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Research Article

Understanding the Influence of Water-Soluble Compounds from Unpretreated Corn Stover Pellets on Enzymatic Hydrolysis of Cellulose

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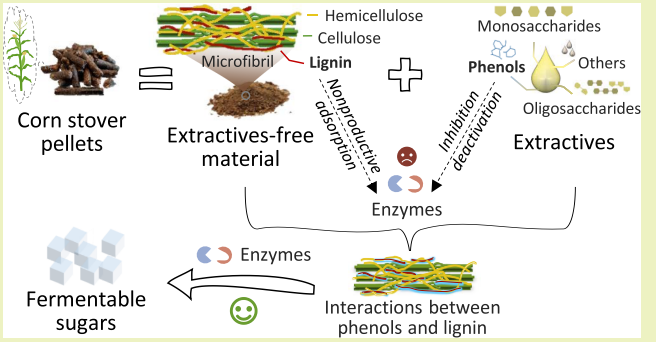
ABSTRACT: Aqueous extractives are minor and nonstructural compounds in biomass that can be extracted using solvents, while contributing significantly to lignocellulose characteristics. Yet their roles in enzyme-mediated processing of lignocellulosic biomass remain elusive. Here, we examine the composition and features of extractives derived from untreated pelleted corn stover as well as their effects on enzymatic hydrolysis. Unlike the observations described in previous reports, we find that water-extractable material improves the enzymatic hydrolysis of extractive-free stover by 67% with a glucose yield increase from 12 to 20% with 6 FPU cellulase per gram of glucan, whereas the enzyme activities are diminished when using microcrystalline cellulose (MCC) as a substrate. The different behavior of corn stover and MCC is likely attributed to the presence of lignin, which may interact with inhibitory compounds, such as phenolics, mitigating the detrimental impacts of soluble inhibitors, insoluble lignin, or both. These findings advance our fundamental understanding of the intrinsic behavior of extractives and help us to optimize the schemes for efficient and cost-competitive enzymatic conversion of lignocellulose.



KEYWORDS: *pelleted corn stover, extractives, phenolics, enzyme activity, inhibition, lignin interaction*

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1. INTRODUCTION

Diminishing petroleum reserves along with increasing economic, environmental, and political concerns about fossil fuel extraction and use necessitate the manufacture of chemicals, materials, and fuels from renewable resources.[1,2] As an abundant and inexpensive carbon-neutral renewable resource, lignocellulosic biomass harbors tremendous potential as a starting material to produce sustainable fuels and commodity chemical feedstocks. Its efficient utilization would address several societal needs.[3−5] In this context, numerous technologies have been developed to extract value from lignocellulosic biomass, with the sugar platform playing a central role, typically involving a combination of physical and chemical pretreatments to overcome biomass recalcitrance followed by enzymatic saccharification of cellulose and hemicellulose to monosaccharides.[6,7]

While enzyme hydrolysis has distinct advantages in terms of minimizing the byproducts that are toxic to the fermentation of resulting sugars, the relatively high cost of enzymes as a catalyst compared to mineral acids continues to hinder their industrial-scale use.[8] Accordingly, considerable efforts have been devoted and remain crucial to understanding and improving the enzymatic saccharification of lignocellulose with low enzyme loadings that result in high sugar yields and concentrations.[6,9]

The inherent recalcitrance of lignocellulosic feedstock is the major obstacle to its efficient transformation.[10,11] Alongside the high molecular weight polysaccharides cellulose and hemicellulose, lignocellulosic biomass comprises lignin that is a polymer of aromatics in a complex three-dimensional structure. These aromatic polymers are considered one of the most recalcitrant factors retarding enzymatic hydrolysis by restricting carbohydrate accessibility physically, unproductively binding enzymes, and releasing soluble inhibitors.[9] Moreover, varying quantities of minerals and other water-soluble compounds are present in lignocellulosic biomass. These water-soluble nonstructural components are a mixture of nonstructural sugars, inorganic matter, nitrogenous compounds, or other minor materials.[12] Although they make up

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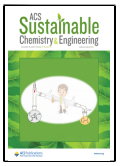

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a small fraction of lignocellulose, their contribution to bioconversion should not be overlooked.[13,14] A number of studies have surveyed the individual effects of insoluble lignin,[15,16] soluble phenolics,[17−19] and inorganic constituents[14] in pretreated lignocellulose on enzymatic saccharification, but there has been limited research that comprehensively integrates these interwinding factors to examine their overall impact without pretreatment.

An understanding of native aqueous extractives may also play a pivotal role in enzyme-mediate processing of biomass prior to pretreatment such as enzymatic liquefaction, which takes advantages of lignocellulolytic enzymes to turn high concentrations of unpretreated biomass solids in water into a slurry with desired rheological properties enabling flow.[20,21] The formation of flowable slurries allows biomass to be transported into a pretreatment reactor using a pump and moved more easily between unit operations, offering the potential to address bottlenecks in solid handling and feeding within a biorefinery.

To better understand and handle aqueous extractives in raw lignocellulosic materials, we herein report the characteristics of extractives in pelleted corn stover and their impact on enzymatic hydrolysis. The chemical composition of corn stover pellets was first examined followed by the preparation of extract and extractive-free stover (EFS), respectively. Various components in the extract potentially contributing to enzyme performance were evaluated for enzymatic hydrolysis of either EFS or microcrystalline cellulose (MCC) or both. These results combined with the impacts of representative phenolics on hydrolysis give new insights into the basic mechanisms by which enzymes interact with soluble inhibitors and insoluble lignin.

2. MATERIALS AND METHODS

2.1. Materials. Pelleted corn stover, generated from a Bliss commercial ring die pellet mill (200B-350, 6 mm die hole diameter, length-to-diameter ratio of eight), was obtained from Idaho National Laboratory, as documented elsewhere.[20] Specifically, corn stover (Pioneer P0157 AMX cultivar) was harvested in Poweshiek County, Iowa, in the fall of 2017.

The pure cellulosic substrate used for enzymatic hydrolysis is Avicel PH-101 MCC. Avicel and all other chemicals and reagents, unless otherwise mentioned, were sourced from Sigma-Aldrich (St. Louis, MO).

Commercial enzyme Celluclast 1.5 L (52 FPU/mL) and β-glucosidase Novozyme 188 (399 IU/mL) were purchased from Sigma-Aldrich. Multifect Pectinase (xylanase, 12 mg protein/mL[22]) was obtained from Genencor, Danisco Division (Palo Alto, CA).

2.2. Preparation of Extract and EFS. Extract used for enzymatic hydrolysis of different substrates was created through the immersion of untreated corn stover pellets in deionized (DI) water with a solid loading of 30% (w/v) at 80 °C. Following a 30-min interval, the resulting slurry was centrifuged (Avanti J-30I, Beckman Coulter inc., Fullerton, CA) at 15,000g for 10 min followed by a filtration of the supernatant with Whatman No. 1 filter paper to obtain the extract.

To prepare EFS samples, corn stover pellets were initially milled using a Wiley mill until they passed through a 20-mesh screen, and then exhaustively Soxhlet extracted with water, as previously described.[12]

2.3. Removal of Phenolics. Phenolics were removed by contacting extract (from 30% (w/v) corn stover slurry) with activated carbon (AC).[23] Mixtures in Erlenmeyer flasks were incubated in a New Brunswick Innova 44 incubator shaker (Eppendorf North America, Hauppauge, NY) at 25 °C and 200 RPM for 24 h followed by 10 min centrifugation at 20,000g. A nylon syringe filter (0.22 μm) was used for further filtration of the supernatant, which was then stored in a freezer until use. The pH of these solutions is around 4 (original pH of the extract), and no further adjustment was made for the adsorption experiment.

2.4. Prehydrolysis of Oligosaccharides. To assess the role of oligosaccharides in enzymatic hydrolysis, oligomeric sugars in the extract were converted into the monomeric form by enzymatic hydrolysis with specific enzymes. Briefly, 10 mL of extract was mixed with 57 μL of Celluclast 1.5 L, 15 μL of Novozyme 188, and 152 μL of Multifect Pectinase[24] under pH 4.8. After 24 h of incubation at 50 °C in an agitated incubator set at 200 RPM to allow adequate hydrolysis, the mixture was then subjected to a 10 min boiling treatment to deactivate the enzymes before further use.

2.5. Analytical Methods. *2.5.1. Compositional Analysis.* The NREL-established analytical procedures were employed to analyze the chemical composition of pelleted corn stover. Quantification of sugars and acetic acid was conducted using high-performance liquid chromatography (HPLC), as described previously.[25]

For the quantification of total sugars including monomers and oligomers in the extractives, the extract was subjected to acid hydrolysis (4% H_2SO_4, 121 °C, and 1 h), and the resulting hydrolysate was analyzed using HPLC with an Aminex HPX-87H column.[26]

2.5.2. Elemental Analysis. The inorganic components of corn stover pellets were analyzed through an elemental analysis. Briefly, 0.5 g of sample was combined with 10 mL of nitric acid and 2 mL of hydrochloric acid and digested at 210 °C for 15 min under microwave heating with an additional temperature ramp time of 21 min. The resultant digest filtrate was analyzed on an inductively coupled plasma atomic absorption spectrometer (ICP-AES) Optima 4300 Dual View (Perkin-Elmer, Waltham, MA) to quantify the constituents of major mineral elements. It should be noted that not all of the silicates can be dissolved in this digestion process, and the ICP-AES determines only the soluble silicates. The insoluble silicates, which are undissolved materials after digestion, were determined gravimetrically. This type of silicate was measured by the filtration of residues after microwave digestion and drying at 105 °C, assuming that all solids except silicates are completely digested.

2.5.3. Total Phenolic Measurement. Total phenolics in various solutions were detected according to Folin-Ciocalteau (FC) procedure[27] by measuring the absorbance at 765 nm with a UV−vis spectrometer (GENESYS 50 UV−vis, Thermo Scientific, USA). The data were expressed in terms of gallic acid equivalents (GAE) with measurement units of mg/L.

2.5.4. Soluble Protein Determination. Total Kjeldahl nitrogen (TKN) was measured to represent the amount of protein assuming the nitrogen to protein factor being 6.25.[28] TKN in extracts was determined using simplified TKN (s-TKN) TNTplus vial test kits in combination with the employment of a Hach DRB 200 block digester and subsequent analysis through a Hach DR6000 spectrophotometer (Hach, Loveland, CO).

2.5.5. Enzyme Activity Assays. Cellulase and β-glucosidase activity measurement was conducted under pH 4.8 condition using 50 mM citrate buffer at 50 °C in triplicates following published protocols.[18,29] Cellulase assays Whatman No. 1 filter paper strip and 4-nitrophenyl β-D-glucopyranoside were employed as substrates for determining the activities of cellulase and β-glucosidase, respectively. Cellulase activity was quantified in terms of filter paper units (FPU). The international unit (IU) was used for the β-glucosidase activity. One unit refers to the enzyme quantity necessary to release 1 μmol of product per minute following specified conditions.

2.6. Enzymatic Hydrolysis. Enzymatic hydrolysis of MCC or nonpretreated stover EFS was carried out at 50 °C and 200 RPM using a New Brunswick Innova 44 incubator shaker (Eppendorf North America, Hauppauge, NY). Commercial enzymes were used for the hydrolysis in a 25 mL Erlenmeyer flask, employing 10 mL of citrate buffer or extract at a cellulose or glucan loading of 5% (w/v). Enzymatic hydrolysis experiments were conducted with both Celluclast 1.5 L individually and a mixture of Celluclast 1.5 L and Novozyme 188. A dose of 6 FPU Celluclast 1.5 L per gram of cellulose was applied for MCC, whereas for EFS, it was 6 FPU/g

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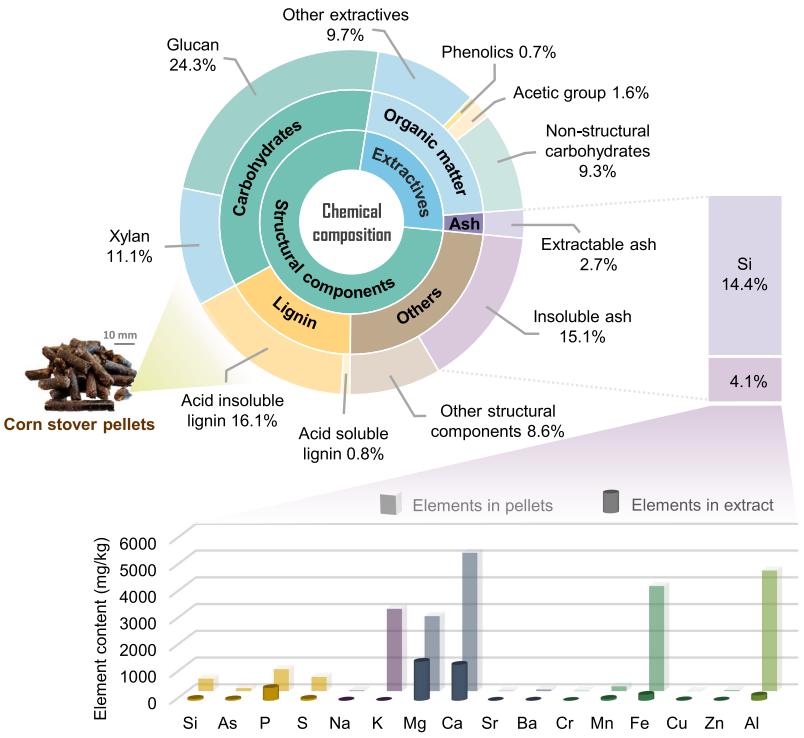

Figure 1. Chemical composition of corn stover pellets.

glucan, and the Novozyme 188 loading (in IU) for involved experiments was twice the dosage of Celluclast 1.5 L dosage (in FPU). The hydrolysates were sampled after 72 h of incubation for sugar concentration analysis.

All extracts were also subjected to enzymatic hydrolysis using the same procedure, except for the MCC or EFS addition. The detected glucose content in the hydrolysate was used as a blank to determine the cellulose or glucan conversion, which was calculated from the net glucose liberated from the cellulose or glucan after subtracting that from the extracts.

EFS was also enzymatically hydrolyzed by incorporating bovine serum albumin (BSA). Prior to enzyme addition, the mixture containing EFS and citrate buffer was preincubated with 10 g/L BSA at 25 °C and 200 RPM for 1 h.[30] Subsequently, enzymes were added to initiate the enzymatic hydrolysis process.

2.7. Statistical Analysis. The statistical analysis involved performing an analysis of variance (ANOVA) using IBM SPSS Statistics 28, with a significance level of $P < 0.05$, to evaluate the significance of the results.

3. RESULTS AND DISCUSSION

3.1. Compositional Characteristics of Corn Stover Pellets. Biomass composition has a profound impact on conversion due to its heterogeneous nature. As depicted in Figure 1, carbohydrates and lignin constitute a major portion of corn stover pellets, representing about 45 and 17% of the dry weight, respectively. The carbohydrate content of corn stover found here is slightly lower than that previously reported.[31,32] Many factors could account for the compositional variability including feedstock species, growing conditions, and physiological state of harvested materials.[14] As the dominant composition of lignocellulosic biomass, carbohydrates are generally categorized into structural and nonstructural components, distinguished by their solubilities in water and other solvents. Structural carbohydrates in corn stover pellets are high-molecular-weight polysaccharides made up of 24% glucan and 11% xylan. These carbohydrate contents are marginally lower than the data from the Idaho National Laboratory (INL)'s Bioenergy Feedstock Library, which reports glucan content in corn stover ranging from 24.54 to 37.61% and xylan levels between 14.60 and 23.56%.[33] Although the majority of carbohydrate portion in corn stover is composed primarily of structural carbohydrates embedded in the cell wall matrix, approximately 9% of nonstructural carbohydrates also exist (Figure 1 and Table S1), which are possibly extracted or washed away. These nonstructural carbohydrates together with structured carbohydrates are the main source of fermentable sugars.[34]

It is noticeable that there is a large amount of inorganic material, namely, so-called ash in the sampled corn stover pellets (18%), with 15.1% insoluble and 2.7% extractable. Insoluble ash can be either structural inorganic compounds that are tightly bound in the solid framework of corn stover or, more likely, extraneous material like soil that was picked up with the stover upon harvest and incorporated into the pellets. Similarly, extractable ash may originate from either corn stover or any soluble substance, such as soil or fertilizer, remaining in pellets. It is also worth noting that the ash present in corn stover pellets analyzed here is markedly higher relative to that commonly documented in previous research.[9,32,35] The considerably high ash content has been explained by the rainy growth conditions and the soil entrainment during the harvest of corn stover.[20] This, in turn, is a reason for the low carbohydrate content observed. Similar variability in composition was noted in other work.[36] Additionally, the INL's Bioenergy Feedstock Library also states a large variation of the ash content of corn stover from 2.29 to 26.93%.[33] Given the potential for equipment wear due to abrasive ash minerals, mechanical separations appear as a viable option for removing high levels of introduced ash content from biomass prior to processing such as pelleting.[37]

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Considering the significant proportion of ash, we further analyzed the mineral constituent and content of corn stover pellets and their extracts. Microwave wet ashing (acid digestion) of corn stover pellets was conducted as a preparation for mineral analysis to simultaneously dissolve minerals and remove organic material via oxidation. Unfortunately, the applied conditions result in noticeable undissolved residues, which are considered silicates and may dissolve after the addition of harsher hydrofluoric acid. While wet digestion with hydrofluoric acid is efficient for silicate dissolution, it was not employed here since it is extremely dangerous to work with and has the potential to corrode analytical instruments, and instead, a gravimetric method was used to individually determine the undissolved silicates. As Figure 1 shows, Si tops all the minerals with over 14% of the total weight of dry corn stover pellets. Such a large amount and most of the insoluble silica likely originates from the soil adhered during harvesting.[38] It should be noted that the abundant silica could not be solely ascribed to introduced ash since it can also be the structural component in plants forming a rigid microstructure to support their tissue structure.[39] This type of bound silica in biomass comes from the uptake of mineral salts from soil into plants as they grow and has been documented previously,[40] where the ash of thoroughly washed corn stover was found to have a similar composition with soil. The rest of the minerals are dominated by potassium, magnesium, calcium, iron, and aluminum, in which Mg and Ca also exist in large amounts in the extract. It is understandable that Mg is partially dissolved in water as it is easy to form soluble compounds.[40] However, a similar mineral K which is in the form of water-soluble salts in biomass is rarely observed in the extract. This observation implies that most of K presents as physiological ash incorporated into the lignocellulosic structure by binding within the cells and cell walls so that it is resistant to washing.[41]

In addition to nonstructural carbohydrates and extractable ash, extractive components in corn stover pellets also include 1.6% acetyl groups, 0.7% phenols, and a certain amount of unknown compounds such as organic acids. These extractives together account for up to 24% of the total mass of the total corn stover pellets, and thus could play a significant role in the bioconversion of stover by potentially influencing its resistance to enzyme attack and/or thermochemical pretreatments.[14]

3.2. Role of Extractives in Enzymatic Hydrolysis of Corn Stover Pellets. To clarify the influence of extractives in corn stover pellets on enzymatic hydrolysis, EFS without any pretreatment, prepared by exhaustive Soxhlet extraction of milled corn stover pellets, was enzymatically hydrolyzed with 6 FPU Celluclast 1.5 L/g glucan in the presence of either citrate buffer or extract isolated from 30% corn stover slurry. The effect of β-glucosidase (Novozyme 188, 12 IU/g glucan) supplementation was also studied. Figure 2 clearly shows that both enzymes and extractives affect the conversion of glucan. Novozyme 188 supplemented to Celluclast 1.5 L significantly improves the glucan conversion both in citrate buffer and in extract ($P < 0.01$). Specifically, the glucan conversion nearly doubled from 12 to 20% upon adding Novozyme 188 to the citrate buffer. β-glucosidase is a key enzyme that catalyzes the release of glucose by converting cellobiose, which has been identified as a strong inhibitor to cellulase hydrolysis.[19,23] Novozyme 188 contains a significant amount of β-glucosidase (399 IU/mL). Therefore, a remarkable improvement of glucan conversion with β-glucosidase addition indicates that cellobiose inhibition dominates these reactions.[42] This is supported

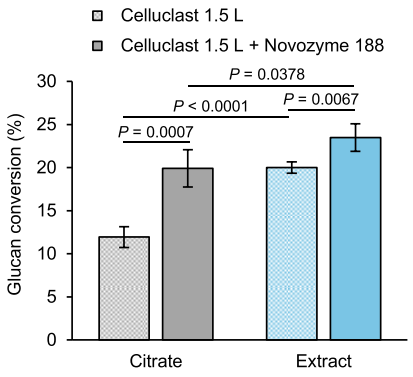

Figure 2. Enzyme-mediated hydrolysis of nonpretreated EFS optionally in the presence of the extract. Reaction conditions: 1.8 g of EFS, 6 FPU Celluclast 1.5 L (and 12 IU Novozyme 188) per g of glucan, 10 mL of solutions, pH 4.8, 50 °C, 72 h. Averages of four replicates are displayed in columns with error bars (standard deviations). The *P* values show the results from pairwise comparison of one-way ANOVA tests.

by the remarkable reduction or even disappearance of cellobiose present in the reaction mixture upon the addition of Novozyme 188 as seen in the HPLC chromatograms (Figure S1). A greater improvement in the citrate buffer reveals that the inhibitory effect of cellobiose is more pronounced in the absence of extractives.

Surprisingly, the results presented in Figure 2 also show that extractives enhance glucan conversion irrespective of whether β-glucosidase is supplemented or not. Up to 67 and 15% relative increases are apparent for Celluclast 1.5 L alone and Celluclast 1.5 L with Novozyme 188, respectively. This observation is opposite to the trend in previous studies that removing water-soluble extractives favors enzymatic saccharification.[14,43] The natural extractive compounds found here and before likely exhibit variances in composition, which could account for the observed differences. Also noteworthy is that the previously reported extractives have undergone various pretreatments, which could lead to detrimental modifications that impair enzymatic hydrolysis.[22] Additional studies on extractives were therefore performed to better understand these results.

3.3. Role of Extractive Components in Enzymatic Hydrolysis. *3.3.1. Inorganic Compounds.* As the dominating constituent of extractives, ash has been recognized as a critical factor affecting enzymatic hydrolysis.[14,40] Its effects on enzymatic hydrolysis were surveyed by hydrolyzing EFS with a citrate buffer in the presence of representative metal chloride salts. Experiments were carried out with four major metal chlorides including $MgCl_2$, $CaCl_2$, $FeCl_3$, and $AlCl_3$ as well as a mixture of them, which were added to the citrate buffer in amounts similar to those existing in extract. It can be found in Figure 3a that the impacts of mineral salts on enzymatic hydrolysis depend on both the metal ions and enzyme types. Although the addition of selected metal ions can slightly improve the glucan conversion catalyzed by Celluclast 1.5 L, it has little effect in the case where supplementing with Novozyme 188, or even reduces the glucan conversion to some extent. These results suggest that minerals have variable influences on different enzymes, in line with prior studies.[44] It should be pointed out that the mineral mixture is remarkably detrimental to enzymatic hydrolysis by combining Novozyme 188 with Celluclast 1.5 L. This detriment is likely due to the

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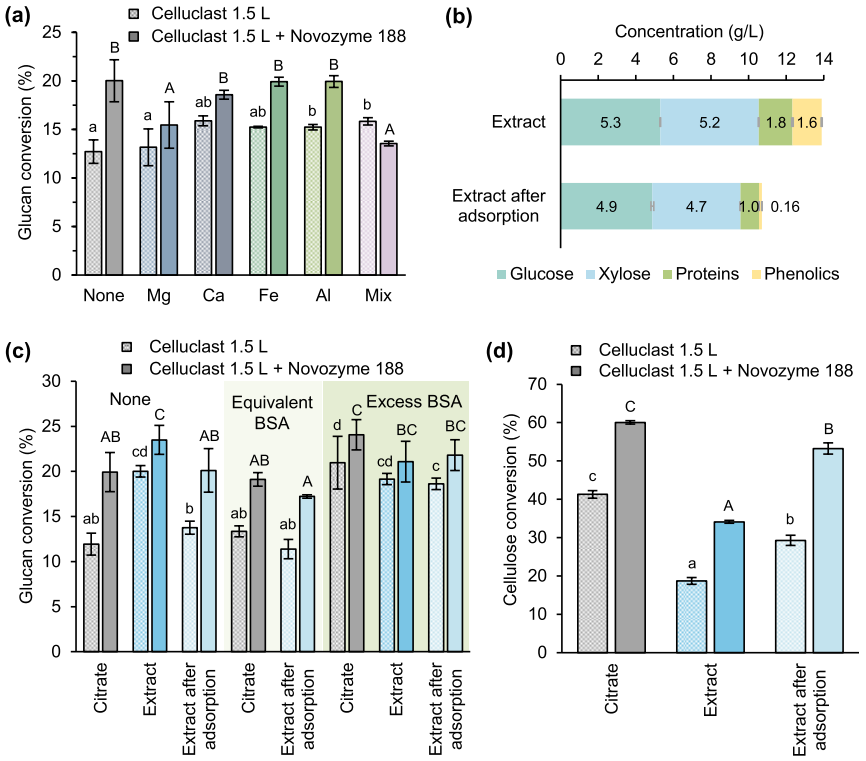

Figure 3. Impact of extractive components. (a) Enzymatic hydrolysis of EFS in the presence of minerals. (b) Major compounds detected in extract before and after adsorption using activated carbon. (c) Enzymatic hydrolysis of EFS in various extracts and with BSA addition. (d) Enzymatic hydrolysis of MCC in different extracts. Averages of triplicate are displayed in columns or bars with standard deviation error bars. Different lowercase letters represent statistically significant differences with Celluclast 1.5 L alone and different capital letters indicate statistically significant differences with Celluclast 1.5 L plus Novozyme 188 at $P < 0.05$ by one-way ANOVA with Duncan test.

synergy of various metal ions and enzymes, resulting in the generation of detrimental substances that affect enzyme performance.

Given the limited beneficial or even adverse effects of the inorganic compounds examined (Figure 3a), the organic fractions of extractives were further assessed to account for their significant benefits for enzymatic hydrolysis. As illustrated in Figure 1, organic materials in the extract have complex compositions including nonstructural sugars, water-soluble proteins, phenolics, and many other unknown components that have been reported previously.[45] With the purpose of assessing their respective influence on enzymatic conversion, we performed the selective removal of specific compounds via activated carbon adsorption. The dark brown color of the raw extract turned pale yellow after adsorption (Figure S2). Superior yields are observed for hydrolysis in the presence of the activated carbon treated extract, which removes about 44% of the soluble proteins and 90% of the phenolic compounds at a loading of 10% (w/v). This observation is consistent with prior reports where the maple pretreatment liquid's phenolics were effectively eliminated by activated carbon with a removal rate of 98%.[23] Another consideration in evaluating a proper adsorbent for removing phenolics is that the adsorption process should avoid and/or minimize the loss of sugars.[46] It can be seen from Figure 3b that most of the major sugars can be retained after adsorption. These results prove that activated carbon demonstrates effectiveness as a highly selective adsorbent for adsorbing phenolics from the extract while removing some soluble proteins.

3.3.2. Soluble Proteins. Noncatalytic proteins are known to play a critical role in the enzymatic saccharification of lignocelluloses, minimizing the nonspecific adsorption of the catalytic enzymes on lignin.[9,16,30] Figure 3c shows a substantial reduction in EFS glucan conversion from 20% in the presence of the untreated extract to 14% in the extract after activated carbon adsorption for Celluclast 1.5 L, confirming the beneficial effects of some extractives. A similar trend is also noticed with the supplemented addition of Novozyme 188. The reduced glucan conversion is likely related to the partial removal of soluble proteins with adsorption (Figure 3b), which are also essential constituents of plant biomass.[28] For a better understanding of how these proteins affect enzymatic hydrolysis, we added equivalent amounts of BSA into citrate buffer and extract after adsorption to achieve the total protein amount equal to that in untreated extract. The glucan conversion in citrate buffer with 1.8 g/L of BSA supplementation reached 13 and 19% without and with Novozyme 188, respectively, compared with 12 and 20% without BSA. However, addition of BSA in extract after adsorption to levels in the untreated extract did not increase the glucan conversion, and even lower conversion was observed, especially in the presence of Novozyme 188 (Figure 3c). In contrast, excessive addition of BSA (10 g/L) could significantly improve the glucan conversion in both citrate buffer and extract after adsorption. These results reveal that the soluble proteins existing in the extract have no significant effect on enzymatic hydrolysis.

3.3.3. Phenolic Compounds. Phenolics, mainly derived from the pretreatments, have been reported as the primary inhibitors and/or deactivators for enzymatic conversion of lignocellulose.[18,19] A certain amount of them can also be observed in the extract of corn stover pellets without any

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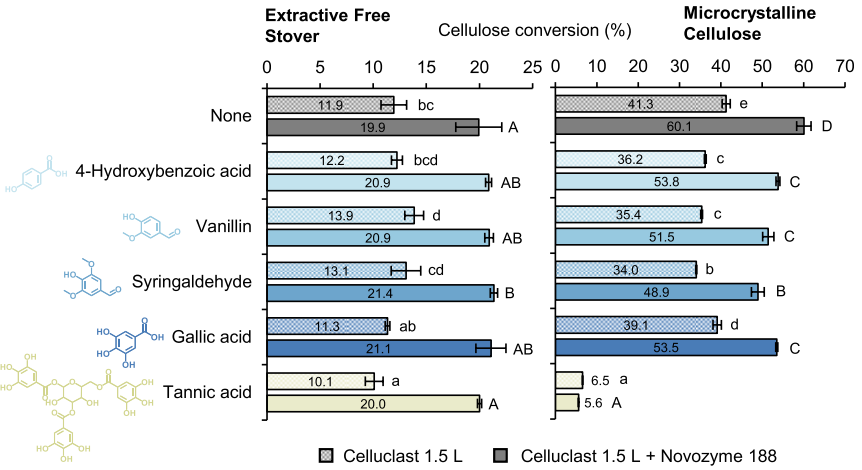

Figure 4. Effect of various phenolic compounds on the enzymatic hydrolysis of EFS (left) and MCC (right). Phenolic concentrations are all 1.5 g/L based on the amount of total phenolics existing in extract. Averages of triplicates are displayed in bars with standard deviation error bars. Different lowercase letters represent significant differences with Celluclast 1.5 L alone and different capital letters indicate significant differences with Celluclast 1.5 L plus Novozyme 188 ($P < 0.05$, one-way ANOVA with Duncan test).

pretreatment (Figure 1). Recently, their promotional roles in enzymatic hydrolysis have been highlighted in recent papers.[47,48] Figure 3b,c indicates that phenolic removal did not improve the glucan conversion; rather, it decreased from 20 to 14% and from 23 to 20% with Celluclast 1.5 L individually and in combination with Novozyme 188, respectively. The significant reduction after removal of phenolics suggests that phenolic compounds in extract may have a beneficial effect on EFS conversion. To gain more insights into the influence of phenolics in extract on enzyme performance, enzymatic hydrolysis of MCC, an indirect measure of enzyme activity, was conducted in citrate buffer and extracts before and after adsorption to avoid the influence of other components in lignocellulosic materials (Figure 3d). The extractives, which enhance the conversion of EFS, substantially reduce the MCC conversion relative to that in citrate buffer. A comparison of Figure 3b,d demonstrates that removing phenolic compounds alleviates the reduction. When the extract was treated with 10% activated carbon, which removes 90% of phenolics, the reduction in cellulose conversion is largely restored to similar levels as observed in citrate buffer (Figure 3d). These results suggest that phenolics are the primary factor negatively influencing enzyme activity, consistent with prior studies.[18,19,23]

Taking into account the divergent influence of such phenolics on the enzymatic hydrolysis of pure cellulose and lignocellulosic material, the chemical composition of feedstocks appears to be influential in determining the enzyme performance. As the major constituents of lignocellulose biomass, lignin not only functions as a physical barrier against enzyme access to polysaccharides but also interferes with the enzymatic hydrolysis through nonproductive binding of enzymes.[9] This is demonstrated by enzymatic hydrolysis of EFS amidst elevated levels of BSA, where adding excessive BSA results in a significant increase in glucan conversion in citrate buffer and extract after adsorption (Figure 3c), demonstrating that nonproductive binding of enzymes to lignin may be the dominating factor in the loss of enzyme activity and reduction in conversion yield. Moreover, it can be deduced that the introduction of nonspecific proteins represents an attractive means of improving enzyme performance. As an extension of the behavior of nonspecific proteins, enhancement in the enzymatic hydrolysis of EFS induced by phenolics could be rationalized from the assumption that phenolics may work as nonspecific additives to reduce the nonproductive adsorption of enzymes on lignin. This is supported by an earlier study in which solvent-extractable lignin was supposed to alleviate the adverse effect of residual bulk lignin by diminishing its nonproductive binding to cellulase.[49]

To validate the impact of phenolics on enzymatic hydrolysis, selected phenol model compounds were used for the hydrolysis of EFS and MCC. As displayed in Figure 4, most of the examined model phenolics show a slight increase in the conversion of EFS, regardless of the presence or absence of Novozyme 188. Although the glucan conversion with Celluclast 1.5 L alone decreases slightly after the addition of gallic acid and tannic acid, the differences are not substantial. These findings provide evidence for the potential advantageous role of phenolics in the enzymatic hydrolysis of EFS, depending on the specific phenolic compounds present. In contrast, after 72 h hydrolysis of MCC, all phenolics studied tend to adversely affect cellulose conversion to different extents, coinciding with the improved cellulose conversion after removing phenolics, as seen in Figure 3d. Notably, a substantial decrease in cellulose conversion is noticed after adding tannic acid, which was also found in a previous study that tannic acid inhibited cellulase activities far more strongly than the other phenols tested.[18] Contrary to expectations, this decrease is more pronounced with the supplementation of Novozyme 188, and the cellulose conversion is even lower than that without Novozyme 188. It is likely that the accessory enzymes in Novozyme 188 such as feruloyl esterase[50] could catalyze the degradation of tannic acid into smaller phenolic compounds, which in turn profoundly inhibit the enzyme activities. Comparison of the impact of these model phenolics on the enzymatic hydrolysis of EFS and MCC confirms that phenolic compounds result in diminished enzyme activities, whereas this detrimental effect is greatly minimized or even eliminated in the presence of lignin due to their potential interactions with lignin to prevent nonproductive adsorption of the cellulase.

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4. CONCLUSIONS

Extractives derived from the corn stover pellets appear to promote the enzymatic hydrolysis of extractives-free biomass. As expected, phenolic compounds dominate enzyme inhibition relative to others, such as oligosaccharides. Unexpectedly, the inhibitory effect is mitigated with EFS in which phenolics may bind to lignin, thus minimizing either nonproductive adsorption or enzyme inhibition. This is also supported by the data with the addition of the selected model phenols. This improved understanding of the roles of extractives in enzymatic hydrolysis contributes to the rational design of biomass processing strategies aimed at the cost-effective conversion of lignocellulose.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.3c04222.

Detailed chemical composition of corn stover pellets, HPLC chromatograms of various enzymatic hydrolysates, and photographs of extract before and after activated carbon adsorption (PDF)

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Notes

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■ REFERENCES

(1) Liu, Y.; Cruz-Morales, P.; Zargar, A.; Belcher, M. S.; Pang, B.; Englund, E.; Dan, Q.; Yin, K.; Keasling, J. D. Biofuels for a sustainable future. *Cell* **2021**, *184* (6), 1636−1647.

(2) Scown, C. D. Prospects for carbon-negative biomanufacturing. *Trends Biotechnol.* **2022**, *40* (12), 1415−1424.

(3) Ragauskas, A. J.; Williams, C. K.; Davison, B. H.; Britovsek, G.; Cairney, J.; Eckert, C. A.; Frederick, W. J.; Hallett, J. P.; Leak, D. J.; Liotta, C. L. The path forward for biofuels and biomaterials. *Science* **2006**, *311* (5760), 484−489.

(4) Liao, Y.; Koelewijn, S.-F.; Van den Bossche, G.; Van Aelst, J.; Van den Bosch, S.; Renders, T.; Navare, K.; Nicolaï, T.; Van Aelst, K.; Maesen, M. A sustainable wood biorefinery for low−carbon footprint chemicals production. *Science* **2020**, *367* (6484), 1385−1390.

(5) Mujtaba, M.; Fernandes Fraceto, L.; Fazeli, M.; Mukherjee, S.; Savassa, S. M.; Araujo de Medeiros, G.; do Espírito Santo Pereira, A.; Mancini, S. D.; Lipponen, J.; Vilaplana, F. Lignocellulosic biomass from agricultural waste to the circular economy: a review with focus on biofuels, biocomposites and bioplastics. *J. Clean. Prod.* **2023**, *402*, No. 136815.

(6) Himmel, M. E.; Ding, S.-Y.; Johnson, D. K.; Adney, W. S.; Nimlos, M. R.; Brady, J. W.; Foust, T. D. Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science* **2007**, *315* (5813), 804−807.

(7) Zhu, J. Y.; Pan, X. Efficient sugar production from plant biomass: Current status, challenges, and future directions. *Renew. Sust. Energ. Rev.* **2022**, *164*, No. 112583.

(8) Klein-Marcuschamer, D.; Oleskowicz-Popiel, P.; Simmons, B. A.; Blanch, H. W. The challenge of enzyme cost in the production of lignocellulosic biofuels. *Biotechnol. Bioeng.* **2012**, *109* (4), 1083−1087.

(9) dos Santos, A. C.; Ximenes, E.; Kim, Y.; Ladisch, M. R. Lignin-Enzyme Interactions in the Hydrolysis of Lignocellulosic Biomass. *Trends Biotechnol.* **2019**, *37* (5), 518−531.

(10) Petridis, L.; Smith, J. C. Molecular-level driving forces in lignocellulosic biomass deconstruction for bioenergy. *Nat. Rev. Chem.* **2018**, *2* (11), 382−389.

(11) Chen, X.; He, D.; Hou, T.; Lu, M.; Mosier, N. S.; Han, L.; Xiao, W. Structure−property−degradability relationships of varisized lignocellulosic biomass induced by ball milling on enzymatic hydrolysis and alcoholysis. *Biotechnol. Biofuels Bioprod.* **2022**, *15*, 36.
(12) Sluiter, A.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. *Determination of extractives in biomass*, 2005; pp 1-9.
(13) Tajmirriahi, M.; Karimi, K.; Kumar, R. Effects of pinewood extractives on bioconversion of pinewood. *Fuel* **2021**, *283*, No. 119302.
(14) Horhammer, H.; Dou, C.; Gustafson, R.; Suko, A.; Bura, R. Removal of non-structural components from poplar whole-tree chips to enhance hydrolysis and fermentation performance. *Biotechnol. Biofuels* **2018**, *11*, 222.
(15) Ko, J. K.; Kim, Y.; Ximenes, E.; Ladisch, M. R. Effect of liquid hot water pretreatment severity on properties of hardwood lignin and enzymatic hydrolysis of cellulose. *Biotechnol. Bioeng.* **2015**, *112* (2), 252−262.
(16) Kim, Y.; Kreke, T.; Ko, J. K.; Ladisch, M. R. Hydrolysis-determining substrate characteristics in liquid hot water pretreated hardwood. *Biotechnol. Bioeng.* **2015**, *112* (4), 677−687.
(17) Michelin, M.; Ximenes, E.; Maria de Lourdes, T.; Ladisch, M. R. Inhibition of enzyme hydrolysis of cellulose by phenols from hydrothermally pretreated sugarcane straw. *Enzyme Microb. Technol.* **2023**, *166*, 110227.
(18) Ximenes, E.; Kim, Y.; Mosier, N.; Dien, B.; Ladisch, M. Deactivation of cellulases by phenols. *Enzyme Microb. Technol.* **2011**, *48* (1), 54−60.
(19) Ximenes, E.; Kim, Y.; Mosier, N.; Dien, B.; Ladisch, M. Inhibition of cellulases by phenols. *Enzyme Microb. Technol.* **2010**, *46* (3−4), 170−176.
(20) dos Santos, A. C. F.; Overton, J. C.; Szeto, R.; Patel, M. H.; Gutierrez, D. M. R.; Eby, C.; Martinez Moreno, A. M.; Erk, K. A.; Aston, J. E.; Thompson, D. N.; Dooley, J. H.; Sharma, P.; Mosier, N. S.; Ximenes, E.; Ladisch, M. R. New strategy for liquefying corn stover pellets. *Bioresour. Technol.* **2021**, *341*, No. 125773.
(21) Serra, L. A.; da Silva Cruz, R. G.; Gutierrez, D. M. R.; Cruz, A. J. G.; Canizares, C. A. T.; Chen, X.; Mosier, N.; Thompson, D.; Aston, J.; Dooley, J.; Sharma, P.; De Marco, J. L.; de Almeida, J. R. M.; Erk, K.; Ximenes, E.; Ladisch, M. R. Screening Method for Enzyme-based Liquefaction of Corn Stover Pellets at High Solids. *Bioresour. Technol.* **2022**, *363*, No. 127999.
(22) Michelin, M.; Ximenes, E.; de Lourdes Teixeira de Moraes Polizeli, M.; Ladisch, M. R. Effect of phenolic compounds from pretreated sugarcane bagasse on cellulolytic and hemicellulolytic activities. *Bioresour. Technol.* **2016**, *199*, 275−278.
(23) Kim, Y.; Ximenes, E.; Mosier, N. S.; Ladisch, M. R. Soluble inhibitors/deactivators of cellulase enzymes from lignocellulosic biomass. *Enzyme Microb. Technol.* **2011**, *48* (4−5), 408−415.
(24) Zhong, C.; Lau, M. W.; Balan, V.; Dale, B. E.; Yuan, Y.-J. Optimization of enzymatic hydrolysis and ethanol fermentation from AFEX-treated rice straw. *Appl. Microbiol. Biotechnol.* **2009**, *84* (4), 667−676.
(25) Chen, X.; Freitas dos Santos, A. C.; Ramirez Gutierrez, D. M.; Song, P.; Aston, J. E.; Thompson, D. N.; Dooley, J. H.; Ladisch, M. R.; Mosier, N. S. Effect of pelleting on the enzymatic digestibility of corn stover. *Bioresour. Technol.* **2023**, *384*, No. 129338.
(26) Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. *Determination of sugars, byproducts, and degradation products in liquid fraction process samples*. Technical Report NREL/TP510-42623; National Renewable Energy Laboratory: Golden, CO, 2006.
(27) Waterhouse, A. L. Determination of total phenolics. *Curr. Protoc. Food Anal. Chem.* **2002**, *6* (1), I1.1.1−I1. 1.8.
(28) Chen, X.; Zhao, G.; Zhang, Y.; Han, L.; Xiao, W. Nitrogen-to-Protein Conversion Factors for Crop Residues and Animal Manure Common in China. *J. Agric. Food. Chem.* **2017**, *65* (42), 9186−9190.
(29) Adney, B.; Baker, J. *Measurement of cellulase activities*. NREL/TP-510-42628; National Renewable Energy Laboratory: Golden, CO, 2008.
(30) Azar, R.; Morgan, T.; Barbosa, M. H. P.; Guimaraes, V. M.; Ximenes, E.; Ladisch, M. Impact of protein blocking on enzymatic saccharification of bagasse from sugarcane clones. *Biotechnol. Bioeng.* **2019**, *116* (7), 1584−1593.
(31) Mosier, N.; Wyman, C.; Dale, B.; Elander, R.; Lee, Y. Y.; Holtzapple, M.; Ladisch, M. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour. Technol.* **2005**, *96* (6), 673−686.
(32) Xiao, W.; Chen, X.; Zhang, Y.; Qu, T.; Han, L. Product Analysis for Microwave-Assisted Methanolysis of Lignocellulose. *Energy Fuels* **2016**, *30* (10), 8246−8251.
(33) Idaho National Laboratory, Bioenergy Feedstock Library. Idaho National Laboratory, 2016. Available from: https://bioenergylibrary.inl.gov/Home/Home.aspx [October 10, 2023].
(34) Jørgensen, H.; Kristensen, J. B.; Felby, C. Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. *Biofuels Bioprod. Biorefining* **2007**, *1* (2), 119−134.
(35) Lyu, Q.; Chen, X.; Zhang, Y.; Yu, H.; Han, L.; Xiao, W. One-pot fractionation of corn stover with peracetic acid and maleic acid. *Bioresour. Technol.* **2021**, *320*, No. 124306.
(36) Liu, H.; Zhang, Y.; Hou, T.; Chen, X.; Gao, C.; Han, L.; Xiao, W. Mechanical deconstruction of corn stover as an entry process to facilitate the microwave-assisted production of ethyl levulinate. *Fuel Process. Technol.* **2018**, *174*, 53−60.
(37) Lacey, J. A.; Aston, J. E.; Thompson, V. S. Wear Properties of Ash Minerals in Biomass. *Front. Energy Res.* **2018**, *6*, 119.
(38) Wu, X.; Tang, W.; Huang, C.; Huang, C.; Lai, C.; Yong, Q. The effects of exogenous ash on the autohydrolysis and enzymatic hydrolysis of wheat straw. *Bioresour. Technol.* **2019**, *286*, No. 121411.
(39) Vassilev, S. V.; Baxter, D.; Andersen, L. K.; Vassileva, C. G.; Morgan, T. J. An overview of the organic and inorganic phase composition of biomass. *Fuel* **2012**, *94*, 1−33.
(40) He, Y.; Fang, Z.; Zhang, J.; Li, X.; Bao, J. De-ashing treatment of corn stover improves the efficiencies of enzymatic hydrolysis and consequent ethanol fermentation. *Bioresour. Technol.* **2014**, *169*, 552−558.
(41) Reza, M. T.; Emerson, R.; Uddin, M. H.; Gresham, G.; Coronella, C. J. Ash reduction of corn stover by mild hydrothermal preprocessing. *Biomass Convers. Biorefinery* **2015**, *5* (1), 21−31.
(42) Pallapolu, V. R.; Lee, Y.; Garlock, R. J.; Balan, V.; Dale, B. E.; Kim, Y.; Mosier, N. S.; Ladisch, M. R.; Falls, M.; Holtzapple, M. T. Effects of enzyme loading and β-glucosidase supplementation on enzymatic hydrolysis of switchgrass processed by leading pretreatment technologies. *Bioresour. Technol.* **2011**, *102* (24), 11115−11120.
(43) Franko, B.; Carlqvist, K.; Galbe, M.; Liden, G.; Wallberg, O. Removal of Water-Soluble Extractives Improves the Enzymatic Digestibility of Steam-Pretreated Softwood Barks. *Appl. Biochem. Biotechnol.* **2018**, *184* (2), 599−615.
(44) de Cassia Pereira, J.; Giese, E. C.; de Souza Moretti, M. M.; dos Santos Gomes, A. C.; Perrone, O. M.; Boscolo, M.; da Silva, R.; Gomes, E.; Martins, D. A. B. Effect of metal ions, chemical agents and organic compounds on lignocellulolytic enzymes activities. *Enzyme inhibitors and activators*, 2017, Vol. 29; pp 139−164.
(45) Chen, S.-F.; Mowery, R. A.; Scarlata, C. J.; Chambliss, C. K. Compositional analysis of water-soluble materials in corn stover. *J. Agric. Food. Chem.* **2007**, *55* (15), 5912−5918.
(46) Freitas, J. V.; Farinas, C. S. Sugarcane Bagasse Fly Ash as a No-Cost Adsorbent for Removal of Phenolic Inhibitors and Improvement of Biomass Saccharification. *ACS Sustain. Chem. Eng.* **2017**, *5* (12), 11727−11736.
(47) Yuan, Y.; Jiang, B.; Chen, H.; Wu, W.; Wu, S.; Jin, Y.; Xiao, H. Recent advances in understanding the effects of lignin structural characteristics on enzymatic hydrolysis. *Biotechnol. Biofuels* **2021**, *14* (1), 205.
(48) Ran, Q.; Li, H.; Liu, J.; Huang, M.; Zhou, Y.; Zhang, L.; Gu, L.; Jiang, Z. An effective strategy for improving the specific activity and saccharification efficiency of cellulase by pre-incubation with phenolic acids. *Bioresour. Technol.* **2022**, *346*, No. 126644.

https://doi.org/10.1021/acssuschemeng.3c04222
ACS Sustainable Chem. Eng. 2023, 11, 17616−17624

(49) Lai, C.; Tu, M.; Yong, Q.; Yu, S. Disparate roles of solvent extractable lignin and residual bulk lignin in enzymatic hydrolysis of pretreated sweetgum. *RSC Advances* **2015**, *5* (119), 97966−97974.
(50) Dien, B. S.; Ximenes, E. A.; O'Bryan, P. J.; Moniruzzaman, M.; Li, X. L.; Balan, V.; Dale, B.; Cotta, M. A. Enzyme characterization for hydrolysis of AFEX and liquid hot-water pretreated distillers' grains and their conversion to ethanol. *Bioresour. Technol.* **2008**, *99* (12), 5216−5225.

https://doi.org/10.1021/acssuschemeng.3c04222
ACS Sustainable Chem. Eng. 2023, 11, 17616−17624