Bioresource Technology 101 (2010) 2076–2083

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Very stable silica-gel-bound laccase biocatalysts for the selective oxidation in continuous systems

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ARTICLE INFO

Article history:
Received 30 August 2009
Received in revised form 13 November 2009
Accepted 18 November 2009

Keywords:
Immobilization
Laccase
Silica-gel
Oxidation reaction
Indigo carmine decolorization

ABSTRACT

Cerrena unicolor laccase was immobilized by adsorption and covalent bonds formation on silica-gel carriers, functionalized with different organosilanes and surface densities. The effects of protein concentration, pH value of the coupling mixture and the enzyme purity on immobilization efficiency of the best carrier, moderately modified (0.75 mmol/g carrier) with 3-aminopropyltriethoxysilane were investigated. Activity of the best biocatalysts, expressed in ABTS oxidation, was 4028 U/mL of the carrier or 3530 U/mg of bound protein. Properties of immobilized laccase were determined to find excellent thermal stability improvement; $t_{1/2}$ for freely suspended enzyme was 2–3 min at 80 °C, whereas after immobilization over 100 min. Kinetic experiments in both batch and packed-bed reactors gave only four times lower k_{cat}/K_m value than for the native enzyme. A packed-bed reactor with silica-gel-bound laccase beads appeared to be very efficient in ABTS oxidation and its exceptional potentials were shown in the continuous decolorization of indigo carmine for 18 days without loss in activity. This system offers perfect ability to degrade recalcitrant dyes, but we can also envisage its use, with ABTS acting as a mediator, in regeneration of nicotinamide cofactors.

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1. Introduction

Oxidizing enzymes such as laccases (EC 1.10.3.2) attract considerable attention because they offer unique solutions to selective oxidation reactions in aqueous, organic and biphasic systems that have been extensively reviewed (Duran et al., 2002; Witayakran and Ragauskas, 2009). Laccases are of particular interest not only because they are easily available at scale, but because their copper containing redox sites catalyze oxidation of important substrates with concomitant reduction of molecular oxygen to two molecules of water, thus meeting the green chemistry criteria (Riva, 2006; Witayakran and Ragauskas, 2009). As the array of substrates for laccases includes alkenes, phenolics, polyamines and lignin-related molecules, and in the presence of mediators also the compounds with high redox potential, these enzymes can be used in bioremediation processes, as adjuvant in textile or paper industries, biosensing elements and also as catalysts in organic synthetic chemistry, in biotransformations of natural compounds and in chemo-enzymatic regeneration. of nicotinamide cofactors (Rodriguez Cauto and Toca Herrera, 2006; Champagne and Ramsay, 2007; Wang et al., 2008; Aksu et al., 2009; Arica et al., 2009; Liebminger et al., 2009; Mathew and Adlercreutz, 2009; Valls and Roncero, 2009; Witayakran and Ragauskas, 2009).

A prerequisite for the widespread use of laccase-based catalysts is stability of their properties, an ease of recovery and possibility of application in continuous operations. All these goals can be achieved by its efficient immobilization on solid supports. Duran et al. (2002) reviewed earlier works on laccase sources, methods of immobilization, useful supports and practical applications. Of various tested supports and immobilization methods the covalent attachment of the enzyme onto modified silica-gels appeared to afford the most efficient catalysts in dyes decolourisation (Peralta-Zamora et al., 2003; Zille et al., 2003; Mittal et al., 2006; Champagne and Ramsay, 2007; Rodriguez Cauto et al., 2007). They appeared to be especially suited for batch operations, due to very rapid adsorption of substrates onto silica-gels, followed by their notably slower enzymatic conversion into products (Peralta-Zamora et al., 2003; Zille et al., 2003). But for the effective continuous processes the prevalence of enzymatic activity over adsorption kinetics is essential and this, by far, could hardly be achieved (Champagne and Ramsay, 2007).

More recently several groups (Pierre, 2004; Kim et al., 2006), including our (Szymańska et al., 2007, 2009; Rekuć et al., 2009a) demonstrated exceptional properties of the mesoporous silica materials as enzymes supports to afford very active biocatalysts.

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0960-8524/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2009.11.077

But, those biocatalysts of a fine powder form perfect for application in slurry systems and hence batch processes, appeared not suited for packed-bed reactors and continuous processes (Rekuć et al., 2009a). Thus, the engineering of efficient laccase-based biocatalysts, suitable for continuous-mode operations, still remains a major challenge to date, and it was addressed in this work.

The efficient biocatalysts were obtained by using more conventional silica-gels, yet with the surface modified similarly as previously reported for the siliceous MCFs (Szymańska et al., 2007; Rekuć et al., 2009a). Amongst laccase sources we chose that from *Cerrena unicolor* strain since it can be produced without inducers and the filtered broth can directly be used for immobilization (Bryjak et al., 2007; Rekuć et al., 2008). Importantly enough, this laccase also shows the ability to degrade recalcitrant dyes without mediators (Michniewicz et al., 2008).

We used two types of silica-gel carriers with controlled pore sizes, the surface of which was modified to host amine or glycidyl groups in two surface densities. Properties of the catalysts were examined in detail in the ABTS oxidation, and practical potentials of the best sample were also confirmed in the continuous decolorization of indigo carmine. Whereas ABTS is well-known as a probe for laccase activity determination, its laccase-catalyzed oxidation may also be of considerable practical importance, inasmuch it is fundamental for the effective operation of laccase-mediator (ABTS) system recently devised for the chemo-enzymatic regeneration of oxidized nicotinamide cofactors, $NAD(P)^+$ (Aksu et al., 2009).

2. Methods

2.1. Materials

Trihydroxymethylaminomethane (tris), glutaraldehyde, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) sodium salt (ABTS), and indigo carmine were purchased from Sigma (USA) whereas 1-chloro-2,3-epoxypropane, chloroacetic acid, 3-aminopropyltriethoxysilane (APTS), 2-aminoethyl-3-aminopropylmethyldimethoxysilane (AEAPMDS), and 3-glicydoxypropyl-triethoxysilane (GPTS) were from Aldrich (USA). One silica-gel was a medium pore size Kieselgel 60 from Degussa, the other one was IE Int. Enzymes Ltd. small-porous type used in gas chromatography. Other reagents, all of analytical grade, were supplied by POCh (Poland).

2.2. Preparation of the carriers

The silica-gels surface was modified as described in details previously (Jarzębski et al., 2007; Szymańska et al., 2007). Before grafting carriers were contacted with water vapour and dried. Direct grafting of organosilanes (AEAPMDS in toluene or GPTS, APTS in hexane) to silanols present on silica surface was directed to obtain the load of amino or epoxy groups of 0.5 or 1.0 mmol/g of silica. Principal characteristics of carriers are given in Table 1. For a selected carrier the extend of surface modification was changed in the range of 0.25–2.00 mmol/g.

2.3. Laccase production and purification

The wood-rotting fungus *C. unicolor* (Bull.ex.Fr.) Murr No. 139, was obtained from the culture collection of the Department of Biochemistry, University of Lublin (Poland). Microorganism cultivation and laccase production was performed according to methods described earlier (Al-Adhami et al., 2002). The laccase containing culture fluid was separated from the mycelium by filtration on a steel sinter, was frosted and defrosted and then microfiltered (Amicon cell with MF-Millipore Membrane Filters GSWP 09000). The permeate was directly used for immobilization (crude preparation) or as a feed for subsequent concentration and diafiltration on the Labscale TFF System with Pellicon XL Device (PLC-10, Millipore). The retentate (laccase preparation) was precipitated with cold acetone (fluid:acetone – 1.0:1.5 v/v), centrifuged (10,000 rpm, – 2 °C, 20 min, Hettich 32R) and precipitate was dissolved in the buffer.

2.4. Enzyme activity

Laccase activity was determined by oxidation of ABTS (207 μM) in 0.1 M citrate–phosphate buffer (pH 5.3), at 30 °C and expressed in U/L. Absorbance (420 nm; ε = 36,000 1/M cm (Eggert et al., 1996)) was measured in time (spectrophotometer Helios α, Unicam) in initial reaction rate region. The enzyme activity unit (U) was defined as the amount of enzyme that required oxidizing 1 μmol of substrate per min. Protein concentration was determined spectrophotometrically by Lowry's method (Sigma procedure P 5656) using bovine serum albumin as a standard.

Immobilized enzyme (0.1–1.0 mL of the settled preparation), suspended in the buffer, was placed in a thermostated reactor and temperature was maintained at 30 °C. Then preheated ABTS solution was added (207 μM end substrate concentration) and several samples were withdrawn from the top of the reactor at 1 min intervals. After absorbance measurement, the sample was turned back into the reactor. Activity was calculated in U/mL of settled immobilized preparation.

2.5. Immobilization of laccase

Functionalized carrier (5 mL) was rinsed by sucking with distilled water and the buffer appropriate to the kind of anchor groups. After activation, which was done as described before (Szymańska et al., 2007), the carrier was suspended in crude or partly purified laccase solutions with protein concentration ranging from 0.202 to 4.06 mg/mL and pH 5.0, 5.8, 7.0 or 8.0. The excess protein was removed by washing and all the eluates were collected and analysed for the presence of protein and activity. The quantity of the bound activity units (expected activity) was calculated by subtracting activity units recovered in the eluates from the amount used for immobilization. Active groups residing on the carrier were blocked in 0.5 M tris–HCl buffer, pH 7.8, for 12 h at 4 °C. Finally, the preparation obtained was rinsed with 0.1 M citrate–phosphate buffer, pH 5.3 and activity was measured. The immobilized preparations were stored at 4 °C and they were washed with the buffer shortly prior to experiments.

2.6. Properties of laccase immobilized onto Z4 carrier

In all cases, control experiments using the native enzyme were carried out.

The effect of temperature on activity of the immobilized enzyme was determined by measuring activity of the preparation at 11–85 °C, pH 5.3. Thermal stability of the enzyme preparations was determined by incubating the samples at 13–85 °C for 1 h. After incubation the preparation was left at room temperature for 1 h and then its activity was measured (irreversible inactivation).

The optimum pH was evaluated in the range of 2.0–9.0 at 30 °C. The pH-stability was determined by incubating the sample at the given pH (ranging from 2.0 to 9.0) for 1 h. The pH of the solution was then adjusted to 5.3 and the preparation was left for 1 h to equilibrate and then its activity was measured (irreversible inactivation).

The effects of temperature and mixing rate on the initial reaction rate were measured at temperatures ranging from 12 to

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