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Nylon 6 film and nanofiber carriers: Preparation and laccase immobilization performance

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ABSTRACT

Enzyme immobilization has attracted continuous attention in the field of fine chemistry, biomedicine and biosensor. Polyamides are promising materials to promote immobilization: thus, nylon 6 film and nanofiber carriers (prepared by electrospinning method) have been investigated. The enzyme (i.e. laccase from *Trametes versicolor*) was covalently immobilized onto spacer-arm attached carriers after acidic hydrolysis. The amount of immobilized enzyme on the nylon film and nanofibers was 59.4% and 71.0% respectively. The maximum activity (V_{max}) and Michaelis–Menten constant (K_m) of laccase immobilized on functionalized carriers were determined. The operational and thermal stabilities of the immobilized laccase were improved compared to free counterpart. The use of nylon carriers for enzyme immobilization has shown interesting properties to be used as biocatalytic material in industrial applications. Furthermore, nylon carriers are cheap and could be produced at large scale.

1. Introduction

Enzyme immobilization is advantageous for commercial application due to convenience in handling, ease of enzyme separation from the reaction mixture, reuse and a possible increase in enzyme thermal and pH stability. An important requirement for protein immobilization is that the matrix should provide a biocompatible and inert environment, i.e. it should not interfere with the native structure of the protein, which thereby could compromise its biological activity [1]. Poor biocatalytic efficiency of immobilized enzymes, however, often limits the development of large-scale bioprocessing to compete with traditional synthetic chemical processes. The effect of immobilization, including the performance of immobilized enzymes, strongly depends on the properties of supports. Improvements of biocatalytic efficiency can be achieved by manipulating the structure of carrier materials [2–4].

In recent decades, nanostructured materials have attracted attention due to their unique properties and interesting applications [5]. They stand out of other supports because of their extremely high surface area-to-volume ratios, which can provide large specific areas for an efficient immobilization as well as stabilization of enzymes. In this context, nanofibers represent excellent supports as: (i) a great variety of polymers can be electrospunned and meet different requirements for support applications, (ii) the high porosity and the interconnectivity endow them with a low hindrance for mass transfer and (iii) the nanofiber surface can be modified accordingly to the functional groups present on the enzyme surface to enhance the amount of supported enzyme [6].

Recently, electrospinning has emerged as a novel tool to prepare fibers and membranes in a cheap, fast and simple way [7,8]. Electrospinning is a highly versatile method to process solutions or melts, mainly of polymers, into continuous fiber with diameters ranging from a few micrometers to a few nanometers [9]. A high electric field is applied to the droplet of a fluid which may be a melt or solution coming out from the tip of a die, which acts as one of the electrodes. This leads to the droplet deformation and finally to the ejection of a charged jet from the tip of the cone accelerating towards the counter electrode leading to the formation of continuous fibers [9]. A straightforward way to increase electrospinning throughout is the usage of multispinneret devices. Among them, needleless device are the most promising technology since it can assure minimal dependency on the fluidic channel numbers (jet repulsions may affect the process) [10]. When magnetic field is applied to the system, several jets are ejected from a rotating roller [11].

In electrospinning, numerous parameters have been identified as affecting the final properties of the electrospun fiber. These parameters are divided into three broad categories [12]: (i) properties of the solution used as the feedstock (solution parameters), (ii) parameters associated with the design, geometry and operation of the electrospinning apparatus (processing parameters) and (iii) atmospheric and other local processing conditions (environmental parameters).

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With respect to continuous films, nanofibers promise low barrier to diffusion, high surface available, a selective barrier from interferences, and improved operational and storage stabilities [7,13]. The porous morphology of the nanofibers allows the analytes to diffuse toward the surface, while the proteins may be retained by physical or chemical bonds within its large surface available [7].

Immobilized enzymes have been used in antibiotic production, drug metabolism, food industry and bioremediation [14–17]. Laccases (EC 1.10.3.2) belong to the polyphenols oxidase family which are able to catalyze the one-electron oxidation of a wide variety of organic compounds, including mono-, di- and polyphenols, aminophenols, methoxyphenols, aromatic amines with the concomitant four-electron reduction of oxygen to water [18–21]. In this work the fungal laccase from *Trametes versicolor* has been immobilized onto produced nylon film and nanofiber carriers. The effect of the morphology of the different supports on the enzyme loading and the activity and stability of the enzyme have been analyzed and discussed. Advantages on the use of supported enzyme in respect to the free form have been highlighted.

2. Experimental

2.1. Materials

ABTS (2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)), nylon 6 pellets and glutaraldehyde were purchased from Sigma–Aldrich Srl (Milan, Italy). Milli-Q water (Millipore, Milford, MA, USA) was used throughout the experiments. Laccase from *T. versicolor* (30.6 U/mg of protein) was purchased by Fluka Chemika (Buchs, Switzerland) and formic acid (85%) was purchased by Carlo Erba (Pisa, Italy).

2.2. Nylon film and nanofiber carriers production

Nylon 6 or polycaprolactam (NY6, Sigma–Aldrich) is a polymer which reproduces the properties of nylon 6,6. Unlike most other nylons, nylon 6 is not a condensation polymer but is formed by ring-opening polymerization. Nylon 6 fibers are tough, possessing high tensile strength, as well as elasticity and lustre. They are wrinkle-proof and highly resistant to abrasion and chemicals such as acids and alkalis. The fibers can absorb up to 2.4% of water, although this decreases its tensile strength. Nylon 6 is used as thread in bristles for toothbrushes, surgical sutures, and strings for acoustic and classical musical instruments. It is also used in the manufacture of a large variety of threads, ropes, filaments, nets, and tire cords, as well as hosiery and knitted garments [22].

Elmarco's NanospiderTM Production Line NS LAB200 based on needle-free electrospinning process has been used for the production of the nanofibrous layer. A polymer solution was prepared at room temperature by dissolving nylon 6 pellets in formic acid (18% w/v). The polymer solution was electrospun with an electric field strength of 55 kV. Fibers were collected on polyethylene terephthalate (PET) nonwoven support. Optimal conditions for the production of a homogeneous nanofibrous layer are listed in Table 1.

In order to verify the effectiveness of the nanostructures in comparison to planar immobilization carriers a thin nylon 6 film has been casted onto the same support. The film was produced from 25% w/v polymer in formic acid. The solution was employed in spin casting at 1000–7000 rpm for 30 s. Finally, the film was oven dried.

Superficial area of the different carriers has been calculated considering the volume for an Amberlite bead ($\approx$0.22 mm^3) as reference value. Thus for nylon 6 film and nanofiber carriers the superficial areas have been calculated according to the following assumptions:

- nylon film: a 0.22 mm^3 parallelepiped with a square base and a thickness of 55 μm (measured by scanning electron microscope (SEM)) has been considered;
- nylon nanofiber: theoretical amount of nanofibers (same volume as for nylon film and nanofiber diameter determined by SEM analysis) has been calculated assuming their alignment in the investigated volume. Moreover, 40% (overestimated, according to SEM analysis by using software IMAGEJ) of the total surface of the nanofibers has not been considered available due to the nanofibers overlapping.

2.3. Morphological measurements

The morphological aspects of the different structures (nanofibers; film; microfibrous layer) and the diameters of the nanofibers were investigated by Scanning Electron Microscope (SEM; Phenom G2 pure desktop apparatus) working in the magnification range 20–17,000×.

2.4. FT-IR characterization

The chemical characterization of the realised polymeric structures, before and after the immobilization procedure, have been analyzed by means of ATR-FTIR spectroscopy (Perkin Elmer Spectrum One spectrometer in HATR reflection mode using a zinc selenide crystal) at a resolution of 4 cm^{-1}.

2.5. Pore size analysis by means of capillary flow porometry

The sample is soaked in a wetting liquid that spontaneously fills the pores. Gas pressure from a non-reacting gas is applied and gradually increased until the pores are cleared and gas can flow through the sample [23]. The pore diameter can be calculated from the differential pressure needed to empty a pore by the following equation:

$$p = \frac{4\gamma\cos\theta}{D} \quad (1)$$

where: p = differential pressure on the sample; γ = surface tension of the wetting liquid; θ = contact angle of the wetting liquid on the sample; D = pore diameter.

2.6. Laccase immobilization on nylon carriers

A surface of 24 mm^2 (3 × 8 mm) for nylon film and nanofiber carriers was used for the laccase immobilization protocol [22].

Nylon was first incubated for 2 h at 25 °C in HCl 3 M for the hydrolytic activation step. Hydrolyzed nylon was rinsed out with sodium acetate buffer 1 mM at pH 4.5 and reacted with 2–5% (v/v) solution of glutaraldehyde in sodium acetate buffer and kept for 20 min incubation time at room temperature. Then, glutaraldehyde-modified nylon was washed off with the sodium acetate buffer. Later, different amounts of enzyme (2–6 mg) were added to the activated nylon, and the coupling reaction was tested for different contact times at room temperature. Finally, the unbound enzyme was rinsed out with the sodium acetate buffer. The washing sodium acetate buffer was kept for activity measurement. Nylon immobilized enzyme was stored in semi-dry condition at 4 °C, without any buffer. The loading enzyme capacity was also tested on nylon nanofiber carrier without the hydrolytic activation step.

To determine the optimum immobilization conditions, the following parameters were analyzed: (a) glutaraldehyde