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Organic Acid-Based Hemicellulose Fractionation and Cellulosic Ethanol Potential of Five *Miscanthus* Genotypes

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Abstract: The pretreatment of lignocellulosic biomass such as *Miscanthus* grown on marginal agricultural land is very challenging and requires severe conditions to fractionate cell wall polymers for further valorization. The current study aimed to determine organic acid-based mild conditions to pretreat contrasting lignocellulosic *Miscanthus* genotypes for the efficient fractionation of cell wall components, with special focus on hemicellulose extraction. In doing so, five *Miscanthus* genotypes were subjected to four different acid treatments (sulfuric acid, oxalic acid, malonic acid, and citric acid) in a vertical high-pressure steam sterilizer. The results demonstrated that, among the organic acids, oxalic acid was identified as the most effective pretreatment solvent for hemicellulose separation, whereas citric acid yielded the highest amount of galacturonic acid, varying from 15 to 17 mg mL^{-1} across genotypes. One best performing genotype was selected for the enzymatic hydrolysis. Overall, *M. floridulus* genotypes exhibited the optimal quality traits for efficient bioconversion with second best in terms of ethanol production potential.

Keywords: bioethanol; biomass composition; biomass utilization; lignocellulose; marginal land; miscanthus; organic acid; platform chemicals; pretreatment; sustainability

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1. Introduction

The industrialization and current economic growth largely depend on non-renewable fossil-based resources [1]; however, the associated pollution and climate change concerns have shifted the focus towards utilization of renewable and sustainable resources for energy and materials production [2]. For energy production, a wide range of renewable resources can be employed, such as hydro, solar, wind, and biomass [3]. However, the multi-functionality of biomass makes it a preferable choice. A wide range of lignocellulosic biomass resources are available, such as agricultural and forest residues, dedicated energy crops [4–7] which can potentially be exploited for the production of biofuels and other value-added products [3,8]. Among the dedicated energy crops, *Miscanthus* is deemed an attractive option due to the high resource efficiency, the adaptability to different conditions, and a high technology-readiness level [9–11].

Miscanthus, a tall rhizomatous perennial grass, is characterized by its fast growth, high biomass yield, good biomass quality, low management cost [12], and long productive production cycle [13,14]. Furthermore, it has the potential to sequester large amounts of CO_2 in comparison with annual crops such as maize [15,16]. The high resource-use efficiency of *Miscanthus* allows the crop to grow on several types of marginal land, such as contaminated [17] and infertile (poor) soils [18], implying that the establishment of such

energy crops will not compete with food production. China has the largest growing area of *Miscanthus* in the world and offers rich germplasm resources [19], which can be exploited to expand the role of *Miscanthus* in developing biobased industry.

Miscanthus can be used for a variety of purposes, including biofuel, heat, electricity, and material production. In Europe, *Miscanthus* is mainly used to obtain heat and electricity by combustion [9,20]. In China, however, *Miscanthus* is mainly used in the paper industry, and the utilization for bioenergy purposes is very limited [21]. Recently, due to environmental pollution concerns, the papermaking enterprises have been shut down by the government, which necessitates the introduction of alternative *Miscanthus*-based value chains. One of them is *Miscanthus*-based ethanol production, because it promises a feasible option to utilize the huge amounts of available *Miscanthus* resources [21]. For instance, the *Miscanthus* biomass production potential from marginal lands alone is estimated to be 13,521.7 $\times$ 10^4 t DM a^{-1} [22].

However, the lack of appropriate pretreatment conditions and poor conversion efficiency are the major challenges to upscaling *Miscanthus*-based ethanol production. Optimal pretreatment of raw materials can break the complex structure of lignocellulose, increase the porosity of biomass, improve the accessibility of enzymes to cellulose and hemicellulose, and subsequently obtain high conversion efficiency [23,24]. The common pretreatment methods can be classified as physical, chemical, physicochemical, and biological. Of these methods, chemical pretreatment has obvious advantages in terms of increasing biomass porosity and facilitating solid separation [25]. Dilute acid pretreatment (inorganic acids predominantly dilute sulfuric acid) is one of the most widely used methods at present. However, it has certain disadvantages, such as the loss of sugars and production of inhibitors (e.g., furan and aldehydes), which can affect the further processing of the substrate [26]. In addition, there are environmental pollution concerns due to the lack of recovery of waste liquid after pretreatment [27,28]. Alternatively, studies have shown that organic acids can effectively promote the degradation of lignocellulose and avoid problems such as poor selectivity, equipment corrosion, and environmental pollution, as in the case of traditional inorganic acid treatment [29]. However, organic acids (citric acid, maleic acid, and oxalic acid) are often used at high concentrations [30–32], making their subsequent handling, such as washing and neutralization, challenging [33]. Moreover, till now, the focus has been largely on the valorization of cellulose, whereas hemicellulose, the second most important fraction of the cell wall, is overlooked. Thus, hemicellulose fractionation in cascade processing of biomass under moderate treatment conditions by using organic acids can improve the economic competitiveness of existing value chains.

The aim of this study was therefore to determine organic acid-based mild conditions to pretreat contrasting lignocellulosic *Miscanthus* genotypes for the efficient fractionation of cell wall components, with a special focus on hemicellulose extraction. Furthermore, the role of biomass composition in the identification of pretreatment conditions was evaluated.

2. Materials and Methods

2.1. Site Conditions and Sample Collection

Field trials with *Miscanthus* genotypes collected across China were set up in a 2 $\times$ 2 m plot size in 2006 on red soil at the *Miscanthus* resource garden of the Hunan Agricultural University (113°4′ E, 28°11′ N). For the year of study, i.e., 2021, the site received 1472.9 mm total annual rainfall, whereas the annual mean temperature was 18.8 °C. Based on the existing dataset developed over the years about biomass yield and quality, five *Miscanthus* genotypes (1× *M. giganteus*, 2× *M. lutarioriparius*, and 2× *M. floridulus*) were selected for this study. Figure 1 depicts the physiological and morphological differences among genotypes at the time of harvest.

Biomass samples were collected in December 2021 by leaving a stubble height of approximately 5 cm with the handheld cutter. The description of the genotypes is presented in Supplementary Table S1.

All samples were dried in an electric blast-drying oven (WGL-230B, Tianjin Taiste instrument Co., Ltd. Tianjin, China) at 60 °C for 24 h–48 h to constant weight. The dried samples were cut into 2 cm particles, crushed in a sample grinder (DLF-70S,

Wenzhou Dingli Medical equipment Co., Ltd. Wenzhou, China), sieved through a 60-mesh sieve (Jian instrument Co., Ltd. Shangyu city, Zhejiang Province, Shaoxing, China), and then stored in a self-sealed bag at room temperature.

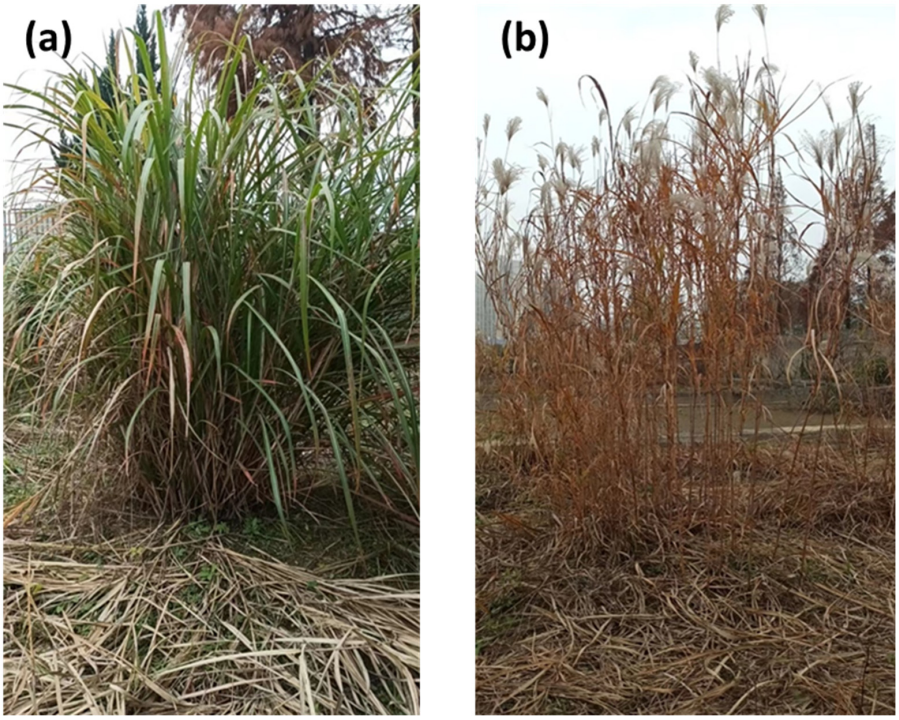

Figure 1. *M. floridulus* (**a**) and *M. lutarioriparius* (**b**) at the time of sample collection (both pictures taken on 13 December 2021) in the *Miscanthus* resource garden of the Hunan Agricultural University.

2.2. Fiber Analysis

Cellulose, hemicellulose, and lignin were determined following the Energy Industry Standard of the People's Republic of China [34]: determination of chemical composition of lignocellulose biomass materials. First, 3 g per sample was taken and wrapped in defatted filter paper to carry out Soxhlet extraction [35]. After drying, a 0.3 g sub-sample was taken and treated with 3 mL 72% sulfuric acid at 30 °C and 150 rpm for 1 h, and then 84 mL distilled water was added. Afterwards, the pretreated sub-sample was transferred to a vertical high-pressure steam sterilizer (Vertical automatic pressure steam sterilizer, GR85DA, Zealway-Xiamen instrument Co., Ltd. Xiamen, China) to react for 30 min at 121 °C. The solid and liquid phases were separated after the reaction was completed. Then, after solid drying and weighing, the acid hydrolysis residues (solid) of the pretreated sub-samples were calcined at 550 °C for 3 h in a muffle furnace (Muffle furnace, MFLC-36/12P, Tianjin Taiste instrument Co., Ltd. Tianjin, China) for the determination of acid-insoluble lignin. A part of the hydrolysate was diluted, and the content of acid-soluble lignin was determined by spectrophotometer at 320 nm (Double-beam UV-vis spectrophotometer A590, Aoyi instrument, Shanghai, Co., Ltd. Shanghai, China).

The contents of the structural carbohydrates were determined by high-performance liquid chromatography (HPLC) (LC-40, Shimadzu Corporation, Shimadzu, Kyoto, Japan with an AminexHPX-87H column, 65 °C column temperature, 5m M H_2SO_4 as eluent at 0.6 mL min^{-1}, and RID-20A detector). The content of cellulose and hemicellulose is calculated by Equations (1), (2), and (3), respectively:

$$C = \frac{(c_1+(c_2\times 1.0526))\times 0.9\times V}{1000\times W\times(1-M)\times 0.944}\times 100\% \quad (1)$$

where C refers to cellulose content in acid hydrolysate (%), c_1 refers to glucose concentration in acid hydrolysate (mg·mL^{-1}), c_2 refers to cellobiose concentration in acid hydrolysate (mg·mL^{-1}), 1.0526 refers to coefficient of conversion of glucose to cellulose, 0.9 refers

to coefficient of conversion of glucose to cellulose, and 0.944 refers to recovery rate of calibrated standard sample for glucose chromatography.

$$H = \left(\frac{c_3 \times 0.88 \times V}{1000 \times W \times (1-M) \times 0.909} + \frac{c_4 \times 0.9 \times V}{1000 \times W \times (1-M) \times 0.916}\right) \times 100\% \quad (2)$$

where H refers to hemicellulose content in acid hydrolysate (%), c_3 refers to xylose concentration in acid hydrolysate (mg·mL^{-1}), c_4 refers to concentration of arabinose in acid hydrolysate (mg·mL^{-1}), 0.88 refers to coefficient of conversion of xylose to hemicellulose, 0.9 refers to coefficient of conversion of arabinose to hemicellulose, 0.909 refers to recovery rate of standard sample corrected by xylose chromatography, and 0.916 refers to recovery of standard sample corrected by arabinose chromatography.

$$L = \frac{U \times V \times D}{\varepsilon \times l \times W \times (1-M)} \times 100\% \quad (3)$$

where L refers to the content of acid-soluble lignin in acid hydrolysate (%); U refers to absorbance of acid hydrolysate under 320 nm (Abs); ε refers to the photoabsorptivity of acid hydrolysate [L/(g cm^{-1})], i.e., 30 L/(g cm^{-1}); and l refers to the optical path, in centimeters (cm), and here its value is 1 cm.

All the chemicals used in the experiment are analytical reagents (distributor: Sinopharm) and fulfilled the chemical reference standards (distributor: Solarbio).

2.3. Pretreatment Condition and Sugar and Inhibitor Analysis

For each sample, 5.0 g weight was added to the acid solution (sulfuric acid 0.1 M, oxalic acid 0.1 M, malonic acid 0.1 M, and citric acid 0.067 M)) of 50 mL, mixed to ensure that the samples were all immersed in acid, and put it into a vertical high pressure steam sterilizer (Vertical automatic pressure steam sterilizer, GR85DA, Zealway-Xiamen instrument Co., Ltd. Xiamen, China) to react for 30 min at 121 °C and 0.1 MPa. After the reaction was completed and cooled, hydrolysate was filtered with filter paper and stored in a centrifuge tube at a low temperature, and the solid was washed to neutral pH with deionized water, followed by drying.

We filtered a diluted sample of the hydrolysate in a 0.22 um filter and determined the concentrations of glucose, xylose, arabinose, furfural, hydroxymethylfurfural (HMF), and formic and acetic acids by using HPLC. The HPLC conditions were consistent with those described in Section 2.2 (fiber analysis). The contents of the sugar and inhibitor were calculated according to Equation (4).

$$C = A \times V \times D/(1000 \times W \times (100 - M/100)) \times 100 \quad (4)$$

where C refers to the content of sugars or inhibitors in hydrolysates, in percent; A is the concentration of sugars or inhibitors in hydrolysates, measured in milligrams per milliliter (mg mL^{-1}); V is the volume of hydrolysate in milliliters (mL); D is the dilution factor for hydrolysate; W is the weight of the sample, in grams (g); and M is the moisture content of the sample, in percent.

The remaining part of the hydrolysate was diluted for a certain number of times to determine the optical density (OD) value by using spectrophotometer at 320 nm, and the content of acid-soluble lignin was calculated according to Equation (5).

$$L = U \times V \times D/(\varepsilon \times l \times W \times (100 - M/100)) \times 100 \quad (5)$$

where L is the content of acid-soluble lignin in hydrolysate, in percent; U is the value of the hydrolysate under 320 nm, in absorbance (Abs); ε is the light absorptivity of the hydrolysate measured in L (g × cm)$^{-1}$, which is 30 L (g × cm)$^{-1}$ here; l is the optical path, in centimeters (cm), and is 1 cm here; W is the weight of the sample in grams (g); and M is the moisture content of the sample, in percent.

2.4. Enzymatic Hydrolysis

The sodium citrate buffer was configured as follows: 4.83 g of citric acid monohydrate was dissolved in 750 mL of distilled water, 7.94 g trisodium citrate was added under stirring, and the volume was raised to 1 L.

According to the predetermined cellulose content, the amount of sample containing 0.5 g cellulose was calculated. Then, 50 mL of sodium citrate buffer was put into a 250 mL flask, together with the pretreatment sample containing 0.5 g cellulose. The flask was sealed to create an oxygen-free environment. For hydrolysis, two kinds of enzymes were used: (1) acid cellulase from SUNSON (SUNSON Industry Group Co., Ltd., Beijing, China), and (2) Cellic CTec3 from Novozyme (Novozyme Biotechnology Co., Ltd., Tianjin, China). Acid cellulase was added from 3 to 6 g, whereas the Cellic CTec3 dosage varied from 1 to 4.5 mL.

Enzymatic hydrolysis was carried out at 50 °C, 180 rpm with pH maintained at 4.8 + 0.05, and loading rate of 1 mL or 1 g depending on enzyme type. A sample of 0.4 mL was taken at 1 h, 3 h, 6 h, 12 h, 24 h, 36 h, and 48 h and centrifuged for 3 min at 8000 rpm (equivalent to relative centrifugal force of 6797 rcf). The supernatant was taken, and the concentration of glucose was determined by HPLC. The HPLC conditions were consistent with those described in Section 2.3 (fiber analysis). The efficiency of enzymatic hydrolysis was calculated according to Equation (6).

$$E = (G_2 \times Vn \times D)/(1000\times G_1) \times 100\% \quad (6)$$

where E is the enzymatic hydrolysis efficiency, in percentage; G_2 is the glucose concentration in the reaction solution, in milligrams per milliliter; Vn is the volume of the reaction solution at different sampling times, in milliliters; and G_1 is the mass of glucose in the substrate (in g).

2.5. Ethanol Production Potential

The equation was followed based on the already available information from the literature [36,37]. Here, the focus is only on the calculation of cellulose-based ethanol production because hemicellulose is already separated at the first step for the production of platform chemicals. The stoichiometric yield calculation for hexose sugar hydrolysis was calculated as follows:

$$\text{Cellulose hydrolysis} = (C_6H_{10}O_5)n + (n-1)H_2O \rightarrow (C_6H_{12}O_6)n \quad (7)$$

$$\text{Fermentation of } C_6 \text{ sugar} = C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + \text{Heat} \quad (8)$$

The cellulose-based ethanol yield was calculated based on the following Equation (9), which was adopted from Scordia et al. [38] and Zheng et al. [37]:

$$\begin{gathered}\text{Ethanol production (L kg}^{-1}\text{)} = \text{Feedstock quantity} \times \{\text{cellulose content (\%)} \times \\ 1.111 \text{ (hydrolytic gainfor } C_6 \text{ sugars)} \times 0.40 \text{ (hydrolysis efficiency for} \\ C_6 \text{ sugars)} \times (1 - \text{inhibitor formation (\%)}\} \times 0.511 \text{ (fermentation} \\ \text{factor for } C_6 \text{ sugars)} \times 0.95 \text{ (fermentation efficiency of} \\ C_6 \text{ sugars)} \times 0.98 \text{ (distillation efficiency)}/0.789 \text{ (ethanol density)}\end{gathered} \quad (9)$$

The hydrolysis efficiency for C_6 sugars is 40%, based on the outcomes of the enzymatic hydrolysis; the inhibitor formation for C_6 sugars was considered 1%, and the fermentation factor was calculated based on the stoichiometric yield from the Equation (5).

2.6. Data Analysis

Analysis of variance was performed as a part of the statistical analysis to determine the level of significance at $p < 0.05$ among genotypes in terms of the compositional traits of the feedstock before pretreatment. Feedstock was subjected to pretreatment, and the quality parameters of solid residue and liquid hydrolysate were compared to find out significant differences among genotypes, as well as to perform a comparison between different acid treatments.

A mixed-model approach was adopted, and the model given below was employed:

$$Yij = u + Gij + Aj + eij$$

where Yij is the response variable, u refers to the mean, G indicates the genotype affect, A denotes the acid treatment affect, and eij is the residual error.

In the case of significant differences ($p < 0.05$), Tukey's Honest Significant Difference (HSD) test was employed to determine the significant differences among the tested groups.

Pearson's correlation coefficients were calculated to determine the strength of antagonistic and synergistic interrelationships between different quality traits. The correlation coefficients were compiled into a heatmap.

All the relevant data analyses and figures were prepared by using open-source R Studio (2023.06.0 Build 421 © 2009–2023 Posit Software, PBC, Boston, MA 02210, USA).

3. Results

3.1. Characterization of Untreated Feedstock

Five *Miscanthus* genotypes with a high biomass-yield potential and contrasting quality traits were selected as a feedstock for this study. In the upcoming results subsections, the selected *Miscanthus* genotypes are compared in terms of the biochemical composition of their cell wall before pretreatment.

Overall, the statistical analyses revealed that significant differences among *Miscanthus* genotypes exist in terms of the biochemical composition of cell wall. The results demonstrated that the cellulose content of *M. floridulus* genotypes (F1 and F2) and *M. giganteus* (G) was significantly higher than that of *M. lutarioriparius* (L1 and L2). The F1 genotype and *M. giganteus* (G) exhibited significantly higher hemicellulose and lignin contents, respectively. Furthermore, similar trend as those of hemicellulose was reported for xylose and galactose, with the highest contents found in the F1 genotype. It is notable that the significantly lowest lignin and ash contents were recorded in L1 and L2, respectively (Figure 2).

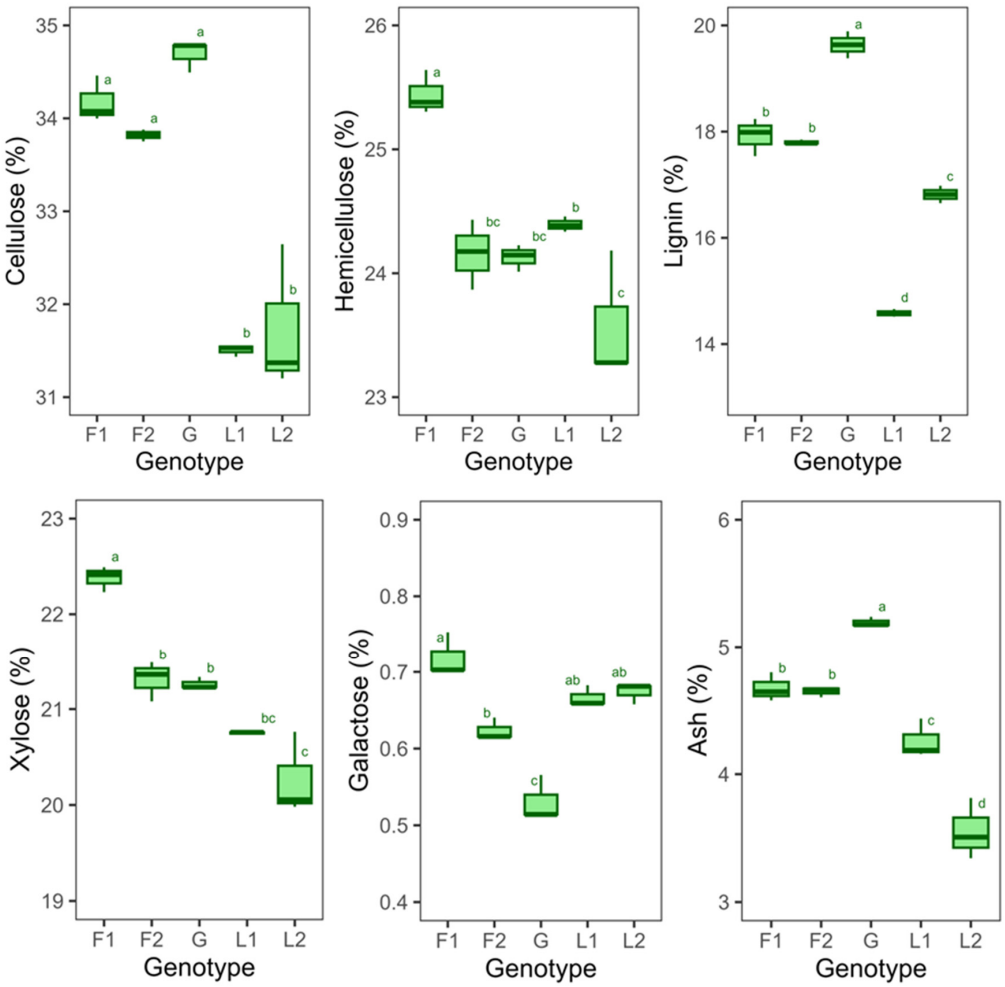

Figure 2. Biochemical composition of different *Miscanthus* genotypes (F1 and F2 = *M. floridulus*; G = *M. giganteus*; and L1 and L2 = *M. lutarioriparius*). Different lowercase letters indicate significant

($p < 0.05$) differences among genotypes for cellulose, hemicellulose, arabinose, lignin, xylose, galactose, and ash content.

3.2. Pretreatment of Feedstock

The following section presents the results of the polysaccharide and monosaccharide analyses of the solid residues and the liquid hydrolysate under tested acid treatments.

3.2.1. Solid Residue

It is evident from the statistical analysis that the genotype, acid treatment, and genotype–acid treatment interaction affects were significant for all parameters except for the genotype affect for xylose and the genotype–acid treatment interaction for ash, as they were not significant (Table 1).

Table 1. Fixed effects of genotype, acid, and their two-fold interaction on biochemical composition of solid residue of pretreated feedstock. Significant codes refer to different *p*-values (*** 0.001, ** 0.01, and * 0.05), and ns denotes non-significant effects.

Response Variables	Explanatory Variables		
	Genotype	**Acid Treatment**	**Genotype × Acid Treatment**
Cellulose	***	***	***
Hemicellulose	*	***	**
Lignin	***	***	***
Ash	***	***	ns
Xylose	ns	***	**
Arabinose	***	***	***

The cellulose content of solid residue was significantly lower for the *M. floridulus* genotypes (F1 and F2) compared with other genotypes under all acid treatments. Pretreatment induced the release of cellulose in *M. floridulus* genotypes, which were among the highest cellulose-yielding genotypes before pretreatment. Depending on the type of acid treatment, the cellulose content in F1 and F2 decreased by 9 to 22% compared with untreated feedstock. However, unexpectedly, the cellulose and lignin contents in *M. lutarioriparius* genotypes (L1 and L2) were increased from 13 to 26% compared with the untreated. The cellulose content of *M. giganteus* (G) did not differ significantly before and after pretreatment. The hemicellulose content decreased rapidly after pretreatment, especially under sulfuric acid (S) and oxalic acid (O). It is pertinent to mention that the release of hemicellulose followed the same pattern in all the genotypes under tested acid treatments. For instance, across genotypes, the hemicellulose content was decreased from 61 to 68% under the sulfuric acid (S) treatment and 48 to 58% under the oxalic acid (O) treatment. Furthermore, both acid treatments (S and O) were more affective in the removal of lignin for all genotypes except *M. lutarioriparius* genotypes (L1 and L2). The separation of hemicellulose for platform chemicals at this stage offers an opportunity to valorize cellulose and lignin in subsequent steps into biofuels and value-added products. In general, acid treatments decreased the ash content in all genotypes. The results revealed that the release of sugars was largely determined by the acid-treatment type and genotype (Figure 3).

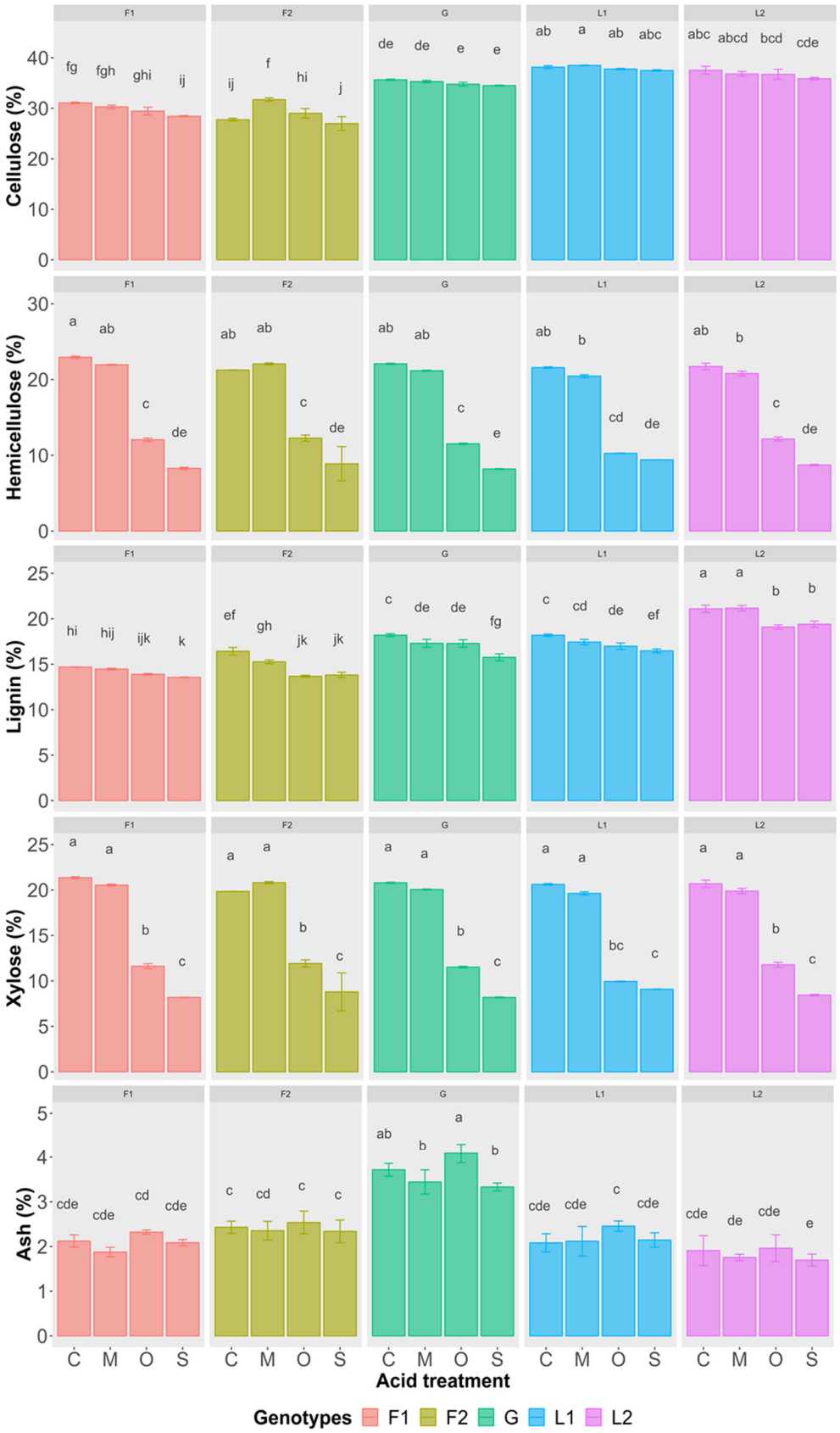

Figure 3. Biochemical composition of solid residue of pretreated feedstock comprising of five different *Miscanthus* genotypes (F1 and F2 = *M. floridulus*; G = *M. giganteus*; and L1 and L2 = *M. lutarioriparius*) and four different acid treatments (C = citric acid, M = malonic acid, O = oxalic acid, and S = sulfuric acid).

Error bars represent standard deviation calculated for replications of each acid treatment. Lowercase letters represent different acid treatment comparisons across genotypes, and the means followed by the same letter are not significantly different at $p < 0.05$.

3.2.2. Liquid Hydrolysate

The outcomes of the statistical analyses show that the sugar content and soluble lignin in the hydrolysate were largely influenced by the genotype, acid treatment, and genotype–acid treatment interaction; all of them had a significant impact on the tested parameters for liquid hydrolysate (Table 2).

Table 2. Fixed effects of genotype, acid treatment, and their two-fold interaction on biochemical composition of liquid hydrolysate separated from pretreated feedstock. Significant codes refer to different *p*-values (*** 0.001).

Parameters	Effect		
	Genotype	Acid Treatment	Genotype × Acid Treatment
Glucose	***	***	***
Xylose	***	***	***
Arabinose	***	***	***
Soluble lignin	***	***	***

The glucose yield in liquid hydrolysate was significantly highest for the sulfuric acid (S) and oxalic acid (O) in most of the tested genotypes, whereas the lowest amount was released in the case of the citric acid (C) treatment (Figure 4). Furthermore, the significantly highest glucose yield was recorded in *M. floridulus* (F1 and F2) compared with other genotypes across all acid treatments. Malonic acid treatment (M) yielded the highest contents of xylose and arabinose in liquid hydrolysate, followed by the sulfuric acid (S) and oxalic acid (O) treatments. Oxalic acid (O) treatment produced significantly high soluble lignin for all genotypes except for *M. floridulus* (F2), but malonic acid (M) yielded the highest. It is important to mention that, only for *M. floridulus* did all treatments lead to the release of galacturonic acid, although concentrations were low (0.37 to 1 mg mL^{-1}), except for citric acid treatment (15.2 mg mL^{-1}). Citric acid (C) treatment induced the highest galacturonic acid levels across all genotypes, varying from 15 to 17 mg mL^{-1}.

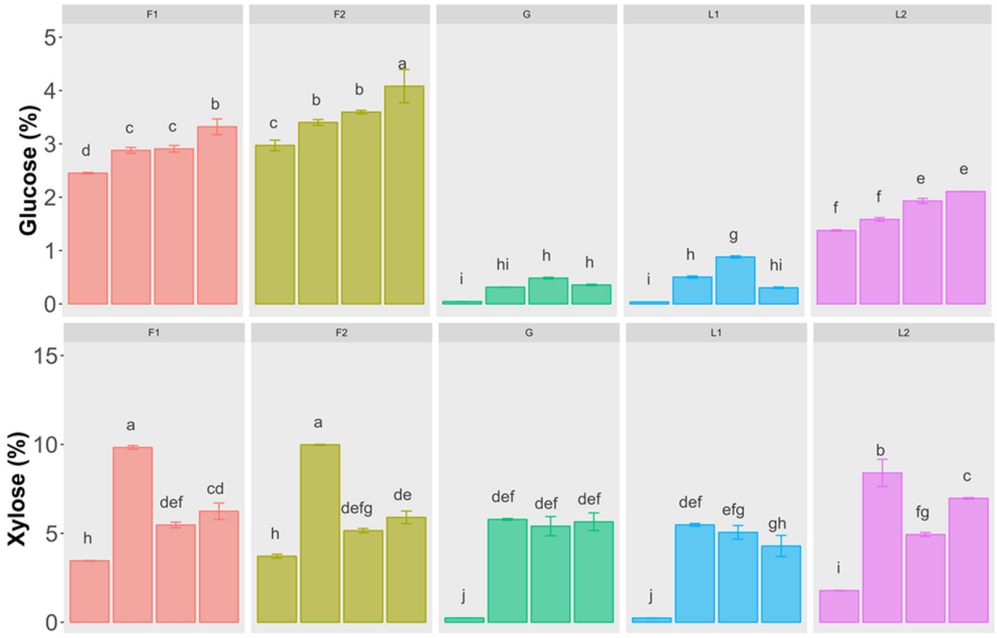

Figure 4. *Cont.*

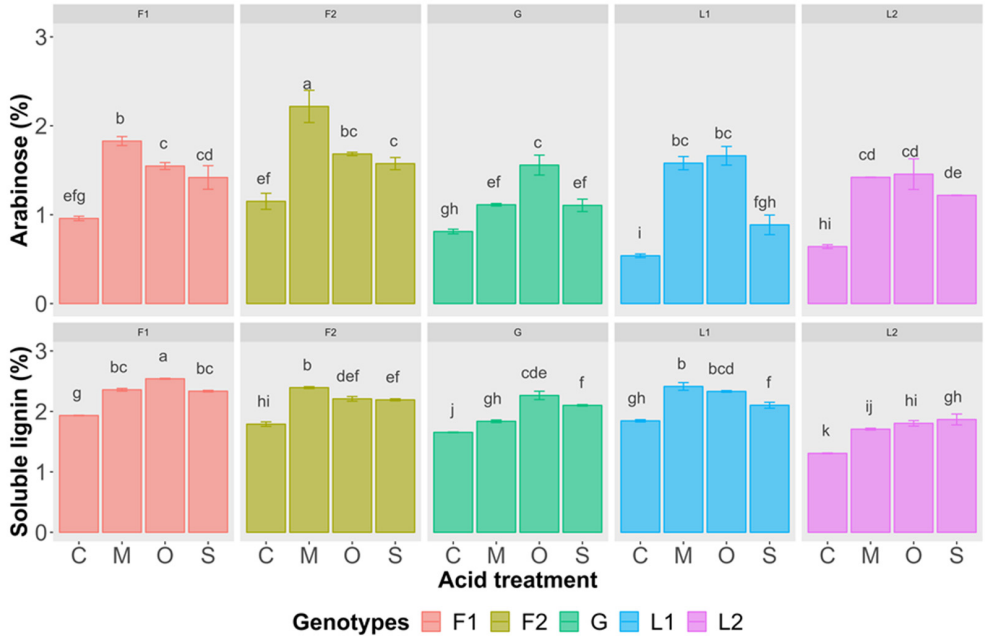

Figure 4. Sugar content in the hydrolysate separated from the pretreated feedstock comprising five different *Miscanthus* genotypes (F1 and F2 = *M. floridulus*; G = *M. giganteus*; and L1 and L2 = *M. lutarioriparius*) and four different acid treatments (C = citric acid, M = malonic acid, O = oxalic acid, and S = sulfuric acid). Error bars represent standard deviation calculated for replications of each acid treatment. Lowercase letters represent different acid treatment comparison across genotypes, and the means followed by the same letter are not significantly different at $p < 0.05$.

3.2.3. Inhibitor Formation in Hydrolysate

The liquid hydrolysate separated from pretreated feedstock was subjected to determination of inhibitor formation under different acid treatments for different genotypes. The effect of genotype, acid treatment, and genotype–acid treatment was highly significant on inhibitor formation in the liquid hydrolysate (Table 3).

Table 3. Fixed effects of genotype, acid treatment, and their two-fold interaction on biochemical composition of liquid hydrolysate separated from pretreated feedstock. Significant codes refer to different *p*-values (*** 0.001).

Parameters	Effect		
	Genotype	Acid Treatment	Genotype × Acid Treatment
Acetic acid	***	***	***
Formic acid	***	***	***
5-hydroxymethyl furfural	***	***	***
Furfural	***	***	***

Malonic acid yielded the highest level of acetic acid across all genotypes, with a significantly high yield for *M. floridulus* (F2). The content of formic acid was highest for oxalic acid (O) treatment, whereas HMF and furfural contents were highest for sulfuric acid (S) and oxalic acid (O) for all genotypes (Figure 5). Overall, the highest inhibitor formation was recorded in *M. floridulus* (F2), whereas it was lowest in *M. lutarioparius.* Across acid treatments, citrus acid (C) treatment yielded the lowest amounts of inhibitors in liquid hydrolysate.

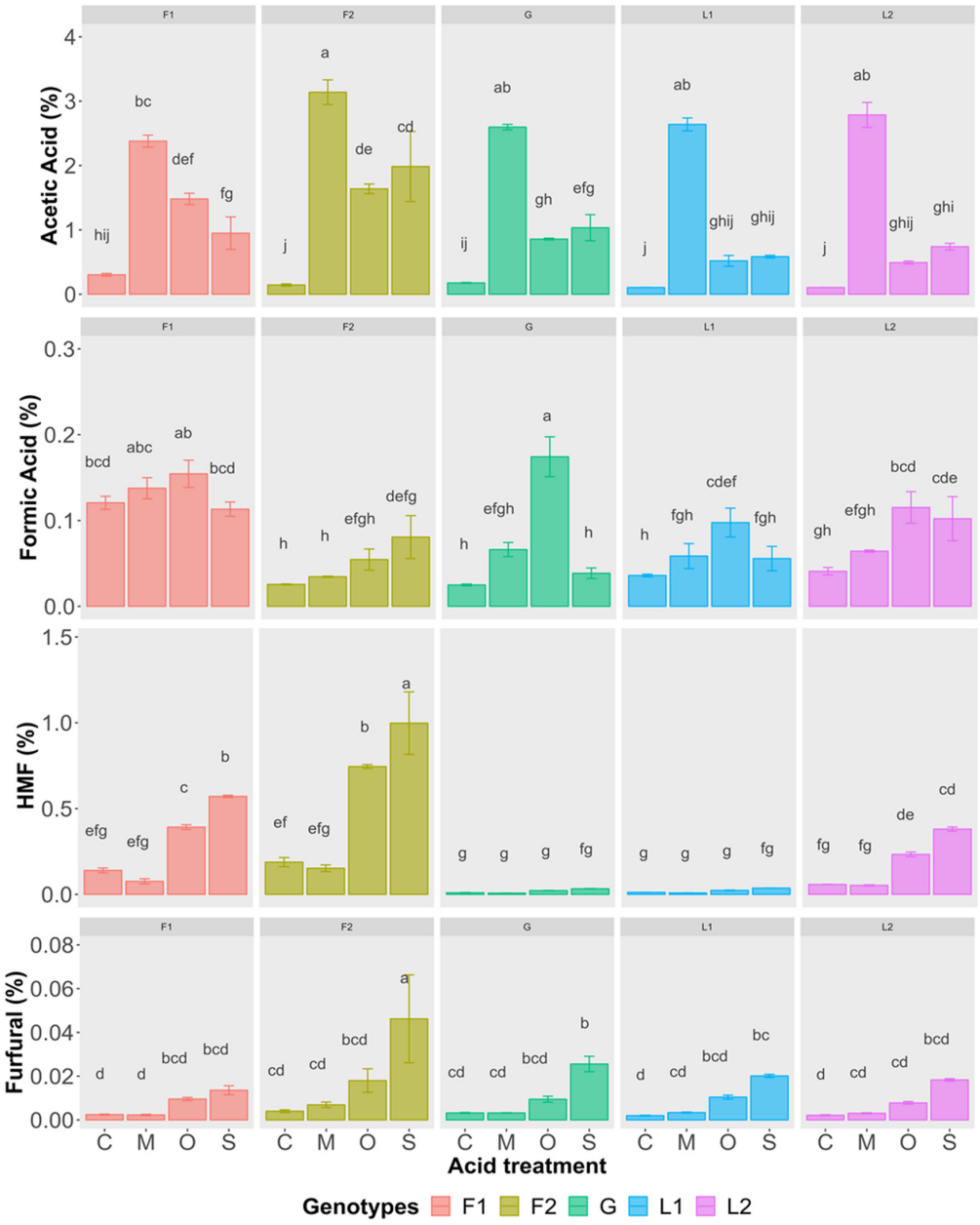

Figure 5. Inhibitor formation in the hydrolysate separated from the pretreated feedstock comprising five different *Miscanthus* genotypes (F1 and F2 = *M. floridulus*; G = *M. giganteus*; and L1 and L2 = *M. lutarioriparius*) and four different acid treatments (C = citric acid, M = malonic acid, O = oxalic acid, and S = sulfuric acid). Error bars represent standard deviation calculated for replications of each acid treatment. Lowercase letters represent different acid treatment comparison across genotypes, and the means followed by the same letter are not significantly different at $p < 0.05$.

3.3. Enzymatic Hydrolysis

The enzyme type had a significant impact on the hydrolysis efficiency, whereas enzyme quantity did not have a significant impact. It is evident from the results that the enzymes sourced from Sunson performed significantly better in terms of glucose yield compared with Novozymes (Supplementary Figure S1). It is important to state that enzymatic hydrolysis was carried out only for one genotype (*M. floridulus*) and largely aimed to feed this information to calculate the theoretical ethanol production potential for the rest of the genotypes. *M. floridulus* was selected over *M. giganteus* because of its better performance in terms of hemicellulose fractionation and overall low recalcitrance.

Depending on the enzyme type, the enzymatic hydrolysis efficiency varied greatly; it was highest (40%) for Sunson, whereas it was 23% for Novozymes (Supplementary Table S2).

3.4. Relationship between Compositional Traits of Cell Wall

The outcomes of correlation analysis show that the contents of cellulose, hemicellulose, and lignin in the pretreated solid residue are positively correlated with each other, whereas the inhibitor formation in the liquid hydrolysate is negatively correlated with the polysaccharides and lignin content of the pretreated solid proportion. It is interesting to note that the glucose and xylose contents in the hydrolysate are strongly negatively correlated with the cellulose, holocellulose, and lignin contents of the solid residue. On the other hand, the release of glucose and xylose was strongly positively correlated with the arabinose content. Furthermore, formic acid exhibited a significant positive correlation with xylose and arabinose, whereas it exhibited a negative correlation with the lignin content in solid residue. Figure 6 highlights the correlation coefficients in a heatmap.

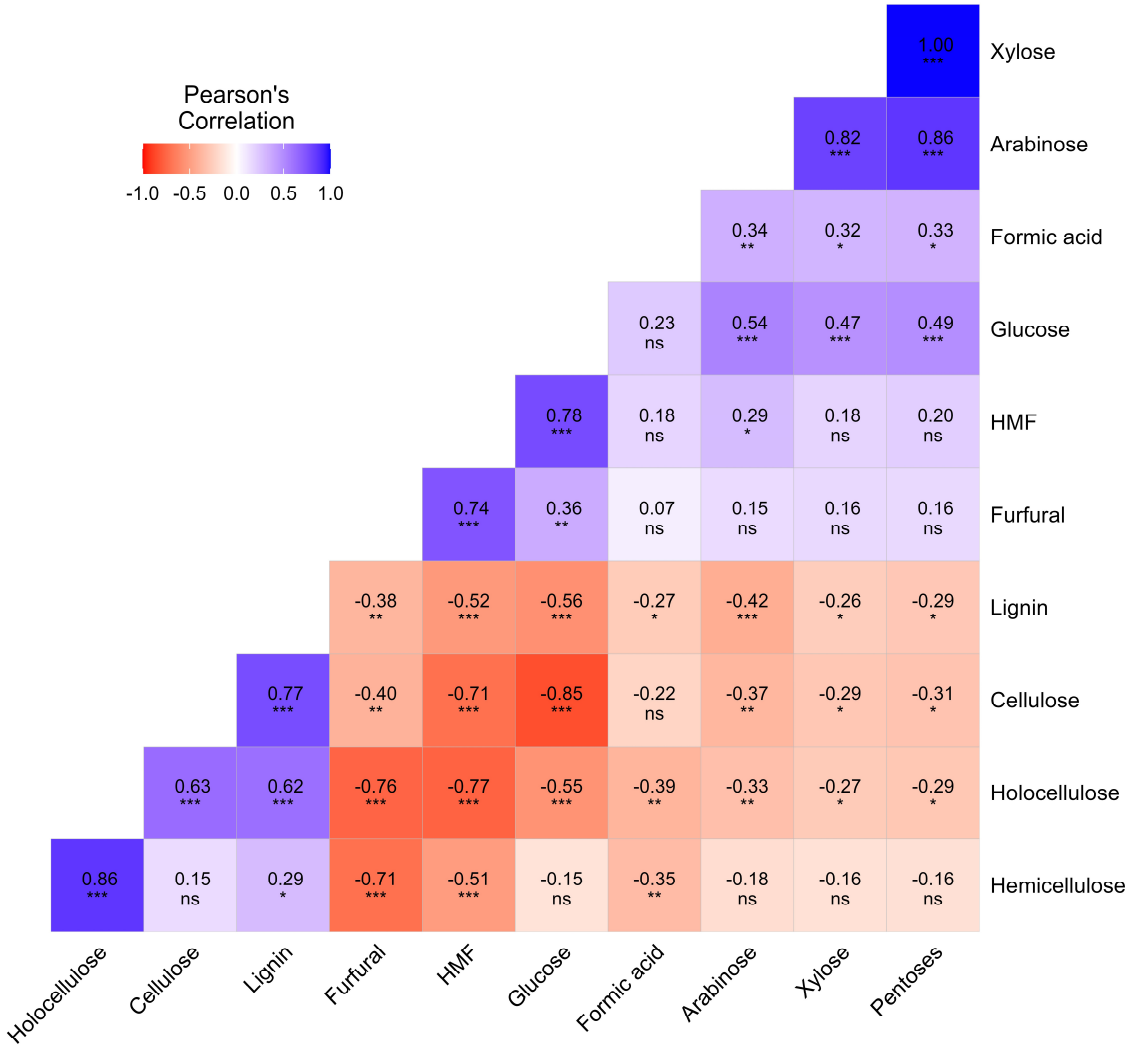

Figure 6. Correlation coefficients of compositional traits of pretreated feedstock, including liquid hydrolysate. Significant codes refer to different *p*-values (ns $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Based on enzymatic hydrolysis efficiency and theoretical fermentation efficiency, the cellulose-based ethanol production potential was estimated for each *Miscanthus* genotype (Table 4). It is important to highlight that only cellulose was considered for ethanol yield because, before the enzymatic hydrolysis during the pretreatment stage, most of the hemicel-

lulose was already separated. The estimated cellulose-based ethanol production potential was highest for *M. giganteus* and *M. floridulus*, whereas it was lowest for *M. lutarioparius*.

Table 4. Estimated cellulose-based ethanol yield for each genotype. Standard deviation was calculated for three genotypes.

Genotype	Ethanol (in L (Mg DM)$^{-1}$)
M. giganteus (G)	92 ± 0.4
M. floridulus (F1)	91 ± 0.7
M. floridulus (F2)	90 ± 0.2
M. lutarioparius (L1)	84 ± 0.2
M. lutarioparius (L2)	84 ± 2.1

4. Discussion

4.1. Variation in Lignocellulosic Components among Genotypes

The abundant *Miscanthus* genetic diversity offers an opportunity to screen the genotypes with optimal quality characteristics for different end uses, which will ultimately extend the role of *Miscanthus* in the developing bioeconomy. The efficient fractionation of cell wall components and valorization under mild pretreatment conditions are a necessary approach to upscale and improve economic competitiveness for lignocellulosic driven biorefineries. Thus, the current study focused on addressing this specific issue by investigating five high-biomass-yielding *Miscanthus* genotypes with diverse lignocellulosic composition under mild pretreatment conditions for hemicellulose extraction as the first step and valorization of solid fraction available at the end of experiment to estimate cellulosic ethanol production potential.

The lignocellulosic content of the selected genotypes for the current study varied from 70 to 79%, which is in agreement with another study conducted with 179 *Miscanthus* accessions across China [39]. These results were also comparable with those of a study where 49 *Miscanthus* genotypes were investigated regarding their cell wall composition [40]. The main sources of lignocellulosic variation among genotypes can be explained by the genetic diversity, environmental factors, plant organs, and harvesting time [41–43]. Genetics define the morphological traits of the crop, which can subsequently influence the biomass quality. For instance, in the current study, the genotypes with highest leaf-to-stem ratio (data not presented) also showed the highest cellulose content, which indicates that the change in the share of leaves can be a source of variation and subsequently influence the overall biochemical composition of biomass. From the literature, it is evident that the lignocellulosic content of *Miscanthus* genotypes varies from organ to organ; also, the polysaccharides composition of leaves is different than stems [44], and it has been investigated to cell or tissue level [45]. In this study, the lignocellulosic content of *M. lutariparius* was lowest, which also coincided with the lowest leaf share compared with other genotypes. This partially explains the variation among genotypes in terms of lignocellulosic content.

The content and composition of lignin are equally important as well in determining the bioconversion efficiency. For example, a high syringyl/guaiacyl (S:G) ratio in lignin can have a negative impact on the digestibility of feedstock [46]. It has been reported that lignin composition, especially the S:G ratio, is directly influenced by the management practices [47]. Furthermore, ferulic acid esters are present in the grass cell wall and are cross-linked with xylans and subsequently influence the process of biomass digestion and enzymatic saccharification [48]. It is clear that the abundance of cross-linkages varies with feedstock type [49]. Thus, both the lignin content and lignin composition can be optimized to maximize pretreatment efficiency through adjusting management practices [14] and screening genotypes with favorable quality traits [50]. From the literature, it is obvious that the lignin composition can be optimized by altering the biosynthesis route of monolignol without a significant effect on plant growth, and such an engineering can facilitate bioconversion of lignocellulosic biomass [51].

4.2. Comparison and Screening of Efficient Acid Pretreatment

The pretreatment with sulfuric acid and oxalic acid led to the highest release of hemicellulose; here, mineral acid was used only as a reference because, traditionally, this is the one used widely in the extraction of cell wall polysaccharides. The separated hemicellulose can be valorized into different end products, such as lactic acid; used in the production of xylitol, a natural food sweetener; or used for the production of platform chemical 2,3-butanediol [52]. Among the organic acids, oxalic acid exhibited the highest selectivity in terms of hemicellulose extraction, which is in accordance with the literature, where similar results were reported in extraction of hemicellulose from corncob [53]. However, it is important to highlight that oxalic acid also yielded the highest cellobiose for all genotypes which is not presented here because the content for other treatments was negligible. It can be explained by the fact that oxalic acid has successfully extracted the hemicellulose in all genotypes and exposed cellulose which subsequently led to the release of cellulose for this acid treatment. Thus, this study recommends that oxalic acid be used for the efficient extraction of hemicellulose in miscanthus genotypes, without losing other polymers. On top of that, oxalic acid, as an organic acid, offers an added value owing to its less corrosive nature and low inhibitor formation [54], which subsequently pave the way for the sustainable processing of lignocellulosic feedstock. In addition to its high pretreatment efficiency, the relatively simple recyclability and the high reuse potential of oxalic acid offer other potential benefit and make it a preferable choice. For instance, it can be separated at the time of ethanol extraction, particularly in the case of hemicellulose fractionation [53].

Furthermore, highest production of galacturonic acid in case of citric acid treatment can add value through introduction of new value chain because it has wide range of applications as a thickening or stabilizing agent from the food to pharmaceutical industry [55]. The literature identifies pectin as a main source of galacturonic acid; however, hemicellulose can also contribute during pretreatment [56]. Generally, *Miscanthus* biomass is characterized by a low pectin content [57], which indicates that high galacturonic acid production can be attributed to both pectin extraction and hemicellulose hydrolysis. From the outcomes of the current study, it is clear that citric acid has induced production of galacturonic acid, which is also evident from the literature where similar results were reported in cocoa pod husks [58]. However, in the context of the current study, follow-up investigations are needed to explore potential of citric acid driven pretreatment of *Miscanthus* biomass to produce galacturonic acid.

4.3. Pretreatment and Response of Genotypes

The response of genotypes varied for same acid treatment; for instance, in *M. floridulus* (F1 and F2), a loss of cellulose was recorded, whereas in *M. giganteus* (G), it stayed intact, and in *M. lutarioparius* (L1 and L2), it even increased. Although in-depth structural differences were not conducted as a part of this study, it can be assumed that the quick release of cellulose in *M. floridulus* can be attributed to comparatively less structural complexities and optimal compositional quality traits, which subsequently facilitated the rapid digestion of biomass [40]. Furthermore, *M. floridulus* is also characterized by high leaf share, which has potentially contributed to improved digestibility because high leaf content in the harvested biomass can alter cell wall structural features significantly and exhibit better recalcitrance [40,59].

In the case of *M. giganteus*, the highest degree of cellulose polymerization contributed towards minimum cellulose losses in pretreatment. A high degree of polymerization plays a key role in providing mechanical strength to the plants; however, it negatively affects biomass digestibility [60]. On the other hand, in *M. lutarioparius*, the proportion of cellulose was increased in the solid residue, likely because pretreatment induced a comparatively greater release of hemicellulose and lignin without losing cellulose. This is in line with the overall aim of this study to successfully fractionate hemicellulose in a way that other biopolymers stay intact for further cascade processing. In addition to the abovementioned factors, structural and compositional diversity in cellulose, hemicellulose, and lignin can

also be responsible for differences in response of genotypes during pretreatment. This can be supported by the literature findings, where a large variation in cell wall components played a key role in defining digestibility [46,48,61]. It indicates that the genotype with relatively less structural complexities is favorable for efficient bioconversion. This leads to the conclusion that *M. floridulus* can be recommended for hemicellulose valorization owing to its low recalcitrance to digestion because of favorable compositional and structural quality traits. The ash content of pretreated solid residue of *M. floridulus* was also among the lowest, whereas the genotype which exhibited the highest recalcitrance was also characterized with the highest ash content. That is why the genotype *M. floridulus* was selected for subsequent enzymatic hydrolysis, and it performed reasonably well. This is also in line with the literature, where *M. floridulus* has been characterized as a less recalcitrant genotype [40]. Furthermore, *M. floridulus* was the second most productive in terms of substrate-specific ethanol yield and leading in biomass dry matter yield (data not presented here). However, structural and compositional interactions still need to be investigated in depth to optimize the processing even further.

4.4. Pretreatment and Inhibitor Formation

This study focused on the implementation of mild pretreatment conditions; thus, the overall inhibitor formation was low, especially for furfural, which was lower than 1 mg g^{-1} and comparable with the previous studies [62,63]. The inhibitor formation in the liquid hydrolysate separated after pretreatment was largely defined by the release of the glucose, arabinose, and xylose content of pretreated solid residue. This is evident from the strong negative correlation between the holocellulose content of solid residue and the inhibitor formation in the liquid hydrolysate. More specifically, the genotypes that preserved more cellulose during the pretreatment subsequently accumulated less glucose and less HMF in the hydrolysate. On the other hand, genotypes with more cellulose and hemicellulose loss during pretreatment consequently accumulated more glucose and pentoses (xylose + arabinose), consequently producing more HMF and slightly more furfural. The HMF formation was directly related to glucose release, which can be supported by the strong positive correlation (0.78) between glucose and HMF, whereas pentoses presence led to the production of furfural. The correlation between pentoses and furfural is positive but non-significant. Overall, this trend can also be supported by the literature, where hexose leads to the formation of HMF, and pentose to furfural [64,65].

This indicates that inhibitor formation not only depends on pretreatment conditions but is also influenced by lignocellulosic content and the substrate composition.

It is important to state that enzymatic hydrolysis and fermentation were not the focus of this study, which is why these aspects are not included in the discussion part. Overall, the outcomes of this study will contribute to the cascade processing of lignocellulosic biomass under mild conditions to deliver a range of advanced biofuels, fine chemicals, and green solvents.

5. Conclusions

One of the key outcomes of this study was to successfully demonstrate that mild organic acids can efficiently deconstruct the lignocellulosic material to facilitate hemicellulose extraction. The results were comparable with the most widely implemented chemical, sulfuric acid, to pretreat the lignocellulosic biomass. The pretreatment can thus be made more environmentally friendly by using mild organic acids without impeding the pretreatment efficiency. Despite the removal of hemicellulose as a first step to produce platform chemicals and extraction of galacturonic acid, an acceptable ethanol yield of approximately 90 L Mg^{-1} of DM was achieved. Follow-up research work is being carried out to develop platform chemicals from hemicellulose and valorization of solid fraction available at the end of experiment.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy14071389/s1, Figure S1: Comparison of two enzyme types on glucose yield of the selected Miscanthus genotype (F2), pretreated with sulfuric acid. Means followed by the same letter are not significantly different at $p < 0.05$; Table S1: Descriptions of *Miscanthus* genotypes used in field trials at the *Miscanthus* resource garden of the Hunan Agricultural University, China; Table S2: Efficiency comparison of enzymatic hydrolysis of pretreated feedstock with different enzymes.

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