Reducing Specific Binding Affinity while Maintaining the Enzyme Activity: Dual Effects of Lignosulfonates on Enzyme Hydrolysis of Sulfite-pretreated Lignocellulose

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It is well known that enzymatic hydrolysis is hampered by soluble inhibitors, while lignosulfonate (LS) generated from the sulfite pretreatment could enhance saccharification under certain conditions. To explain the roles of the LS during the hydrolyzing process, two types of LS were tested on selected lignocellulosic substrates and investigated through surface activity analysis and designed hydrolyzing experiments. The results showed that the LS with higher surface activity bound to and saturated the enzyme at a lower dosage and more effectively influenced the enzymatic hydrolysis. Both lignosulfonates, irrespective of their molecular weight and sulfonation degree, inhibited or enhanced the enzymatic saccharification related to two opposing mechanisms, *i.e.*, competitive inhibition by the LS and its beneficial role on the enzyme activity. According to the Michaelis-Menten equation, the rate of cellulase-substrate complex conversion into product did not change with the introduction of the LS, whereas the specific binding affinity of the enzyme to the substrate was noticeably altered. With the introduction of LS, the stability of the enzyme increased, which increased the final hydrolysis yield. The hypothesis that the inhibition effects of LS could be effectively overcome by increasing the substrate content and the buffer concentration of the hydrolysates was confirmed through additional experiments.

Keywords: Lignosulfonate; Enzymatic hydrolysis; Surface activity; Competitive inhibition; Enzyme activity

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INTRODUCTION

Enzymatic saccharification of cellulose is the primary step in biorefinery processes for converting lignocellulosic biomass into biofuels and other chemicals (Zhu and Pan 2010; Guo *et al.* 2018). Cellulose in the lignocellulosic biomass is physically and chemically protected by the highly robust heterogeneous ultrastructure of lignin and hemicellulose that can effectively resist the biodegradation completed through enzymatic saccharification (Ahmad *et al.* 2016; Holwerda *et al.* 2019). In a biomass biorefinery, substantial loading of enzyme is required to break down the lignocellulose into fermentable sugars. The high enzyme content greatly increases the cost of the process, and enzymatic hydrolysis can account for approximately 27% to 40% of the total cost of the overall bioconversion process (Ma *et al.* 2008; Ferreira *et al.* 2009; Yang *et al.* 2017).

Introducing surfactants to the hydrolysis reaction is a promising technique for increasing the rate and yield of the enzyme hydrolysis and consequently reducing the enzyme loading and operating costs (Winarni *et al.* 2013; Holmberg 2018; Lou *et al.*

2018). Surfactants can enhance both the enzyme solubility and enzyme activity during the hydrolysis, thereby increasing the efficiency of the hydrolysis reaction (Krickl *et al.* 2018; Silva *et al.* 2018). Due to their strong interaction with hydrophobic surfaces, surfactants can bind to the lignin residuals of the substrate and thereby reduce the non-productive enzyme adsorption and to increase the amount of available enzymes (Chen *et al.* 2018; Kamsani *et al.* 2018). Surfactants can also protect enzymes from heat and agitation, thereby helping to maintain their activity in the hydrolysate (Okino *et al.* 2013; MacKenzie and Francis 2014). One plausible mechanism of this protection effect is that the "reverse micelles" formed by the surfactants can reduce the detrimental effects of the ambient environment on the enzymes (Chen *et al.* 2006). Meanwhile, surfactants may enhance the enzyme stability by reducing the surface tension of the substrate and facilitate enzyme adsorption and desorption (Holmberg 2018; Zhou *et al.* 2019).

Lignosulfonates (LS) are lignin-derived products that are present in hydrolysates generated during sulfite pretreatment (Serna-Diaz *et al.* 2016; Dong *et al.* 2018b; Zhou *et al.* 2013, 2018). Despite the well-accepted concept that pretreatment hydrolysate exhibits an inhibitory role with respect to enzymes (Del Rio *et al.* 2019; Zhong *et al.* 2019), some studies have recently demonstrated the beneficial effects of LS on the cellulose saccharification. This finding has rendered the whole slurry fermentation process a one-pot reaction, where the pretreated substrate can be hydrolyzed and fermented with the pretreatment liquor to increase the yield and reduce the water consumption (Dong *et al.* 2018a; Zhou *et al.* 2018). The inhibitory or enhancing effect of the pretreatment liquor has been related to the properties and amount of the LS (Cai *et al.* 2017; Lin *et al.* 2017). Wang *et al.* (2013) found that the enzymatic digestibility of cellulose rapidly decreased when a small amount of LS was introduced in the hydrolysates, but the performance of the hydrolysis reaction improved when the dose of LS surpassed a critical concentration. Other studies showed that LS with high molecular weight and low degree of sulfonation inhibited the saccharification process, while low molecular weight LS with a high degree of sulfonation enhanced the enzymatic hydrolysis (Zhou *et al.* 2013; Lou *et al.* 2014). At high LS doses, the inhibition effect of the LS with low molecular weight and high sulfonation was also observed (Lou *et al.* 2014). Despite several sporadic hypotheses, to the best of current knowledge, no systematic study has been conducted on the effect of LS to enhance or inhibit the saccharification process. To properly design the LS-assisted saccharification system, a more solid understanding of the mechanism of the LS influence on the enzymatic hydrolysis may be necessary.

In this study, the enzymatic activities and reaction kinetics of the hydrolysis process under a well-controlled LS-enzyme complex were investigated. The objective of this study was to explain the inhibitory or enhancing mechanism of enzymatic saccharification by examining the specific binding affinity of enzyme to substrate and product formation using the appropriate models as well as the enzyme activity. Strategies to overcome the inhibitory effects of the LS for whole slurry fermentation process have also been provided based on the proposed mechanism.

EXPERIMENTAL

Materials

Monterey pine wood chips were purchased from the Tianjin Ji Xing Wood Processing Factory (Tianjin, China). Commercial cellulase enzymes, including Cellic Ctec2, were generously provided by Novozymes Investment Co., Ltd. (Beijing, China). Two types of LS with different characteristics were purchased. The first type of LS

(denoted as LS1) was purchased from Shandong Xiya Chemical Industry Co., Ltd. (Shandong, China). The other type of LS (denoted as LS2) was purchased from the Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). The LS used in the present study was centrifuged to remove water insoluble material. It was then separated and purified using dialysis membrane with a cutoff molecular weight of 500 Da. Table 1 lists the sulfur content and molecular weights of both lignin samples.

Table 1. Molecular Weight Distribution and Elemental Sulfur Content of the LS

Lignosulfonate	M_n (Da)	M_w (Da)	M_w/M_n	Sulfur (mmol/g)
LS1	1265	1331	1.05	2.8
LS2	6592	31230	4.74	2.2

Methods

Sulfite pretreatment

The laboratory-scale sulfite pretreatment process was conducted in a 4-L rotary-type electric heating digester (KRK 2611; Kyoraku Co., Ltd., Tokyo, Japan) (Zhu *et al.* 2009; Zhou *et al.* 2018). In each batch, a total of 500 g of wood chips were pretreated using the sulfite pretreatment method under three predesigned conditions: DA with 2.2% (w/w) sulfuric acid, SP10 with 10% (w/w) sodium bisulfite and 2.2% (w/w) sulfuric acid, and SP20 with 20% (w/w) sodium bisulfite and 3.5% (w/w) sulfuric acid. The fixed dosages of acid and sodium bisulfite in sulfite pretreatment could maintain the initial pH of system at 2.3. The temperature profile of the heating process was monitored using a thermocouple. The digester was heated to 165 °C at a rate of 5 °C/min and was maintained at that temperature with a fluctuation of ± 1 °C for 75 min. After the heating process was completed, the digester was water-cooled to reach room temperature before the substrate and the liquor were collected. The substrate and the spent liquor were separated using a nylon cloth and then weighed separately. The substrate was thoroughly washed to remove the spent liquor absorbed by the substrate. The spent liquors from pretreatment of SP10 and SP20 were denoted as H-SP10 and H-SP20, respectively. Additionally, U and W were used to designate the unwashed and washed samples, respectively.

Chemical composition of substrates and liquors

The chemical compositions of the solid samples were measured according to the procedures established by the National Renewable Energy Laboratory (Sluiter *et al.* 2008). Briefly, the solid samples were ground to pass through a 20-mesh sieve before a two-stage acid hydrolysis (72% H_2SO_4, 30 °C, 1 h dilution to 4%, 121 °C, and 1 h). Concentrations of sugars and inhibitors in the hydrolysates were analyzed on a high-performance liquid chromatograph (HPLC) (Shimadzu Corp., Kyoto, Japan) equipped with a refractive index detector. The monosaccharides (glucose, xylose, arabinose, and galactose) and cellobiose in all the hydrolysates were analyzed using an Aminex HPX-87P column (Bio-Rad Laboratories, Hercules, CA, USA) at 85 °C at an eluent (deionized water) flow rate of 0.6 mL/min. Acid insoluble lignin content was determined gravimetrically after hydrolysis.

Sulfur content analysis and determination of lignin molecular weight

The sulfur content of LS was analyzed using the bomb-washing method as per the ASTM E775-87 (2008) standard. Gel permeation chromatography (GPC) coupled with

Shodex OHpak SB-804 HQ and SB-806 HQ columns (Showa Denko, Tokyo, Japan) were employed to determine the molecular weight of LS.

A $NaNO_3$ aqueous solution of 0.10 M was used as eluent at a flow rate of 1.0 mL/min. The eluent was determined by the means of an evaporative light scattering detector (ELSD) (DAWN®, Wyatt Technology Corporation, Goleta, CA, USA), and dextran was used as the criterion.

Surface activities of lignosulfonates

Surface activities of the lignosulfonates were examined by recording the alterations in the surface tension of the enzyme solutions from increasing LS concentration using a DCAT 21 tensiometer (DataPhysics Instruments, Filderstadt, Germany). The enzyme concentration was kept at the same amount as used in the hydrolysis experiment.

Hydrolysis experiment

Enzymatic hydrolysis was conducted in 150-mL flasks at 50 °C with a total working volume of 50 mL. The pH level of the solution was adjusted to 5.5, because the nonproductive binding of cellulase to lignin on the substrate can be greatly reduced at this pH value (Lou *et al.* 2013). The Ctec2 loading was fixed at 5 FPU/g glucan. At regular intervals, a 500 μL sample was taken and centrifuged at 7000 rpm for 5 min. The glucose contents of the supernatants were analyzed *via* HPLC, as described in the preceding sections. The substrate enzymatic digestibility (SED) of the solid samples was calculated by taking the mass fraction of the dissolved sugars over the total glucan of the substrates.

Enzyme activity assay

Filter paper activity (FPU) was measured according to according to the National Renewable Energy Laboratory (NREL) technical report (Adney and Baker 1996). Enzyme activity (EA, %) was calculated by Eq. 1,

$$EA = \frac{FPU_t}{FPU_{initial}} \times 100 \quad (1)$$

where FPU_t is the enzymatic hydrolysis (g/L) measured at time t (h).

Michaelis-Menten equation

Using the Langmuir isotherm model, it has been well documented that lingosulfonates can reduce the nonproductive binding of enzyme to lignin (Ko *et al.* 2015; Li *et al.* 2016). However, due to the inability of this model to distinguish between the specific and nonspecific binding of the enzyme to the substrate, the effect of lignosulfonate on the specific binding of enzymes has not yet been explored.

Therefore, the Michaelis-Menten model, which is based on the specific binding affinity of enzyme to substrate and can analyze the rate of product formation, was used to study the enzyme kinetics (Bezerra *et al.* 2013). The initial rate of hydrolysis (v_0) can be expressed as Eq. 2,

$$v_0 = \frac{V_{max}[S]_0}{K_m + [S]_0} \quad (2)$$

where V_{max} is the maximum rate of hydrolysis (g/Lh), $[S]_0$ is the initial concentration of substrate (w/v, %), and K_m is the Michaelis constant physically representing the concentration of substrate when the hydrolysis rate reaches $V_{max}/2$; K_m is also considered as an index of affinity between the substrate and enzyme.

RESULTS AND DISCUSSION

Effects of Pretreatment Severity on Enzyme Hydrolysis

In this study, sulfite pretreatment was applied to break down the robust lignocellulose structure and enhance the enzyme accessibility to cellulose. The trends of the SED change in different lignosulfonate and acid concentrations are presented in Fig. 1. When no bisulfite was available in the pretreatment process, the biomass-to-sugar conversion was low (39%). As shown in Table 2, pretreating the feedstock with acid could only dissolve the major fraction of the hemicellulose from the lignocellulose, and no delignification could occur (*i.e.*, lignin was enriched from 25.20% to 40.26%), although lignin condensation was likely to take place.

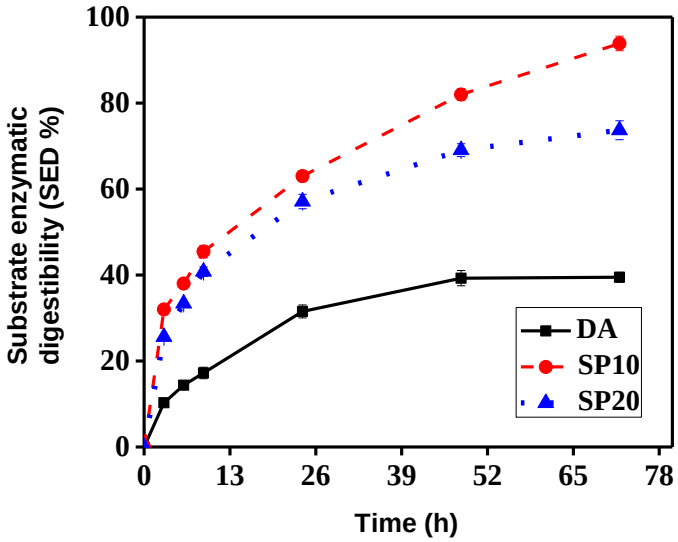

Fig. 1. Effect of sulfite loading on the enzymatic digestibility (SED) of sulfite-pretreated substrates. Substrate content and acetate buffer (pH=5.5) concentration were 2% (w/v) and 0.1 M, respectively; DA, SP10, and SP20 are the pretreated substrates without sulfite, 10% (w/w) sulfite, and 20% (w/w) sulfite, respectively.

Physical blockage of the cellulose by the lignin in lignocellulose and non-productive binding of the enzyme to lignin could limit the enzyme hydrolysis and result in the low SED when no sodium bisulfite was utilized in the pretreatment process (Zhu *et al*. 2009). As the concentration of sodium bisulfite was increased up to 10%, the SED value drastically rose to approximately 93%. Sulfite pretreatment method introduces sulfonic groups on the benzylic carbons of the lignin, partially dissolves the lignin, and increases its hydrophilicity (Shuai *et al.* 2010). After sulfonation, the hydrophobic interaction between the lignin residues and the enzymes is reduced (Lou *et al.* 2013). Lignosulfonate present in the pretreated hydrolysate can also reduce the nonproductive binding of the cellulase to the lignin leading to the enhanced lignocellulose saccharification (Lou *et al.* 2014). It was interesting to note that the SED of the whole slurry SP20 substrate was lower than the whole slurry SP10 (75% *vs* 93%, respectively) (Fig. 1). Zhu *et al.* (2009) reported that at a fixed acid concentration of 2.2% (w/w), 8% to 10% (w/w) sodium bisulfite was the optimal dosage in the pretreatment of softwood chips (Shuai *et al.* 2010). However, when the dosage of sodium bisulfite was increased, the pH value in the pretreatment system also increased.

Table 2. Pretreatment Conditions and Chemical Compositions of Monterey Pine and the Substrate's Bisulfite Pretreatment Method

Pretreatment Label	Pretreatment Conditions	Samples	Glucan	Xylan	Galactan	Mannan	Klason lignin
Monterey pine	N/A	N/A	41.83%	3.71%	4.60%	12.10%	25.20%
DA	165 ºC, 75 min, 2.2% (w/w) H_2SO_4	Washed solid substrate (w/w)	54.96%	ND	ND	ND	40.26%
SP10W	165 ºC, 75 min, 2.2% (w/w) H_2SO_4, 10% (w/w) $NaHSO_3$	Washed solid substrate (w/w)	59.81%	0.18%	ND	ND	29.27%
SP10U		Unwashed solid substrate (w/w)	47.92%	1.57%	1.25%	6.84%	25.14%
H-SP10		Pretreatment hydrolysate (g/L)	13.93	6.97	5.84	26.75	N/A
SP20W	165 ºC, 75 min, 3.5% (w/w) H_2SO_4, 20% (w/w) $NaHSO_3$	Washed solid substrate (w/w)	79.09%	0.22%	ND	ND	10.68%
SP20U		Unwashed solid substrate (W/W)	48.28%	1.55%	1.16%	6.77%	15.23%
H-SP20		Pretreatment hydrolysate (g/L)	13.23	6.70	5.84	26.75	N/A

N/A: Not applicable; ND: Not detectable

Table 3. Regression Lines of Surface Tensions and Concentrations of LSs

Label	Regression lines	R^2
Line 1	ST=-7.72LogC+32.01	0.99
Line 2	ST=-18.42LogC+54.40	0.97
Line 3	ST=-2.86LogC+48.30	0.99

ST: Surface tension (mN/m); C: Concentration (g/L);
Label of line are marked in Fig. 2.

The pH rise resulted in a lower hemicellulose removal, decreased the mean pore size of the substrate, and limited the accessibility and probability of the cellulose to be hydrolyzed (Zhou *et al.* 2018). Therefore, it was practical to relate the changes in the SED solely to sodium bisulfite concentration, as the hemicellulose concentration was not identical in different experimental conditions. In this work, to avoid any pH (~2.3) increase, as the sodium bisulfite dosage of the pretreatment process was increased from 10% to 20% (w/w), more acid (2.2% to 3.5%, w/w) was simultaneously introduced to the pretreatment process. According to Table 2, Klason lignin was reduced from 29.27% to 10.68%, and almost all the hemicellulose was removed, so the decrease of the SED from SP20 to SP10 (Fig. 1) could not be attributed to the blockage of hemicellulose and lignin. Rather, the enzyme saccharification of lignocellulose was inhibited by higher degrees of lignin sulfonation, whereas it was previously believed that higher sulfonation was always a favorable strategy (Zhou *et al.* 2013; Lou *et al.* 2014). This often-misinterpreted phenomenon will be dealt with in more detail in subsequent sections.

Surface Activity of Lignosulfonates

Surface activity is related to the efficiency of surfactants in increasing enzyme hydrolysis (Holmberg 2018). Figure 2 shows the surface tension of the enzyme solution as a function of lignosulfonate concentration. The specifications of the regression lines are listed in Table 3. For each lignosulfonate, two break points were obtained at intersections of regression lines.

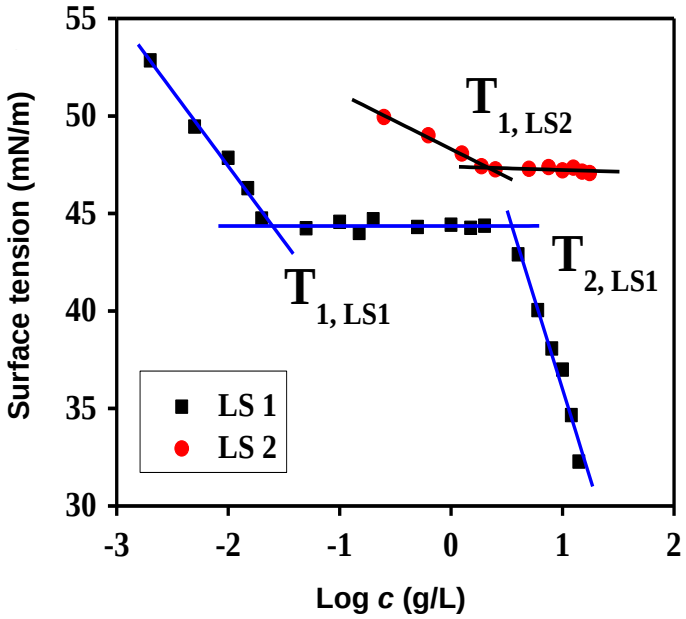

Fig. 2. Surface tension-concentration isotherm for two lignosulfonates: T_1: critical aggregation concentrations; T_2: critical micelle concentration

The intersection point T_1 (LS1: 1.6 × 10^{-2} g/L, LS2: 2.3 g/L) represented the critical aggregation concentrations (CAC) that corresponded to the onset of micelle formation on the enzyme (Jain *et al.* 2004). The intersection point T_2 (LS1: 3.6 g/L) signified that the enzymes were saturated with lignosulfonates. At low concentrations, lignosulfonates were adsorbed at the air-liquid interface of the solution surface, and consequently the surface tension exhibited a decreasing trend. When the air-liquid

interface was crowded with lignosulfonate (T_1), the lignosulfonate was bound to the enzyme and the surface tension of the solution was not noticeably altered, and thus reached a plateau (between T_1 and T_2) until a critical micelle concentration (CMC) was achieved (Jain *et al.* 2004). At excessive concentrations of the lignosulfonates, the enzymes were saturated with the lignosulfonate micelles, which resulted in the adsorption of lignosulfonates to air-liquid interface. This posed further reduction in the surface tension of the solution when the lignosulfonate concentration exceeded the CMC. Because the value of $T_{1,\,LS1}$ was smaller than $T_{1,\,LS2}$ and $T_{2,\,LS2}$ was not achieved (*i.e.*, the enzyme was not saturated with the LS2 in the range of the tested concentrations), it could be concluded that LS1 had a higher surface activity than LS2. The lower molecular weight and higher degree of sulfonation for LS1 compared with LS2 accounted for the higher surface activity of the former lignosulfonate.

Effect of Lignosulfonate Dosage on Enzyme Hydrolysis

It is well documented that the efficiency of enzymatic saccharification is highly dependent on both the properties and dose of lignosulfonates (Wang *et al.* 2013; Zhou *et al.* 2013; Lou *et al.* 2014). Nonetheless, some contradictory trends on the effect of lignosulfonates on the enzymatic saccharification have been reported. While low-molecular weight lignosulfonates with higher degrees of sulfonation were shown to be more effective in enhancing the enzymatic saccharification (Zhou *et al.* 2013; Lou *et al.* 2014), both enhancing and inhibitory effects were found when the dose of the introduced lignosulfonates varied in the hydrolysates (Zhou *et al.* 2013). LS was produced in sulfite pretreatment, which acted as a surfactant and further enhanced the enzymatic hydrolysis, enabling the whole sulfite pretreated slurry (solid plus the liquid fraction) to be directly used in saccharification and fermentation. The amount and properties of LS varied with pretreatment conditions, which both affect inhibitory or enhancing of LS on enzymatic hydrolysis. In order to elucidate the controversial phenomenon and maximize the LS beneficial effects, SP10W substrate was selected to perform the enzymatic hydrolysis with different dosage of LS. According to Fig. 3, the enzymatic hydrolysis was not only a function of the lignosulfonate loading, but also of the hydrolysis time.

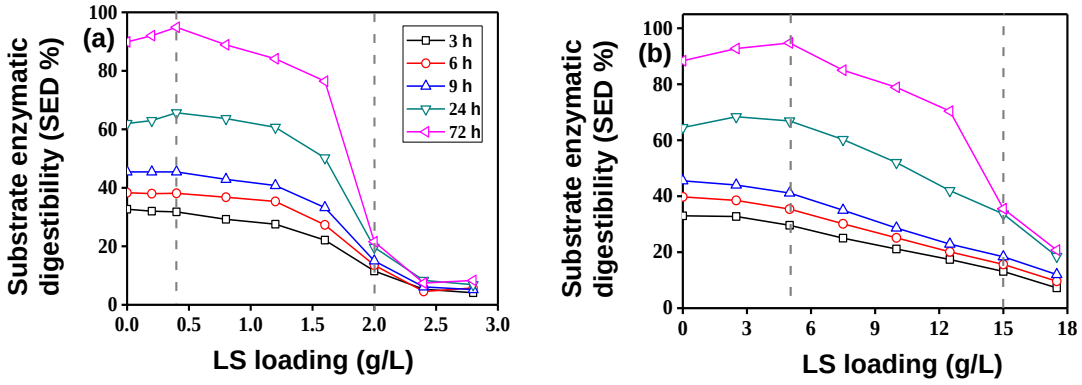

Fig. 3. Effects of LS1 (a) and LS2 (b) on the enzymatic digestibility of the SP10W substrate; substrate content and acetate buffer (pH=5.5) concentration were 2% (w/v) and 0.1 M, respectively

The effect of lignosulfonate loading on the SED could be categorized into three stages: Stage I (low LS loading), Stage II (medium LS loading), and Stage III (high LS loading). At Stage I (< 0.4 g/L for LS1 and < 5 g/L for LS2), the introduction of the lignosulfonate had a negligible impact on the enzymatic saccharification of the substrate in the first 9 h. When the hydrolysis time was extended to over 24 h, an enhancement in the enzymatic saccharification was observed at low LS concentrations. At a hydrolysis time of 72 h, the introduction of 0.4 g/L LS1 and 5 g/L LS2 increased the SED to over 95%. Introduction of LS beyond the optimum loading amount (Stage II), however, had an adverse impact on the conversion of lignocellulose to sugars. This trend was more intense at prolonged hydrolysis durations, where the SED decreased to 78% and 70% at LS1 and LS2 loadings of 1.6 g/L and 13 g/L, respectively. Excessive LS loading (Stage III) resulted in a drastic reduction in the lignocellulose-to-sugar conversion.

Despite the similar trends observed for the SED changes with the two lignosulfonates, the range of the LS loading for each stage was very different for the two types of the LS, where the LS1 loadings window for various stages was almost ten times lower than that of the LS2. For instance, the optimum LS1 and LS2 loadings to achieve the highest SED were 0.4 g/L and 5 g/L, respectively. This implied the importance of surface activity of lignosulfonate in the conversion efficiency of lignocellulose.

Lou *et al.* (2014) and Zhou *et al.* (2013) showed that lignosulfonates with lower molecular weights and higher degrees of sulfonation can have an enhancing effect on the SED, whereas higher molecular weight and less sulfonated lignosulfonates may lead to an inhibiting effect. Nevertheless, this study verified that lignosulfonates could exhibit either enhancing or inhibiting behavior depending on their dosage. As demonstrated in Fig. 2, the LS1 with lower molecular weight and higher degree of sulfonation had higher surface activity. Therefore, it could bind to the enzymes ($T_{1,\,LS1}$), saturate it ($T_{2,\,LS1}$), and influence the enzyme hydrolysis more effectively at a lower dosage.

Mechanistic Insight into the LS Inhibition Effect for Enzymatic Saccharification in the Initial Reaction Period

Wang *et al.* (2013) asserted that formation of lignosulfonate-cellulase complexes could diminish the nonproductive binding of cellulase to lignin and therefore enhance the glucan conversion into sugars. However, it was not clarified whether the complexes could also influence the specific binding of enzyme and affect the enzyme activity. To shed light on the mechanistic impact of lignosulfonates on enzymes, a series of hydrolysis experiments under controlled conditions were performed at different lignosulfonate dosages and substrate contents (Table 4). The initial hydrolysis rates (v) in the experimental conditions were calculated according to the method developed by Lee and Fan (1982). As summarized in Table 4, the initial hydrolysis rate increased when the initial substrate concentration increased and decreased when more lignosulfonate was added. Particularly, the initial hydrolysis rate became low when the dosages of LS1 and LS2 were set at 2.8 g/L and 17.5 g/L, respectively.

To clarify the inhibition mechanism of the lignosulfonates, initial rates were incorporated in the Lineweaver-Burk equation, and the kinetic parameters, V_{max} and K_m, were calculated (Fig. 4). Generally, in substrate saccharification processes, inhibitors can affect the reaction *via* three different routes: competitive, noncompetitive, or uncompetitive (Yeh *et al.* 2010). In competitive inhibition, the inhibitor is bound to the active site of the enzyme and prevents the binding of enzyme to substrate, but the ability of enzyme-substrate to form a product is not affected. Hence, the maximum velocity

(V_{max}) of the reaction does not change due to the presence of the inhibitor, while the apparent affinity of the substrate to the specific binding site is decreased. In contrast, non-competitive inhibition reduces the maximum velocity (V_{max}) without changing the specific binding affinity (K_m) of the enzyme to the substrate. Additionally, the Lineweaver-Burk plot for an uncompetitive inhibitor produces a line parallel to the original enzyme-substrate plot (Bezerra *et al.* 2013).

Table 4. Initial Rate (v: g/Lh) of Enzyme Hydrolysis at Different Conditions

Lignosulfonate (g/L)		Substrate content (w/v, %)			
		2%	2.5%	3.3%	5%
Control	0	1.54	1.81	2.06	2.62
LS1	0.4	1.50	1.73	2.03	2.56
	1.2	1.35	1.56	1.86	2.39
	2.8	0.10	0.13	0.20	0.70
LS2	5	1.51	1.76	2.03	2.58
	7.5	1.39	1.62	1.94	2.41
	17.5	0.14	0.20	0.27	0.61

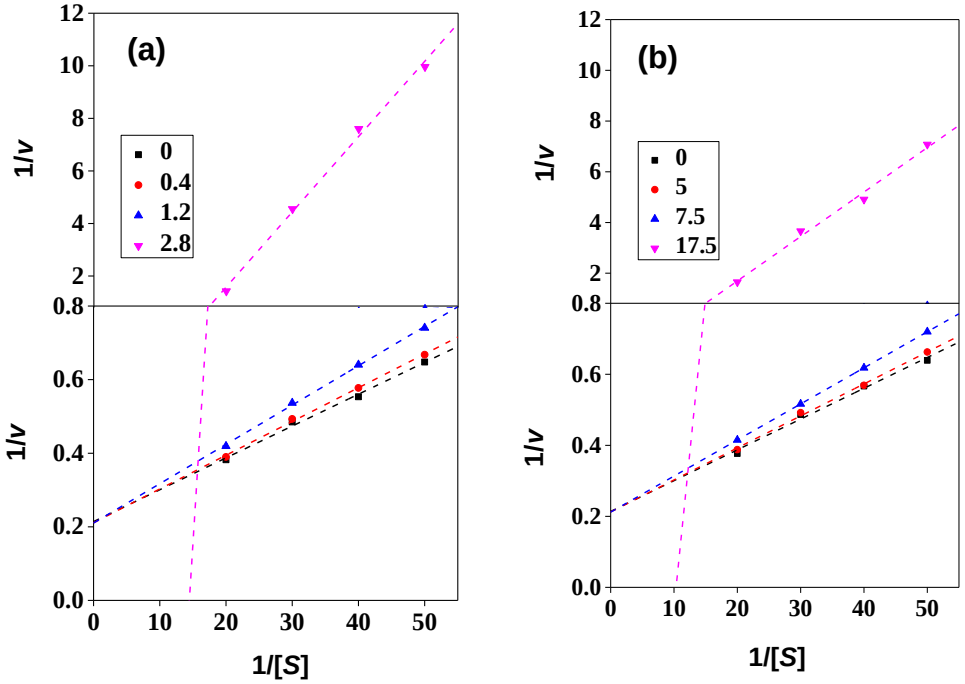

Fig. 4. Kinetic analysis of SP10W substrate enzyme hydrolysis in the presence of several concentrations of the inhibitor at different substrate concentrations: LS1 (a) and LS2 (b); the legend represents the dosages (g/L) of LS, *v* is the initial rate (g/Lh) of enzymatic hydrolysis, and [*S*] is the substrate concentration (w/w, %)

When excessive amounts of LS (2.8 g/L for LS1 and 17.5 g/L for LS2) were introduced into the reaction, negative V_{max} values were obtained, which implied the immediate deactivation of the enzymes upon the introduction of the LS into the hydrolysates. Meanwhile, as shown in Table 4, the half-reaction constant, K_m, increased from 4% (w/v) for the control sample with no lignosulfonate to 5% (w/v) when 1.2 g/L LS1 was introduced. Despite a considerable change in the K_m value, the rate of the cellulase-substrate complex conversion into glucose (V_{max}) did not change with the introduction of LS1. This suggested that competitive inhibition occurred between the inhibitor and the substrate in the enzyme hydrolysis, which demonstrated that the LS and the substrate combined with the same enzyme site. When LS2 was introduced into the system, a similar trend was observed, where the reaction rates were not altered, but the K_m values changed remarkably. This further verified the hypothesis of competitive inhibition.

Effects of the Lignosulfonate Content on the Enzyme Activities

Enzyme deactivation as a result of nonproductive binding of cellulase to lignin and other unfavorable components can be reduced by using surfactants for enhanced enzyme stability (Di Pasqua *et al.* 2014; West *et al.* 2014; Silva *et al.* 2018). Lignosulfonates might play two distinct opposing roles in the enzymatic hydrolysis: they could be considered either as lignin-derived substances that might deactivate the cellulase or as surfactants that might stabilize the cellulase. To explain which role was more dominant in the hydrolysis reaction, it was necessary to determine the enzyme activity as lignosulfonates bonded to cellulase nonproductively. According to Fig. 5, excessive loading of lignosulfonate completely deactivated the enzyme due to the fact that high loading of anionic surfactants could damage the structure of the enzymes. However, the enzyme stability was slightly enhanced with the introduction of low concentrations of lignosulfonates. While the enzyme activities dropped to 48% and 18% of their initial activity after 24 h and 72 h, respectively, incubation in the absence of lignosulfonate, enzyme activity loss was slightly enhanced to 58% and 25% as 1.6 g/L LS1 was used as surfactant (Fig. 5a). A similar trend was also observed as the concentration of LS2 increased (Fig. 5b). The introduction of 15 g/L LS2 deactivated the enzyme after 72 h, 12.5 g/L LS2 led to enhancement of the enzyme activity to 57% and 26% after 24 h and 72 h, respectively.

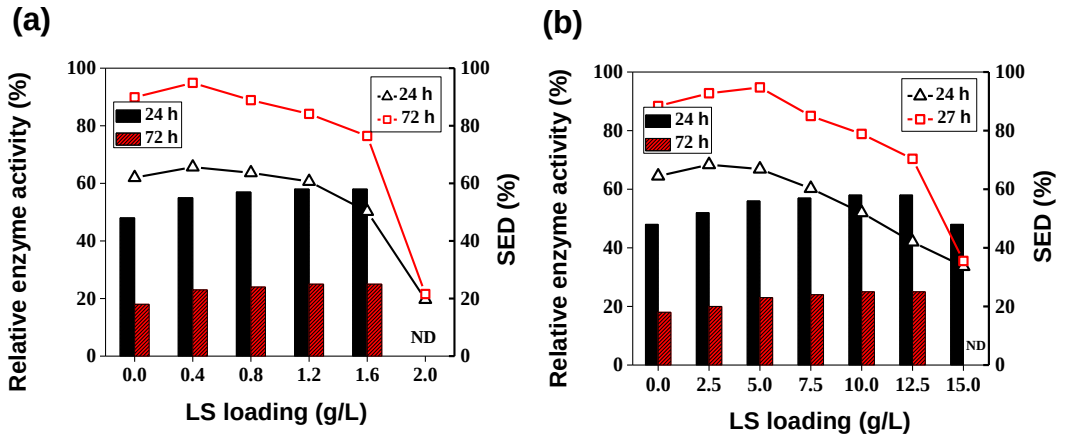

Fig. 5. Effects of LS1 (a) and LS2 (b) on enzymatic activity (column) and SED (line) at different LS loadings; substrate content and acetate buffer (pH=5.5) concentration were 2% (w/v) and 0.1 M, respectively

Several mechanisms accounted for the enzyme stabilization by employing an appropriate amount of lignosulfonate. In the absence of lignosulfonate, enzymes can easily be exposed to the air-liquid interface, which results in a significant enzyme deactivation (Kim *et al.* 1982). Nonetheless, when lignosulfonates were introduced, they occupied the surface of the solution and thus reduced the enzyme exposure to the air/liquid interface and protected it from deactivation. In addition to the surface exposure of enzyme, the adsorption and desorption of enzymes to lignin can also lead to their activity loss (Silva *et al.* 2018). Lignosulfonates can block the nonspecific binding of cellulase by binding on lignin in the same manner as a nonionic surfactant (Zhou *et al.* 2015). Moreover, lignosulfonates can employ their ionic nature and mitigate the nonproductive binding of cellulase to lignin as a result of the electrostatic repulsion between the LS-cellulase complex and lignin (Wang *et al.* 2013).

Notably, although the enzyme activity was increased by increasing the LS loading up to 1.6 g/L LS1 and 12.5 g/L LS2, the trend was not identical to the lignocellulose-to-sugar conversion, where the SED decreased by increasing the LS content over the optimum loading (Fig. 5). Retention of the enzyme activity implied that competitive inhibition was mainly responsible for the decrease of SED in Stage II. Additionally, if no inhibition occurred, the enzyme activity should theoretically follow a steadily increasing trend by increasing the LS loading. However, the rate of increase in enzyme activity was greatly reduced after the LS1 and LS2 loadings were increased over 0.4 g/L and 5.0g/L, respectively, which indicated a "relative" decrease in enzyme activity compared with the theoretical one. The reason for this was that competitive inhibition also affected the enzyme activity results obtained by the filter paper method. This trend was in line with the already-discussed mechanism of competitive inhibition, which was mainly responsible for the decrease of SED at medium dosage of lignosulfonate (Stage II).

Effects of the Substrate Content and Buffer Concentration on the Optimum Content of Lignosulfonate

In the previous sections it was explained that competitive inhibition was the main reason for the reduction in lignocellulose conversion. To overcome this challenge, three approaches can be employed to increase the substrate content (Bezerra *et al.* 2013) and adjust pH and buffer concentration. As the substrate content was increased from 2% (w/v, Fig. 3) to 5% (w/v, Fig. 6a and Fig. 6b), the optimum dosage of LS1 increased from 0.4 g/L (Fig. 3a) to 1.2 g/L (Fig. 6a), and the optimum dosage of LS2 increased from 5 g/L (Fig. 3b) to 7.5 g/L (Fig. 6b). Additionally, the SED of the SP10W substrate recovered from 27% (Fig. 3a) to 79% at LS1 loading of 2.0 g/L (Fig. 6a), which confirmed that the deactivation of enzyme could also be prevented as the substrate content increased. This was related to the fact that the insoluble lignin could adsorb lignosulfonate and thus could mediate the interaction between lignosulfonate and enzyme.

The effect of pH and buffer concentrations on enzyme hydrolysis was also investigated in this study. The optimal dosages of LS1 and LS2 were unchanged when pH of buffer decreased from 5.5 to 4.8. SED at elevated hydrolysis pH of 5.5 was higher than pH of 4.8 (Fig. 3a, b and Fig. 6c, d), which can be attributed to the shielding effect of electrostatic interactions between enzyme and sulfonated lignin in SP10W (Lou *et al.* 2013). However, the optimal dosage of LS1 increased from 0.4 g/L (Fig. 3a) to 2.0 g/L (Fig. 6e), and the optimal dosage of LS2 increased from 5 g/L (Fig. 3b) to 7.5 g/L (Fig. 6f) when the buffer concentration increased from 0.1 M (Fig. 3) to 0.4 M (Fig. 6e and Fig. 6f).

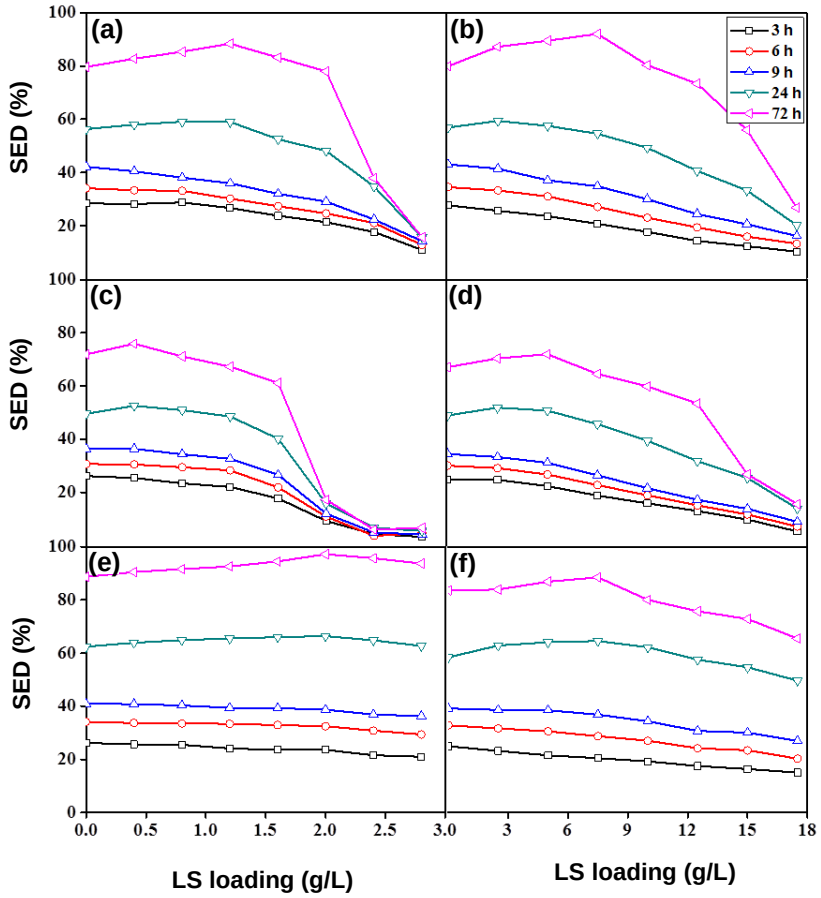

Fig. 6. Effects of LS1 (a, c, e) and LS2 (b, d, f) on enzymatic digestibility of SP10W substrate. In (a) and (b), substrate content and acetate buffer (pH=5.5) concentration were 5% (w/v) and 0.1 M, respectively. In (c) and (d), substrate content and acetate buffer (pH=4.8) concentration were 2% (w/v) and 0.1 M, respectively. In (e) and (f), substrate content and acetate buffer (pH=5.5) concentration were 2% (w/v) and 0.4 M, respectively.

Additionally, enzyme deactivation was not observed even at excessive LS loading for both types of lignosulfonate. Liu *et al.* (2010) discovered that metal compounds can deactivate the adsorption sites of lignin for enzymes to reduce or eliminate nonproductive enzyme adsorption. Consequently, sodium ions from the buffer solution could form complexes with the lignosulfonate and reduce the amount of the lignosulfonate binding to the enzyme. Thus, adding salt to the solution is an effective way to eliminate the inhibition of the lignosulfonate.

Effects of Lignosulfonates on Enzyme Hydrolysis at their Optimal Dosage

A conceptual model was introduced to explain the contradictory inhibiting and enhancing effects of lignosulfonate on enzymatic hydrolysis of sulfite-pretreated lignocellulosic substrate at its optimal dosage (Fig. 7). The enzyme-lignosulfonate interactions could have two distinct effects: competitive inhibition and stabilization. Most cellulose-degrading enzymes have a two-domain structure that consists of a catalytic domain and a cellulose-binding domain connected by a linker region (Linder *et al.* 1996). Lignosulfonate could bind with the two domains randomly after the surface of the

hydrolysate was crowded with lignosulfonate (Fig. 2). The parameters determined by the Michaelis-Menten equation showed that only competitive inhibition was exerted by the lignosulfonates at their optimal dosage. It was indicated that lignosulfonates that covered the binding domain could decrease the specific binding affinity of enzymes to cellulose. As a result, the initial rates of hydrolysis were decreased when lignosulfonates were incorporated. The ability of the catalytic domain to convert the cellulose to glucose was not inhibited when the lignosulfonates bound to the domain unproductively. In contrast, the negatively charged lignosulfonate-enzyme complex was more hydrophilic than the pure enzyme. Additionally, the sulfonated lignin residue from the sulfite pretreatment process was hydrophilic and negatively charged. Thus, the decrease in the hydrophobic interaction between the LS-enzyme and sulfonated lignin, as well as the electrostatic repulsion, could reduce the nonproductive interaction. Furthermore, lignosulfonate adsorbed at the air-liquid interface prevented the enzyme from being exposed to the air. Thus, enzyme stabilization was promoted and the final yield of the hydrolysis experiment was enhanced.

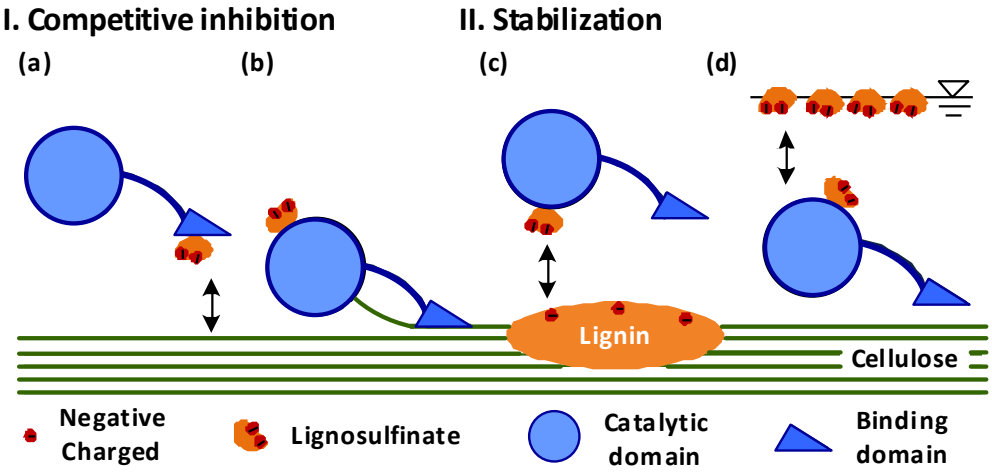

Fig. 7. Conceptual schematic of the effect of the LS on the enzyme hydrolysis at its optimal dosage: (a) Covering the specific binding site; (b) No inhibition of product formation; (c) Reduction in non-productive binding of enzyme to lignin; and (d) Preventing the exposure of enzyme to the air

CONCLUSIONS

This study shed light into the contradictory information about the inhibitory or enhancing effect of lignosulfonates in enzymatic hydrolysis.

1. Lignosulfonates with higher surface activity influenced the enzyme hydrolysis more effectively through two opposing mechanisms.
2. Competitive inhibition of enzyme by LS reduced the specific binding affinity between enzyme and cellulose, which inhibited the enzyme hydrolysis. The presence of LS resulted in enzyme stability and enhanced the digestibility.

3. Competitive inhibition was the dominating factor at excess LS loading. Increasing the solid substrate content and buffer concentration proved to be promising in overcoming the inhibition effect of LS in the whole slurry hydrolysis process.

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