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Enzymatic polymerization on the surface of functionalized cellulose fibers

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Abstract

Enzymatic coating of functionalized cellulose fibers with catechol was performed in the presence of *Trametes hirsuta* laccase. Cellulose functionalization was done by covalent fixation of aromatic amines onto the cellulose surface using a dyeing procedure with C.I. Reactive Black 5 (RB5) followed by reduction with sodium hydrosulfite. Cellulase enzymes were used on coated and control samples to obtain the analytes linked with the soluble sugars in solution, to prove the reaction concepts described in this paper. Hydrolyzed coated-cellulose showed lower concentration of reducing sugars (1188 mg/L) than control samples (2011 mg/L). The structures of these compounds were checked by LC/MS analysis confirming the presence of functionalized glucose and cellobiose units coupled to poly(catechol) molecules (*m/z* 580 and *m/z* 633). Alkali extraction method showed to be very promising to coat cellulose fibers with phenols in the presence of enzymes, at mild conditions of temperature and pH.

Keywords: Enzymes; Biopolymers; Radical polymerization; Coatings; Mass spectrometry

1. Introduction

The enzymatic coating of synthetic and natural materials has provided several advantages and a new strategy for obtaining useful polymers under mild reaction conditions with regard to temperature, pressure and pH without using toxic reagents [1–4]. Therefore, the enzymatic polymerization can be regarded as an environmentally friendly synthetic process of polymeric materials, providing a good example of "green polymer chemistry" [5].

In this type of coating processes, forming a covalently bound organic coating system (rather than merely physically) is preferred in order to improve coating adhesion and resistance to degradation [6]. Moreover, since the length of the chains for coating is usually directly related to the solubility and durability of the polymers, long chain and water resistant polymers would be desirable [7–9].

Recently, the potential of laccase enzymes for polymerizing, crosslinking and functionalizing various compounds was studied [10]. Thus, increasing interest has been focused on the application of this enzyme as a new biocatalyst in organic synthesis [11]. Laccases (EC 1.10.3.2) are a class of multi-copper-containing oxidoredutases enzymes produced by plants, fungi, insects and bacteria [12–14]. Laccases are able to catalyze the transformation of various aromatic compounds, specifically phenols and anilines, through an oxidative coupling reaction and of concomitantly reducing molecular oxygen to water. The oxidation of reduced substrate typically involves the formation of a free cation radical after the transfer of a single electron to laccase. The radical can further react on non-enzymatic oxidation [15,16]. The range of substrates that various laccases can catalyze is very wide. Basically any substrate with characteristics similar to a *p*-diphenol will be oxidized by laccases. The phenolic derivatives resulting in the production of polymeric aggregates are usually less soluble and much stable than their parent compounds [17–23]. These abilities of laccase for the synthesis of new compounds can be also used for chemical coating and modification of the fibers surface [24].

In this study, C.I. Reactive Black 5 was covalently attached onto cellulose fibers by a nucleophilic addition reaction and then chemically reduced to obtain aminic functional groups on the fiber surface [25]. Reactive Black 5 was chosen because is cheap and it is one of the most widely used dye in the textile industry [26]. Afterwards, the functionalized cellulose was coated "in situ" with enzimatically-synthesized poly(catechol) in the presence of *Trametes villosa* laccase. The enzymatic

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oxidation of catechol, a known laccase substrate, typically involves the formation of a free (cation) radical, which may undergo further non-enzymatic reactions resulting in colored dimeric, oligomeric, and polymeric molecules [18,27,28]. These reactions were confirmed by LC/MS analysis of the solutions obtained after short hydrolysis with cellulases of the coated cellulose fiber samples and their controls.

This enzymatic coating process, performed at lower temperatures and using less water, could be applied to several natural substrates and appears to be a promising environmental alternative technology [29].

2. Materials and methods

2.1. Materials

The cellulose fibers used in the experiments were alkaline scoured and bleached 100% cotton fabric. Laccase (EC 1.10.3.2) from *Trametes hirsuta* (5.6 mg protein/mL, 250 U/mL, supplied from Graz University of Technology, Austria) was applied at pH 5 (0.1 M Na–citrate buffer). Cellulase (Cellusoft L, 24.6 mg protein/mL) was purchased from Aquitex, Portugal. The non-ionic detergent Hostapal was purchased from Clariant. All other reagents and dyes were purchased from Sigma–Aldrich and used without further purification.

2.2. Cellulose functionalization

The covalent attachment of Reactive Black 5 (RB5) on cellulose fibers was performed in three consecutive steps at 40 °C in an AHIBA Spectradye apparatus (Datacolor) in a bath ratio of 1:20. In the first step (15 min), the fibers were treated with 0.1 g/L of RB5 and 50 g/L of NaCl solution. The second step (15 min) was the addition of 5 g/L of Na_2CO_3 in the same solution and the last step (75 min) was the addition of 1 mL/L of 10 M NaOH solution. Treated fibers were washed at the same liquor ratio with 2.5 g/L nonionic detergent Hostapal (Clariant) for 30 min at boiling temperature to remove the unfixed RB5, then washed and air dried. Afterwards, treated cellulose was reduced in a solution of 8 g/L of sodium hydrosulfite at 70 °C for 2 h in AHIBA Spectradye dyeing apparatus in order to obtain aminic functional groups on the cellulose surface. Functionalized fibers were washed with 2.5 g/L non-ionic detergent Hostapal for 30 min at boiling temperature, washed and air dried. White cellulose fibers, as control, were also treated at the same reductive conditions.

2.3. Enzymatic coating

The experiments were performed in an Ahiba Spectradye apparatus in 0.1 M Na–citrate buffer pH 5 in a final volume of 100 mL. Functionalized-cellulose coating was carried out with 2.5 U/mL *T. hirsuta* laccase in a 50 mL of 10 mM catechol solution at 50 °C for 2 h. Controls functionalized-cellulose and controls white-cellulose samples were performed in buffer, buffer/catechol and buffer/laccase solutions at the same operational conditions. After the treatment the fibers were thoroughly washed at boil for 2 h with non-ionic detergent Hostapal to remove unlinked polymers.

2.4. K/S measures

Color differences on the cellulose fibers were determined using a reflectance-measuring apparatus Spectraflash 600 (Datacolor) according to the CIELab color difference concept, at standard illuminant D65 (LAV/Spec. Excl., d/8, $D_{65}/10°$). The results were summarized by the overall color difference (ΔE^*) value [30].

2.5. Measure of reducing sugars

Coated-cellulose fiber samples were hydrolyzed in 30 g/L of cellulase solution (24.6 mg protein/mL in 0.1 M Na–citrate buffer pH 5) and incubated in a shaking bath at 50 °C for 4 days. Measure of reducing sugar was performed spectrophotometrically at 475 nm with Neocuproine-Cu method as previously described in reference [31].

2.6. LC/MS analysis

The analysis were performed on an Agilent MSD (Waldbronn, Germany) with direct injection using the following parameters: Drying gas temperature 350 °C, drying gas pressure 40 psi, drying gas flow (nitrogen) 10 L/min, capillary voltage 3500 V, fragmentor voltage 70 V, mass range 70–2000 *m/z*. The data were analyzed using the software Chemstation Rev. A 10.01 (Agilent, Waldbronn, Germany). For measurement in positive mode 0.01% acetic acid was added to the acetonitrile, which was used for the negative ion detection.

3. Results and discussion

A variety of chemical surface modification techniques have been developed to improve cellulose fiber–matrix adhesion [32,33]. Aminization was the first chemical modification performed on cellulose in order to increase its "dyeability" [25]. The aromatic amines were formed by chemical reduction on cellulose with covalent attached RB5 molecules. RB5 is a diazo vinyl sulphonic dye. The reduction on the two azo groups of the RB5 produces three compounds containing amino groups. One of these remains covalently linked to cellulose and the other two compounds containing amino groups being solubilized in water. The linkage of bifunctional reactive dyes (like RB5) to cotton cellulose was demonstrated by others: the digested cotton with cellulase enzymes and soluble reaction products were characterized by ^{13}C NMR [34,35]. Based on these results and in our LC/MS experiments, a model reaction was proposed. The reduction of the RB5 on cellulose is shown schematically in Fig. 1. During the reduction process, decolorization of the fibers was observed due to the azo bond disruption and consequent formation of the colorless amines. The functionalization was confirmed by LC/MS analysis. The LC/MS analyses were performed on the supernatants obtained after hydrolysis of the aminized cellulose by cellulase. Cellulases are enzymes which hydrolyze cellulose polymers to the smaller oligosaccharides, cellobiose and glucose [36,37]. The identified compounds are in majority products of the cellulose hydrolysis (Fig. 1). Compound **I** has been identified as one glucose molecule ($m/z = 180$) and compound **II** as one cellobiose molecule ($m/z = 342$) while compound **III** is a cellobiose molecule linked to the amine through an ether linkage ($m/z = 525$).

After confirmation of the cellulose functionalization, the fibers were reacted "in situ" with catechol and laccase. The amino groups on cellulose were coupled with poly(catechol) obtained through oxidation by laccase. The polymerization reaction involves continuous initiation and coupling steps during which the two kinds of radicals, the initially produced phenoxy radicals and subsequently formed phenyl radicals, co-exist and propagate simultaneously [38]. Generally, the enzymatically obtained polyphenols have structures normally composed of a mixture of phenylene and oxyphenylene units, which are formed by C–C and C–O coupling of phenols, respectively [20]. For the results of coupling, less soluble, high molecular weight compounds are formed [17].

At the same time, in the reaction medium, the coupling between the oxidized catechol and the amine take place [28,39].

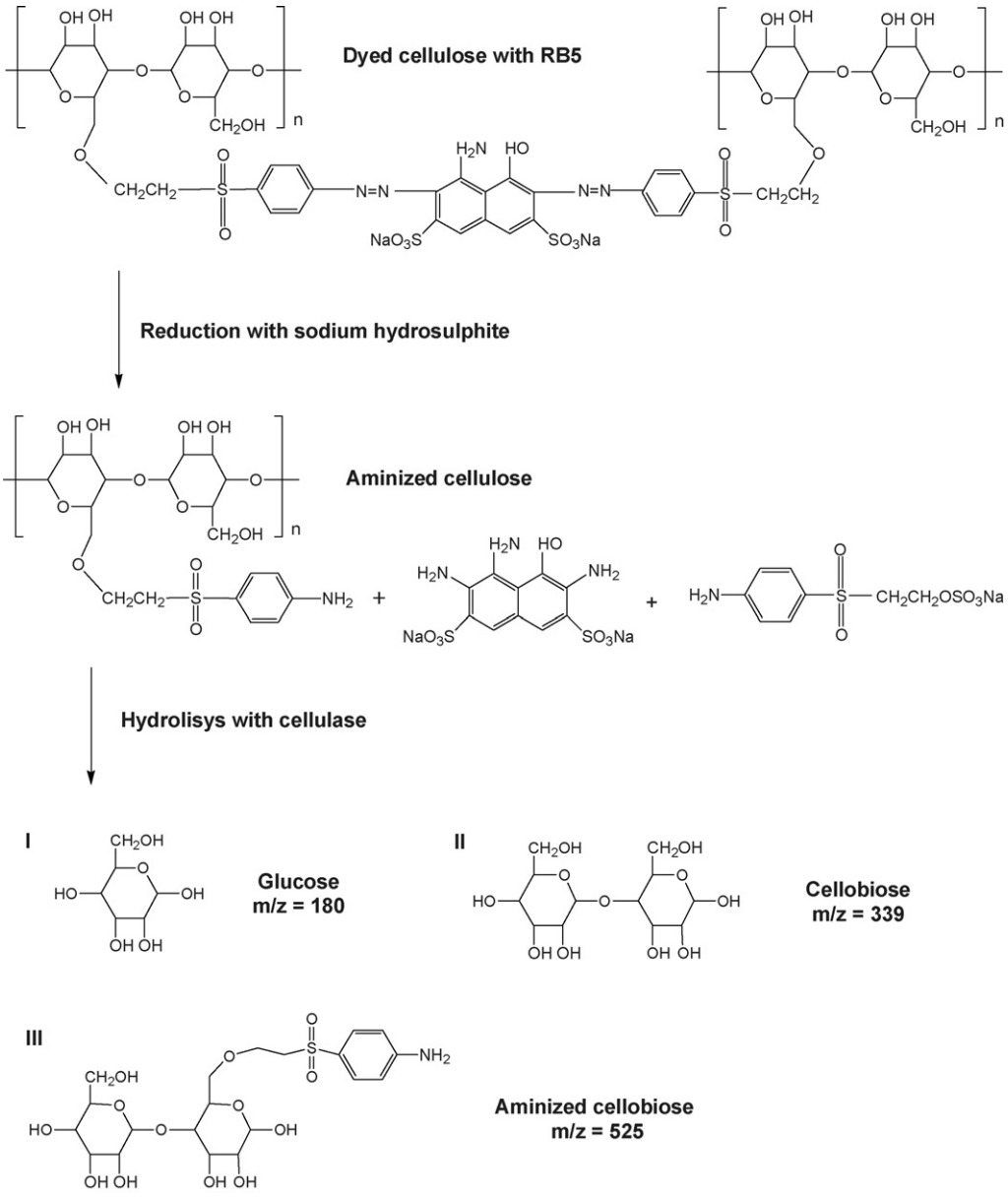

Fig. 1. Mechanism of chemical-reductive cellulose functionalization and identified mass spectra after cellulase hydrolysis by LC/MS analysis.

Niedermeyer et al. have previously demonstrated that employing laccase-catalyzed reactions, mono- and diaminated quinones can be synthesized in good to very good yields starting from *p*-dihydroxylated benzoic acid derivatives or alkylated hydroquinones and primary aromatic amines [40]. We also in a previously work have performed ^{13}C NMR experiments that confirmed the differences in the structures of the polymers obtained from the laccase-catalyzed processes between catechol and aromatic amine. The differences in peak positions between catechol, aromatic amine, and aromatic amine/catechol polymers allowed an interpretation of the inhibition effect of the catechol on aromatic amine self-coupling with the formation of a copolymer between the oxidized anilines and catechol from the simultaneous nonenzymatic coupling and enzymatic polymerization reactions [41]. The coupling between the aminized cellulose and the poly(catechol) is shown schematically in Fig. 2. The products obtained from the coupling processes of aminized cellulose with the products obtained from catechol oxidation by laccase were identified by LC/MS analysis (Fig. 2). As previously described, to provide liquid samples the coated cellulose fibers were hydrolyzed by cellulases. Due to the property of quinones to form stable structures by addition reactions with the radicals in the reaction environment, compound **IV** has been identified as the coupling reaction between a aminized glucose molecule and a quinone molecule ($m/z = 469$) [42,43].

Compound **V** has been identified as the product of the coupling reaction of a molecule of poly(catechol) ($n = 1$) with one aminized glucose molecule ($m/z = 580$) while, compound **VI** is obtained from coupling of one molecule of catechol ($n = 0$) with one cellobiose molecule ($m/z = 633$). In addition, another two compounds were identified, denominated as compound **VII** and compound **VIII**, which are a molecule of catechol ($m/z = 109$)

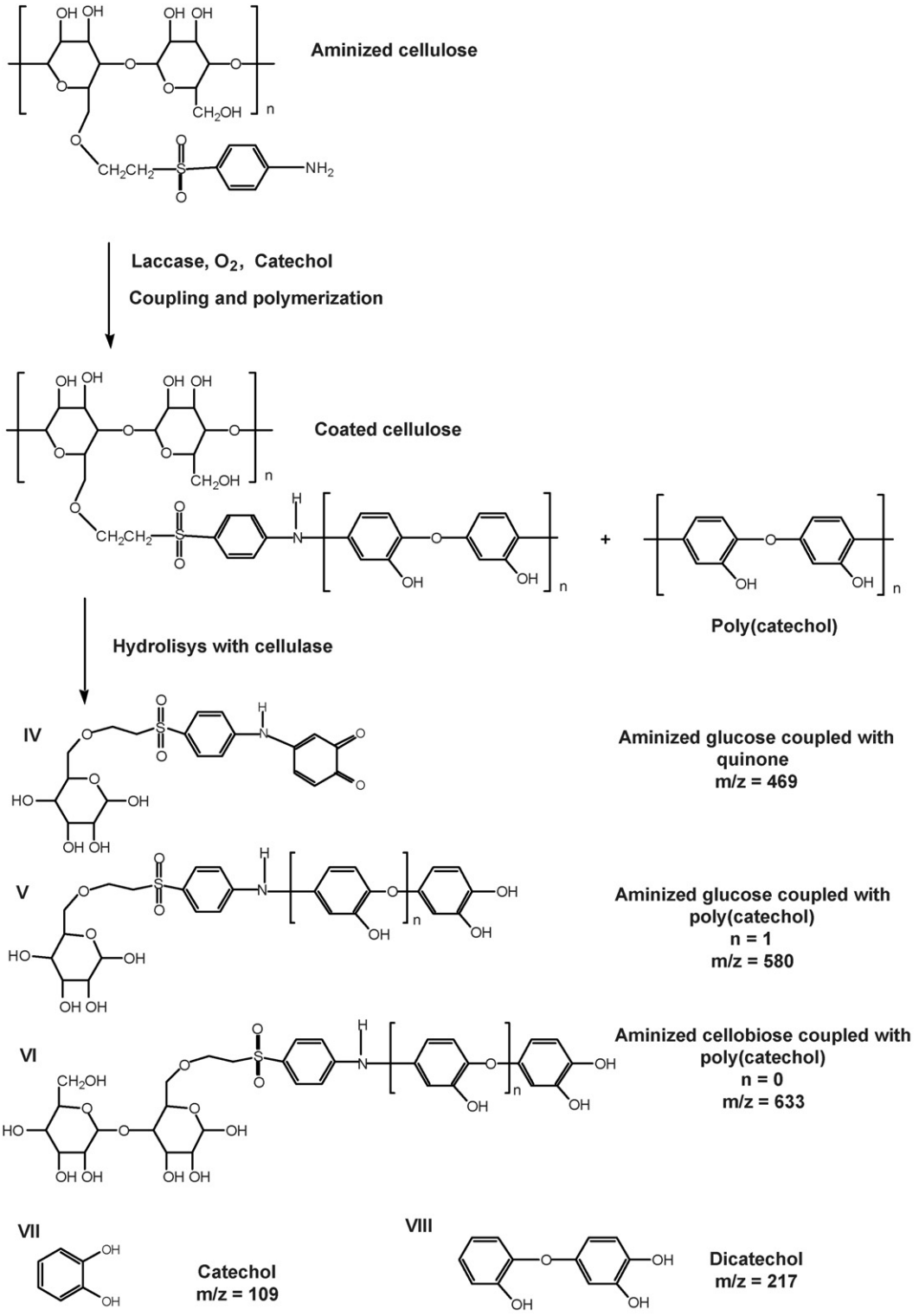

Fig. 2. Mechanism of coupling reaction between aminized cellulose and laccase-catalyzed poly(catechol) and identified mass spectra after cellulase hydrolysis by LC/MS analysis.

and a its dimer ($m/z = 217$), respectively. In accordance with literature, laccase catechol oxidation produces higher molecular weight poly(catechol) polymers than the observed ones in this study in the LC/MS analysis [44]. Those polymers cannot be seen on buffer solutions after cellulose hydrolysis by the simple fact that they are insoluble. High molecular weight poly(catechols) are soluble in 0.5 M NaOH yielding dark brown solutions [44].

White cellulose and cellulose with covalently linked RB5 were chemically reduced as previously described and treated with buffer, buffer/catechol, buffer/laccase and in a mixture of buffer/laccase/catechol. Afterwards, the color strength of the

Table 1
Qualitative degree of coating expressed as color difference (ΔE^*) values respect to the control treated in 0.1 M pH 5 Na–citrate buffer

	Catechol	Laccase	Laccase + catechol
Reduced white cellulose fibers	0.1	0.7	20.1
Reduced dyed cellulose fibers	4.5	5.9	47

fibers, in term of *K*/*S* values, was measured and compared to the buffer-treated sample. The results were summarized by the overall color difference (ΔE^*) value in order to provide a qualitative degree of enzymatic coating. The ΔE^* values in the laccase and catechol separately treated fibers showed slight differences compared to the buffer control, while the fibers treated with the catechol and laccase at the same time showed higher ΔE^* values (Table 1). The white cellulose fibers, even if not functionalized, when treated with the buffer/catechol/laccase solution showed high ΔE^* values providing evidence that the same oxidized poly(catechol) is able to link on the untreated fiber surfaces. However, coated functionalized fibers showed higher ΔE^* values ($47\Delta E^*$) than the coated white fibers ($20\Delta E^*$) confirming the high level of coupling sites on the functionalized-cellulose surface.

In addition, the amount of reducing sugars after cellulase hydrolysis of the fibers was also determined. Reducing sugars concentration values for white cellulose fibers and enzimatically-coated samples is reported in Table 2. No considerable differences among white cellulose treated in buffer solution, white cellulose treated in buffer/catechol/laccase solution and the functionalized cellulose treated in buffer (2.01, 1.98, and 1.91 g/L, respectively) were observed. On the other hand, the functionalized cellulose coated in buffer/catechol/laccase solution showed a significant difference, compared to the other fibers (1.19 g/L). Coated functionalized-cellulose provides high level of resistance to cellulase hydrolysis due to steric hindrance of the poly(catechol) molecules [45]. Cellulases generally have a catalytic domain and a cellulose binding domain (CBD). The catalytic domain has an active site in the shape of a tunnel or a cleft where the hydrolytic reaction takes place. However, the cellulose binding domain is essential for the degradation of crystalline cellulose and enhances the catalysis, increasing the concentration of the enzyme nearto the insoluble cellulose [46]. Therefore, the polymeric network formed on the fiber surfaces prevents an efficient interaction of the cellulase molecules with the cellulose surface through steric blocking of the catalytic domain and at the same time reducing the formation of the cellulose binding domain.

Table 2
Reducing sugar resulting from celulase treatment (see experimental part for details)

	Concentration of reducing sugar (g/L) ± S.D.
White cellulose fibers (buffer)	2.01 ± 0.04
White cellulose fibers (laccase + catechol)	1.98 ± 0.06
Aminized cellulose fibers (buffer)	1.91 ± 0.9
Aminized cellulose fibers (laccase + catechol)	1.19 ± 0.12

LC/MS analysis confirmed the cellulose fibers functionalization and the coupling between the enzymatically synthesized poly(catechol) and the aminized cellulose fibers. The colored coating of cellulose fibers showed significant color difference ($47\Delta E^*$ values) confirming the high level of coupling sites on the functionalized-cellulose surface. White untreated cellulose fibers were also enzimatically dyed with poly(catechol) but a lower degree of coating and lower resistance to hydrolysis were attained, this was confirmed by *K*/*S* and reducing sugars measurements. It can be concluded that the enzymatic coating carried out using aromatic amines covalently immobilized on cellulose fibers provides a new route for the generation of biocomposite materials. Enzymatic reaction occurs under mild conditions that completely preserve fiber structure. Extension of this method to other phenol polymers, using appropriate and cost-efficient functionalization methods, may provide a new route to environmentally friendly materials with predefined structures and properties.

References

[1] Kobayashi S, Higashimura H. Oxidative polymerization of phenols revisited. Prog Polym Sci 2003;28:1015–48.
[2] Gübitz GM, Cavaco-Paulo A. New substrates for reliable enzymes: Enzymatic modification of polymers. Curr Opin Biotechnol 2003;14:577–82.
[3] Zille A, Munteanu FD, Gubitz GM, Cavaco-Paulo A. Laccase kinetics of degradation and coupling reactions. J Mol Catal B: Enzym 2005;33:23–8.
[4] Distel KA, Zhu G, Wang P. Biocatalysis using an organic-soluble enzyme for the preparation of poly(lactic acid) in organic solvents. Bioresour Technol 2005;96:617–23.
[5] Kobayashi S, Uyama H, Kimura S. Enzymatic polymerization. Chem Rev 2001;101:3793–818.
[6] Foitzik RC, Kaynak A, Beckmann J, Pfeffer FM. Soluble poly-3-alkylpyrrole polymers on films and fabrics. Synth Met 2005;155:185–90.
[7] Marsh J, Scantlebury JD, Lyon SB. The effect of surface/primer treatments on the performance of alkyd coated steel. Corros Sci 2001;43:829–52.
[8] Abdelmouleh M, Boufi S, Belgacem MN, Duarte AP, Salah A, Gandini A. Modification of cellulosic fibres with functionalised silanes: development of surface properties. Int J Adhes Adhes 2004;24:43–54.
[9] Li Y, Cheng XY, Leung MY, Tsang J, Tao XM, Yuen MCW. A flexible strain sensor from polypyrrole-coated fabrics. Synth Met 2005;155:89–94.
[10] Grönqvist S, Suurnäkki A, Niku-Paavola ML, Kruus K, Buchert J, Viikari L. Lignocellulose processing with oxidative enzymes. ACS Symp Ser 2003;855:46–65.
[11] Mayer AM, Staples RC. Laccase: new functions for an old enzyme. Phytochemistry 2002;60:551–65.
[12] Claus H. Laccases: structure, reactions, distribution. Micron 2004;35:93–6.
[13] Gianfreda L, Xu F, Bollag J. Laccases: a useful group of oxidoreductive enzymes. Biorem J 1999;3:1–25.
[14] Chivukula M, Renganathan V. Phenolic azo dye oxidation by laccase from *Pyricularia oryzae*. App Environ Microbiol 1995;61:4374–7.
[15] Johannes C, Majcherczyk A. Laccase activity tests and laccase inhibitors. J Biotechnol 2000;78:193–9.
[16] Soares MB, Pessoa Amorim MT, Oliveira AM, Hrdina R, Ferreira M. Specificity of phenolic disazo dyes in relation to transformation by laccase. Enzyme Microb Technol 2002;30:607–12.
[17] Gianfreda L, Sannino F, Rao MA, Bollag JM. Oxidative transformation of phenols in aqueous mixtures. Water Res 2003;37:3205–15.
[18] Aktas N, Tanyolac A. Kinetics of laccase-catalyzed oxidative polymerization of catechol. J Mol Catal B: Enzym 2003;22:61–9.
[19] Ikeda R, Sugihara J, Uyama H, Kobayashi S. Enzymatic oxidative polymerization of 4-hydroxybenzoic acid derivatives to poly(phenylene oxide)s. Polym Int 1998;47:295–301.

[20] Mita N, Tawaki S, Uyama H, Kobayashi S. Laccase-catalyzed oxidative polymerization of phenols. Macromol Biosci 2003;3:253–7.
[21] Aktas N, Cicek H, Unal AT, Kibarer G, Kolankaya N, Tanyolac A. Reaction kinetics for laccase-catalyzed polymerization of 1-naphthol. Bioresour Technol 2001;80:29–36.
[22] Karamyshev AV, Shleev SV, Koroleva OV, Yaropolov AI, Sakharov IY. Laccase-catalyzed synthesis of conducting polyaniline. Enzyme Microb Technol 2003;33:556–64.
[23] Guresir M, Aktas N, Tanyolac A. Influence of reaction conditions on the rate of enzymic polymerization of pyrogallol using laccase. Process Biochem 2005;40:1175–82.
[24] Barfoed M, Kirk O, Salomn S. U.S. Patent 6,296,672;2001.
[25] Chhagani RR, Iyer V, Shenai VA. Modifying cotton for dyeing. Colourage 2000;47:27–32.
[26] Mohorcic M, Friedrich J, Pavko A. Decoloration of the diazo dye reactive black 5 by immobilised Bjerkundera adusta in a stirred tank bioreactor. Acta Chim Slov 2004;51:619–28.
[27] Pilz R, Hammer E, Schauer F, Kragl U. Laccase-catalysed synthesis of coupling products of phenolic substrates in different reactors. Appl Microbiol Biotechnol 2003;60:708–12.
[28] Anderson JS. The chemistry of hair colorants. J Soc Dyers Colour 2000;116:193–6.
[29] Tzanov T, Basto C, Guebitz GM, Cavaco-Paulo A. Laccases to improve the whiteness in a conventional: bleaching of cotton. Macromol Mater Eng 2003;288:807–10.
[30] Harold RW. Textiles: appearance analysis and shade sorting. Textile Chem Color 1987;19:23–31.
[31] Cavaco-Paulo A, Almeida L, Bishop D. Hydrolysis of cotton cellulose by engineered cellulases from *Trichoderma reesei*. Textile Res J 1998;68:273–80.
[32] Zhou Q, Greffe L, Baumann MJ, Malmstrom E, Teeri TT, Brumer III H. Use of xyloglucan as a molecular anchor for the elaboration of polymers from cellulose surfaces: a general route for the design of biocomposites. Macromolecules 2005;38:3547–9.
[33] Tsubokawa N, Iida T, Takayama TJ. Modification of cellulose powder surface by grafting of polymers with controlled molecular weight and narrow molecular weight distribution. Appl Polym Sci 2000;75:515–22.
[34] Meyer U, Muller S, Rys P. Enzymatic degradation and nuclear magnetic resonance spectroscopy as cornerstones in the analysis of dyeings with bifunctional reactive dyes. Textilveredlung 1991;26:80–6.
[35] Dilger P, Meyer U, Rys P. Fixing of bifunctional reactive dyes on cellulose [Fixierung von bifuntionellen reaktivfarbstoffen aut cellulose]. Textilveredlung 1998;33:14–21.
[36] Criquet S. Measurement and characterization of cellulase activity in sclerophyllous forest litter. J Microbial Methods 2002;50:165–73.
[37] Cao Y, Tan H. Effects of cellulase on the modification of cellulose. Carbohydr Res 2002;337:1291–6.
[38] Wu X, Liu W, Kumar J, Samuelson LA, Cholli AL. Effect of temperature on the enzymatic polymerization of 4-propylphenol: an in situ 1H-NMR study. J Macromol Sci 2003;40:1423–31.
[39] Thorn KA, Pettigrew PJ, Goldenberg WS, Weber E. Covalent binding of aniline to humic substances, 2. 15N NMR studies of nucleophilic addition reactions. J Environ Sci Technol 1996;30:2764–75.
[40] Niedermeyer THJ, Mikolasch A, Lalk M. Nuclear amination catalyzed by fungal laccases: reaction products of *p*-hydroquinones and primary aromatic amines. J Org Chem 2005;70:2002–8.
[41] Zille A, Gornacka B, Rehorek A, Cavaco-Paulo A. Degradation of azo dyes by *Trametes villosa* laccase over long periods of oxidative conditions. Appl Environ Microbiol 2005;71:6711–8.
[42] Thurston CF. The structure and function of fungal laccases. Microbiology 1994;140:19–26.
[43] Ulbricht J. Grundlagen der synthese von polymeren. Heidelberg, Germany: Hüthig and Wepf Verlag; 1992.
[44] Aktas N, Sahiner N, Kantoglu Ö, Salih B, Tanyolaç A. Biosynthesis and characterization of laccase catalyzed poly(catechol). J Polym Environ 2003;11:123–8.
[45] Offord DA, Soane DS, Ware Jr.W, Linford MR. U.S. Patent 6,617,268;2003.
[46] Cavaco–Paulo A, Morgado J, Juergen Andreaus J, Kilburn D. Interactions of cotton with CBD peptides. Enzyme Microbial Technol 1999;25:639–43.