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Enzymatic behavior of laccase following interaction with γ-CD and immobilization into PCL nanofibers

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A B S T R A C T

This study examines the effects of CD use on enzymatic activity, following enzyme immobilization into nanofibers. There is almost no research available on the change in enzyme activity following interaction with cyclodextrin and electrospun nanofiber mats together. Laccase enzyme was immobilized into nanofibrous structures by various techniques, with and without γ-CD addition, and the enzymatic activity of the laccase was analyzed. SEM, XRD, and FTIR analyses were used for the characterization of the resulting structures. Our results showed that cyclodextrin use has a positive effect on the enzyme's activity, and increases its stability. The enzymes treated by cyclodextrin showed activation after complex formation trials, and no activation loss or enzyme denaturation was detected. Our conclusions were supported by the enzyme activity test results, which also showed that immobilization by encapsulation methods gave better activity results than layering methods. Another important finding concerned the laccase's stable characteristics that helped to maintain its enzyme activation after the freeze drying process. Among all test groups, the best activity result was recorded by laccase-γ-CD complex encapsulated PCL nanofibers with 96.48 U/mg.

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Introduction

Enzymes, as biological components, are preferred as catalysts in several different fields, due to their high selectivity and activation characteristics [1]. Enzymes used as bio-catalysts have opened a door to the use of eco-friendly, green, and sustainable processes in the field of synthetic chemistry, and thereby obtaining results by using natural production methods has been possible [2]. Since enzymes are unaffected by the reactions that they catalyze and are very expensive compounds, in order to enable their reuse and avoid undesirable mixtures with the post-production products, immobilization processes should be carried out [3]. Immobilization offers inherent advantages including: convenient handling, continuous processing, cost effectiveness, efficient recovery and reuse of the biocatalysts, and elimination of product contamination [4–6]. In the immobilization process, diverse materials are used as structures (substrates) for enzyme attachment, i.e., inorganic substances, organic polymers, hydrogels, etc. The selection of the required structure is performed by considering parameters such as mechanical strength, chemical and physical stability, maximum carrying capacity of the enzymes, and leakage behavior [7]. It is accepted by some researchers that one of the best immobilization techniques are the encapsulation of enzymes by a sol gel process or an electrospinning process. Although encapsulation may have steric problems for the entrance of the substrate and may have strong diffusion problems, encapsulation by electrospinning process presents remarkable results such as substantially reduced damage to the structure and the activity loss [8–11].

Electrospinning is a common and versatile method for nanofiber spinning from pure polymer solutions, polymer mixtures, or blends; it can produce fibers from the nanometer level to the micron level. Nanofibers produced by electrospinning are formed by the creation of polymer droplets, followed by the whipping, stretching, and thinning of the viscoelastic liquid under the application of a high electrical voltage; the fibers form after the electrical field surpasses the surface energy of the droplet. The fibers have an extremely thin diameter and accumulate randomly over a grounded collector plate in a continuous manner, creating nanofibrous nonwoven mesh layers [12,13]. There are many factors that affect

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the quality of the produced nanofiber layers: parameters related to the polymer solutions (viscosity, surface tension, and electrical conductivity), production parameters (applied voltage, distance between tip and collector plate, feed rate), and environmental parameters (temperature and humidity) [14,15]. In general, the properties of nanofibers, such as high porosity, large surface area to volume ratio, good mechanical strength, and small fiber diameter, make them ideal for several application fields, including biocatalysis, tissue engineering, technical textiles, filtration, and strengthened composites [16–18]. In particular, nanofibrous mats with interconnected porous structure, fairly small fiber size and easy production method are very appealing for large scale enzyme catalyzed industrial applications. This is mainly because of high ratio enzyme attachment onto nanofibers which increase efficiency of the reaction and eliminated diffusion problems due to interconnected porous structure [19].

In recent years, cyclodextrins have been proposed as alternative support for enzyme immobilization processes [20]. Cyclodextrins (CDs) offer functional solutions by creating complex structures (e.g., inclusion complexes, ICs) that have unique properties, e.g., nontoxic natures, improved solubilities, and reduced undesirable odor. Due to their superior properties, they can be used in several industrial fields, such as foods, pharmaceuticals, cosmetics, environmental protection, and textiles [21].

Studies, as yet at an early stage, examining the interactions between enzymes and CDs, have not yet examined the subject of enzyme immobilization on CDs contained within nanofiber structures. This study examines the effects of CD use on enzymatic activity, following enzyme immobilization into nanofibers. Analysis was conducted on enzyme stability, enzyme activity, and the reaction performance potential of enzymes. The well-known and broadly used laccase enzyme, which belongs to the phenol oxidase group [22,23] was chosen as a reference material for this study. Laccase catalyzes the oxidation of a broad range of substrates, and is found in various species, mostly in a variety of plants and mushrooms. The laccase enzyme was supplied to us in the form of a solid powder; we attempted to immobilize it into PCL nanofibers in the presence of γ-CD, which possesses a large cavity. Enzyme activation tests and characterization of the structures were then performed.

Materials and methods

Materials

The following materials were used in this study: poly(ε-caprolactone) (PCL) (Mn 80,000), laccase (Trametes versicolor, ≥10 U/mg), dimethyl formamide (DMF), chloroform, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), acetic acid, sodium acetate, and potassium bromide (KBr) were supplied by Sigma Aldrich. Gamma cyclodextrin (γ-CD)(Cavamax brand) was supplied by Wacker Chemie AG.

Method

Enzyme immobilization

Laccase enzyme was supplied to us in the form of a powder, and we tried to immobilize it into nanofiber structures by different techniques. In the first technique, 60 mg pure laccase enzyme was added to a PCL polymer solution, which had been previously prepared by dissolving PCL granules in a solvent mixture consisting of 30% DMF and 70% Chloroform. The mixture was magnetically stirred at room temperature until a homogenous solution was achieved. The laccase was then added, and the PCL polymer solution was mixed again, and the prepared mixture was electrospun in order to generate enzymes encapsulated by nanofiber meshes. In the second technique, 30 mg laccase enzyme and 30 mg γ-CD were added to the previously prepared PCL polymer solution, and the resulting laccase-γ-CD physical mixture was encapsulated into the nanofiber structure by electrospinning of the mixture solution. As another alternative technique, 5 mg purified laccase enzyme and a physical mixture of 5 mg laccase enzyme and 5 mg γ-CD were separately added (sandwiched) between nanofibrous layers (two layers of nanofibrous mats). In the last technique, it was intended to produce laccase-γ-CD complex, and then immobilize it into nanofibrous structures by both encapsulation (electrospinning of laccase-γ-CD complex containing PCL polymer solution) and entrapment (introducing or sandwiching laccase-γ-CD complex into nanofiber layers) methods. In the production of laccase-γ-CD complex, freeze drying was applied. For that purpose, 0.3 g laccase and 3 g γ-CD were added to 20 ml distilled water and mixed on the stirrer plate for two days until a turbid solution was obtained. All the produced structures were characterized by appropriate methods (SEM, FTIR, and XRD), and enzyme stabilization, enzyme activity and its reuse properties were tested.

Nanofiber production by electrospinning

The distance between the tip to the collector plate during the nanofiber production was kept at 15 cm, while the feeding rate was adjusted to 0.5 ml/h, and the applied voltage was chosen as 12.5 kV. Nanofiber production for encapsulation purposes was continued for approximately 1 h 30 min in each case. On the other hand, nanofiber production for layering purpose was carried out for the time required to ensure the production of durable top and bottom nanofiber mat layers, without blocking the enzyme's diffusion.

Enzyme activity test

For the determination of enzyme activity, 50 mM, pH 4.5 acetate buffer, prepared from acetic acid and sodium acetate, was used for maintaining the medium at a constant pH. As a substrate, 5 mM ABTS (Sigma Aldrich) was used. Acetate buffer was used as a blank sample. ABTS gives a maximum absorbance at 420 nm, thus spectrophotometric measurements were conducted at a wavelength of 420 nm. Samples containing laccase enzyme were added to the reactive test tubes, and absorbance measurements of the solutions inside the tubes were carried out spectrophotometrically at various time intervals. UV–Vis spectrophotometer (Shimadzu-UV 1601, Japan) was used for the absorbance measurements.

Characterization

The morphology of the produced nanofiber structures, and the fiber arrangements in the nanofibrous mat layers were investigated by scanning electron microscope (SEM) (FEI Quanta 200 FEG, Netherlands). Fibers were first sputter coated with 5 nm of Au/Pd, and observed with a field emission scanning electron microscope at 5 kV.

Analysis of the laccase-γ-CD complex formation and crystalline structure formation was performed by X-Ray diffraction spectroscopy (XRD) (PANalytical X'Pert powder diffractometer, Netherlands). Pure γ-CD, laccase-γ-CD complex, and laccase-γ-CD complex encapsulated by PCL nanofibers were examined under XRD using Cu Kα radiation in a range of $2\theta = 5-30°$.

The infrared spectra of samples of the laccase encapsulated by nanofibers and the laccase-γ-CD encapsulated by nanofibers were recorded using a Fourier transform infrared spectrometer (FTIR) (Bruker-VERTEX 70, Germany). First, the samples were mixed with potassium bromide (KBr). They were then thoroughly homogenized, ground in a mortar, and converted into pellets by pressing under a high pressure cylinder. The spectral measurements were recorded with a 4 cm^{-1} resolution and within a range between 4000 cm^{-1} and 400 cm^{-1}.

Results and discussion

The laccase enzyme, which had been supplied in powder form, was first tested for inclusion complex (IC) formation after interaction with γ-CD, which was followed by freeze-drying process. IC formation was analyzed by X-ray diffraction (XRD). According to data from the XRD results, IC formation was not detected, and crystalline structure formation was not observed. In the XRD pattern of laccase-γ-CD complex encapsulated by PCL polymer, there were some distinct peaks observed which might be attributed to the PCL polymer. However, it was unclear whether PCL polymer peaks suppressed the laccase-γ-CD complex peaks or not. In this regard, it was thought that instead of IC formation, some other kind of complex formation, i.e., the attachment of several CDs to various parts of the laccase enzyme, might have occurred [24,25]. It is a well-known fact that CDs with their unique chemistry have the potential to form ICs in which one polar guest molecule enters into a slightly apolar cavity of a host CD molecule in aqueous solution. However, in the last few years, it was claimed by several studies that CDs can also form non-inclusion complexes, which have the capability to dissolve drugs [26–28]. On the other hand, supramolecular assemblies have emerged as an important topic in CD based chemical studies; these assemblies have multiple CD cavities that bind strongly to substrates [29–31]. Since we couldn't verify the IC formation clearly, we called the produced complex structures lyophilized complexes (LC) (see Fig. 1).

In a further step, for clarification purposes, FTIR analysis was performed. Pure PCL nanofibers, the laccase-γ-CD complex, laccase encapsulated by nanofibers, laccase-γ-CD complex encapsulated by nanofibers, and laccase-γ-CD physical mixture encapsulated by PCL nanofibers were all examined by FTIR spectroscopy. Considering Fig. 2, even though no big difference was observed between purified PCL and enzyme encapsulated PCL, some characteristic peaks for the laccase enzyme were encountered. For instance, a substantial extension of the 3440 cm^{-1} band and a marked increase in the peak intensity were identified, which is generally interpreted as a characteristic stretching vibration due to OH groups. Moreover, a characteristic peak of 1639 cm^{-1}, which was assigned to the CO stretch, was detected [32–35]. It is important to note that, in the literature, recorded values might show some discrepancies due to the origin of the laccase enzyme and its production from different sources. Comparing the FTIR results of laccase encapsulated by PCL nanofibers to laccase-γ-CD complex encapsulated by PCL nanofibers revealed that for some peak values, i.e., 733, 960, 1048, 1105, 1170, and 3444 cm^{-1}, there was a 4 cm^{-1} unit peak shift. In general, it can be concluded that peaks belonging to structures other than PCL might have been suppressed by PCL peaks. However, when a different scale was in use, explicit peaks of 2350–2400 cm^{-1} were noted for the laccase-γ-CD complex encapsulated by PCL nanofibers, which showed differences from normal PCL peaks and those from the laccase–γ-CD physical mixture encapsulated by PCL nanofibers. The shifting of the peaks also informed us about the interaction of PCL and the laccase-γ-CD complex. It means that there might be another complex structure (lyophilized complex) occurring between laccase and γ-CD, as mentioned previously.

In order to comprehend the morphologies of PCL nanofibers following encapsulation procedures with pure laccase, laccase-γ-CD physical mixture, and laccase-γ-CD complex, micrographs of the produced structures were recorded with a scanning electron microscope (SEM). SEM micrographs of laccase, laccase-γ-CD physical mixture encapsulated PCL nanofibers, and laccase-γ-CD complex encapsulated PCL nanofibers were taken with 10 μm and 20 μm scales (Fig. 3). Interestingly, the fiber diameters were further thinned in cases of encapsulation. In all encapsulations, nanofibers containing beaded structures were encountered, due to large size of the laccase or laccase-γ-CD mixtures. It was revealed that the fiber and bead dimensions of laccase-γ-CD complex encapsulated nanofibers were larger than the other two encapsulations. This situation can be interpreted as the sample dimensions of laccase-γ-CD complex being larger than the dimensions of separate pure

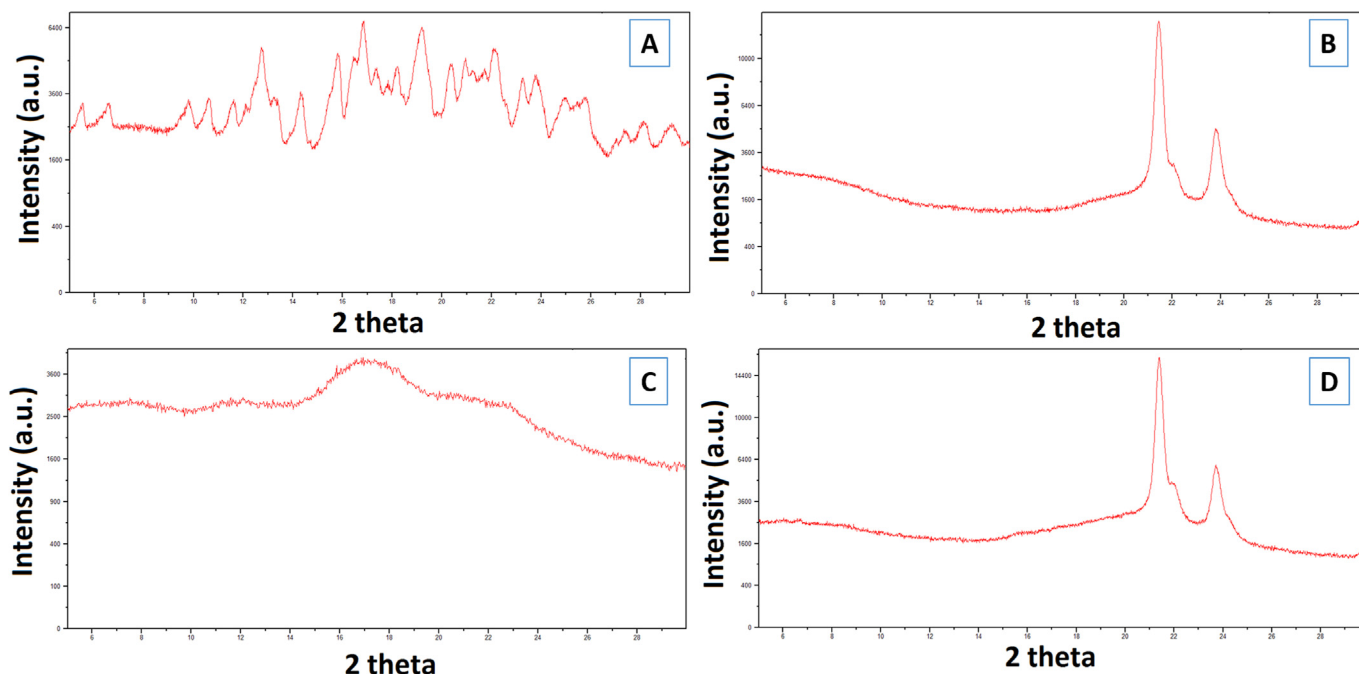

Fig. 1. XRD patterns of pure γ-CD (A), pure PCL nanofibers (B), laccase-γ-CD complex (C), laccase-γ-CD complex encapsulated PCL nanofibers (D).

Fig. 2. FTIR spectra of pure PCL nanofibers (A), laccase encapsulated PCL nanofibers (B), laccase and γ-CD physical mixture encapsulated PCL nanofibers (C), laccase-γ-CD complex encapsulated PCL nanofibers.

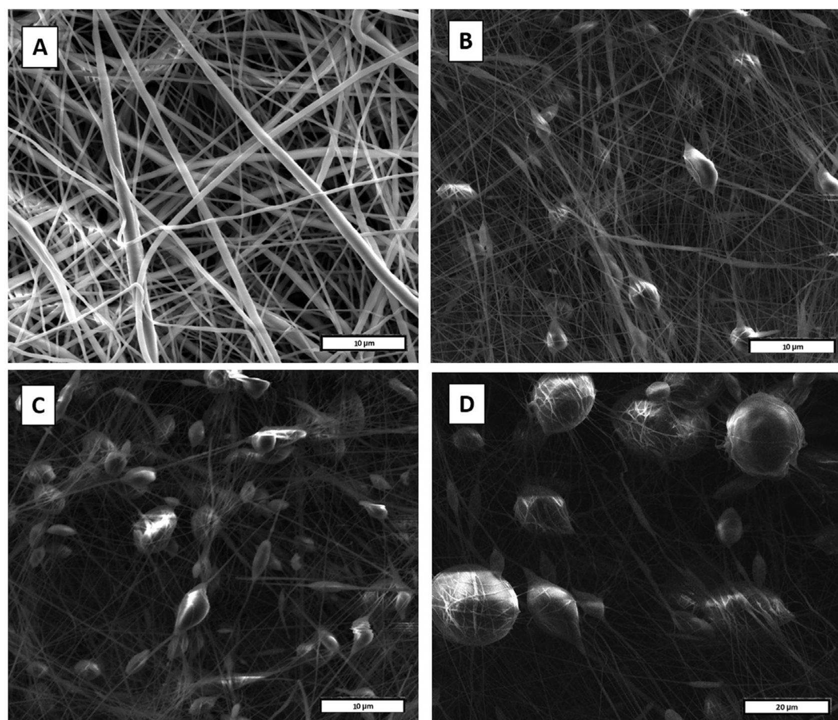

Fig. 3. SEM micrographs of pure PCL nanofibers (A), laccase encapsulated PCL nanofibers (B), laccase and γ-CD physical mixture encapsulated PCL nanofibers (C), laccase-γ-CD complex encapsulated PCL nanofibers.

Table 1
Enzyme activation test results of laccase which was immobilized into PCL nanofibers by various techniques.

Laccase activation measurement results		
Sample type	Activation	Unit
Laccase encapsulation into PCL nanofibers	23.3	U/mg
Laccase-γCD physical mixture encapsulated into PCL nanofibers	71.6	U/mg
Laccase-γ-CD complex encapsulated into PCL nanofibers	96.48	U/mg
Laccase immobilization into PCL nanofibrous layers	10.78	U/mg
Laccase-γCD physical mixture immobilization into PCL nanofibrous layers	11.28	U/mg
Laccase-γ-CD complex immobilization into PCL nanofibrous layers	29.81	U/mg

laccase and γ-CD.

In this study, the substrate and buffer were sometimes used freshly prepared, and sometimes used after being kept in the refrigerator for a while. When stored in the refrigerator, it was observed that the enzyme activity test did not produce meaningful output data before reaching room temperature. The activity test measurements were set to include 4 groups, with 6 repeats in each group. Following the measurement of a series of the first three sets, the fourth and last period measurements were carried out approximately after half an hour to obtain clearer activity results. In this manner, the testing of both enzyme activity and reuse properties was possible.

The enzyme activation tests conducted with a large number of samples and extended periods of time came up with interesting results. The basic result was that the immobilization of both laccase—γ-CD physical mixture and laccase-γ-CD complex into PCL polymer nanofibers by encapsulation method exhibited significant increases in the enzymatic activity values, 71.60 U/mg and 96.48 U/mg respectively (Table 1). Among all test groups, the best activity result was recorded by laccase-γ-CD complex encapsulated PCL nanofibers with 96.48 U/mg as seen in Table 1. The encapsulation method produced better results than the layering method for laccase immobilization studies. Layering laccase even with cyclodextrin into nanofibers significantly hinder the activity of the enzyme and the least activity was obtained with the laccase-γCD physical mixture entrapped PCL nanofibrous layers as 11.28 U/mg. Another important finding concerned the laccase's stable characteristics that helped to maintain its enzyme activation after the freeze drying process. Although laccase has a sensitive structure, and storage at −20 °C is advised, it was frozen at −80 °C during laccase-γ-CD complex formation, and no loss of enzyme activity occurred. In addition, obtaining improved activity test results following the long standing, long lasting, and complicated procedures of preparing enzyme containing mixtures and the application of the electrospinning process is one other extremely important finding of the study. The enzyme was tested several times, and then kept stored, embedded inside the polymer structures in a liquid environment, for up to 45 min. It was then tested with a fresh substrate-buffer mixture. In all cases, a high level of enzyme activity was recorded. The enzyme activity was not measured after 45 min, since the substrate-buffer liquids change their colors to dark green with the lapse of time, which did not enable us to perform reliable activity determination as a result of the insufficient color difference detection of the spectrophotometer device. In general, the results obtained were compatible with each other, and these results have been interpreted as meaningful.

Conclusions

The main purpose of this study was to develop methods to perform enzyme immobilization more easily, economically, and effectively, by using nanofibers and cyclodextrins, and so benefit from the high efficiency and selectivity of enzymatic reactions. The analyses that have been carried out have not exactly confirmed the formation of laccase-γ-CD IC, but it was concluded that different types of complex formations, or different structure formations (lyophilized complex), were created by enzyme-γ-CD interactions. Both the FTIR data and the images of the laccase-γ-CD lyophilized mixture have confirmed this conclusion. Subsequently, uniform nanofiber formation by electrospinning and the morphologies of the produced structures were proven by means of SEM micrographs. The enzymes treated by cyclodextrin showed activation after complex formation trials, and no activation loss or enzyme denaturation was detected. The general results obtained from the conducted studies have indicated that the cyclodextrin use has a positive effect on the enzyme's activity, and increases its stability. This conclusion was supported by the enzyme activity test results, which showed that immobilization by encapsulation methods gave better activity results than layering methods.

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