Comparison of performances of different fungal laccases in delignification and detoxification of alkali-pretreated corncob for bioethanol production

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Abstract: The performance of the alkaline fungal laccase PIE5 (pH 8.5) in the delignification and detoxification of alkali-pretreated corncob to produce bioethanol was evaluated and compared with that of the neutral counterpart (rLcc9, 6.5), with the acidic laccase rLacA (4.0) was used as an independent control. Treatment with the three laccases facilitated bioethanol production compared with their respective controls. The lignin contents of alkali-pretreated corncob reduced from 4.06%, 5.06%, and 7.80% to 3.44%, 3.95%, and 5.03%, after PIE5, rLcc9, and rLacA treatment, respectively. However, the performances of the laccases were in the order rLacA > rLcc9 > PIE5 in terms of decreasing total phenol concentration (0.18, 0.36, and 0.67 g/l), boosting ethanol concentration (8.02, 7.51, and 7.31 g/l), and volumetric ethanol productivity (1.34, 0.94, and 0.91 g/l hr), and shortening overall fermentation time. Our results would inform future attempts to improve laccases for ethanol production. Furthermore, based on our data and the fact that additional procedures, such as pH adjustment, are needed during neutral/alkaline fungal laccase treatment, we suggest acidic fungal laccases may be a better choice than neutral/alkaline fungal laccases in bioethanol production.

Keywords: Fungal laccase, Delignification, Detoxification, Alkali-pretreated corncob, Bioethanol

Introduction

Lignocellulosic feedstocks are low-cost and renewable raw materials that are abundant and have no competition on food crops (Balan et al., 2013). Bioethanol production from lignocellulosic biomass is considered promising alternatives to mitigate global climate change and reduce dependence on petroleum-based fuels (Fillat et al., 2017). However, lignocellulosic materials have a complex and recalcitrant structure. Thus, a pretreatment step is needed to depolymerize cellulose, hemicellulose, and lignin to make the biomass more accessible to hydrolytic enzymes (Binod et al., 2010). Various pretreatment methodologies, including alkaline pretreatment, acid pretreatment, and their combinations (Pedersen et al., 2010), have been developed and applied to pretreat a wide range of lignocellulose feedstocks. Compared with acid or oxidative reagents, the alkaline treatment appears to be the most effective method in breaking the ester bonds between lignin, hemicellulose, and cellulose (Gáspár et al., 2007).

Unfortunately, the toxic monomeric lignin compounds released during the lignin degradation process, which comprise phenolic compounds, such as aromatic acids, catechol, 4-hydroxybenzaldehyde, and vanillin (Pedersen & Meyer, 2010), as well as furan derivatives and weak acids from pentose and hexose sugars degradation, limit the subsequent saccharification and fermentation processes. Different remediation treatments for detoxification, including physical, chemical, and biological treatments, have been employed to reduce the effects of inhibitory compounds (Rodrigues et al., 2001; Yang & Wyman, 2008; Ranjan et al., 2009). However, most chemical and mechanical methods are costly, make the biomass-to-ethanol process more complicated, and produce additional waste products (Liu et al., 2005). As an alternative to physico/chemical methods, the biological technology of employing fungal laccase has gained considerable attention in the last several years (De La Torre et al., 2017; Fang et al., 2015; Moreno et al., 2012, 2015; Moreno et al., 2016; Moreno et al., 2013; Suman et al., 2018).

Fungal laccases (benzenediol–oxygen oxidoreductases, EC1.10.3.2) are a family of blue multicopper oxidases that can oxidize a wide range of phenolic and aromatic compounds and concomitantly reduce molecular oxygen to water as the only end-product (Hoegger et al., 2006). Its distinguished oxidative capacity makes fungal laccase a potential tool for modifying or partial removal of lignin monomer and its derivatives from the pretreated biomass to improve saccharification yields (Fillat et al., 2017). Several laccases of fungal origin have been evaluated on their abilities to delignify and detoxify differentially pretreated materials during the bioethanol production process. Overall, these laccases improved the performances of cellulases and yeast to different extents during saccharification and fermentation processes. Fungal laccases have pH optimums at pH 3–5.5 and become essentially inactive as the pH approaches to neutral and alkaline (Petr, 2006). Thus, the application potential of fungal laccases on bioethanol production was evaluated only at acidic conditions (De La Torre et al., 2017; Fang et al., 2015; Moreno et al., 2012, 2015; Moreno et al., 2016; Suman et al., 2018). Due to

the fact that very few fungal laccases with optimum pH at neutral/alkaline conditions have been reported, the performances of neutral/alkaline fungal laccases on the delignification and detoxification of different pretreated materials during bioethanol production have not been evaluated. By comparison, the performances of several alkaline bacterial laccases, such as that from *Klebsiella pneumoniae* (Gaur et al., 2018), *Amycolatopsis* sp. 75iv3 (Singh et al., 2017), and *Streptomyces ipomoea* (De La Torre et al., 2017), have been already evaluated on delignification and detoxification of pretreated materials.

rLcc9 is a fungal laccase from *Coprinopsis cinerea* but expressed in *Pichia pastoris*. It has a pH optimum of 6.5 toward guaiacol (Xu et al., 2019). PIE5 is the first fungal laccase with an alkaline pH optimum of 8.5 obtained by the directed evolution of Lcc9 at sites E116K, N229D, and I393T (Yin et al., 2019). The objective of this study was to assess the application potential of the alkaline fungal laccase PIE5 (pH opt. 8.5) on bioethanol production and compare its performance with that of the neutral fungal laccase rLcc9 (pH opt. 6.5) (Xu et al., 2019). Simultaneously, a classical acidic fungal laccase rLacA (pH opt. 4.0) from *Trametes hirsuta* AH28-2 but expressed in *P. pastoris* was used as a separate control (Hong et al., 2006). The laccases were employed for the delignification and detoxification of alkali-pretreated corncob. In addition, their effects on enzymatic hydrolysis and fermentation were also evaluated.

Materials and Methods
Laccase Enzyme Preparation, Purification and Enzyme Activity Assay

rLacA from *Trametes hirsuta* AH28-2, rLcc9 from *C. cinerea*, and PIE5 were expressed in *P. pastoris* in 3-l fermentation tanks. The laccases were purified and biochemically characterized according to the protocols reported by Hong et al. (2006), Xu et al. (2019), and Yin et al. (2019), respectively. Laccase activity was determined using the assay mixture consisted of 10 μl of the purified enzyme and 990 μl of appropriate buffer [50 mM citrate/phosphate buffer (4.0–8.0) and 50 mM Tris/HCl buffer (8.0–9.5)] containing 5 mM guaiacol. Laccase activity was measured at 465 nm after incubation the mixture at 30°C for 5 min (ε_{465} = 12 000 M^{-1} cm^{-1}) (Hong et al., 2006). The homogeneity of the purified protein was determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) with a 12% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Native PAGE was conducted using guaiacol as the substrate.

Alkali Pretreatment of Corncob

The alkali pretreatment of raw corncob was carried out at 86°C with 7% NaOH for 1 hr using a solid:liquid (wt/vol) ratio of 1:10 (Kong et al., 2019). Then, pH was adjusted to 8.5, 6.5, and 4.0 using 75% H_2SO_4 (Fig. S1).

Laccase Treatment of Solid Fraction

The pH-adjusted whole slurries were employed for laccase treatment. Assays were carried out in 250-ml Erlenmeyer flasks containing appropriate amounts of alkali-pretreated solid fractions (converted to 2.5 g dry weight of each pH-adjusted sample) in a total volume of 50 ml and incubated at 50°C in a water bath shaker (200 rpm) for 24 hr. The enzyme loading of 5 U/g dry substrate was added. Laccase activity was determined at 50°C and pH 4.0, 6.5, or 8.5 for rLacA, rLcc9, or PIE5, respectively, using 5 mM guaiacol as

the substrate to make sure that the same amount of laccase activity was added (Froehner & Eriksson, 1975). Control assays were performed under the same conditions without laccase addition. All the experiments were carried out twice, and each sample was repeated in triplicate.

Enzymatic Hydrolysis and Fermentation of Solid Fractions

Samples with and without laccase treatment were used directly as the substrates for enzymatic hydrolysis and fermentation experiments. The pH of the samples was adjusted to 4.8 before enzymatic hydrolysis. The Cellulase UTE-1500, of which the filter paper activity, β-glucan activity, β-glucosidase activity, and xylanase activity were 100 FPU/ml, 5,000 CMCU/ml, 500 CBU/ml, and 30 U/ml, respectively, was obtained from Youtell Bio (Hunan, China). A filter paper activity of 15 FPU/g dry weight was added for enzymatic hydrolysis (Fig. S1). Hydrolysis was performed at 50°C in a water bath shaker at 200 rpm for 72 hr. In a subsequent step, the pH of the samples was adjusted to 4.0 (Fig. S1). A medium that contained 2.0 g/l $(NH_4)_2SO_4$, 4.0 g/l KH_2PO_4, 1.0 g/l $MgSO_4$, and 0.2 g/l $CaCl_2$ was added into each hydrolysate. *Saccharomyces cerevisiae* was inoculated at a final concentration of 1.0 (optical density at 600 nm). The fermentation was performed at 30°C in an orbital shaker at 200 rpm. Samples were withdrawn at different time points and centrifuged at 15 000 × g for 5 min. Supernatants were filtered through 0.22 μm Millipore filters (Millipore, Bedford, MA, USA), and glucose consumption and ethanol concentration were analyzed. Samples without laccase treatment were used as the controls.

Analytical Methods

The chemical compositions of raw material, pretreated solid fraction, and laccase-treated samples were determined using the National Renewable Energies Laboratory (NREL) standard methods for the determination of structural carbohydrates and lignin in biomass (LAP-002, LAP-003, and LAP-019) (Sluiter et al., 2010). Glucose and ethanol concentrations were quantified by high-performance liquid chromatography (Agilent, USA) using a refractive index detector and a Bio-Rad Aminex HPX-87H column (Bio-Rad, USA), which was maintained at 45°C with a mobile phase (5 mmol/l H_2SO_4) at a flow rate of 0.5 ml/min (Zhang et al., 2010). Total phenolic content was determined according to the Folin–Ciocalteu procedure using vanillin as standard (Folin & Ciocalteu, 1927). Formic acid, acetic acid, and 5-HMF were analyzed and quantified by HPLC (Moreno et al., 2016). All analytical values were calculated from triplicate experiments. Where appropriate, analysis of variance (ANOVA) was used for comparisons between assays. The level of significance was set at $p < .05$.

Results and Discussion
Biochemical Properties of Laccases

Three laccases, namely, rLacA, rLcc9, and PIE5, were successfully expressed in *P. pastoris* upon induction with methanol. The proteins were purified based on ion-exchange chromatography, as shown by SDS–PAGE and native-PAGE (Fig. 1). The purified rLacA had optimum pH and temperature of 4.0 and 50°C toward guaiacol, respectively (Hong et al., 2006). In addition, rLacA laccase showed a redox potential of 680 mV. This laccase represents classic fungal laccase, which has optimum acidic pH and middle/high-level redox potential. By comparison, the optimum pH and temperature of rLcc9 toward guaiacol were 6.5 and 60°C,
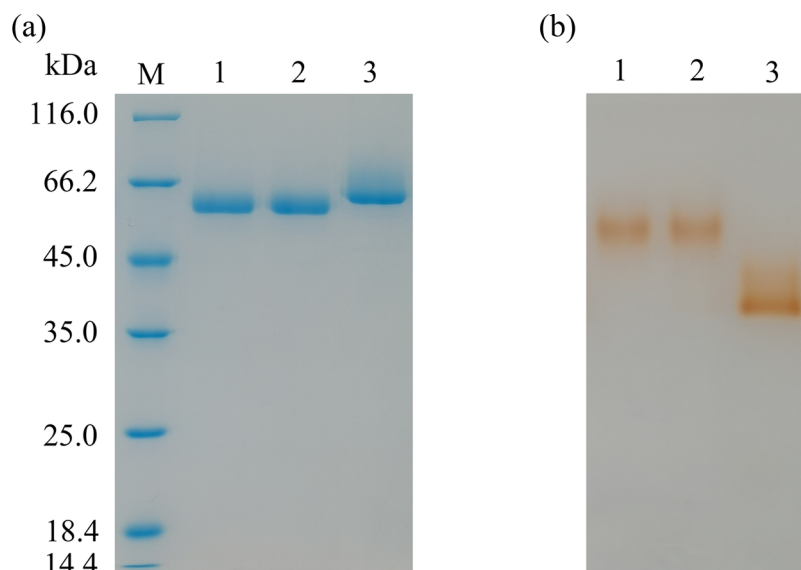
Fig. 1. Eelectrophoresis of three purified fungal laccases. (a) SDS–PAGE of purified laccases. Lanes: M: protein marker; 1: PIE5; 2: rLcc9; 3: rLacA. (b) Native-PAGE of purified laccases. Lanes: 1: PIE5; 2: rLcc9; 3: rLacA. Staining of laccase activity was performed using 5 mM guaiacol as the substrate.

Table 1. Summary of Reported Laccases Studied for Detoxification and Delignification in Lignocellulosic Ethanol Production

Laccase origin		Feedstocks	Pretreat strategy	pH	Activities	Redox potential	Decrease in phenol content	Reference
Bacterial laccases	*Klebsiella pneumoniae*	Wheat and rice bran	Acid	5.0	123 U/mg	–	–	Gaur et al. (2018)
	Amycolatopsis sp. 75iv3	Poplar	Steam	8.0	–	–	15%	Singh et al. (2017)
	MetZyme	Wheat straw	Steam	5.5	284 IU/g	+450 mV	21%	Moreno et al. (2016)
	Streptomyces ipomoeae	Wheat straw	Steam	8.0	8 IU/ml	+450 mV	35%	De La Torre et al. (2017)
Fungal laccases	*Trametes hirsuta* AH28-2	Corncob	Alkali	4.0	1085 U/mg	+680 mV	82%	This study
	Coprinopsis cinerea	Corncob	Alkali	6.5	310 U/mg	+506 mV	63%	This study
	PIE5	Corncob	Alkali	8.5	320 U/mg	+599 mV	28%	This study
	Trametes villosa	Wheat straw	Steam	4.0	370 IU/ml	>730 mV	71%	De La Torre et al. (2017)
	Coriolopsis rigida	Wheat straw	Steam	5.0	49 U/mg	–	70–75%	Jurado et al. (2009)
	Ganoderma lucidum 77002	Corn stover	Steam	5.0	186 U/mg	–	84%	Fang et al. (2015)
	Pycnoporus cinnabarinus	Wheat straw	Steam	5.0	60 IU/ml	–	67–80%	Oliva-Taravilla et al. (2015)
	Trametes maxima IIPLC-32	Sugarcane bagasse	Acid	5.5	1610 IU/mg	–	79.28%	Suman et al. (2018)

respectively, and those of PIE5 were 8.5 and 60°C, respectively. The redox potentials of rLcc9 and PIE5 were 506 and 599 mV, respectively (Yin et al., 2019). Compared with classic fungal laccases, these two laccases possess neutral and alkaline pH activities and stabilities (Petr, 2006). rLcc9 and PIE5 showed higher redox potentials (506 and 599 mV) and specific activities (310 and 320 U/mg) compared with their bacterial counterparts (De La Torre et al., 2017; Gaur et al., 2018; Moreno et al., 2016; Singh et al., 2017), because fungal laccases usually have higher redox potential (470–790 mV vs. 340–490 mV) and specific activities (>200 U/mg protein toward ABTS vs. <100 U/mg protein) (http://www.brenda-enzymes.org/) (Table 1), which ensure the wider substrate ranges and higher activities of fungal laccases (Pezzella et al., 2015; Rodgers et al., 2010).

Chemical Composition of Solid Fractions After Laccase Treatment

Alkaline extraction was employed to pretreat the corncob substrate because of its higher efficiency in delignification than other pretreatment strategies (Gáspár et al., 2007). After alkaline pretreatment, the cellulose proportion of the solid fraction increased (>52.74%) compared with that of the untreated substrate (44.16%) (Table 2). This increase was attributed to the extensive solubilization and degradation of lignin and hemicellulose as pointed out by the lower proportions of remaining lignin (<7.80% vs. 15.01%) and hemicellulose (<28.16 vs. 37.56%, Table 2). In accordance with this fact, the concentration of total phenols in the supernatant was 0.98 g/l (Table 3). These compounds are derivatives of lignin (Pedersen & Meyer, 2010).

Table 2. Composition of Alkaline Pretreatment Corncob Treated With Different Strategies

Substrates	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)	Other components (%)
Crude corncob	44.16 ± 4.12	37.56 ± 0.13	15.01 ± 0.36	1.70 ± 0.33	1.58 ± 0.19
pH 4.0 + C	52.74 ± 0.54	28.16 ± 0.15	7.80 ± 0.26	7.53 ± 0.26	3.77 ± 0.18
pH 4.0 + rLacA	58.65 ± 0.49**	31.10 ± 0.32**	5.03 ± 0.01	5.14 ± 0.19**	0.07 ± 0.00*
pH 6.5 + C	53.26 ± 1.07	27.35 ± 0.49	5.06 ± 0.27	6.43 ± 0.14	7.90 ± 0.15
pH 6.5 + rLcc9	59.09 ± 0.16*	30.31 ± 0.22*	3.95 ± 0.04*	5.13 ± 0.28*	1.51 ± 0.06*
pH 8.5 + C	55.04 ± 0.90	27.89 ± 0.45	4.06 ± 0.08	5.63 ± 0.14	7.37 ± 0.13
pH 8.5 + PIE5	59.02 ± 1.54	29.59 ± 0.71	3.44 ± 0.02**	4.93 ± 0.09*	3.02 ± 0.14

pH 4.0 + C, NaOH-pretreated corncob with pH adjusted to 4.0; pH 4.0 + rLacA, rLacA laccase treatment of (pH 4.0 + C) sample; pH 6.5 + C, NaOH-pretreated corncob with pH adjusted to 6.5; pH 6.5 + rLcc9, rLcc9 laccase treatment of (pH 6.5 + C) sample; pH 8.5 + C, NaOH-pretreated corncob with pH adjusted to 8.5; pH 8.5 + PIE5, PIE5 laccase treatment of (pH 8.5 + C) sample. Results were calculated from two times of independent experiments performed in triplicate ($n = 6$). Difference in means is significant at the *$p < .05$ or **$p < .01$ level.

Table 3. Content of Total Phenols With Different Assay Procedures

Substrates	After laccase treatment (g/l)	Adjusted to pH 4.8 (g/l)	After 72 hr saccharification (g/l)	Fermentation (2 hr) (g/l)
pH 4.0 + C	0.98 ± 0.04	1.09 ± 0.07	1.04 ± 0.07	0.98 ± 0.01
pH 4.0 + rLacA	0.18 ± 0.01***	0.15 ± 0.00**	0.16 ± 0.02**	0.16 ± 0.02***
pH 6.5 + C	0.97 ± 0.02	0.86 ± 0.00	0.78 ± 0.00	0.77 ± 0.01
pH 6.5 + rLcc9	0.36 ± 0.01***	0.34 ± 0.00***	0.29 ± 0.00***	0.29 ± 0.00***
pH 8.5 + C	0.93 ± 0.05	0.83 ± 0.04	0.74 ± 0.04	0.74 ± 0.05
pH 8.5 + PIE5	0.67 ± 0.05*	0.62 ± 0.11	0.49 ± 0.04*	0.51 ± 0.07

pH 4.0 + C, NaOH-pretreated corncob with pH adjusted to 4.0; pH 4.0 + rLacA, rLacA laccase treatment of (pH 4.0 + C) sample; pH 6.5 + C, NaOH-pretreated corncob with pH adjusted to 6.5; pH 6.5 + rLcc9, rLcc9 laccase treatment of (pH 6.5 + C) sample; pH 8.5 + C, NaOH-pretreated corncob with pH adjusted to 8.5; pH 8.5 + PIE5, PIE5 laccase treatment of (pH 8.5 + C) sample. Results were calculated from two times of independent experiments performed in triplicate ($n = 6$). Difference in means is significant at the *$p < .05$ or **$p < .01$ or ***$p < .001$ level.

The water-insoluble fraction of pretreated material is usually separated by filtration, washed with water, and resuspended in a suitable buffer to meet the pH demand of acidic fungal laccases after alkali pretreatment (De La Torre et al., 2017). In this study, pH was adjusted directly using the mixture as the substrate to avoid the filtration and washing steps and decrease operational costs and wastewater. Results from three control groups suggested that the pH adjustment affected the proportion of cellulose but not hemicellulose, in which the former decreased (55.04%, 53.26%, and 52.74%, respectively, $p < .05$) and the latter kept unchanged (27.89%, 27.35%, and 28.16%, respectively, $p > .05$) with decreasing pH (8.5, 6.5, and 4.0). Along with adjusting pH from extremely alkaline condition to acidic condition by using H$_2$SO$_4$, the phenolic units of lignin could be polymerized to yield oligomers or undergo grafting reactions onto pretreated solid fraction, because the H$_2$SO$_4$ used as a catalyst would reduce phenol solubility at harsh conditions (Jurado et al., 2009). As a support to this hypothesis, the lignin content increased remarkably from 4.06% to 7.8% as the pH decreased from 8.5 to 4.0 (Table 2).

The changes in concentrations of other toxic compounds, including furan derivatives and weak acids, were determined and compared among three control samples. The concentration of formic acid kept unchanged (246.2 ± 9.7 mg/l) in the three samples. By comparison, acetic acid concentrations were 114.1 ± 4.6, 346.8 ± 13.3, and 413.7 ± 15.1 mg/l in pH 4.0, 6.5, and 8.5 samples. Slight decrement for furan derivatives, of which the concentrations were 127.4 ± 4.9 mg/l (pH 8.5), 121.8 ± 3.6 mg/l (6.5), and 120.2 ± 3.1 mg/l (4.0), respectively, was detected along with the pH increment. It was reported that alkaline pH could affect furan derivatives' concentration. For example, in an investigation of detoxification of steam-pretreated wheat straw, the content of

5-HMF and furfural was slightly higher in samples at pH 4 than that at pH 8 (De La Torre et al., 2017).

The laccase action on alkali-pretreated corncob results in polymeric lignin oxidation and/or soluble phenolic compounds oxidation (Kudanga & Le Roes-Hill, 2014). Furthermore, laccase catalyzed oxidation gives rise to radical species that can evolve degradation processes, of which oxidative coupling is the primary mechanism resulting in homo- and/or cross-coupling of molecules. Radical species can also act as mediators for further oxidation of other compounds, causing bond cleavage, or undergo rearrangement per se to result in dead-end products (Pezzella et al., 2015). As a result, treating the pretreated corncob with laccase may cause the reduction of lignin content. In fact, the lignin content of the pretreated solid fraction decreased about 21.9% and 15.3% after rLcc9 and PIE5 pretreatment (Table 2). Thus, it was reasonable that the cellulose and hemicellulose proportions in alkaline-pretreated solid fraction increased by 10.9% and 7.2%, and 10.8% and 6.1%, respectively, when compared with their respective controls after the addition of rLcc9 and PIE5. Similar results were obtained when alkali-pretreated *Brassica campestris* straw was treated with laccase from *Ganoderma lucidum* Tr16 (Yang et al., 2011). By contrast, the lignin content of steam-pretreated spruce increased slightly after laccase treatment (Moilanen et al., 2011).

Redox potential determines the ability and the substrate range of laccase to oxidize lignin compounds (De La Torre et al., 2017). It was reported that the lignin content of unwashed steam-exploded wheat straw had a slight increment after treatment with a fungal laccase from *Trametes villosa* with high redox potential (730 mV) (De La Torre et al., 2017). By comparison, lignin content was unchanged when laccases with lower redox potentials (around 450 mV) were employed to treat the samples (De La Torre et al.,

2017; Moilanen et al., 2011; Yang et al., 2011). As an independent control in this work, after adding the laccase rLacA, which showed a moderate redox potential of 680 mV, into the alkaline-pretreated corncob, the lignin content decreased 35.5% when compared with its control (Table 2). Thus, we suggest that the redox potential of a laccase determines the change in lignin content after laccase treatment, that is, laccases with high redox potential (e.g., >730 mV) increase lignin content by catalyzing the grafting and polymerization of most soluble phenolic compounds in samples. By comparison, laccases with moderate and low redox potentials (e.g., <710 mV) decrease or do not affect lignin content because of their limited abilities to oxidize lignin compounds. However, more evidence is needed to prove this hypothesis.

The treatment of the alkaline-pretreated solid fraction with rLacA resulted in the highest delignification efficiency (35.5%) among the three fungal laccases when compared to their respective controls. The higher redox potential of rLacA (680 mV) compared to rLcc9 (506 mV) and PIE5 (599 mV) may facilitate the action of laccase toward phenolic units of lignin and result in a variety of reactions such as ether and C–C bonds cleavage in polymeric lignin (Moreno et al., 2016). However, after the addition of laccases, rLcc9 removed more lignin from the pretreated solid fraction than the PIE5 (21.9% compared to 15.3%), although the former possessed lower redox potential than the later. The difference found in the delignification between the two laccases could be explained by the sophisticated physicochemical characteristics of these laccases. For example, PIE5 showed K_m values of 3.3×10^{-4} and 5.7×10^{-3} M on guaiacol and 2,6-DMP, respectively, compared to 0.9×10^{-4} and 2.3×10^{-3} M of rLcc9 (Yin et al., 2019).

Effect of Laccase Treatment on Phenols in Pretreated Solid Fractions

Phenol concentrations in pretreated solid fractions with and without laccase treatment were determined and compared with their respective controls (Table 3). Although the total phenol concentrations in the rLcc9 and PIE5 control samples were similar, it should be noted that the compositions and concentrations of phenols in samples with different pH values were different because alkaline pH can affect the concentrations of furan derivatives and phenols and the solubilization of phenolic compounds (De La Torre et al., 2017; Jurado et al., 2009). As a result, the total phenols decreased by 62.89% (pH 6.5) and 27.96% (8.5), respectively, after rLcc9 and PIE5 treatment because of the different affinities of laccases toward different phenolic compounds. As an independent experiment, the total phenols decreased by 81.63% (pH 4.0) after rLacA treatment (Table 3). Several acidic laccases (mainly fungal origin) and alkali laccases (mainly bacterial origin) have been evaluated on their abilities to decrease phenol content. Similar to our results, acidic laccases removed more phenols than alkali laccases regardless of the lignocellulose feedstock and pretreatment strategies used (Table 1). As shown in these cases, the pH of the slurry and the characteristics of the laccase used can affect the phenol removal from the pretreated lignocellulose. In addition, the redox potential of laccases play a key role in the range of phenol removal (De La Torre et al., 2017). The concentrations of total phenols in all the samples did not change too much after adjusting the pH to 4.8 in this study (Table 3).

Effect of Laccase Treatment on Enzymatic Hydrolysis of Pretreated Corncob

Laccase treatment affects the saccharification process. The relative glucose (RGR) and xylose concentration recoveries (RXR) in the rLcc9 and PIE5 control samples decreased gradually after 72 hr of saccharification (Fig. 2). The phenomenon occurred probably because of the different degrees of erosion and exposure of pretreated materials by the harsh conditions when the pH was adjusted to 6.5 and 8.5 using H_2SO_4 (Jurado et al., 2009; Saha et al., 2005). As a support to this conclusion, the surface morphology of the differentially treated corncob was quite different (Fig. 3). After alkaline pretreatment, the external surface of the corncob became plicated and slightly dehiscent (Fig. 3), which resulted from the removal of lignin and the breakage of the lignocellulosic complex construction during the pretreatment. Moreover, the toxicity of different phenols to cellulosic enzyme activity cannot be ignored (Table 3). The saccharification of the rLcc9- and PIE5-treated samples showed a substantial increase in RGR and RXR compared with the corresponding controls. The RGR and RXR in rLcc9-treated samples were enhanced by 8.81% and 7.00%, respectively, whereas those in PIE5-treated samples increased by 5.99% and 5.81%, respectively (Fig. 2). Saccharification efficiency can be affected by many factors, including the phenolic compounds content, the lignin content, and the available surface area of substrate (Fig. 3). The increased saccharide recovery after the rLcc9 and PIE5 treatments could be attributed to lower lignin content as well as an increase in the porosity and the available surface area. Moreover, according to Palonen and Viikari, laccase treatment increased carboxyl groups of lignin, reducing the hydrophobicity, and increasing surface charge, which led to the decrease of the nonspecific adsorption of cellulases to lignin and the improvement of saccharification yields (Palonen & Viikari, 2004).

Compared with the samples with pH adjusted to 6.5 and 8.5, the surface morphology of the sample with pH adjusted to 4.0 seemed to be more plicated and exposed, which triggered a further increase in the surface area and accessibility of cellulose to enzymatic hydrolysis (Fig. 3). However, the saccharification of the rLacA-treated sample showed 3.98% and 2.87% decrements in RGR and RXR, respectively, compared with its corresponding control despite the significant phenolic content reduction observed. As ligninolytic enzymes, in addition to soluble phenol removal, laccases have the ability to interact with phenolic units present in lignin polymer. Laccase treatment could increase the nonspecific adsorption of cellulases to lignin and decrease glucose and xylose yields. In addition, Tejirian et al. observed that oligomeric phenols formed after the laccase treatment of lignocellulosic materials could inhibit, to a greater extent, the enzymatic hydrolysis than single soluble phenols (Tejirian & Xu, 2011). Furthermore, a grafting effect may explain the lower saccharification yields of rLacA because it could covalently couple some phenols, such as p-coumaric acid or ferulic acid, to the lignin component of the fibers, limiting the accessibility of cellulose (Oliva-Taravilla et al., 2015). Moreover, the formation of phenol-derived compounds by laccase could inhibit hydrolytic activities, especially β-glucosidase activity (Ximenes et al., 2010; Ximenes et al., 2011). The enhancement of the nonproductive binding of cellulolytic enzymes onto the lignocellulosic fibers and a major strengthening of lignin-carbohydrate complexes might be involved in this reduction.

Effect of Laccase Treatment on Cell Viability and Ethanol Fermentation
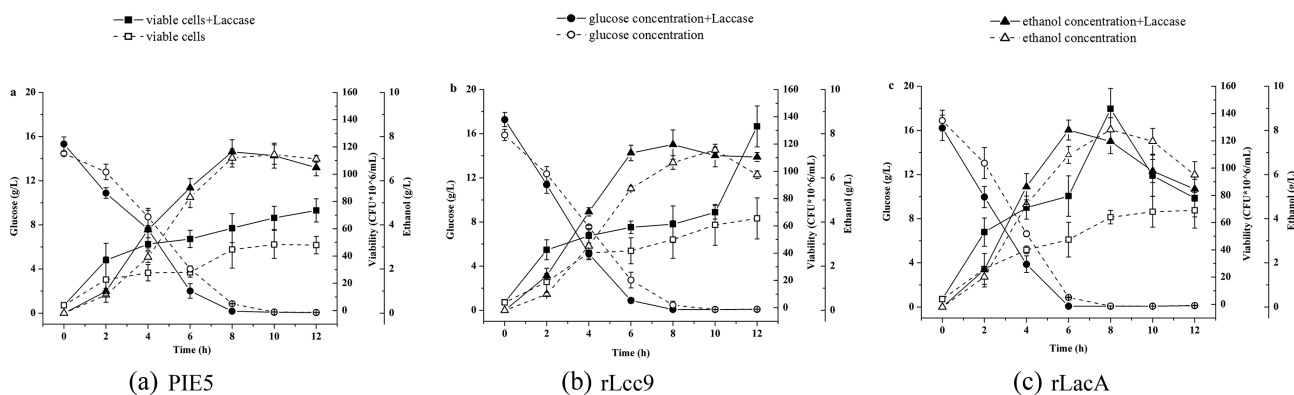
The viable cells, glucose consumption, and ethanol production of S. cerevisiae were improved by PIE5 and rLcc9 treatment compared with the corresponding control sample because of the removal of phenols from the pretreated substrate (Fig. 4 and Table 4). However, the level of improvement was quite different between
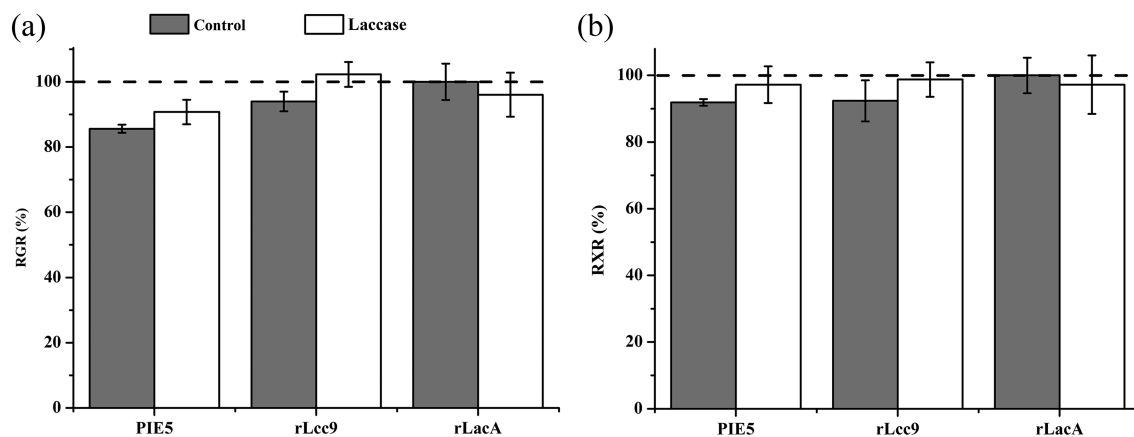
Fig. 2. RGR(a) and RXR (b) of corncob residues at 72 hr of enzymatic hydrolysis after alkaline pretreatment and different laccase treatments (white, different laccase treatments; gray, control sample with corresponding pH). rLacA was set as a separate control experiment.
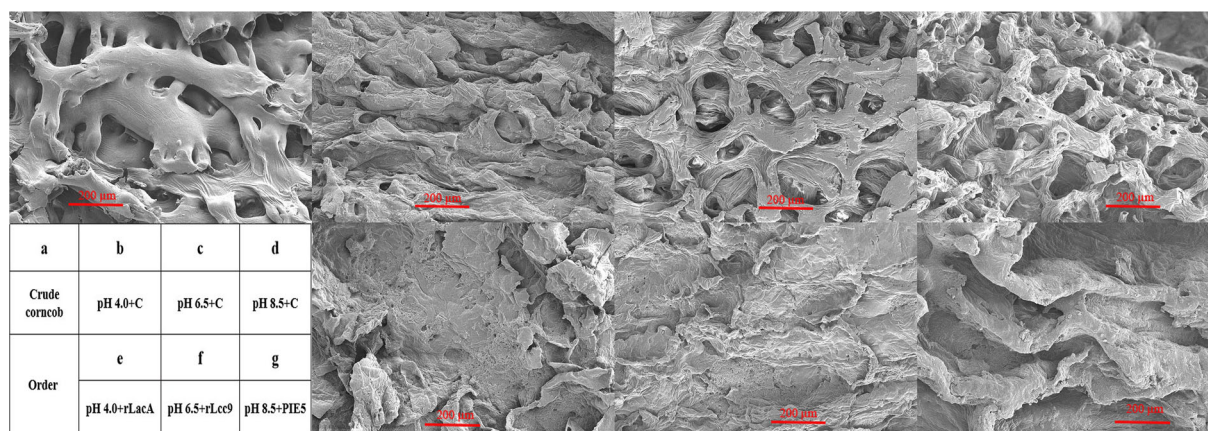
Fig. 3. Scanning electron microscopy photomicrograph of the surface of corncob samples. pH 4.0 + C, NaOH-pretreated corncob with pH adjusted to 4.0; pH 4.0 + rLacA, rLacA laccase treatment of (pH 4.0 + C) sample; pH 6.5 + C, NaOH-pretreated corncob with pH adjusted to 6.5; pH 6.5 + rLcc9, rLcc9 laccase treatment of (pH 6.5 + C) sample; pH 8.5 + C, NaOH-pretreated corncob with pH adjusted to 8.5; pH 8.5 + PIE5, PIE5 laccase treatment of (pH 8.5 + C) sample. rLacA was set as a separate control experiment.

Fig. 4. Time course for viable cells (squares), glucose consumption (circles), and ethanol production (triangles) during the fermentation of hydrolysates from alkali-pretreated corncob residues. (a) PIE5 treatment, (b) rLcc9 treatment, (c) rLacA treatment. rLacA was set as a separate control experiment.

samples. During the fermentation process, the yeast in samples treated with PIE5 or rLcc9 showed a remarkable increase in cell viability, reaching the value of 0.74×10^8 CFU/ml and 1.33×10^8 CFU/ml (Fig. 4 and Table 4). Yeast growth increased slower in PIE5-treated samples compared with that in rLcc9-treated samples. The ethanol production rates increased from 0.73 and 0.72 g/l hr

in control samples to 0.94 and 0.91 g/l hr in rLcc9- and PIE5-treated samples, respectively (Table 4). These increases are probably due to the lower phenolic content of PIE5- or rLcc9-treated samples compared with their respective controls. Moreover, the final ethanol concentrations of rLcc9 and PIE5 laccases were 7.51 ± 0.66 and 7.31 ± 0.54 g/l compared with their respective

Table 4. Summary of Saccharification and Fermentation Parameters from Samples With Different Laccase Treatments

Sample	EtOH$_M$ (g/l)	Glu (g/l)	YE/G (g/g)	QG (g/l hr)	QE (g/l hr)	Cell viability$_M$ (CFU × 10^8 per ml)
pH 4.0 + C	8.04 ± 0.23	16.90 ± 0.94	0.48 ± 0.02	2.10† ± 0.12	1.01† ± 0.09	0.69 ± 0.13
pH 4.0 + rLacA	8.02 ± 0.19	16.23 ± 1.14	0.49 ± 0.01	2.69§ ± 0.19	1.34§ ± 0.06	1.44 ± 0.15**
pH 6.5 + C	7.26 ± 0.23	15.88 ± 0.51	0.46 ± 0.03	1.58‡ ± 0.05	0.73‡ ± 0.06	0.66 ± 0.15
pH 6.5 + rLcc9	7.51 ± 0.16	17.28 ± 0.64	0.43 ± 0.02	2.15† ± 0.08*	0.94† ± 0.08	1.33 ± 0.15*
pH 8.5 + C	7.18 ± 0.15	14.46 ± 0.21	0.49 ± 0.00	1.44‡ ± 0.02	0.72‡ ± 0.01	0.49 ± 0.06
pH 8.5 + PIE5	7.31 ± 0.15	15.34 ± 0.63	0.48 ± 0.01	1.89† ± 0.06*	0.91† ± 0.06**	0.74 ± 0.08*

EtOH$_M$, maximum ethanol concentration; Glu, glucose produced after 72 hr of enzymatic hydrolysis; Y$_{E/G}$, ethanol yield based on total glucose content present in the hydrolysate of pretreated corncob; Q$_G$, volumetric glucose consumption rate based on time when maximum ethanol concentration is achieved: 6 hr (§), 8 hr (†), and 10 hr (‡); Q$_E$, volumetric ethanol productivity based on time when maximum ethanol concentration is achieved: 6 hr (§), 8 hr (†), and 10 hr (‡). Cell viabilityM, maximum viable cells during fermentation. Results were calculated from two times of independent experiments performed in triplicate ($n = 6$). Difference in means is significant at the *$p < .05$ or **$p < .01$ level.

controls (7.26 ± 0.23 and 7.18 ± 0.15 g/l). Several studies have also reported an enhancement of the *S. cerevisiae* performance after detoxification treatments with different laccases. Moreno et al. reported higher glucose consumption rates, ethanol volumetric productivities, and ethanol yields when the whole slurry from steam-exploded wheat straw was submitted to laccase treatments and fermented with *S. cerevisiae* (Moreno et al., 2012; Moreno et al., 2016). Fang et al. reported that the addition of the *G. lucidum* laccase Glac15 before cellulase hydrolysis increased ethanol yield by 10% (Fang et al., 2015). All these changes may be explained by the adaptation of yeast to fermentation conditions, which depends on different factors, such as the type and concentration of inhibitory compounds and their synergistic effects (Klinke et al., 2004; Moreno et al., 2013; Palmqvist & Hahn-Hägerdal, 2000).

Yeast cell viability was improved remarkably after treatment with rLacA and reached the highest number of 1.43 × 10^8 CFU/ml after 8 hr of fermentation compared with the 0.69 × 10^8 CFU/ml obtained after 12 hr of fermentation in control samples. Thus, faster glucose consumption and ethanol production rate were observed. Ethanol productivity increased from 1.01 g/l h at 8 hr in the control samples to 1.34 g/l h at 6 hr in rLacA-treated samples. These increases are probably due to the lower phenolic content of rLacA-treated samples. Nevertheless, maximum ethanol concentrations (8.02–8.04 g/l) and ethanol yields (0.48–0.49 g/g) obtained were similar for both control- and rLacA-treated samples (Table 4) .rLacA laccase treatment did not improve the final ethanol concentration and ethanol yield (0.49 g/g, Table 4). When treated the steam-exploded whole slurry with *T. villosa* laccase, faster glucose consumption and ethanol production rates were observed. However, the ethanol production yield kept unchanged when compared to the control (De La Torre et al., 2017).

Conclusions

The potentials of the alkaline fungal laccase PIE5 in the delignification and detoxification of alkali-pretreated corncob to produce bioethanol were evaluated and compared with other fungal laccases, including rLcc9 and rLacA. The laccases decreased phenolic compounds and lignin contents in slurries and improved the performance of *S. cerevisiae* by shortening the adaptation time and enhancing the production rate, cell viability, and volumetric ethanol productivity. The comprehensive performances of rLcc9 were better than that of PIE5, with rLacA performed the best among the three laccases. Based on these data, we concluded that acidic fungal laccases may be a better choice than neutral/alkaline fungal laccases in bioethanol production.

Supplementary Material

Supplementary material is available online at *JIMB* (*www.academic. oup.com/jimb*).

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Conflict of Interest

The authors declare no conflict of interest.

Data availability

All the required links or identifiers for the data are present in the manuscript as described.

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