Mild Acid-Catalyzed Atmospheric Glycerol Organosolv Pretreatment Effectively Improves Enzymatic Hydrolyzability of Lignocellulosic Biomass

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ABSTRACT: Conventional atmospheric glycerol organosolv pretreatment is energy-intensive with the requirement of long time and/or high temperature. Herein, acid-catalyzed atmospheric glycerol organosolv (ac-AGO) pretreatment was developed under a mild condition to modify the sugarcane bagasse structure for improving enzymatic hydrolyzability. Using single factor and central composite design experiments, ac-AGO pretreatment was optimized at 200 °C for 15 min with 0.06% H₂SO₄ addition, wherein the hemicellulose and lignin removal rates were 82 and 52%, respectively, with extremely high cellulose retention of 98%. The ac-AGO-pretreated substrate exhibited good enzymatic hydrolyzability at a modest cellulase loading, affording a 70% glucose yield after 72 h. Multiple analysis tools were used to correlate the hydrolyzability of the substrate with its structural features. The results indicated that the mild ac-AGO pretreatment can modify the lignocellulosic biomass structure to achieve good hydrolyzability, mainly resulting in significant hemicellulose removal.

1. INTRODUCTION

Lignocellulose is a promising renewable resource that can replace petroleum-derived fuels or decrease our dependence on them, but lignocellulosic recalcitrance remains a significant challenge for the competitive production of bioethanol fuel. This is because lignocellulosic plant cell walls are composed of crystalline cellulose nanofibrils embedded in an amorphous matrix of cross-linked lignin and hemicelluloses that impede enzyme and microbial accessibility.1 These structures complicate the depolymerization of cellulose into simple fermentable sugars. Therefore, development of an efficient and environment-friendly method capable of deconstructing the recalcitrance of lignocellulosic materials and improving the overall process for biorefinery industries is essential.2

More than 10 years ago, the atmospheric glycerol organosolv (AGO) pretreatment was developed by us using industrial glycerol as a cooking solvent.3,4 The AGO pretreatment performed at 240 °C for 4 h resulted in 95% cellulose recovery and >70% delignification, and the pretreated wheat straw released 92% of the theoretical reducing sugar content after 48 h of enzymatic hydrolysis.4 Modified AGO pretreatment (220 °C, 3 h) retained 98% of the cellulose and removed >65% of the lignin from wheat straw, resulting in 90% of enzymatic substrate hydrolysis (48 h).5 The method performed at 220 °C for 2 h removed approximately 70% of the lignin content of sugarcane bagasse while leaving most of the cellulose (94%) intact. In addition, the pretreated substrate exhibited high cellulose purity (>50%), with a small amount of hemicellulose (<15%) and lignin (<15%).6 These earlier studies on the AGO pretreatment demonstrated that this process exhibits notable selectivity for deconstructing lignocellulosic biomass, significantly improving the hydrolyzability of lignocellulosic substrates. To date, the conventional AGO pretreatment typically involves5−7 (1) organosolv pretreatment; (2) atmospheric operation process; (3) value-added organosolv lignin; (4) nontoxic solvent; (5) residual solvent usable as microbial carbon source; (6) reduced production of furan inhibitors; and (7) a bridge between bioethanol and biodiesel production. Briefly, the glycerol is a desirable solvent to cook the lignocellulosic feedstock in an operationally simple and environmentally benign organosolv pretreatment process. However, the current AGO pretreatment process is achieved
autocatalytically by catalysis of organic acids and acid derivatives produced in the reaction system without catalyst addition, which is energy-intensive and requires high temperatures (220–240 °C) and long reaction durations (2–4 h). Therefore, a relatively mild AGO pretreatment process would be beneficial for the enzyme-based lignocellulosic bioenergy industry.

After the AGO pretreatment development, tens of similar studies have been performed regarding the glycerol—water organosolv (GWO) pretreatment of various lignocellulosic biomasses.7,8 In particular, GWO pretreatment with excess acid addition has become extremely attractive because the process can be carried out under industrially relevant conditions similar to those for fiber pulping.9–10 Martin et al.11 showed that GWO pretreatment at 187.7 °C for 2.3 h with 0.64% H2SO4 in an 80% glycerol solution resulted in a maximized delignification of 53% from sugarcane bagasse, whereas the highest enzymatic hydrolysis yield of substrates was observed at 194.1 °C, 1.67 h, and 1.1% H2SO4. Hilares et al.12 evaluated the glycerol acid pretreatment of sugarcane bagasse and determined the optimal pretreatment conditions of 0.15 g acid/g dried mass, 130 °C, and 57.5 min, thereby achieving a 68.5% glucose yield from the pretreated substrate. Ebrahimi et al.13 obtained a GWO-pretreated rice husk substrate containing 40% cellulose, 1.6% xylan, and 17.6% lignin under a selected pretreatment condition (130 °C, 60 min, and 1.2% HCl). The acid-catalyzed GWO pretreatment disrupted the lignocellulosic biomass recalcitrant structure, effectively improving substrate hydrolyzability. The addition of excess acid catalyst would likely be helpful to achieve a mild AGO pretreatment process.

Herein, an acid-catalyzed atmospheric glycerol organosolv (ac-AGO) pretreatment was developed. The main variables (H2SO4 addition, pretreatment temperature, and pretreatment time) of the ac-AGO pretreatment process were first selected with single factor experiments, followed by optimization using central composite design. Subsequently, the hydrolyzability of ac-AGO-pretreated sugarcane bagasse was evaluated via batch enzymatic hydrolysis. Finally, the susceptibility of the pretreated substrates towards enzymatic saccharification was correlated with its structural features via characterization using modern analytical equipment.

### 2. RESULTS AND DISCUSSION

#### 2.1. Construction of the ac-AGO Pretreatment Process

Numerous studies have demonstrated that the key variables of the process include pretreatment temperature, time, and catalyst addition during thermochemical pretreatment.9–10 Herein, considerable efforts were made to optimize these key variables and establish an ideal ac-AGO pretreatment process.

##### 2.1.1. Single Factor Experiments

Table 1 describes the key variables selected individually in the single factor experiments. For the pretreatment temperature, the process reached a high pretreatment yield (63%) at a low temperature (140 °C) owing to the significant degradation of hemicellulose and lignin. Increasing temperature caused an acute removal of hemicellulose, resulting in an obvious reduction of the pretreatment yield from 63 to 56%. In addition, the cellulose content increased from 58% (140 °C) to 67% (200 °C) with hemicelluloses removal. When the temperature was increased to 160 °C, the pretreated biomass achieved the highest cellulose retention of 95 with 85% hemicellulose and 29% lignin removal. Thereafter, temperatures of >160 °C caused significant cellulose degradation and the pretreatment temperature of 160 °C was determined to be optimal. A long pretreatment time resulted in low pretreatment yield where after 30 min of pretreatment, the ac-AGO-pretreated sugarcane bagasse exhibited a high cellulose content of 65% with a small fraction of hemicellulose (6%). Prolonging the pretreatment resulted in an obvious cellulose loss, so 30 min of pretreatment time was determined to be optimal for future experiments. The addition of H2SO4 exerted a significant effect on the substrate chemical composition. The hemicellulose content was lowered from 12 to 2% after increasing the H2SO4 content from 0.02 to 0.1%, which was likely the main reason for the significantly increased cellulose content (54–70%) and slightly increased lignin content (23–29%). At 0.04% H2SO4 addition, the pretreatment showed maximum cellulose retention of 97%, resulting in good cellulose (63%) and hemicellulose (6%) contents of the pretreated substrate. Excess acid addition degraded the cellulose and 0.04% H2SO4 addition was selected for future experiments. In summary, the selected ac-AGO pretreatment parameters were 160 °C, 30 min, and 0.04% H2SO4 addition.

| H2SO4 (%) | T (°C) | time (min) | yield (%) | substrate composition (%) | C retention | H removal | delignification (%) |
|----------|--------|------------|-----------|--------------------------|------------|-----------|---------------------|
| 0.06     | 140    | 20         | 63        | 57 7 25                 | 93         | 81        | 27                  |
| 160      |        |            | 61        | 64 6 24                 | 95         | 85        | 29                  |
| 180      |        |            | 58        | 65 5 22                 | 90         | 87        | 38                  |
| 200      |        |            | 56        | 67 4 26                 | 90         | 90        | 31                  |
| 0.06     | 160    | 5          | 65        | 60 8 25                 | 87         | 80        | 27                  |
|          | 10     |            | 62        | 62 7 25                 | 93         | 82        | 29                  |
|          | 20     |            | 61        | 64 6 24                 | 95         | 85        | 29                  |
|          | 30     |            | 60        | 67 6 25                 | 97         | 85        | 29                  |
|          | 40     |            | 55        | 67 5 24                 | 90         | 90        | 36                  |
| 0.02     | 160    | 30         | 66        | 54 12 23                | 85         | 67        | 28                  |
| 0.04     |        |            | 64        | 63 6 22                 | 97         | 83        | 31                  |
| 0.06     |        |            | 61        | 64 6 24                 | 95         | 85        | 29                  |
| 0.08     |        |            | 55        | 69 3 27                 | 93         | 94        | 29                  |
| 0.1      |        |            | 50        | 70 3 29                 | 84         | 95        | 33                  |

C, cellulose; H, hemicellulose; L, lignin.

### Table 1. Single Factor Experiments of ac-AGO Pretreatment Process

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2.1.2. Optimization of the ac-AGO Pretreatment Process.

To understand the effects of pretreatment temperature, H₂SO₄ concentration, and time on the ac-AGO pretreatment, a central composite design with lignin removal as the response value was performed (Table 2). The analysis of variance showed that the model built by the central composite design was precise and the predicted response was reliable (Model F-value = 2.33, lack of fit F-value = 18.79%, P value < 0.05).

As shown in Figure 1, a clear interaction between the pretreatment temperature and acid addition, as well as between pretreatment temperature and time, whereas no interaction between acid addition and pretreatment time was observed. The pretreatment temperature showed the greatest influence.

Table 2. Results of the Central Composite Design Experiments

| run # | T (°C) | H₂SO₄ (%) | time (min) | C (%) | H (%) | L (%) | retention (%) |
|-------|--------|-----------|------------|-------|-------|-------|---------------|
| 1     | 120    | 0.02      | 15         | 91    | 89    | 93    |
| 2     | 120    | 0.06      | 50         | 94    | 59    | 77    |
| 3     | 160    | 0.04      | 32.5       | 92    | 41    | 64    |
| 4     | 160    | 0.04      | 32.5       | 88    | 33    | 61    |
| 5     | 160    | 0.07364   | 32.5       | 93    | 11    | 62    |
| 6     | 200    | 0.06      | 15         | 72    | 7     | 18    |
| 7     | 92.728 | 0.04      | 32.5       | 90    | 92    | 98    |
| 8     | 200    | 0.02      | 50         | 95    | 30    | 52    |
| 9     | 227.27 | 0.04      | 32.5       | 82    | 3     | 40    |
| 10    | 200    | 0.06      | 50         | 95    | 16    | 54    |
| 11    | 160    | 0.04      | 32.5       | 92    | 40    | 60    |
| 12    | 160    | 0.00636   | 32.5       | 85    | 80    | 70    |
| 13    | 160    | 0.04      | 3.06863    | 91    | 56    | 66    |
| 14    | 200    | 0.02      | 15         | 96    | 36    | 48    |
| 15    | 120    | 0.02      | 50         | 89    | 86    | 91    |
| 16    | 160    | 0.04      | 61.9314    | 91    | 39    | 66    |
| 17    | 160    | 0.04      | 32.5       | 91    | 42    | 61    |
| 18    | 160    | 0.04      | 32.5       | 93    | 46    | 79    |
| 19    | 120    | 0.06      | 15         | 91    | 49    | 89    |
| 20    | 160    | 0.04      | 32.5       | 89    | 43    | 68    |

C, cellulose; H, hemicellulose; L, lignin.

Figure 1. Response surface plot (three-dimensional and two-dimensional) representing the interaction of variables for lignin removal: (a) temperature and H₂SO₄ addition over 15 min; (b) temperature and time at a fixed H₂SO₄ addition of 0.06%; (c) H₂SO₄ addition and time at a constant temperature of 200 °C.
on the chemical composition of the pretreated sugarcane bagasse (Table 2 and Figure 1). At low temperatures, the mild pretreatment at run 7 resulted in a low hemicellulose degradation of 8%. In contrast, a severe pretreatment process featuring high temperature (run 9) solubilized almost all hemicelluloses and >50% of the lignin, resulting in significant cellulose loss. Consequently, the main variables in the ac-AGO pretreatment process were optimized to 200 °C pretreatment temperature, 0.06% H2SO4 addition, and 15 min of pretreatment time, which is overlapped with run 6. Under the abovementioned optimized conditions, the substrate after the pretreatment contained 72% cellulose, 7% hemicelluloses and >50% of the lignin, achieving significant cellulose retention and 80% hemicellulose removal. The results indicate that the ac-AGO pretreatment was capable of solubilizing hemicelluloses effectively from the substrate without damaging the cellulose.

Table 3 lists main studies on the AGO and GWO pretreatment of various lignocellulosic biomasses. Compared with the pretreatment without acid catalysis, acid catalysis allowed for mild pretreatment at a lower temperature (Table 3). Interestingly, the ac-AGO pretreatment studied herein was achieved with very dilute acid (0.06%) at a relatively short pretreatment time (15 min), which is of economic interest for enzyme-based lignocellulosic bio refineries. Consequently, acid addition was helpful for constructing a mild AGO pretreatment process typically requiring low pretreatment temperatures and/or relatively short pretreatment times, wherein the acid action contributed to hemicellulose removal from lignocellulosic biomass.7 In contrast, the acid-catalyzed pretreatment resulted in a large proportion of lignin residues in the pretreated substrate, though this was accompanied by overwhelming hemicellulose removal.15 It is widely recognized that high lignin content negatively affects subsequent enzymatic saccharification of the pretreated substrates as it can physically restrict polysaccharide accessibility and irreversibly adsorb cellulase enzymes.16−18 Moreover, large lignin residues in the pretreated substrates often represent relatively low cellulose purity and can necessitate additional high-solid enzymatic saccharification to supply sufficient fermentable sugars. This tends to deteriorate the saccharification environment with mixing and heat–mass transferring difficulty in the sticky slurry, which is a major problem for the enzyme-based lignocellulosic bio refining industry.19,20

2.2. Hydrolyzability of the ac-AGO-Pretreated Sugarcane Bagasse. It has been reported that acid-catalyzed glycerol pretreatment enhanced the digestibility of cellulose into fermentable sugars.11−13 Herein, the hydrolysis of the raw and ac-AGO-pretreated sugarcane bagasse was performed at 2% solid content with different enzyme loadings (3, 6, and 10 FPU/g dry substrate) to assess hydrolyzability. As shown in Figure 2, the glucose yields from the hydrolysis of both substrates increased with higher enzyme loading, indicating that the current substrate hydrolysis was dominated by enzyme loading. Increased enzyme loading should contribute to higher glucose yield. In terms of single substrates, the glucose yield from raw sugarcane bagasse was <15% after 72 h at 10 FPU/g dried substrate, indicating very weak hydrolyzability. The hydrolysis of the ac-AGO-pretreated substrates increased sharply with hydrolysis time, resulting in an increased glucose yield from 38% at 12 h to 70% at 72 h at 10 FPU/g enzyme loading. These results indicate that the ac-AGO-pretreated substrate released notably high glucose titer compared to the
raw feedstock at the same enzyme loading. Furthermore, the hydrolyzability of the ac-AGO-pretreated substrates was compared to other studies (Table 3). Similar to the other reports, the ac-AGO-pretreated substrate obtained herein was enriched with lignin in addition to cellulose. At 10 FPU/g cellulase loading, the glucose yields from various pretreated substrates were relatively low (∼70%). Most substrates exhibited >90% glucose yields at 72 h when the cellulase loading was 20 FPU/g. These results indicate that the cellulase acting on the substrate predominantly influenced the glucose yield. It is likely that the ac-AGO-pretreated substrate released a significant amount of fermentable sugars with high enzyme loadings. Thus, the ac-AGO pretreatment effectively improved the hydrolyzability of the sugarcane bagasse.

2.3. Enzymatic Hydrolysis of ac-AGO-Pretreated Sugarcane Bagasse at a High Solid Content. Recently, many studies have demonstrated that performing enzymatic hydrolysis at high solid contents is crucial for cost-effective and competitive bioethanol fuel production.19,20 Therefore, the enzymatic hydrolyzability of the ac-AGO-pretreated sugarcane bagasse was further evaluated at 10, 12, 15, 18, and 20% solid contents with 3, 6, and 10 FPU/g cellulase (Figure 3). At 3 FPU/g enzyme loading, the substrates at 10, 12, and 15% solid contents did not liquefy for 12 h and released 29, 34, and 41 g/L of glucose at 72 h, respectively. When the solid contents were 18 and 20%, the liquefaction time of the substrate hydrolysis was significantly delayed due to the high viscosity during the early stages of hydrolysis, releasing 48 and 52 g/L glucose, respectively, at 70 h. With additional enzymes loading from 3 to 10 FPU/g, the glucose titer and yield from the substrate hydrolysis at all solid contents increased. The addition of 10 FPU/g dried substrate enabled the ac-AGO-pretreated sugarcane bagasse at 10, 12, 15, 18, and 20% solid contents to liquefy rapidly and produce 48, 58, 64, 78, and 82 g/L glucose, respectively, at 72 h. This indicated that the titer of fermentable sugars released during the enzymatic hydrolysis increased with higher solid contents. In contrast, the glucose yield from substrate hydrolysis gradually decreased with higher solid content at 3, 6, and 10 FPU/g. At 10 FPU/g enzyme loading, the hydrolysis yield declined from 60 at 10% solid content to 51 at 20% solid content. These results suggested that glucose production was strongly dependent on the solid content regardless of enzyme loading and hydrolysis time.19,21 Evidently, high solid contents were beneficial for the titer of glucose released from the substrate hydrolysis while sacrificing glucose yield. It is likely that the high solid content would contribute to a simultaneously desirable sugar titer and yield at very high cellulase loadings.21 However, at low cellulase...
loading, the high solid content can result in inconsistency of the glucose titer and yield. This inconsistency is likely due to the “high-solids effect” that reduces the mixing and heat–mass transfer of the substrate and cellulase due to its rheological behavior.20−22 In these scenarios, a fed-batch mode, namely substrate feeding in a stepwise manner is reasonable for the high-solids enzymatic hydrolysis with low cellulase loading.23

Additionally, the ac-AGO-pretreated substrate contained a high lignin content (~20%), resulting in the following effects that were adverse to the high-solids enzymatic hydrolysis: (1) physically coating cellulose to impede cellulase accessibility; (2) chemically binding the enzyme to form a nonproductive binding of cellulase; (3) providing more substrate content to potentially supply sufficient fermentable sugars; and (4) partially contributing to enzyme deactivation.24,25 Accordingly, strategies including extended delignification of substrates and use of additives/accessory enzymes during enzymatic hydrolysis are promising to reduce the high-solids effect, which is both substrate and enzyme dependent.26−28 Briefly, the high-solids batch enzymatic hydrolysis of substrates at low enzyme loading remains a significant challenge for the enzyme-based lignocellulosic biorefining industry.

2.4. Structural Features. To determine the mechanism of ac-AGO modification of the sugarcane bagasse structure for improved hydrolyzability, scanning electron microscopy (SEM), confocal laser scanning microscopy (CLSM), thermogravimetric analysis (TGA), Fourier transform infrared (FTIR), and X-ray diffraction (XRD) were used to characterize any structural modification. SEM images of the feedstock and ac-AGO-pretreated biomass were obtained (Figure 4). The feedstock exhibited a smooth surface with compact fiber bundles, whereas the ac-AGO-pretreated substrate was disrupted, distorted, and curled into a mass, exhibiting a coarse and loose structure with an increased specific surface area. Thus, the modified structure should be more accessible and susceptible to the cellulases, enhancing enzymatic hydrolysis.25,26 A previous study showed that CLSM can be used to determine the distribution of lignin on lignocellulosic biomass.29 As shown in Figure 4, the feedstock possessed an intact web-like structure, while the ac-AGO pretreated substrate exhibited a significant breakage of the sclerenchyma and middle lamella of the plant cell walls, as well as an obvious separation of cell walls and middle lamella. The observations show that the ac-AGO pretreatment dissociated the natural cell structure of sugarcane bagasse, which is consistent with the above SEM images. Compared with the feedstock, ac-AGO pretreated substrate displayed almost the same fluorescence intensity, indicative of the substrate still enriched with lignin. This comparison is in accordance with the above compositional analysis.

Figure 5a shows the TGA curve of the feedstock and ac-AGO-pretreated substrate. The feedstock began to degrade at approximately 225 °C, with 20% degradation at 310 °C and 75% degradation at 390 °C. The ac-AGO-pretreated sample presented a relatively high initial decomposition temperature of 275 °C and became 20% degraded at 365 °C and 75% degraded at 390 °C. Thus, the ac-AGO-pretreated substrate exhibited a much higher degradation temperature and thermal stability due to the significant removal of constituents (i.e., hemicellulose and lignin). The substrates before and after ac-AGO pretreatment were also examined by FTIR spectroscopy from 4000 to 400 cm⁻¹ (Figure 5b). After ac-AGO pretreatment, the intensity of the C−H ester/C−O bond at 1375 cm⁻¹ significantly diminished, and the peak corresponding to the COO ester bond at 1736 cm⁻¹ weakened, indicating the dissociation of chemical bonds between lignin and hemicellulose in the pretreated substrate. The disappeared peaks at 1240 cm⁻¹ (β-ether bond) and 1600 cm⁻¹ (benzene ring of lignin) as well as the weakened signals at 1325 cm⁻¹ (syringyl and guaiacyl condensed lignin) and 834 cm⁻¹ (CH⁻ of guaiac lignin adsorption) indicated significant lignin removal. These results indicate that ac-AGO pretreatment removed most hemicelluloses while preserving the main cellulose in the sugarcane bagasse by selective breakdown of key chemical bonds and functional groups.5,6 Figure 5c shows the XRD curves of the ac-AGO-pretreated substrate that were
used to determine crystallinity. Both curves presented major crystalline peaks at 2θ = 16° (101), 21.9° (002), and 34.6° (040), indicating that the crystal form of the substrate belonged to cellulose I. The ac-AGO pretreatment method did not result in crystal transfer but increased the crystallinity of the feedstock from 41.8 to 59.0%. The data suggested that the amorphous components, i.e., hemicellulose and lignin, of the sugarcane bagasse were at least partially removed during the ac-AGO pretreatment.

The above described structural features indicate that the ac-AGO pretreatment deconstructed and modified the recalcitrant architecture of the sugarcane bagasse. The pretreatment-dissociated key chemical bonds (i.e., β-ether, β-ester, and hydrogen bonds) and functional groups (i.e., syringyl and guaiacyl), resulting in micro- and macro-structural changes in the substrates. The ac-AGO-pretreated substrates mainly consisted of shrunk and defibrillated fibrils with increased roughness and surface area, as well as lignin relocalization. The structural modification of the substrate was conducive to enzymatic hydrolysis, which is in good agreement with previous studies regarding AGO pretreatment.

3. CONCLUSIONS
A relatively mild AGO pretreatment process was developed with acid catalysis at low temperature and short duration. The ac-AGO pretreatment modified the lignocellulosic biomass structure towards a good hydrolyzability by dissociation of key chemical bonds and functional groups, which resulted in significant hemicellulose removal. The high lignin residue in the ac-AGO-pretreated substrate is evidently adverse to the batch enzymatic hydrolysis at high solid contents. Strategies including extended substrate delignification, use of additives/accessory enzymes, and fed-batch mode may compromise the high-solids effect in the high-solids enzymatic hydrolysis of ac-AGO-pretreated substrates.

4. MATERIALS AND METHODS
4.1. Materials. Sugarcane bagasse, consisting of 40.78% cellulose, 23.91% hemicelluloses, and 29.45% lignin, was obtained from Guangxi Province, China and was manually sieved into scraps approximately 10 mm in length. The scraps were subsequently dried to constant weight at 105 °C and stored in polyethylene plastic containers. Industrial glycerol (Technical grade, 99.0% purity) was purchased from a chemical plant in Jiangsu Province, China. The cellulase (Technical grade, 99.0% purity) was purchased from a chemical plant in Jiangsu Province, China. The cellulase was neutralized by the addition of CaCO₃, followed by dilution. All samples were passed through a G3 glass column (300 μm pore size) before HPLC analysis. Glucose and xylose were quantified at 65 °C using an Aminex HPX-87H column (300 × 7.8 mm, BioRad) at a flow rate of 0.6 mL/min with 5 mM H₂SO₄ as the mobile phase. The pretreatment yields and components were designated, respectively, as: pretreatment yield (%) = 100 (g of insoluble solid component of feedstock); Hemicellulose removal (delignification) (%) = 100 (g of component of insoluble solid fiber fraction)/(g in component of feedstock); Hemicellulose removal (delignification) (%) = 100 (g in component of feedstock − g in component of insoluble solid fiber fraction)/(g in component of feedstock).

4.2. ac-AGO Pretreatment. A set of single factor experiments were performed to optimize the three key variables of pretreatment temperature (140–200 °C), sulfuric acid addition (0.02–0.1%), and pretreatment time (5–40 min). For central composite design optimization, 14 different runs were conducted by randomization and six replicates at the central point were performed to evaluate the pure error. The model was analyzed using variance and statistical significance determined by F and P values. After analysis, set of experiments were performed in triplicate to confirm the model.

In a typical run, 10 g of dry sugarcane bagasse was suspended in a three-necked round bottom flask containing 100 g (solid/liquid ratio, 1:10 (w/w)) of industrial glycerol and a certain volume of dilute H₂SO₄ solution (0.1 g/mL) in the above addition ratio was added to the flask. The pretreatment procedure was performed as described previously.

4.3. Enzymatic Hydrolysis. The sugarcane bagasse feedstock (2%) and ac-AGO-pretreated substrate (2–20% (w/v) dry substrates) were subjected to enzymatic hydrolysis at three enzyme loadings of 3, 6, and 10 FPU/g dry substrates in citrate buffer (0.05 M, pH 4.8). The enzymatic hydrolysis mixture was incubated at 50 °C and 150 rpm in a 250 mL Erlenmeyer flask with a 45 mL working volume. Samples of 0.5 mL were periodically withdrawn during hydrolysis and centrifuged at 11 200 rpm and 4 °C for 1 min. The supernatant was stored in the freezer (<−18 °C) prior to glucose determination.

4.4. Structural Characterization of the Substrates. Before analysis, all wet samples were parted manually into smaller fragments and dried to a constant weight at 60 °C. All characterizations were performed according to the methods described previously. Changes in sample morphology were observed via scanning electron microscopy (SEM; Quzmfa-200, FEI, Netherlands) operated at a 10 kV acceleration voltage, and confocal laser scanning microscopy (CLSM; LSM 710, Zeiss, Germany). The thermal stabilities of the samples were analyzed using thermogravimetric analysis (TGA-STDA851e, Mettler-Toledo, Switzerland). Changes in chemical bonding and functional groups before and after ac-AGO pretreatment were determined by Fourier transform infrared (FTIR) spectroscopy. The X-ray diffraction (XRD) pattern of the sample was measured using a D8 (AXS, Germany) X-ray diffractometer equipped with Ni-filtered Cu Kα1 radiation (λ = 0.154 nm) at room temperature and the crystallinity index (Crl) of the samples was calculated.

4.5. Analytical Methods. The chemical composition of sugarcane bagasse feedstock and the ac-AGO-pretreated sugarcane bagasse was determined using standard laboratory analytical methods. Acid-insoluble lignin was measured by weighing after overnight drying at 105 °C of the residue obtained after acid hydrolysis. The fraction of acid-insoluble ash was determined by heating the samples at 550 °C until a constant weight was obtained. The sugar contents in the hydrolysates were analyzed by high-performance liquid chromatography (HPLC; Japan HITACHI). The hydrolysates were neutralized by the addition of CaCO₃, followed by dilution. All samples were passed through a G3 glass filter (100 mL, 15–40 μm pore size) before HPLC analysis. Glucose and xylose were quantified at 65 °C using an Aminex HPX-87H column (300 × 7.8 mm, BioRad) at a flow rate of 0.6 mL/min with 5 mM H₂SO₄ as the mobile phase. The pretreatment yields and components were designated, respectively, as: pretreatment yield (%) = 100 (g in insoluble solid fiber fraction)/(g in feedstock); cellulose retention (%) = 100 (g in component of insoluble solid fiber fraction)/(g in component of feedstock); Hemicellulose removal (delignification) (%) = 100 (g in component of feedstock − g in component of insoluble solid fiber fraction)/(g in component of feedstock). And the glucose yield was used to evaluate the hydrolyzability of the substrate, which is calculated as below: glucose yield (%) = 100 × 0.9 (g of glucose in hydrolysate)/(g of glucose in substrate). All samples were analyzed in duplicate and the mean values were calculated with <4% standard deviation.
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