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Laccase pretreatment for agrofood wastes valorization

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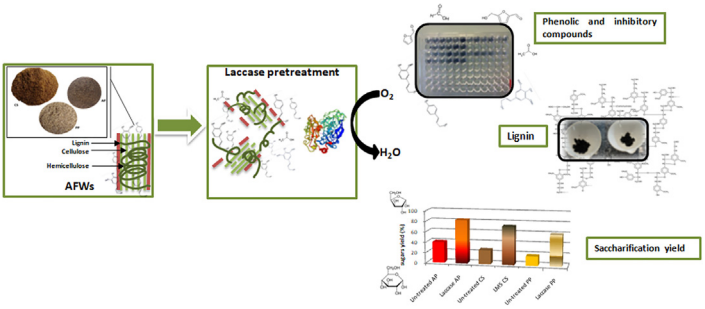
GRAPHICAL ABSTRACT



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ABSTRACT

Apple pomace, potato peels, and coffee silverskin are attractive agrofood wastes for the production of biofuels and chemicals, due to their abundance and carbohydrate content. As lignocellulosic biomasses, their conversion is challenged by the presence of lignin that prevents hydrolysis of polysaccharides, hence demanding a pretreatment step. In this work, the effectiveness of *Pleurotus ostreatus* laccases (with and without mediator) to remove lignin, improving the subsequent saccharification, was assessed. Optimized conditions for sequential protocol were set up for all agrofood wastes reaching delignification and detoxification yields correlated with high saccharification. Especially noteworthy were results for apple pomace and coffee silverskin for which 83% of and 73% saccharification yields were observed, by using laccase and laccase mediator system, respectively. The herein developed sequential protocol, saving soluble sugars and reducing the amount of wastewater, can improve the overall process for obtaining chemicals or fuels from agrofood wastes.

1. Introduction

In recent years, the concept of linear economy is started to switch towards a circular economy (CE) one, since the linear model is mainly based on the use of fossil resources. The main problem related to the fossil resources is their limitation in supply due to their non-renewability. Moreover the linear economy is also based on "take-make-dispose" linear flow, making it nowadays unsustainable. In contrast, the circular economy model endorses the "reduce-reuse-recycle" approach closing the loop of product lifecycles (Hennig et al., 2016; Liguori and Faraco, 2016; Nizami et al., 2017).

In this contest the utilization of wastes, mainly agrofood feedstocks, in biotechnological processes for the production of value added chemicals and fuels represents an application of the CE concept, linked to the bio-based economy (Pleissner et al., 2016).

Many industrial food wastes, rich in cellulose and hemicellulose,

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represent secondary raw materials to be valorized through biorefineries, as reported by different studies in literature (Amore et al., 2014; Mirabella et al., 2014). Nevertheless, as lignocellulosic materials, they contain also a variable amount of lignin, and their biochemical conversion into chemicals and fuels requires a pretreatment step to remove/modify lignin with consequently increase of (hemi)cellulose accessibility and reduction of hydrolytic enzyme adsorption to residual lignin. An ideal pretreatment should be cheap, effective against various substrates without increase of inhibitors, and should prevent loss of polysaccharides. Several pretreatment methods have been developed, including steam explosion, ammonia fiber expansion (AFEX), dilute acid, biological, enzymatic treatment and ionic-liquid pretreatment (Kumar et al., 2009; Woiciechowski et al., 2013; Amore et al., 2014). Among all reported processes, the enzymatic pretreatment, by ligninolytic enzymes, represents an attractive method to both delignify and detoxify lignocellulosic materials, such as agrofood wastes (AFWs), preventing the occurrence of side reactions or formation of by-products.

Laccases (EC 1.10.3.2) are ligninolytic enzymes, which can oxidize phenolic substrates with a concomitant reduction of oxygen to water (Giardina et al., 2010) and are suitable for industrial applications thanks to their broad substrate specificity (Pezzella et al., 2015). In particular, laccase enzymes play an important role in lignin degradation and modification processes offering the possibility to increase the yield of both hydrolysis and fermentation phases due to alteration of lignin hydrophobicity and porosity (Giardina et al., 2010; Piscitelli et al., 2011; Plácido and Capareda, 2015; Fillat et al., 2017). Several bacterial and fungal laccases have been used for detoxification and/or delignification of various pretreated and milled un-pretreated feedstocks, alone or in the presence of a mediator, the laccase mediator system (LMS) (Fillat et al., 2017). All previous studies have reported “separate” laccase delignification and saccharification (Kuila et al., 2011a, 2011b; Mukhopadhyay et al., 2011; Moreno et al., 2016a; Moreno et al., 2016b) in which filtration and washing steps are applied between the two phases to remove inhibitory compounds and mediator, and to change the operative conditions. Laccases alone have resulted able to delignify materials with a lignin content ranging from 17 to 24%, reaching a delignification yield of up to 89% with a consequential increase of saccharification (Kuila et al., 2011a, 2011b; Mukhopadhyay et al., 2011; Rico et al., 2014; Rencoret et al., 2016; Rajak and Banerjee, 2016). When the LMS has been tested, different results have been reported, depending on the nature of the used mediator (natural or chemical one) (Fillat et al., 2017).

The use of laccases on already pretreated feedstocks have yielded high detoxification, although contrasting results on delignification have been observed, due to the occurrence of the covalent coupling of phenolic radicals to the aromatic lignin fibers, the so-called grafting phenomenon (Moreno et al., 2016a; Oliva-Taravilla et al., 2015).

In this study the ability of two laccase preparations from *Pleurotus ostreatus* to delignify and detoxify three different un-pretreated AFWs was evaluated. The treatment was applied on apple pomace (AP), potato peels (PP) and coffee silverskin (CS), selected on the basis of their European availability and carbohydrate content. To the best of our knowledge, no study has been reported regarding laccase pretreatment of these AFWs. Moreover, to the aim of reducing cost and environmental impact of the process, the effect of LMS was investigated exploiting a lignin-derived natural mediator, supposed to not produce toxic side-products (Cañas and Camarero, 2010; Rico et al., 2014).

2. Materials and methods

2.1. Materials

Reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO), unless otherwise specified.

The commercial enzymes used in this study were:

- Commercial cellulolytic enzyme cocktail Cellic® CTec2 (kindly supplied by Novozyme);
- *endo*-1,4-β-Xylanase M1 from *Trichoderma viride* (purchased from Megazyme);
- α-amylase from *Bacillus licheniformis* (purchased from Megazyme).

AP, CS and PP used in this study were kindly supplied by Spanish and Italian companies, in the frame of the Waste2fuels project. The supplied biomass was oven-dried at 40 °C and milled. Solids collected in the range 1–0.5 mm were stored under dry conditions at room temperature until further use.

AFWs characterizations were performed following the National Renewable Energy Laboratory (NREL) Laboratory Analytical Procedures (LAPs) standard protocols (Laurens, 2013; Sluiter et al., 2008a, 2012).

2.2. Laccase enzymes

Two different preparations of laccases from *P. ostreatus* were used in this work: rPOXA1b laccase recombinantly expressed in *Pichia pastoris* (Pezzella et al., 2017) and a mix of native laccases ($mix_{P.o.}$) produced after 10 days of growth in PDY supplemented with 150 μM $CuSO_4$ and 2 mM ferulic acid (Pezzella et al., 2013).

2.2.1. Assay of laccase activity

Laccase activity against ABTS was assayed as reported by Macellaro et al. (2014).

2.2.2. Stability at pH and temperature

The pH and temperature stabilities of both laccase preparations were evaluated at two different pH by using McIlvaine buffer adjusted at pH 5.0 and in 50 mM sodium phosphate buffer adjusted at pH 6.5 at 28 °C, 40 °C and 50 °C. The activity was assayed as described above (Section 2.2.1).

2.2.3. Effect of laccase on cellulase activity

The effect of laccases on cellulases was analysed by measuring endo- and eso-cellulolytic activity against two different substrates, carboxymethyl cellulose-Remazol Brilliant Blue R (AZO-CM-Cellulose) (Megazyme Co., Bray Ireland), and *p*NP-β-D-glucopyranoside (pNPG), respectively.

In both assays laccases were added at time zero (active laccase), after incubation at 50 °C for 5 h (partially deactivated laccase), and after incubation at 50 °C for 24 h (deactivated laccase).

Endo-Cellulase activity was determined spectrophotometrically utilizing the soluble chromogenic substrate AZO-CMC following the manufactories instructions with some modifications. The assay mixture, containing 500 μL of 0.2% (w/v) substrate in 50 mM sodium acetate buffer pH 4.6, 250 μL of properly diluted cellulase enzyme solution and 250 μL of laccase enzymes, was incubated for 30 min at 50 °C. The reaction was stopped by adding 2.5 mL of 96% ethanol to the mixture, followed by incubation at room temperature for 10 min and centrifugation. The activity was measured at 590 nm. The β-glucosidase activity of the cellulases was evaluated as reported by Marcolongo et al. (2014) with some modifications. 25 μL of diluted enzyme solution and 25 μL of laccase enzymes were added to 450 μL of 50 mM sodium acetate buffer pH 5.0 containing 2 mM *p*NPG. After heating the assay mixture at 50 °C for 10 min, the reaction was stopped by adding 1 mL of 1 M sodium carbonate and the release of *p*-nitrophenol was measured at 405 nm.

Cellulase activity reduction was estimated as percentage respect to control samples containing only cellulase enzymes.

2.2.4. Laccase pretreatment

The pretreatments were carried out at 10% (w/v) in case of AP and CS, while at 5% (w/v) in the case of PP, due to its high water