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Effect of Laccase Detoxification on Bioethanol Production from Liquid Fraction of Steam-Pretreated Olive Tree Pruning

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Abstract: During lignocellulosic bioethanol production, the whole slurry obtained by steam explosion is filtered, generating a water-insoluble fraction rich in cellulose which is used for saccharification and ethanol fermentation, as well as a liquid fraction containing solubilised glucose and xylose but also some inhibitory by-products (furan derivatives, weak acids and phenols), which limits its use for this purpose. Since utilization of this liquid fraction to ethanol is essential for an economically feasible cellulosic ethanol process, this work studied a laccase from *Myceliophthora thermophila* to detoxify the liquid fraction obtained from steam-pretreated olive tree pruning (OTP) and to overcome the effects of these inhibitors. Then, the fermentation of laccase-treated liquid fraction was evaluated on ethanol production by different *Saccharomyces cerevisiae* strains, including the Ethanol Red, with the capacity to ferment glucose but not xylose, and the xylose-fermenting recombinant strain F12. Laccase treatment reduced total phenols content by 87% from OTP liquid fraction, not affecting furan derivatives and weak acids concentration. Consequently, the fermentative behavior of both Ethanol Red and F12 strains was improved, and ethanol production and yields were increased. Moreover, F12 strain was capable of utilizing some xylose, which increased ethanol production (10.1 g/L) compared to Ethanol Red strain (8.6 g/L).

Keywords: bioethanol; detoxification; laccase; lignocellulose; liquid fraction; olive tree pruning; *Saccharomyces cerevisiae*; steam explosion; xylose fermentation

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1. Introduction

Recent initiatives such as the European Green Pact set the goal of making Europe the first climate-neutral continent by 2050 through a decoupling of the economy from fossil resources [1]. In this scenario, the Circular Bioeconomy plays a key role, using natural resources as feedstock for the sustainable production of energy, chemicals and materials. Among them, the use of agricultural residues is gaining attention because, on the one hand, they can be used for this purpose, and on the other hand, their industrial valorization also helps to alleviate the problem of the generation of high-volume residues.

An interesting and abundant agricultural residue is olive tree pruning (OTP). Spain, with about 2.3 m ha of olive trees cultivated, is one of the most prolific olive oil producers worldwide (1.8 million tons) [2], generating 1.5 tons ha^{-1} $year^{-1}$ (3.8 tons ha^{-1} biennial pruning) of this residue [3]. OTP, including a main woody fraction and a remaining fraction of leaves and thin branches, has been widely evaluated for valorization due to its high carbohydrate (cellulose and hemicelluloses) content. Then, cellulose has been employed for cellulosic pulp and nanocellulose [4,5] and bioethanol production [6], whereas hemicelluloses have been explored for production of xylitol and bioethanol [7].

Bioethanol is the most important biofuel worldwide in the transport sector, with a production (1,842 thousand barrels of oil equivalent per day) representing 62% in 2019 of the total value (52% of total bioethanol production in USA and Brazil) [8]. Besides its use

as a biofuel in the transport sector, bioethanol has excellent potential to be converted into chemicals, having been defined as a chemical building block for biorefineries [9]. Currently, bioethanol is mainly produced from certain sugar crops and starch-based feedstocks (first-generation), although second-generation bioethanol produced from lignocellulosic biomass, such as agriculture residues, offers economic and environmental benefits compared to food crops. The biochemical conversion of lignocellulosic biomass to bioethanol is one of the most favorable routes among all developed technologies [10]. The process comprises pretreatment, enzymatic hydrolysis and fermentation steps.

Pretreatment is necessary to increase the accessibility of hydrolytic enzymes to carbohydrates through an extensive alteration of the lignocellulosic structure [11,12]. Several pretreatment technologies, mainly physical and/or chemical, have been developed and used for a large range of raw materials [11,12]. Among them, steam explosion is considered a cost-effective method for lignocellulosic bioethanol production. However, the high pressures and temperatures required by this hydrothermal technology lead to partial degradation of carbohydrates and lignin, generating some soluble inhibitory by-products (furan derivatives and weak acids from carbohydrates and phenolic compounds from lignin) that limit the subsequent enzymatic hydrolysis and fermentation steps [13]. Several detoxification approaches have been developed to overcome the inhibitory effects of these compounds [14]. Among them, the use of laccases (benzenediol/oxygen oxidoreductases, EC 1.10.3.2) represents a suitable choice since they can be added in the same hydrolysis and fermentation vessel under mild reaction conditions, require low energy and generate few by-products [15,16]. Laccases are multicopper-containing phenoloxidases that catalyze the oxidation of a wide range of substituted phenols, anilines and aromatic thiols at the expense of molecular oxygen [17]. This capacity allows laccases to act specifically on phenolic compounds present in steam-exploded materials [18–21].

Efficient fermentation of all the carbohydrates contained in lignocellulosic biomass is essential to increase the profitability and competitiveness of biochemical conversion of lignocellulose into bioethanol [22]. Since xylose is the second most abundant carbohydrate in agriculture residues, such as OTP, its fermentation is essential to improve the global economy of the process. The wild-type *Saccharomyces cerevisiae* is one of the preferred microorganisms for lignocellulosic bioethanol production since it shows high bioethanol productivity and high tolerance to ethanol and to inhibitory compounds [23]. However, since wild-type *S. cerevisiae* strains are not able to ferment xylose, great efforts have been made to develop efficient engineered xylose-fermenting *S. cerevisiae* strains. In this sense, *S. cerevisiae* F12 has been genetically engineered by over-expressing the endogenous gene-encoding xylulokinase (XK) and by introduction of the genes for xylose reductase (XR) and xylitol dehydrogenase (XDH) from Pichia stipites, and it has been evolved to ferment xylose [6,24,25].

Usually, the whole slurry resulting from steam explosion pretreatment is filtered, generating a water-insoluble fraction rich in cellulose, which is used for enzymatic hydrolysis and ethanol fermentation, and a liquid fraction containing glucose and xylose but also some inhibitory by-products that limit its use for this purpose. This liquid fraction should also be employed for bioethanol production, improving the profitability and competitiveness of bioethanol production. In this study, OTP, a widely available renewable agricultural residue in Spain, was subjected to steam explosion pretreatment. After filtration of the whole slurry generated, the liquid fraction, with high content of glucose and xylose but also some inhibitory by-products, was detoxified by a laccase enzyme from *Myceliophthora thermophila* in order to be used for bioethanol production. For that, the liquid fraction was firstly subjected to enzymatic hydrolysis to convert the oligomeric sugars present into available fermentable monomers, and laccase enzyme was added after 20 h of enzymatic hydrolysis, and for 4 h previously, to initiate fermentation. After that, the liquid fraction detoxified was evaluated for bioethanol production by *S. cerevisiae* Ethanol Red. Moreover, due to high concentrations of xylose present in the liquid fraction, a xylose-fermenting strain, *S. cerevisiae* F12, was also studied to increase the bioethanol production.

2. Materials and Methods

2.1. Raw Material and Steam Explosion Pretreatment

OTP, provided by Universidad de Jaen (Jaen, Spain), was subjected to water extraction in an autoclave at 121 °C for 60 min [26]. After the water extraction, the material was filtered, and two fractions, a solid and a liquid fraction, were obtained. The solid fraction was used as substrate for steam explosion pretreatment in a steam explosion unit (2 L reactor, ATI Sistemas, A Coruña, Spain) at 187 °C for 30 min, according to Ballesteros et al. [26]. Steam explosion pretreatment generated a pretreated whole slurry that was separated by filtration into a water-insoluble fraction rich in cellulose and a liquid fraction, the latter analyzed for sugars and inhibitory compounds. Most of the sugars present in the liquid fraction were in the oligomeric form, and therefore, a mild acid hydrolysis (4% (*v*/*v*) H_2SO_4, 120°C and 30 min) was required to determine monomeric sugars concentration.

2.2. Enzymes

Novozymes (Bagsvaerd, Denmark) provided a cellulose-hydrolyzing enzyme with an activity of 65 FPU/mL, a glucoamylase from *Aspergillus niger* with an activity of 755 AGU/mL, and a laccase from *Myceliophtora thermophila* with an activity of 235 IU/mL to be used for enzymatic hydrolysis and detoxification assays.

2.3. Microorganisms and Media

Two strains of *S. cerevisiae* were used. *S. cerevisiae* Ethanol Red (Lessafre, Marcq-en-Barœul, France) is an industrial strain used in the fuel alcohol industry, which ferments glucose but not xylose. *S. cerevisiae* F12 is an engineered strain that has been modified by overexpressing the endogenous gene-encoding xylulokinase and by introduction of the genes for xylose reductase and xylitol dehydrogenase from *Picchia stipitis* [24]. This strain is able to ferment both glucose and xylose to ethanol.

Active cultures of *S. cerevisiae* Ethanol Red was prepared in 100 mL Erlenmeyer flasks with 25 mL of growth medium containing 30 g/L glucose, 5 g/L yeast extract, 2 g/L NH_4Cl, 1 g/L KH_2PO_4 and 0.3 g/L $MgSO_4$ 7H2O. Preinoculum of *S. cerevisiae* F12 was prepared taking a colony from YPD (yeast extract, peptone, dextrose) agar plates and inoculating into 500 mL flasks containing 200 mL of Delft medium, as follows: 20 g/L glucose, 20 g/L xylose, 7.5 g/L $(NH_4)_2SO_4$, 14 g/L KH_2PO_4, 0.5 g/L $MgSO_4 7H_2O$, 2 mL/L trace metal (EDTA, $CaCl_2$ $2H_2O$, $ZnSO_4$ $7H_2O$, $FeSO_4$ $7H_2O$, H_3BO_3, $MnCl_2$ $2H_2O$, Na_2MoO_4 $2H_2O$, $CoCl_2$ $6H_2O$, $CuSO_4$ $5H_2O$, KI) solution and 1 mL/L vitamin solution [27]. The precultures were grown in a shaking incubator at 150 rpm and 35 °C for 16 h (Ethanol Red) and 24h (F12). After incubation, the preculture was centrifuged and the supernantant discarded. The cells were weighed using a laboratory balance and diluted to obtain an inoculum size of 1 g/L for starting the fermentation.

2.4. Enzymatic Hydrolysis

The enzymatic hydrolysis of liquid fraction was performed using the undiluted media adjusted to pH 5 by addition of 2M NaOH. The process was carried out at 50 °C, 150 rpm and for 24 h prior to initiating fermentation. A dosage of 0.61 FPU/mL of cellulose-hydrolyzing enzyme and 1.72 AUG/mL of glucoamylase was used. All the experiments were carried out in triplicate.

2.5. Laccase Detoxification

For the laccase detoxification treatment, a dosage of 10 UI/ml of *M. thermophila* laccase was used. The treatment was started after 20 h of enzymatic hydrolysis of liquid fraction and for 4 h previously to initiating fermentation, with the same conditions fixed for the enzymatic hydrolysis, i.e., 50°C and 150 rpm. Control assay was performed under the same conditions, without the addition of laccase. To evaluate the effect of laccase treatment on inhibitory compounds, samples from control and laccase-treated hydrolysates were taken prior to starting fermentation. The samples were centrifuged (10,000 rpm for 5 min), and then the supernatants were analyzed for identification and quantification of inhibitory compounds. Glucose and xylose concentrations were also determined.

2.6. Fermentation

In a first set of experiments, a synthetic medium with the same sugar concentration as the liquid fraction was used. Moreover, the synthetic medium contained 5g/L yeast extract, 2g/L NH_4Cl, 1g/L KH_2PO_4 and 0.3g/L $MgSO_4$ 7H2O in the case of *S. cerevisiae* Ethanol Red; and 3g/L KH_2PO_4, 5g/L $(NH_4)_2SO_4$, 0.5g/L $MgSO_4$ $7H_2O$, 1mL/L vitamins solution and 2mL/L trace elements solution in the case of *S. cerevisia*e F12. The temperature was 35 °C in both cases, and 1 g/L (dry weight) of inoculum from Ethanol and F12 strains was added. The fermentations were carried out in a rotatory shaker (150 rpm) for 64 hours under nonsterile conditions. During fermentation assays, samples were periodically withdrawn (16, 20, 25, 40, 44, 48 and 64 h) and centrifuged (10,000 rpm for 5 min), and then the supernatants were analyzed for glucose, xylose, xylitol, glycerol and ethanol concentrations.

In a second set of experiments, laccase-detoxified hydrolysates and their controls were supplemented with nutrients, vitamin solution and trace elements, as explained above. The temperature was reduced to 35 °C in both cases and 1 g/L (dry weight) of inoculum from *S. cerevisiae* Ethanol Red and *S. cerevisiae* F12 was added. The fermentations were carried out in a rotatory shaker (150 rpm) for 50 hours under nonsterile conditions. During fermentation assays, samples were periodically withdrawn (6, 18, 22, 25, 28, 32, 45 and 50 h in the case of *S. cerevisiae* Ethanol Red; and 6, 18, 24, 42 and 50 h in the case of *S. cerevisiae* F12) and centrifuged, and then the supernatants were analyzed for glucose, xylose and ethanol concentrations.

2.7. Analytical Methods

Ethanol concentration was quantified by gas chromatography using a 7890A GC System (Agilent, Waldbronn, Germany) equipped with an Agilent 7683B series injector, a flame ionization detector and a Carbowax 20 M column at 85 °C. Injector and detector temperature was maintained at 175 °C.

Glucose and xylose concentrations were quantified by high-performance liquid chromatography (HPLC) equipped with a refractive index detector (Waters, Mildford, MA, USA). A CarboSep CHO-682 column (Transgenomic, San Jose, CA, USA) with 0.5 mL/min of ultrapure water as a mobile-phase at 80 °C was used for the separation. This column and these conditions were also used to quantify xylitol and glycerol.

Furfural, 5-hydroxymethylfurfural (5-HMF), 4-hydroxybenzaldehyde, vanillin, syringaldehyde and *p*-coumaric acid concentrations were also determined by HPLC equipped with a 1050 photodiode-array detector (Agilent, Waldbronn, Germany). A Coregel 87H3 column (Transgenomic, San Jose, CA, USA) with 0.7 mL/min of 89% 5 mM H_2SO_4 and 11% acetonitrile as a mobile phase at 65 °C was used for analysis and quantification.

Formic acid and acetic acid were quantified by HPLC equipped with a 2414 refractive index detector (Waters, Mildford, MA, USA). A Bio-Rad Aminex HPX-87H (Bio-RadLabs, Hercules, CA, USA) column with 0.6 mL/min of 5 mmol/L H_2SO_4 as a mobile phase at 65 °C was used for analysis and quantification.

Total phenolic content was analyzed according to the Folin–Ciocalteu methodology at 765 nm with a spectrophotometric microplate reader (Anthos Zenyth 200rt, Biochrom, UK) [28]. Firstly, 20 µL of sample and the serial standard solution were diluted with 88 µL of water on a 96-well microplate. After the addition of 12 µL Folin–Ciocalteu reagent, the plate was incubated for 5 min at room temperature in dark conditions. The reaction was stopped with 80 µL of 7.5% sodium carbonate solution. Before reading, the plate was incubated for 2 h at room temperature in the dark.

All analytical values were measured in duplicate or triplicate, and average results are shown. When appropriate, one-way analysis of variance (ANOVA) was performed to evaluate the effect of laccase detoxification. The level of statistical significance was set at $p < 0.05$.

3. Results and Discussion

3.1. Steam Explosion

Steam explosion bases its mode of action on the extensive solubilization of the hemicellulosic fraction from lignocellulosic biomass in order to increase the accessibility of hydrolytic enzymes to carbohydrates contained in the water-insoluble solid fraction [12,29]. Then, a high xylose content and glucose, both mostly in the oligomeric form, were measured in the liquid fraction obtained after filtration of the whole slurry generated from steam explosion of OTP (Table 1). Different degradation by-products were also identified and quantified as soluble compounds (Table 1). Among them, furan derivatives (furfural and 5-HMF), weak acids (acetic and formic acids) and phenolic compounds (vanillin, syringaldehyde, and *p*-coumaric acid) were found. Furfural and 5-HMF are formed from pentoses and hexoses decomposition, respectively, and their subsequent degradation leads to the production of formic acid. The hydrolysis of acetyl groups from hemicelluloses forms acetic acid, and phenolic compounds are derived from lignin degradation [13]. Among phenols, vanillin, formed by degradation of guaiacyl propane lignin units; syringaldehyde, produced by degradation of syringyl propane lignin units; and *p*-coumaric acid from cynnamic acids were identified (Table 1).

Table 1. Composition of liquid fraction after steam explosion pretreatment.

	Monomeric form	Total
Sugars (g/L)		
Glucose	2.0	17.3
Xylose	1.5	14.5
Galactose	0.7	3.1
Arabinose	1.7	2.6
Mannose	nd	1.1
Inhibitors (g/L)		
Furfural	0.6	
5-HMF	0.2	
Formic acid	1.0	
Acetic acid	4.0	
Vanillin	0.01	
Syringaldehyde	0.02	
p-Coumaric acid	0.003	

nd, no detected.

The mentioned degradation by-products have already been identified by some authors in the liquid fraction from OTP [6], as well as other agricultural residues such as wheat [30], barley straw [31] and sugarcane bagasse [32]. These by-products act as inhibitory compounds since they can alter the growth of the fermenting microorganisms and

inhibit hydrolytic enzymes, decreasing final bioethanol yields and volumetric productivities [13]. Furan derivatives can alter different intracellular pathways, prolonging the microorganism lag phase. Weak acids decrease microorganism growth by modifying the intracellular pH that promotes an imbalance in the ATP/ADP ratio; and finally, phenolic compounds can affect biological membranes, thus reducing growth rates and inhibiting or deactivating hydrolytic enzymes.

3.2. Laccase Detoxification

As previously mentioned, the high content of glucose and xylose present in the liquid fraction obtained after steam explosion pretreatment of OTP should also be used as substrate for bioethanol production. However, the use of this medium is challenging due to the presence of inhibitory compounds, which affect the downstream enzymatic hydrolysis and bioethanol production. In order to overcome the inhibitory effects of these by-products, this liquid fraction was subjected to laccase detoxification.

M. thermophila laccase produced a huge reduction in the phenols content (87% of the total phenols content, from 3.67±0.17 g/L in control samples to 0.48±0.06 g/L in laccase samples (the mean difference is significant at the 0.05 level)) of liquid fraction enzymatic hydrolysate. However, the concentration of furan derivatives and weak acids was not affected by laccase treatment (Table 2). This effect shows some advantages over chemical or physical methods, which have the ability to reduce the overall inhibitor concentration to a greater or lesser extent [13,14]. Nevertheless, the substrate specificity described by laccases allows mild reaction conditions, lowering both by-products' formation and energy necessities [16].

Table 2. Inhibitory compounds concentration of control and laccase-treated liquid fraction enzymatic hydrolysates.

	Control	Laccase
Inhibitors (g/L)		
Furfural	0.466 ± 0.033	0.436 ± 0.011
5-HMF	0.139 ± 0.051	0.120 ± 0.032
Formic acid	1.014 ± 0.022	0.926 ± 0.012
Acetic acid	3.878 ± 0.041	3.778 ± 0.023
Vanillin	0.093 ± 0.015	0.001 ± 0.002 *
Syringaldehyde	0.015 ± 0.001	0.000 ± 0.000 *
p-Coumaric acid	0.003 ± 0.001	0.000 ± 0.001 *

* The mean difference is significant at the 0.05 level.

Detoxification by laccase enzymes involves the oxidation of soluble phenols resulting in unstable phenoxy radicals [16,33]. Then, these newly formed radicals can polymerize between them to yield less toxic oligomeric compounds. Nevertheless, some soluble phenols identified herein were resistant to laccase oxidation, such as 4-hydroxybenzaldehyde (Figure 1). This effect depends on the reactivity of the different lignin phenols towards laccase. Phenols such as syringaldehyde and cinnamic acids, i.e., *p*-coumaric acid, can be easily oxidized, whilst other phenolic compounds are oxidized at lower rates, as is the case for vanillin, or are not oxidized at all, as with hydroxybenzaldehyde [34]. In this sense, it has been described that an extra methoxy group at the benzene ring (the difference between syringaldehyde and vanillin) shows a greater sensitivity towards laccase. Moreover, para-substituted phenols with ethylene group, such as *p*-coumaric acid, increase the reactivity of these phenolic compounds towards laccase [34].

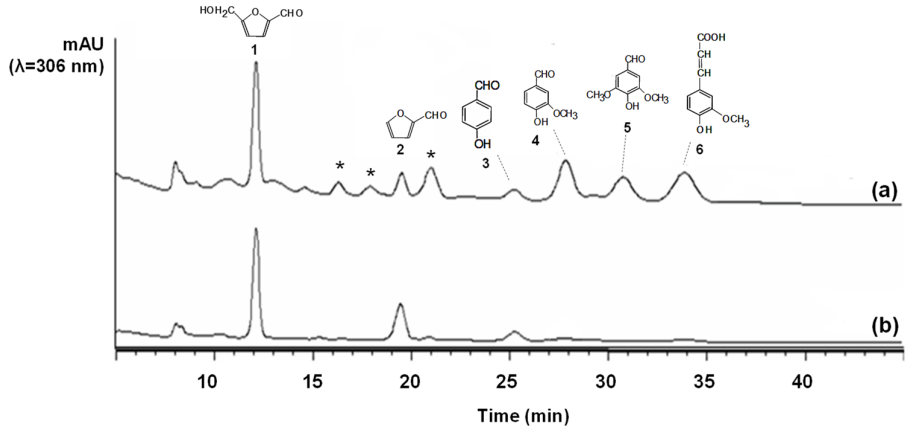

Figure 1. Comparison of high-performance liquid chromatography profiles of inhibitory compounds of liquid fraction enzymatic hydrolysates (**a,** control; **b**, laccase). Identified peaks: 1, 5-HMF; 2, Furfural; 3, 4-hydroxybenzaldehyde; 4, vanillin; 5, syringaldehyde; 6, *p*-coumaric acid. * Non identified phenols.

Results shown in this study about the phenols removal by *M. thermophila* laccase are comparable to or greater than those of studies conducted with other laccase sources on steam-preatreated materials or their liquid fractions after filtration. For example, a phenols removal of 71−75% was described when a fungal laccase from *Trametes villosa* was used to detoxify the whole slurry obtained from steam explosion of wheat straw [20]. In the same study, a bacterial laccase from *Streptomyces ipomoeae* also showed its capacity to reduce the phenols content of this pretreated material, albeit to a lesser extent (35-37%) compared to *T. villosa* laccase. Steam-exploded eucalypt was also detoxified by laccase obtained from the secretome of *Marasmiellus palmivorus*, removing 70% of phenolic compounds [35]. Kalyani et al. [36] also described the capacity of a laccase from *Coltricia perennis* to reduce the phenols content of steam-exploded rice straw by 76%. Regarding the laccase detoxification of liquid fraction, Esteves et al. [37] reported the detoxification of the liquid fraction obtained from diluted sulfuric acid pretreatment of sugarcane bagasse using a laccase from *Trametes versicolor*, resulting in a lignin phenols reduction between 49−53%. Chandel et al. [38] showed the capacity of a laccase from *Cyathus stercoreus* to detoxify the liquid fraction produced from dilute chloride acid pretreatment of sugarcane bagasse, observing a total phenols reduction of 77.5%. Moreno et al. [39] also described the detoxification of the liquid fraction resulting from steam explosion of wheat straw using a laccase from *P. cinnabarinus* (73% of the phenol content was removed). Laccase treatment of hydrolysate from rice straw subjected to hot water treatment with *T. versicolor* enzyme demonstrated 76% removal of phenolic compounds after 12 h of treatment [40]. In the same study, the same enzyme was used to detoxify the hydrolysate from poplar subjected to dilute acid pretreatment, resulting in a 94% reduction in inhibitors [40]. However, *M. thermophila* laccase has been already used to detoxify a liquid fraction obtained from sugarcane bagasse subjected to liquid hot water pretreatment [41], albeit unsuccessfully in this case.

3.3. Fermentation

3.3.1. Fermentation of Synthetic Medium

Fermentation assays using a synthetic medium, with the same composition of sugars as liquid fraction obtained from steam explosion of OTP prior to enzymatic hydrolysis but without inhibitors, were performed to evaluate the fermentative behavior of *S. cerevisiae* Ethanol Red and the xylose-fermenting *S. cerevisiae* F12.

Time courses for glucose and xylose consumption, fermentation by-products formation (i.e., xylitol and glycerol) and ethanol production by *S. cerevisiae* Ethanol Red and

the xylose-fermenting *S. cerevisiae* F12 are displayed in Figures 2 and 3, respectively, and concentrations are shown in Table 3. Glucose was the first sugar to be exhausted by both strains, observing a total depletion at 16 h of fermentation, and no differences in consumption rates were found. Afterwards, xylose started to be consumed. In the presence of both sugars, a lower xylose consumption is usually described since they share the same transporter by which both sugars are introduced into the cells [42], and because of competitive inhibition of xylose transport by glucose [43]. In the case of Ethanol Red, xylose was consumed with a linear rate, slightly decreasing by time, with a total xylose consumption of 36.4% during the fermentation process. However, a much more pronounced xylose consumption was observed in the case of F12, with a total xylose consumption of 86.2%. Then, owing to its xylose fermenting ability, the F12 strain produced a higher maximum ethanol concentration (10.7 g/L, with yield of 0.32 g/g), resulting in 16% more ethanol produced than with the Ethanol Red strain (8.0 g/L, with yield of 0.24 g/g).

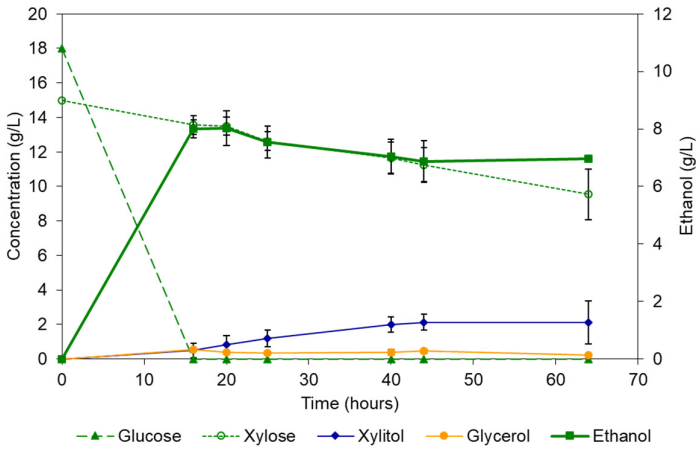

Figure 2. Time course for fermentation of synthetic medium using *S. cerevisiae* Ethanol Red. Concentrations (g/L) of glucose, xylose, xylitol and glycerol and production (g/L) of ethanol are shown.

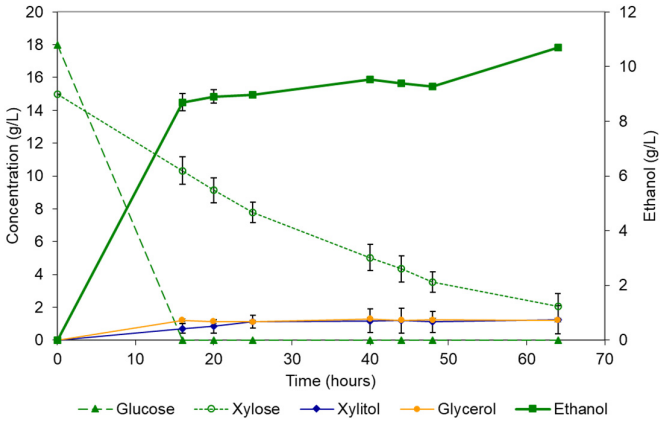

Figure 3. Time course for fermentation of synthetic medium using *S. cerevisiae* F12. Concentrations (g/L) of glucose, xylose, xylitol and glycerol and production (g/L) of ethanol are shown.

Table 3. Summary of fermentation assays with *S. cerevisiae* and *S. cerevisiae* F12 using synthetic medium.

	Strain	
	***S. cerevisiae* Ethanol Red**	***S. cerevisiae* F12**
Residual sugars		
Glucose (g/L)	0.0 ± 0.0	0.0 ± 0.0
Xylose (g/L)	9.5 ± 1.4	2.1 ± 0.7
$EtOH_{max}$ (g/L)	8.0 ± 0.6	10.7 ± 0.0
$Xylitol_{max}$ (g/L)	2.1 ± 1.3	1.2 ± 0.8
$Glycerol_{max}$ (g/L)	0.6 ± 0.1	1.3 ± 0.1
$Y_{E/S}$ (g/g)	0.24	0.32
$Y_{E/ET}$ (%)	47.5	63.5

$EtOH_{max}$, maximum ethanol concentration during fermentation; $Y_{E/S}$, ethanol yield based on total sugars (glucose and xylose) content present in the synthetic medium prior to fermentation; $Y_{E/ET}$, theorical ethanol yield assuming ethanol yields on glucose and xylose by both *S. cerevisiae* strains 0.51 g/g.

Xylitol formation was also observed in both strains. Although the inability of wild-type *S. cerevisiae* to ferment xylose is well known, the xylose consumption observed herein by Ethanol Red could be explained by the presence of certain xylose reductase activity, attributed to an endogenous non-specific aldose reductase enzyme, GRE-3 [44], and xylitol dehydrogenase [45], resulting in xylitol formation from xylose. Then, when glucose was exhausted, xylose started to be consumed and xylitol was formed (Figure 2), achieving a maximum xylitol production of 2.1 g/L during fermentation process. In this sense, Malfredi et al. [46] described xylitol production by Ethanol Red when alkaline-pretreated sugarcane straw material without inhibitors was subjected to simultaneous saccharification and fermentation. Certain xylitol formation was also observed by F12 strain (Figure 3), although it was lower in this case (1.2 g/L). Xylitol formation has been also described by *S. cerevisiae* F12 using a synthetic medium without inhibitors [47]. Its production is due to a redox imbalance because of different redox dependence during the initial xylose conversion step. Finally, low amounts of glycerol were also formed by both strains (0.6 g/L by Ethanol Red strain and 1.3 g/L by F12 strain), reflecting that glucose was mostly used for ethanol production [47].

3.3.2. Fermentation of Laccase Liquid Fraction Enzymatic Hydrolysates

Control and detoxified liquid fraction enzymatic hydrolysates were also subjected to fermentation using *S. cerevisiae* Ethanol Red and the xylose-fermenting *S. cerevisiae* F12. Time courses for glucose and xylose consumption and ethanol production for both strains are displayed in Figures 4 and 5, respectively. The presence of inhibitory compounds in the liquid fraction affected the fermentation behavior of Ethanol Red significantly. As can be seen for the control sample (Figure 4), glucose consumption was slower (glucose depletion at 45 h) than in the case of synthetic medium (glucose exhaustion at 16 h), and consequently, ethanol production was delayed. This delay in glucose consumption and ethanol production is due to the adaptation of the microorganism to fermentation medium, which depends on different factors such as the inhibitory by-products type, their quantity, the synergistic effects between them, and the fermenting microorganism used [48]. During this time, the microorganisms have the capacity to assimilate part of these inhibitors, mainly furfural, 5-HMF and aromatic aldehydes (4-hydroxybenzaldehyde, vanillin, syringaldehyde) to their less toxic alcohol forms, which largely determines the extension of this delay or lag phase [48]. After overcoming the lag phase, *S. cerevisiae* Ethanol Red showed a great glucose consumption and ethanol production between 32 h and 45 h (Figure 4), attaining a maximum ethanol concentration of 6.8 g/L (Table 4), lower than that produced with synthetic medium (8.0 g/L) (Table 3). Regarding *S. cerevisiae* F12, the

effects of inhibitory compounds were not so evident in the case of glucose consumption, observing a glucose exhaustion at 18 h (Figure 5), similar to that observed with synthetic medium. Nevertheless, the maximum ethanol concentration achieved (8.6 g/L, Table 4) was lower compared to that obtained with synthetic medium (10.7 g/L, Table 3). Tomás-Pejó et al. [47] already described the faster glucose consumption by *S. cerevisiae* F12 than the robust hexose-fermenting strain Red Star, which was clearly reflected in higher volumetric ethanol productivities at 3 h (0.7 g/L h and 0.3 g/L h, respectively).

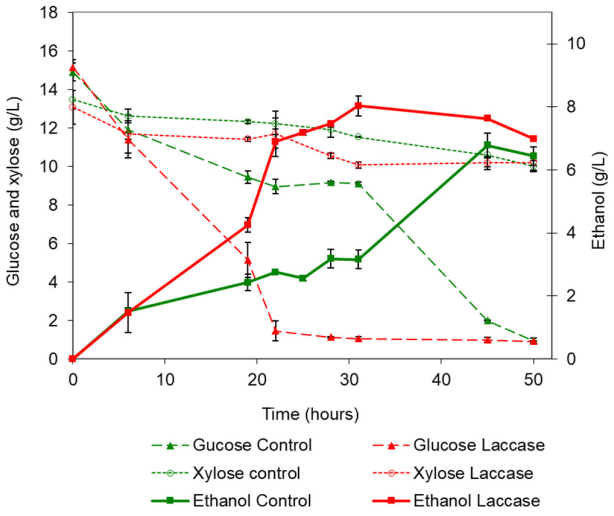

Figure 4. Time course for fermentation of control and laccase-treated liquid fraction enzymatic hydrolysates using *S. cerevisiae* Ethanol Red. Concentrations (g/L) of glucose and xylose and production (g/L) of ethanol are shown.

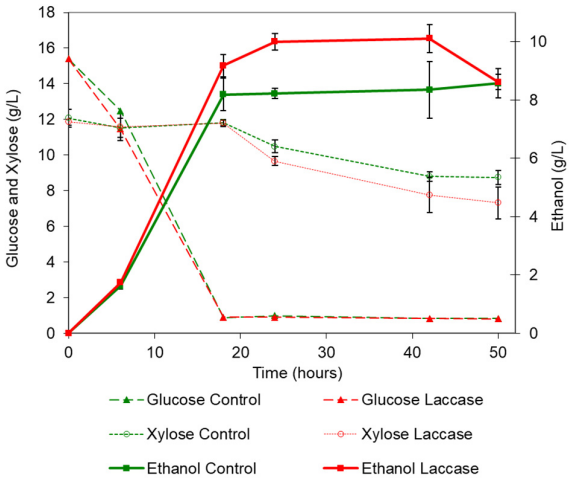

Figure 5. Time course for fermentation of control and laccase-treated liquid fraction enzymatic hydrolysates using *S. cerevisiae* F12. Concentrations (g/L) of glucose and xylose and production (g/L) of ethanol are shown.

Table 4. Summary of fermentation assays with *S. cerevisiae* Ethanol Red and *S. cerevisiae* F12 using laccase-treated liquid fractions.

	Sample	$EtOH_{max}$ (g/L)	$Y_{E/S}$ (g/g)	$Y_{E/ET}$ (%)	Q_E (g/L h)
S. cerevisiae	Control	6.8 ± 0.4	0.21	41.8	0.12
	Laccase	8.0 ± 0.2	0.25	49.7	0.22
S. cerevisiae F12	Control	8.6 ± 0.5	0.27	52.9	0.43
	Laccase	10.1 ± 0.5	0.32	62.4	0.48

$EtOH_{max}$, maximum ethanol concentration during fermentation; $Y_{E/S}$, ethanol yield based on total sugars (glucose and xylose) content present in the liquid fraction prior to saccharification and fermentation; $Y_{E/ET}$, theorical ethanol yield assuming ethanol yields on glucose and xylose by both *S. cerevisiae* strains 0.51 g/g; Q_E, volumetric ethanol productivity at 18 h of fermentation. Differences in means are not statistically significant.

Regarding xylose, the inhibitory effects of the liquid fraction also affected its consumption. Ethanol Red showed a total xylose consumption of 25.4% during the fermentation process, slightly lower than that observed with synthetic medium (36.4%), without xylitol formation. This inhibitory effect was much more marked when F12 strain was used, showing a 26% of xylose consumption during fermentation compared to 86.2% observed with synthetic medium. Similar results were reported by Tomás-Pejó et al. [47], when a liquid fraction obtained by filtration of the whole slurry from steam-exploded wheat straw was used for fermentation by F12 strain. Nevertheless, despite the lower xylose consumption shown by F12 with the liquid fraction, most of it was probably used for ethanol production [47]. The presence of inhibitory by-products such as furfural and HMF can decrease xylitol formation (not detected herein) and increase ethanol yield on xylose. These inhibitory compounds are reduced by the yeast and therefore act as external electron acceptors during anaerobic fermentation of xylose [49], supplying NAD^+ for the XDH reaction and directing product formation toward ethanol at the expense of xylitol. This effect could explain the higher ethanol concentration (8.6 g/L, with yield of 0.27 g/g) obtained compared to that produced by Ethanol Red (6.8 g/L, with yield of 0.21 g/g).

Phenols removal by *M. thermophila* laccase improved the fermentation performance of both strains. Compared to control, the fermentation of laccase-treated liquid fraction hydrolysate by Ethanol Red showed a faster glucose consumption (Figure 4), increasing the ethanol productivity value from 0.12 g/L h, for control liquid fraction, to 0.22 g/L h for laccase-treated liquid fraction hydrolysate (Table 4). Moreover, laccase treatment improved the ethanol production (8.0 g/L, for laccase sample at 32 h; 6.8 g/L, for control sample at 45 h), obtaining higher ethanol yield (0.25 g/g, for laccase sample; 0.21 g/g, for control sample), comparable to the values obtained during the fermentation of synthetic medium. Similar improvements in the *S. cerevisiae* behavior have also been attributed to different laccase enzymes in several detoxification studies. Moreno et al. [28] reported higher glucose consumption rate, ethanol productivity and ethanol yield when the whole slurry from steam-exploded wheat straw was subjected to *P. cinnabarinus* laccase and fermented with *S. cerevisiae*. The ethanol productivity of *S. cerevisiae* was also enhanced when steam-exploded wheat straw was detoxified by *T. villosa* and *S. ipomoeae* laccases [20]. Nevertheless, in this case, ethanol concentrations and ethanol yields were similar for both control and laccase-treated samples. Regarding laccase-treated liquid fraction studies, Jönsson et al. [50] reported the fermentation by *S. cerevisiae* of a liquid fraction from acid steam-exploded willow subjected to *T. versicolor* laccase, resulting in higher glucose consumption rate, ethanol productivity value and ethanol yield. Similar results were described on liquid fraction from acid steam-exploded spruce treated with *T. versicolor* laccase [51].

In the case of *S. cerevisiae* F12, a total glucose depletion at 18 h was observed for both laccase and control liquid fraction hydrolysates (Figure 5). Nevertheless, a slightly higher ethanol productivity (0.48 g/L h) was observed by the effect of laccase compared to control

(0.43 g/L h). Regarding xylose consumption, it was around 11.9% higher compared to control, augmenting ethanol production from 8.6 g/L (0.27 g/g) for control liquid fraction hydrolysate to 10.1 g/L (0.32 g/g) for laccase-treated liquid hydrolysate fraction, a value comparable when synthetic medium without inhibitors was used. Moreno et al. [39] already described a higher xylose consumption when a liquid fraction obtained from steam-exploded wheat straw was detoxified by *P. cinnabarinus* laccase and fermented by the evolved xylose-recombinant *S. cerevisiae* KE6-12 strain. Nevertheless, this effect did not translate into higher ethanol production. In another work, Martín et al. [52] also reported improvements in ethanol yield and ethanol volumetrics using the xylose-utilizing *S. cerevisiae* TMB 3001 on steam-exploded sugarcane bagasse detoxified by *T. versicolor* laccase.

4. Conclusion

A substantial elimination of phenolic compounds (87% removal) by *M. thermophila* laccase, comparable to or greater than that of other laccases, reduced the inhibitory effects of the liquid fraction from steam-exploded OTP. It improved the fermentation performance of both Ethanol Red and F12 strains, increasing ethanol production and yields. Moreover, a higher ethanol production was achieved for F12 (10.1 g/L) due to its capacity to utilize some xylose for ethanol fermentation in comparison to Ethanol Red strain (8.6 g/L), which is able to ferment glucose but not xylose. According to this, detoxification by laccase of the liquid fraction generated during steam explosion pretreatment of lignocellulosic materials should improve the profitability and competitiveness of bioethanol production.

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