EFFECT OF LACCASE DOSAGE ON ENZYMATIC HYDROLYSIS OF STEAM-EXPLODED WHEAT STRAW

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When laccases are applied for detoxifying steam-exploded slurries, enzymatic hydrolysis is affected. In this study, 10% (w/v) of dry weight (DW) slurry or water insoluble solid fraction were subjected to 48 h enzymatic hydrolysis in the presence of different laccase loadings (0.1, 0.5, 5 or 10 IU/g DW). All laccase dosages higher than 0.1 IU/g DW significantly inhibited glucose recovery. Nevertheless, half of the amount of phenolic compounds present in steam-exploded slurries was removed with 0.1 IU/g DW of laccase, while 75% of phenolic compounds were efficiently eliminated when employing 0.5 IU/g DW laccase. These findings suggested that, in order to avoid enzymatic hydrolysis inhibition, laccase dosage has to be kept as low as possible for detoxifying steam-exploded wheat straw slurry.

Keywords: lignocellulose, wheat straw, enzymatic hydrolysis, laccase detoxification, ATR-FTIR

INTRODUCTION

Phenols are well-known inhibitors for cellulase activity and yeast viability in bioethanol production processes.[1-5] The presence of these inhibitory compounds in steam-explosion pretreated lignocellulosic materials limits the direct use of whole steam-exploded slurries. In this context, the removal of phenolic compounds has been investigated, and the use of laccase enzymes has been proved as an interesting approach to detoxify steam-exploded wheat straw.[3] Laccases act on phenolic substrates by catalyzing the oxidation of their hydroxyl groups to phenoxyl radicals, while molecular oxygen (O_2) is reduced to water.[6] Laccase detoxification of steam-exploded wheat straw enhances the fermentability of slurries, reducing the lag phase of the fermentative microorganism and therefore, increasing ethanol production.[7]

However, the application of laccases for detoxifying steam-exploded wheat straw reduces glucose release during the enzymatic hydrolysis step.[8-10] In a previous work, Oliva-Taravilla and co-workers showed that phenoxyl radicals and phenolic oligomers formed by laccase oxidation, together with lignin modifications, are involved in the inhibition of enzymatic hydrolysis.[11] The utilization of laccases as detoxifying agents would allow the use of higher substrate loadings or even the use of whole pretreated slurries, reducing the global ethanol production cost. Therefore, it is crucial to determine the optimal conditions of laccase treatment to increase the fermentability of steam-exploded pretreated substrates without affecting enzymatic hydrolysis. In this sense, laccase dosage should be optimized regarding the saccharification rate and phenol removal.

In the present study, the effect of different dosages of *Myceliophthora thermophila* laccase (MtL) on the enzymatic hydrolysis of whole slurry and water insoluble solid fraction (WIS) of steam-exploded wheat straw was evaluated.

EXPERIMENTAL

Raw material and pretreatment

Wheat straw was supplied by CEDER-CIEMAT (Soria, Spain). The slurry obtained after steam explosion pretreatment at 200 ºC for 10 min was directly used as substrate or after a vacuum-filtration step in order to obtain: the solid fraction or WIS and the liquid fraction or prehydrolysate. Substrates were analyzed as described in the analytical methods.

Enzymes

The cellulolytic enzymes employed in this study were NS50013 (Celluclast 1.5L), containing 60 FPU/mL of total cellulase activity and NS50010 (Novo188) with 510 CBU/mL of β-glucosidase activity. Laccase from *M. thermophila* (MtL) showed 127 IU/mL activity using ABTS substrate. All these enzymes were kindly supplied by Novozymes (Denmark).

Enzymatic hydrolysis

10% (w/v) DW of slurry or WIS were diluted in 50 mM citrate buffer at pH 5 and were used as substrates for enzymatic hydrolysis. For all assays, cellulolytic enzymes, Celluclast 1.5L and Novo188, were loaded at 5 FPU/g DW and 5 CBU/g of substrate, respectively. All assays were run in triplicate in 100 mL-flasks at 50 ºC and 180 rpm for 48 h.

Laccase treatment

The different laccase dosages were 0.1, 0.5, 5 and 10 IU/g DW of substrate. Laccase was added simultaneously to cellulolytic enzymes during enzymatic hydrolysis.

Analysis by ATR-FTIR

Residual solids obtained after enzymatic hydrolysis were recovered by centrifugation and analyzed by ATR-FTIR using a Thermo Nicolet 6700 FT-IR spectrometer. Spectra were collected at room temperature in the 2000-800 cm^{-1} range with 1.9 cm^{-1} resolution and as an average of 32 scans. In order to make the spectra clearer, each FTIR spectrum obtained from an assay without laccase was subtracted to the corresponding FTIR spectrum from a laccase-treated assay.

Analytical methods

The chemical composition of the slurry and WIS fraction was analyzed using the National Renewable Energy Laboratory (NREL) standard methods for structural carbohydrates and lignin in biomass.[12] Glucose, xylose and ethanol concentration were quantified according to a previous work.[8] Furfural, 5-hydroxymethylfurfural (HMF), acetic and formic acids in prehydrolysate were quantified according to a previously reported procedure.[13] Total phenolic content in the prehydrolysate and supernatants after enzymatic hydrolysis was analyzed according to a modified version of the Folin–Ciocalteau method.[8]

RESULTS AND DISCUSSION

Effect of laccase dosage on phenolic content

As WIS is the solid vacuum-filtered fraction of whole slurry, it possesses lower phenolic content. Therefore, the phenolic content was only analyzed using slurry as substrate. To evaluate the effect of different laccase dosages on the removal of phenolic compounds, the total phenolic content was measured after 4, 6 and 24 h enzymatic hydrolysis (Figure 1). For all laccase dosages, a significant phenols decrease was observed after 6 h. Longer incubations did not result in higher detoxification. This fact indicates that phenols removal by laccase was only effective at the early stages of enzymatic hydrolysis, which is in agreement with Jurado *et al*. (2009).[10] The slight decrease of the phenolic content in the laccase untreated control sample after 24 h could be attributed to phenolic oxidation by natural reaction with atmospheric oxygen.[14]

Laccase treatment at the lowest enzyme dosage tested (0.1 IU/g DW) showed a reduction of 45% of total phenols at 6 h, whereas higher reduction, of 76%, 79% and 85%, was quantified using 0.5, 5 and 10 IU/g DW laccase, respectively (Figure 1).

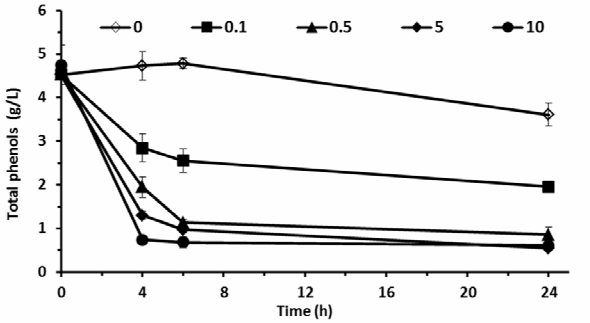

Figure 1: Time course of total phenols content during enzymatic hydrolysis of 10% (w/v) DW slurry when treated or not with laccase; (0: laccase untreated; 0.1: 0.1 IU/g DW laccase; 0.5: 0.5 IU/g DW laccase; 5: 5 IU/g DW laccase; 10: 10 IU/g DW laccase)

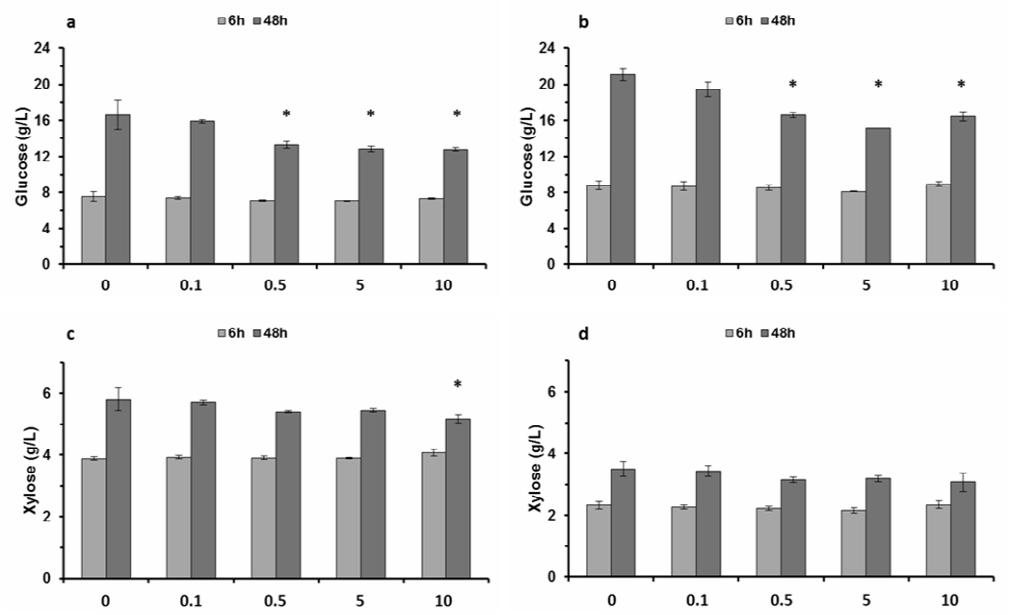

Figure 2: Glucose and xylose release after 6 h (clear bars) and 48 h (dark bars) in enzymatic hydrolysis of 10% (w/v) slurry or WIS treated with 0, 0.1, 0.5, 5 or 10 IU/g DW of laccase (a: Glucose content on slurry; b: Glucose content on WIS; c: Xylose content on slurry; d: Xylose content on WIS; *significantly different from corresponding control without laccase (*p-values* < 0.05))

From these results, it can be concluded that low laccase dosage, of 0.1 IU/g DW, was able to remove half of the amount of phenolic compounds present in steam-exploded slurries, while 0.5 IU/g DW laccase was efficient enough to eliminate more than 75% of the phenolic compounds. A slight improvement in phenols removal was obtained at higher laccase doses, of 5 and 10 IU g/DW.

Effect of laccase loading on enzymatic hydrolysis

10% (w/v) DW of slurry or WIS fraction were subjected to 48 h enzymatic hydrolysis in the presence of different laccase loadings (0, 0.1, 0.5, 5 or 10 IU/g DW). Figure 2 shows the glucose and xylose recovered at early (6 h) and late stages (48 h) of enzymatic hydrolysis of both substrates in the presence of several laccase dosages. After 6 h of enzymatic hydrolysis of both substrates, sugar production (glucose and xylose) was not affected by the laccase treatment at any dose tested. At a late stage of enzymatic hydrolysis (48 h), the xylose content was not affected by the presence of laccase at any dosage tested (Figure 2c and 2d). However, a significant decrease in glucose production was observed at 48 h. These results are in accordance with several previous studies.[8-10,15] Oliva-Taravilla and co-workers (2015) reported that the xylanase components of the cellulolytic enzymatic cocktails (Celluclast 1.5L) were less susceptible to be inhibited by the action of laccase.[8] The results presented in Figure 2 show that the inhibitory effect of laccase on glucose production during enzymatic hydrolysis was time-dependent.

Glucose production by the enzymatic hydrolysis of both substrates was not affected significantly in the experiments at the lowest laccase dosage of 0.1 IU/g DW. Nevertheless, a significantly lower glucose release was observed in the assays with 0.5, 5 and 10 IU/g DW of laccase (Figure 2a and 2b). At a laccase dosage from 0.5 to 10 IU/g DW, the same levels (in the range of 23%) of inhibition were measured for both substrates.

Laccase detoxification of 10% (w/v) DW slurry and WIS using 0.1 IU/g DW affected the glucose recovery of 4.4% and 7.9%, respectively, compared to the laccase untreated sample (Figure 2a and 2b). Lignin content in the pretreated substrates plays a key role in the inhibition of the cellulose enzymatic hydrolysis by laccases.[11] The lignin fraction present in WIS is higher (35.6%) than that in the slurry (18.53%), thus laccase oxidation of phenolic units forming lignin has a

greater impact on WIS hydrolysis even at low laccase dosages, compared to slurry hydrolysis.

In the present study, the maximum saccharification rate of steam exploded wheat straw treated with MtL was obtained with 0.1 IU/g DW of laccase, the minimal amount tested.

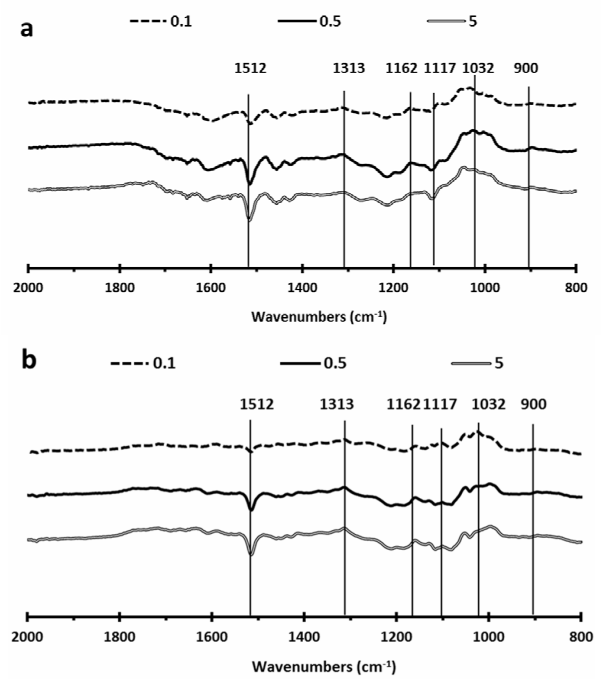

Figure 3: Subtractions of FTIR spectra obtained from the residues of enzymatic hydrolysis assays of slurry or WIS and resulting from the subtraction of sample treated with 0.1, 0.5 or 5 IU/g DW of laccase minus the corresponding control sample without laccase (a: slurry; b: WIS).

When using 0.5 IU/g DW laccase dosages, glucose release was significantly affected in both substrates. On the contrary, Qiu and coworkers obtained the maximum saccharification rate of steam exploded wheat straw treated with laccase from *Sclerotium* sp. using 0.55 U/g of laccase, while the conversion rate decreased with further increase of the laccase dosage.[15] These results indicate that the efficient detoxification of pretreated lignocellulosic materials without affecting enzymatic hydrolysis is dependent on laccase dosage, which has to be optimized for each specific case.

Effect of laccase dosage on lignin structural changes

Residual solids obtained after enzymatic hydrolysis were analyzed by ATR-FTIR to evaluate the potential structural changes on lignin fiber generated by the different laccase dosages. With both substrates, the corresponding FTIR spectrum subtractions (Figure 3) did not show any difference in the 1160-900 cm^{-1} region, attributed to the vibration of carbohydrates. The main variable intensity was observed at 1512 cm^{-1}, which is attributed to the aromatic ring vibrations of lignin due to aromatic skeletal vibration (C═C).[16] While laccase dosage increases, the intensity of the band at 1512 cm^{-1} decreases. Laccase oxidation of phenolic subunits of lignin generates modifications at its surface and gives rise to higher hydrophobic interactions between lignin and cellulose.

CONCLUSION

The effect of laccase dosage on enzymatic hydrolysis and on phenols removal was mainly time-dependent. The laccase dosage that allowed avoiding the inhibition of enzymatic hydrolysis in pretreated whole slurry of wheat straw was found to be 0.1 IU/g DW. Although this low laccase dosage seems to be the most suitable regarding

enzymatic hydrolysis, the enhanced fermentability of half detoxified slurries needs to be evaluated. The application of the optimal laccase dosage in ethanol production processes is expected to allow the use of whole slurries and the increase of substrate loadings, improving glucose yields and therefore, ethanol production.

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