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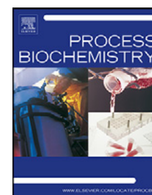

Immobilization of commercial laccase on spent grain

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ABSTRACT

The aim of this work was to assess the possibility of using beer spent grain (a byproduct of beer's brewing industry) as a carrier for laccase immobilization. Both adsorption (on spent grain – SG and on digested spent grain – DSG) and covalent binding (using glycidol and glycidol followed by ethylenediamine on DSG) were used. The effect of different immobilization conditions on the immobilization yields and recovered activities such as contact time, enzyme concentration and pH was evaluated. For the best conditions, immobilization yields, recovered activities and thermal, operational and storage stabilities were also evaluated. Finally, the Michaelis–Menten mechanism was applied and the parameter with respect to ABTS oxidation was determined.

Enzyme immobilization on DSG led to the best enzyme activities (recovered activities as high as 90%) and to high storage and operational stabilities (10 cycles). Thermal stability was also improved and the half-life of immobilized laccase in SG increased from 0.64 h to 1.1 h at 70 °C.

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1. Introduction

Over the last decades, progress in understanding the environmental impacts of pollutants has promoted the development of new treatment technologies. To overcome limitations of traditional treatment processes, research has focused on the use of enzymes for the elimination of a wide range of contaminants. In order to improve the efficiency and cost of the enzymatic process, the enzyme can be reused by an immobilization technique. This capacity to retain the enzyme on a support also allows an easy biocatalyst separation from product permitting continuous processes and prevents the deactivation of protein or loss of activity.

Laccases (EC 1.10.3.2) are multi copper oxidases that can catalyse the oxidation of phenolic substrates with concomitant reduction of O_2 and do not require hydrogen peroxide as co-substrate or any cofactor for the enzymatic reaction [1]. Due to their capability of catalysing the oxidation of aromatic compounds, laccases are attracting increasing attention as potential industrial enzymes in various applications, such as pulp delignification [2], dye decolorization [3,4], and contaminated water or soil remediation [5].

The choice of the most suitable method to immobilize the desired enzyme depends on both the reaction and environmental conditions, the configuration of the reaction apparatus, as well as the carrier to be used. Also, the choice of the most suitable carrier on which to perform immobilization, besides depending on the immobilization method, is influenced by its chemical properties (composition; hydrophilic or hydrophobic nature; stability in the conditions of the reaction medium; etc.), its mechanical stability and geometric properties (size, shape, thickness, porosity, etc.), the characteristics of the enzyme to be immobilized (chemical composition, physical properties, structure, etc.) and its ecological and biological properties (non-toxic neither to the environment or to humans; easily disposable; etc.) [6]. In order to achieve an economically viable application at industrial level, some works on effective cheap supports for enzyme immobilization have been reported, like functionalized rice husk for invertase immobilization [7] and rice straw for lipase immobilization [8] and coconut fibre for laccase immobilization [9].

Spent grain (SG) is the most important by-product of the brewing industry which makes it economically attractive. In Portugal, SG is a disposable material of the Unicer group (beer producer) with no associated costs. It is an abundant waste consisting of the solid residue generated after mashing and lautering processes [10]. Its composition contains many functional groups such as carboxyl, hydroxyl and amino [11] that makes it a potential support for enzyme immobilization. Although large amounts are generated

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throughout the year, SG has received little attention as a low-cost raw material and its use is still limited in large scale. The main application of SG is for animal feed; however it is possible to find in the literature the following applications [10]: energy production, paper manufacture, adsorbent, source of added-value products and substrate for cultivation of microorganisms. Specifically, the use of SG as supports for immobilization of enzymes or microorganisms is still poorly studied. Some studies about the use of SG as a solid support for yeast immobilization in fermentation processes have been reported. Brányik et al. [12,13] studied the conditions of immobilization of brewing yeast onto a cellulose based carrier obtained from SG in a continuous bioreactor. In another study, the fermentation efficiency of yeast cells immobilized on delignified brewers' spent grains was evaluated with acceptable yields [14].

It is well known that laccases have been immobilized on various types of supports, such as silica [15], activated carbon [16–18], Eupergit C [16], chitosan [19], alginate [20] among others. The laccase substrates immobilization by covalent attachment onto siloxanes was also evaluated by Rollett et al. [21]. To date, no research about the laccase immobilization on spent grain has been reported. So, considering the advantages of enzyme immobilization and the features of laccase, the objective of this work was to evaluate the laccase catalytic behaviour under different medium conditions, at laboratorial scale, when it is immobilized by adsorption or covalent bond on brewer's spent grains.

2. Materials and methods

2.1. Chemicals and enzyme

2.1.1. Enzyme

Pure commercial laccase (DeniLite base; 800 U/g) from genetically modified *Aspergillus* was kindly provided by Novozymes.

2.1.2. Support

Brewer's spent grain (SG) was kindly donated by UNICER, S.A. (Porto, Portugal). It was washed with distilled water and dried at 60 °C before being used as an immobilization matrix.

2.2. Immobilization technique

In all experiments, 1.0 mL of enzyme solution was put in contact with 0.1 g of SG or modified-SG under stirring. The specific conditions are detailed below. After immobilization, spent grains were washed several times with phosphate buffer (0.1 M, pH 7.0).

2.3. Functionalization of spent grain

The modifications of SG were carried out as follows. First, SG was acid/base treated to obtain digested spent grain (DSG). DSG carrier was prepared by mixing dry spent grains (50 g) in 0.75 L of 3% (v/v) HCl solution at 60 °C for 2.5 h in order to hydrolyse the residual starchy endosperm and embryo of the barley kernel present in the spent grains. The mixture was then cooled, washed with water, and dried. The remaining solids were partially delignified by shaking (120 rpm) in 500 mL of 2% (w/v) NaOH solution at 30 °C for 24 h. After that, the carrier (5 g) was washed several times with water until neutral pH was reached and dried.

Glycidol-modified spent grains (GSG) were prepared by incubation of DSG in NaOH (1.7 M) with $NaBH_4$. The 1% glycidol was gently poured into this suspension up to a final concentration of 2 M and was kept under stirring for one night at 4 °C. The support was washed with distilled water and $NaIO_4$ (0.1 M) and diluted 10 times and left to oxidize for 2 h. The support was then washed with distilled water, dried and stored at 4 °C.

Ethylenediamine-modified glycidol spent grain (EAGSG) was prepared as described by Rocha et al. [22]. The glyoxyl-spent grains were activated with 1 M ethylenediamine at pH 10.0 for 2 h. Sodium borohydride was added and the support was washed for an additional 2 h and then washed with sodium acetate buffer (pH 4.0) followed by sodium borate buffer (pH 9.0) and distilled water.

The immobilization conditions for modified SG were the optimal conditions obtained for SG (Section 2.5). For modified SG, both pH 7.0 and pH 10.0 were tested because for glyoxyl-carriers immobilization occurs at alkaline pH, via the richest area in lysines on the proteins surface [23]. EAGSG carriers were previously activated with 1% glutaraldehyde (w/v) for 2 h in 0.05 M pH 7 phosphate buffer.

The enzyme was immobilized overnight.

2.4. Measurements of activity of free and immobilized laccase

The free laccase activity was assayed spectrophotometrically (Thermo Electron, model UV1 spectrophotometer) with ABTS as substrate (0.4 mM) in 0.05 mM citrate/0.1 mM phosphate buffer at pH 4.5. To measure the laccase activity, 0.1 mL of the incubated enzyme solution was added to 1.9 mL of the ABTS solution at 40 °C [24]. The change in absorbance at 420 nm (ε = 36,000 M^{-1} cm^{-1}) was recorded automatically by the spectrophotometer for 90 s and the catalytic activity was determined by measuring the slope of the initial linear portion of the kinetic curve. One unit (U) was defined as the amount of enzyme that oxidized 1 μmol of ABTS per min and the activities were expressed in U/L.

To measure laccase activity when using immobilized enzyme, spent grains were mixed with 7 mL of citrate/phosphate buffer 0.05 M/0.1 M, pH 4.5 at 40 °C and 2.5 mL of ABTS 0.4 mM, under magnetic stirring at 100 rpm, for 4 min. Samples were taken every minute, filtered with 0.45 μm polypropylene filters and absorbance was measured spectrophotometrically (Thermo Electron, model UV1 spectrophotometer) at 420 nm. After linear regression of the data obtained, enzyme activity was determined using Eq. (1):

$$\frac{\mathrm{U}}{\mathrm{kg}} = \frac{\mathrm{abs}/\min \times f_{dilution} \times V_{reaction} \times 10^6}{\varepsilon \times m_{carrier}} \quad (1)$$

where U/kg is the quantity of enzyme capable to oxidase 1 μmol of ABTS per minute and per mass unit of carrier; abs/min is the absorbance per minute determined by linear regression; $f_{dilution}$ is the dilution factor of the sample; $V_{reaction}$ (L) is the volume of reaction; 10^6 is the conversion factor from M to μM; ε is the molar extinction coefficient (36,000 M^{-1} cm^{-1} at 420 nm); $m_{carrier}$ (kg) is the mass of SG with immobilized enzyme.

2.5. Optimization of the conditions for laccase immobilization on SG

The optimal laccase concentration determination was performed immobilizing laccase solution in phosphate buffer (0.05 M, pH 7.0) at different concentrations of laccase: 0.001–0.015 g/mL (800–12,000 U/L) for 2 h.

To test the influence of pH on laccase immobilization, the laccase solution (0.005 g/mL or 4000 U/L) was mixed with 0.1 g of SG for 2 h using different buffers: citrate/phosphate buffer 0.05 M for pH values of 3.0, 4.0 and 5.0; phosphate buffer 0.05 M for pH 6.0, 7.0 and 8.0.

To determine the optimum contact time, laccase immobilization was carried out at 30 min to 8 h at the optimum conditions (pH 7.0; 4000 U/L of laccase solution).

Immobilization yield (%) is defined as the difference obtained between the initial free enzyme activity of the laccase solution before the immobilization and the activity of free laccase obtained in the supernatant after immobilization divided by the enzyme activity before immobilization.

The recovered activity (%) of the immobilized enzyme is defined as the ratio between the activity of the immobilized enzyme times the mass of carrier used in the immobilization process (corresponding to the activity of the amount of laccase that was in fact immobilized) and the difference obtained between the initial enzyme activity of the laccase solution before the immobilization and the activity of free laccase obtained in the supernatant after immobilization times the volume used for the activity assay (corresponding to the activity that the same amount of immobilized laccase had when it was free).

2.6. Thermal stability of free and immobilized laccase

The thermal inactivation of the free and SG and DSG immobilized laccase was investigated by incubating the free and immobilized enzyme in phosphate (100 mM) buffer pH 7.0 at different temperatures. Immobilized laccase was suspended in 3 mL of the buffer. The sample was removed regularly from the water bath and enzymatic activity was quickly determined according to the methods described above.

The thermal parameters were calculated according to the simplified deactivation model proposed by Arroyo et al. [25]:

$$E \xrightarrow{k_1} E_1 \xrightarrow{k_2} E_2 \quad (2)$$

$$A = \left[100 + \frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_2}{k_2 - k_1}\right] e^{-k_1 t} + \left[\frac{\alpha_2 k_1}{k_2 - k_1} - \frac{\alpha_1 k_1}{k_2 - k_1}\right] e^{-k_2 t} + \alpha_2 \quad (3)$$

where A is the residual enzyme activity, α_1 and α_2 ratios of specific activities E_1/E and E_2/E, respectively to the different states (see Eq. (2)), k_1 and k_2 the thermal inactivation parameter and t the time.

Analysing the data obtained, it was considered that laccase suffers a conformational transition due to the high temperatures according to the model of Eq. (2), thus the inactivation followed a single exponential decay, in which $\alpha_2 = 0$ and $k_2 = 0$:

$$A = [100 - \alpha] \times e^{-kt} + \alpha \quad (4)$$

The thermal parameters α and k of the model were calculated by a non-linear fit ting of the experimental data, using MATLAB v7.5.0 (MathWorks, Inc. USA).

2.7. Operational and storage stability of immobilized laccase

The operational stability of immobilized laccase was determined by incubating 0.1 g of the SG or DSG immobilized laccase with 2.5 mL of ABTS (4 mM) in 7 mL of citrate–phosphate buffer (pH 7.0) and by incubating 0.1 g of the DSG immobilized laccase with 2.5 mL of ABTS (4 mM) in 7 mL of citrate–phosphate buffer (pH 7.0) with NaCl to obtain a final concentration of 1 M, at room temperature and under moderate mixing. 10 cycles of operational stability were carried out. At each cycle, a sample was withdrawn in 1 min intervals (over a 4 min period), absorbance was measured and then it was returned to reactor (initial reaction rate measurements). Afterwards, the reaction was stopped by substrate removal. Then the immobilized enzyme was collected by filtration, washed twice with phosphate buffer (100 mM pH 7.0) and resuspended in a fresh substrate solution to begin the next cycle.

The storage stability of DSG immobilized laccase was determined incubating the 0.5 g DSG in sodium carbonate buffer (pH 10.0) or phosphate buffer (pH 7.0) at 4 °C for 35 days.

2.8. Determination of kinetic parameters of free and immobilized laccase

To determine the kinetic parameters of free laccase, enzymatic activity was measured for concentration of ABTS ranging from 0.003 to 0.105 mM, during 15 min, according to the method described before. Similarly, the kinetic parameters of SG and DSG immobilized laccase were determined measuring the enzymatic activity, during 10 min, for the catalysis of ABTS at concentrations ranging from 0.25 to 7.0 mM.

Since the data obtained followed the pattern predicted by the Michaelis–Menten model (Eq. (5)), MATLAB v7.5.0 (MathWorks, Inc. USA) was used to perform a non-linear fit of the experimental values in order to determine K_M and v_m.

$$v = \frac{v_m\,[S]}{K_M + [S]} \quad (5)$$

where v (mM min^{-1}) is the velocity of catalysis; v_m (mM min^{-1}) is the maximal velocity of the reaction; [S] (mM) is the concentration of substrate; and K_M is the Michaelis–Menten constant (mM).

2.9. Diffuse reflectance infrared Fourier transformed – DRIFT analyses for SG and DSG characterization

The presence of functional groups in SG and DSG were determined by DRIFT analysis. The DRIFT analysis of the supports was performed on a Nicolet 510P FTIR Spectrometer with a KBr beam splitter for mid-IR range and a DTGS with KBr windows equipped with a special beam collector (COLLECTOR from Spectra Tech), fixed on a plate for consistent experimental conditions. The interferograms were converted by the instrument software (OMINC) to equivalent absorption units in the Kubelka–Munk scale. The spectra of the supports were measured within the range of 500–4000 cm^{-1} wavelength.

3. Results and discussion

3.1. Optimization of the immobilization conditions of laccase on spent grain

The effect of enzyme concentration, pH and contact time between laccase solution and SG on the activity of immobilized commercial laccase was studied and optimized. Fig. 1 shows the effect of laccase concentration from 0.001 g/mL to 0.015 g/mL, pH and contact time in the immobilization of laccase on SG. No oxidation of ABTS (laccase substrate) was detected when the pure SG (without enzyme – control) was used as catalyst. According to the results obtained from Fig. 1a, a hyperbolic curve is obtained. For laccase solutions with concentrations up to 0.005 g/mL, initial slope, the enzymatic activity of the immobilized laccase rises fast, while for concentrations above it, enzymatic activity does not differ significantly. This is the typical behaviour of adsorption phenomena that can be described by Langmuir isotherm. So, the experimental data has been modelling using the Langmuir isotherm, represented by the following equation:

$$q = \frac{q_{\max} C}{k + C} \quad (6)$$

where C is the laccase concentration in solution (g/mL) and q is the amount of adsorbed laccase per kilogram of adsorbent (U/kg). The $q_{\max}$ and k are the maximum adsorption capacity (U/kg) and k is the Langmuir adsorption equilibrium constant (g/mL), respectively.

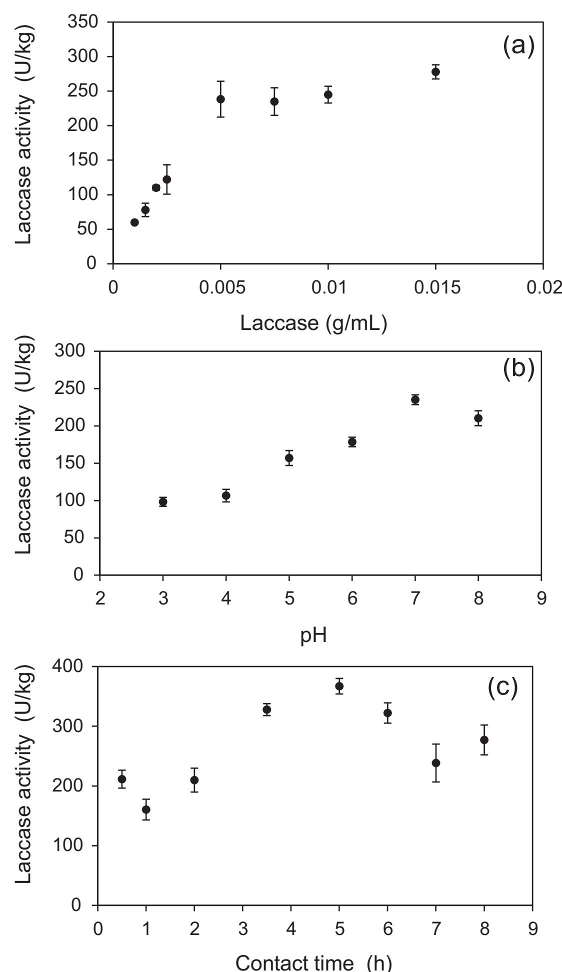

Fig. 1. Optimization of the laccase immobilization on spent grain. (a) Effect of the initial laccase concentration in the supernatant upon enzyme immobilization at pH 7.0 for 2 h; (b) effect of the pH medium upon immobilization of laccase 0.005 g/mL for 2 h and (c) effect of contact time upon immobilization of laccase 0.005 g/mL at pH 7.0.

The Langmuir isotherm gave a good fit to the adsorption data of laccase on the support with a good correlation coefficient (R^2 = 0.987). The maximum capacity ($q_{\max}$) was 371 U/kg. The k value, the binding strength between enzyme and support, was 224 g/mL. However, as Langmuir isotherm considers only monolayer coverage [26] and normally the enzyme molecule presents high size and can present charges in different parts of the molecule, it is not possible to conclude that only a monolayer of enzyme is adsorbed into the support. Also it cannot be concluded which is the nature of the linkage (adsorption or covalent linkage) between the enzyme and the support.

As showed in Fig. 1b, when immobilization is performed in acidic media laccase activity is lower than that for a neutral or alkaline medium. As a matter of fact, little difference was observed in the enzymatic activity at pH 7.0 and 8.0, so it can be said that the immobilized laccase is stable both at neutral and slight alkaline pH. The maximum laccase activity may be explained by the maintenance of the active site native structure of the immobilized laccase at these pH values. On the other hand, at a too low pH, the interaction of the available protons in the medium may change the ionization state of some amino acids and, consequently, the structure of the enzymes immobilized, affecting their function. The critical effect of low pH values over laccase are further evidenced by the negative values obtained for immobilization efficiency and recovered activity at pH 3.0 and 4.0 (data not shown). On the other hand, Silva et al.

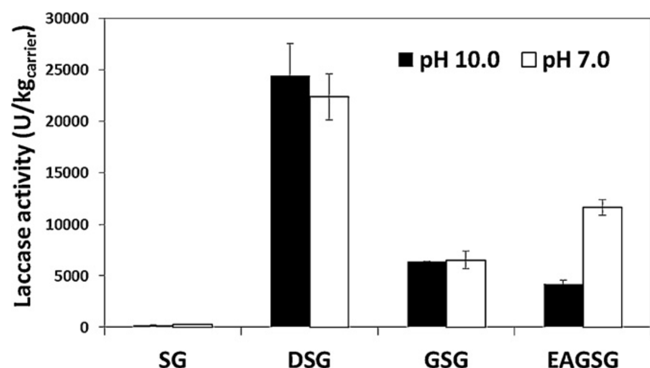

Fig. 2. Effect of spent grain modification: digested spent grain (DSG), glycidol-modified spent grain (GSG) and ethylenediamine-modified glycidol spent grain (EAGSG) on laccase activity. Laccase 0.005 g/mL was immobilized at pH 7.0 for 3.5 h at room temperature, and at pH 10.0, overnight at 4 °C.

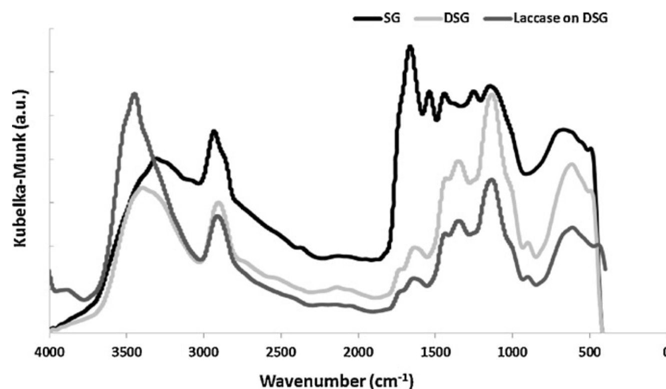

Fig. 3. FTIR spectra of spent grain (SG), digested spent grain (DSG) and immobilized laccase on DSG. Immobilization was performed with laccase 0.005 g/mL, at pH 10.0, overnight at 4 °C.

[27] studied the influence of pH (from 4.0 to 7.0) on the immobilization of laccase with polyamide matrices, and showed none or small significance on the immobilization of laccase.

The contact time also affects the probability of interactions between enzymes and free space on the carrier. For the time intervals tested, immobilization with laccase solution at optimal concentration during 3.5 h seemed to be the most appropriate time to obtain maximum activity. For shorter time intervals, observed enzymatic activity is lower because there was not enough time for enzymes to firmly adsorb onto the carrier. The first slope probably corresponds to the formation of a monolayer of enzyme molecules. For long-lasting intervals, two layers or more of enzyme are possibly formed over spent grains. Indeed, despite performing this test considering the optimal concentration of enzymatic solution already determined, contact times above 7 h allow the enzyme to interact with enzymes adsorbed directly on the carrier, forming another layer of enzymes that diminishes the number of free enzymes, as was observed for immobilization with concentrated enzymatic solutions. In this case, the maximum recovered activity was registered for contact periods near 3.5 h, while the maximum immobilization efficiency was registered for longer periods of contact (6 h and 8 h). So, it was concluded that it isn't worth to perform immobilization for periods longer than 3.5 h, since it has no practical effects on the recovered activity of the immobilized enzyme (data not shown).

Considering these results, the optimum conditions for laccase immobilization on SG are pH 7.0, initial laccase concentration of 0.005 g/mL and a contact time of 3.5 h. These conditions were selected to perform the following tests with laccase immobilized on modified SG.

3.2. Immobilization of laccase on modified spent grain

The results obtained for laccase activity after immobilization of DSG with glycidol and with glycidol plus ethylenediamine are shown in Fig. 2. Comparing the results with SG, a very high increase on laccase activity was observed, particularly for DSG immobilized laccase. An increase of laccase activity of 20, 35 and 68 times was obtained for GSG, EAGSG and DSG, respectively. The activation of DSG with glycidol and with glycidol plus ethylenediamine did not promote a better immobilization of laccase. The high attachment of laccase, when DSG was used, can be explained considering the modifications on the support. The alkaline digestion delignifies SG, thus opening the structure of the remaining cell walls in the support [13]. This treatment also probably opens the glucose structure, exposing the functional groups. In order to evaluate the differences of the functional groups between SG and DSG, FTIR analysis were carried out for SG before and after digestion, and also for DSG with immobilized laccase, as shown in Fig. 3. The spectrum of DSG obviously changed as compared to the SG, due to the change in the functional groups of SG. The spectrum of DSG revealed a large increase in the peak intensity around 1120 cm^{-1} and a new peak appears around 1370 cm^{-1}, corresponding to the presence of OH groups [13,28]. On the other hand, the peaks 1540 and 1670 cm^{-1} observed in SG were missing in DGS. These results indicate that these bonds, which correspond to the amide groups [28] were removed during the acid-base digestion of the SG. In fact this digestion removes the proteins content and delignifies SG, thus making the OH groups more accessible for the laccase immobilization. The FTIR spectrum of laccase immobilized on DSG shows that the peak at 1120 cm^{-1}, which increases after digestion, decreased after immobilization of laccase. This indicates that laccase was covalently bound to the free OH groups in DSG.

The lower activity of immobilized laccase on GSG (Fig. 2) can also be explained by the accessibility of the OH groups. Glycidol reacts with the free OH groups of DSG [22], thus promoting a lower availability of OH groups for laccase, also showing that laccase does not have a strong affinity for glycidol, probably because it may not have a very rich region in lysines. However the immobilization results improve for pH 7.0 when ethylenediamine was added to the GSG. The carrier was modified to have amine groups that can be activated with glutaraldehyde, allowing the immobilization to occur at mild pH values. Yield of laccase immobilization and recovered activity (corresponding to the % of activity expressed by the enzyme that was in fact immobilized when compared to the activity of the same amount of free enzyme) were also evaluated as shown in Table 1. The digestion of SG led to the greatest improvement in yield, around 94%, for both immobilization conditions. Laccase activity recovery was also maximized when using DSG, especially for pH 10.0

Table 1
Recovery activity and immobilization yield of laccase immobilized on spent grain (SG), digested spent grain (DSG), glycidol-modified spent grain (GSG) and ethylenediamine-modified glycidol spent grain (EAGSG) at pH 7.0 and 10.0.

Immobilization conditions	Recovered activity (%)	Immobilization yield (%)
pH 7.0		
SG	1 ± 0.3	36 ± 4
DSG	75 ± 7	94 ± 5
GSG	25 ± 3	82 ± 2
EAGSG	73 ± 7	51 ± 3
pH 10.0		
DSG	99 ± 1	95 ± 4
GSG	63 ± 4	40 ± 7
EAGSG	50 ± 6	33 ± 3

with 99% of recovery. The addition of glycidol and ethylenediamine decreased the laccase activity recovery and immobilization yield. Some data is available in the literature on raw biomass for the immobilization of laccase. Al-Adhami et al. [29] studied the immobilization of laccase, from different sources, on modified cellulose and acrylic supports and only the DEAE–Granocel 500 (cellulose) showed a high immobilization yield of 92.7%. The other supports, DEAE–Granocel 500 and copolymer of butyl acrylate and ethylene glycol dimethacrylate (acrylic carrier) did not present any enzymatic activity. Laccase was also immobilized on green coconut fiber, however, in this support, the maximum yield was 69% for the optimized conditions [9]. Yang et al. [30] reported the value of 56% and 70% for laccase immobilization yields on chitosan microsphere and water-soluble chitosan, respectively. Zhang et al. [19] obtained 52.2% of its original activity for laccase immobilized on chitosan using glutaraldehyde as a cross-linking agent.

From the data reported on the available literature, it can be concluded that the immobilization on DSG presented very high yield for laccase. It was superior to other types of raw biomass. Comparing the same support (modified spent grain), but with different enzyme, trypsin, the enzyme activity retention was only 46% [22].

It is also interesting to compare immobilized laccase activity and yield with different supports of immobilization (not biomass). However, it is not simple due to the use of different substrates, reaction conditions and definition of enzyme activity unit. As many different supports and techniques of immobilization have been published for laccase, some relevant results were compared. Rekúc et al. [31] showed a maximum yield of immobilization of 66.7% of laccase immobilized covalently on the mesostructured siliceous cellular foams functionalized using various organosilanes with amine and glycidyl groups. The yield of laccase immobilized in SBA-15 mesoporous silica was 98% of the initial enzyme amount [15], whilst for controlled porosity carrier silica the yield was 89% [17]. When laccase was immobilized on four different supports: activated carbon, Eupergit® C, Cu-alginate, Ca-alginate, the immobilization yields were 4.8%, 39%, 65% and 43%, respectively [16]. For laccase immobilized on alumina pellets and coated with polyelectrolytes, the immobilization yield was 70% [32].

DSG and SG (for comparison) were then used in further experiments. Since better results were obtained at condition 2, pH 10.0, 4 °C and contact time of 24 h, these conditions were used to prepare the complexes carrier-enzyme used in the following experiments.

3.3. Thermal stability of SG and DSG immobilized laccase

Thermal stability experiments were carried out with free and SG and DSG immobilized laccase, which were incubated at different temperatures. The results show that the high temperatures (80 and 90 °C) lead to total inactivation of both free and DSG immobilized laccase (data not shown) and a high decrease (90 °C) for SG immobilized laccase along time, Fig. 4. This inactivation is probably due to a higher vibration of the atoms of the protein, which may break some chemical bonds inside the molecule and, as consequence, change its structure. Then, enzymes acquire a conformation which does not allow them to maintain their catalytic capacity at very high temperatures. At 70 °C and 80 °C the SG immobilized laccase was more stable during the analysis period. Considering these results along with those obtained for free laccase and DSG immobilized laccase, it can be concluded that immobilization on SG makes the enzyme much more stable than when it is free in solution, as was expected. Hu et al. [33] studied the effect of temperature on immobilized laccase on nanoparticles and kaolinite at 40–70 °C. In this case, the activity of the immobilized laccase did not decrease significantly up to 180 min and therefore, this immobilized laccase appeared to be resistant to thermal inactivation due to the protective effect of the support.

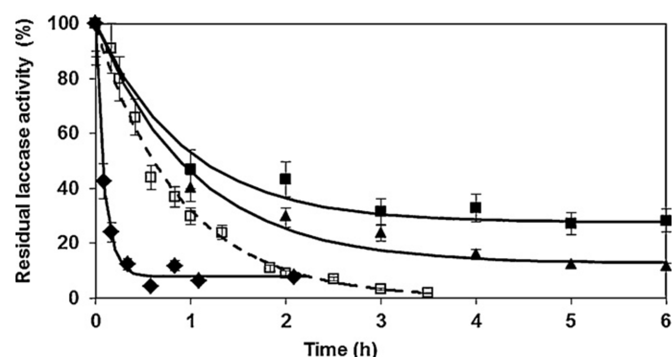

Fig. 4. Thermoinactivation of free laccase at 70 °C (□), and immobilized laccase on spent grain at different temperatures: (■) 70 °C; (▲) 80 °C and (♦) 90 °C. During inactivation the medium was kept at pH 7.0. Laccase 0.005 g/mL was previously immobilized at pH 7.0 for 3.5 h.

The thermal parameters of free and immobilized laccase are presented in Table 2. For immobilized laccase, it can be seen that $t_{1/2}$ decreases with increasing temperatures, which means that the enzyme is less stable, as was expected. Comparing the same temperature, 70 °C, the $t_{1/2}$ was higher for SG immobilized laccase (1.8 times) than for free and DSG immobilized laccase, which means that a longer period of time is necessary to denaturate the SG immobilized laccase. DSG immobilized laccase lost all its activity after 5 min of incubation at 70 or 80 °C. This is probably due to structural changes promoted by SG digestion. According to Mussatto et al. [34], acid and base digestions remove hemicellulose and lignin from SG, respectively, which results in a transition of the support fibrils from a rigid and highly ordered structure to a more disorganized and porous one, where the remaining cellulose fibres are separated and fully exposed. Consequently, the enzymes adsorbed in DSG are more susceptible to the external conditions and hence can undergo more conformational changes due to the temperature rising than in SG.

Considering these results along with those obtained for free laccase, it can be concluded that immobilization renders the enzyme much more stable than when it is in free solution, as was expected. Indeed, immobilization constrains atomic vibration and the enzyme can maintain its structure for longer periods of time.

3.4. Operational and storage stability of immobilized laccase

Considering Fig. 5, the repeated use of SG immobilized laccase promoted a decrease in the enzymatic activity, which may be due to the loss of laccase weakly adsorbed onto the carrier. This can be supported by the initial decrease denoted between the first and second cycle, where the enzymes loosely attached to the carrier were lost, and those more strongly linked remained active for the following cycles. Since laccase activity was maintained above 50% up to cycle six, it can be considered that laccase immobilized on spent grains is reasonably stable when repeatedly used for catalysis. Comparing the results with DSG immobilized laccase, it can be seen that laccase activity never falls below 60% of its initial activity. Besides, the values of enzymatic activity registered for the last cycles of catalysis are similar to those obtained in the initial cycles. Considering these results it can be assumed that laccase immobilized on DSG is stable during all the cycles of this study, with around 87% of its initial activity after the 10 consecutive cycles. The same tendency was obtained for laccase immobilized on Sepabeads EC-EP3 activated with epoxy groups where the immobilized enzyme retained 84% of its initial activity after 17 cycles of oxidation of ABTS [35]. It is clear that immobilization on DSG greatly increases the operational stability of the enzyme. This may be due to an increase of the links established between the enzyme and the modified carrier. This is

Table 2
Thermal parameters obtained fitting the experimental data for the thermal inactivation of free and immobilized laccase on spent grain (SG).

Laccase	Temperature (°C)	α	R^2	k (h^{-1})	$t_{1/2}$ (h)
Free	70	0	0.994	1.12 ± 0.06	0.62 ± 0.08
SG immobilized	70	27.6 ± 1.3	0.998	1.05 ± 0.02	1.11 ± 0.12
SG immobilized	80	12.7 ± 0.4	0.999	0.96 ± 0.04	0.88 ± 0.11
SG immobilized	90	8.11 ± 0.6	0.998	11.2 ± 0.3	0.07 ± 0.01

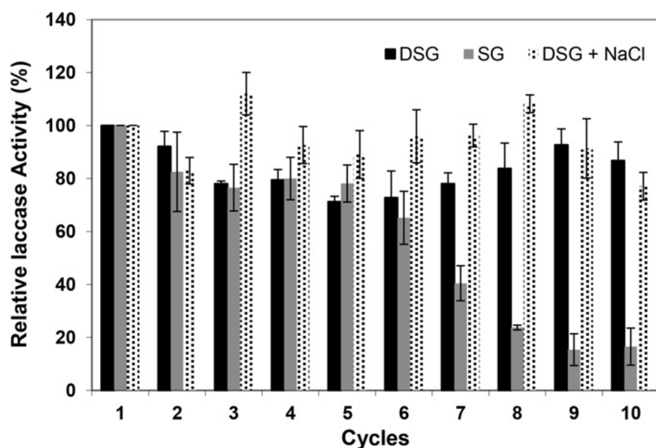

Fig. 5. Operational stability of immobilized laccase on spent grain (black), immobilized laccase on digested spent grain (gray) and immobilized laccase on digested spent grain (light gray) in the presence of NaCl 1 M. 10 subsequent cycles in batch reactor was carried out. Between each cycle of laccase activity measurement, the immobilized enzyme was recovered and washed with phosphate buffer 100 mM pH 7.0 Laccase 0.005 g/mL was immobilized on SG at pH 7.0 for 3.5 h, and on DSG at pH 10.0, overnight at 4 °C.

an advantage that may allow their reuse several times in batch processes as well as the application of this system to continuous processes. Very high operation stability was obtained here when compared for example with acrylic carrier, where approximately 12.5% of activity was maintained after the sixth cycle [36]. More severe operational stability conditions were investigated similar to that applied in the textile finishing, wine cork and other environmental and industrial fields. This investigation entailed conducting an experiment in a reaction medium with a higher ionic strength (1 M NaCl). From the results, it was concluded that the enzyme does not leach from the support. The immobilized laccase had a very high stability as well as a higher ionic strength (Fig. 5).

The results of storage stability of DSG immobilized laccase in pH 7.0 and pH 10.0 are presented in Fig. 6. The laccase activity takes values between 6000 and 8000 U/kg for a period of 35 days

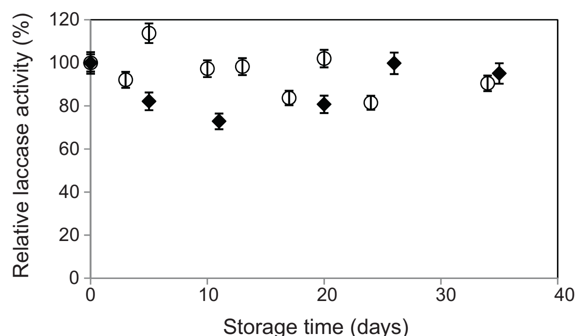

Fig. 6. Storage stability of the immobilized laccase on digested spent grain. Laccase 0.005 g/mL was immobilized at pH 10.0 (♦) and at pH 7.0 (○), overnight, at 4 °C, and then several samples of DSG were resuspended in sodium carbonate buffer 0.1 M, pH 10.0. All tubes were stored at 4 °C and samples were taken regularly to measure enzymatic activity.

Table 3
Kinetic parameter (K_M) of catalysis of ABTS for free and immobilized laccase on spent grain (SG) and digested spent grain (DSG).

Laccase	K_M (mM)	R^2
Free	0.028 ± 0.003	0.979
Immobilized on SG	0.748 ± 0.08	0.987
Immobilized on DSG	0.355 ± 0.06	0.984

for both values of pH. Comparing this stability with the storage stability of laccase immobilized on poly(vinyl alcohol) cryogel, which presented a decrease of about 40% of stability after 48 h [37], it can be considered that laccase immobilized on DSG is very stable when stored at 4 °C for a large period of time.

3.5. *Kinetic study of free and immobilized laccase*

In order to determine the affinity of ABTS substrate oxidation for free laccase, SG and DSG immobilized laccase, kinetic tests were carried out. The enzymatic kinetic studies were based on the evaluation of the kinetic parameters from initial velocity data. The Michaelis–Menten model was followed for the kinetics of free enzyme and those immobilized on spent grain and digested spent grain acting on ABTS. The values of the Michaelis–Menten kinetic parameter are summarized in Table 3. The performance of free and immobilized laccase was very different. It is well known that the parameter K_M gives an indication of the affinity of the enzyme to the substrate: a higher K_M registered for a certain substrate, means a lower affinity for that substrate. Then, it can be concluded that immobilization diminishes the affinity of laccase to ABTS for SG and DSG, with an increase of K_M value of 12.7 and 26.7 times for DGS and SG respectively. This can be due to: (i) some limitations of mass transfer of the substrate to the surface of spent grains; (ii) slight changes in the three-dimensional conformation of the enzymes when they adsorb onto the carrier that, directly or indirectly, affect the active site of the enzyme. Rekúc et al. [38] also immobilized laccase in cellulose-based carrier Granocel. They reported that catalytic efficiency of immobilized laccase was higher than catalytic efficiency of free laccase and the immobilized enzyme had five-fold higher K_M value than free enzyme. Cabana et al. [39] also reported for the same enzyme that the K_M parameter for free laccase was lower than for immobilized laccase.

4. Conclusion

Spent grain appears to be an efficient support for immobilization of commercial laccase. A very significant improvement in activity and operational stability of laccase was achieved after the immobilization of the enzyme on DSG. FTIR analysis indicated that the OH groups are responsible for the high immobilization of laccase in DSG. The half-life of immobilized laccase in SG could be increased from 0.64 h to 1.1 h at 70 °C. A high storage and operational stability (10 cycles) was obtained with the laccase immobilized on DSG. The kinetics parameters indicate that laccase immobilized on DSG has a high affinity and maximum velocity for ABTS than laccase immobilized on SG.

Therefore, the potential use of immobilized laccase on DSG for different applications can be achieved by its immobilization.

The immobilized laccase preparation offers an interesting tool to increase the applicability, stability and reusability of laccase in biocatalytic reactions.

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References

[1] Mayer AM, Staples RC. Laccase: new functions for an old enzyme. Phytochemistry 2002;60:551–65.
[2] Gamelas JAF, Tavares APM, Evtuguin DV, Xavier AMB. Oxygen bleaching of kraft pulp with polyoxometalates and laccase applying multi-stage process: comparative study. J Mol Catal B Enzyme 2005;33:57–64.
[3] Amaral PFF, Fernandes DLA, Tavares APM, Xavier ABMR, Cammarota MC, Coutinho JAP, et al. Decolorization of dyes from textile wastewater by *Trametes versicolor*. Environ Technol 2004;25:1313–20.
[4] Tavares APM, Cristóvão RO, Loureiro JM, Boaventura RAR, Macedo EA. Optimization of reactive textile dyes degradation by laccase-mediator system. J Chem Technol Biot 2008;83:1609–15.
[5] Chiacchierini E. Bioremediation of food industry effluents: recent applications of free and immobilised polyphenoloxidases. Food Sci Technol Int 2004;10:373–82.
[6] Brady D, Jordaan J. Advances in enzyme immobilization. Biotechnol Lett 2009;31:1639–50.
[7] D'Souza SF, Godbole SS. Immobilization of invertase on rice husk using polyethylenimine. J Biochem Biophys Methods 2002;52:59–62.
[8] Castro HF, Lima R, Rice Roberto IC. Straw as a Support for Immobilization of Microbial Lipase. Biotechnol Progr 2001;17:1061–4.
[9] Cristóvão RO, Tavares APM, Brígida AI, Loureiro JM, Boaventura RAR, Macedo EA, et al. Immobilization of commercial laccase onto green coconut fiber by adsorption and its application for reactive textile dyes degradation. J Mol Catal B Enzym 2011;72:6–12.
[10] Mussatto SI, Dragone G, Roberto IC. Brewers' spent grain: generation, characteristics and potential applications. J Cereal Sci 2006;43:1–14.
[11] Chai L, Li Q, Zhu Y, Zhang Z, Wang Q, Wang Y, et al. Synthesis of thiol-functionalized spent grain as a novel adsorbent for divalent metal ions. Bioresource Technol 2010;101:6269–72.
[12] Brányik T, Vicente AA, Machado Cruz JM, Teixeira JA. Spent grains – a new support for brewing yeast immobilization. Biotechnol Lett 2001;23:1073–8.
[13] Brányik T, Vicente A, Oliveira R, Teixeira J. Physicochemical surface properties of brewing yeast influencing their immobilization onto spent grains in a continuous reactor. Biotechnol Bioeng 2004;88:84–93.
[14] Tsaousi K, Koutinas AA, Bekatorou A, Loukatos P. Fermentation efficiency of cells immobilized on delignified brewers' spent grains after low and high-temperature thin layer thermal drying. Appl Biochem Biotechnol 2010;162:594–606.
[15] Salis A, Pisano M, Monduzzi M, Solinas V, Sanjust E. Laccase from *Pleurotus sajor-caju* on functionalised SBA-15 mesoporous silica: Immobilisation and use for the oxidation of phenolic compounds. J Mol Catal B Enzym 2009;58:175–80.
[16] Brandi P, D'Annibale A, Galli C, Gentili P, Pontes ASN. In search for practical advantages from the immobilisation of an enzyme: the case of laccase. J Mol Catal B Enzym 2006;41:61–9.
[17] Areskogh D, Henriksson G. Immobilisation of laccase for polymerisation of commercial lignosulphonates. Process Biochem 2011;46:1071–5.
[18] Davis S, Burns RG. Covalent immobilisation of laccase on activated carbon for phenolic effluent treatment. Appl Microb Biotechnol 1992;37(4):474–9.
[19] Zhang J, Xu Z, Chen H, Zong Y. Removal of 2,4-dichlorophenol by chitosan-immobilized laccase from *Coriolus versicolor*. Biochem Eng J 2009;45:54–9.
[20] Niladevi KN, Prema P. Immobilization of laccase from *Streptomyces psammoticus* and its application in phenol removal using packed bed reactor. World J Microbiol Biotechnol 2008;24:1215–22.
[21] Rollett A, Schroeder M, Schneider KP, Fischer R, Kaufmann F, Schöftner R, et al. Covalent immobilisation of protease and laccase substrates onto siloxanes. Chemosphere 2010;80:922–8.
[22] Rocha MPG, Teixeira JA. Immobilization of trypsin on spent grains for whey protein hydrolysis. Process Biochem 2011;46:505–11.
[23] Mateo U, Palomo JM, Fuentes M, Betancor L, Grazu V, Lopez-Gallego F, et al. Glyoxyl agarose: a fully inert and hydrophilic support for immobilization and high stabilization of proteins. Enzyme Microb Technol 2006;39:274–80.
[24] Ander P, Messner K. Oxidation of 1-hydroxybenzotriazole by laccase and lignin peroxidase. Biotechnol Tech 1998;12:191–5.
[25] Arroyo M, Sánchez-Montero J, Sinisterra J. Thermal stabilization of immobilized lipase B from *Candida antarctica* on different supports: Effect of water activity on enzymatic activity in organic media – a practical procedure for organic chemists. Enzyme Microb Technol 1999;24:3–12.
[26] Sohn S, Kim D. Modification of Langmuir isotherm in solution systems-definition and utilization of concentration dependent factor. Chemosphere 2005;58:115–23.
[27] Silva C, Silva CJ, Zille A, Guebitz GM, Cavaco-Paulo A. Laccase immobilization on enzymatically functionalized polyamide 6,6 fibres. Enzyme Microb Technol 2007;41:867–75.
[28] Qing-zhu L, Li-yuan C, Jing Z, Zhi-hui Y, Qing-wei W. Lead desorption from modified spent grain. Trans Nonferrous Met Soc China 2009;19:1371–6.
[29] Al-Adhami AJH, Bryjak J, Greb-Markiewicz B, Peczynska-Czoch W. Immobilization of wood-rotting fungi laccases on modified cellulose and acrylic carriers. Process Biochem 2002;37:1387–94.
[30] Yang WY, Min DY, Wen SX, Jin L, Rong L, Tetsuo M, et al. Immobilization and characterization of laccase from Chinese *Rhus vernicifera* on modified chitosan. Process Biochem 2006;41:1378–82.
[31] Rekúc A, Bryjak J, Szymanska K, Jarzebski AB. Laccase immobilization on mesostructured cellular foams affords preparations with ultra high activity. Process Biochem 2009;44:191–8.
[32] Osma JF, Toca-Herrera JL, Couto SR. Biodegradation of a simulated textile effluent by immobilised-coated laccase in laboratory-scale reactors. Appl Catal A Gen 2010;373:147–53.
[33] Hu X, Zhao X, Hwang H-min. Comparative study of immobilized *Trametes versicolor* laccase on nanoparticles and kaolinite. Chemosphere 2007;66:1618–26.
[34] Mussatto SI, Fernandes M, Milagres AMF, Roberto IC. Effect of hemicellulose and lignin on enzymatic hydrolysis of cellulose from brewer's spent grain. Enzyme Microb Technol 2008;43:124–9.
[35] Kunamneni A, Ghazi I, Camarero S, Ballesteros A, Plou FJ, Alcalde M. Decolorization of synthetic dyes by laccase immobilized on epoxy-activated carriers. Process Biochem 2008;43:69–178.
[36] Bryjak J, Kruczkiewicz P, Rekúc A, Peczynska-Czoch W. Laccase immobilization on copolymer of butyl acrylate and ethylene glycol dimethacrylate. Biochem Eng J 2007;35:325–32.
[37] Stanescu MD, Fogorasi M, Shaskolskiy BL, Gavrilas S, Lozinsky VI. New potential biocatalysts by laccase immobilization in PVA cryogel type carrier. Appl Biochem Biotechnol 2010;160:1947–54.
[38] Rekúc A, Jastrzembska B, Liesiene J, Bryjaka J. Comparative studies on immobilized laccase behaviour in packed-bed and batch reactors. J Mol Catal B Enzym 2009;57:216–23.
[39] Cabana H, Alexandre C, Agathos SN, Jones JP. Immobilization of laccase from the white rot fungus *Coriolopsis polyzona* and use of the immobilized biocatalyst for the continuous elimination of endocrine disrupting chemicals. Bioresource Technol 2009;100:3447–58.