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Continuous enzymatic regeneration of redox mediators used in biotransformation reactions employing flavoproteins

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Abstract

Oxidoreductases are a group of enzymes that have been regarded uneconomical for industrial processes due to their dependence on cofactors or prosthetic groups for activity and the difficulties of regenerating these. Especially, flavoproteins have long been neglected for biocatalytical applications. The prosthetic group of some of these enzymes, but not all, can be regenerated by oxygen, resulting in hydrogen peroxide formation, which is detrimental to enzyme stability. As a contribution to alleviating this problem, a novel concept for the regeneration of electron acceptors (redox mediators) for flavoenzymes is described. Flavin-containing enzymes such as cellobiose dehydrogenase (CDH) or pyranose oxidase (P2O) are used in conjunction with laccases and a redox mediator. The flavin of the synthetic enzyme is reduced while the oxidized product of interest is formed, in turn, the flavin is reoxidized with the help of an electron acceptor, which then is regenerated using a laccase. Laccases are copper containing phenol oxidases that can transfer four electrons to oxygen, producing two molecules of water. Preliminary screening experiments with different redox mediators, and a coupled enzyme system of CDH and laccase, showed that a wide variety of different substances can efficiently shuttle electrons between these two enzymes. Among them are substituted and unsubstituted *ortho*- and *para*-quinones, benzoquinone imines, cation radicals such as 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), redox dyes such as phenothiazines or phenoxazines, as well as iron complexes.

Experiments in which CDH completely oxidizes lactose to lactobionic acid and P2O entirely converts glucose to 2-keto-glucose are presented. Catalytic amounts of redox mediators are used and continuously regenerated by a laccase. Specific productivities of up to 19.3 $g \cdot (h \cdot kU)^{-1}$ and 72 $g \cdot (h \cdot kU)^{-1}$ for CDH and P2O, respectively, were found. The total turnover numbers (TTNs) for the two enzymes used were in the range of 10^5–10^6. Oxygen supply for the laccase is a crucial factor in avoiding rate limitation. Undeniably, this system facilitates the efficient use of a hitherto underexploited group of enzymes for preparative purposes.

Keywords: Enzymatic regeneration; Quinone electron acceptors; Redox mediators; Laccase; Cellobiose dehydrogenase; Pyranose oxidase

1. Introduction

Only a decade ago hydrolases were considered the only game in town for larger-scale biotransformations with glucose isomerase as a major exception [1,2]. In fact, the use of isolated oxidoreductases for

Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); CDH, cellobiose dehydrogenase; DCIP, 2,6-dichloro-indophenol; P2O, pyranose oxidase; pO_2, dissolved oxygen tension; TTN, total turnover number

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this purpose has been regarded as intrinsically uneconomic [3], since this latter class of enzymes depends on either freely dissociated cofactors (coenzymes) or on more or less tightly bound redox centers (prosthetic groups) that have to be continuously regenerated to transfer oxidation or reduction equivalents. The last decade, however, has brought considerable progress in the field of enzymatic oxidoreductive biotransformations, and by now at least one industrial process has been described in which a soluble NADH-dependent dehydrogenase is utilized as the key biocatalyst [1]. This progress in employing nicotinamide coenzyme dependent enzymes for biocatalytic purposes has mainly been made possible by establishing efficient, reliable methods for continuously regenerating the coenzyme. Several strategies have been developed for the regeneration of both NAD(H) and NADP(H) which can be classified into (i) enzymatic, (ii) electrochemical, (iii) chemical and photochemical, and (iv) biological methods [4]. Among these methods, enzymatic regeneration of the coenzymes offers clear advantages due to its selectivity, its efficiency, as well as its excellent compatibility with other components of the enzyme-catalyzed reaction [5].

It seems, however, as if at present almost exclusively NAD-dependent dehydrogenases are employed for oxidoreductive biotransformations [6,7], whereas other oxidoreductases such as flavoproteins or quinoproteins are rarely used for this purpose, even though they represent a vast biocatalytic potential. One reason for this negligence is the necessity of an efficient and stable regeneration method of the prosthetic group that is compatible with a biocatalytic process after electron equivalents have been transferred between the enzyme and the substrate. Typically, this regeneration is performed by an appropriate electron acceptor, which can range from oxygen to various quinones, redox mediators or metal ions. During these reactions, the electron acceptors are consumed in a stoichiometric amount, which provides no disadvantages to the biotransformation process when the electron acceptor is cheap and readily available such as oxygen. However, some flavoproteins, e.g., cellobiose dehydrogenase (CDH), show negligible activity with oxygen [8,9]. Furthermore, oxygen is reduced to hydrogen peroxide, which as a highly reactive compound can irreversibly inactivate enzymes and hence has to be removed by catalase, metal catalysts or by a H_2O_2-consuming reaction [10–12]. For biosynthetic purposes employing flavoenzymes, some electron acceptors such as benzoquinone have been added in equimolar amounts or even in excess, which in turn means that the cosubstrate or coproduct has to be removed after completion of the reaction. Alternatively, the redox mediator can be regenerated and therefore has to be added in catalytic amounts only. This regeneration can be done either by in situ reoxidation with oxygen [13], by microbiological methods [14], or by electrochemical methods [15–17]. It is especially these latter electrochemical methods that are predominantly used for preparative synthesis of various biomolecules [18].

In the present study, a novel concept of enzymatic regeneration of a range of electron acceptors (redox

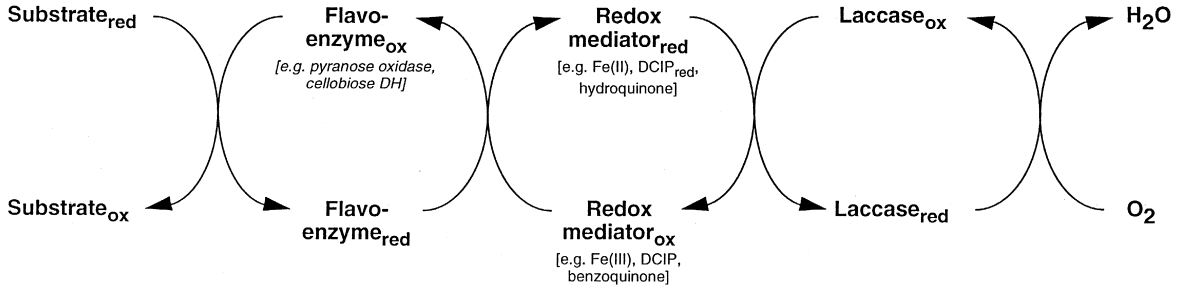

Fig. 1. Continuous, laccase-catalyzed regeneration of electron acceptors (redox mediators) that are employed in oxidation reactions of flavoproteins.

mediators) used by various flavoenzymes is introduced (Fig. 1). This regeneration is catalyzed by laccases, which are multi-copper oxidases and have been detected in various plant species, several insects as well as in a number of fungi. Laccases can catalyze the oxidation of various substances such as inorganic or organic metal complexes, anilines, thiols, and especially phenols with the concomitant reduction of molecular oxygen to water. Although laccases strongly prefer O_2 as their oxidizing substrate, they are typically highly unspecific towards their reducing substrate [19]. These properties render laccase an ideal enzyme for regenerating a wide range of electron acceptors that are used by flavoenzymes during their catalytic action.

2. Experimental

2.1. Chemicals

All chemicals (quinones, dyes) used in this study were of the highest grade available and were obtained from Aldrich (Vienna, Austria), Fluka (Vienna, Austria) or Sigma (Vienna, Austria).

2.2. Enzymes

Unless otherwise stated, the laccase used in this study was produced by the white-rot fungus *Trametes pubescens* MB 89. The organism was cultivated at 25°C in a 20-l stirred tank reactor (working volume 15 l) using a medium based on glucose (20 $g \cdot l^{-1}$). Laccase production was induced by addition of 1.5 mM $CuSO_4$. Typically, 15.3 $U \cdot ml^{-1}$ laccase were obtained after 7 days of cultivation, corresponding to a specific activity of 85 $U \cdot mg^{-1}$. The laccase preparation used in this study was partially purified by anion exchange chromatography using a Q-Sepharose fast flow (Amersham-Pharmacia, Uppsala, Sweden) column previously equilibrated with 20 mM sodium acetate buffer pH 5.0. The laccase isoenzyme of interest was eluted with a linear gradient of 0–0.25 M NaCl of the same buffer. The specific activity of this partially purified enzyme was 422 $U \cdot mg^{-1}$; it contained no additional, contaminating laccase isoenzymes. Alternatively, a partially purified enzyme from *Coriolus (Trametes) versicolor*, which was a generous gift of Shin Nihon Chemical, Anjyo, Japan, was used.

CDH was produced by the basidiomycete *Athelia (Sclerotium) rolfsii* CBS 191.62. This fungus was cultivated on a medium based on cellulose (42 $g \cdot l^{-1}$) for 13 days as described previously [20]. On the average, 3.8 $U \cdot ml^{-1}$ of CDH activity (specific activity of 0.95 $U \cdot mg^{-1}$) was produced in these cultivations. The preparation of CDH used throughout this study was partially purified by a single anion exchange chromatography step using DEAE Sepharose fast flow (Amersham-Pharmacia) with 20 mM histidine buffer (pH 5.5) and elution with a linear NaCl gradient (0–0.5 M).

Pyranose oxidase (P2O) from *T. multicolor* was produced on a lactose-based medium as described elsewhere [21]. The average P2O yield in these fed-batch laboratory fermentations was approximately 1.6 U P2O activity/ml. For biotransformation experiments, a partially purified enzyme preparation obtained by anion-exchange chromatography was employed. Crude mycelial extract was loaded on a Source 30Q column (Amersham-Pharmacia) pre-equilibrated with 20 mM BisTris buffer, pH 6.5 (buffer A). Subsequently, P2O was eluted with a linear gradient of 0–100% 1 M KCl in buffer A.

2.3. Enzyme activity assays

The reaction mixture for assaying laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) activity contained 5 mM 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) ($\varepsilon_{436} = 29{,}300$ $M^{-1} \cdot cm^{-1}$) in 20 mM acetate buffer, pH 3.5 [22]. *T. versicolor* laccase was assayed in 20 mM acetate buffer, pH 4.0. One unit of laccase activity was defined as the amount of enzyme oxidizing 1 μmol of ABTS/min at 25°C. CDH [cellobiose:(acceptor) 1-oxidoreductase, EC 1.1.99.18] activity was assayed by following the decrease in absorbance of 2,6-dichloro-indophenol (DCIP) at 520 nm ($\varepsilon_{520} = 6800$ $M^{-1} \cdot cm^{-1}$), pH 4.0 and 37°C. The reaction mixture contained DCIP (0.3 mM) and lactose (30 mM)

in 100 mM sodium acetate buffer. One unit of enzyme activity is defined as the amount of enzyme reducing 1 μmol of DCIP/min under the above reaction conditions. In case that laccase interfered with the determination of CDH activity by rapidly reoxidizing the reduced form of DCIP, it was completely inhibited by the addition of fluoride (4 mM) to the assay [23]. P2O (pyranose:oxygen 2-oxidoreductase, EC 1.1.3.10) activity was determined spectrophotometrically at 420 nm and 30°C by measuring the formation of H_2O_2 in a peroxidase-coupled assay using ABTS ($\varepsilon_{420} = 43{,}200\ M^{-1} \cdot cm^{-1}$) as the chromogen [24]. The standard assay mixture (1 ml total) contained 1 μmol ABTS in potassium phosphate buffer (50 mM, pH 6.5), 2 U horseradish peroxidase, 100 μmol D-glucose and a suitable amount of the P2O sample. One unit of P2O activity is defined as the amount of enzyme necessary for the oxidation of 2 μmol of ABTS/min under the given conditions. When measuring P2O activity in the presence of laccase, 8 mM sodium fluoride (final concentration) was added to the assay to completely inhibit laccase activity.

2.4. Biotransformation experiments

Preliminary small-scale screening experiments were performed in 12-well microtiter plates to determine which redox mediators are suitable for the conjugated enzyme couple laccase/CDH. The reaction contained 1.0 U laccase, 0.5 U CDH, 30 μmol lactose, and 0.2 μmol of the respective redox mediator in 2 ml sodium acetate buffer (30 mM, pH 4.5). When necessary, redox mediators were first dissolved in a small volume of DMSO before adding the appropriate amount of buffer. The microtiter plates were continuously shaken at 30°C and 75% relative humidity for 2 h. Samples were then boiled for 10 min to inactivate the biocatalysts. Redox mediators were removed by adsorption to activated charcoal prior to HPLC analysis.

The CDH-catalyzed oxidation of lactose was performed at 30°C using 50-ml beakers with a working volume of 20 ml. Unless otherwise indicated, the reaction system contained 50 mM lactose, 1.5 mM ABTS, 1.4 $U \cdot ml^{-1}$ CDH, and 5 $U \cdot ml^{-1}$ laccase (*T. pubescens*) in 20 mM Na acetate buffer, pH 4.0. The reaction system was stirred and oxygenated by sparging the solution with air or oxygen (3.5 vol/vol solution/min). The pH was held constant by automatically adding 300 mM NaOH. As an alternative, DCIP (0.6 mM) was used as the electron acceptor for CDH. Reaction conditions for this transformation experiment were: 200 mM lactose, 1.4 $U \cdot ml^{-1}$ CDH, 2.8 $U \cdot ml^{-1}$ laccase (*T. versicolor*) in 200 mM Na acetate buffer, pH 4.5, which was flushed with pure oxygen. In addition, polyethylene glycol 35,000 (7 $mg \cdot ml^{-1}$) was added to avoid foaming due to aeration. The dissolved oxygen concentration was measured by using a microoxygen electrode (Microelectrodes, Londonderry, NH, USA). At the times stated, samples were taken to monitor chromatographically the course of the reaction.

The P2O-catalyzed conversion of D-glucose into 2-keto-D-glucose was performed at 30°C in 50-ml flasks with a total working volume of 25 ml. The reaction system contained 400 mM D-glucose in sodium citrate buffer (50 mM, pH 5.0) and 1.0 $U \cdot ml^{-1}$ P2O. The reaction system was continuously stirred and oxygenated using pure oxygen (0.9 vol. oxygen/vol. solution/min) which was bubbled through a porous sintered glass disk. Hydroquinone (0.5 mM), used as the redox mediator, was continuously oxidized by laccase (*T. versicolor*, 10 $U \cdot ml^{-1}$). Alternatively, the electron acceptor for P2O was oxygen in which case 10,000 $U \cdot ml^{-1}$ catalase were added to destroy the by-product hydrogen peroxide. Controlling the pH value during the transformation was not necessary.

2.5. Analytical methods (HPLC)

Lactose and lactobionic acid were analyzed by HPLC using an Ostion LGKS 0800 Ca column (250 × 8 mm; Watrex, Prague, Czech Republic) at 80°C with 10 mM $Ca(NO_3)_2$ as eluent (0.7 ml min^{-1}) and refractive index detection. Glucose and 2-ketoglucose were quantitated by HPLC employing an Ostion LGKS 0800 Na column (250 × 8 mm; Watrex) and refractive index detection. Water was used as a mobile phase at a flow rate of 0.5 ml min^{-1} and a column temperature of 70°C.

3. Results and discussion

3.1. Screening for suitable redox mediators

Preliminary transformation experiments were performed to determine which redox mediators, employed in low, catalytic concentrations (0.1 mM), can be used efficiently by the conjugated enzyme system laccase/CDH for the oxidation of lactose to lactobionic acid. Because of its very low activity with oxygen, the flavoheme enzyme CDH (formerly cellobiose oxidase), which is believed to be a constituent of the cellulose-degrading enzyme system of fungi [25], was selected as the biocatalyst of choice for these experiments. It specifically oxidizes a small number of β-1,4-linked di- and oligosaccharides (e.g., cellobiose, cellotriose, lactose) to the lactones, which are then hydrolyzed to the corresponding aldonic acids. In contrast, it is highly unspecific towards the electron accepting substrates, which include a variety of *ortho*- and *para*-quinones, several cation radicals, and a number of metal ion complexes [9]. This simple screening procedure using the complete system laccase/CDH/mediator was chosen rather then investigating the activity of the enzymes individually with each mediator, since it was found to be rapid and convenient, and gave preliminary indications whether all components of the reaction system were compatible with each other. In addition, the performance of two laccases produced by different fungal strains was compared in these initial experiments since it is known that fungal laccases can vary significantly in their substrate spectrum [19].

The relative amount of lactose (initial concentration 15 mM) that was converted to lactobionic acid within 2 h by the reaction system is listed in Table 1. Out of approximately 100 redox mediators tested, those results are shown, in which a significant conversion was obtained. It should be noted that a short reaction time was deliberately chosen in order to get preliminary information about the productivity with which the product is formed when using the different redox mediators with the conjugated enzyme system. A blank that contained the respective redox mediator but lacked laccase was treated in a similar way to determine the reoxidation of reduced electron acceptors by molecular oxygen. The performance and the specificities of both laccases, i.e., from *T. pubescens* and *T. versicolor*, are well comparable. Close to complete conversion of the substrate and high product concentrations were obtained when a number of differently substituted benzoquinones/hydroquinones were used as the redox mediator. In general, *ortho*-quinones seem to be better suited for the investigated biotransformation than *para*-quinones. The reason for this could be that the latter seem to be somewhat worse substrates (lower v_{max} values) for CDH [9]. Substituting hydrogens of the quinones with an increasing number of methyl groups drastically decreased the efficiency of the redox mediator, resulting in lower product yields. This effect was even more pronounced when bulkier alkyl substituents, e.g., *tert*-butyl groups, replaced the hydrogens, whereas chloro or bromo substituents increased the product yield, showing that the various chloro- and bromobenzoquinones are excellent redox mediators for the proposed enzyme couple laccase/CDH. In addition, the use of the two benzoquinone imines indophenol and dichloro-indophenol (DCIP), the latter a common electron acceptor for a number of flavoenzymes as well as for quinoenzymes, resulted in high lactose conversion. This indicates that both of the reduced forms of these compounds are readily reoxidized by the laccases compared in this study. On the contrary, various naphthoquinones and anthraquinones were not suitable for shuttling electrons between CDH and laccase as can be seen by the very low product yields.

Additionally, a number of redox dyes were tested for their ability to serve as redox mediators between laccase and CDH (Table 1). However, yields for more than 15 triphenylmethane dyes tested were negligible (data not shown) and the use of only a few dyes from this group (e.g., bromocresol purple, cresol red) resulted in moderate yields. In contrast, some phenazine (e.g., phenazine methosulfate), phenothiazine (e.g., methylene green) and phenoxazine dyes (e.g., Meldola's blue) were good or excellent mediators with conversion of up to 90%. In addition, ABTS, which is routinely used as substrate in standard laccase assays, was a very suitable mediator for the coupled system laccase/CDH. It has been recently reported that the ABTS cation radical is an excellent electron acceptor for CDH from *T. versi-*

Table 1
Screening for redox mediators capable of shuttling electrons between CDH and two different laccases. Values given are relative amounts of lactose converted to lactobionic acid within 2 h under the selected reaction conditions

Redox mediator	Laccase, *T. pubescens*	Laccase, *T. versicolor*	Blank
1,4-Benzoquinone (hydroquinone)	72.3	74.1	0.6
Catechol	> 98	82.0	0
Methyl-1,4-benzoquinone	92.5	76.1	1.9
2,6-Dimethyl-1,4-benzoquinone	30.0	28.1	2.6
Tetramethyl-1,4-benzoquinone	2.7	26.8	1.2
2,5-Di-*tert*-butyl-1,4-hydroquinone	2.4	1.2	0.7
3,5-Di-*tert*-butyl-1,2-benzoquinone	31.2	21.8	0
2,6-Dimethoxy-1,4-benzoquinone	28.9	27.9	0
2-Hydroxymethyl-6-methoxy-1,4-benzoquinone	39.8	34.7	1.5
2,3-Dimethoxy-5-methyl-1,4-benzoquinone	19.3	19.9	4.7
1,2,4-Trihydroxybenzene	57.3	47.8	4.9
Tetrahydroxy-1,4-benzoquinone	4.7	3.1	1.1
2-Chloro-1,4-benzoquinone	> 98	83.8	1.9
Tetrachloro-1,2-benzoquinone	> 98	80.3	1.4
Tetrachloro-1,4-benzoquinone	> 98	74.8	0
2,5-Dichloro-3,6-dihydroxy-1,4-benzoquinone	1.8	1.3	0.5
2,3-Dichloro-5,6-dicyano-1,4-benzoquinone	70.8	> 98	20.3
Tetrafluoro-1,4-benzoquinone	75.8	68.6	3.5
Tetrabromo-1,4-benzoquinone	> 98	> 98	1.6
2,6-Dichloroquinone-4-chloroimide	92.6	> 98	2.6
1,4-Benzoquinone dioxime	11.6	6.5	0
Indophenol	82.6	80.9	5.0
2,6-Dichloro-indophenol	> 98	95.3	3.1
Anthraquinone	1.5	0.6	0.1
Phenantrene-9,10-quinone	12.7	13.4	11.2
Cresol purple	3.8	2.0	0
Cresol red	12.0	9.4	0.4
Bromocresol green	5.0	3.5	0
Bromocresol purple	33.7	31.3	0.6
Methylene blue (Basic Blue 9)	41.4	44.2	2.9
Methylene green (Basic Green 5)	84.0	85.8	4.1
Meldola's blue	74.4	69.0	3.3
Phenazine methosulfate	90.6	92.9	32.4
2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)	94.6	97.4	0.8
$FeSO_4$	8.2	5.0	1.5
Ferricyanide	32.1	29.8	0
Ferricenium hexafluorophosphate	47.4	28.8	4.2
Ferricenium tetrafluoroborate	47.8	25.1	5.0
Cytochrome *c*	4.8	5.3	3.8
$CuSO_4$	nd	8.6	0
Oxygen	1.1	0.4	–
Oxygen + catalase	5.0	3.0	–

color, in fact, it is even more readily accepted by CDH than DCIP or various quinones [9].

Several metal ions and metal ion complexes such as Fe(III), Cu(II), Mn(III) or ferricyanide have also been shown to be substrates for CDH from *T. versicolor* [9]. Since at least ferrocyanide has been reported to be oxidized by laccase from, e.g., *T. hirsuta*, with high efficiency [26], these inorganic ions as well as two different ferricenium salts were included in the screening experiments for suitable redox mediators. In accordance with the reported specificities of both enzymes used, significant con-

version of the substrate was observed. Especially ferricyanide and the two ferricenium compounds were found to effectively shuttle electrons between the two enzymes, thereby sustaining the conversion of 30–50% of the substrate in the 2-h reaction period selected for this experiment. Particularly the ferrocenes (ferrodicyclopentadienyls), which currently have almost exclusively been employed in bioelectrochemical preparative processes, could be very attractive redox mediators for the laccase regeneration system. These compounds interact with a wide range of flavo- and quinoproteins, and a large number of derivatives with diverse functionalities are available which could vary in their activity towards different biocatalysts [3,18,27]. They can also be modified by polymers, thereby increasing their water solubility and enlarging their molecular weight. This makes possible their retainment in an enzyme reactor together with the enzyme biocatalysts by a simple ultrafiltration membrane, so that these biocatalytic processes can also be performed in a continuous mode with constant feeding of the substrates and removal of the low molecular weight products [17].

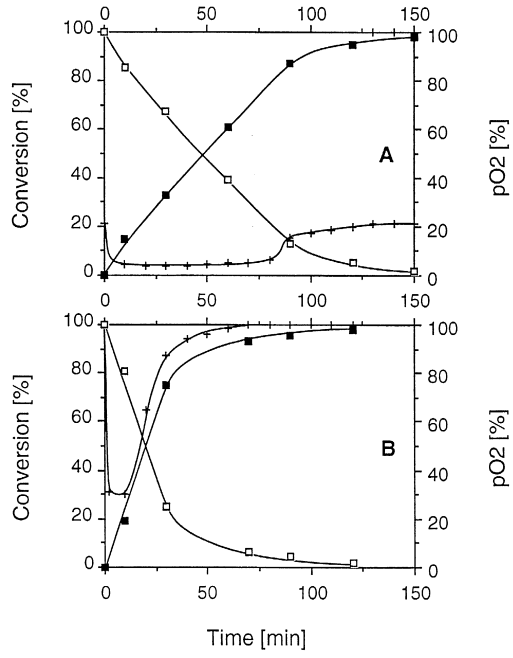

Fig. 2. Conversion of lactose to lactobionic acid by CDH. The ABTS radical, which serves as the electron acceptor for this oxidation reaction, was continuously regenerated by laccase. (A) Oxygenation by using air (3.5 vvm); (B) oxygenation by using oxygen (3.5 vvm). Symbols: □, lactose; ■, lactobionic acid; +, dissolved oxygen tension (relative to saturation with pure oxygen). Conditions: 50 mM lactose, 1.5 mM ABTS, 1.4 $U \cdot ml^{-1}$ CDH, and 5 $U \cdot ml^{-1}$ laccase (*T. pubescens*) in 20 mM Na acetate buffer, pH 4.0. The pH was held constant by adding 300 mM NaOH.

3.2. CDH-catalyzed oxidation of lactose with continuous regeneration of the redox mediator

In order to investigate the laccase-catalyzed regeneration of suitable redox mediators in more detail, discontinuous biotransformation experiments for the oxidation of lactose to lactobionic acid by CDH were performed, in which two different electron acceptors, i.e., the ABTS radical and DCIP, were employed in catalytic amounts. Results for the oxidation of 50 mM lactose by 1.4 $U \cdot ml^{-1}$ CDH using 1.5 mM ABTS and 5 $U \cdot ml^{-1}$ laccase for its regeneration are shown in Fig. 2. As is evident, laccase efficiently catalyzed the continuous oxidation of ABTS to the ABTS cation radical which in turn served as the electron acceptor for CDH during the oxidation of lactose to lactobionic acid. This conversion was close to complete within 150 min when air was sparged through the reaction mixture to supply oxygen for the laccase (Fig. 2A). The productivity and specific productivity of this process were calculated to be 7.2 $g \cdot (l \cdot h)^{-1}$ and 5.2 $g \cdot (h \cdot kU)^{-1}$, respectively. Due to the relatively low concentrations of lactose used in this experiment, the maximum reaction rate of CDH, which has a Michaelis constant of 2.6 mM for lactose, could only be attained during the initial phase of substrate conversion. The values for productivity and specific productivity calculated for this phase of the reaction were more favorable and were found to be 10.9 $g \cdot (l \cdot h)^{-1}$ and 7.8 $g \cdot (h \cdot kU)^{-1}$, respectively. When adding the biocatalyst to the reaction solution, the pO_2 decreased very rapidly and then stayed relatively constant during substrate conversion. With the depletion of the substrate in the reaction solution the pO_2 rises again to its initial value. As is evident from Fig. 2, the pO_2 is an excellent indicator for the progress of the enzymatic oxidation reaction.

One critical factor for the efficient utilization of laccase in bioconversion processes seems to be the gas–liquid mass transfer of oxygen, which is only poorly soluble in aqueous solutions. Assuming that in these experiments CDH (1.4 $U \cdot ml^{-1}$) is fully active and oxidizes lactose with its maximum catalytic capacity, 0.7 mmol of oxygen will be consumed per liter and minute under the selected reaction conditions. Thus, a sufficient oxygen supply is crucial for avoiding possible limitations of the reaction [28]. Furthermore, the K_m-values for oxygen for the *Trametes* laccases used in this study are approximately 0.2 mM (Galhaup and Baminger, unpublished results). These values are in the range of the oxygen concentration dissolved in aqueous solutions under 1 atm of air at 30°C which amounts to approximately 0.25 mM [29]. Because of its low solubility, this laccase cosubstrate is present in the reaction system in nonsaturating concentrations and hence the enzyme-catalyzed reaction will not proceed at its maximum velocity. In order to increase both the oxygen concentration and the oxygen transfer into the reaction system, the substrate solution was sparged with pure oxygen in a subsequent biotransformation experiment, in which all conditions were otherwise held constant (Fig. 2B). The use of pure oxygen can increase the initial rate of the biocatalytic reaction considerably, as is also evident from the maximal productivity and specific productivity of 27.0 $g \cdot (l \cdot h)^{-1}$ and 19.3 $g \cdot (h \cdot kU)^{-1}$, respectively, which are approximately 2.5 times higher than the values obtained in the experiment with air. The enzymatic, laccase-catalyzed regeneration of the redox mediator is well compatible with the CDH reaction system which is not only indicated by the complete conversion of the substrate but also by the residual enzyme activities determined after completion of the reaction. After a reaction time of 150 min, approximately 90% of the initially employed CDH activity and 98% of laccase activity were found. These two enzymes can easily be recovered from the product solution by ultrafiltration to be reutilized for subsequent substrate conversions.

The conversion of 200 mM lactose when using DCIP as the redox mediator is shown in Fig. 3. Again, the laccase regeneration system proved to be very efficient and compatible with the biocatalytic system chosen. Lactose was completely converted in

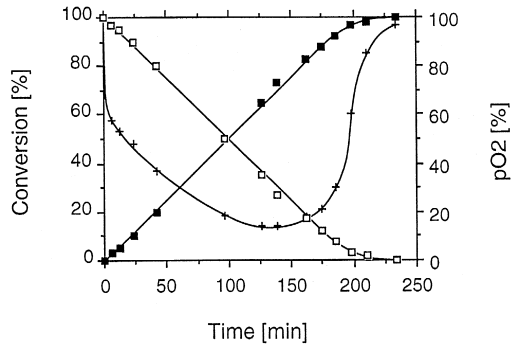

Fig. 3. Conversion of lactose to lactobionic acid by CDH. DCIP, which serves as the electron acceptor for this oxidation reaction, was continuously regenerated by laccase. Symbols as in Fig. 2. Reaction conditions: 200 mM lactose, 0.6 mM DCIP, 1.4 $U \cdot ml^{-1}$ CDH, 2.8 $U \cdot ml^{-1}$ laccase (*T. versicolor*), 7 $mg \cdot ml^{-1}$ polyethylene glycol 35,000 in 200 mM Na acetate buffer, pH 4.5, which was flushed with pure oxygen.

less than 4 h [max. productivity 23.4 $g \cdot (l \cdot h)^{-1}$, max. specific productivity 16.0 $g \cdot (h \cdot kU)^{-1}$]. Both enzymes were found to be very stable under operational conditions with $>95\%$ of the initially employed CDH and $>98\%$ of the laccase still active after complete substrate conversion. The total turnover number (TTN), defined as mol product formed per mol CDH employed, for this process was 2.3×10^5, while the TTN for the redox mediator was 330. The TTN for the enzyme is well comparable with data published for another flavoheme enzyme used in a biocatalytic process, in which the regeneration was done by electrochemical reactivation [17]. This process, however, was continuously operated and a similar mode of operation should greatly increase the efficiency with which the biocatalyst is utilized in the CDH-catalyzed process.

3.3. P2O-catalyzed oxidation of glucose with continuous regeneration of the redox mediator

P2O, which catalyzes the oxidation of several aldopyranoses at the position *C*-2 to yield the corresponding aldos-2-uloses (2-keto sugars, osones), is widely distributed among wood-degrading basidiomycete fungi. Recently, P2O has found increased use in carbohydrate chemistry as valuable biocatalysts for the synthesis of dicarbonyl sugar derivatives [12,30,31]. Typically, P2O is a rather large, homote-

trameric protein which contains covalently bound flavin adenine dinucleotide. The in vivo substrates of P2O are most probably glucose and xylose, which are abundant in lignocellulose and which are oxidized to D-*arabino*-hexos-2-ulose (2-ketoglucose) and D-*threo*-pentos-2-ulose (2-ketoxylose), respectively. During these oxidation reactions electrons are transferred to molecular oxygen and hydrogen peroxide is formed. In addition to oxygen, P2O also transfers electrons to a number of quinones and redox dyes (Leitner and Haltrich, unpublished results). Interestingly, the activity of P2O from *T. multicolor* with 1,4-benzoquinone is significantly higher than with oxygen (air) as is also expressed by some of the kinetic constants determined for this enzyme. The catalytic efficiency K_{cat}/K_m is more than six times higher for benzoquinone; furthermore, the K_m value is significantly lower for benzoquinone than for oxygen (120 and 650 μM, respectively). Because of these properties of the enzyme, the use of benzoquinone as cosubstrate should be favorable for biocatalytic purposes and should result in faster substrate conversion. Fig. 4 shows the P2O-catalyzed oxidation of glucose to 2-ketoglucose with either benzoquinone or oxygen as electron acceptor. When benzoquinone was used, it was again continuously regenerated by laccase, when using O_2 as the only cosubstrate catalase was added to remove H_2O_2 which can rapidly inactivate the biocatalyst [12]. The substrate glucose was completely oxidized after 60 min when using the system benzoquinone/laccase (Fig. 4A), whereas the reaction proceeded considerably slower when O_2 was the electron acceptor and was completed after 150 min (Fig. 4B). This longer reaction time is also reflected by the specific productivities determined for these two conversion experiments, which were calculated to be 72.0 and 28.8 $g \cdot (l \cdot h \cdot kU)^{-1}$, respectively. Residual P2O activity after complete substrate conversion was in the range of 80–85% for both reaction systems compared. The TTN determined for the enzyme was 1.1×10^6, while the TTN for the cosubstrate benzoquinone was found to be 800, indicating the efficiency of the novel enzymatic regeneration system for reduced electron acceptors.

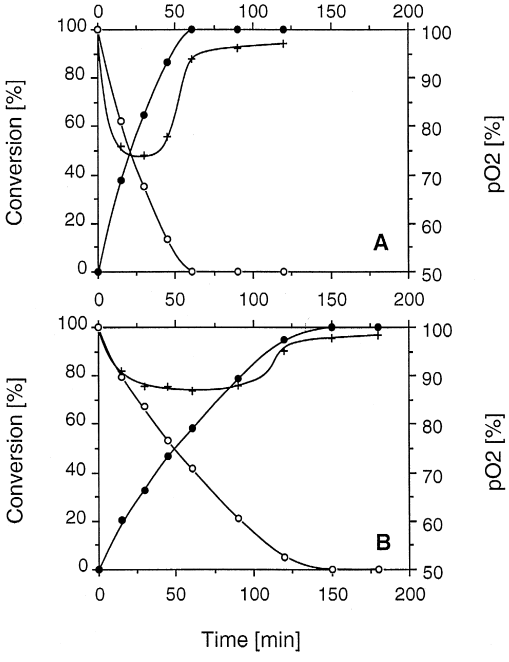

Fig. 4. Conversion of glucose into 2-ketoglucose by P2O. The electron acceptor for this oxidation reaction was (A) benzoquinone, which was continuously regenerated by laccase, and (B) oxygen. Symbols: ○, glucose; ●, 2-ketoglucose; +, dissolved oxygen tension. Reaction conditions: 400 mM glucose, 1.0 $U \cdot ml^{-1}$ P2O in Na citrate buffer (50 mM, pH 5.0), oxygenation with pure oxygen (0.9 vvm). (A) 0.5 mM hydroquinone, 10 $U \cdot ml^{-1}$ laccase (*T. versicolor*). (B) 10,000 $U \cdot ml^{-1}$ catalase.

4. Conclusions

In this study we describe a novel enzymatic method employing fungal laccases for the regeneration of electron acceptors used by various flavoenzymes in biotransformations. By using this enzymatic regeneration method, the electron acceptor (redox mediator) can be continuously reoxidized and hence has to be added in small, catalytic amounts only. Advantageously, the regenerative enzyme laccase transfers electrons to the terminal acceptor O_2, which is reduced to H_2O, therefore no additional product is formed which facilitates downstream processing. This novel regeneration system is widely applicable since laccases oxidize a very wide spectrum of different compounds that in their oxidized form can serve as electron acceptors for flavo- and quinoenzymes. Furthermore, laccases from different organisms can also widely vary with respect to their

properties such as substrate specificity, pH optima, stability, etc. They can therefore be selected for certain, specific applications depending on the requirements of the synthetic biocatalyst. Several laccases from diverse sources are commercially available by now. The compatibility of the complete system, i.e., synthetic enzyme, regenerative enzyme and redox mediator, can easily be tested in a simple screening procedure as described in this study. In contrast to electrochemical regeneration of redox mediators, no special equipment is needed when using the novel enzymatic regeneration method introduced in this study.

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