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Laccase immobilization on enzymatically functionalized polyamide 6,6 fibres

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Abstract

Polyamide matrices, such as membranes, gels and non-wovens, have been applied as supports for enzyme immobilization, although in literature the enzyme immobilization on woven nylon matrices is rarely reported. In this work, a protocol for a *Trametes hirsuta* laccase immobilization using woven polyamide 6,6 (nylon) was developed. A 2^4 full factorial design was used to study the influence of pH, spacer (1,6-hexanediamine), enzyme and crosslinker concentration on the efficiency of immobilization. The factors enzyme dosage and spacer seem to have played a critical role in the immobilization of laccase onto nylon support. Under optimized working conditions (29 U mL^{-1} of laccase, 10% of glutaraldehyde, pH = 5.5, with the presence of the spacer), the half-life time attained was about 78 h (18% higher than that of free enzyme), the protein retention was 30% and the immobilization yield was 2%. The immobilized laccase has potential for application in the continuous decolourization of textile effluents, where it can be applied into a membrane reactor.
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Keywords: Polyamide; Nylon; Immobilization; Crosslinking; Glutaraldehyde; Laccase

1. Introduction

Enzymes are usually stable when stored at low temperatures and neutral pHs in aqueous media. Generally, they are soluble and consequently can only be used once in free solutions [1–5]. The application of immobilization processes allows that enzymes can be recovered from solution and reused. Moreover, the enzyme stability can usually be enhanced as well as the ability to run a process on a continuous basis [1,3].

Enzymes immobilized on synthetic non-woven supports like polyester and nylon have been applied for the gentle treatment of sensitive surfaces, like sensitive skin regions, wounds with difficult healing, valuable documents or paintings [6–8]. A severe drawback, however, is the frequently unfavourable interaction between the enzyme and the surface of the support, which must fulfil some specific criteria such as chemical reactivity, compatibility with the enzyme, insolubility and stability in process solutions [1,9]. Several studies report the immobilization of various enzymes like proteases, glucosidases, endocellulases and laccases onto different nylon matrices [1,6,7,10–12]. However, until now, the state-of-the-art does not report the immobilization of enzymes on woven nylon supports. The application of woven nylon as an immobilization matrix could offer several advantages for enzyme immobilization when compared with other materials such as nylon membranes. Woven nylon is inexpensive, chemically inert, non-toxic, mechanically stable, insoluble in water, readily available and can be obtained in a number of forms [6]. In addition, the immobilization takes place only in the external surface of the support, allowing a better expression of the enzymatic activity [10].

Nevertheless, two severe restrictions hamper the extensive preparation of enzyme-immobilized polyamide surfaces: (1) the absence of strongly reactive groups and (2) the unfavourable interactions of the enzyme with the weak polar surface [8,11]. To overcome these problems, a partial enzymatic hydrolysis of the nylon surface can be performed, without loss of mechanical strength, to generate reactive amino groups, which can be coupled to proteins using glutaraldehyde [6,13,14].

In this study, an immobilization procedure was developed to immobilize *Trametes hirsuta* laccase onto woven nylon supports using glutaraldehyde as the crosslinking agent (Fig. 1). The immobilization procedure also included a spacer (1,6-hexanediamine) in order to increase the distance between the enzyme and the hydrophobic surface of the nylon [15], and

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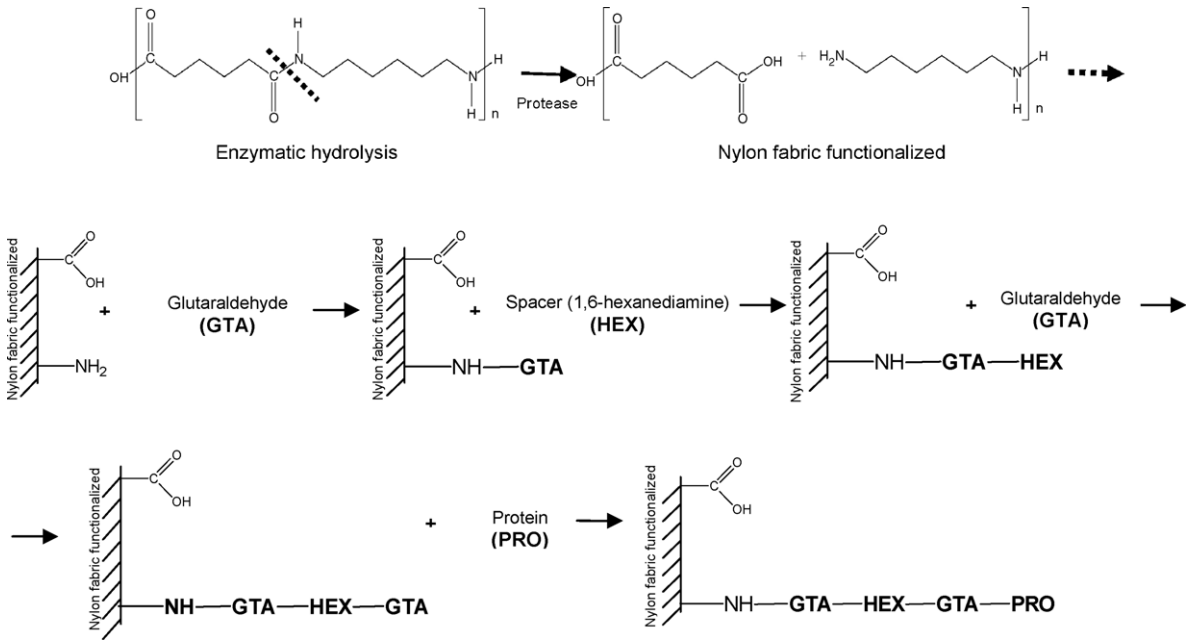

Fig. 1. Possible crosslinking reactions between glutaraldehyde, spacer and protein, on immobilization procedure.

consequently to obtain a higher conformational flexibility for the enzyme, which is usually a prerequisite for higher activity [16]. The possible crosslinking reactions between nylon fabric, glutaraldehyde, spacer and protein are described below (Fig. 1).

Laccases are multi-copper oxidase proteins that use molecular oxygen to oxidize various aromatic and non-aromatic compounds by a radical-catalyzed reaction mechanism [17,18]. The low substrate specificity of these enzymes, associated with their good intrinsic stability properties, has prompted interest for application in biobleaching, wastewater treatment, dye decolourization, cathode fuel cells and biosensors [12,19,20]. Immobilization can protect laccase from denaturation by organic solvents, extend its half-life time and allows enzyme reuse in several reaction cycles [20–22].

A two-level factorial design was adopted in this study for a complete understanding of the effects of pH, spacer, glutaraldehyde and enzyme concentration in the immobilization procedure, and their possible interactions. This method is ideal for the identification of the vital variables that significantly affect the immobilization process and has been applied successfully to study and optimize a different number of biocatalytic and bioseparation processes [23–30]. The major advantage of studying the influence of several parameters by means of factorial design methodology is to distinguish possible interactions among factors, which would not be possible by classical experimental methods, such as the one-factor-at-a-time approach [31].

2. Materials and methods

2.1. Enzymes and reagents

Commercial polyamide 6,6 fabric (nylon), a plain woven structure with 63 g m^{-2}, was supplied by Rhodia (Switzerland). Esperase, a subtilisin from *Bacillus* sp. (E.C. 3.4.21.62), was a commercial protease purchased from Sigma (St. Louis, USA) and the laccase from *T. hirsuta* (E.C. 1.10.3.2) was obtained from Institute of Biotechnology of Graz (TUG). Glutaraldehyde 50% solution in water and the azine-2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were purchased from Sigma (St. Louis, USA) and Lutensol AT 25 was acquired from BASF (Ludwigshafen Germany). All other reagents used were of analytical grade.

2.2. Support preparation and activation

The polyamide fabric used in this work was submitted to a previous washing with 2 g L^{-1} of a non-ionic agent, Lutensol AT 25 that belongs to the group of $C_{16}C_{18}$ fatty alcohol ethoxylates and distilled water for 1 h, followed by washing with a 2 g L^{-1} of Na_2CO_3 solution for 1 h, both at 50 °C.

2.2.1. Enzymatic functionalization of nylon

Cleavage of the amide bonds of the polymer surface was achieved by hydrolysis with a protease (Esperase from *Bacillus* sp.). In 100 mL of Tris–HCl buffer (0.3 M Tris; 3 M HCl; pH = 7.5), 0.7 g ± 0.1 g (5 × 5 cm^2) of washed polyamide fabrics were incubated with 18 U mL^{-1} of Esperase (activity measured according to the method described by Silva et al. at 37 °C for 24 h under continuous orbital agitation using an Erlenmeyer held in a shaking water bath, operating at 90 rpm min^{-1} [32]. After 24 h of incubation, the fabrics were removed from the liquor and washed in sodium carbonate solution (2 g L^{-1}) for 2 h to stop the enzymatic reaction, followed by washing with 2 g L^{-1} of Lutensol AT 25 solution for 1 h. After that, the samples were rinsed in running cold tap water for 5 min and allowed to air dry.

2.2.2. Activation of nylon

Woven nylon pieces (0.23 ± 0.05 g) were place in different glutaraldehyde solutions (2%, 15% and 28%) in borate buffer (0.1 M; pH = 9) at room temperature (25 °C) for 2 h. The non-reacted glutaraldehyde was removed by washing with a 0.1 M of potassium phosphate (pH = 7.5).

2.2.3. Introduction of a spacer (1,6-hexanediamine)

After activation of nylon samples with glutaraldehyde, half of the samples were placed in 40 mL of 0.5 M 1,6-hexanediamine in 0.1 M Na_2CO_3/$NaHCO_3$ buffer, pH = 9.5, for 4 h at room temperature. Then, the samples were washed thoroughly with distilled water followed by washing with 0.1 M of potassium phosphate (pH = 7.5).

Table 1
Factor levels used according to the 2^4 factorial design

Variable	Level		
	−1	0	1
A: pH	4	5.5	7
B: Spacer	Without	Without/with	With
C: Glutaraldehyde (%)	2	15	28
D: Enzyme (U mL^{-1})	10	49	88

2.2.4. Reactivation of nylon

All the samples were reactivated with different glutaraldehyde solutions (2%, 15% and 28%) in borate buffer (pH = 9) at room temperature (25 °C) for 2 h. The non-reacted glutaraldehyde was removed by washing with a 0.1 M of potassium phosphate (pH = 7.5) buffer.

2.2.5. Laccase Immobilization

Treated woven nylon pieces were incubated with laccase solutions containing 10, 49 or 88 U mL^{-1} of enzyme at different pHs (acetate buffer 0.2 M for pH = 4 and 5.5; phosphate buffer 0.1 M for pH = 7). Nylon fabric was maintained in laccase solutions overnight at 4 °C. After that, the supernatant was kept for protein measurements and the nylon samples were washed three times with 0.1 M of potassium phosphate (pH = 7.5) buffer. The solutions produced from the three washing steps were stored for protein determination.

2.3. Experimental design

The influence of pH (A), spacer (B), glutaraldehyde concentration (C) and enzyme concentration (D) on laccase immobilization was studied using a 2^4 full factorial design with four repetitions at the central point (Table 2). The variable (B) was categorical and therefore two conditions were tested: without the spacer and with the spacer. The range and the levels of the variables investigated in this study are given in Table 1 and were chosen based on preliminary studies. For statistical calculations, the variables were coded according to Eq. (1):

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

Table 2
Values for half-life time of immobilized laccase (HLT), protein retention (PR) and immobilization yield (IY), according to the 2^4 factorial design

Assay	Variables				Responses		
	A	B	C	D	HLT (h)	PR (%)	IY (%)
1	−1	−1	−1	−1	38	47.2	2.2
2	1	−1	−1	−1	35	39.2	2.4
3	−1	1	−1	−1	70	37.5	4.3
4	1	1	−1	−1	60	32.5	4.2
5	−1	−1	1	−1	41	36.4	2.2
6	1	−1	1	−1	36	26.0	2.1
7	−1	1	1	−1	61	30.2	5.2
8	1	1	1	−1	65	32.1	5.1
9	−1	−1	−1	1	50	22.6	0.2
10	1	−1	−1	1	70	15.3	0.1
11	−1	1	−1	1	63	33.3	0.2
12	1	1	−1	1	78	20.4	0.2
13	−1	−1	1	1	41	10.0	0.3
14	1	−1	1	1	43	16.5	0.3
15	−1	1	1	1	134	13.8	0.5
16	1	1	1	1	139	5.3	0.5
17	0	0	0	0	62	18.5	0.8
18	0	0	0	0	95	17.4	0.8
19	0	0	0	0	45	16.4	0.1
20	0	0	0	0	63	14.4	0.5

where x_i is the independent variable coded value, X_i the independent variable real value, X_0 the independent variable real value on the centre point and ΔX_i is the step change value.

The Design-expert version 7.0 – free evaluation version (Stat-Ease Inc., Minneapolis, USA) was used for regression and graphical analysis of the data. The half-life time of immobilized laccase (HLT, hours), the protein retention (PR, %) and the immobilization yield (IY, %) were taken as the responses of the design experiments. The statistical significance of the regression coefficients was determined by Student's *t*-test and the model equation was determined by Fischer's test. The proportion of variance explained by the model obtained was given by the multiple coefficient of determination, R^2. The optimum conditions were obtained by the graphical analysis using the Design-expert program.

2.4. Laccase activity and protein determination

Laccase activity was measured as the oxidation of ABTS according to the method described by Childs and Bardsley [33]. In the case of free enzyme solution, 1 mL of diluted enzyme (in acetate buffer, pH = 5, 0.1 M) was mixed with 1 mL of ABTS (0.5 mM) solution in water, in a disposable cuvette. The increase in absorbance was followed at 420 nm ($\varepsilon = 36\,\text{mM}^{-1} \times \text{cm}^{-1}$) for 2 min. The spectrophotometer was zeroed with the $\text{ABTS}_{\text{zero}}$ sample, which contained 1 mL of acetate buffer (0.1 M, pH = 5) and 1 mL of ABTS solution. The experiment was performed at 25 °C. Activity in units (U) was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute [33].

A similar procedure was adopted to measure the activity of laccase bounded to the woven nylon. The immobilized sample (0.073 ± 0.011 g) was soaked in a stirred cell, outside the range of the laser light, containing 1 mL of acetate buffer (0.1 M, pH = 5). This solution was mixed with 1 mL of ABTS and the absorbance of the supernatant was measured at 420 nm ($\varepsilon = 36\,\text{mM}^{-1} \times \text{cm}^{-1}$) for 10 min. The final activity of immobilized laccase in U/mL was converted to activity in U/mg of fabric used in the assay. The immobilization yield was determined by dividing the activity value of immobilized laccase, obtained immediately after the immobilization procedure, by the value of activity of the initial laccase solution, converted to U/mg. A conversion factor of 0.086 mL/mg was used, considering the ratio of total volume of the immobilization solution (20 mL) by the total weight of nylon fabric used to immobilize the enzyme (233 mg).

The half-life time for the native and immobilized enzymes was determined by measuring the remaining activity of the fabrics after 24, 48 and 96 h of incubation at 25 °C. The decay curves attained fitted well a first-order exponential curve, and the time at which half of the initial activity was lost was assigned as half-life time.

The protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as a standard [34]. The amount of bound protein was determined indirectly by the difference between the amount of protein introduced into the reaction mixture and the amounts of protein in the supernatant and washing solutions.

3. Results and discussion

Laccase from *T. hirsuta* was immobilized on woven nylon supports, previously hydrolyzed with a protease for amine activation, based in a methodology described by Silva et al. [32]. It is known that the cleavage of the secondary amide linkages reduces the mechanical strength of the woven nylon. Therefore, the extent of amide bond cleavage represents a compromise between the adequate supply of free amino groups and the structural integrity of the polymer [32,35]. In the case studied, the total strength loss obtained after enzymatic surface treatment was lower than 3%, consequently the integral structure of the polymer was well preserved.

In the immobilization procedure, the concentrations of laccase and glutaraldehyde were varied, as well as the pH. In addition, two different approaches were used by immobilizing the enzyme in the absence and in the presence of a spacer (1,6-

hexanediamine). The introduction of a spacer enables a decrease in the steric hindrance effects and consequently increases enzymatic activity.

After the immobilization procedure and according to the variations of the factors imposed by the design (Table 2), the activity of the immobilized laccase was measured as previously described. As expected, the immobilized enzyme presented absolute activity values lower than those obtained for the free enzyme, presenting also a slower kinetics (data not shown) for the oxidation of the soluble substrate ABTS. This evidence can be explained by the mass transfer limitations of the substrate. In this system, where the enzyme is covalently incorporated onto a nylon matrix, the consumption of substrate and the release of the product resulting from enzymatic activity to the aqueous medium, is limited by external and internal constrains that can be related with the steric hindrance effect of the woven nylon support [9]. Therefore, the activity of the immobilized laccase on woven nylon depends on several variables such as: area distribution of immobilized enzyme, level of support activation, density of enzyme binding sites, the surface charge, the hydrophilicity of the support, the bulk mass transfer and local diffusion of the system [9]. The polymeric microenvironment and the covalent interactions connecting the biocatalyst to the support usually lead to a reduction in the enzyme mobility. Despite these drawbacks, the half-life time of immobilized laccase could be improved when compared with the free enzyme. The inclusion of a spacer, 1,6-hexanediamine, was essential to improve enzyme conformational flexibility and enhance enzymatic activity.

Table 2 shows the designed experiment matrix, together with the experimental results. Regression analysis was performed to fit the response functions (half-life time, HLT; protein retention, PR and immobilization yield, IY) with the experimental data (Table 3).

Both the Student's *t*-test and *P*-value statistical parameters were used to verify the significance of the considered factors. In this study, factors having a confidence level higher than 95% were considered to a further analysis of the responses in the area studied.

According to the Student's *t*-test results, the most significant parameter for the responses PR and IY was the amount of enzyme, which was significant at a probability level of less than 0.0005. Analysing the values attained for IY in Table 2, it is clearly seen that the higher values were attained in assays 1–8 (were the enzyme dosage was in the lowest level) comparing to assays 9–16. Also, the presence of the spacer (factor B) increased the IY (assays 3–4 and 7–8), confirming therefore its positive effect. The response half-life time was also increased by the presence of the spacer, where the highest values for this response were attained when the factors glutaraldehyde concentration and enzyme dosage were in the higher level (assays 15–16).

Surprisingly, for the response HLT, the most influencing parameter was the presence/absence of the spacer. This variable had a positive effect, meaning that its presence could significantly increase the half-life time for the immobilized enzyme. This fact can be justified by the increase in the stability of the immobilized laccase. It is known that immobilization often brings an increase in the operational stability of enzymes [36,37]. Silva et al. found an increase in half-life time of 46 days at room temperature, when a protease (Esperase) was immobilized on the polymer Eudragit S-100 [38]. Enhanced stability seems to depend on the rigid conformation of the enzyme modified by the crosslinker and/or by covalent binding to the polymer, where the introduction of a spacer can further help in stabilizing the tertiary structure of the enzymes

Its effect was also positive for the immobilization yield, basically for the same reasons. Note that without the spacer, the

Table 3
Estimated coefficients, standard errors and Student's *t*-test for half-life time (HLT), protein retention (PR) and immobilization yield (IY) using the 2^4 full factorial design

Factors	HLT (h)			PR (%)			IY (%)		
	Effects	Standard errors	*t*-values	Effects	Standard errors	*t*-values	Effects	Standard errors	*t*-values
Intercept	64.000	±3.71	–	26.14	±0.77	–	1.87	±0.079	–
A: pH	1.75	±3.71	0.47	−2.730	±0.77	−3.55[c]	−0.007	±0.079	−0.09
B: Spacer	19.75	±3.71	5.32[b]	−0.500	±0.77	−0.65	0.660	±0.079	8.35[c]
C: Glutaraldehyde	6.000	±3.71	1.62	−4.85	±0.77	−6.30[b]	0.160	±0.079	2.03
D: Enzyme	13.25	±3.71	3.57[c]	−9.000	±0.77	11.69[a]	−1.58	±0.079	−20.00[a]
AB	0.000	±3.71	0.00	−0.320	±0.77	−0.42	−0.014	±0.079	−0.18
AC	−1.000	±3.71	−0.27	1.420	±0.77	1.84	−0.014	±0.079	−0.18
AD	3.500	±3.71	0.94	−0.039	±0.77	−0.05	0.015	±0.079	0.19
BC	10.000	±3.71	2.69[c]	−0.440	±0.77	−0.57	0.160	±0.079	2.03
BD	6.500	±3.71	1.75	1.560	±0.77	2.03	−0.590	±0.079	−7.47[c]
CD	6.000	±3.71	1.62	−0.890	±0.77	−1.15	−0.046	±0.079	−0.58
ABC	1.500	±3.71	0.40	−0.002	±0.77	−0.003	0.021	±0.079	0.27
ABD	−0.250	±3.71	−0.07	−2.240	±0.77	−2.90[c]	0.016	±0.079	2.03
ACD	−2.500	±3.71	−0.67	0.860	±0.77	1.12	0.025	±0.079	0.32
BCD	11.000	±3.71	2.96[c]	−2.460	±0.77	3.19[c]	−0.120	±0.079	−1.52
Centre point (1)	9.25	±11.73	0.79	−9.200	±2.43	−3.78[c]	−0.760	0.25	−3.04
Centre point (2)	−4.75	±11.73	−1.28	−9.720	±2.43	−4.00[c]	−1.850	0.25	−7.40[c]

[a] $P < 0.0005$; [b] $P < 0.0050$; [c] $P < 0.0500$.

maximum half-life time attained for immobilized laccase was 70 h, when the maximum amount of enzyme was used, which is quite close to the half-life time of native enzyme (64 h) in the same working conditions. With the introduction of 1,6-hexanediamine, the half-life time increased significantly, and it reached 139 h when factors C and D were in the higher level.

The main factor concentration of crosslinker was significant only for PR. This response also presented as significant the amount of enzyme added, both showing negative effects. Glutaraldehyde is an effective crosslinker of proteins, able to link the amino groups of the enzyme to the fabric and moreover it can promote self-oligomerization reactions [13]. If the nylon surface is saturated with enzyme and crosslinker, the recovery will be lower, as the study confirms.

The other factor studied, pH (factor A), showed no significance at less than 95% confidence level for the responses HLT and IY and it showed a small effect in the response PR, at less than 95% confidence level.

3.1. Effect of parameters on half-life time

The model expressed by Eq. (2), where the variables take their coded values, represents the half-life time (HLT) of the prepared immobilised conjugates as a function of the spacer (B), glutaraldehyde concentration (C) and enzyme dosage (D).

$$\text{HLT (h)} = 64 + 19.75\text{B} + 13.25\text{D} + 10\text{BC} + 11\text{BCD} \quad (2)$$

The statistical significance of the linear model equation (Table 4) was evaluated by the *F*-test analysis of variance (ANOVA), which revealed that this regression is statistically significant ($P < 0.0001$) at a 99% confidence level. The model did not show lack of fit and presented a determination coefficient of $R^2 = 0.82$, that explains 82% of the variability in the response.

The contour plot attained for the half-life time in the area studied is presented in Fig. 2. It shows a different behaviour when the spacer is either present (Fig. 2b) or absent (Fig. 2a). Without the spacer, the maximum half-life time predicted by the model is around 73 h, in a good agreement with the measured data, which was attained when the lower level of glutaraldehyde and the higher level of enzyme were used. With the introduction of the spacer, the profile of this response changes and the predicted half-life times increase considerably, varying from 73 h to 110 h. The best conditions to maximize this response are therefore attained when both enzyme and glutaraldehyde are used in the higher levels.

Table 4
Analysis of variance (ANOVA)[a] for the representative model of half-life time for immobilized laccase at 25 °C, in the area studied

Source	SS	d.f.	MS	*F*-value	*P*
Model	13079.45	4	3269.86	14.73	<0.0001
Curvature	173	2	86.50	0.39	0.6850
Residual	2886.50	13	222.04		
Lack of fit	*2230*	*11*	*202.73*	*0.62*	*0.7581*
Pure error	*656.50*	*2*	*328.25*		
Total	16138.95	19			

SS = sum of squares; d.f. = degrees of freedom; MS = mean square.
[a] $R^2 = 0.82$; CV = 23.12%.

Analysing the contour plots obtained for this response (Fig. 2), it is possible to see, by the shape of the curve, that the interaction effect among enzyme and glutaraldehyde becomes significant for a higher concentration of crosslinker.

3.2. Effect of parameters on protein retention

The response PR, which measures the retained protein in the nylon fabric, was also measured. According to the Student's *t*-test, factor D (enzyme dosage) was extremely significant for this response, having a negative effect. The concentration of glutaraldehyde was also significant at less than 95% of confidence level. Although pH was the less significant factor, it was decided to include it in the model to minimize the error. Again, the interaction of the spacer, glutaraldehyde and the enzyme was significant. It seems that these three factors are extremely dependent on each other, justifying the third order interaction as significant. The model to represent PR in the studied region is

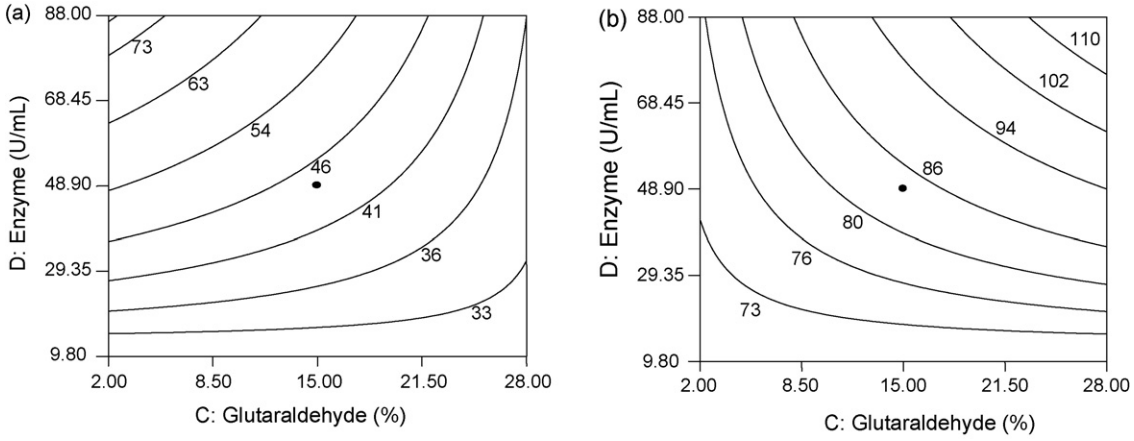

Fig. 2. Contour plot showing the effect of laccase and glutaraldehyde concentration: (a) without the spacer and (b) with the spacer, on the half-life time of the immobilized enzyme at 25 °C. The other factors were kept at the central level.

Table 5
Analysis of variance (ANOVA)[a] for the representative model of protein retention, in the area studied

Source	SS	d.f.	MS	*F*-value	*P*
Model	1967.52	5	393.50	35.54	<0.0001
Curvature	288.78	2	144.39	13.04	0.001
Residual	132.88	12	11.07		
Lack of fit	*126.13*	*10*	*12.61*	*3.74*	*0.2295*
Pure error	*6.75*	*2*	*3.38*		
Total	2389.18	19			

SS = sum of squares; d.f. = degrees of freedom; MS = mean square.
[a] $R^2 = 0.94$; CV = 13.72%.

Table 6
Analysis of variance (ANOVA)[a] for the representative model of immobilization yield, in the area studied

Source	SS	d.f.	MS	*F*-value	*P*
Model	51.69	3	17.23	173.12	<0.0001
Curvature	6.42	2	3.21	32.24	<0.0001
Residual	1.39	14	0.100		
Lack of fit	*1.10*	*12*	*0.092*	*0.63*	*0.7576*
Pure error	*0.29*	*2*	*0.15*		
Total	59.50	19			

SS = sum of squares; d.f. = degrees of freedom; MS = mean square.
[a] $R^2 = 0.97$; CV = 19.61%.

therefore:

$$\mathrm{PR}\,(\%) = 26.14 - 2.73\mathrm{A} - 4.85\mathrm{C} - 9\mathrm{D} - 2.24\mathrm{ABD} - 2.46\mathrm{BCD} \quad (3)$$

Table 5 shows the analysis of variance for the response PR. It shows that the considered model is extremely significant in the area studied ($P < 0.0001$) and presents a high determination coefficient ($R^2 = 0.94$), thus explaining 94% of the total variation in the response, the rest (6%) being explained by the residues. This is proof that the model describes the studied region well.

Although this model presented significant curvature ($P = 0.001$), showing that the studied area should be extended to perform a correct analysis, our goal was to study the influence of these parameters on the three evaluated responses (HLT; PR, IY) and to maximise them in this range, so the model was accepted. Another proof that validates the model, as can be seen from the ANOVA table, is that it presents no lack of fit and its significance ($P < 0.0001$) is much higher than the curvature's probability level ($P = 0.001$), having also the residuals distributed along a well-randomised straight line.

The contour plots attained for this model are shown in Fig. 3. The protein retention was similar for the two considered situations: with and without the spacer, confirming the non-significance of factor B. This response showed higher values when both glutaraldehyde concentration and enzyme dosage were in the lower levels. Factor pH was negative and therefore, lower pHs increase protein retention. This fact might be explained by the increase in the electrostatic interactions of the enzyme and crosslinkers with nylon fabric, therefore increasing the amount of protein retained.

3.3. Effect of parameters on immobilization yield

Finally, the last response studied was the immobilization yield (IY). From Table 6 it can be seen that the main effects B and D and their interaction were significant at less than 5% probability. The linear model then obtained was:

$$\mathrm{IY}\,(\%) = 1.87 + 0.66\mathrm{B} - 1.58\mathrm{D} - 0.59\mathrm{BD} \quad (4)$$

Analysing the ANOVA table it can be seen that the model was highly significant, presenting no lack of fit and the R^2 value, being the measure of the goodness-of-the-fit, indicates that 97% of the total variation is explained by the model.

Nevertheless, this model also presented significant curvature. By the same reasons stated above, the model will be accepted to optimize the immobilization methodology in the area studied. A rotation of the experimental plan had to be considered to account for curvature, if the objective of the study was the modelling of the responses. In that situation, a quadratic model would be attained.

The contour plots attained for the immobilization yield are presented in Fig. 4. The graphs clearly show the dependence of this response on enzyme dosage, since glutaraldehyde con-

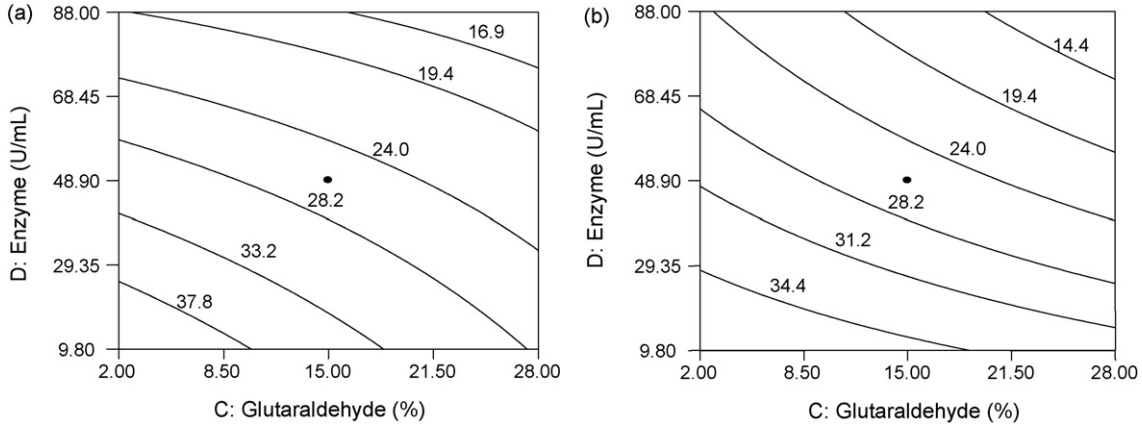

Fig. 3. Contour plot showing the effect of laccase and glutaraldehyde concentration: (a) without the spacer and (b) with the spacer, on the protein retention at 25 °C. The other factors were kept at central level.

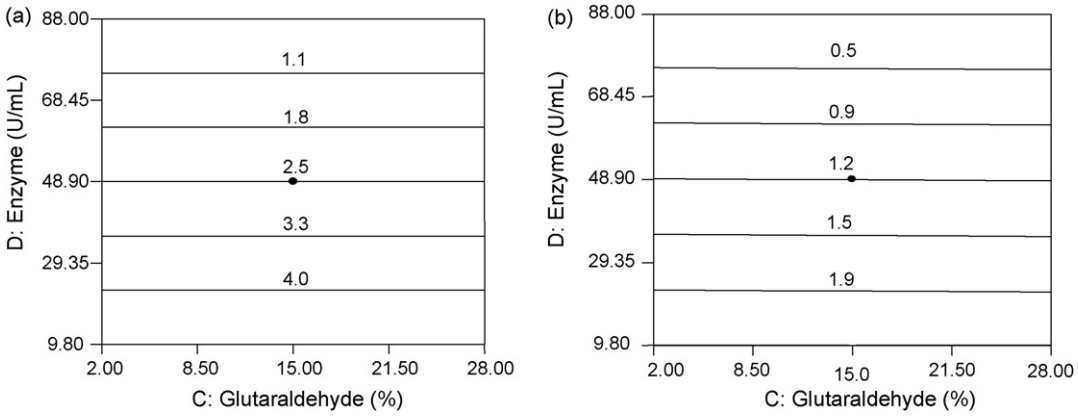

Fig. 4. Contour plot showing the effect of laccase and glutaraldehyde concentration: (a) without the spacer and (b) with the spacer, on the immobilization yield at 25 °C. The other factors were kept at central level.

centration was not significant and therefore not included in the model. The contour plots, by a matter of uniformity, were built with the same two factors (C and D). Again, factor D, enzyme dosage, has a negative effect and consequently, the immobilization yield is improved at lower levels of enzyme added to the media. The influence of the spacer is positive, as previously observed in the other analysed responses. As a result, higher immobilization yields are attained in the presence of the spacer.

Given that the intention of the study was to optimise the immobilisation procedure in order to maximise all the analysed responses, the graphical optimisation of the statistical program 'Design-expert' was performed. The method basically consists of overlaying the curves of the models according to the criteria imposed [27]. Based on the three models obtained, a graphical optimisation was conducted using the statistical program 'Design-expert', defining the optimal working conditions to attain high half-life times, protein retention and immobilization yields. The criteria imposed for the preparation of the enzyme conjugates were: (a) the half-life time at 25 °C should be no less than 75 h, (b) the protein retention should be more than 30% and (c) the immobilization yield should be above 3%. The overlay plot attained (Fig. 5) shows a shaded area where all these criteria are satisfied simultaneously. The pH was kept at the central level and the optimization was investigated when the spacer was present, since this effect was always positive for the three responses evaluated.

Thus, a point was chosen on the graph (marked by the circle), which was assigned as optimum point corresponding to 10% (v/v) of glutaraldehyde and 29 U/mL of laccase. Analysing the plot attained for the graphical optimization, one can see that the same responses can be attained using medium levels of enzyme and lower levels of glutaraldehyde, or contrarily, using lower levels of enzyme and higher amounts of crosslinker. These factors were chosen assuming a compromise between their lowest possible values, for economic reasons. Under the optimized conditions, the models predicted a half-life time of 78 h (a variation of 64–87 h being possible), protein retention of 34% (a variation of 30% to 34% being possible) and immobilization yield of 3.4% (a variation of 3.4% to 3.9% being possible) in the confidence range of 95%.

To confirm these results, a validation assay was conducted in the conditions imposed as the optimum, i.e., immobilization conducted in the presence of the spacer, using 10% of glutaraldehyde, 29 U/ml of laccase and at pH 5.5.

In this assay, a half-life time of 78 h, protein retention of 34% and immobilization yield of 2% were attained. Fig. 6 shows the activity decay for native and laccase immobilized at optimized conditions.

The values reached in the validation assay for HLT and PR are in good agreement with the predicted values for the analysed responses, validating the mathematical linear models attained in the studied region, except for IY.

For the response immobilization yield, the attained value was only 2%, which was out of the interval of prediction in the 95% confidence level. This fact can be explained by the high determination errors in the analysis of this parameter. First,

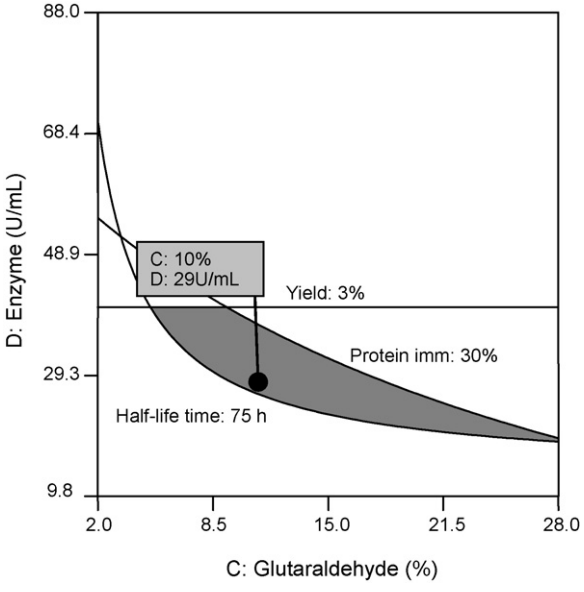

Fig. 5. The optimum region by overlay plots of the three responses evaluated (half-life time, protein retention and immobilization yield) as a function of glutaraldehyde and enzyme concentration. Factor pH was kept at the central level and factor spacer was in the upper level.

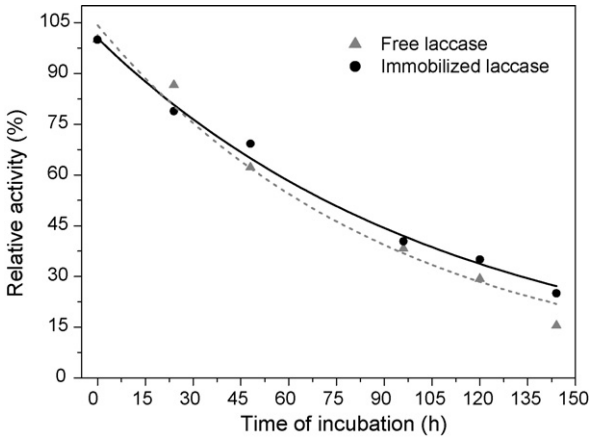

Fig. 6. Half-life time for native laccase (HLT = 64 h) and laccase immobilized at the optimized conditions onto a nylon support (HLT = 78 h), measured at different periods of incubation at 25 °C, pH = 5.

it represents a ratio of the immobilized enzyme at the fabric surface to the native enzyme initially present in solution. Secondly, the model shows a high significant curvature (see Table 6) and consequently the linear model is not adequate. Finally, one has to consider the method of analysis. The initial activity was measured with the enzyme free in the solution, completely capable of interacting with the soluble substrate, ABTS. After the immobilization of the laccase, the method to determine the remaining active enzyme consisted in using a small piece of nylon fabric, which was introduced into the sample cell in the spectrophotometer, therefore enabling the determination of the activity of the laccase in U/mg. The release of nylon micro fibrils to the medium can interfere with the spectrophotometer measurements, weakening the signal (∼1%), which in turns might induce higher values for the activity of the immobilized laccase. Therefore, to overcome this systematic error, all the activity measurements were expressed in terms of relative activity.

4. Conclusions

In this study, a multi-step procedure to immobilize laccase, from *T. hirsuta*, onto a woven nylon matrix was developed. Four variables that could influence the immobilization procedure, namely pH, spacer, enzyme and glutaraldehyde concentration were studied and an optimum work region was achieved.

Woven nylon matrix seems to have high potential as an immobilization support since it is inexpensive and readily available. At the same time glutaraldehyde, used as a crosslinking agent, is much less hazardous and easy to handle, when compared with other reticulating agents.

The application of a heterogeneous biocatalysis via immobilization can lead to a reduction of downstream processing costs and the solid biocatalyst can be reusable. Moreover, the laccase showed a high ability to be used as biocatalyst and its immobilization onto woven nylon presents new insights about future applications. The laccase immobilization onto nylon matrices seems to be a promising system for bioremediation of contaminated soils, wastewater treatment, wine and other beverages stabilization. Nylon woven can also probably be applied as support of laccase, used on biosensors applications [39].

In conclusion, woven nylon matrices show a high ability to act as supports for immobilizing several enzymes like laccases or proteases and other different products such as perfumes or medical drugs. In this area a more exhaustive investigation is being carried out.

Acknowledgments

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