Spent grain as a sustainable and low-cost carrier for laccase immobilization

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Abstract

Spent grain is promising lignocellulosic by-product support for laccase immobilization. The waste digestion with two different approaches (HCl/NaOH and H_2SO_4/NaOH) was performed. Different procedures (soaking and dropping), based on chemical and physical reactions, were also used to obtain the highest immobilized activity. Results showed that H_2SO_4/NaOH digestion guaranteed an immobilized activity five times higher than HCl/NaOH digestion. The best immobilization conditions with physical dropping procedure resulted in the highest immobilized activity on digested spent grain (2500 U/Kg). Good reusability (42% of activity retained after four cycles), and lower catalytic efficiency (V_{max}/K_m) of 0.053 min^{-1} than free laccase (0.14 min^{-1}) with ABTS as substrate, were also obtained. Besides, when 20 mg of biocatalyst (0.02 U) were tested for syringic acid removal, complete oxidation of the phenol was achieved in just 4 hours.

Keywords: laccase immobilization, spent grain, syringic acid, eco-sustainable procedure.

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1.Introduction

the COVID-19 crisis has fundamentally changed our way of thinking, with a significant effect in four main domains of the food sector: food safety, bioactive food ingredients, food security and sustainability (Galanikis et al., 2021, Galanikis et al., 2020). For example, in the food safety trend, non-thermal technology, as ultrasounds, has become a viable alternative to the conventional thermal processes since they can destroy the nutritional and sensory components (Zinoviadou et al., 2015).

In the sustainability scenario, the enormous amount of food waste from agro-industrial production is a hot topic due to the high amount disposal problem. Therefore, the crops' valorization as sources of functional compounds (Galanikis et al., 2020) or new sustainable material for new emerging technologies (Bilal et al., 2020) is today proposed in the food industry. In particular, the dumping or open ground tipping is prohibited for the brewer wastes disposal regulation with a total organic carbon (TOC) exceeding 5%, and industry is encouraged to look for new waste utilization possibility (Zanker et al., 2007). The major by-products of the brewer industry (85 %) is spent grain which is the solid residue generated after mashing and lautering processes (Mussatto et al., 2006) with 3.4 million tons produced in the European Union (Lynch et al., 2016). Brewer spent grain (BSG) is a lignocellulosic material composed of four major components: 12-25% cellulose, 20-25% hemicellulose,12-28% non-sugar lignin, and proteins 19-30% (Lynch et al., 2016; Mendis and Simsek, 2014; Mandalari et al., 2005). Cellulose microfibrils are coated with hemicellulose matrices and protected by lignin outside, so their structures are rigidly packed and strictly accessible (Calvo-Flores and Dobado, 2010; Kumar et al., 2009; Perez et al., 2002).

Due to its composition, BSG has a high nutritive value that could be introduced in several applications, but it is basically used as animal feed with a market value of ~€ 35 per tons

(Buffington, 2014). However, since it contains many functional groups such as carboxyl, hydroxyl, and amino, BSG can be considered a good potential support for enzyme attachment (Girelli et al., 2020). Recently, BSG has been successfully used as a carrier for trypsin (Rocha et al. 2011a, 2005b), lipase (Pospiskova and Safarik, 2013), and laccase (Da Silva et al., 2012) employing either chemical or physical immobilization methods.

Enzyme immobilization is a promising approach that can overcome the bottlenecks of free enzymes, such as the impossibility of reuse and the high cost of the protein (Homaei et al., 2013). The immobilization procedure combines the stability and selectivity of enzymes with the peculiarities of supports (Bilal and Iqba, 2019). In this way, it is possible to have maximal stability and performance of the biocatalyst with the possibility of the enzyme recovery, high precision for catalytic process control, high enzyme stability against denaturing agents, and use of the system in continuous and batch mode, useful for industrial application (Apriceno et al., 2019; Bommarius and Payeb, 2013). Therefore, immobilized enzymes' success depends on choosing the suitable protocols and supports, which must be inexpensive and available (Zdarta et al., 2018). The use of several supports such as inorganic, organic (polymers and biopolymers), and hybrid material is currently explored to develop immobilized enzymes (Sheldom and Van Pelt, 2013). Between them, agro-industrial wastes have gained particular attention as enzymatic support thanks to the increasing request of scientific interest on sustainability. In fact, they are biodegradable, biocompatible, non-toxic, and easily available (Ranganathan et al., 2020).

Therefore, this study aimed to obtain a new sustainable material with the re-utilization of a brewer by-product processing and applied to the immobilization of laccase enzyme from *Trametes Versicolor* which is an aspecific oxidoreductase glycoprotein with a high redox potential. It is extensively applied in the food, textile, and paper industry (Fernàndez-Fernàndez et al., 2013) and has a great potential in the bioremediation process. In this way, reducing the

spent grain disposal problem and overcoming bottlenecks of the conventional process like thermo-chemical treatments, advantages from both economic and environmental points of view may be obtained. For example, the classical treatment as incineration generates toxic gases, causing severe environmental and human health hazards (Evangelisti et al., 2015).

In this study, to increase the access of laccase to microfibrils cellulose chains a digestion step of spent grain was required (Dehnavi et al., 2011). Thus, the lignocellulosic material was firstly subjected to an acid step that broke down hemicelluloses into monomeric sugars. Then, the obtained solid residue was treated with an alkaline solution to solubilize the lignin and obtain a material with more accessible cellulose (Mussatto et al., 2006). Different methods were tested to optimize the laccase immobilization procedure on spent grain: the DSG-soaking in enzyme solution or enzyme-dropping on DSG. The dropping procedure was tested because a minor volume and enzyme activity units than other protocols were involved.

Finally, the optimized biocatalyst was applied to remove a model phenol compound, syringic acid, which is toxic to animals and the microbial population of the soil when present at high levels (Cheemanapalli et al., 2018).

2.Experimental

2.1. Chemical and reagents

Laccase from *Trametes Versicolor* with a nominal activity of 136 U/mg protein, 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonicacid) diammonium salt (ABTS), and salts for buffer solutions were purchased from Sigma-Aldrich. Spent grain was kindly donated by “Birreria Peroni srl” (Rome, Italy). The fresh material was washed with distilled water and dried at 100 °C before the digestion procedure.

2.2 Digestion procedure

103 The digestion procedure with HCl/NaOH reported by Da Silva et al., (2012) was followed. In
104 a 1000 mL glass flask, 20 g of BSG were first mixed with 300 mL of HCl (3% v/v) at 60°C for
105 2.5 h to hydrolyze the hemicellulose component and remove the starchy endosperm present in
106 the raw material. The solution with BSG was cooled, washed with distilled water until neutral,
107 and dried for 24 h at 100 °C. Then digested spent grain (DSG) was treated with 200 mL of
108 NaOH (2% w/v) solution at 30 °C for 24 h. After that, the carrier (8 g) was washed several
109 times with water until neutral pH and dried at 105 °C for 24 h.
110 The digestion procedure with H_2SO_4/NaOH was made following the method reported by
111 Mussatto et al., (2006). Firstly, 26 g of BSG were treated with 240 mL H_2SO_4 (1.25% v/v) for
112 17 min at 120 °C; then, the obtained solid residue (basically cellulignin) was cooled, washed
113 with distilled water, and dried at 100 °C for 24 h.
114 Finally, the residue dried material (22 g) was treated with 500 mL of NaOH (2% w/v) solution
115 at 120 °C for 90 min. After that, the carrier (5 g) was washed several times with water until
116 neutral pH and dried at 100 °C for 24 h.
117 *2.3 Laccase immobilization on spent grain digested by-soaking procedure*
118- For the covalent immobilization 2 g of BSG digested with HCl/NaOH were treated with 50 mL
119 $NaIO_4$ 0.047 M for 2 h in the dark to oxidize the hydroxyl groups of cellulose to carbonyl groups
120 and to link the enzyme by imine binding formation. Then, 50 mg of DSG_{ox} were directly
121 immersed for 24 h, at 4°C in the laccase solution 2U at pH 7 (DSG_{ox}-LAC) or activated with 1
122 M ethylenediamine (EDA) at pH 9 for 2 h and then with glutaraldehyde (GA) (1.25% v/v) at
123 pH 8 for 5 h at 25 °C (DSG_{ox}-EDA-GA) and successively soaked in laccase solution (DSG_{ox}-
124 EDA-GA-Lac). Both biocatalysts were washed and stored at pH 7.
125 For the physical immobilization 50 mg of DSG were soaked in laccase solution 2 U at pH 7 for
126 24 h at 4°C (DSG-LAC_{soak}). After the biocatalyst removal, it was washed several times with

phosphate buffer until no enzyme activity was detected in the washing solution. Then, it was stored at 4 °C in a 0.05 M phosphate buffer at pH 7.

The physical immobilization procedure was also performed on BSG digested with the procedure reported by Mussatto et al., (2006) based on H_2SO_4/NaOH treatment to define the best operative conditions. The comparison was performed taking into account the results of SEM scanning electron microscopy (SEM; LEO 1450 VP; Carl Zeiss, Oberkochen, Germany) and ATR-FTIR (Perkin Elmer 1600 ATR) in transmittance mode.

2.4 Laccase immobilization on spent grain digested by enzyme-dropping procedure

In this protocol, the immobilization of laccase was performed on spent grain digested with H_2SO_4/NaOH using chemical and physical methods.

For the covalent immobilization, 20 μL of glutaraldehyde solution (25 % v/v), as cross-linking agent, were dropped on 20 mg of DSG and left to stand for 5 min. A glass rod was used to gently spread the glutaraldehyde solution thoroughly on the DSG surface (DSG-GA). Then, 100 μL of free laccase were dropped and immobilized on DSG-GA, obtaining DSG-GA-LAC biocatalyst.

For the physical immobilization100 μL of laccase (1.1 U) native or oxidized were dropped on 20 mg of DSG and left to stand at 25°C. After 48 h the biocatalysts (DSG-LAC_{drop} and DSG-LAC_{ox}) were washed with 0.05 M phosphate buffer pH 7 solution and stored at 4 °C.

The laccase oxidation was performed by 0.02 M potassium periodate for 30 min at 4 °C to oxidize the enzyme's glycosidic part to aldehydes groups. The reaction was stopped by adding 3 μL of ethylene glycol and left to stand 10 min in the dark at 4 °C. Finally, the oxidized enzyme (LAC_{ox}) solution was transferred in a cellulose membrane (cut-off 12-14 KDa) for dialysis in order to remove the unreacted periodate, as previously reported (Apriceno et al., 2018).

2.5 Laccase activity assay

Free and immobilized laccase activities were assayed spectrophotometrically (Model T60, PG Instrument Limited, Leicester, United Kingdom) with ABTS as substrate (0.18 mM) in 0.1 mM citrate/0.2 mM phosphate buffer at pH 3 and 30 °C. To measure the laccase activity, 10 μL of enzyme solution or 5 mg of immobilized enzyme were added to 2 mL of ABTS solution, reaching a final volume of 2.7 mL with 0.1 M citrate-0.2 M phosphate buffer at pH 3. The change in absorbance at 420 nm (ε = 36000 L/mol x cm) (Kenzom et al., 2014) was recorded automatically by the UV-vis spectrophotometer every 30 s for 5 min. One unit (U) was defined as the amount of enzyme that oxidized 1 μmol of ABTS per min. Immobilized enzyme activity was determined using the following equation:

$$\text{Immobilized activity } \left(\frac{U}{Kg}\right) = \frac{\Delta A}{min}\ x\ \frac{V_{reaction} x 10^6}{\varepsilon x m}$$

ΔA/min is the change of absorbance; $V_{reaction}$ is the volume of reaction (L); 10^6 is the conversion factor from M to μM; ε is the molar extinction coefficient of radical cation $ABTS^{+\cdot}$ at 420 nm (36000L/mol x cm,); m (Kg) is the biocatalyst mass.

2.6 Parameters for enzyme immobilization

Activity yield (%) describes the percentage of enzyme activity immobilized, taking considering the initial activity of the incubated enzyme.

$$\text{Activity yield (\%)} = \frac{Ui - Uf}{Ui}\ x\ 100$$

Efficiency (%) describes the percentage of immobilized enzyme in the function of the residual activity.

$$\text{Efficiency (\%)} = \frac{Us}{Ui - Uf}\ x\ 100$$

Recovery (%) describes the immobilized activity compared to incubated enzyme activity.

$$\text{Recovery (\%)} = \frac{Us}{Ui}\ x\ 100$$

where U_i and U_f are enzyme activity in the solution before and after the immobilization process, respectively, and U_s is the immobilized enzyme activity.

2.7 Optimization of adsorption immobilization conditions

The evaluation of the optimal laccase concentration in both the procedures was performed at pH 7 by immobilizing laccase in the range 0.1-2 U for DSG-soaking and between 0.1 U to 1.7 U for enzyme-dropping procedures.

The immobilization time optimization in both cases was performed by following the reaction up to 48 h.

2.8 Comparison between DSG-LAC$_{soak}$ and DSG-LAC$_{drop}$ biocatalysts

The comparison between DSG-LAC$_{soak}$ and DSG-LAC$_{drop}$ biocatalysts was performed considering the operational stability, immobilized activity, and recovery. The operation stability was evaluated by repeated utilization of the biocatalyst to catalyze ABTS oxidation at 30 °C and pH 3. The activity obtained in each round was compared with the initial activity (defined as 100 %) to calculate the relative activity.

2.9 Kinetic parameters determination of DSG-LAC$_{drop}$ biocatalyst

The kinetic study of the optimized DSG-LAC$_{drop}$ biocatalyst was performed employing ABTS as substrate. The kinetic parameters (K_m and V_{max}) for DSG-LAC$_{drop}$ were evaluated by extrapolation the Lineweaver-Burk double-reciprocal plot obtained varying the final concentration of ABTS in the reaction medium at pH 3 from 0.018–0.25 mM at 30 °C.

2.10 Application of DSG-LAC$_{drop}$ biocatalyst: syringic acid removal

The phenol bio-removal study was carried out by adding 2 mL of syringic acid (50 mg/L) at pH 5 to 4 mg of immobilized laccase on (0.02 U). The syringic acid oxidation was monitored for 4 h with UV-Vis spectrophotometer in 250-450 nm range, using quartz cells of 1 cm and with HPLC-UV system using as stationary phase a C18 column (15 cm x 4.6 mm) and with a mobile phase of H_2O:MeOH (70:30 v:v) and a flow rate of 1 mL/min.

3.Results

The digestion was made using the method reported by Da Silva et al., (2012) with HCl/NaOH and by Mussatto et al., (2006) with H_2SO_4/NaOH to guarantee the highest lignin removal and the highest immobilized activity. In both cases, the predisposition of digested spent grain as a carrier for laccase immobilization, was tested.

Then, to select the immobilization process's appropriate conditions, factors such as enzyme concentration and immobilization time were optimized. In the following research step, the immobilized biocatalysts preparation was also subjected to comparative studies between the DSG-soaking and dropping-enzyme procedures (fig.1).

3.1 Optimization of laccase immobilization by soaking procedure on spent grain digested with HCl/NaOH.

Since a critical step in developing a biocatalyst is the choice of enzyme immobilization method, a preliminary study was made comparing the immobilization yield and immobilized activity of DSG_{ox}-LAC, DSG_{ox}-EDA-GA-LAC, and DSG-LAC_{soak}. Taking into account that a key reaction in covalent immobilization (Feeney et al., 1975) is the formation of Shiff's bases between aldehyde and amine groups of support and enzyme, DSG was oxidized by $NaIO_4$. Therefore, the oxidized hydroxyl groups of cellulose to 2,3-dialdehyde and aldehyde groups, can react to N-terminal amino group and lysine ε-amino groups of the protein.

In DSG_{ox}-EDA-GA-LAC, a new strategy was employed. In this case DSG was activated with a spacer made from ethylenediamine/glutaraldehyde in order to exploit the peculiarities of GA that, being a bi-functional reagent, can react with the activated support (DSG_{ox}-EDA) and with laccase, involving primary amine groups.

In DSG-LAC_{soak}, the adsorption can be due, generally, to hydrogen bond, hydrophobic and ionic interactions. The activity yield and immobilized activity of all the biocatalysts obtained

with the incubation of laccase 2 U are reported in table 1. The lowest results are unexpectedly obtained when the covalent immobilization reactions were involved.

The catalytic process rate is dramatically reduced upon immobilization even though the activity yield % of DSG_{ox}-EDA-GA-LAC is almost equal to that of DSG-LAC_{soak}. These results may be due to structural changes introduced into enzyme molecules by the covalent immobilization procedure and the creation of a microenvironment in which the enzyme acts different by bulk solution (Homaei et al., 2013). In general, it is not easy to explain the effects of a chemical modification on enzyme properties because it is usually dependent on certain experimental conditions such as pH, temperature, and cross linker concentration. In some cases, chemical modifications may improve in the enzyme properties but, in other cases, it may cause a decrease in the enzyme reactivity and selectivity (Nguyen et al., 2019). Although the immobilized enzyme activity is low for all biocatalysts, the value increases tenfold with the enzyme absorption. Physical protocol results to be the preferred one also because it is cheap, easy, and tends to be less disruptive to the enzyme than chemical immobilization (Datta et al., 2013). This method is the simplest way of preparing heterogeneous enzymes; it guarantees a non-specific physical interaction between the enzyme and the surface of the support since it occurs mainly by hydrogen bonds, hydrophobic interaction and Van der Waal's forces (Jesionowski, et al., 2014). Therefore, the physical immobilization procedure was set up and used to investigate of the immobilization parameters such as enzyme concentration and immobilization time.

For the study of the enzyme concentration, 50 mg of DSG-LAC_{soak} were soaked in laccase solution at different concentrations between 0.1-2 U for 24 h and pH 7. The depicted figure in the supplementary material (figure S1a) shows that the activity yield % increases until 2U while the relative activity % rises fast up to 1 U, then it decreases. The lack of immobilized activity and the increase of activity yield after 1 U can be attributed to several factors that can reduce

247 the accessibility of the substrate to the active sites. In particular, the support saturation can cause
248 the formation of multilayers of enzymatic molecules on the surface, and the DSG porosity
249 induces a high number of enzyme molecules on the outer surface that may hinder the access of
250 the substrate to the inner surface (Klein et al., 2012).
251 The laccase immobilized amount on the carrier is also affected by the contact time (figure S1b).
252 For a time higher of 24 h the influence of the above factors caused the decrease in immobilized
253 activity. Thus, the chosen conditions are 1 U of incubated laccase and 24 h.
254 *3.2 Choice of the digestion procedure*
255 As reported in the literature (Millati et al., 2020), the chemical digestion process of SG,
256 obtained with acidic and alkaline pretreatments, is strongly influenced by experimental
257 conditions. Acid hydrolysis is generally performed for breaking inter-and intra-molecular bonds
258 between hemicellulose and lignin. The alkaline step involves saponification of the
259 intermolecular ester bond cross-linking xylan, hemicellulose and other compounds causing a
260 decrease of polymerization degree, a release of lignin and an increase of internal surface of
261 cellulose (Martin et al., 2012). The efficiency of hydrolysis is strongly correlated to the nature,
262 concentration, temperature, and reaction type. Thus, two different approaches (HCl/NaOH and
263 H_2SO_4/NaOH in different experimental conditions) were performed to obtain cellulose chains
264 more accessible to the enzyme.
265 The selection of the most appropriate digestion method for spent grain was performed taking
266 into account the results of FT-ATR and SEM analysis and considering the immobilized activity,
267 efficiency, and recovery.
268 The ATR-FTIR absorption spectra of BSG as control (a), DSG delignified with HCl /NaOH
269 (b), and DSG delignified with H_2SO_4/NaOH (c) are reported in Fig. 2. The main bands appear
270 to be the same in all samples but with different intensities. In details, at 3300 cm^{-1} is attributed
271 to the axial deformation of the O–H and N-H groups; a strong absorption between 2900-2700

272 cm^{-1} is related to the axial deformation of C–H group; a band at 1744 cm^{-1} is associated to the
273 ester groups or to the ester linkage of the ferulic and p-coumaric acid bonded together with
274 lignin and hemicellulose; a strong absorption band at 1033 cm^{-1} is related to C-O vibration of
275 cellulose fibers (Raspolli Galletti et al., 2015; Kahar, 2013). In particular, to quantify the
276 chemical change related to hemicellulose and lignin removal, the ratios between the absorbance
277 at two representative wavenumbers (2900cm^{-1} and 1744 cm^{-1}) and that of 3300cm^{-1} (OH
278 vibration) were determined. It appears that the absorbance ratios relative to 2900 and 1744 cm^{-1}
279 tend to decrease from 0.77 and 0.39 (BSG) to 0.5 and 0.2 (HCl/NaOH BSG treatment) and to
280 0.4 and 0.05 (H_2SO_4/NaOH BSG treatment), respectively, indicating that the lignin linked to
281 branched hemicellulose was more efficiently removed with H_2SO_4. This is also confirmed from
282 the absorbance increase at 1033 cm^{-1} (C–O, C–C stretching, or C–OH bending in xylan
283 cellulose) in the digested samples (Raspolli Galletti et al., 2015).
284 The digestion treatment promoted morphological changes of spent grain (BSG), as shown in
285 micrographs SEM (figure 3). In particular, BSG (fig 3a) presents a homogeneous structure with
286 some globular aggregates associated with protein (Han et al., 2020) while the spent grain
287 digested with H_2SO_4/NaOH (fig 3c) presents a structure without any spots of proteins and
288 unpacked fibers with an open structure. These fibers appear to be more accessible to the
289 enzymes, confirming the elimination of integrating lignin. The treatment with HCl/NaOH (fig
290 3b) partially removed lignin from the surface, and protein spot bundles remained in the
291 structure, supposedly leaving the internal lignin. Another confirmation was made in terms of
292 color; in fact, the BSG and DSGwith HCl/NaOH had a dark brown coloration while digested
293 spent grain with H_2SO_4/NaOH presented light brown coloration, indicating that the lignin was
294 cleaved and solubilized in the solution that becomes dark brown. In order to correlate the
295 digestion procedures to the immobilization parameters, immobilized activity (U/Kg), activity
296 yield (%), efficiency (%), and recovery (%) are reported in Figure 4. In the case of spent grain

297 digested with HCl/NaOH, laccase was not adsorbed on the support after a long exposure time.
298 The biocatalyst showed a very low immobilized activity (U/Kg = 130), efficiency (1.3 %), and
299 recovery (0.22 %). Therefore, the use of this procedure was not economically justified.
300 Nevertheless, the DSG, obtained with H_2SO_4/NaOH digestion process, enabled a significant
301 increase of all the parameters. In particular, the immobilization activity and the efficiency were
302 five and eighteen-fold higher than the other procedure, respectively. Therefore, the higher
303 exposition of cellulose chains, obtained with H_2SO_4/NaOH pretreatment, unequivocally
304 allowed better laccase adsorption.
305
306 *3.3. Optimization of laccase immobilization by dropping procedure on spent grain digested*
307 *with H_2SO_4 /NaOH.*
308 The immobilization was made by dropping the enzyme with a syringe on the surface of BSG
309 digested only with H_2SO_4/NaOH because, as above reported, this digestion guaranteed higher
310 cellulose accessibility to laccase. The idea of this new procedure was made because: i) it was a
311 very simple procedure, ii) the immobilization method was very quick, and iii) it guaranteed
312 some economic benefits thanks to the little volume (μL) of enzyme employed.
313 The selection of appropriate immobilization conditions is the crucial step in producing of the
314 stable and applicable biocatalyst. Thus, in order to confirm that the adsorption is the best
315 method also for this procedure, the immobilization on digested spent grain of native enzyme
316 (DSG-LAC), oxidized laccase (DSG-LAC_{ox}), and with glutaraldehyde as cross linker agent
317 (DSG-GA-LAC) was made. Therefore, the immobilized activity (U/g) and recovery (%) of
318 three biocatalysts, obtained in the same experimental condition (1.1 U incubated activity, 20
319 mg of DSG, pH 7, 48 h), were compared. The results of immobilized activity and recovery were
320 for DSG-LAC 2500 U/Kg and 4.6 %, for DSG-GA-LAC 1100 U/Kg and 3.3 %, and for DSG-
321 LAC_{ox} 210 U/Kg and 0.6 %, respectively, confirming that the direct adsorption of laccase on
322 DSG is the best immobilization procedure, again.

To select the immobilization process's appropriate conditions, factors such as laccase concentration and enzyme volume and time were examined. The suitability of each of the above-mentioned factors was determined on the optimal biocatalyst (DSG-LAC_{drop}) and considering the immobilized enzyme activity. According to the enzyme loading, the study was made dropping a range of catalytic activity of native laccase between 0.1 and 1.7 U on DSG and results are shown in the supplementary material (Figure S2a). The enzyme loading effect is similar to that of the soaking procedure (figure S1a). In fact, the same optimal enzyme concentration (1 U) and an activity decrease at a high amount of enzyme are obtained. Thus, the procedure did not influence the laccase immobilization process. The contact investigation (fig. S2b) shows that the immobilized activity rises fast until 48 h, and above remains almost constant, indicating that support saturation is reached. Another important factor influencing the laccase adsorption is the volume to drop on DSG since it determines the support surface's homogenous covering. The best condition from the data shown in figure S2c is obtained employing 100 µL of native laccase. The volume of 50 µL was not cover the support uniformly, while 180 µL was too much volume and a liquid film remained over the surface.

3.3 Comparison between DSG-LAC_{soak} and DSG-LAC_{drop} *biocatalysts*

The soaking and dropping immobilization procedures, obtained under the same optimal immobilization conditions (laccase 1.0 U, spent grain digested with H_2SO_4/NaOH, adsorption method), are compared here. The drop method resulted in higher laccase activity (2500 U/Kg) and recovery (4.6 %) than the soak approach (700 U/Kg and 0.60%). In addition, taking to account that it is an important feature to reuse biocatalyst for many cycles retaining the activity, the operational stability was tested. In order to quantify this peculiarity, six repeated immobilized activity measurements with 0.18 mM of ABTS were carried out for both the biocatalyst. Between cycles, biocatalysts were washed several times with the reaction buffer to remove any products' remaining level. As shown in figure 5, as the number of analyses

increases, a decrease in activity was obtained. This generally depends on the interaction force to keep the enzyme fixed to the carrier for many cycles. Comparing two biocatalysts appeared that the drop procedure guaranteed a higher interaction force, maintaining 58% of its initial activity at sixth use for 10% in the biocatalyst obtained with soaking procedure at fourth use. These features emanating from the immobilization procedures made the drop method potentially more attractive for biotechnology applications.

3.5 Kinetic parameters study of DSG-LAC$_{drop}$ biocatalyst

To estimate the affinity of ABTS substrate towards free and immobilized laccase, kinetic tests (K_m and V_{max}) were carried out. A low K_m value indicates a high substrate binding ability thanks to the good orientation of site actives of the enzyme. The V_{max} depends on the enzyme amount since more enzymes will convert more substrate moles into the product. In figure 6, the kinetic behavior of the immobilized laccase in function of ABTS concentration (0.018-0.25 mM range) and Lineweaver-Burk plot (in the insert), at pH 3 and 30°C, are reported. From the double reciprocal plot, K_m 0.079 mM and V_{max} 4.2 μM/min, are obtained. A comparison of K_m between immobilized and free laccase shows that K_m value of 0.079 mM was two-fold higher than that of 0.041 mM, respectively, indicating a lower affinity for ABTS. A similar result was also found for laccase immobilization on cellulose nanofiber (Sathiskumar et al., 2014). In this study, the ratio of V_{max}/K_m, a measure of catalytic efficiency of the enzyme-substrate pair, appeared to be 0.053 min^{-1} and 0.14 min^{-1} for immobilized and free laccase. The decreased catalytic efficiency upon immobilization (2.5-fold) was lower value respect other previous studies on laccase immobilization on magnetic chitosan beads (Bayramoglu et al., 2010), on amberlite (Spinelli et al., 2013), and on MANAE-agarose (Brugnari et al., 2021). The difference in the values between free and immobilized laccase could be attributed to diffusional substrate limitations

for the enzyme immobilization in the inner part of the pores and decreased enzyme flexibility after immobilization.

3.4. *Application of DSG-LAC$_{drop}$ biocatalyst: syringic acid removal*

Degradation of syringic acid (50 mg/L) at pH 5 was carried out in batch mode with a very low immobilized activity of DSG-LAC$_{drop}$ biocatalyst (0.02 U) in order to reduce the overall enzyme costs. The reaction was spectrophotometrically followed by UV analysis for 4 h (figure 7). The time course degradation, reported in the region 230-410 nm, shows that the maximum absorbance of syringic acid (260 nm) decreases with time and a contemporary formation of two new peaks at 288 nm and 360 nm is observed (figure 7a). As reported by Shin, 1995, the presence of two isosbestic points at 232 nm and 276 nm evidenced that syringic acid was totally converted to the final products without detectable accumulation of any additional intermediate. The syringic acid residual at 4 h, expressed as the absorbance ratio at the final and initial time, appeared to be 38% without considering the influence of the product absorbance at 260 nm. To evaluate this interference and establish the real percentage of syringic acid removal, HPLC analysis was carried out at the initial and final time of enzyme degradation. The absence of the phenol peak (4.55 min) and the presence of peak product (7.93 min) evidenced that syringic acid is completely removed (figure 7b). Finally, to determine if the syringic acid removal was only due to the enzymatic oxidation, the analyte adsorption on DSG alone was followed with UV-Vis spectrophotometry. A value of 12% was obtained at 4h. For this reason, a synergic action of DSG and laccase on syringic acid removal was hypothesized.

4. Conclusion

Until recently, the high value added of spent grain was not considered; it was generally used for animal feed. In a current trend, the attention of research on this by-product is re-considered since it represents a cheap, sustainable, and valuable raw material. For this reason, in this paper,

spent grain was chosen as support for immobilizing laccase and obtaining an economic and ecofriendly biocatalyst with high operational stability and similar catalytic behavior of free laccase. The research and evidence on the effectiveness of reducing agro-industrial wastes on a small scale are achieved with this research proposal. This finding could increase the market uptake of spent grain, on the hand reducing its disposal problem and on the other hand converting it into a new product as the biocatalyst, which may be then recycled. In addition, the proposed strategies could be an attractive solution to apply the 'green' biocatalyst in different fields (food, bioremediation, and industrial textile) on a larger scale with the proposal to combine a green enzyme (laccase from *Trametes Versicolor*) with an easily available cheap support, making an attractive biocatalyst to apply in different biotechnological applications. Furthermore, spent grain disposal may be minimized by exploiting waste as added product value. Although further studies should be recommended, the system may be a promising research tool in the bioremediation fields thanks to the absents of sophisticated equipment, not toxic product formation, lower energy consumption, and higher sustainability than the advanced oxidation processes (AOPs).

Acknowledgements

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Table1: Comparison of immobilized activity obtained by DSG-soaking procedure. Experimental conditions: laccase activity 1 U, 50 mg digested spent grain (DSG with HCl/NaOH) immobilization time 24 h at 4 °C. The immobilized activity assay conditions: 420 nm, 30 °C, pH 3, and 0.18 mM ABTS as substrate.

Laccase immobilization method	**Activity yield (%)**	**Immobilized activity (U/Kg)**
Adsorption on DSG	39	130±15
Covalent immobilization on oxidized DSG activated with ethylendiammine/ glutaraldheyde	38	20±3.4
Covalent immobilization on oxidized DSG	2.5	10±2.3

Figures

Figure 1: Scheme of immobilization procedures used in the current work chemical and physical immobilization, was analyzed.

Figure 2: ATR-FTIR of spent grain (a), spent grain digested with HCl/NaOH (b), and spent grain digested with H_2SO_4/NaOH (c).

Figure 3: Scanning electron microscope micrographs of spent grain (a) spent grain digested with HCl/NaOH (b) and spent grain digested with H_2SO_4/NaOH (c). SEM images conditions: electron tension of (a) and (c) 2.5 kV and of (b) 3 kV, magnification 1.00 kx.

Figure 4: Comparison of immobilization parameters obtained with HCl/NaOH and with H_2SO_4/NaOH digestion procedures obtained in the same conditions.

Figure 5: Comparison of operative stability between soaking (grey histogram) and dropping (dotted histogram) procedures for laccase immobilization on digested spent grain with H_2SO_4/NaOH.

Figure 6: Michaelis-Menten plot for laccase's catalytic activity immobilized on spent grain digested with H_2SO_4/NaOH. In the insert, Lineawever-Burk plot is reported. Reaction conditions: 30°C, 0.1 M citrate-0.2 M phosphate buffer at pH 3, and 0.018-0.25 mM of ABTS as substrate.

Figure 7: UV-vis spectra of syringic acid during enzymatic degradation with DSG-LAC_{drop} device at different time intervals (a) and HPLC profiles at the initial time after 4 h (b). Chromatographic conditions: stationary phase C 18 3µM (15 cm x 4.66 mm); mobile phase H_2O:MeOH= 70:30 v/v. flow rate 1 mL/min, λ= 260 nm.

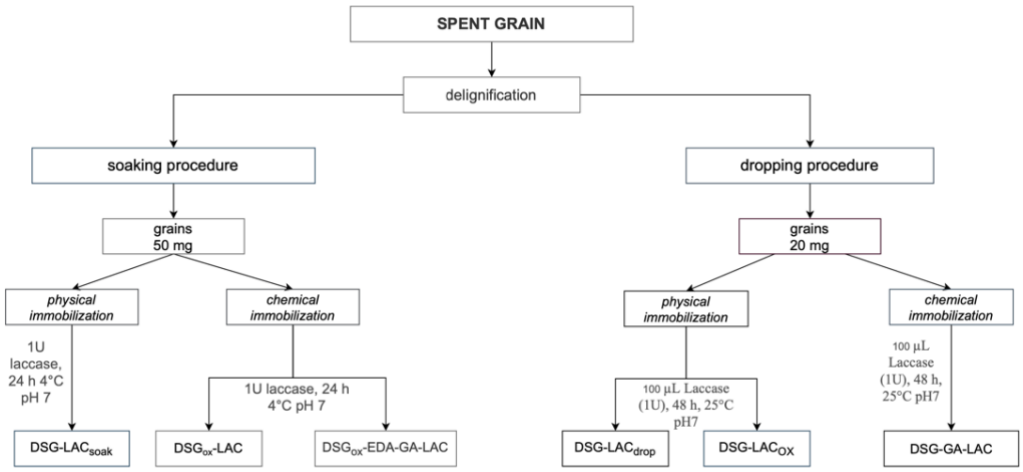
SPENT GRAIN
delignification
soaking procedure
dropping procedure
grains
50 mg
grains
20 mg
physical immobilization
chemical immobilization
physical immobilization
chemical immobilization
1U laccase, 24 h 4°C pH 7
1U laccase, 24 h 4°C pH 7
100 µL Laccase (1U), 48 h, 25°C pH7
100 µL Laccase (1U), 48 h, 25°C pH7
DSG-LAC$_{soak}$
DSG$_{ox}$-LAC
DSG$_{ox}$-EDA-GA-LAC
DSG-LAC$_{drop}$
DSG-LAC$_{OX}$
DSG-GA-LAC

Figure 1

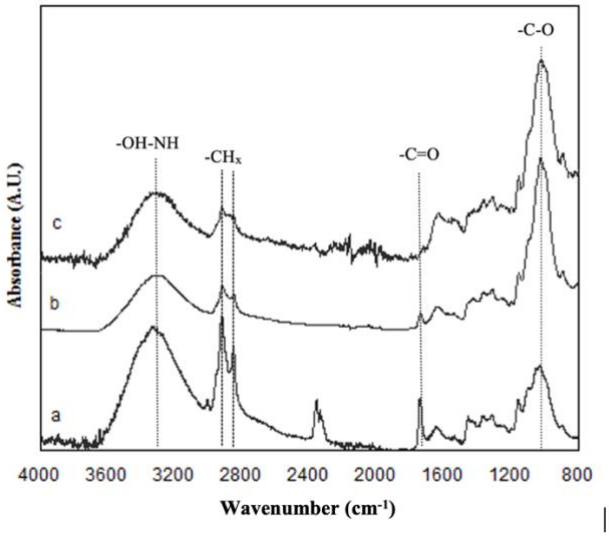
-C-O
-OH-NH
-CHx
-C=O
Absorbance (A.U.)
c
b
a
4000
3600
3200
2800
2400
2000
1600
1200
800
Wavenumber (cm-1)

Figure 2

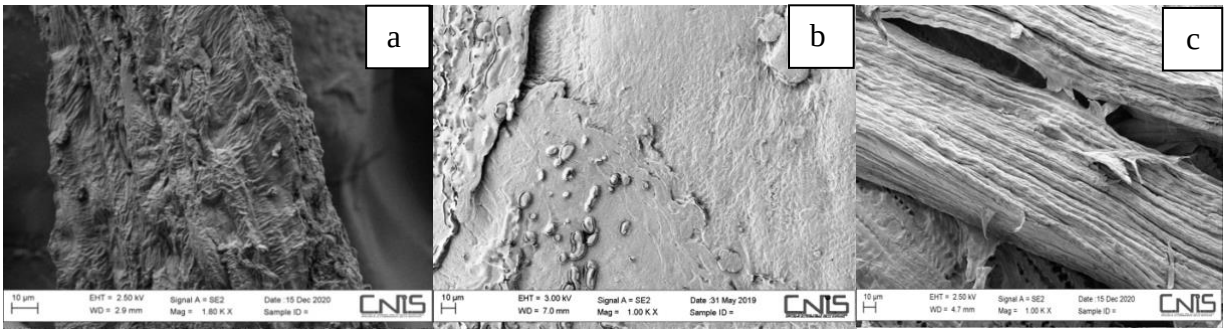

Figure 3

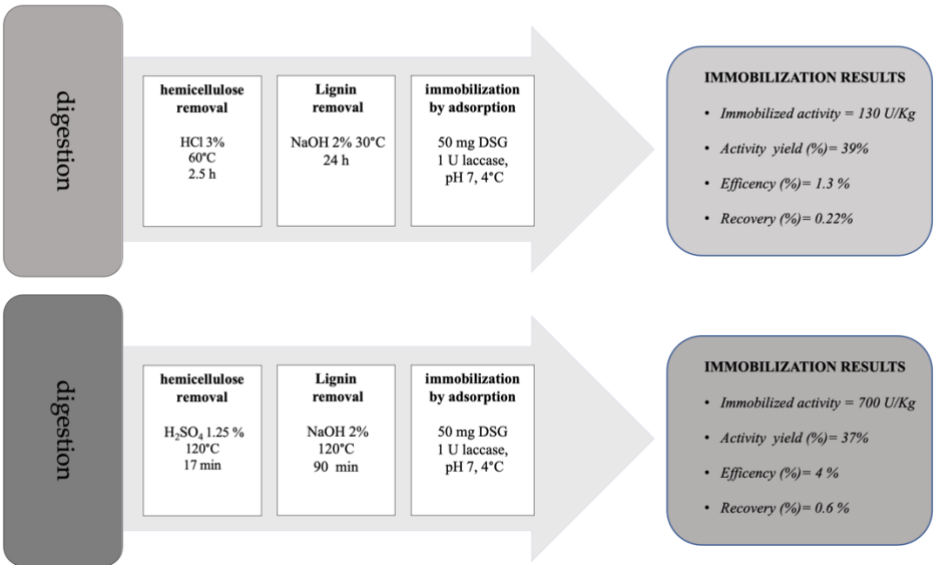
digestion
hemicellulose removal
HCl 3%
60°C
2.5 h
Lignin removal
NaOH 2% 30°C
24 h
immobilization by adsorption
50 mg DSG
1 U laccase,
pH 7, 4°C
IMMOBILIZATION RESULTS
Immobilized activity = 130 U/Kg
Activity yield (%)= 39%
Efficency (%)= 1.3 %
Recovery (%)= 0.22%
digestion
hemicellulose removal
H_2SO_4 1.25 %
120°C
17 min
Lignin removal
NaOH 2%
120°C
90 min
immobilization by adsorption
50 mg DSG
1 U laccase,
pH 7, 4°C
IMMOBILIZATION RESULTS
Immobilized activity = 700 U/Kg
Activity yield (%)= 37%
Efficency (%)= 4 %
Recovery (%)= 0.6 %

Figure 4

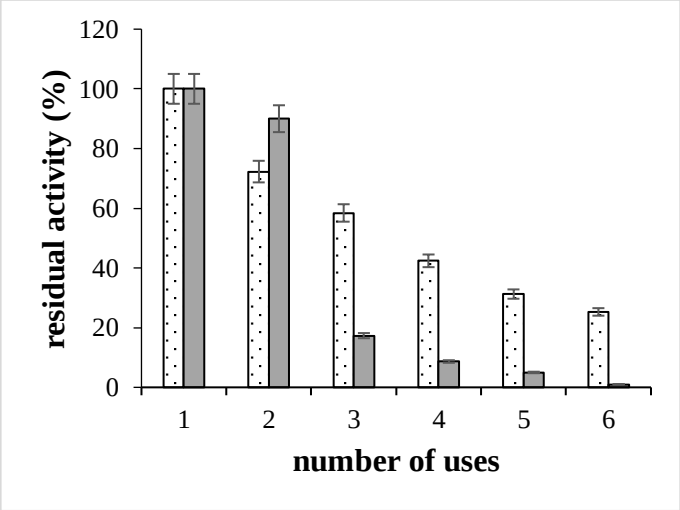

Figure 5

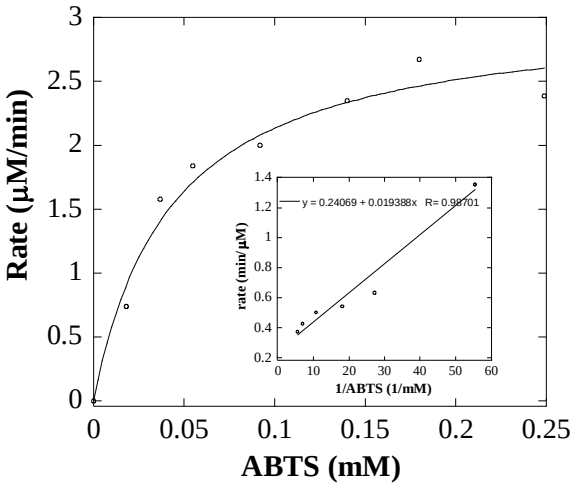
Rate (µM/min)
ABTS (mM)
rate (min/µM)
1/ABTS (1/mM)
y = 0.24069 + 0.019388x R= 0.98701

Figure 6

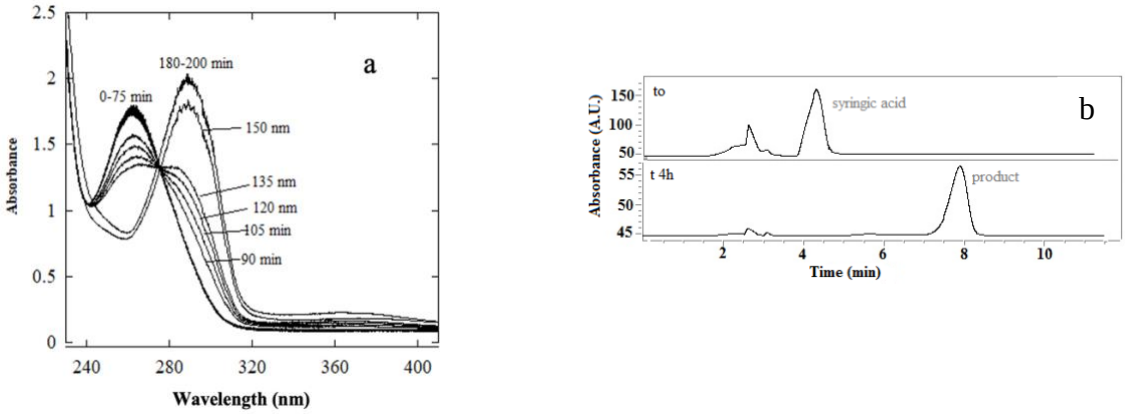
a
180-200 min
0-75 min
150 nm
135 nm
120 nm
105 min
90 min
Absorbance
Wavelength (nm)
b
to
syringic acid
t 4h
product
Absorbance (A.U.)
Time (min)

Figure 7