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The response surface methodology for optimization of tyrosinase immobilization onto electrospun polycaprolactone–chitosan fibers for use in bisphenol A removal

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Abstract

Composite polycaprolactone–chitosan material was produced by an electrospinning method and used as a support for immobilization of tyrosinase by mixed ionic interactions and hydrogen bonds formation. The morphology of the fibers and enzyme deposition were confirmed by SEM images. Further, multivariate polynomial regression was used to model the experimental data and to determine optimal conditions for immobilization process, which were found to be pH 7, temperature 25 °C and 16 h process duration. Under these conditions, novel type of biocatalytic system was produced with immobilization yield of 93% and expressed activity of 95%. Furthermore, as prepared system was applied in batch experiments related to biodegradation of bisphenol A under various remediation conditions. It was found that over 80% of the pollutant was removed after 120 min of the process, in the temperature range 15–45 °C and pH 6–9, using solutions at concentration up to 3 mg/L. Experimental data collected proved that the stability and reusability of the tyrosinase were significantly improved upon immobilization: the immobilized biomolecule retained around 90% of its initial activity after 30 days of storage, and was still capable to remove over 80% of bisphenol A even after 10 repeated uses. By contrast, free enzyme was able to remove over 80% of bisphenol A at pH 7–8 and temperature range 15–35 °C, and retained less than 60% of its initial activity after 30 days of storage.

Keywords: enzyme immobilization; biocatalysts, immobilization optimization; bisphenol A removal

1. Introduction

The widespread use of bisphenols in industry, and their adverse impact on living organisms, including mutagenic and cancerogenic effects, make it essential to monitor their circulation in the environment [1–4]. Many studies are focused on searching for efficient and environmentally friendly methods of degradation of phenolic compounds and their derivatives [5, 6]. One of such approaches concerns biodegradation of toxic pollutants using enzymes: natural biocatalysts having the ability to oxidize phenols, dyes, pharmaceuticals and estrogens [7, 8]. The most widely used enzymes in biodegradation

are oxidoreductases, including peroxidases such as horseradish, lignin or chloroperoxidase, and polyphenol oxidases such as laccase and tyrosinase [9–11].

Recently published scientific reports suggest that tyrosinase is capable for efficient degradation of phenols and bisphenols by catalytic conversion into corresponding quinones, which tend to form oligomeric and polymeric compounds that can be easily separated from solution by precipitation [12, 13]. Mechanism of such catalytic performance consist of hydroxylation and subsequent oxidation reaction of phenols into ortho-quinones with simultaneous reduction of oxygen, as a co-substrate, to water. The mushroom *Agaricus bisporus* tyrosinase, which was reported to be a glycosylated, tetrameric protein with a molecular mass of around 120 kDa, composed of two heavy subunits of ~43 kDa (H subunit) and two lights subunits of ~14 kDa (L subunit), is mostly used [14, 15]. This tyrosinase possesses isoelectric point at pH 4.7–5, whereas shows its maximum activity at pH around 7 and at mild temperature of around 25 °C [16]. In general, tyrosinase consists of three various domains, among which, the central domain, responsible for catalytic action contains two Cu binding sites, called CuA and CuB, which interact both with molecular oxygen and phenolic substrate. Moreover, the mushroom tyrosinase has 11 cysteine residues at N-terminal domain and 1 cysteine residue at C-terminal domain that stabilize protein structure by internal disulfide linkages [17].

Application of free enzymes, due to their low activity, poor stability and lack of reusability is limited. In order to improve it, immobilization process might be used, after which the protein is bound to a solid support [18,19]. The immobilization of biomolecules provides some operational stability, higher resistance against adverse effects of the reaction conditions, as well as facilitate reuse of the enzymes [20]. In this context, a controlled immobilization, particularly using porous supports, has a positive effect on the enzyme maintenance and performance, reduces diffusional limitations and partitions as well as enzyme aggregation and its inhibition and prevents against internal conformational changes in its structure [21, 22]. Furthermore, immobilization results in significant enzyme stabilization, especially when enzyme is multipointly immobilized [23], as well as when favorable environment for the enzyme is generated upon immobilization [24]. A properly performed immobilization also leads to enhancement of enzyme catalytic activity under harsh process conditions

and its selectivity and/or specificity [22]. Finally, examples of enzyme purification by immobilization have been also reported [25, 26].

Among others immobilization strategies, adsorption approach based on formation of ionic, hydrogen or mixed interactions, is the most commonly used. This is mainly due to the simplicity of the immobilization procedure, its low-cost and usually observed retention of high catalytic activity by the immobilized biomolecules [27, 28]. Adsorption immobilization is characterized by low-force interaction, however, physical adsorption of biomacromolecules is a multipoint process having advantages and disadvantages [29, 30]. On the one hand, the structure of the enzyme is insensibly altered and there is a possibility to reuse the support [22]. On the other hand, weak interactions lead to elution of the enzyme from the support, and thus fast decrease of catalytic properties and limited reusability might be observed [31]. It should be highlighted that such enzymes as oxidoreductases have been also immobilized via adsorption and other techniques in order to improve their properties in environmental applications. For instance, lignin peroxidase immobilized onto chitosan was able to degrade over 95% of textile dyes, and laccase immobilized onto nanoporous silica beads was capable for removal of over 90% of 2,4-dinitrophenol [32, 33]. In another study, tyrosine immobilized onto silica alginate was reported as very efficient in removal of bisphenol A with an efficiency up to 100% [34].

It is important to note, however, that successful immobilization is highly dependent on a proper selection of a suitable support, as well as determination of optimal process conditions, which affect the final parameters of the immobilized enzymes [35, 36]. The numerous research, concerning enzymes immobilization and supports selection, was presented in the literature in the past decade. In recent years, electrospun supports have raised particular interest, due to the possibility to prepare tailor-made, highly porous materials that exhibit affinity towards enzymes [37]. However, bearing in mind the specificity of enzymes and the nature of the process, it is extremely important to select the most favorable conditions for the immobilization of a given protein [38]. Parameters that require analysis include pH and temperature of the immobilization process, which may inactivate the biocatalyst, and the process time and initial enzyme concentration, which may affect the quantity of immobilized enzyme and its activity [39]. In this context, the one-factor-at-a-time (OFAT) method,

response surface methodology (RSM) and Box–Behnken design (BBD) are techniques used for modeling of the immobilization process and selection of its optimal conditions [40, 41]. However, methods of data matching have also been used to optimize enzyme immobilization [42]. The criteria listed above have a great impact on attainment of the maximum enzyme activity and on the possible applications of the immobilized biocatalysts.

Therefore, to address the issues related to the efficient immobilization of tyrosinase and optimization of this process, in presented study, to the best of our knowledge for the first time, an attempt was made to produce polycaprolactone–chitosan (PCL–chitosan) electrospun materials for tyrosinase immobilization in order to obtain novel type of biocatalytic systems capable for bisphenol A removal. These being the key and novel objectives of the study. The hypothesis that supports this research approach is that biocompatible PCL–chitosan materials facilitate tyrosinase immobilization by adsorption and improve enzyme stability as well its reusability over repeated use. The produced PCL–chitosan material, beside relatively good mechanical stability, is characterized by the presence of hydroxyl, amine and carbonyl groups on its surface. These groups are capable for creation of hydrogen bonds and ionic interactions during enzyme immobilization process with functional groups of biomolecule, including mainly amine, hydroxyl and carboxyl moieties (Fig. 1). Moreover, this support material was selected due to its open three-dimensional structure, high volume to mass ratio and relatively low-cost of its production. A thorough analysis of the produced electrospun support was performed and the response surface methodology was applied for modelling of the immobilization process and optimization of its conditions to obtain a biocatalytic system offering high catalytic activity. The stability and reusability of the immobilized biomolecule were also examined. Finally, the possible practical application of the produced biocatalytic systems was validated in the biodegradation of bisphenol A under various reaction conditions. The research approach concerning immobilization of tyrosinase and its use for removal of bisphenol A is an overlooked research area in recently published articles.

Figure 1

2. Materials and Methods

2.1. Materials

Polycaprolactone (PCL) with molecular weight of 80 kDa, chitosan with molecular weight of 1000 kDa, trifluoroacetic acid (TFA), mushroom tyrosinase (polyphenol oxidase, activity ~2000 U/mg solid, EC 1.14.18.1), 100 mM acetate buffer (pH 3–5), 100 mM phosphate buffer (pH 6–8), 100 mM bicarbonate buffer (pH 9, 10), bisphenol A (BPA) (purity $\geq 99\%$) and L-catechol, were supplied by Sigma-Aldrich (USA).

2.2. Fabrication of PCL–chitosan materials

PCL–chitosan electrospun fibers were prepared using a NANON-01A device (MECC Co. Ltd., Japan) by mixing two polymers solutions. To prepare chitosan solution, it was dissolved in TFA to obtain 5% (w/v) solution. The obtained mixture was then stirred for 4 h at 75 °C. To obtain second solution, polycaprolactone was dissolved in TFA to obtain 10% (w/v) solution - it was stirred for 4 h at room temperature. Next, both solutions were mixed together to obtain PCL:chitosan ratio of 15:85 (w/w) and further stirred for 1 h. The resulting PCL–chitosan solution was placed in a plastic syringe and electrospun. The applied voltage was set at 10 kV, the distance between nozzle and collector was 150 mm, and the ejection rate was 1 mL/h. The obtained PCL–chitosan fibers were dried in a vacuum drier at 25 °C.

2.3. Tyrosinase immobilization

Immobilization of mushroom tyrosinase was performed by adsorption under various process conditions in order to obtain immobilized enzymes with enhanced catalytic activity. For immobilization, 10 mg of PCL–chitosan material (two pieces 1 cm x 1 cm in size), washed with distilled water, was placed in 10 mL of tyrosinase solution at the desired concentration (from 0.5 to 5.0 mg/mL), prepared in 100 mM buffer solution at an appropriate pH (ranging from 4 to 9). The samples were then placed in a shaker (IKA Werke GmbH, Germany) and agitated at 150 rpm for a specified period of time ranging from 1 h to 24 h, at various temperatures (from 5 °C to 55 °C). After immobilization, samples were separated from the reaction mixture and were washed with 100 mM phosphate buffer (pH 7) to remove unbounded enzyme.

2.4. The effect of process conditions on the activity of immobilized enzyme – modeling study

The mathematical model proposed was formulated using Mathcad software. For the six distinct data sets, with different independent variables, the *polyfit* function was used to generate the response surface. Based on the least squares modelhood, the problem of fitting the surface consisted of searching of such parameters as defined by equation (1), in order to obtain the smallest value of the sum of squared distances between data points and the surface. Calculating the partial derivatives of F is straightforward for the formulation chosen, although a numerical procedure might be required in the case of more complex surface functions. In presented example, symbolic solutions for the optimal parameters A_{opt} and B_{opt} were derived analytically.

2.5. Immobilization yield, enzyme activity and stability

The activity and stability of the free and immobilized enzyme were examined based on a model reaction using L-catechol as a substrate, by measuring the rate of formation of ortho-quinones, according to slightly modified methodology presented by Lincer et al. [43]. Briefly, 50 mg of free enzyme or an appropriate amount of the biocatalytic system produced containing 50 mg of tyrosinase or immobilized tyrosinase was added to 10 mL of 20 mM L-catechol solution in 100 mM phosphate buffer (pH 7). The process was carried out for 60 min at 25 °C with a continuous oxygen supply at a flow rate of 1 mL/min. One unit of free and immobilized tyrosinase activity was defined as the amount of enzyme that liberates 1 mM of ortho-quinones per minute under assay conditions. Analyzing the storage and thermal stability, the initial value of enzyme relative activity was defined as 100% activity. Measurements of relative activity were performed at regular time intervals.

The storage stability of free and immobilized tyrosinase was examined by spectrophotometric measurements, over 20 days of storage at 4 °C in 100 mM phosphate buffer at pH 7, based on the above-mentioned model reaction.

The thermochemical stability of free and immobilized enzyme was determined over 120 min of incubation under optimal temperature and pH conditions (25 °C and pH 7), based on the same model reaction using 20 mM L-catechol as a substrate. Spectrophotometric measurements were performed after a specified heating time. Then the relative activity was calculated. In addition, inactivation parameters of the free and immobilized enzyme—the inactivation constant (k_D) and enzyme half-life ($t_{1/2}$)—were calculated based on the linear regression slope of inactivation curves.

The immobilization yield and expressed activity were calculated following eq. 1 and eq. 2:

$$\text{Immobilization yield (\%)} = \frac{A_i - A_f}{A_i} \cdot 100\% \quad (\text{eq. 1})$$

$$\text{Activity recovery (\%)} = \frac{A_t}{A_i} \cdot 100\% \quad (\text{eq. 2})$$

where: A_i denotes the initial activity of tyrosinase added to the immobilization medium, A_f denotes the total activity of the enzyme in the supernatant and washing solution after the immobilization and A_t denotes the activity of the immobilized tyrosinase. The activities of the supernatant and reference samples were determined under standard conditions, based on the above-presented reaction.

The kinetic parameters of free and immobilized tyrosinase—the Michaelis–Menten constant (K_m) and maximum reaction rate (V_{max})—were determined against L-catechol, by measuring the initial reaction rates of L-catechol oxidation under optimal process conditions with a continuous oxygen supply with a flow rate 1 mL/min using various substrate solutions at concentrations ranging from 0.5 to 20 mM. The apparent kinetic parameters of free and immobilized tyrosinase (K_m and V_{max}) were calculated from Hanes–Woelf plot.

2.6. Removal of bisphenol A

Degradation of BPA by free and immobilized tyrosinase was performed using an IKA KS 4000i Control incubator (IKA Werke GmbH, Germany). For the BPA removal experiments, 10 mg of free or an appropriate amount of the biocatalyst produced containing 10 mg of immobilized enzyme was introduced to BPA solution at desired concentration. The samples were shaken under specified process conditions at 150 rpm for 120 min with a continuous oxygen supply at a flow rate of 1 mL/min, and furthermore analyzed at specified time intervals to determine the BPA removal efficiency.

The effect of initial BPA concentration on the removal efficiency was examined using solutions at concentrations of 0.1, 0.5, 1.0, 3.0 and 5 mg/L, pH 7 and 25 °C. The effect of pH on the percentage removal of bisphenol A was examined over a wide pH range of 3–10, using 100 mM buffer solutions to adjust an appropriate pH value. The process was performed at 25 °C using BPA solution in concentration of 3 mg/L. To assess the effect of temperature on the removal of BPA, biodegradation was performed at temperature ranging from 5 °C to 65 °C (with a 10 °C step), using BPA solution in concentration of 3 mg/L and at pH 7.

The reusability of the immobilized tyrosinase was examined over ten repeated cycles of bisphenol A removal. Briefly, each cycle was performed within 120 min at 25 °C, using BPA solution in an initial concentration of 3 mg/L and at pH 7. After each biodegradation step, the immobilized tyrosinase was separated from the reaction mixture, washed several times with 100 mM phosphate buffer (pH 7), and placed in a fresh BPA solution.

2.7. Analytical techniques

The morphology of the fabricated PCL–chitosan electrospun fibers, before and after tyrosinase immobilization, was presented in SEM images (EVO40, Zeiss, Germany). The average diameter of the obtained fibers, before and after enzyme attachment, was calculated using the Image J program (National Institute of Health, USA).

The porous structure parameters of the materials before immobilization were determined using an ASAP 2020 instrument (Micromeritics Instrument Co., USA). The surface area was evaluated according to the multipoint BET (Brunauer–Emmett–Teller) method using low temperature nitrogen (77K) adsorption under relative pressure (p/p_0). The mean size and total volume of pores were calculated based on the BJH (Barrett–Joyner–Halenda) algorithm.

The Fourier transform infrared spectra (FTIR) of electrospun material, before and after tyrosinase immobilization, were recorded using Vertex 70 spectrometer (Bruker, Germany) working in attenuated total reflectance (ATR) mode. The measurements were conducted in the wavenumber range of 4000 to 400 cm^{-1} , at a resolution of 0.5 cm^{-1} and 64 scans.

The percentage mass contribution of selected elements present in the samples before and after immobilization was analyzed by means of energy dispersive X-ray spectroscopy (EDS) using a Princeton Gamma-Tech unit with a prism digital spectrometer (UK).

The efficiency of bisphenol A removal, as well as the stability and reusability of free and immobilized enzyme, were determined based on spectrophotometric measurements using a Jasco V-750 spectrophotometer (Jasco, Japan), measuring the absorbance during ABTS oxidation at 420 nm (stability study) and during BPA removal at $\lambda=480$ nm. The final concentration of ortho-quinones after oxidation process, and the concentration of BPA after biodegradation, were obtained based on a proper calibration curves. The efficiency (%) of bisphenol A removal was calculated by considering the initial

and the final concentration of the pollutant in the solution, before and after degradation performed under specified conditions.

2.8. Statistical analysis

All experiments and measurements were performed in triplicate, and error values are presented as means $\pm$ standard deviation. Statistically significant differences were determined by one-way ANOVA using Tukey's test, performed in SigmaPlot 12 software (Systat Software Inc., USA). Statistical significance was established at a level of $p < 0.05$.

3. Results and Discussion

3.1. Morphological characterization of fibers before and after immobilization

In order to characterize the morphology of the obtained materials and confirm effective enzyme immobilization, in the first stage of the investigation, SEM images were taken for PCL–chitosan electrospun fibers before and after tyrosinase immobilization (Fig. 2). Electrospun fibers, before attachment of tyrosinase (Fig. 2a) are branched, with a smooth surface, and have an average diameter of 344 ± 121 nm. By contrast, after enzyme deposition a significant increase in fiber diameter (average diameter of 689 ± 365 nm) was observed, indicating attachment of the enzyme to the fibers surface. Mushroom tyrosinase is characterized by the molecular weight of around 120 kDa thus, irregular shapes almost uniformly covered surface of the fibers (Fig. 2b). This fact confirms effective enzyme immobilization and suggests formation of enzyme aggregates [44]. This might lead to the partition and diffusional problems upon immobilization that reduce enzyme activity. However, it should be noted that electrospun material has a multidimensional structure with relatively large pores irregular in shape and size, which reduce diffusional resistance and facilitate efficient transport of reaction substrates and products between active centers of the immobilized enzyme and the reaction mixture [45]; this makes such material a suitable support for the immobilization of biomolecules. BET surface area of the produced PCL–chitosan material was found to be $72.3 \text{ m}^2/\text{g}$. In addition, the average pore size of the single fiber was 19.64 nm, whereas its volume was $0.874 \text{ cm}^3/\text{g}$. Such values indicate porous nature of the produced support, making it suitable for enzyme immobilization. In the FTIR spectrum of PCL–chitosan material (Fig. 2c) the most important is the presence of signals noted at 3420 and 1720 cm^{-1} ,

assigned to hydroxyl and carbonyl functional groups, capable for enzyme binding. In the spectrum of produced biocatalytic system additional signals can be observed. Among them, those at 1645 and 1080 cm^{-1} are the most important ones and confirm effective enzyme deposition onto electrospun material. Additionally, results of elemental analysis confirm the effective enzyme adsorption, as higher percentage mass contribution of elements such as carbon, nitrogen, oxygen and sulfur, in the samples after immobilization was noticed.

Figure 2

3.2. Modeling the effect of process conditions on the activity of immobilized enzyme

As one the most important objectives of the study was to produce highly active biocatalytic system for removal of bisphenol A, a proper selection of immobilization conditions, including initial concentration of enzyme solution, pH and process duration, is of key importance. Therefore, in presented study, the effect of process conditions on the activity of immobilized tyrosinase was modeled in order to determine the optimal ones.

The response surface methodology was used for this purpose, leading to a model that serves to find the optimum immobilization conditions. Additionally, for specified pH and process time t , the influence of other parameters such as temperature T , initial concentration of enzyme solution C , and their dependencies vs. catalyst activity, were estimated and analyzed. The analysis consisted of selecting an appropriate multidimensional function $F(A, B)$ exhibiting a similar character to the data measured; see equation (1). The usual measure of the quality of model fit and data variance is standard deviation. It is important that mathematical model describing the data should not be too complex, to prevent overfitting and consequently to avoid identifying false trends. The model shouldn't also be too simple, so that it reflects all of the relevant relationships in the obtained data. The choice of the order of multivariate polynomial F in the form given by equation (1) is justified by the fact that for higher, even third-degree formulations the resultant models exhibit several extrema, which cannot be explained by the measured data variance. Besides, the existence of several maxima and saddle points in the model makes the problem more difficult to solve. Numerous extrema which might exist in a model fitted to the data will always lead to problems regarding the localness of the optimum; for example, the results obtained might not reflect a global optimum for the process parameters.

Moreover, mathematical solution of the optimum search problem stated in terms of higher-order polynomials becomes impossible by analytical methods, and can only be done by a numerical approach, which should be avoided if possible due to the greater complexity involved. The higher quality of a fit obtainable using polynomials of higher degree than two does not compensate the risk of obtaining false positive optimization results.

For each model presented, the calculated value of standard deviation describes the amount of variation in the data that is not explained by the model; the lower this value, the better. The model's quality of a fit is also indicated by the determined value of R^2 coefficient. This indicates the fraction (or percentage) of the total variance of the data that is explained by the model; the closer this value is to 1 (or 100%), the better. The data fit procedure utilizes linear statistical estimation to obtain a nonlinear model of the dependence of activity on selected explanatory variables A and B . The explanatory variables A and B may denote pH, process time, temperature or concentration, according to the analysis selected.

$$F(A, B) = a_1 + a_2A + a_3B + a_4A \cdot B + a_5A^2 + a_6B^2 \quad (1)$$

The response surface methodology is implemented on the continuous domain over which the fitted model is defined, by applying partial derivative operators to a model mathematical function. The partial derivatives of model F are given by:

$$\frac{dF}{dA} = a_2 + a_4B + 2a_5A \quad (2)$$

$$\frac{dF}{dB} = a_3 + a_4A + 2a_6B \quad (3)$$

The resulting formulations for the analytical solution of equations obtained by setting the expressions in (2) and (3) equal to zero can be solved analytically, representing the optimization problem defined in terms of surface response methodology. The solutions to the problem are given by equations (4) and (5).

$$A_{opt} = \frac{-a_3a_4 + 2a_6a_2}{a_4^2 - 4a_5a_6} \quad (4)$$

$$B_{opt} = -\frac{a_4 a_2 - 2a_5 a_3}{a_4^2 - 4a_5 a_6} \quad (5)$$

The advantage of the response surface methodology optimization presented in the analytical form of solutions (4) and (5) is that it is general in the sense of the model used, and does not require a numerical approach to find the optimal process conditions. It is important to note, however, that this solution is tailored only to the model F presented in (1).

A typical view of the response surface, based on the pH vs. process time model for catalyst activity, is shown in Fig. 3. The model F exhibits only one global maximum, which is justified by observing the measured experimental data. The response surface is a statistical fit, and therefore it extends far beyond the experimental values, in the range $(-\infty, \infty)$. The meaningful range of F is located inside the convex hull containing experimental data points. Although the formal convex hull is not calculated here, it can easily be estimated by examining the minimum and maximum values of the measured data, that is, the explanatory (A and B) variables. Only within this range and in its close neighborhood the response surface exhibits physically meaningful results.

Figure 3

The detailed results obtained by calculation are given in Table 1. Six distinct sets of experimental values were used, which relate selected independent variables A and B to the measured activity of the catalyst. The actual process variables denoted by A and B are given in the first two columns.

Table 1

The values a_i represent the coefficients of a model F for a given relation between variables that affect the catalyst's activity. Their meaning is statistical only, and they have no physical significance. Nonetheless, they play a key role in the model formulation. The optimal values A_{opt} and B_{opt} for process parameters represent the results from the response surface analysis. These provide the values of process parameters—pH, time t , concentration C , and temperature T —that lead to the highest activity of the catalyst. The quality of the models is confirmed by high values of the determined coefficient R^2 , close to one, and relatively low values of the standard deviation in comparison to the activity values.

3.3. Characterization of immobilized enzyme

Based on the result of modelling study and preliminary experiments, immobilization conditions allowing for obtaining the biocatalytic system with the highest catalytic activity were found to be: pH 7, temperature 25 °C, initial enzyme concentration 3 mg/mL and 16 h of immobilization duration. Under these conditions, immobilization yield reached 93% whereas activity recovery attained 95%. Slight decrease in enzyme activity, as compared to free counterpart, might be related to the formation of diffusional limitation in transport of L-catechol and oxygen [46] as well as some distortion of enzyme structure due to its binding to the support [21, 47]. Immobilization of tyrosinase performed under process conditions differ than optimal one, results in a lower activity recovery due to the negative effect of the process conditions on enzyme structure and its activity. As produced immobilized tyrosinase is also characterized by the highest stability among all systems obtained and tested at various immobilization conditions. It should be noted that enzyme loading, beside its influence on enzyme activity [48], may also affect its stability due to possible changes in the distances of enzyme-support interactions in a highly loaded enzymatic systems [49]. It has been reported that high immobilization yield, corresponding with high enzyme loading, leads to improvement in enzyme stability due to crowding formation and reduction of enzyme mobility [50,51]. On the other hand, internal enzyme hydrophobic interactions as well as enzyme-enzyme interactions in the highly loaded support may lead to the irreversible conformational changes resulting in the drop in enzyme activity [52]. Furthermore, the occurrence of diffusional limitations in highly loaded biocatalytic systems cause that apparent activity of these enzymes is higher as compared to the systems where diffusional restrictions did not occur [53].

In addition, to follow the immobilization progress a proper curve was plotted under optimal immobilization conditions (Fig. 4). After 3 h, less than 40% of the activity was immobilized that might be due to the homogenous distribution of support functional groups onto its surface [54]. After 16 h most of the enzyme's activity was immobilized and expressed activity of the immobilized tyrosinase reached 95%. Prolongation of the immobilization increased supernatant residual activity probably due to enzyme elution from the support.

Figure 4

3.4. Removal of Bisphenol A

Free tyrosinase and biocatalytic system produced under optimal immobilization conditions and exhibiting the highest catalytic activity were further applied and compared in the removal of bisphenol A from model water solution under various remediation conditions. Tyrosinase was used in this study because this enzyme is capable for efficient degradation/conversion of mono- and bisphenols [55], and because biomolecules deposited on solid supports, unlike free enzymes, offer improved resistance against inactivation by harsh process conditions and can be easily separated from the reaction mixtures [56], which is of great importance for the treatment of large volume of wastewaters.

Figure 5a shows the percentage removal of bisphenol A by free and immobilized enzyme over time. It can be seen that irrespective of the biocatalyst used, removal efficiency increased gradually over the initial stage of the process, reaching 100% after 60 min and 90 min of the process performed using free and immobilized tyrosinase, respectively. Nevertheless, over analyzed time intervals, the biocatalytic system produced exhibited slightly lower removal rate of BPA than the free enzyme, which may be due to the lower activity of PbCl_2 -chitosan-bonded tyrosinase (an activity recovery of 95%) and accumulation of the products of enzymatic conversion, that lead to the inhibition of the biocatalyst [57]. Furthermore, the results obtained are in agreement with the kinetic parameters obtained for model reaction concerning L-catechol oxidation. The values of the Michaelis–Menten constant (K_m) for free and immobilized enzyme were 1.46 mM and 2.53 mM, respectively. Moreover, the maximum velocity rate (V_{max}) of free enzyme (46.8 U/mg) was higher than the V_{max} of the immobilized enzyme (31.4 U/mg). These data indicate the lower substrate affinity towards tyrosinase after immobilization and may be explained by the formation of diffusional limitations upon immobilization due to partial blocking of enzyme active sites and aggregation of tyrosinase molecules [58]. The changes in the values of the kinetic parameters upon immobilization are also attributed to the fact that immobilized enzyme is located in an environment different from that of its free form, leading to alterations in its microenvironment and causing changes in the enzyme kinetic. Furthermore, changes in the substrate affinity may be related to the changes in enzyme structure and formation enzyme-support interactions [59, 60]. Similar observations were made by Dincer et al. (2012), who immobilized tyrosinase onto chitosan–clay composite beads and noticed high activity

retention accompanied by pronounced changes in enzyme kinetic. Two times lower substrate affinity and 40% lower $V_{\max}$ value of the immobilized tyrosinase were explained by diffusional limitations, steric effects and alteration of enzyme structure [43].

The effect of various process parameters on BPA removal process efficiency was further investigated. As bisphenol A is commonly used as an additive and intermediate product in polymer processing, it frequently occurs in wastewaters at different concentration [61]. Therefore, it was decided to examine the percentage removal of BPA over a wide range of its solution concentrations (from 0.1 mg/L to 5 mg/L), (Fig. 5b). It was evidenced that after 120 min of the process, both, free and immobilized tyrosinase demonstrated excellent catalytic activity, as BPA was totally removed from the solution in concentrations up to 3 mg/L. For solution in the highest concentration (5 mg/L), a drop in the total removal rate of bisphenol A was recorded. Although, higher degradation efficiency of the immobilized tyrosinase was attained, as compared to its performance in lower concentrated BPA solutions, the 5 mg of BPA per 1 L of the solution was too high to be fully converted by the enzyme. Furthermore, some distortions of the enzyme structure caused by such high substrate concentration, may lead to the production of semiinactivated forms of BPA that also decrease the removal efficiency of bisphenol A. Nevertheless, even from the 5 mg/L solution, over 80% of the BPA was removed. For comparison, in our previous study concerning immobilization of laccase onto spongin-based scaffolds, we demonstrated that the immobilized enzyme was capable to remove over 95% of bisphenol A from a solution in concentration of 2 mg/L after 24 h of the treatment process [62].

Another important parameter strongly influencing the stability and catalytic activity of the biocatalysts is the process temperature. The effect of this parameter on the efficiency of BPA removal was investigated over a temperature ranging from 5 °C to 65 °C (Fig. 5c). As shown, the optimum process temperature, for both, free and immobilized enzyme, was found to be 25 °C, as total removal of bisphenol A was noted. Changing the temperature to higher or lower values led to a significant drop in the removal rate of the pollutant by free enzyme. By contrast, immobilized tyrosinase exhibited higher activity over a wider temperature range; it removed over 80% of BPA at temperatures ranging from 15 °C to 55 °C. Even at 65 °C, around 70% of bisphenol A was converted by the immobilized biomolecule, over 30% more as compared to free enzyme. This clearly demonstrates that immobilized

enzyme, protected against thermal inactivation, was more stable as its free counterpart, even at higher temperatures. The improvement in the enzyme's stability against harsh temperature conditions upon immobilization may be explained by the formation of enzyme–support interactions which stabilize the structure of the biomolecule, as well as rigidization of the enzyme, which facilitates its application at higher operational temperatures [63, 64]. Similar observations have been reported by Tamura et al. [65] and Nicolucci et al. [66], who immobilized tyrosinase using ion-exchange resins and polyacrylonitrile beads, for use in the removal of alkylphenols and bisphenol A, respectively. Irrespective of the support material type, enzyme stabilization upon immobilization caused that pollutants were removed more efficiently over a wider temperature range than by free enzyme.

Another key parameter affecting the catalytic activity of biomolecule is pH. The effect of pH on the removal of BPA (pH range 3–10) was investigated (Fig. 5d). As the results demonstrate, pH 7 was found to be the optimum one for both free and immobilized tyrosinase; this is in agreement with previously published studies concerning immobilization and application of mushroom tyrosinase [67, 68]. Beyond this value, BPA removal by the free enzyme gradually decreased: only at pH 7 and 8 it removed more than 80% of the pollutant. By contrast, immobilized tyrosinase displayed improved stability over the whole analyzed pH range. Furthermore, PCL–chitosan-bonded tyrosinase exhibited a less pronounced decline in the BPA % removal under harsh pH conditions - it enabled the removal of over 80% of bisphenol A in the pH range 6–9. The improvement in the activity of tyrosinase upon immobilization, over a wide pH range, is attributed to the stabilization of the enzyme structure and protection of the biomolecule against the dissociation of amino acids caused by adverse effects of acidic and basic conditions (H^+ and OH^- ions) [69]. Similar observations have been reported by Wu et al. [70], who immobilized tyrosinase on polyacrylonitrile beads and used them in the biodegradation of phenol. Authors observed an improvement in enzyme stability over a pH range 6–9; however, total removal of the phenol was not achieved, indicating the higher capability of the biocatalytic system obtained in presented study for the removal of phenolic compounds under various pH conditions.

Figure 5

3.5. Stability and reusability of free and immobilized tyrosinase

It has been reported that immobilization improves the stability of enzymes and facilitates their reuse [7, 36]. As thermochemical and storage stability, as well as reusability, are among the key criteria affecting the practical application of immobilized enzymes, these parameters for free and PCL–chitosan-immobilized tyrosinase we examined and compared (Fig. 6).

The free and immobilized biocatalysts were stored at 4 °C over 20 days, and the storage stability was examined at specified time intervals. As shown in Fig. 6a, the free tyrosinase gradually lost its activity during storage, and after 20 days of the test retained less than 50% of its initial activity. As expected, the decrease in the activity of the immobilized enzyme was lower. Over 5 days of storage no activity loss was observed, and after 20 days of storage PCL–chitosan-immobilized tyrosinase retained over 85% of its initial activity. Although loss of enzymatic activity is a naturally occurring phenomenon, the presented data clearly indicate that upon immobilization of tyrosinase on PCL–chitosan electrospun fibers, followed by the stabilization of the enzyme structure and multiple enzyme attachment [71], this effect may be effectively reduced. Similar observations have been reported in other studies concerning immobilization of various polyphenol oxidases on chitosan-based materials, indicating the positive effect of this biomaterial on enzyme stability [34,72,73].

Evaluation of thermochemical stability of immobilized enzyme over time, under process conditions, is one of the most important criteria to be considered. The stability of free and immobilized tyrosinase over 120 min of a process carried out at pH 7 and 25 °C was examined and compared (Fig 6b). The relative activities of both free and immobilized enzyme declined over time, and after 60 min of incubation reached 70% and 85%, respectively. Prolongation of the incubation time led to a further drop in the relative activity; however, a significantly smaller decrease was observed in case of immobilized tyrosinase. After 120 min of the test the relative activity of free enzyme was around 40%, while PCL–chitosan-bonded tyrosinase retained over 80% of its activity. Observed drop in the catalytic activity may be attributed to changes in the tertiary structure of the enzyme caused by the reaction environment [74]. Nevertheless, the enzyme's thermal stability improved significantly upon immobilization. These results correspond well with the values of the inactivation parameters. The inactivation constant (k_d) and half-life ($t_{1/2}$) of free tyrosinase were measured as 0.0083 1/min and 84.5 min, respectively. In the case of immobilized enzyme, the inactivation constant and half-life were

improved more than fivefold as compared to free tyrosinase, taking values of 0.0015 1/min and 462 min, respectively. The higher thermal stability of immobilized tyrosinase may be explained by several factors, including stiffening of the enzyme structure upon immobilization [75], its protection against distortion and conformational changes of the amino acids in the enzyme structure [76], and the protective effect of the support material against process conditions [77]. All of these factors facilitate the retention of high catalytic activity by immobilized enzyme [78] and improve its thermal stability.

The reusability of immobilized enzyme over repeated reaction cycles is the most important parameter affecting its practical application, as it can provide advantages—including cost reductions—for treatment processes. Thus, the reusability of the PCL–chitosan-immobilized tyrosinase over ten repeated cycles of bisphenol A removal, and under optimal process conditions was tested (Fig. 6c). The efficiency of BPA removal slightly decreased after repeated use, and reached approximately 90% after five reaction cycles and around 80% after ten repeated use. The decline in the removal efficiency is related mainly to the inhibition of the enzyme by the macromolecular products of catalytic conversion, but due to adsorption interaction, partial enzyme elution should not be excluded [79]. The maintenance of high removal of BPA by immobilized tyrosinase, over numerous treatment steps is attributed mainly to the stabilization of the enzyme structure, and improvement of its durability under reaction conditions that facilitate the retention of catalytic activity, as previously reported [80, 81]. Similar conclusions were reached by Veismoradi et al. [82] and Wu et al. [70], who immobilized tyrosinase using polyaniline membrane coated with chitosan, and polyacrylonitrile beads. The biocatalytic systems were used for the removal of azo dyes and phenols over repeated cycles, and because of the stabilization of the enzyme upon immobilization, the pollutants removal of 80% and 60%, after ten repeated uses, were noted, respectively.

Figure 6

4. Conclusions

Experimental data collected proved that PCL–chitosan electrospun composite material is well-suited for the efficient immobilization of mushroom tyrosinase. Data modeling has been applied to determine the optimal immobilization conditions. Multivariate regression analysis based on the least squares

method provided values of polynomial coefficients which produced the statistical best fit to the experimental values, and gave a process parameters such as its duration - 16 h, pH 7 and temperature 25 °C as the optimal ones. The biocatalytic systems produced were further tested in biodegradation of bisphenol A. Obtained dependencies showed that PCL–chitosan-immobilized laccase was capable to efficiently remove BPA over a significantly wider pH and temperature range than free enzyme, clearly indicating improvement of the enzyme stability upon immobilization. Furthermore, the reusability and stability of the immobilized biomolecule over time were also analyzed. The enhanced stability and reusability of immobilized tyrosinase provide economic advantages for large-scale practical applications of the produced systems, as the high biodegradation of bisphenol A suggests that the obtained biocatalytic systems may be considered as a promising tool for the removal of hazardous compounds from wastewaters. Moreover, the obtained electrospun PCL–chitosan material may be a promising alternative to synthetic and natural polymers commonly used as supports in enzyme immobilization; however, further study is still required.

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Table 1. Calculated parameters of models F for selected relations between explanatory (measured) variables A and B .

A	B	a_1	a_2	a_3	a_4	a_5	a_6	A_{opt}	B_{opt}	R^2	Std. dev.
pH	t, min	-111.80	39.404	4.226	0.234	-2.631	-0.146	8.433	21.187	0.944	6.598
pH	$C, g/L$	-128.46	40.853	37.696	0.363	-2.616	-5.874	8.048	3.458	0.906	8.814
pH	$T, ^\circ C$	-145.20	53.332	1.956	-0.043	-3.265	-0.024	7.946	33.669	0.979	3.163
$T, ^\circ C$	$C, g/L$	8.57	1.483	39.145	-0.0050	-0.02	-5.532	36.064	3.522	0.891	8.202
$T, ^\circ C$	t, min	4.592	1.774	7.001	-0.0037	-0.025	-0.188	33.725	18.294	0.96	5.19
$C, g/L$	t, min	4.303	27.46	4.631	0.326	-4.749	-0.146	3.57	19.78	0.893	8.842

Figure captions:

Fig. 1. Scheme presenting fabrication of polycaprolactone–chitosan (PCL–chitosan) electrospun material and immobilization process, with detailed insight into mechanism of performed process.

Fig. 2. SEM images of PCL-chitosan electrospun materials before (a) and after (b) enzyme immobilization and (c) FTIR spectra of tyrosinase, PCL-chitosan electrospun material and PCL-chitosan with immobilized enzyme.

Fig. 3. Response surface for catalyst activity as a function of pH and process time. Dots indicate rescaled experimental values.

Fig. 4. Immobilization course of tyrosinase onto PCL-chitosan electrospun support material. Immobilization was performed at pH 7 and 25 °C using enzyme solution in concentration of 3 mg/mL.

Fig 5. Percentage removal of BPA using free and immobilized tyrosinase vs. process parameters: time (a), initial BPA concentration (b), process temperature (c) and pH of the solution (d).

Fig. 6. Storage (a), thermochemical (b) stability of free and PCL-chitosan immobilized tyrosinase and reusability (c) of the immobilized enzyme over repeated reaction cycles.

Figure 1

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Figure 2

Figure 3

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Figure 4

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Figure 5

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Figure 6

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical abstract

Highlights

- Effective immobilization of tyrosinase onto PCL-chitosan electrospun material
- Optimization of immobilization conditions using response surface methodology
- Application of biocatalytic systems produced for removal of bisphenol A
- Total removal of BPA after 90 min of the process at temperature 25°C and pH 7
- Improvement of enzyme stability and reusability upon immobilization