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Research Article

Polyvinylamine-coated polyester fibers as a carrier matrix for the immobilization of peroxidases

Biocatalytic transformations that employ immobilized enzymes become increasingly important for industrial applications. Synthetic or natural textile fiber materials such as polyester, polyamide or viscose are support materials that are comparatively inexpensive. Contrary to traditional support materials, their flexibility enables their use in reactors of any geometry and a fast and residue-free removal from batch reactors. In this study a permanently immobilized peroxidase (Baylase®) has been investigated on polyester felt as a solid support as a new heterogeneous catalyst system. The polyester felt was functionalized by coating with polyvinylamine and subsequent activation with glutaraldehyde as a crosslinking agent. The enzyme load on the textile surface, the activity of the immobilized protein after repeated use as well as the storage stability was evaluated. Scanning electron micrographs and UV Vis spectroscopy made it possible to verify the enzyme immobilization on the textile surface. Furthermore, the load of immobilized peroxidase was determined by ICP OE spectrometry to be 9–12 mg per gram of textile. The activity of immobilized Baylase® remained high over 35 reaction cycles and a storage period of 8 weeks.

Keywords: Immobilization / Peroxidase / Polyvinylamine / Textile

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1 Introduction

The majority of globally produced chemicals are obtained by catalytic processes [1]. Inorganic, organometallic or organic catalysts in industrial applications may be replaced by biocatalysts, because of the focus on energy saving and environmentally friendly production procedures [2–4]. Enzymatic catalysis is possible under mild conditions: close to ambient temperatures, physiological pH and atmospheric pressure. Further benefits of biocatalysts are their biocompatibility, biodegradability and resource efficiency [5]. The immobilization of the biocatalyst on an adequate carrier material leads to the possibility to reuse enzymes. The separation and thus the elimination of protein contamination from the products are simplified and it enables the use in fixed-bed operations or even continuous applications [6–10]. We have shown previously that textiles are suitable as carrier materials for enzyme immobilization on textile structures made of polyethylene terephthalate, polyamide or cotton.

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Abbreviations: GDA, glutaraldehyde; PET, polyester; POD, peroxidase; PVAm, polyvinylamine

These textiles have a lot of advantages compared to other conventional carrier materials such as low weight, flexibility and stability and are inexpensive in the production [11, 12]. A covalent immobilization of proteins by crosslinking on a suitable textile carrier material using peroxidase as a model enzyme will be discussed in the following sections. Peroxidases are able to use hydrogen peroxide to catalyze different oxidative reactions. They are ubiquitously present in the flora and fauna as well as in microorganisms located in peroxisomes and usually contain one or more heme molecules as prosthetic groups. In technical processes, peroxidases are used in wastewater treatment. For example, horseradish peroxidases have the ability to bleach lignin pulp and wastewater in the paper industry [13]. They are also capable to dismantle sulfonated and non-sulfonated azo dyes such as Remazol Brilliant Blue R [14] and are used for bleaching cotton [15]. In the future, novel peroxidases can also be used for the synthesis of high quality flavors from carotenoids or for the enzymatic digestion of lignified biopolymers [16]. Particularly the use of peroxidases for bleaching foods such as dairy products or bakery products is a promising application. For example, in the production of various kind of cheese like Cheddar or Gouda, food-colorants such as Annatto or β -Carotene are added to intensify and standardize the color of the cheese. After manufacturing the color is found to be one-third in the whey [17]. For further treatment the whey is

either disposed of over the sewage treatment or bleached for further use as food. Conventionally, benzoyl peroxide or high quantities of hydrogen peroxide is used in chemical bleaching [18]. A gentle and environmentally friendly alternative could be the enzymatic bleaching by peroxidase. Ideally, the enzymes should be available as immobilized catalysts to allow a repeated or continuous use, without contaminating the products. The present work describes a new way to immobilize peroxidases on unconventional materials for use in the bleaching process of colored whey.

2 Materials and methods

2.1 Reagents and materials

The experiments were carried out using two types of polyester (PET) felt as support material: E20102 0/G (PET-0/G) and E20102 0/0 (PET-0/0) with an area weight of 350 g/m², donated by Heimbach Filtration GmbH (Düren, Germany). Polyvinylamine (PVAm, Lupamin® 9095), produced by BASF (Ludwigshafen, Germany), was used to modify both types of PET textiles. The solution contains 10–15 wt% PVAm with an average molecular weight of 340 000 g/mol and a degree of hydrolysis >90%. Glutaraldehyde (GDA, 25 %) by Roth (Karlsruhe, Germany) was used as the crosslinker for peroxidase immobilization. The peroxidase (POD; Baylase® RP-system) from horseradish was a kind gift from Lanxess (Cologne, Germany) and had a molecular weight of above 37 000 g/mol and a temperature optimum close to 30°C at pH 6. ABTS (2,2'-azino-di-(3-ethylbenzthiazolin-6-sulfonic acid)) by AppliChem Panreac (Darmstadt, Germany) was used as substrate for activity measurements. The food colorant Annatto was kindly given by ASA Spezialenzyme GmbH; (Wolfenbüttel, Germany) and was used to dye commercial whey (Real, Germany).

2.2 Polymer modification

Lupamin® 9095 was diluted with the same amount of deionized water to obtain a solution of 5–7.5%. Next, the pH was raised to 10 by the addition of a 1 M sodium hydroxide solution. Afterwards, the PET felts were immersed for 30 min in this solution. After squeezing the fabrics to approximately 170–190% of wet pick-up, they were dried at 80°C. To fix PVAm covalently to the functional groups on the PET surface, the samples were heated to 150°C for 15 min. Afterwards the fabrics were washed thoroughly with hot water and air-dried overnight at room temperature. By weighing the textiles before and after the fixation it is possible to calculate a mass difference and thus the mass of fixed polyvinylamine.

2.3 Functionalization of the modified polymer

The finished textiles were shaken for 2 h in a 5% glutaraldehyde solution at 19°C and 50 rpm. Subsequently, the modified textiles were rinsed in water to remove the unbound GDA from the

textile. Afterwards, the fabrics were squeezed vigorously to get the fabric as dry as possible.

2.4 Protein immobilization

In the next step the Baylase® solution was pipetted onto the textile directly. To each gram of fabric were added 20 mg of enzyme. The samples were dried overnight at room temperature (Fig. 1).

2.5 Sample washing

To remove non-covalently attached enzymes, the samples treated with POD were first stirred three times for 10 min in deionized water. Then, the textiles were stirred three times for 10 min in 1.5 M NaCl solution to disrupt ionic binding. After this treatment, the samples were washed 1 min in deionized water and 10 min in an ultrasonic bath, squeezed, and air-dried overnight.

2.6 Analysis

For a qualitative determination of the textile modification, a TNBA (2,4,6-trinitrobenzene sulfonic acid) assay was performed. A 0.1% TNBA solution was prepared in borate buffer (pH 9.5). The textiles were immersed in this solution for 2 min at 80°C. After this fixation step, the samples were washed 30 min in deionized water. Finally, the samples were stirred for 2 min in borate buffer.

Furthermore, the number of saturated carboxyl groups gives a hint of the amount of bounded PVAm. The carboxyl groups were measured before and after the modification step. For that, the samples were dyed with a 0.05% methylene blue-solution (phosphate-borate acetate buffer pH 7.1–7.4) and immersed for 10 min at room temperature. After washing with deionized water and air-drying the samples were weighed and shaken in 20 mL acetic acid (84%) to remove the color from the textile. The dyed solution was measured at 650 nm. The calculated concentration corresponds to the concentration of carboxyl groups on textile.

The immobilization of POD on the surface of the textile carrier materials was analyzed qualitatively using UV Vis spectroscopy (Lambda 950 S, Perkin Elmer) with an integrating sphere (250–800 nm). Scanning electron microscopy images of fabrics were made using a Hitachi S-3400N. The POD load on the samples was determined quantitatively using ICP OES (Varian; Salt Lake City, USA) by measuring the iron concentration of solutions, obtained by microwave digestion of 0.2 g of textile in 8 mL of concentrated nitric acid.

2.7 Enzyme activity and reuse of immobilized POD

The activity of the immobilized POD was determined by the ABTS (2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay in presence of hydrogen peroxide. Baylase® catalyzes the

transformation of ABTS to a radical ion (see Fig. 5), that was detected by UV Vis measurements at 420 nm. The enzymatic reaction with immobilized POD were carried out at 19°C with 0.02 g of treated fabric in 2 mL of reaction medium which at least contains 0.4 mL of a 2 mM ABTS solution along with 0.4 mL of a 1.4 mM solution of hydrogen peroxide, 0.64 mL of a 100 mM phosphate buffer and 0.16 mL of deionized water. Kinetic experiments were carried out by photometrical measurements in a time scan (measurement point at every 10 s over a period of 2 min). The absorbance was measured photometrically in comparison to a blank value. The immobilized POD was reused 35 times in fresh reaction solution. Between each repetition, the samples were washed or stored at 4°C.

2.8 Bleaching of whey

The bleaching of whey with immobilized POD were carried out at 30°C with 0.5 g of treated fabric in 100 mL of colored whey, which at least contains 0.4 mL of Annatto solution (see Fig. 8 at 0 h). During the bleaching process 14 mM hydrogen peroxide was added step by step (0.025 mL/min). Kinetic experiments were carried out by photometrical measurements in a time scan (measurement point at every 5 min over a period of 6 h). The absorbance was measured photometrically in comparison to uncolored whey.

3 Results and discussion

3.1 Polyester modification

The specific functional groups of a textile's surface allow the covalent immobilization of enzymes. On the fiber surface polyester has easily accessible free carboxyl groups, which can be used for further modification steps [19]. However the amount of free carboxyl groups on PET surface is not enough for an adequate enzyme immobilization. The measurements show an amount of 0.16 μmol per 1 g PET (see Table 1). In a first modification step the number of functional groups was therefore increased, in order to be able to obtain a high enzyme load on the PET felt. Figure 1 shows schematically how PVAm reacts with the reactive groups of the fiber to generate a significantly increased number of new functional groups. At elevated temperatures the amino groups of PVAm react with the terminal carboxyl groups on the surface on the PET surface and with the ester groups of the PET chain, to form amide groups [20]. After the modification the amount of carboxyl groups decreased by half from 0.16 μmol to 0.09 μmol per 1 g of textile. So PVAm saturated the half of carboxyl groups in a covalent bound. By gravimetric measurements, the PVAm loading was determined. On both types of used textile substrates, an average load of 7–8% PVAm was found (see Table 1). The amount of polyvinylamine coating is adjustable by the variation of the pH during the modification step. The

Figure 1. Preparation of PET fibers carrying enzyme on its external surface. From top to bottom: untreated polyester; coated polyester with PVAm; activating the modified polyester with glutaraldehyde and POD-immobilization on the modified fibers.

Table 1. Determination of carboxyl groups on modified and unmodified PET, as well as fixed amount of polyvinylamine on textiles. And, ICP OES analysis for the determination of immobilized POD amount based on the iron concentration on the textile through denatured peroxidase

Sample	Average carboxyl group [μmol] per 1 g textile	Average carboxyl group [μmol] per 1 g modified textile	Average PVAm load [%] per 1g textile	Amount of Baylase® [mg] per 1g textile
PET-0/G	0.16 ± 0.33	0.09 ± 0.30	7.86 ± 0.22	9.09
PET-0/0			6.90 ± 0.25	11.77

PET-0/0			PET-0/G

PET blank	PET-PVAm	PET-PVAm-GDA	PET blank	PET-PVAm	PET-PVAm-GDA

PET blank TNBA	PET-PVAm TNBA	PET-PVAm-GDA TNBA	PET blank TNBA	PET-PVAm TNBA	PET-PVAm-GDA TNBA

Figure 2. TNBA staining for the qualitative determination of amino groups on both modified PET-felts with polyvinylamine and following glutaraldehyde treatment.

lower the pH value, the lower the amount of polyvinylamine coating [21, 22].

The amino groups introduced on the surface of the textile can be detected by the TNBA assay. TNBA is a highly sensitive reagent to observe free amino groups on the surface. The reaction of primary amines with TNBA produces a chromogenic product, which is detectable at 335 nm [23]. The substrate turns to a deep orange if free amino groups are present (Fig. 2: PET-PVAm-TNBA). Indirectly, it is also possible to assess the second modification with glutaraldehyde (Fig. 1) by this assay. The color reaction is significantly less efficient when most of the amino groups are masked by GDA after the modification (Fig. 2: PET-PVAm-GDA-TNBA).

3.2 Peroxidase immobilization

GDA reacts under mild conditions (room temperature, neutral pH) with the amino groups of the modified textile and the N-terminal amino groups of the enzymes. The imine groups formed provide a covalent fixation of the enzyme to the PVAm-coated polyester via the GDA linker (Fig. 1).

The immobilization of POD can be investigated qualitatively by using UV Vis spectroscopy. In Fig. 3, UV Vis spectra of the unmodified material as well as those of the modified fibers with PVAm, GDA and POD, respectively, are shown. The enzyme shows a characteristic absorbance maximum close to 400 nm (see Fig. 3, inset). At the same time, both the pristine textile substrate and the reagents PVAm and GDA show no absorbance at 400 nm. The spectrum of diffuse reflection of the textile after the cross-

linking reaction with POD (PET/PVAm/GDA/POD) shows a significant remission close to 400 nm, compared to the PVAm modified textile (PET/PVAm). This signal can be considered as evidence of the successful immobilization of peroxidase on the textile carrier.

Figure 4 shows scanning electron micrographs of the different samples. The micrograph of untreated PET (a) shows the individual fibers in accordance aligned the tissue structure along the axis. The coating of PVAm (b) leads to a structure surrounding the polyester fibers, which is also located between the individual fibers. The use of glutaraldehyde (c) results in a similar coating surrounding the fiber material. Following the addition of enzyme (d), some particulate deposits formed preferably in the fibers interstices. SEM images show in a high resolution the fibers surface and the single modification steps additional to the UV Vis spectra, and create a further overview about the enzyme immobilization.

3.3 Determination of protein load

Because of the presence of iron in the prosthetic group of the peroxidase, ICP OES analysis allows the quantitative determinations of the protein load directly on the textile samples (following a digestion with acids). A corresponding calibration curve that has been calculated from the digestion of unbound Baylase® allows the calculation of bounded POD on textile. Table 1 summarizes the fiber-fixed enzyme load per gram of textile. The data shows that it was possible to achieve an enzyme load of 9 mg (PET-0/G) and 12 mg (PET-0/0) Baylase® per

Figure 3. UV-Vis remission spectra of PET-PVAm samples before and after POD immobilization. Inset: absorbance spectrum of Baylase®.

Figure 4. SEM micrographs of PET felts: (A) untreated; (B) after modification with PVAm; (C) after additional cross-linking with GDA; (D) after immobilization of Baylase®.

gram of textile. These calculated Baylase® amounts were then used to calculate the specific enzyme activity fixed on gram of textile, which is described in Section 3.4.

3.4 Activity and reuse of immobilized peroxidase

The biocatalytic activity was measured photometrically by using the ABTS assay after repeated use (up to 35 reaction runs) in the presence of hydrogen peroxide. The active peroxidase uses colorless ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) as a substrate and converts it to a green ABTS radical ion (ABTS•+) (Fig. 5). The amount of developed dye is then determined by measuring the absorbance at a wavelength of 420 nm.

Figure 6A shows the activity profile of the immobilized peroxidase of 19 catalytic reactions over a period of eight weeks. In the beginning of the reaction cycles a loss of activity is noticeable, which results from a loss of unbound enzyme. Following, a

significant biocatalytic activity about 15 units was achieved until the end of the experiment. A significant catalytic activity in the period of 74 days and 19 reaction cycles of one sample could be obtained without a significant activity loss after the fifth reaction. Additional in Fig. 6B the determination of stock stability is represented for the immobilized Baylase® after previous storage over eight weeks at 4°C. 35 experimental reaction cycles could be determined and the catalytic activity stays stable over all reaction cycles even after initially observed enzyme desorption. Noticeable is that the immobilization on PET-0/G has no major impact on the storage, because the starting activity is comparable to the starting activity in experiment A. Different is the stock stability in case of PET-0/0, here some loss of starting activity compared to experiment A is visible. After the third reaction cycle activities remain constant on the same level as in experiment A. Despite previous storage, the activity stays almost constant at a relatively high level. Crosslinking with GDA leads to a covalent and stable binding to the textile substrate. Noticeable is the significant loss of activity after the first test repetition, which indicates a first

Figure 5. Catalytic conversion of the substrate ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) to a radical ion.

Figure 6. Determining the activity of bounded peroxidase, cross-linked by GDA to PET PVAm modified textile, over several reaction cycles and weeks (A) and after a previous storage over eight weeks at 4°C (B) (determined the reaction of 0.5 mL ABTS to ABTS^{•+} radical in the presence of hydrogen peroxide, 1 g of textile).

desorption of adsorptive bounded protein. However, the activity remains stable on any further reaction cycles. The presented immobilization method exhibits a considerable activity even after 35 reaction cycles.

First bleaching experiments were done using Annatto-colored whey. The commercial whey has a pH value close to 6, which is also the optimum of our used POD. The PVAm cross-linked coating stay stable under that condition. Figure 7 shows the

Figure 7. Kinetic measurement of the enzymatic bleaching of Annatto-colored whey with immobilized POD on PET textile (pointed graph) and the control without POD but addition of hydrogen peroxide (crossbred graph).

Figure 8. Enzymatic bleaching steps of Annatto-colored whey with immobilized POD on PET textile. From left to right: standard whey; Annatto-colored whey; bleaching after 2 h; bleaching after 5 h; bleaching after 6 h.

kinetic degradation of Annatto in whey using textile-fixed POD, in comparison to addition of hydrogen peroxide (control). Supplementary the images in Fig. 8 clarify the bleaching process, which finished after 6 h. Hydrogen peroxide is given step by step in small quantities during the bleaching process. In time peroxidase is able to convert it so that it could not break the PVAm bound.

4 Concluding remarks

Permanent binding of peroxidases on a textile carrier material such as polyester is possible by increasing the required functional groups of the fabric with polyvinylamine followed by crosslinking with glutaraldehyde, a methodology that is simple and cost-efficient. The biocatalytic activity was maintained for a large number of reaction cycles even after a storage time of eight weeks under simple storage conditions. The activity of one

single sample stays stable over 35 successive times without losing its activity. Additionally, first experiments show the success of bleaching an Annatto-colored whey by immobilized POD on a PET felt carrier.

Practical application

Bleaching processes with chemical additives could be replaced by the technique of 'white biotechnology' especially in food industries. For instance, some peroxidases are able to catalyze a gentle enzymatic degradation of food colors, e.g. β -carotene or norbixin, which added to food during production. The economical use of expensive enzymes can be improved by immobilization on a suitable support material. In principle the fixation allows a multiple or even continuous use for example in flow-through reactors. Conventional carrier materials are usually inorganic and the immobilization is often complex and expensive. In contrast natural or synthetic textile materials for example polyester, polyamide or viscose are comparatively inexpensive. Polyester as a suitable support material has a flexible structure, which allows the use in reactors of any geometry and a fast and residue-free removal after the end of each reaction.

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5 References

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