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Research Article

Effect of various aromatic compounds with different functional groups on enzymatic hydrolysis of microcrystalline cellulose and alkaline pretreated wheat straw

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ABSTRACT

Low molecular aromatic compounds are detrimental to the enzymatic hydrolysis of lignocellulose. However, the specific role of their functional groups remains unclear. Here, a series of nine aromatic compounds as additives were tested to understand their effect on the hydrolysis yield of microcrystalline cellulose (MCC) and alkaline pretreated wheat straw. Based on the results, the inhibition of aldehyde groups on MCC was greater than that of carboxyl groups, whereas for the alkaline pretreated wheat straw case, the inhibitory effect of aldehyde groups was lower than that of carboxyl groups. Increased methoxyl groups of aromatic compounds reduced the inhibitory effect on enzymatic hydrolysis of both substrates. Stronger inhibition of aromatic compounds on MCC hydrolysis was detected in comparison with the alkaline pretreated wheat straw, indicating that the substrate lignin can offset the inhibition to a certain extent. Among all aromatic compounds, syringaldehyde with one aldehyde group and two methoxyl groups improved the glucan conversion of the alkaline pretreated wheat straw.

1. Introduction

The intensive consumption of fossil fuels by humans has a serious impact on global climate. Biorefining technology, which uses renewable biomass to produce chemicals, materials, and biofuels, is a crucial tool for generating sustainable alternatives to fossil energy sources (Manzanares, 2020). Lignocellulose, as an important component of biomass feedstock, has been considered as an important raw material for the production of bioethanol due to its low cost, renewability, and availability (Mabee and Saddler, 2010; Haldar and Purkait, 2020). Cellulose, hemicelluloses and lignin are the main components of lignocellulose. Among them, cellulose and hemicelluloses are important polysaccharides in the cell wall. The production of bioethanol from cellulose and hemicelluloses generally involves enzymatic hydrolysis of lignocellulose to monosaccharides, and the fermentation of monosaccharides to bioethanol (Shen et al., 2020). However, the complex assembly of lignocellulosic biomass is naturally recalcitrant material to enzymatic hydrolysis. Lignin serves as a barrier preventing the cellulase from entering the cellulose and hemicellulose. It is mainly formed by the coupling of three phenylpropanoid monolignols. The three monolignols give rise to three aromatic lignin units, guaiacyl, syringyl, and hydroxyphenyl, respectively (Yoo et al., 2020). Lignin can precipitate on the surface of

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carbohydrate and block the binding of enzyme to the cellulose and hemicellulose, causing the steric hindrance (Zhang et al., 2020). Furthermore, lignin was reported to adsorb cellulase irreversibly caused by hydrophobic and electrostatic interactions as well as hydrogen bond. Generally, the irreversible cellulase adsorption is regarded as the main reason for reducing the enzymatic digestibility of lignocellulose (Liu et al., 2016). Pretreatment is considered as an effective method for reducing the recalcitrant nature of lignocellulose by removing the lignin, thus liberating cellulose and hemicellulose for subsequent enzymatic saccharification (Chen et al., 2021; Orejuela-Escobar et al., 2021; Patel and Shah, 2021; Vignesh and Chandraraj, 2021; Banu Jamaldheen et al., 2022; Fan et al., 2022).

Recently, biological (biodegradation), physical (irradiation and milling), chemical (acid, basic and oxidation process, etc.), and physicochemical (steam explosion) pretreatment processes has been developed. Among these techniques, acid and base pretreatments are noted for their high lignin removal rate (Silveira et al., 2015; Gillet et al., 2017). However, certain promising pretreatment methods, such as acid pretreatment, have the capability to catalyze the degradation of lignin, leading to the formation of aromatic compounds. The literatures have acknowledged that various aromatic aldehydes or acids, such as vanillin, syringaldehyde, 4-hydroxybenzaldehyde, syringic acid, ferulic acid, 4-hydroxybenzoic acid, etc. were detected in prehydrolysate (Du et al., 2010; Li et al., 2014). Some literature suggests that these soluble aromatic compounds hinder the enzymatic digestion. The inhibition of aromatic compounds on enzymatic hydrolysis is because they can reduce the cellulase activity and even lead to the precipitation of cellulase (Boukari et al., 2011; Kellock et al., 2017). Pertinently, the degree of inhibition depends on the structure of the inhibitors (Ximenes et al., 2010; Zhao and Chen, 2014). The use of different pretreatment methods results in phenolic compounds with distinct structures during lignin degradation. Particularly, differences in functional groups and side chains have significant effects on the structure-activity relationship between phenolic compounds and their inhibitory effect on hydrolysis. The presence of phenolic hydroxyl groups in aromatic compounds is the primary reason for inhibitory effect on enzymatic hydrolysis. For instance, different degrees of dilute acid pretreatment of wheat straw lead to an increase in the concentration of phenolic compounds in the hydrolyzate as the pretreatment severity increases, and this increase is associated with a greater degree of enzyme inhibition (Jiang et al., 2022). Existing studies have shown a positive correlation between the presence of carbonyl groups in aromatic compounds and inhibitory effect, while there is a negative correlation between the presence of carboxyl groups and inhibitory effect (Qin et al., 2016; Oliveira et al., 2020). There is relatively limited literature on the relationship between methoxyl groups and inhibitory effect. Pan (2008) suggested that the number of methoxyl groups in phenolic compounds is not correlated with their inhibition on enzymatic hydrolysis. Qin et al. (2016) pointed out that the impact of methoxyl groups in phenolic compounds on cellulase activity depends on the presence of other functional groups within the phenolic compounds.

In studies examining the inhibitory effect of aromatic compounds on enzymatic hydrolysis, pure cellulose has been widely utilized by many researchers as the substrate for enzymatic hydrolysis (Ximenes et al., 2011; Chen et al., 2020). However, only a few studies have explored the effects of aromatic compounds on the enzymatic saccharification of lignin-containing substrates. The complexity of lignocellulose substrates and the diversity of pretreatment methods contribute to the elusive nature of the impact of aromatic compounds on enzymatic hydrolysis of lignin-containing substrates. Understanding the inhibition mechanism of functional groups of small molecular aromatic compounds on enzymatic hydrolysis of lignin-containing substrates is instrumental in avoiding the negative effect of aromatic compounds produced during lignin degradation.

The present work aims at examining the inhibitory effect of various aromatic compounds on the enzymatic saccharification of non-lignin-contained (microcrystalline cellulose) or lignin-contained substrates (alkaline pretreated wheat straw). Specifically, the comparative study using low molecular weight nine aromatic compounds as additives during enzymatic hydrolysis as to identify the effect of functional group on phenyl ring of aromatic compounds on hydrolysis yield of microcrystalline cellulose (MCC) and alkaline pretreated wheat straw (APW).

2. Materials and methods

2.1. Materials

Wheat straw stem was obtained by manually removing the leaves and sheaths from the wheat straw (*Triticum aestivum* L.) collected from Nanjing, China. Prior to use, the air-dried stem length was controlled between 3 and 5 cm; the stems were then stored in a sealed bag at room temperature. Cellic CTec2 used for cellulose hydrolysis was purchased from Novozymes (Bagsværd, Denmark). Microcrystalline cellulose (Avicel PH-101, Sigma-Aldrich, USA) was used as the pure cellulose substrate. Nine low molecular weight aromatic compounds (>99.0 %), as listed in Fig. 1, were provided by Tokyo Chemical Industry (Tokyo Japan). Analytical grade sodium acetate (99.3 %) was purchased from Nanjing Chemical Reagent (Nanjing, China).

2.2. Sodium hydroxide pretreatment

Sodium hydroxide pretreatment was carried out in stainless steel cooking equipment with 61.25 L cooking pots. The 60 g of dried wheat straw samples were put into the tank containing 8 % NaOH solution (based on oven-dry feedstock) with the liquid-to-solid ratio of 10:1. The wheat straw was preheated in the NaOH solution to 80 °C and equilibrated for 30 min. There after, the temperature was raised to 150 °C and equilibrated for 1 h. The NaOH-treated wheat straw was separated from the pretreated solution (black liquor) through a cheese cloth filtration. The pretreated wheat straw was washed with distilled water to neutrality and to remove other dissolved straw compounds. In order to prepare the substrate for subsequent experiment, the pretreated wheat straw was crushed

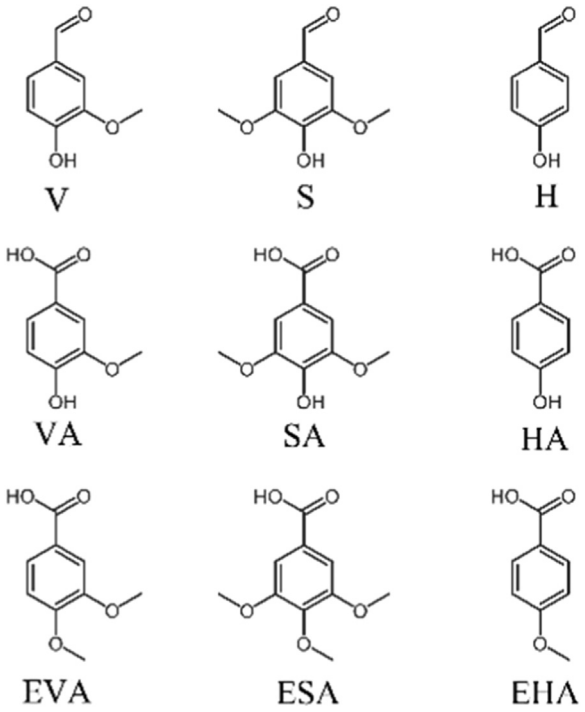

Fig. 1. Aromatic compounds used in this work, including three phenolic aldehydes: vanillin (V), syringaldehyde (S) and *p*-hydroxybenzaldehyde (H); three phenolic acids: vanillic acid (VA), syringic acid (SA) and *p*-hydroxybenzoic acid (HA); three etherified phenolic acids: 3,4-dimethoxybenzoic acid (EVA), 3,4,5-trimethoxybenzoic acid (ESA) and *p*-methoxybenzoic acid (EHA).

Table 1
Chemical composition of raw material and pretreated solids (%).

	Lignin			Carbohydrate				
Sample	Klason	Acid soluble	Total	Glucan	Xylan	Arabinan	Total	Ash
Wheat straw[a]	20.2 ± 1.2	2.1 ± 0.1	22.3 ± 1.2	40.4 ± 0.7	18.4 ± 0.8	2.8 ± 0.6	61.6 ± 0.8	6.4 ± 0.4
Pretreated wheat straw	13.5 ± 0.1	1.5 ± 0.0	15.0 ± 0.1	58.9 ± 0.6	19.2 ± 0.5	3.6 ± 0.0	81.7 ± 0.6	1.9 ± 0.1

Note: a, the benzene/ethanol extractives content is 2.5 %.

using a laboratory disk refiner (KRK, *Φ* 300 mm, Jilin, China) with the plate gap of 0.5 mm. The size-reduced pretreated wheat straw was then stored at 4 ˚C till further analysis. The chemical composition of the raw material and pretreated solids are given in Table 1.

2.3. Enzymatic hydrolysis

Enzymatic hydrolysis of pretreated wheat straw was conducted with a concentration of 2 % (*w/V*) and 50 mmol/L acetate buffer (pH 4.8) on a shaker (IS-A, Jiangsu Qixuan, China) at 50 ˚C and 180 r/min for 72 h. The reaction mixture was added with Cellic CTec2 at 20 FPU per g-cellulose, while as, 80 μg/mL tetracycline was used to prevent bacterial infections. The filter enzyme activity and protein content were 200 FPU/mL and 125.6 mg/mL, respectively. Various aromatic compounds ranged from 0 % to 16 % (based on the dry weight of the substrate) were added into the hydrolysis system. Aliquots of 0.5 mL were taken periodically (3, 6, 12, 24, 48, and 72 h) from the hydrolysate mixtures for monomeric sugar measurement. The samples were centrifuged at 10 000 r/min for 5 min (Thermo Fisher, USA) before analysis.

2.4. Enzyme activity

Cellulase activity was analyzed by incubating the enzyme in the absence and presence of aromatic compounds at 50 ˚C for 24 h. Filter paper activity (FPU/mL) was measured using the method described in a previous study (Ghose, 1987). Glucose concentration was analyzed with a dinitrosalicylic acid (DNS) method using Ultraviolet-visible (UV–Vis) spectrophotometer. The relative activity (%) was determined by comparing the measured activity at a specific time to the activity at time 0 h (Yao et al., 2022b).

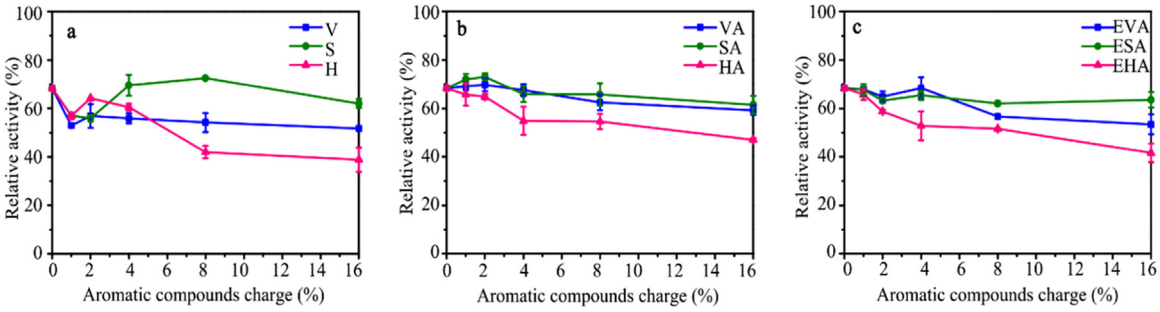

Fig. 2. Relative activity of cellulase in the presence of different aromatic compounds (V-EHA) at 50 ˚C for 24 h.

2.5. Fluorescence spectroscopic

Fluorescence measurements were carried out using a spectrofluorimeter (LS-55, PerkinElmer, Foster City, USA) with a 1 cm quartz cell. Stock solutions of cellulase were prepared with acetate sodium buffer (50 mmol/L, pH 4.8) at a concentration of 0.1 mg/mL. Fixed concentrations of cellulase and varying concentrations of aromatic compounds were allowed to react for 1 hour at room temperature.

The fluorescence emission spectra of the mixture of aromatic compounds and cellulase were recorded using an excitation wavelength of 280 nm, and the emission wavelengths ranged from 300 to 500 nm. The excitation and emission slits were set to 2.5 nm. The spectra were corrected using an acetate sodium buffer spectrum as a baseline (Zhang et al., 2013).

2.6. Analytical methods

The chemical compositions of feedstock and pretreated substrate were analyzed according to the method by Sluiter et al. (2008). The glucose in the enzymatic hydrolysate were determined using a high-performance liquid chromatographic (HPLC) system (ACQUITY Arc series, Waters) equipped with the refractive index detector (2414, Waters) using the Aminex column (HPX-87H). The temperature of column and detector were set as 55 and 45 ˚C. The mobile phase contains 5 mmol/L sulfuric acid with a flow rate of 0.6 mL/min. The sample was filtrated through 0.22 μm nylon membranes before HPLC analysis (Ling et al., 2022). The glucan conversion was calculated from the glucan in enzymatic supernatant as the percentage of that in pretreated wheat straw or MCC. The glucan was corrected by multiplying the glucose content by 0.9 (conversion coeffcient of glucose to glucan) (Zheng et al., 2020).

3. Results and discussion

3.1. Effect of aromatic compounds on cellulase activity

We investigated that the effect of three groups of aromatic compounds on enzyme activity. Specifically, it was found that low concentrations of syringaldehyde (S) inhibited the cellulase activity, but at a concentration of 8 g/L, S slightly improved the activity. The vanillin (V) and *p*-hydroxybenzaldehyde (H) showed a negative effect on the enzyme activity at all concentration. At concentrations above 4 g/L, S had almost no negative effect on enzyme activity, while V had a negative effect but to a lesser extent than H, which had the greatest inhibitory effect (Fig. 2a). The *p*-hydroxybenzoic acid (HA) had a more significant inhibitory effect on cellulase activity compared to vanillic acid (VA) and syringic acid (SA) (Fig. 2b), while *p*-methoxybenzoic acid (EHA) had a greater inhibitory effect than 3,4-dimethoxybenzoic acid (EVA) and 3,4,5-trimethoxybenzoic acid (ESA) (Fig. 2c). In addition, the VA and HA had a lower negative impact compared to their corresponding aldehyde compounds (V and H). And the low concentration of VA improved the cellulase activity. These findings suggested that carboxyl group is beneficial to the enzyme activity. Overall, functional groups of aromatic compounds, such as methoxyl groups and carboxyl groups, can play an important role in determining the impact of these compounds on enzyme activity.

3.2. Effect of aromatic compounds on fluorescence spectra of cellulase

Fluorescence spectra has been used to examine the interaction between cellulase and phenolic compounds (Tian et al., 2013). The fluorescence quenching observed in spectra indicated changes in binding strength of cellulase to these aromatic compounds. Maximum fluorescence intensity (I_{max}) was used to access conformation change of cellulase (Zhao et al., 2022). The results suggested that when the cellulase was excited by a light of 280 nm wavelength, its maximum emission wavelength was 330 nm. When the cellulase was incubated with increasing concentrations of the nine aromatic compounds, the fluorescence intensity decreased significantly. Additionally, depending on the type of aromatic compound, the maximum emission wavelength shifted either towards longer wavelengths (red shift) or towards shorter wavelengths (blue shift). The decrease of concentration-depending fluorescence intensity illustrated that there is some interaction occurring between cellulase and these compounds. Based on the observed significant drop in fluorescence intensity induced by aromatic compounds, we analyzed the effect of nine different aromatic compounds on the I_{max} of

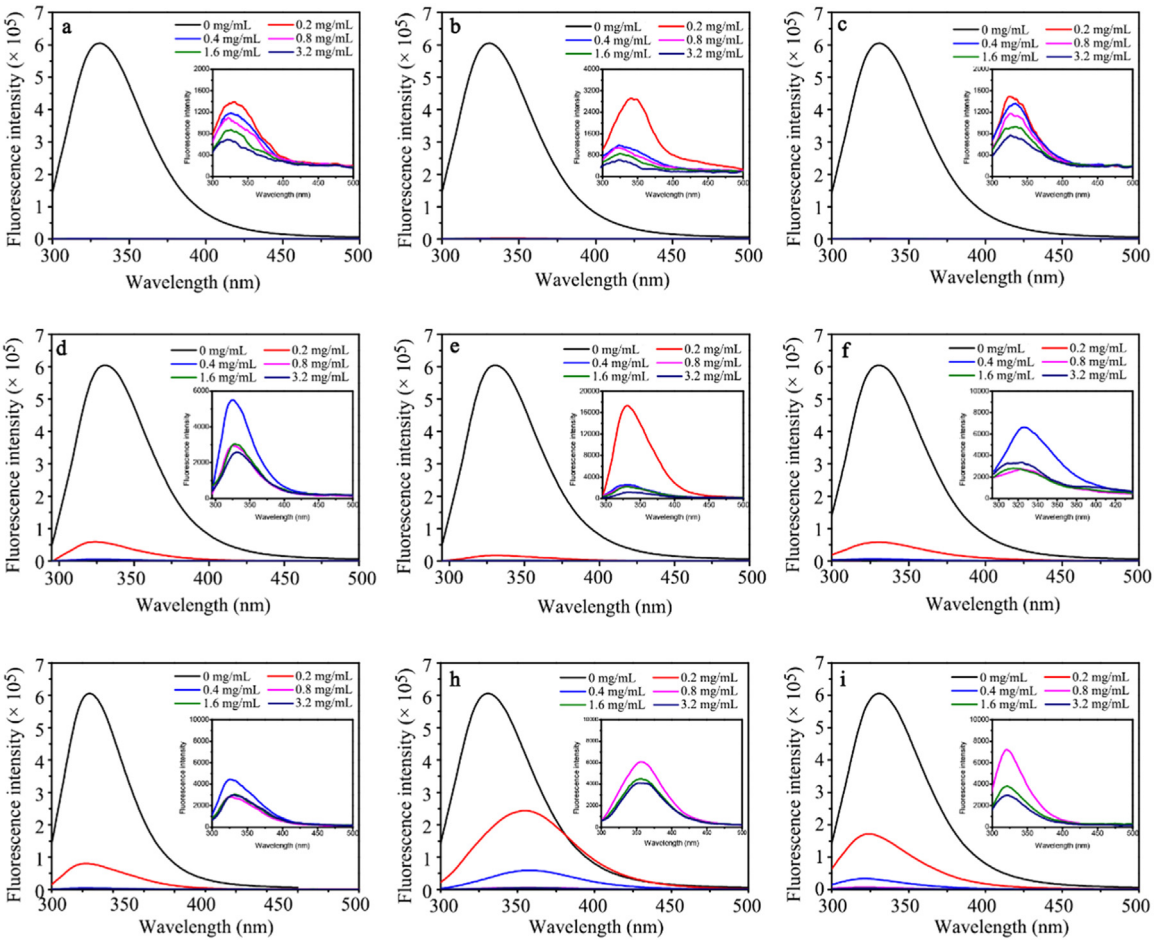

Fig. 3. Fluorescence spectra of cellulase treated with different concentrations of V (a), S (b), H (c), VA (d), SA (e), HA (f), EVA (g), ESA (h), and EHA (i). The aromatic compounds (V-EHA) concentrations in each figure are 0, 0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml. The fluorescence intensities of the aromatic compounds were disregarded.

cellulase at a minimum concentration of 0.2 g/L. The I_{max} of cellulase in V and H solution was lower compared to that in S solution which showed that interaction of cellulase with S was weaker than with V and H (Fig. 3a, b, c). Similar, the I_{max} of cellulase incubated with EVA and EHA was lower compared to that with ESA (Fig. 3g, h, i). This revealed that EVA and EHA adsorbed more cellulase than ESA. The S and ESA contained more methoxyl groups compared to their corresponding aldehyde and non-phenolic acid compounds, resulting in weaker interactions with cellulase. This weaker interaction was associated with a mildly inhibitory effect on cellulase activity. These findings support the previous view that increased methoxyl content can reduce the inhibitory effect of aromatic compounds on cellulase. On the other hand, for phenolic acid compounds (VA, SA, and HA), the I_{max} of cellulase in SA solution was lower compared to that in VA and HA solutions, indicating that SA had a strong binding capacity with cellulase (Fig. 3d, e, f). Interestingly, in our study, we found that low concentration SA could improve cellulase activity, indicating that the binding of cellulase to low concentration SA was conducive to enzymatic hydrolysis. Taking into account the effect of aromatic aldehyde/acid compounds on the I_{max} of cellulase, we observed that the aromatic aldehyde compounds exhibited a stronger interaction with cellulase. This finding confirms previous research suggesting that aldehyde groups have a greater inhibitory effect on enzyme activity than carboxyl groups.

In our study, we attempted to explain the relationship between the structure of aromatic compounds and their inhibitory effect on enzymatic hydrolysis using fluorescence spectroscopy. Therefore, we did not select single-component cellulases. However, elucidating the interaction between different components of cellulases and aromatic compounds is crucial for reducing the inhibitory effect of aromatic compounds on enzymatic hydrolysis. This requires further research in the future.

3.3. Effect of aromatic compounds on enzymatic hydrolysis of lignin-free substrate

Fig. 4 shows that the effect of nine different aromatic compounds on the enzymatic hydrolysis of MCC, which is a lignin-free substrate. The inhibitory effect of these compounds on the conversion of cellulose to glucose was measured by hydrolysis yield. All

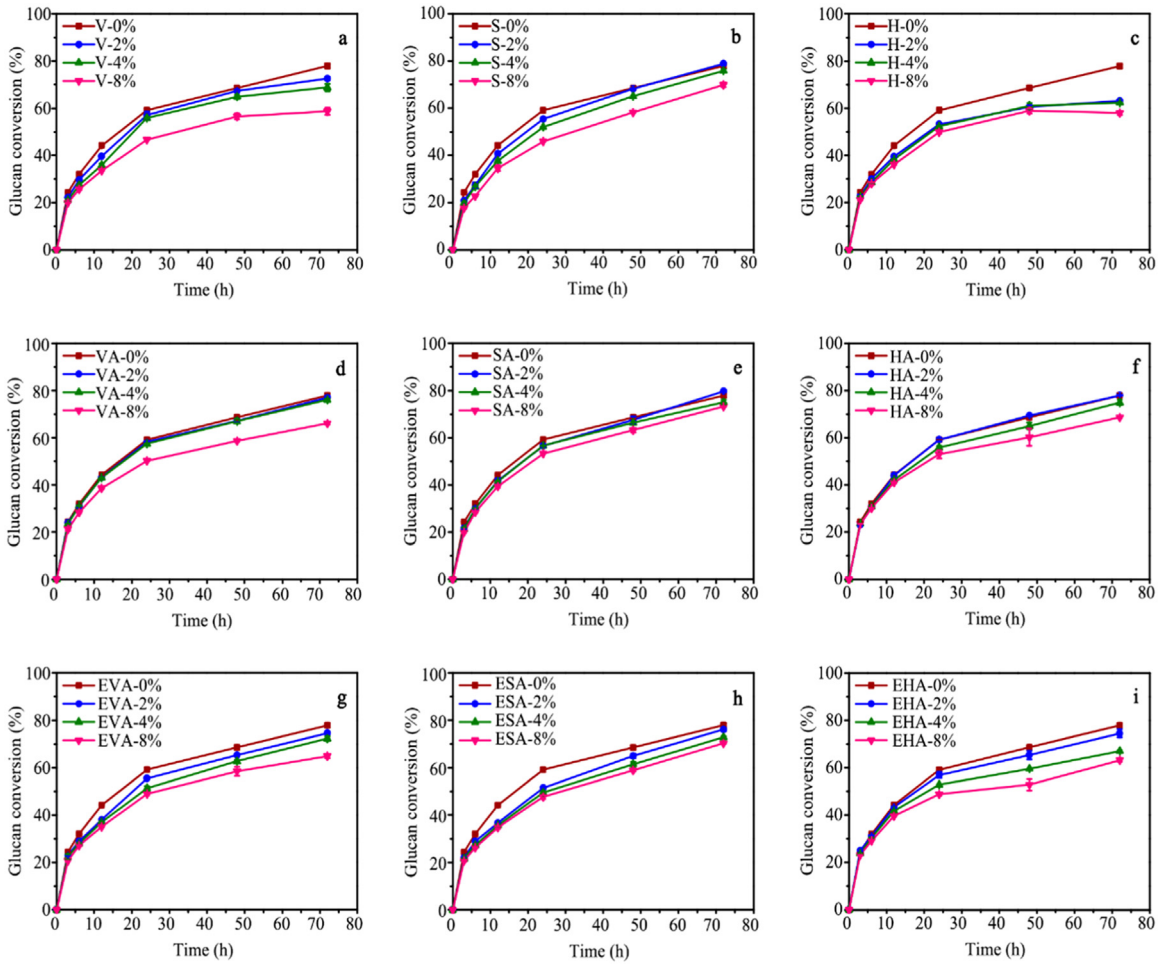

Fig. 4. Effect of aromatic compounds dosage on enzymatic hydrolysis of microcrystalline cellulose (MCC) at 72 h. Several representative concentrations (2 %, 4 %, and 8 % based on the oven dried Avicel) was selected to investigate the effect of aromatic compounds dosage. In the *x*-axis are reported the time of enzymatic hydrolysis. The sample was taken at 3, 6, 12, 24, 48 and 72 h respectively for the glucose detection. The *y*-axis shows the percentage of the amount of glucan in the enzymatic supernatant of MCC over the amount of the maximum theoretical glucan from initial MCC.

the aromatic compounds exhibited a concentration-dependent inhibitory effect on the hydrolysis of MCC. Among the compounds tested, phenolic aldehyde compounds had the strongest inhibitory effect, followed by etherified phenolic acid compounds, while phenolic acid compounds had the lowest inhibitory effect. For example, compound H showed the strongest inhibitory effect on MCC hydrolysis. When the concentration of compound H was increased to 8 %, the hydrolysis efficiency of MCC decreased from 77.9 % to 58.0 % (as shown in Fig. 4c). On the other hand, compound SA had the weakest inhibitory effect on enzymatic hydrolysis, with a smaller decrease in glucose conversion from 77.9 % to 73.2 % when 8 % SA was added (Fig. 4e). Interestingly, at low concentrations, some aromatic compounds had a slightly positive effect or no significant effect on MCC hydrolysis. For instance, the addition of 2 % compound SA slightly increased the enzymatic hydrolysis efficiency of MCC from 77.9 % to 79.7 % (Fig. 4e). The addition of 2 % compounds VA and HA did not show a significant inhibitory effect on MCC hydrolysis (Fig. 4d, f).

Similar studies have previously reported the inhibitory effects of lignin-derived aromatic compounds on cellulose hydrolysis (Jing et al., 2009; Kumar et al., 2018). Tejirian and Xu (2011) found that compound S demonstrated a concentration-dependent inhibitory effect on the enzymatic saccharification of Avicel. Zhai et al. (2018a) observed that phenolics generated from steam pretreated lodgepole pine and poplar exhibited a higher inhibition effect on the enzymatic hydrolysis of cellulose. Additionally, the addition of aromatic compounds or lignin to cellulase can lead to the formation of a complex between cellulase and the aromatic compounds/lignin, resulting in cellulase precipitation (Oh et al., 1980; Kim et al., 2011).

Literature reports have also highlighted that the phenolic hydroxyl groups present in lignin can contribute to nonproductive cellulase adsorption (Huang et al., 2016; Song et al., 2020; Xu et al., 2020). The phenolic hydroxyl groups in lignin have the ability to form hydrogen bonds with cellulase. Greater numbers of phenolic hydroxyl groups lead to stronger hydrogen bond interactions between lignin and cellulase. This increased hydrogen bonding consequently intensifies the inhibitory effect on cellulase. Moreover,

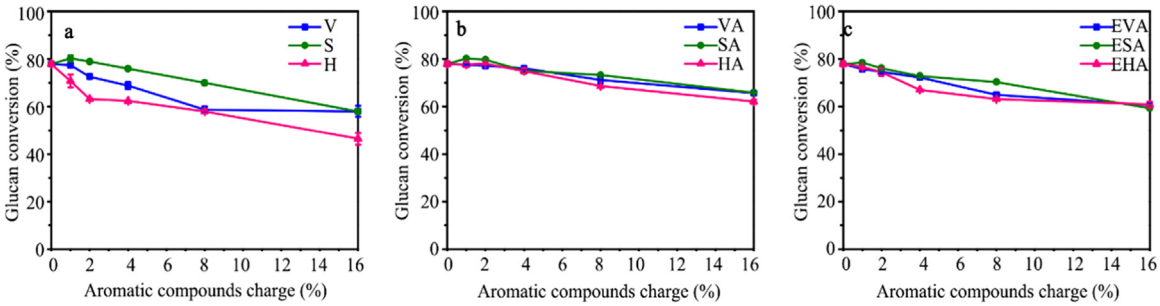

Fig. 5. Effect of the functional groups of various aromatic compounds on enzymatic hydrolysis of MCC at 72 h. In the *x*-axis are reported the addition of aromatic compounds (1 %, 2 %, 4 %, 8 %, and 16 % of oven dried Avicel). The *y*-axis shows the percentage of the amount of glucan in the enzymatic supernatant of MCC over the amount of the maximum theoretical glucan from initial MCC.

higher contents of phenolic hydroxyl groups contribute to enhanced nonproductive adsorption caused by hydrophobic interactions between the phenolic hydroxyl groups and cellulase. Therefore, the presence of phenolic hydroxyl groups derived from aromatic compounds may have a negative effect on the enzymatic hydrolysis of MCC.

The study also investigated the impact of different functional groups (methoxyl, aldehyde, carboxyl, and phenolic hydroxyl groups) present in various aromatic compounds on the enzymatic hydrolysis of MCC. Fig. 5a, b, c illustrate that the inhibitory effect among the three groups of aromatic compounds decreased as the number of methoxyl groups increased. It was observed that the compounds H, HA, and EHA exhibited greater inhibitory effects on MCC hydrolysis compared to their counterparts V, VA, and EVA, as well as S, SA, and ESA, respectively. For instance, with the addition of 16 % aromatic compounds (HA, VA, and SA), the glucan conversion of MCC was reduced by 15.9 %, 12.2 %, and 12.0 %, respectively (Fig. 5b). Furthermore, by observing the spacing between the different colored lines in Fig. 5a, b, c, it can be noted that the reduction in inhibition caused by an increase in methoxyl groups in phenolic aldehyde compounds (V, S, and H) is more pronounced than that caused by an increase in methoxyl groups in phenolic acid (VA, SA, and HA) and etherified phenolic acid compounds (EVA, ESA, and EHA). These findings align with the inhibitory effects reported by Akimkulova et al. (2016) for three aromatic compounds, namely coumaric acid, ferullic acid, and sinapic acid, on the enzymatic hydrolysis of filter paper, where the order of inhibition was coumaric acid (non-methoxylated compound) > ferullic acid (monomethoxylated compound) > sinapic acid (dimethoxylated compound). Qin et al. (2016) also suggested that higher methoxyl groups in phenolic acid-based aromatic compounds weakened the inhibitory effect on the enzymatic hydrolysis of Avicel. This effect may be attributed to the electron-donating nature of methoxy groups, which can interact with the negatively charged cellulase. Consequently, an increase in methoxy groups can enhance the electrostatic repulsion between cellulase and aromatic compounds, thereby reducing non-productive adsorption of cellulase and aromatic compounds. Additionally, the steric hindrance effect of the additional methoxy groups may also contribute to the observed experimental results.

Fig. 5a, b demonstrate the effect of aldehyde and carboxyl groups on the enzymatic saccharification of MCC. The results indicate that aldehyde groups exert a much stronger inhibitory effect on the enzymatic saccharification of MCC compared to carboxyl groups. For instance, when three different phenolic aldehyde compounds (V, S, and H) were added at a concentration of 16 %, the glucan conversion of MCC decreased by 19.9–31.4 %. On the other hand, at the same concentration, the three different phenolic acid compounds (VA, SA, and HA) led to a reduction in glucan conversion of MCC by 12.0–15.9 % (as shown in Fig. 5a, b). These findings are consistent with the stronger inhibitory effect of aldehyde group-based aromatic compounds on the enzymatic hydrolysis of Avicel compared to those containing carboxyl groups (Qin et al., 2016). In previous study by Chen et al. (2020), it was discovered that the presence of aldehyde groups in phenolic compounds generated from biomass during steam pretreatment was the primary factor responsible for their inhibitory effect on cellulose hydrolysis. However, they found that if these aldehyde groups were reduced to hydroxyl groups, the inhibitory effect was significantly reduced. Furthermore, Jiang et al. (2022) recently conducted a study to compare the inhibitory effects of phenolic compounds with different functional groups on cellulose hydrolysis and found that the degree of inhibition decreased in the order of aldehyde > carboxyl > hydroxyl.

The three pairs of aromatic compounds (VA-EVA, SA-ESA, and HA-EHA) was tested the effect of phenolic hydroxyl groups on the enzymatic saccharification of MCC. As shown in Fig. 5c, the phenolic acid compounds (VA, SA, and HA) exhibited a slightly lower inhibitory effect on the enzymatic hydrolysis of MCC compared to the etherified phenolic acid compounds (EVA, ESA, and EHA) at a concentration of 16 %. The inhibitory effect of the phenolic acid compounds ranged from approximately 1.1 % to 6.1 % lower than that of the corresponding etherified phenolic acid compounds. These results contradict some reports that suggest phenolic hydroxyl groups have a significantly higher inhibitory effect on enzymatic hydrolysis (Yao et al., 2022a). This discrepancy can be explained by considering the electron-donating ability of phenolic hydroxyl groups, which is stronger than that of methoxyl groups. As a result, the electron cloud density of the non-etherified aromatic compounds was higher than that of the etherified compounds. Since cellulase is negatively charged (Sang et al., 2022), the non-etherified aromatic compounds exhibited stronger electrostatic repulsion with cellulase compared to the etherified compounds. This led to less loss of cellulase activity. Therefore, the inhibitory effect on the enzymatic digestion of MCC increased when phenolic hydroxyl groups were substituted with methoxyl groups in aromatic compounds.

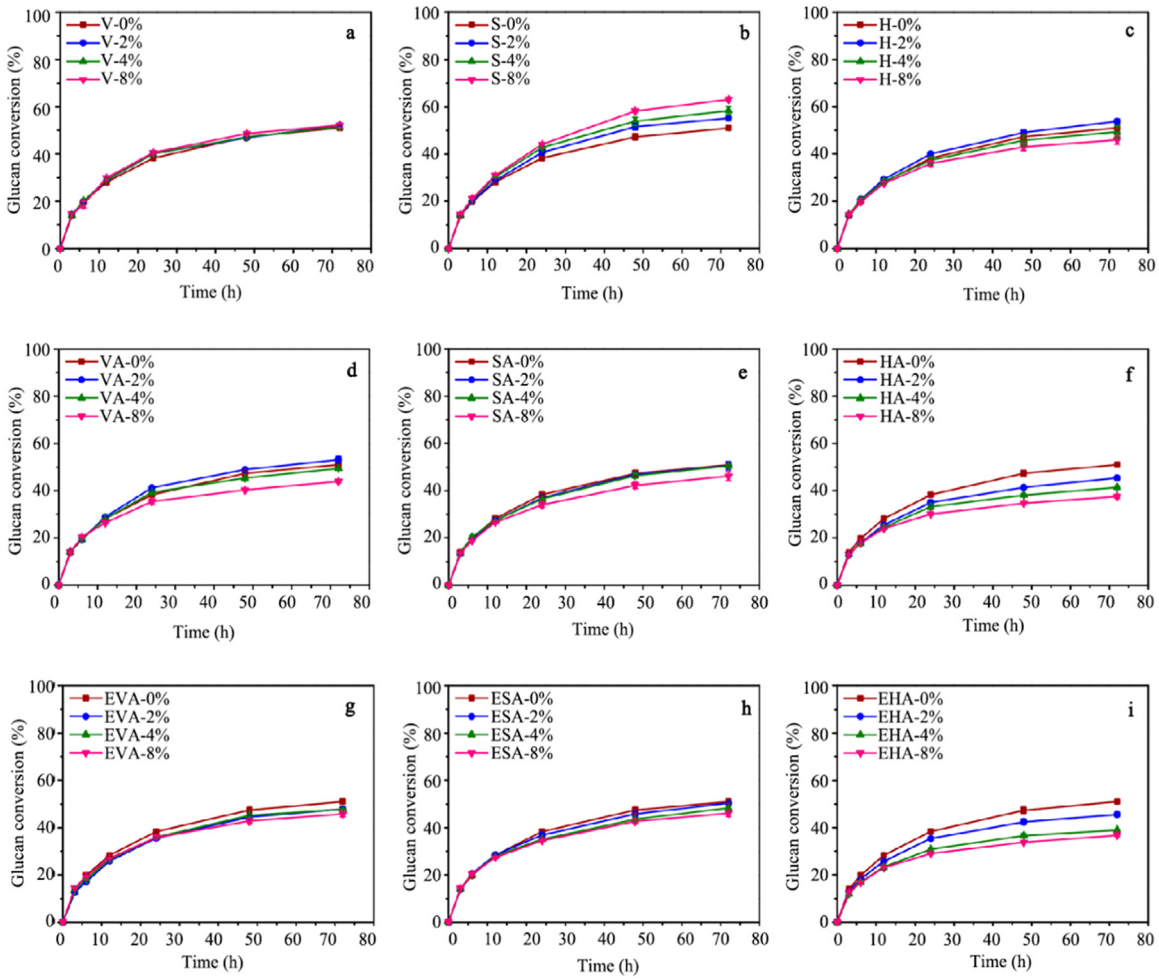

Fig. 6. Effect of aromatic compounds dosage on enzymatic hydrolysis of alkaline pretreated wheat straw (APW) at 72 h. Several representative concentrations (2 %, 4 %, and 8 % based on the oven dried APW) was selected to investigate the effect of aromatic compounds dosage. In the *x*-axis are reported the time of enzymatic hydrolysis. The sample was taken at 3, 6, 12, 24, 48 and 72 h respectively for the glucose detection. The *y*-axis shows the percentage of the amount of glucan in the enzymatic supernatant of APW over the amount of the maximum theoretical glucan from initial APW.

3.4. Effect of aromatic compounds on enzymatic hydrolysis of lignin-containing substrate

Nine aromatic compounds with distinctive functional groups were utilized to investigate their impact on the enzymatic saccharification of alkaline pretreated wheat straw (APW), a lignin-containing substrate. As depicted in Fig. 6, all of the aromatic compounds demonstrated a concentration-dependent inhibitory effect on the enzymatic hydrolysis of APW. For instance, the addition of HA in the range of 2–8 % resulted in a decrease in glucan conversion of APW from 51.0 % to 37.6 %, respectively (Fig. 6f). Additionally, the inhibitory effect of etherified phenolic acid and phenolic acid compounds on the enzymatic hydrolysis of APW was more pronounced compared to phenolic aldehyde compounds. At a higher dosage of 8 %, the respective aromatic compounds, namely H, HA, and EHA, reduced the glucan conversion of APW by 4.9 %, 13.4 %, and 14.1 % respectively. Interestingly, it was observed that the addition of 2 % H slightly increased the glucan conversion by 2.9 % (Fig. 6c). Some researchers have suggested that aromatic compounds at low concentrations could form a hydrophobic monolayer on the surface of proteins, thereby increasing the hydrophobicity of cellulase. This enhanced hydrophobicity facilitated the binding of cellulase to the substrate through hydrophobic interactions (Zhao and Chen, 2014; Kumar et al., 2018). Furthermore, compound V exhibited slightly reduced inhibition activity on the enzymatic hydrolysis of APW at all concentrations (Fig. 6a). On the other hand, the addition of 0–4 % SA did not show any significant inhibition on enzymatic hydrolysis (Fig. 6e); however, the addition of 8 % SA notably inhibited the enzymatic hydrolysis of APW.

Interestingly, the addition of compound S had a significant positive effect on the glucan conversion of APW, as shown in Fig. 6b. For instance, compared to the control experiment, the addition of 8 % S resulted in a 12.2 % increase in glucan conversion, from 51.0 % to 63.2 %. In contrast, S inhibited the enzymatic hydrolysis of MCC, as discussed in Section 3.3 (Fig. 4b). The interaction between aromatic compounds and enzymes is considered to be the main reason for their inhibitory effect on enzymatic hydrolysis.

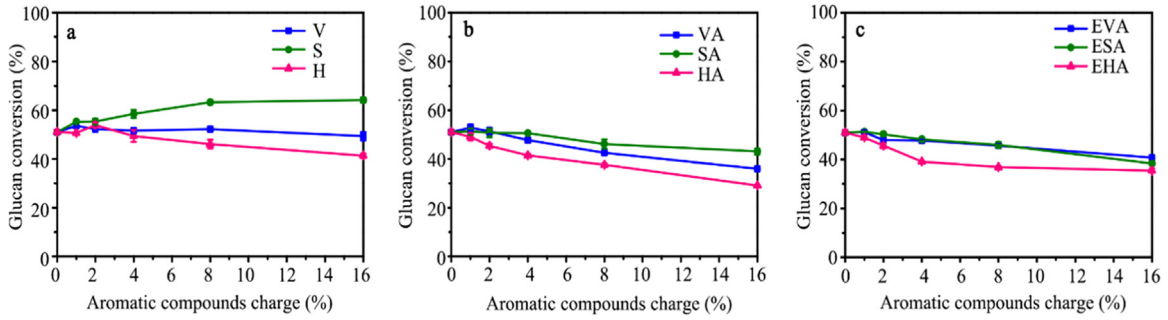

Fig. 7. Effect of the functional groups of aromatic compounds on enzymatic hydrolysis of APW at 72 h. In the *x*-axis are reported the addition of aromatic compounds (1 %, 2 %, 4 %, 8 %, and 16 % of oven dried APW). The *y*-axis shows the percentage of the amount of glucan in the enzymatic supernatant of APW over the amount of the maximum theoretical glucan from initial APW.

However, Stamogiannou et al. (2021) suggests that the interaction between aromatic compounds and substrates also has a significant impact on cellulase adsorption on the substrate. The aromatic compounds can adsorb onto cellulose, reducing substrate accessibility and blocking cellulase binding sites. Based on these findings, it is hypothesized that S may adsorb onto the substrate during the enzymatic hydrolysis process, impeding cellulase binding to cellulose. However, it may also prevent nonproductive adsorption of cellulase and substrate lignin. Therefore, it is speculated that substrate lignin plays a crucial role in the enhancement of S on the enzymatic efficiency of APW. The effect of S on enzymatic digestion might be associated with factors other than cellulase, which requires further research.

The inhibitory effect of various aromatic compounds on the hydrolysis of APW was correlated with their functional groups, as depicted in Fig. 7. In the presence of S, SA, and ESA (green lines), the glucan conversion of APW was higher compared to V, VA, and EVA (blue lines), as well as H, HA, and EHA (pink lines). Among the three phenolic acid compounds (VA, SA, and HA) tested at a 16 % addition (Fig. 7b), HA demonstrated the highest inhibitory effect on glucan conversion (21.8 %), followed by VA (15.1 %), and SA (7.9 %). Furthermore, EHA, which contains a single methoxyl group, exhibited a stronger inhibitory effect (ranging from 2.9 % to 5.3 %) on the enzymatic hydrolysis of APW when compared to EVA and ESA, which contained 2 and 3 methoxyl groups, respectively (Fig. 7c). These results indicated that an increase in the methoxyl group content in aromatic compounds resulted in a decreased inhibitory effect on enzymatic hydrolysis. Interestingly, the reduction in inhibition due to increased methoxyl groups was more pronounced in phenolic aldehyde compounds than in phenolic acid and etherified phenolic acid compounds. For example, at a 16 % addition, the glucan release in the presence of S, SA, and ESA was higher than that observed in the presence of H, HA, and EHA, which were 22.8 %, 13.9 %, and 2.9 %, respectively. These observations were consistent with the findings concerning the effect of methoxyl groups on MCC hydrolysis, as discussed in Section 3.3.

As depicted in Fig. 7, the inhibitory effect of aromatic compounds containing aldehyde groups on the enzymatic hydrolysis of APW was found to be lower than that of compounds containing carboxyl groups. This result contradicted the findings discussed in Section 3.3, where aldehyde groups exhibited a higher inhibitory effect on the enzymatic digestion of MCC compared to carboxyl groups. For instance, when 16 % of V and H were added to the reaction mixture, the glucan conversion of APW was reduced by 1.7 % and 9.7 %, respectively. However, at the same 16 % addition rate, the presence of VA and HA resulted in a larger reduction of glucan conversion in APW, specifically by 15.1 % and 21.8 %, respectively. These results indicate that the impact of aldehyde and carboxyl groups on enzymatic efficiency might be closely related to the presence of residual lignin in the substrate. In conclusion, aromatic compounds exhibited weaker inhibitory effects on the enzymatic saccharification of lignin-containing substrates compared to pure cellulose.

In the evaluation of the effect of phenolic hydroxyl groups in aromatic compounds on the enzymatic efficiency of APW, three pairs of compounds were used: VA-EVA, SA-ESA, and HA-EHA, as depicted in Fig. 7b, c. The results showed that the inhibitory effect of 16 % EVA and EHA (etherified phenolic compounds) on the enzymatic hydrolysis of APW was 4.8 % and 6.2 %, respectively, which was significantly lower than that of 16 % VA and HA (non-etherified lignin phenolic compounds), respectively. These findings align with previous conclusions that phenolic hydroxyl groups in lignin are detrimental to enzymatic hydrolysis due to the strong non-productive cellulase adsorption through hydrogen bond interactions (Zhai et al., 2018a; Lai et al., 2022). Yang and Pan (2016) also reported that blocking the phenolic hydroxyl groups of lignin through hydroxypropylation reactions can reduce the inhibitory effect of lignin on enzymatic digestion. However, the etherified compounds (ESA) exhibited higher inhibitory effects on enzymatic saccharification compared to non-etherified compounds (SA) at 16 % addition. Therefore, the exact impact of phenolic hydroxyl groups on the enzymatic saccharification of APW remains inconclusive.

Furthermore, it is noteworthy that the inhibitory effect of phenolic hydroxyl groups on the enzymatic efficiency of APW and MCC differed significantly, indicating that the inhibition effect of phenolic hydroxyl groups on enzymatic saccharification varied with different substrates. Many studies have reported the impact of phenolic hydroxyl groups on enzymatic saccharification, but the relationship between phenolic hydroxyl groups and enzymatic hydrolysis remains uncertain due to the diversity of biomass sources and pretreatment methods. Therefore, further investigation is necessary to understand the effect of phenolic hydroxyl groups on enzymatic saccharification more comprehensively. This study primarily adopted alkaline pretreatment as the method to prepare

lignocellulosic substrates. The acid pretreatment is also a common and easy-to-operate technique. However, the extent of lignin removal and changes in its structure differ between these two pretreatment methods. Therefore, it is necessary to study typical acid and alkali pretreatment processes. Subsequent research will also explore the influence of these compounds on the enzymatic hydrolysis of acid-pretreated residues.

The impact of aromatic compounds on cellulase activity is related to their concentration and structure. The effect of the same compound on enzyme activity varies at different concentrations. Zhao and Chen (2014) found that phenolic acid compounds exhibited inhibitory effects on enzyme activity at 0.05 g/L, while they showed promoting effects at 2–4 g/L. The functional groups of aromatic compounds are closely related to their impact on enzyme activity. Generally, aromatic compounds containing carboxyl groups have less inhibitory effect on enzyme activity and may even promote it (Tian et al., 2013). The presence of methoxyl groups is believed by some studies to reduce the inhibition of aromatic compounds on enzyme activity or be necessary for promoting enzyme activity (Qin et al., 2016; Zhai et al., 2018b). In this study, most aromatic compounds showed an inhibitory effect on enzymatic hydrolysis, with only a few compounds exhibiting mild promotion at low concentrations. Moreover, aromatic compounds containing carboxyl groups had lower inhibitory effects on enzymatic hydrolysis compared to those containing aldehyde groups. It was observed that the presence of more methoxy groups in aromatic compounds led to a weaker inhibition of enzymatic hydrolysis. Therefore, the inhibitory effects of some phenolic compounds on enzymatic hydrolysis in this study are related to their concentration and other functional groups (methoxyl and carboxyl groups).

Previous studies suggest that interaction between aromatic compounds and cellulase result in reduced or even deactivated cellulase activity, which is considered the primary reason for inhibitory effect on enzymatic hydrolysis (Ximenes et al., 2010; Tejirian and Xu, 2011, 2011; Li et al., 2014). To our knowledge, there has been limited research on the interaction between aromatic compounds and substrate so far. However, it is undeniable that the type of substrate plays a crucial role in the influence of aromatic compounds on enzymatic hydrolysis. Currently, many advanced methods are used to study the interaction between cellulases and lignin, such as quartz crystal microbalance, atomic force microscopy, and nuclear magnetic resonance spectroscopy. In the future, these techniques will be employed to further elucidate the interaction between substrates and aromatic compounds, revealing the mechanism by which aromatic compounds affect enzymatic hydrolysis.

4. Conclusions

The effect of nine different aromatic compounds on enzymatic hydrolysis were evaluated in this study. The increased methoxyl groups in aromatic compound reduced the inhibitory impact on enzymatic hydrolysis. The addition of syringaldehyde (dimethoxylated compounds) increased the enzymatic efficiency of alkaline pretreated wheat straw. Furthermore, the aldehyde groups exhibited stronger inhibitory effect than carboxyl groups on glucan conversion of microcrystalline cellulose. Nevertheless, the carboxyl groups displayed stronger inhibitory effect than aldehyde groups on glucan conversion of alkaline pretreated wheat straw. Therefore, the substrate lignin played a vital role on the different behavior of aromatic compounds towards the enzymatic hydrolysis of two substrates.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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