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Optimization of the laccase detoxification step in hybrid hydrolysis and fermentation processes from wheat straw by *K. marxianus* CECT 10875

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Abstract: The addition of laccase enzymes reduces the amount of phenols present in lignocellulosic pretreated materials and increases their fermentability. However, laccase addition in combination with cellulases reduces hydrolysis yields. In this work, hybrid hydrolysis and fermentation (HHF) configuration allowed overcoming the negative effect of laccase treatment on enzymatic hydrolysis. Furthermore, the effects of different laccase dosages, length of detoxification time and inoculum size on ethanol production were evaluated. In the evaluated configurations, the different laccase dosages did not show any significant effect on enzymatic hydrolysis. The lowest laccase dosage (0.5 IU/g DW) removed ~70% of total phenols which was enough to reach the highest ethanol production yields (~10 g/L) using *K. marxianus* CECT 10875. Shorter detoxification times and larger inoculum sizes had a positive impact on both ethanol production and volumetric productivity. These optimal detoxification conditions enable the fermentation of inhibitory slurries by reducing the overall time and cost of the process.

Keywords: Wheat straw; slurry; laccase detoxification; thermotolerant yeast; HHF process

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1 Introduction

Lignocellulosic biomass is a suitable candidate for bioethanol production due to its worldwide distribution, low cost and non-competition with food crops. However, the recalcitrant structure of lignocellulosic biomass hampers the release of sugars contained in both cellulose and hemicellulose polymers. To break down the tight organization of lignocellulose, a pretreatment step to disrupt the structure and increase the accessibility of cellulolytic enzymes is needed [1]. Steam explosion is one of the most extensively applied pretreatment methods for ethanol production from wheat straw [2,3]. However, the harsh conditions during steam explosion treatment lead to the formation of inhibitory compounds, such as furans, weak acids and phenolic compounds. These compounds are released to the liquid phase during pretreatment and can affect the enzymes and fermentative microorganism [4,5]. Because of that, the removal of the inhibitors before the fermentation step is beneficial to improve ethanol yields.

The use of oxidoreductases, such as laccases, is a good approach for specifically removing phenolic compounds [6,7]. Laccases are gaining interest as detoxification enzymes in the biofuel industry and their use is very promising to replace complex and costly physical and chemical detoxification methods. Phenols removal by laccase allows the use of whole slurries and high substrate loadings in ethanol production processes [8]. However, several studies proved that when laccases are supplemented to the enzymatic hydrolysis of pretreated wheat straw, lower glucose recoveries are attained [6,9]. Oliva-Taravilla et al. [10] showed that phenoxyl radicals, phenolic oligomers and modifications of the phenolic subunits of lignin all produced by laccase action, are involved in the inhibition of enzymatic hydrolysis. In this context, the efficient integration of the detoxification step by laccases is required to minimize the adverse effects on enzymatic hydrolysis.

In a bioconversion scheme to produce ethanol from lignocellulose, the enzymatic hydrolysis and fermentation steps can either be performed separately, in separate hydrolysis and fermentation (SHF) or simultaneously, in simultaneous saccharification and fermentation (SSF). In an SSF the enzymatic hydrolysis and fermentation are performed at the same time in one single step. SSF processes, however, are usually conducted at lower temperatures than the optimum for cellulolytic enzymes that is around 50°C [11]. Additionally, a third option combining presaccharification with subsequent SSF is referred as hybrid hydrolysis and fermentation (HHF). This last configuration has been shown to be particularly advantageous [12]. Optimum temperature is an important factor to take into account when considering SHF, SSF or HHF. In SHF configuration, enzymatic hydrolysis and fermentation are performed at their respective optimum temperatures. However, the main drawback of SHF is the end-product inhibition produced by glucose and cellobiose which reduces the rate and yields of enzymatic hydrolysis.

Since most of the fermenting yeasts have an optimum temperature ranging from 30 to 38°C, the use of thermotolerant yeasts in SSF processes would imply higher enzymatic hydrolysis yields [3,13]. *Kluyveromyces marxianus* CECT 10875, capable of growing and fermenting at temperatures above 42°C, has been successfully used in SSF processes from wheat straw with high yields [14]. It is worth to mention that high temperatures increase yeast susceptibility to inhibitory compounds due to changes in cell membrane [14]. This fact makes the use of thermotolerant yeasts on slurries very challenging. In this sense, the use laccases as detoxification enzymes is even more important to increase slurries fermentability when employing thermotolerant yeasts. In this study, the effect of the laccase dosage, the duration of the detoxification step and the inoculum size was evaluated in terms of ethanol production, yields and productivities achieved by *K. marxianus* in HHF processes.

2 Materials and methods

2.1 Raw material and pretreatment

Wheat straw was supplied by CEDER-CIEMAT (Soria, Spain). Autocatalyzed steam explosion of wheat straw was performed at 200°C and 7 min in a steam explosion pilot plant. These conditions were optimized in a previous work [15]. After pretreatment, the slurry was recovered and vacuum filtered in order to obtain: (i) the Water Insoluble Solid fraction or WIS and (ii) the liquid fraction or prehydrolysate. Both fractions were analyzed as described in the analytical methods.

2.2 Enzymes

Celluclast 1.5L (NS50013) and Novozym 188 (NS50010) containing 60 FPU/mL of cellulase activity and 510 CBU/mL of β-glucosidase activity, respectively, were used as cellulolytic enzymes. For presaccharification and subsequent HHF, 15 FPU/g dry weight (DW) substrate of Celluclast 1.5L and 15 CBU/g DW of Novo188 were added. Laccase from *Myceliophthora thermophila* (NS51003) with 127 IU/mL was used as detoxification catalyst. All these enzymatic cocktails were kindly provided by Novozymes A/S (Denmark).

2.3 Microorganisms and media

The thermotolerant *K. marxianus* CECT 10875 strain [16] was used as fermenting microorganism. For inoculation, active cultures were obtained in 250 mL flasks with 100 mL of growth medium containing 20 g/L glucose, 5 g/L yeast extract, 2 g/L NH_4Cl, 1 g/L KH_2PO_4, and 0.3 g/L $MgSO_4 \cdot 7H_2O$. After 16h on a rotary shaker at 180 rpm at 42°C, the cultures were centrifuged at 5000 rpm for 5 min. Supernatant was discarded and cells were diluted to obtain the desired inoculum size according to the HHF conditions. HHF assays were carried out on the above described media, replacing glucose by the pretreated wheat straw.

2.4 Hybrid hydrolysis and fermentation

The slurry obtained after pretreatment was diluted to 10% (w/v) of totals solids in 50 mM citrate buffer at pH 5 under unsterile conditions.

In HHF assays presaccharification step was conducted at 50°C and 180 rpm. In HHF experiments without laccase (control) the presaccharification step lasted 20 h. In case of assays with detoxified slurries, laccase were applied after 12h presaccharification, Laccase was added at 0.5, 2.75 or 5 IU/g DW substrate to the diluted slurry according to the process configuration and experimental design (Table 1). Detoxification step stood at 50°C, 180 rpm for 4, 8, or 12h before yeast addition. Thus, the whole presaccharification process in these assays was 16, 20 or 24h long depending on the duration of the detoxification step.

Table 1. Coded and actual levels of variables chosen for the statistical design of experiment. Factorial design at two levels with 1 central point to study the effect of laccase dosage, detoxification time and inoculum size.

Assay	Laccase dosage (IU/g DW)	Detoxification time (h)	Inoculum size (g/L DW)
1	0.5	4	1
2			3
3	5	4	1
4			3
5	0.5	12	1
6			3
7	5	12	1
8			3
9	2.75	8	2

Control assays were inoculated with 1 or 3 g/L DW of *K. marxianus* CECT 10875. Laccase treated assays were inoculated with 1, 2 or 3 g/L DW of *K. marxianus* according to the factorial design (Table 1). HHF experiments were run at 42°C for 72 h. Samples were withdrawn periodically for glucose and ethanol analysis.

2.5 Analytical methods

The chemical composition of the WIS fraction was analyzed using the National Renewable Energy Laboratory (NREL) standard methods for determination of structural carbohydrates and lignin in biomass (LAP-002, LAP-003, and LAP-019) [17]. Total phenolic content of the supernatants was determined according to a modified version of the Folin–Ciocalteau method [2].

Furfural and 5-hydroxymethylfurfural (5-HMF) content in the prehydrolysate was analyzed by high-performance liquid chromatography (HPLC) (Hewlett Packard, Palo Alto, CA USA), using an Aminex ion exclusion HPX-87H cation-exchange column (Bio-Rad Laboratories Inc., Hercules, CA USA) at 55°C. As mobile phase, 89% 5 mM H_2SO_4 and 11% acetonitrile at a flow rate of 0.7 mL/min was used. For detection, a 1040A Photodiode Array Detector (Agilent Technologies, Waldbronn, Germany) was employed. Acetic and formic acids were quantified by HPLC with a 410 Waters Refractive Index Detector (HPLC-RID) (Waters Corporation, Milford, MA USA). The Aminex HPX-87H column was maintained at 65°C with a flow rate of 0.6 mL/min mobile phase (5 mM H_2SO_4). Glucose and ethanol concentration from HHF were quantified by HPLC-RID employing the Aminex HPX-87H column at 50°C and 5 mM H_2SO_4 as a mobile-phase (0.5 mL/min) for separation.

2.6 Experimental design

The effect of the laccase detoxification time (4, 8 and 12 h), laccase dosage (0.5, 2.75 and 5 IU/g DW of substrate), and inoculum size (1, 2 and 3 g/L DW) on ethanol production, ethanol yield and ethanol productivity was evaluated using statistical analysis standardized Pareto charts. The response surface plots were constructed later with the regression model. To determine the number of experiments, a factorial design at two levels with one central point was carried out using the Software Statgraphics Plus 5.0. The effects of the 3 parameters were statistically analyzed in nine assays including one central point (Table 1).

Analysis of variance (ANOVA) followed by the multiple range test at 95% confidence level (Statgraphics Plus 5.0) was applied to evaluate the statistical significance of laccase dosages, duration of detoxification step and inoculum size.

3 Results and discussion

3.1 Steam explosion pretreatment

Table 2 shows chemical composition of the WIS fraction and prehydrolysate of steam-exploded wheat straw. After steam explosion pretreatment, slurry with a total solids content of 19.7% (w/v) was obtained wherein WIS accounted for 11.3% (w/v). WIS fraction is composed mainly of cellulose and lignin. In the prehydrolysate, 27 g/L xylose (mainly in oligomeric form) was obtained due to the extensive solubilization of hemicellulosic sugars. Acetic acid (5.2 g/L), formic acid (3.1 g/L), and phenolic compounds (4.9 g/L) were found to be the main degradation products. Acetic acid is formed primarily by hydrolysis of acetyl groups of hemicellulose, while formic acid arises as degradation products from polysaccharides. Formic acid is a product of furfural and 5-HMF degradation. The content of aliphatic acids in slurries varies strongly upon the type of feedstock and the severity of the pretreatment. Feedstocks with high content of acetylated xylan, typically agricultural residues and hardwood, result in higher concentrations of aliphatic acids than softwood. Furfural (1 g/L) and 5-HMF (0.1 g/L), coming from sugar degradation, were also present in lower concentration. These results are in accordance to previous works that shown similar inhibitors composition and content in steam-exploded wheat straw prehydrolysates [2,18].

Among all the degradation products released during steam explosion pretreatment, phenolic compounds are the most potent inhibitors [8,9,19,20]. More specifically, phenolic compounds have been reported to inhibit or deactivate the cellulolytic enzymes [19]. Phenol concentrations depend on the biomass type, the pretreatment conditions and the biomass to water ratio. Although the level of inhibition is highly dependent on the type and concentration of phenol, these compounds are potent cellulase inhibitors even at low concentrations [10,19-21]. Concentrations of phenolic compounds of 1.3 g/L were reported to strongly inhibit cellulases during the hydrolysis of microcrystalline cellulosic substrate [21]. Similarly, García-Aparicio et al. [22] found 50% cellulase inhibition in presence of prehydrolysate from steam-exploded barley straw with 4.9 g/L total phenols.

Table 2. Composition of steam-exploded wheat straw at 19.7% (w/v) totals solids.

Composition of WIS	**% (w/w) DW**
Cellulose	55.1 ± 0.5
Hemicellulose	7.3 ± 0.7
Lignin	32.6 ± 1.7
Ash	3.8 ± 0.1
Composition of prehydrolysate (g/L)	
Total sugars*	
Glucose	4.8 ± 0.1
Xylose	27.0 ± 0.3
Galactose	2.5 ± 0.1
Arabinose	1.9 ± 0.1
Degradation products	
5-HMF	0.1 ± 0.01
Furfural	1.0 ± 0.03
Acetic acid	5.2 ± 0.2
Formic acid	3.1 ± 0.1
Total phenols	4.9 ± 0.1

*Total sugars determined after acid hydrolysis

Phenols do not only affect enzymatic hydrolysis but also the fermentation process [8,23]. In many cases, the mechanism of toxicity that could be related to specific functional groups of phenols has not been elucidated [24]. Concentration of aromatic phenols, such as vanillin, in the range of 0.9-1.2 g/L caused oxidative stress and mitochondrial fragmentation in *Saccharomyces cerevisiae* [25]. Although this range of concentrations did not inhibit the growth of *S. cerevisiae* [26], the combination of different degradation products, inhibited the growth of *K. marxianus* [4].

Phenolic compounds act cumulatively. In this sense, their elimination or reduction is needed to increase enzyme and yeast performance. In this study, to optimize laccase detoxification step, slurry was diluted at 10% (w/v) of totals solids which implied a reduction of phenolic concentration by half (2.5 g/L).

3.2 Ethanol production in HHF process with non-detoxified slurries

To test the fermentability of steam-exploded wheat straw without any detoxification step, preliminary HHF experiments were carried out without laccases. In absence of laccase, no glucose consumption nor ethanol production were observed independently of the inoculum size (1 and 3 g/L) after 72h of HHF (data not shown). These results confirmed the high toxicity of slurries obtained at 200°C for 7 min (even when diluted at 10% (w/v) total solids) for *K. marxianus* CECT 10875. These results were in agreement with Moreno et al. [27] and confirmed that under these conditions, cell growth and ethanol production were completely inhibited.

3.3 Integration of laccase detoxification in the HHF process

Since laccase treatment results in significant inhibition of enzymatic hydrolysis yield [10], it seems crucial to separate enzymatic hydrolysis and laccase treatment. In this sense, a configuration consisting in a presaccharification step followed by laccase detoxification at 50°C and then the HHF process at 42°C was evaluated in terms of different ethanol production parameters (ethanol production, yield and productivity).

3.3.1 Presaccharification and laccase detoxification step

Operational conditions given by the statistical experimental design are shown in Table 1. After 12h of presaccharification, laccase at different dosages was added, and detoxification was performed for 4, 8 or 12h. Total phenols were measured before and after the presaccharification and detoxification step (Figure 1). Pretreated slurry at 10% (w/v) totals solids contained 2.5 g/L of total phenols. After 20h of presaccharification, the concentration of phenols increased to 4 g/L (Control,

Figure 1). This result showed that phenols were released during enzymatic hydrolysis. In commercial enzymatic cocktails such as Novo188, several types of enzymes are present. This cocktail is a cellulase preparation from *Aspergillus niger* containing mainly β-glucosidase activity. However, Novo188 cocktail has been shown to comprise also xylanases and feruloyl esterase activities [28]. The feruloyl esterase enzymes could be responsible for the phenols increase due to phenol release from hemicellulose and lignin [29]. As a consequence, higher content of phenols are usually recovered after enzymatic hydrolysis, making the substrate even more toxic for the fermentation step [2,14].

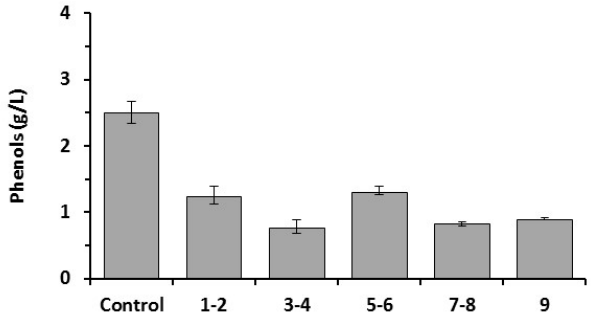

Figure 1. Total phenols content after presaccharification plus detoxification step. Control: no laccase addition.

The detoxification step using 0.5 UI/g DW of laccase resulted in ~70% phenols removal regardless of the incubation time (Figure 1). At the highest laccase dosages (2.75 and 5 IU/g DW), ~80% phenols removal was obtained. Extending the detoxification time did not increase phenols removal. The latter only depended on laccase dosage. Previous works showed a similar reduction of total phenols close to 80% in presence of higher laccase of 10 IU/g DW of substrate, even though initial phenol concentration was lower (2.5 g/L versus 4 g/L) [4]. Herein, the saturation of phenol removal was achieved from 2.75 IU/g DW laccase dosage. This result indicated that laccase detoxification reached the highest level that may not be increased due to the recalcitrant nature of the 20% phenols left or to laccase saturation/deactivation over the time. The latter is consistent with the fact that laccase action does not improve with longer detoxification times. Indeed, Kunamneni et al. [30] observed that free *M. thermophila* laccase lost nearly 20% of residual activity at pH 5 after 12h of incubation.

In terms of glucose released, after 12h of presaccharification, all the assays contained approximately 15 g/L glucose, which corresponds to 40% of hydrolysis efficiency. After 20h presaccharification, the glucose reached 23 ± 0.5 g/L in the control assay without laccase treatment. On the other hand, in the assays treated with laccase, the glucose concentration was around 20 g/L after 16 and 24h of presaccharification plus detoxification and 22.9 ± 1.5 g/L glucose after 20 h of presaccharification plus detoxification (Table 3). Therefore, addition of laccase after 12h of presaccharification did not result in a significant hydrolysis inhibition. These results indicated that this configuration, namely 12h of presaccharification and 4 or 8 h of detoxification is a promising strategy that avoids a significant enzymatic hydrolysis inhibition.

3.3.2 Effects on ethanol production in HHF

After the presaccharification plus detoxification step, the temperature was reduced to 42°C (optimal temperature for *K. marxianus* CECT10875 fermentation). Then, yeast inoculum was added at 1, 2 or 3 g/L DW according to the factorial design (Table 1). According to the Pareto chart (Figure 2a), the addition of different laccase dosages (0.5, 2.75 or 5 IU/g DW) did not have a significant effect on ethanol production, whereas the detoxification time had a statistically significant negative impact. Although 0.5 IU/g DW resulted in the lowest phenol removal (1-2; Figure 1), 4 h of detoxification time was enough to avoid the inhibitory effect on yeast fermentation, leading to higher ethanol concentrations (10.2 ± 0.8 and 10.8 ± 0.8 g/L) compared with higher laccase dosage or longer detoxification time (Table 3). This result highlighted the importance of optimizing the laccase dosage and operating conditions in order to improve ethanol yields without increasing the cost of the process.

In the case of inoculum size, a statistically significant positive effect on maximum ethanol concentration was observed (Figure 2a and b). Indeed, assays at low laccase dosage with a detoxification time of 12 h and 3 g/L DW of inoculum showed the same levels of ethanol concentration at 24 h of HHF than the assays with 4 h of detoxification and 1 or 3 g/L DW of inoculum (Table 3). This proved the positive impact of inoculum size on ethanol production which can relieve the negative effect of prolonged detoxification periods. The negative effect of the detoxification length on ethanol production could be due to the release of acetyl groups from hemicelluloses and phenols from lignin during the presaccharification [2,31]. In this context, prolonged laccase treatment also implied longer hydrolysis times that contributed to increase the presence of other inhibitors in the media.

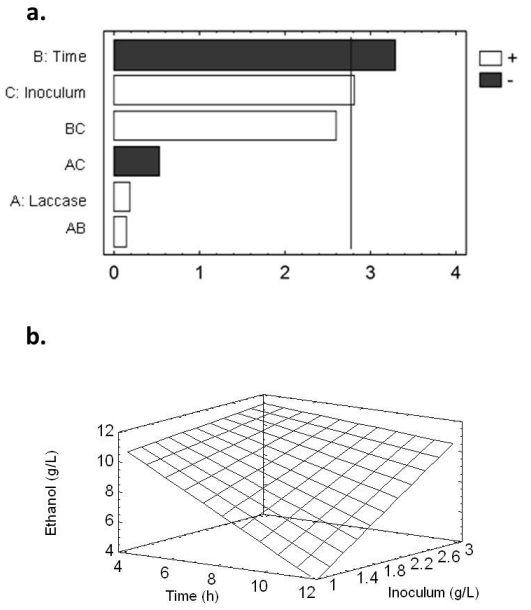

Figure 2. Standardized Pareto chart (a) and Surface response plots (b) of the effect of laccase dosage fixed at 0.5 UI/g DW, detoxification time and inoculum size on ethanol concentration at 24h of HHF process.

3.3.3 Effect on volumetric productivities of ethanol in HHF

According to Figure 3a, only the inoculum size had a statistically significant positive effect on volumetric productivity at 6 h of HHF process (Q_{e6h}). At 24 h of HHF process, in addition to the positive effect of the inoculum size, a statistically significant negative impact due to the detoxification time was observed on volumetric productivity (Q_{e24h}) (Figure 3b). Higher inoculum size implied faster glucose consumption and higher ethanol production rates. Thus, even though the ethanol productivity was lower, the ethanol concentration was around 10 g/L in almost all cases.

During presaccharification and subsequent detoxification, acetic acid could be released from residual acetyl groups of hemicelluloses by cellulolytic enzymes. In this work, acetic acid was measured before and after presaccharification and after laccase treatment. After 12h of presaccharification, a slight increase in acetic acid concentration (8.8%) was observed in all assays. This increment was even more obvious after laccase treatment. An increase of 23.8% and 27.2% acetic acid concentration was measured after 16h and 24h of presaccharification plus detoxification, respectively. Despite the removal of 70-80% of totals phenols, this elevated concentration of acetic acid could affect the yeast fermentation capacity. Rugthaworn et al. [32] determined that ~1.8 g/L of acetic acid was the half maximal inhibition concentration (IC_{50}) of the growth of *K. marxianus* strain TISTR5925 at 42°C. In our work, the initial acetic acid concentration in the slurry was 2.5 g/L demonstrating that *K. marxianus* CECT 10875 was more resistant to acetic acid than others *K. marxianus* strains. It is known that robustness of yeast strain is favored when the size of the inoculum is maximized [33,34]. In this context, the development of robust inocula becomes crucial in ethanol production processes. The optimization of the propagation strategy or the development of recombinant strains that overexpress important genes which confer toxic and stress resistance have been proved to be efficient strategies for overcoming the low bioethanol yields attained when slurries are used as substrate [33,35].

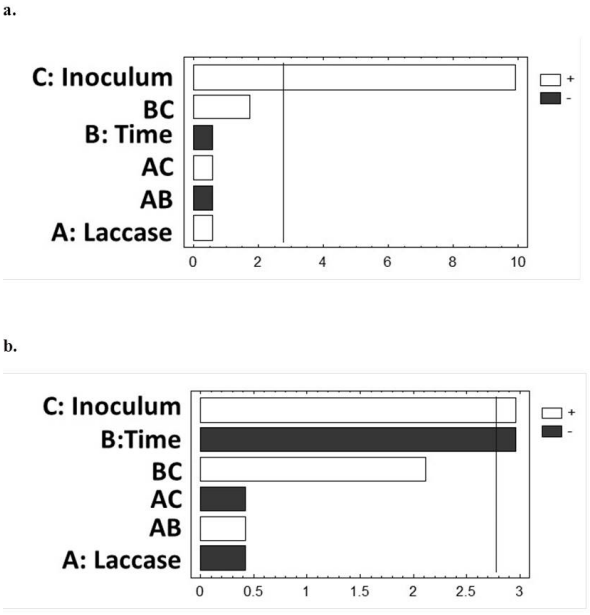

Figure 3. Standardized Pareto chart of the effect of laccase dosage, detoxification time and inoculum size on ethanol volumetric productivity at 6h (Qe6h) (a) and 24h (Qe24h) (b) of HHF process.

4 Conclusions

Laccase treatment decreased the inhibitory profile of slurries by reducing total phenolic content. Optimized detoxification conditions allowed overcoming the inhibitory effect of the laccase treatment on the enzymatic hydrolysis. However, prolonged detoxification times

caused lower ethanol concentration and productivity than short ones when low inoculum sizes were applied. This fact reveals the high influence of other toxics on the yeast and thus, the use of robust and tolerant yeast strains becomes necessary. The HHF process configuration assayed in this work improved the ethanol volumetric productivities by adding the lowest laccase dosage.

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