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Characterization of Laccase Adsorption on Lignocellulosic Biomasses

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Biorefinery is a valid alternative to fossil-based processes through the transformation of lignocellulosic biomasses (LB) into a spectrum of chemicals and biofuels (Cherubini, 2010). The sugar-based platform consists of I) one or more biomass pretreatments aimed at modifying/removing lignin; II) cellulose and hemicellulose enzymatic hydrolysis; III) the fermentation of the released pentose and hexose sugars. Pretreatment of lignocellulosic biomass is a key step. Biochemical pretreatments may be the best alternative in terms of process sustainability. Laccases – multicopper oxidoreductases - are among the most proposed enzymes for this purpose. The present study reports the adsorption of a recombinant fungal laccase - PoxA1b, previously proposed for the delignification of several LBs - on coffee silverskin and Cardoon stalks. The milled and sieved biomasses (0.5 - 1 mm) were dispersed in PoxA1b solutions at several initial enzyme concentrations. The preliminary characterization pointed out two occurring phenomena: the adsorption of the laccases on the biomass surface, and its effect on the further adsorption of cellulases in the enzymatic hydrolysis step. Perspectives on future studies include tuning the adsorption-related phenomena and maximising the impact of the biochemical pretreatments in the delignification of LBs for biorefinery purposes.

1. Introduction

The exploitation of waste lignocellulosic biomass as a non-fossil carbon source via biorefinery processes is an effective strategy for producing biofuels and high-value-added bio-based products (Alberts et al., IRENA, 2016). The saccharification of lignocellulosic biomass (LB) requires several steps to efficiently produce liquid hydrolysate at a high monomeric sugar titre and to maximize the (hemi)cellulose conversion. Among the steps, the pre-treatment is necessary to modify the structure and composition of the LB through the increase of available specific surface and the decrease of lignin content. All actions aimed to increase the polysaccharides accessibility for enzymatic hydrolysis (EH). EH step is catalysed by cellulases and results in the production of sugars that can be used as substrates for industrial fermentation processes (Cherubini, 2010). Biological/biochemical pre-treatments are the most eco-friendly alternative among the pretreatment strategies proposed so far (Fillat et al., 2017). Indeed, the modification of lignin fraction can be obtained by using microorganisms (e.g. fungi) and enzymes (e.g. laccases, phenol oxidases). All actions are aimed at minimising the production of inhibitors that could affect the EH and fermentation stages under mild operating conditions that do not impact the overall costs and energy duty of the biorefinery process (Sharma et al., 2019). Laccases are among the most used and well-known 'lignin-active' enzymes. They are multicopper oxidoreductases that act on lignin by oxidizing phenolic subunits and produce water as a by-product (Plácido and Capareda,2015). Laccases are produced by a wide range of microorganisms, including fungi, bacteria and archaea and have been widely studied against lignin model substrate and LB substrate with a lignin content between 17 to 24% (Giacobbe et al., 2018). The heterogeneous nature of the process catalysed by laccases in the case of lignin-substrate embedded in LB solid particles, the homology between the lignin-destructive process and the EH catalysed by cellulases can be considered to set the methodologies for the investigation of phenomena responsible of the biochemical modification of lignin chains. Among these phenomena, the adsorption of the

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biocatalyst (laccases) on the surface of LB particles is expected to have effects in regulating the effective loading of enzymes in the LB slurry as it is already proved for the EH process.
The present contribution reports a preliminary characterization of the adsorption of PoxA1b - a laccase from *Pleurotus ostreatus* - was performed using two LBs: coffee silverskin (an agro-food waste of the coffee bean-roasting process), and Cardoon stalks (a crop spread in the Mediterranean area and used as energy resource).

2. Materials and methods

2.1 Lignocellulosic biomass

Coffee silverskin (CSS) and *Cynara cardunculus* (Cardoon) stalks were milled and sieved in a steel blade mill (MF 10, IKA, Staufen, Germany) in the range 0.5-1 mm. The powder was stored in boxes at room temperature until use. The composition of the raw biomass was assessed according to the acid hydrolysis method (Sluiter et al., 2008) and data are reported in Table 1.

2.2 Enzymes

The recombinant laccase PoxA1b from *P. ostreatus* expressed in *Pichia pastoris* was kindly donated by Biopox s.r.l. (Napoli, Italy). Cellic® Ctec2 is a commercial cellulase cocktail (Novozymes Latin America, Araucária, PR, Brazil) used for the enzymatic hydrolysis tests and composed of high concentrations of cellulases, hemicellulases, and β-glucosidases. The total enzyme concentration was assessed using Bradford's assay (Bradford, 1976), and the cellulase activity was assessed in terms of Filter Paper Units (FPU) (Adney and Baker,1996). The activity of laccases was assayed following the oxidation of 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Macellaro et al.,2014).

2.3 Biomass pretreatment with PoxA1b

Raw LBs were washed with 0.05 M sodium citrate buffer pH 4.8 in a continuous flow column at room temperature to allow the desorption of proteins present on the surface of the particles.
The laccase activity for delignification tests was set between 0.8 and 31.2 U/mL. Delignification tests were performed with 5% w/v substrate (washed LB) in 30 mL of 0.05 M sodium citrate buffer pH 4.8 in 100 mL glass bottles at 28°C in an incubator (bioSan PSU-10i) equipped with an orbital shaker set at 200 rpm for 24h. The initial protein concentration in the slurry was assessed, then aliquots were prepared as substrate control samples and pretreatment samples were PoxA1B solution was added at the desired concentration.
The liquid samples were centrifugated at 13000 rpm for 10 min to separate solid and liquid phases at times 0, 30, and 60 min and the adsorption of PoxA1b on LB samples was monitored by measuring the protein concentration and the laccase activity in the liquid phase. The free PoxA1b content of the liquid was calculated subtracting the protein content of the substrate control to the total dissolved protein in the liquid at any time. The amount of adsorbed enzyme (E_{ads}) was assessed according to the mass balance referred to the PoxA1b content and extended to the reactor closed system (liquid and solid LB).
Any effect of compounds released by the LB on PoxA1b activity was assessed also incubating the enzyme with the liquid equilibrated with the cardoon biomass. Pre-washed LB was suspended in 0.05 M sodium citrate buffer at 5% w/v final substrate concentration and located on the orbital shaker incubator at 200 rpm, 28°C for 30 min. The release of proteins in the liquid phase was checked by protein concentration assay at time 0 and 30 minutes. The liquid phase was separated by centrifugation and mixed with a PoxA1b stock solution at 0 5.8 IU/mL final activity (≈ 16 mg/L). The solution was kept at 28°C and agitated with an orbital shaker at 200 rpm. The activity of PoxA1b in the liquid phase was monitored for 60 min.

2.4 Enzymatic hydrolysis

The LB samples recovered after the pretreatment with laccases were incubated for 4 hours at 50°C to deactivate laccases (Niglio et al., 2019). The solid phase was harvested by centrifugation and vacuum filtration, pooled together, and resuspended in the Celli® CTec2 solution to reach a sufficient volume for the hydrolysis tests set at 5%w/v biomass in 50 mL centrifuge tubes, and 1 g/L protein. The slurry was kept for 30 min on a rotary shaker at 40 rpm and 25°C to allow adsorption of cellulases. The hydrolysis step was carried out at 5% w/v for CSS and up to 10% w/v in the case of Cardoon according to a previous optimization study (Posilipo et al., 2024). The slurry was transferred in glass bottles and put at 50°C, on an orbital shaker (120 rpm) for 48h to allow the onset and progress of the enzymatic hydrolysis of cellulose and hemicellulose. Sugar concentration in the liquid phase was assessed at 1, 24, and 48h by HPLC of the liquid phase recovered by filtration of the LB slurry (13000 rpm (Minispin, Eppendorf) and PVDF 13 mm, 0.22 μm filters, Millex).
The glucose and xylose yields were assessed as the ratio between the sugar concentration in the liquid phase and the theoretical sugar concentration at the onset of the hydrolysis test.

2.5 Analytical methods

The concentration of monomeric sugars – glucose, xylose, arabinose- in the liquid samples was assessed by HPLC (Agilent 1100 Series), equipped with Refractive Index Detector, using Rezex RHM-Monosaccharide H^+ Column, at room temperature, with 0.6 mL/min flow of HPLC grade water as mobile phase.

3. Results and discussions

3.1 Lignocellulose substrates composition

The composition of the lignocellulosic biomasses is reported in Table 1. CSS is characterized by lignin and sugar concentrations smaller than those measured for Cardoon stalks. The method by Sluiter et al. (2008) provides a percentage of detected compounds between 57.4 (CSS) and 67.1 % (Cardoon) (Posilipo and Russo, 2022; Posilipo et al., 2024).

Table 1: polysaccharides fractions of lignocellulosic biomasses. AIR= Acid Insoluble Residue, ASL= Acid Soluble Residue

	AIR, % dry basis	ASL, % dry basis	Glucans, % dry basis	Xylans, % dry basis	Arabinans, % dry basis
CSS	27.3 ± 0.6	1.0 ± 0.02	19.5 ± 0.008	7.7 ± 0.001	1.9 ± 0.001
Cardoon	30.7 ± 0.8	0.9 ± 0.01	29.8 ± 0.04	12.8 ± 0.001	0.4 ± 0.001

3.2 Characterization of laccase adsorption on lignocellulosic biomasses

The LBs were washed/pre-conditioned before the laccase adsorption tests in the citrate buffer. The biomass was placed in a column and flowed continuously with the buffer solution until the concentration of proteins released by the biomass approached a value low enough to allow the assessment of PoxA1B variations during pretreatment and adsorption tests. Accordingly, the washing was stopped when the released protein concentration was between 2.7 and 6.6 mg/L. The operating conditions of PoxA1b adsorption on LB substrates were selected considering the results of previous studies on CSS (Giacobbe et al., 2019; Niglio et al., 2018) and the initial laccase concentration was set at values larger than the initial protein concentration released by the LB (Table 2). The selected buffer and pH were consistent with a possible application of sequential laccase pre-treatment and enzymatic hydrolysis (section 2.4).

Tests with CSS were carried out at PoxA1b concentration set in the range 4.2 – 27.2 mg/L (15.9 to 136.1 U/g $_{biomass}$) (Table 2). Laccase concentration and activity decreased over 60 min due to enzyme adsorption on the CSS particles (Figure 1A-B). At low laccase loading (15.9 - 20 U/g) the laccase concentration in the liquid phase was almost stable up to 30 min and then decreased until 60 min. At high PoxA1b loading (136.1 U/g), a steep decrease of the enzyme concentration was measured since the beginning of the test. The analysis of Table 2 points out the increase of the adsorbed enzyme amount with the initial enzyme concentration.

Tests with Cardoon were carried out at a high PoxA1b loading (123.3-623.5 U/g) because of the high adsorption capacity against cellulase cocktails, assessed in previous studies (Posilipo et al., 2024). The increase in adsorbed enzyme amount with the initial PoxA1b concentration was confirmed in the case of Cardoon (Figure 1 C - D).

Tests carried out with both CSS and Cardoon samples - in a wide range of initial concentrations of PoxA1b – were characterized by E_{ads} quite low and almost lower than 1 mg/g. A substantial decrease of laccase activity over 60 min adsorption tests was observed (Figure 1 B-D) for both LBs. The activity decrease is due to both the adsorption of the protein on the biomass surface and the deactivation of the laccases. To assess the occurrence of PoxA1b deactivation, tests were carried out with the liquid phase equilibrated with Cardoon biomass without the solids (section 2.3). The decrease of PoxA1b activity after 60 minutes of incubation with proteins released by Cardoon was between 1.4 and 10.3% of the initial value. This quite small decrease was low enough to be neglected in the assessment of laccases adsorption. Therefore, the decrease in activity reported in Figure 1 can be justified with the transport of the enzyme to the solid phase. Further tests were made on the pretreated samples to assess any effect of PoxA1b on the adsorption of cellulases and their activity in the hydrolysis of polysaccharides.

Table 2: PoxA1b adsorption tests. E_{eq}= enzyme equilibrium concentration in the liquid phase, E_{ads}= amount of equilibrium adsorbed enzyme per unit mass of biomass.

Substrate	Initial conditions					Results	
	Substrate concentration, % w/v	Biomass released proteins, mg/L	PoxA1b, mg/L	PoxA1b, U/mL	PoxA1b, U/g biomass	E_{eq}, mg/L	E_{ads}, mg/g
CSS	5	3.5	4.2 ± 0.3	1.0 ± 0.0	20 ± 0.0	2.7 ± 0.9	0.03 ± 0.01
			7.8 ± 0.2	0.8 ± 0.04	15.9 ± 0.9	0.9 ± 0.1	0.14 ± 0.01
			9.4 ± 1.5	2.1 ± 0.4	41.7 ± 8.4	3.1 ± 0.5	0.13 ± 0.04
			27.2 ± 1.3	6.8 ± 0.4	136.1 ± 8.1	19.4 ± 1.3	0.18 ± 0.07
Cardoon		2.5	27.9	6.2	123.3	16.1	0.24
			32.5 ± 0.7	8.6 ± 0.2	172.5 ± 4.2	16.7 ± 1.6	0.32 ± 0.02
			158.6 ± 8.4	31.2 ± 0.8	623.5 ± 16.5	58.9 ± 3.4	1.00 ± 0.47

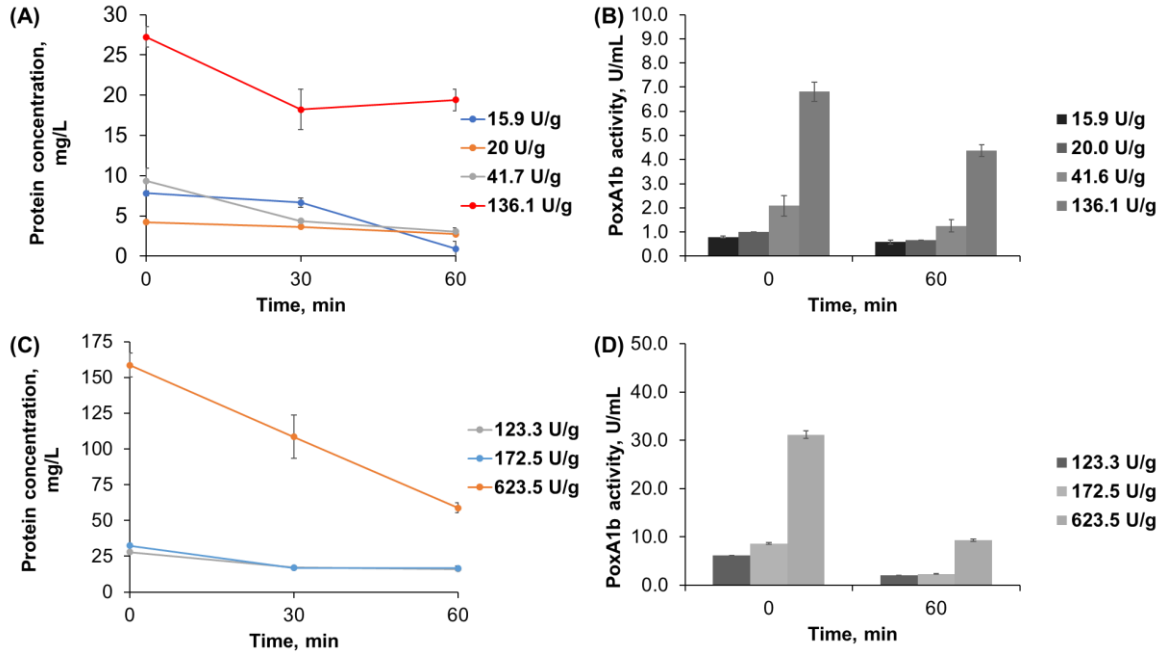

Figure 1: Results of adsorption tests of PoxA1b vs. time: A and B) CSS (5% w/v); C and D) Cardoon (5% w/v). A and C) total protein concentration; B and D) laccase activity. Tests were made as duplicates according to the enzyme stock availability. Single initial PoxA1b concentrations are reported in Table 2.

3.3 Enzymatic hydrolysis of laccase-pretreated biomasses

Enzymatic hydrolysis tests were carried out with CSS and cardoon pre-treated with PoxA1b (average loading 17.2 U/g and 136.1 U/g). Cellulase adsorption and maximum monomeric glucose yield (assessed at 24h and 48h for cardoon and CSS, respectively) are reported in Table 3. Xylose yields followed the same trend as glucose yield (data not reported).

For tests carried out with CSS, as the concentration of the PoxA1b set in the pretreatment increased from 17.2 to 136.1 mg/L the adsorbed cellulases decreased. Accordingly, a substantial decrease in glucose yield upon enzymatic hydrolysis was assessed. The lowest concentration of PoxA1B (17.2 U/g) provided an increase (17-32%) of the glucose yield compared to the value obtained from enzymatic hydrolysis of raw CSS. For tests carried out with Cardoon, no significant variation in cellulase adsorption and maximum glucose yield upon PoxA1b pretreatment was observed and they were about those assessed for raw samples. The increase of PoxA1b initial concentration (from 168-176 to 607-640 mg/L) provided the increase in the amount of adsorbed cellulases for biomass weight unit (from about 5 to 8 mg/g). Despite being strongly influenced by the complex formulation of the commercial cellulase cocktail Cellic® CTec2, this evidence suggests that any chemical modification of the lignin fraction by laccase (Fillat et al., 2017) may affect the adsorption of cellulases.

Therefore, efforts should be focused on assessing if the action of laccases drives toward a lower affinity of lignin to cellulases (more generally proteins) adsorption. This type of pretreatment can be applied to both raw LBs or to LBs pretreated with physico-chemical processes, hence on the residual lignin that still contributes to the unproductive binding of cellulases.

Table 3: Hydrolysis of the investigated LBs pretreated with PoxA1b.

Biomass	PoxA1b U/g	Initial Cellulase g/L	Adsorbed cellulases mg/g	Maximum glucose yield %
CSS	0*	0.02*	n.a.*	34*
	17.2 ± 1.8	0.85 ± 0.01	1.55± 0.07	42.6 ± 2.6
	136.1 ± 8.1	0.73 ± 0.02	0.61 ± 0.1	9.3 ± 0.6
Cardoon	0	1.17	8.07	21.1
	172.5 ± 4.2	1.1 ± 0.03	5.19 ± 0.18	17.4 ± 0.3
	623.5 ± 16.5	1.3 ± 0.02	8.70 ± 0.22	18.5 ± 0.5

**data from Niglio et al., 2020, initial cellulases as 1FPU/g_cellulose.*

4. Conclusions

A preliminary characterization of the adsorption of the laccase PoxA1b on two relevant LBs among agro-industrial waste and energy crops - CSS and Cardoon stalks – was carried out. Adsorption tests showed an increase of adsorbed enzyme loading at increasing initial PoxA1b concentration for both CSS and Cardoon. The decrease of laccase activity and concentration in the liquid phase was proved to be related to the occurrence of the adsorption on the LB surface even though it is limited to less than 1 mg/g adsorbed enzyme due to the low dosage of PoxA1b applied according to the literature (large specific activity). The effect of PoxA1b pretreatment on the adsorption of cellulase and their performances in the enzymatic hydrolysis of polysaccharides is strongly dependent on the type of biomass and the initial dosage of PoxA1b. The assessed values of adsorbed cellulases (below 8.7 mg/g) upon PoxA1b pretreatment and related glucose yields (below 42.6%) upon enzymatic hydrolysis suggested combining the laccase-mediate biological pretreatment with other processes promoting the exposure of cellulose fibres. In conclusion, further investigation should be focused on the chemical modifications occurring on the lignin fraction of selected raw and pretreated lignocellulosic biomasses because of PoxA1b activity and on the effects of the lignin chemical modification on the adsorption of single processive exo- and endo-glucanases as the main components of hydrolytic cocktails is also to be investigated.

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References

Alberts G., Ayuso M., Bauen A., Boshell F., Chudziak C., Gebauer JP., German L., Kaltschmitt M, Nattras L., Ripken R., Robson P., Taylor R., Wagner H., 2016, Innovative Outlook Advanced Liquid Biofuels, IRENA.

Bradford, M. 1976, A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, Analytical Biochemistry, 72, 1–2.

Cherubini F., 2010, The biorefinery concept: Using biomass instead of oil for producing energy and chemicals, Energy Conversion and Management, 51, 1412-1421.

Fillat Ú., Ibarra D., Eugenio M.E., Moreno A.D., Tomás-Pejó E., Martín-Sampedro R., 2017, Laccases as a Potential Tool for the Efficient Conversion of Lignocellulosic Biomass: A Review., Fermentation; 3(2):17.

Giacobbe S., Pezzella C., Lettera V., Sannia G., Piscitelli A.,2018, Laccase pretreatment for agrofood wastes valorization, Bioresource Technology, 265, 59-65.

Giacobbe S., Piscitelli A., Raganati F., Lettera V., Sannia G., Marzocchella A., Pezzella C., 2019, Butanol production from laccase-pretreated brewer's spent grain. Biotechnol Biofuels, 12, 47.

Macellaro G., Baratto M.C., Piscitelli A., Pezzella C., Fabrizi de Biani F., Palmese A., Piumi F., Record E, Basosi R., Sannia G.,2014, Effective mutations in a high redox potential laccase from *Pleurotus ostreatus*, Appl Microbiol Biotechnol 98, 4949–4961.

Niglio S., Procentese A., Russo M. E., Piscitelli A., Marzocchella A., 2019, Integrated enzymatic pretreatment and hydrolysis of apple pomace in a bubble column bioreactor, Biochemical Engineering Journal, 150,107306.

Niglio S., Procentese A., Russo M. E., Sannia G., Marzocchella A., 2020, Combined pretreatments of coffee silverskin to enhance fermentable sugar yield, Biomass Conversion and Biorefinery. https://doi.org/10.1007/s13399-019-00498-y

Plácido J., Capareda S., 2015, Ligninolytic enzymes: a biotechnological alternative for bioethanol production. Bioresour. Bioprocess, 2, 23.

Posilipo A., Russo M.E., 2022, Initial Rate of Hydrolysis of Coffee Silverskin by a Commercial Cellulase Cocktail, Chemical Engineering Transactions, 93, 25-30.

Posilipo A., 2024, Optimization of Biochemical Saccharification of Lignocellulosic Biomass. PhD Thesis *Università degli Studi di Napoli Federico II*

Sharma H. K., Xu C., Qin W., 2019, Biological Pretreatment of Lignocellulosic Biomass for Biofuels and Bioproducts: An Overview. Waste Biomass 10, 235–251.

Sluiter A., Hames B., Ruiz R., Scarlata C., Sluiter J., Templeton D., 2008, Determination of Structural Carbohydrates and Lignin in Biomass - Laboratory Analytical Procedure.

Suhag M., Sharma H. R., 2015, Biorefinery Concept: An Overview of Producing Energy, Fuels and Materials from Biomass Feedstocks, International Advanced Research Journal in Science, Engineering and Technology 2,12.