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Immobilization of laccase and tyrosinase on untreated and plasma-treated cellulosic and polyamide membranes

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ABSTRACT

Laccase and tyrosinase were immobilized by adsorption and covalent attachment onto microfiltration membranes made of cellulosic and polyamide material. Amine, hydroxyl and carboxylic functional groups for covalent attachment were generated by plasma polymerization of allylamine, allyl alcohol and acrylic acid using mild plasma parameters. Mass analysis of the modified membranes, surface tension and FTIR-ATR spectra were used to show the presence of stable plasma polymer on the membrane surface. It was shown that untreated and plasma treated cellulosic membranes were unsuitable for laccase and tyrosinase immobilization. Both, immobilization of laccase onto polyamide membrane modified with $AlNH_2$ and adsorption on the untreated membrane at pH 5.2 gave satisfactory and comparable results with better operational stability in 10 consecutive batch processes for covalently bound enzyme. In the case of tyrosinase, adsorption of the enzyme on the untreated PA at pH 7.0 was as effective as covalent binding onto PA–$AlNH_2$ (in pH 7.0). Operational stability was tested in the presence of diphenolic substrate, which exhibits strong suicide inactivation towards the enzyme. It was shown that immobilized tyrosinase seems to be exceptionally stable in the presence of diphenolic substrate.

1. Introduction

Phenol oxidases are very appealing group of enzymes that catalyze reduction of dioxygen to water with production of substrate radicals, which are non-enzymatically converted to dimers, oligomers and polymers [1]. Increasing numbers of oxidative biotransformations of phenol oxidases, especially laccases (EC 1.10.3.2) and tyrosinases (EC 1.14.18.1), have been extensively reviewed [1–5]. They show fairly wide specificity to substrates such as alkenes, mono- and diphenols, aromatic amines, polyphenols, polyamines and lignin-related molecules. Although these enzymes are widespread in nature, mainly fungi laccases and mushroom tyrosinase have been employed in textile or stain bleaching, decolorization, bio-bleaching of pulp, detoxification of xenobiotics, synthesis of polymers and modification of biopolymers, chemical synthesis, bioremediation and biosensor production [1–9]. Although these processes were carried out in mild conditions (pH 6–8 and 20–30 °C) the biocatalyst instability and relatively high cost of the protein are the main obstacles in practical applications. Therefore, immobilization is very often applied in order to ease this limitation, as was summarized by Duran et al. [2,10]. Authors of the paper [10] reviewed methods of immobilization of laccase and tyrosinase as well as useful supports and practical applications. From various immobilization methods tested covalent attachment of the enzymes onto modified water insoluble polymers appeared to give the most efficient catalysts. More recently membranes seem to attract attention as supports in laccase and tyrosinase immobilization for affinity separation, construction of biosensors, or in processes with catalytic membranes [6,11–15]. The membranes usually have to be modified by introducing the functional groups that make possible covalent bonding with proteins. Because of variety of applied supports, enzymes, immobilization and analytical methods, the comparison of the results of published works is very difficult.

In order to be able to select an adequate immobilization method for a particular application, it would be necessary to have a broad database covering the performance of different immobilized preparations [16]. To fulfill this requirement a systematic study of laccase and tyrosinase immobilization was started. First, as a support, the aminated poly(ethylene terephthalate) films were taken [17]. In most of the aminolysis conditions, however, serious degradation of the modified films was observed, resulting in decrease of their mechanical properties. In this study the plasma polymerization technique was used to introduce the functional groups on the polymer surface. In contrast to chemical modification, plasma-treated material is altered only on the surface with no changes introduced to the bulk properties. This paper describes the results of studies on the covalent and adsorptive immobilization of *Cerrena unicolor* laccase and *Agaricus bisporus* tyrosinase onto cellulose- and polyamide-based membranes. Membranes were surface-modified by plasma polymerization of allyl alcohol

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(AlOH), allylamine ($AlNH_2$) and acrylic acid (AAc) in mild plasma conditions that make possible partial retention of the monomer structure used in the deposited layer. The main goal of the study was to learn about the relative preferences of laccase and tyrosinase towards the same membrane, but modified with different polymers and activators and towards both membranes but modified in the same way.

Carrying out enzymatic reactions in organic solvents, covalent immobilization is often unnecessary, as the adsorbed enzymes are unable to desorb from the carrier surface in non-aqueous media. Thus, simple adsorption was used for control experiments. Particular attention was given to operational stability of the immobilized preparations that were checked in the consecutive batch operations.

2. Materials and methods

2.1. Materials

Disc membranes (19.63 cm^2) composed of cellulose acetate (11107-50-N) and poly(amide) (25007-50-N) with 0.2 μm pore size were purchased from Sartorius (Germany). Cellulose acetate membranes were purified from glycerin by extraction in distilled water (with stirring and changing the water a few times) for 30 h. Allylamine (Sigma-Aldrich), allyl alcohol (Loba Chemie) and acrylic acid (Fluka) were used as received. Trihydroxymethylaminomethane (tris), glutaraldehyde (GA), divinyl sulfone (DVS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (CDI), protein assay kit (P 5656), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) sodium salt (ABTS), L-3-(3,4-dihydroxyphenyl)alanine (L-DOPA) were purchased from Sigma (USA). Other reagents, all of analytical grade, were supplied by POCh (Poland).

2.2. Plasma treatment

A plasma generator of 75 kHz frequency (Dora Power System, Wroclaw, Poland) was used throughout the study. The weighed membrane (with accuracy of 10^{-5} g) was attached to the lower electrode and the system was evacuated to reach a vacuum of 0.02 Torr. Then argon was introduced to the reactor to reach the pressure 0.06 Torr and then the monomer was introduced. The distance between the electrodes was 28 mm. The parameters of plasma polymerization of the chosen compounds are shown in Table 1. After the plasma membranes were weighed again, the plasma polymerization yield was calculated as an increase of membrane weight. The data in Table 2 are average values of at least 8 measurements.

2.3. Analysis of the membranes after plasma treatment

Fourier Transform Infrared Spectroscopy (FTIR) spectra were obtained by the total reflection technique (ATR), using a Perkin-Elmer System 2000 spectrometer with horizontal ATR device (Ge, 45°). 64 scans were taken with 4 cm^{-1} resolution.

Dynamic contact angles of water and diiodomethane were measured with the help of a goniometer PG-X (FibroSystem AB). Average values (from 10 to 15 droplets) of contact angle at time 0 were assumed to be equal to static ones. From these values the surface tension and its polar and dispersive components were calculated according to harmonic averaging [18]. Data of surface tension for testing liquids were taken from work of Kuznietsov [19]. Sample polarity was defined as the ratio of the polar component of surface tension to the total value of surface tension given in percent.

A home-made dead-end cell with a membrane filtration surface area of 11.64 cm^2 was used to measure the water flow through the membrane. The measurements were made under a pressure of 100 cm^3 of water, without outer pressure. Before the measurement, the dry membranes were hydrophilized by immersion in ethyl alcohol for 0.5 h then in an aqueous solution of ethyl alcohol (1:1) for 1 h.

Table 1
Parameters of plasma polymerization of chosen monomers.

Parameter	Allyl alcohol	Allylamine	Acrylic acid
Plasma power [W]	14–20	18–22	18–22
Total p [Torr]	0.5–0.6	0.2	0.2
Treatment time [min]	3	4	3

Table 2
Mass increase and permeate flux of cellulose and polyamide membranes functionalized by allyl alcohol (AlOH), allylamine ($AlNH_2$) and acrylic acid (AAc) plasma.

Membrane	Mass increase [μg·cm^{-2}]			Water flux [dm^3 m^{-2} h^{-1}]			
	AlOH	$AlNH_2$	AAc	-	AlOH	$AlNH_2$	AAc
Cellulose	61.5 ± 3.2	11.2 ± 0.8	72.9 ± 3.5	108.2	95.5	112.4	152.5
Poly(amide)	31.5 ± 2.9	12.4 ± 0.8	60.4 ± 3.4	55.9	70.8	94.0	123.5

2.4. Enzymes

The wood-rotting fungus *Cerrena 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 were performed according to a method described earlier [20]. The laccase containing culture fluid was separated from mycelium by filtration. An averaged activity of the crude preparation was 550 000 U cm^{-3}.

The fresh mushrooms for tyrosinase isolation were obtained from a local producer (W. Idasiak, Wrocław, Poland). Tyrosinase preparation was done according to [21]. Briefly, young and fresh mushrooms (300 g) were cut into small pieces, homogenized for 1–2 min in 550 mL of cold acetone (−26 °C), mixed at 0–4 °C for 30 min and then centrifuged at 7000 rpm for 20 min. The pulp was suspended in 200 mL of the buffer and put under a vacuum pump to remove the remaining acetone. The suspension was centrifuged at 10 000 rpm for 20 min and the supernatant was used as a crude preparation. An averaged activity of the crude preparation was 16 402 U cm^{-3}.

2.5. Activity assay

Laccase activity was determined by oxidation of ABTS (0.21 mM) in 0.1 M citrate-phosphate buffer (pH 5.3), at 30 °C [22]. Tyrosinase activity was determined in 0.1 M phosphate Buffet (pH 7.0) containing L-DOPA (1.0 mM), at 30 °C [17]. The absorbance of 420 nm (laccase) or 475 nm (tyrosinase) was measured against time (spectrophotometer Helios α, Unicam) in the region of the initial reaction rate. The enzyme activity unit (U) was defined as the amount of enzyme required for oxidizing substrates to colored products and increases of absorbance by 0.001 per min at 30 °C. The mean analytical error for both enzymes was less than ± 2.8%.

The activity of the immobilized enzyme was measured in an efficiently stirred (200 rpm) reactor at 30 °C in a batch regime. The enzyme immobilized on the membrane (two discs for laccase or four discs for tyrosinase) in the appropriate buffer was placed in the reactor, the temperature of which was maintained at 30 °C. Then, preheated ABTS (0.21 mM end substrate concentration) or L-DOPA (1 mM) in the buffer was added and several samples were taken from the reactor at one-minute intervals. After measurement of the absorbance, the sample was returned to the reactor. Activity was calculated from linear dependence of absorbance versus time and recalculated per 1 cm^2 of both sides of the membranes. All the