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Unraveling the effects of laccase treatment on enzymatic hydrolysis of steam-exploded wheat straw

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HIGHLIGHTS

- The activity of β-glucosidase was slightly reduced by laccases.
- Total phenolic content decreased 80% points after laccase treatment.
- Laccase treatment increased the Klason lignin in the lignocellulosic substrate.
- Laccases modified the infrared adsorption spectra of the lignocellulosic substrate.
- Grafting process is limiting the accessibility of cellulolytic enzymes to cellulose.

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ABSTRACT

Laccase enzymes are promising detoxifying agents during lignocellulosic bioethanol production from wheat straw. However, they affect the enzymatic hydrolysis of this material by lowering the glucose recovery yields. This work aimed at explaining the negative effects of laccase on enzymatic hydrolysis.

Relative glucose recovery in presence of laccase (10 IU/g substrate) with model cellulosic substrate (Sigmacell) at 10% (w/v) was almost 10% points lower ($P < 0.01$) than in the absence of laccase. This fact could be due to an increase in the competition of cellulose binding sites between the enzymes and a slight inhibition of β-glucosidase activity. However, enzymatic hydrolysis and infrared spectra of laccase-treated and untreated wheat straw filtered pretreated residue (WS-FPR), revealed that a grafting process of phenoxy radicals onto the lignin fiber could be the cause of diminished accessibility of cellulases to cellulose in pretreated wheat straw.

1. Introduction

Concerns about climate change and uncertainties about fuel supply make renewable biofuels, such as bioethanol and biodiesel, attractive options for oil replacement in the short-term perspective. Presently, international governments have adopted some policies, such as the European Union Directive 2009/28/CE (2009), to gradually replace fossil fuels by biomass-based fuels or biofuels. Moreover, innovative technologies and advanced biorefineries based on lignocellulosic biomass are expected to play an important role in future bio-economy in Europe. Lignocellulosic biorefineries are expected to provide different forms of energy such as bioethanol and create new markets for bio-based products such as food, feed, chemicals and materials.

Lignocellulosic biomass is an abundant, low-cost and widely distributed feedstock. Among different lignocellulosic raw materials, wheat straw is an ideal candidate for bioethanol production worldwide (Tomás-Pejó et al., 2008). The biochemical conversion of lignocellulose into ethanol is performed via enzymatic hydrolysis of the carbohydrates to monomeric sugars, which are subsequently converted into ethanol by a fermentation step. The process is, however, hindered by the complex and recalcitrant structure of the lignocellulosic materials and thus, a pretreatment step is needed to increase biomass digestibility. Hydrothermal methods, such as steam explosion, are cost-effective technologies to increase the accessibility of enzymes to cellulose by solubilization of hemicelluloses and lignin

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Nomenclature

ANOVA	analysis of variance
ATR–FTIR	attenuated total reflectance–Fourier transform infrared spectroscopy
DW	dry weight
FPU	filter paper unit
IU	international unit
PEG	polyethylene glycol
Raw material	milled wheat straw (particle size: 2–10 mm)
RGR	relative glucose recovery
SF	severity factor
Sigmacell	model cellulosic substrate Sigmacell Type 50® (Sigma S5504)
Slurry	whole steam-exploded wheat straw
WS-FPR	wheat straw filtered pretreated residue (vacuum-filtered but not washed)

redistribution (Alvira et al., 2010). Nevertheless, harsh conditions applied in these pretreatments also promote sugar and lignin degradation, triggering the formation of weak acids, furan derivatives and phenolic compounds which inhibit enzymes and microorganisms performance (Palmqvist and Hahn-Hägerdal, 2000; Ximenes et al., 2010). The removal of the inhibitors from the fermentation broth, also known as detoxification, is an interesting alternative to overcome the negative effect of these compounds. As a detoxification method, *in situ* laccase treatment represents an appropriate option since the process is carried out in the same vessel under mild conditions (Parawira and Tekere, 2011; Moreno et al., 2014). Laccases are multicopper-containing phenoloxidases that catalyze the oxidation of substituted phenols, anilines and aromatic thiols at the expense of molecular oxygen. Laccase addition to pretreated lignocellulosic materials selectively removes the phenolic compounds formed during the hydrothermal pretreatment (Jönsson et al., 1998; Jurado et al., 2009; Moreno et al., 2012, 2013a). This phenol removal by laccases leads to improved fermentation performance of microorganisms, shortening the lag phase and boosting cell viability. In consequence, ethanol volumetric productivities are enhanced and the overall process time is reduced. Nevertheless, in case of steam-exploded wheat straw, laccase-detoxified biomass shows lower glucose hydrolysis yields during the saccharification step, which implies a reduction in the final ethanol concentrations (Jurado et al., 2009; Moreno et al., 2012, 2013a,b).

Several hypotheses have been proposed to explain the lower glucose recovery yields after laccase treatment: (1) laccases increase the non-specific adsorption of hydrolytic enzymes onto the lignocellulosic fibers by catalyzing reactions on lignin (Moilanen et al., 2011); (2) laccases cause strengthening of carbohydrate-lignin bonds (Moilanen et al., 2011) and (3) formation of laccase-derived compounds can inhibit the hydrolytic enzymes (Jurado et al., 2009; Tejirian and Xu, 2011). Although all these inhibition mechanisms seem to be accepted, there is no information about the alternatives to overcome this limitation. The present work attempts to establish a better understanding of the effects observed during the enzymatic hydrolysis of laccase-treated steam-exploded wheat straw for the optimum integration of the detoxification step into the current lignocellulosic bioethanol production process.

In this work, the effect of laccase on enzymatic activities of cellulases was examined to discard any direct effect of laccases on those hydrolytic enzymes. Furthermore, the effect of laccase was studied in enzymatic hydrolysis experiments of model cellulosic substrate (Sigmacell) without lignin polymer using different substrate concentration and laccase loadings, which could give information about a potential physical interaction or hindrance between the enzymes. Concurrently, in order to reduce a possible non-specific adsorption of cellulases on to the lignin fiber, steam-exploded wheat straw was enzymatically hydrolyzed in the presence of laccase adding polyethylene glycol (PEG). The chemical composition of the wheat straw filtered pretreated residue (WS-FPR) was studied after 3 h incubation with laccase without hydrolytic enzymes. Finally, attenuated total reflectance–Fourier transform infrared (ATR–FTIR) spectra were determined for laccase treated or untreated WS-FPR after the enzymatic hydrolysis.

2. Methods

2.1. Raw material and pretreatment

Wheat straw was supplied by CEDER-CIEMAT (Soria, Spain). This material presented the following composition (% (w/v) dry weight (DW)): cellulose, 40.5; hemicelluloses, 26.1 (xylan, 22.7; arabinan, 2.1; and galactan, 1.3); Klason lignin, 18.1; ash, 5.1; and extractives, 14.6 (Alvira et al., 2011).

The biomass was milled until a particle size between 2 and 10 mm using a laboratory hammer mill. The milled wheat straw was pretreated by steam explosion in a 2 L reactor without acid impregnation, considering a severity factor (SF) of 3.94 (200 °C for 10 min). The whole steam-exploded wheat straw was vacuum filtered and the wheat straw filtered pretreated residue (WS-FPR) was used as substrate. Since the WS-FPR was unwashed, low amount of phenolic compounds remained soaked in the material. The chemical composition of the WS-FPR was characterized as described in the Section 2.6. having the following composition (% DW (w/v)): hemicellulose, 8.4; cellulose, 55.0 and Klason lignin 33.6. The material was kept at 4 °C until use.

The model cellulosic substrate used was Sigmacell Type 50® (Sigma S5504). This substrate is considered as high purity cellulose according to manufacture information.

2.2. Enzymes

Pycnoporus cinnabarinus laccase (60 IU/mL; 8 g/L), produced by Beldem (Belgium), was used for the evaluation of laccase treatment. Laccase activity was measured by the oxidation of 5 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) to its cation radical (ε_{436} = 29300 M^{-1} cm^{-1}) in 0.1 M sodium acetate (pH 5) at 24 °C.

A mixture of the preparations NS50013 (60 FPU/mL cellulase activity; 150 g/L total protein content) and NS50010 (510 IU/mL β-glucosidase activity; 200 g/L total protein content), both produced by Novozymes (Denmark), was used for the enzymatic hydrolysis.

Total protein content from all enzymatic preparations was analyzed by BCA protein assay kit (Pierce Ref. 23225), using bovine serum albumin as standard.

2.3. Enzymatic activities of hydrolytic enzymes

Overall cellulase activity of NS50013 was determined using filter paper (Whatman No. 1 filter paper strips), while β-glucosidase activity of NS50010 was measured using cellobiose as substrate