified MATLAB model. The influence of different laccase:GDH activity ratios, redox mediator or coenzyme concentrations were modeled and useful activities and concentrations selected for the conversion experiments based on the model. The productivity plots show that the initial concentration of redox mediator and coenzyme together with the activity of regenerating and synthetic enzyme govern STY (Fig. 4). At high concentrations of the coenzyme and redox mediator (500 μM each, Fig. 4A), which are significantly above the K_M values of laccase and GDH, neither r_1 nor r_3 are limited. Also the bimolecular rate constant for the oxidized acetosyringone/NADH (294 M^{-1} s^{-1}) ensures that for the selected concentrations r_2 is high enough (73.5 μM s^{-1}) to support enzymatic activities up to 4400 U L^{-1}. With such a high efficiency of the mediator system, the activities of both enzymes and their ratio become dominant for the STY and specific productivities. Enzyme ratios for Batch A and B were selected to investigate the sensitivity of one enzyme from the other. For a reduced coenzyme concentration (100 μM, Fig. 4B) a limiting effect of r_3 was predicted due to a NAD^+ concentration below the K_M of GDH for its coenzyme. This results in a low predicted STY (4.06 mM h^{-1}) and a reaction that is little sensitive toward changes in GDH activity and even less toward changes in laccase activity. The conditions of Batch C were chosen from this

plot. To study the effect of a reduced redox mediator concentration (100 μM) the conditions for Batch D were derived from Fig. 4C. The reduced redox mediator concentration shows only a moderate effect in the simulation which is based on the laccase activity of Batch A and the GDH activity of Batch B. The predicted STY for Batch D (10.17 mM h^{-1}) results from the optimized enzymatic activities. The relative insensitivity toward the reduced redox mediator concentration compared to the coenzyme is explained by the lower K_M value of laccase for acetosyringone than in the case of GDH for NAD^+.

Specific productivities of enzymes under the three selected redox mediator/coenzyme concentrations are plotted in Figs. 5 and S2 By following the isoactivity curves for laccase and GDH the critical activities below which the STY drops can be found. The maximal specific productivity of laccase at 500 μM acetosyringone and 500 μM NADH is predicted to be 20.1 mmol kU^{-1} h^{-1} and the maximum specific productivity of GDH is 33 mmol kU^{-1} h^{-1}. A good compromise to achieve high specific productivities for laccase and GDH can be reached when the activity ratio of laccase:GDH is about 2:1. In the case of 500 μM acetosyringone and 100 μM NADH, the maximum specific productivity of laccase is unchanged, but the specific productivity of GDH is reduced to 14 mmol kU^{-1} h^{-1}, caused by the low possible NAD^+ concentration. Under these conditions, the best compromise to achieve high specific productivities for laccase and GDH is reached when the activity ratio of laccase:GDH is about 0.6:1. For the last case (100 μM acetosyringone, 500 μM NADH) the maximum specific productivity of laccase is lower (14 mmol kU^{-1} h^{-1}), whereas the specific productivity of GDH is the same as for Batch A and B (33 mmol kU^{-1} h^{-1}). The reason is the low possible concentration of reduced acetosyringone. Under these conditions, the best compromise to achieve high specific productivities for laccase and GDH is reached when the activity ratio of laccase:GDH is about 1.5:1.

For the activity ratios used in Batches A-D the following initial acetosyringone and NADH concentrations were predicted to be necessary to obtain a good STY (Figs. 6 and S3). Batch A (activity ratio laccase:GDH 4.1:1): 100 μM acetosyringone and 1000 μM NADH; Batch B (activity ratio 0.8:1) 500 μM acetosyringone and 200 μM NADH; Batch C (activity ratio 3:1) 200 μM acetosyringone and 1000 μM NADH; Batch D (activity ratio 1.8:1) 300 μM acetosyringone and 1000 μM NADH. Of course, these concentrations were not applied, but given here to demonstrate that at activity ratios of laccase:GDH >1.5:1 the availability of the coenzyme governs the reaction rate, whereas in the case of a limiting laccase activity the redox mediator concentration governs the reaction rate.

These predictions were tested by two Batch reactions: Batch C employing a limiting coenzyme concentration and Batch D with a limiting redox mediator concentration. For Batch C, an available NAD^+ concentration of 92 μM from the initially added 100 mM was predicted. Despite the efficient regeneration of NADH the low coenzyme concentration is the rate-limiting step in the reaction (r_3). In Batch D, the predicted concentration (~60 μM) of the oxidized redox mediator acetosyringone was sufficient to keep r_1 high. However, in contrast to the model, the experiment showed an unexpected effect of the acetosyringone phenoxy radical. A polymerization reaction of the redox mediator occurred, which reduced the amount of the redox mediator drastically during the first 3 h and stopped the reaction.

The enzyme deactivation observed in the batch conversion processes is relatively low and high total turnover numbers of $>10^6$ were obtained. However, certain deactivation patterns are observed. In Batches A and B the measured enzymatic activities decreased linearly, which indicates that enzyme deactivation is proportional to product formation. The loss of activity was moderate. Enzyme consumption numbers of 0.76–1.08 U per mM formed product were observed. Only the inactivation of laccase in Batch A was higher (2.81 U mM^{-1}), which is probably an effect of the higher concentration of the acetosyringone phenoxy radical. Under conditions, which favor high radical concentrations even more (Batch C), the enzyme consumption number for both enzymes increases to 4 and 4.6 U mM^-. The higher enzyme consumption is also observed for GDH and indicates that the deactivation caused by acetosyringone phenoxy radicals is concentration dependent and not connected with substrate turnover. Even more interesting results were found under a limiting redox mediator concentration in Batch D. After a short time a color change in the reaction vessel was observed, which indicates a degradation of the redox mediator acetosyringone. The most probable cause is a dimerization/polymerization by coupling of the formed phenoxy radicals or coupling to proteins. The low concentration of the redox mediator made this process obvious. A fast reduction of the acetosyringon phenoxy radical by NADH should stabilize the redox mediator in the process. Together with the destruction of the redox mediator also a deactivation of laccase slows down after the first hours and follows similar to GDH a time-dependent process.

In conclusion, an optimized process using the laccase/mediator system should fulfill the following criteria: (i) A high concentration of NAD^+ to achieve a high specific GDH productivity. The actual NAD^+ concentration depends mostly on the initial coenzyme concentration and laccase activity; (ii) a two-fold excess of laccase activity over GDH activity to ensure efficient regeneration of the coenzyme and high specific productivities of regenerating and synthetic enzymes. When the ratio drops, the NAD^+ concentration is low regardless of the added coenzyme concentration. (iii) The enzyme activity ratio also affects the concentration of the oxidized redox mediator. High concentrations should be avoided to reduce enzyme deactivation. Also the redox mediator itself is susceptible to degradation and more stable in the reduced form. If necessary, additional redox mediator has to be added during the reaction. (iv) The enzyme activities can be increased to increase STY until the oxygen concentration becomes a limiting factor. (v) Modeling can be elegantly used to optimize the enzymatic activities as well as redox mediator and coenzyme concentrations to improve the productivity, stability and economics of the reaction.

When comparing the excellent obtained productivity and enzyme total turnover numbers of the optimized laccase/mediator regeneration system with recently reported requirements for feasible industrial biocatalytic processes [17,26] the investigated regeneration reaction appears to be very useful to regenerate $NAD(P)^+$-dependent reactions.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2015.06.011

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