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

Ngoc Hung Pham[a,b], Frank Hollmann[c], Daniel Kracher[a], Marita Preims[a], Dietmar Haltrich[a], Roland Ludwig[a,*]

[a] Department of Food Science and Technology, BOKU – University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria
[b] School of Biotechnology and Food Technology, Hanoi University of Science and Technology, 1 Dai Co Viet, Hanoi, Viet Nam
[c] Department of Biotechnology, Biocatalysis and Organic Chemistry Group, Technical University Delft, Julianalaan 136, 2628 BL Delft, The Netherlands

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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}).

* Corresponding author.
E-mail address: roland.ludwig@boku.ac.at (R. Ludwig).

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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.

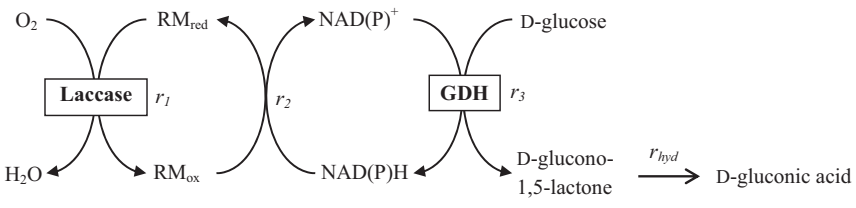

Fig. 1. Reaction scheme for the bi-enzymatic system employing laccase as regenerating enzyme and glucose dehydrogenase (GDH) as synthetic enzyme. The redox mediator in its reduced form RM_{red} is oxidized by laccase to RM_{ox} with the rate r_1. The bimolecular rate observed for the reaction between 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}.