Two-Step Saccharification of the Xylan Portion of Sugarcane Waste by Recombinant Xylanolytic Enzymes for Enhanced Xylose Production

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Cite This: ACS Omega 2021, 6, 11772−11782

ABSTRACT: Sugarcane bagasse (SB) and sugarcane trash (SCT) containing 30% hemicellulose content are the waste from the sugarcane industry. Hemicellulose being heterogeneous, more complex, and less abundant than cellulose remains less explored. The optimized conditions for the pretreatment of SB and SCT for maximizing the delignification are soaking in aqueous ammonia (SAA), 18.5 wt %, followed by heating at 70 °C for 14 h. The optimization of hydrolysis of SAA pretreated (ptd) SB and SCT by the Box–Behnken design in the first step of saccharification by xylanase (CtXyn11A) and ε-α-L-arabinofuranosidase (PsGH43_12) resulted in the total reducing sugar (TRS) yield of xylooligosaccharides (TRS(XOS)) of 93.2 mg/g ptd SB and 85.1 mg/g ptd SCT, respectively. The second step of saccharification by xiosidase (BoGH43) gave the TRS yield of 164.7 mg/g ptd SB and 147.2 mg/g ptd SCT. The high-performance liquid chromatography analysis of hydrolysate obtained after the second step of saccharification showed 69.6% xylan-to-xylose conversion for SB and 64.1% for SCT. This study demonstrated the optimization of the pretreatment method and of the enzymatic saccharification by recombinant xylanolytic enzymes, resulting in the efficient saccharification of ptd hemicellulose to TRS by giving 73.5% conversion for SB and 71.1% for SCT. These optimized conditions for the pretreatment and saccharification of sugarcane waste can also be used at a large scale.

1. INTRODUCTION

Indian economy largely depends on agriculture; major population of India relies on agriculture for their living. Wheat, rice, sugarcane, maize, barley, etc. are the major crops grown in India. Approximately, 200 billion tonnes of lignocellulosic biomass is produced from the agriculture sector.1 In 2018, the worldwide production of sugarcane was 1.9 billion tonnes, where Brazil contributed 39% followed by the contribution of India, 20%, to the world’s total production. Sugarcane stems are used for the production of sugarcane juice, which is further used for sugar and bioethanol production. The remaining biomass, sugarcane bagasse (SB), and sugarcane trash (SCT) are considered as solid wastes. Due to the lack of proper management, the wastes produced are burnt for energy production that causes environmental pollution. Instead of burning and causing environmental pollution, both biomasses, SB and SCT, can be used for the production of value-added products such as xylitol, furfural, lactic acid, biobutanol, and bioethanol.

The major plant carbohydrate polymers, such as cellulose and hemicellulose, can be converted into their respective monosaccharides by treatment with cocktail of enzymes.2 Saccharification of pretreated (ptd) banana peels using cocktail of cellulase and pectinase for the production of bioethanol was reported earlier.3 First of all, pretreatment of the biomass is an essential step before the enzymatic saccharification as it increases the surface area and decreases the degree of polymerization.4 Acid pretreatment (H2SO4, HCl, and HNO3) causes the loss of hemicellulosic sugars.5 In general, pretreatment with alkali (NaOH or KOH) and ammonia is preferred for hemicellulosic polymer exposure. Cellulose is a homopolymer of glucose, but hemicellulose is a complex plant polymer because of the presence of side-chain substitutions. Owing to the complexity and less abundance in comparison with cellulose, the saccharification of the hemicellulosic part of lignocellulosic biomass is not considered and that leads to the lower yield of the total reducing sugar (TRS).
The results of SAA pretreatment obtained were similar to the reports published earlier, in which corn stover and elephant grass on SAA pretreatment retain 80%\(^{14}\) and 68.7%\(^{15}\) of hemicellulose, respectively. The chemical bonds of hemicellulose with cellulose and lignin vary with the type of biomass and their structure complexity.\(^{16}\) Therefore, the pretreatment conditions are needed to be optimized for each biomass.

### 2.2. Purification and Assay of Recombinant Xylanolytic Enzymes

The purified fractions after dialysis, analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% (w/v) gel, displayed a single band of molecular mass approximately 65 kDa for \(P_s\)GH43\(_{12}\) (3.4 mg/mL), 40 kDa for \(Bo\)GH43 (1.8 mg/mL), and 25 kDa for \(Ci\)Xyn11A (2.1 mg/mL) (Figure 1). The specific activities determined were 2716 U/mg for \(Ci\)Xyn11A against xylan (Carbosynth), 83.4 U/mg for \(Ps\)GH43\(_{12}\) against rye arabinoxylan (Megazyme), and 78.7 U/mg for \(Bo\)GH43 against xylan (Carbosynth).

### 2.3. Selection of the Best Pretreatment Method Based on Saccharification by \(Ci\)Xyn11A

The enzyme saccharification of ptd biomass by all methods was performed using endo-1,4-\(\beta\)-xylanase, \(Ci\)Xyn11A (100 U/g) at 65 °C, and 50 mM sodium-phosphate buffer, pH 7, for 3 h, and the resulting TRS yield was estimated. Among all the pretreatments, the unoptimized SAA (15 wt %) gave the maximum TRS yield of 8.3 mg/g for SB and 7.8 mg/g for SCT, while the minimum TRS yield was observed with harsh MAIS pretreatment (Table 1). Therefore, SAA pretreatment might have caused the swelling and significant disruption of lignocellulosic biomass with comparatively less hemicellulose loss, leading to enhanced accessibility to the hydrolyzing enzymes.

### 2. RESULTS AND DISCUSSION

#### 2.1. Hemicellulose Content Determination

The hemicellulose content of raw SB and SCT was found to be 28.6% and 27.4%, respectively (Table 1). The maximum hemicellulose content of 23.9% for SB and 22.3% for SCT (percentage is calculated using ptd biomass as base percentage) was retained after soaking in aqueous ammonia (SAA) (15 wt %) pretreatment, although it removed relatively lesser amount of lignin as compared with NaOH or microwave-assisted inorganic salt (MAIS) pretreatment (Table 1). The higher delignification was obtained with harsh MAIS pretreatment with \(Fe\)Cl\(_3\) and \(NaCl\), but both significantly lowered the hemicellulose content, which is required in higher amounts for its saccharification. In another study, 100% hemicellulose loss was reported on pretreatment of olive tree biomass with 0.26 M \(Fe\)Cl\(_3\) at 152.6 °C for 30 min.\(^{13}\) The results of SAA pretreatment obtained were similar to the reports published earlier, in which corn stover and elephant grass on SAA pretreatment retain 80%\(^{14}\) and 68.7%\(^{15}\) of hemicellulose, respectively.

### Table 1. Hemicellulose and Lignin Content and TRS Yield of Untreated and ptd SB and SCT Samples

| pretreatment | hemicellulose (% w/w) | hemicellulose recovery (% w/w) | lignin (% w/w) | TRS yield (mg/g)\(^{14}\) | hemicellulose (% w/w) | hemicellulose recovery (% w/w) | lignin (% w/w) | TRS yield (mg/g)\(^{14}\) |
|--------------|----------------------|-------------------------------|---------------|-------------------|----------------------|-------------------------------|---------------|-------------------|
| untreated biomass | 28.6 ± 0.8 | 21.1 ± 0.75 | 0.7 ± 0.03 | 27.4 ± 0.9 | 19.8 ± 0.85 | 0.4 ± 0.01 |
| 0.6% (w/v) NaOH | 20.1 ± 0.6 | 70 ± 1.1 | 12.7 ± 0.5 | 16.1 ± 0.65 | 67 ± 0.9 | 11.8 ± 0.4 | 2.8 ± 0.08 |
| unoptimized SAA (15 wt %) | 23.9 ± 0.6 | 83 ± 1.3 | 14.2 ± 0.5 | 22.3 ± 1.05 | 79 ± 1.2 | 15.1 ± 0.6 | 7.8 ± 0.25 |
| optimized SAA (18.5 wt %) | 22.4 ± 0.9 | 78 ± 0.8 | 13.2 ± 0.2 | 20.7 ± 0.5 | 75 ± 0.9 | 12.4 ± 0.25 | 14.1 ± 0.5 |
| 1 M NaCl | 16.1 ± 0.45 | 66 ± 0.9 | 9.6 ± 0.35 | 13.2 ± 0.55 | 61 ± 1.1 | 9.8 ± 0.3 | 1.9 ± 0.07 |
| 1 M FeCl\(_3\) | 13.8 ± 0.2 | 58 ± 0.8 | 6.9 ± 0.15 | 9.2 ± 0.35 | 57 ± 0.7 | 7.8 ± 0.2 | 1.4 ± 0.06 |

\(^{14}\) After hydrolysis by endo-1,4-\(\beta\)-xylanase.

SB and SCT contain 33–46% cellulose, 18–29% hemicellulose, and 19–41% lignin.\(^{6}\) The hemicellulose of SB contains 55.2% (w/w) xylose and 10.7% (w/w) arabinose of the total hemicellulose content.\(^{6}\) The enzyme xylanase (endolytically) and xylosidase (exolytically) act on the main chain of xylan polysaccharides.\(^{5–11}\) The use of accessory enzymes, such as \(\alpha\)-L-arabinofuranosidase, to hydrolyse side chains synergistically acts and enhances the rate of overall hydrolysis and increases the production of monosaccharides.\(^{12}\)

The monosaccharides, arabinose, and xylose act as anti-glycemic agents and are of immense importance to the human health. These monosaccharides can be fermented in the food industry for the production of xylitol and arabitol or in fuel industries for enhanced bioethanol production.\(^{7,9}\) The aim of this study was to facilitate the saccharification of the xylan portion of hemicellulose by including an additional enzyme, \(\alpha\)-L-arabinofuranosidase, along with xylanase and xylosidase of sugarcane waste and achieve enhanced TRS yield by optimizing the various conditions of the process. The pretreatment conditions were optimized to reduce the hemicellulose loss during pretreatment. The saccharification of xylan was carried out using recombinant xylanolytic enzymes. The optimized process conditions thus can be used for higher-scale efficient hydrolysis. The hemicellulosic portion of sugarcane waste (SB and SCT) is not explored. This is the first report showing the efficient hemicellulosic saccharification of sugarcane wastes.
enzyme. A similar study reported that SAA pretreatment is a superior method over alkali pretreatment because SAA pretreatment removes lignin more efficiently with a higher recovery of hemicellulose by removing the acetyl and uronic acid substitutions.  

2.4. Optimization of SAA Pretreatment. The optimized conditions for the SAA pretreatment process using the Box–Behnken design were aqueous ammonia concentration of 18.5 wt%, temperature of 70 °C, the solid–liquid (S/L) ratio of 1:9, and the incubation time period of 14 h (Figure S1). After the optimized SAA pretreatment, from 100 g of raw SB and 100 g of raw SCT, the remaining solid biomass residue recovered was 78 g for SB and 75 g for SCT. The hemicellulose content of SB and SCT after optimized SAA pretreatment was 22.4% (17.5 g) and 20.7% (15.5 g), respectively (Tables 1 and S5). This after the saccharification by CtXyn11A gave the TRS yield of 15.3 mg/g of ptd SB and 14.1 mg/g of ptd SCT. In an earlier report, the pretreatment of barley hull at 1:12 S/L ratio, with 15 wt% aqueous ammonia at 75 °C for 48 h, reduced the lignin (w/w) from 19.3% to 7.5% and increased the xylan content by 10–20.5%.  

2.5. FTIR Spectroscopy Analysis of Untreated and ptd SB and SCT. The relative change in the absorbance pattern was observed between the untreated and optimized SAA ptd biomasses (Figure 2). The peak at position 1732 cm⁻¹ attributes to the ester-linked acetyl, feruloyl groups between hemicellulose and lignin, and it started disappearing in ptd SB and SCT. Thus, it showed lignocellulose matrix disruption, leading to delignification. The diminished peak in ptd SB and SCT at 1512 cm⁻¹ also represented lignin removal as this peak occurred due to C=C stretching vibrations in phenol rings in lignin, as also reported earlier. The band at 1378 cm⁻¹ displayed the presence of hemicellulose in the ptd SB and SCT, as also previously reported. The prominent peak obtained in ptd SB and SCT at 897 cm⁻¹ was assigned to the β-glycosidic linkage, which represented a higher exposure of the polysaccharide portion after the pretreatment, as reported earlier.  

2.6. FESEM Analysis of Untreated and ptd SB and SCT. The FESEM images of raw biomasses, such as SB and SCT, showed that their structural integrity was intact as the surface evenness and insignificant surface porosity were observed (Figure 3A,C). However, the SAA ptd SB and SCT showed significant structure disruption, resulting in increased surface roughness and considerable pore formation (Figure 3B,D). The high surface porosity and irregularities are required.
to enhance biomass accessibility to hydrolyzing enzymes, which ultimately improves reducing sugar concentration. The similar result on structure disruption after pretreatment of barley hull and rapeseed straw with SAA was observed by field emission scanning electron microscopy (FESEM).18,23

2.7. Optimization of Hemicellulose Hydrolysis of ptd Sugarcane Biomass (SB and SCT) in the First Step by CtXyn11A and PsGH43_12. The percent biomass loadings, CtXyn11A and PsGH43_12 loadings, varied under defined levels, as mentioned in Section 3.9, were optimized at 40 °C and pH 7.0 for both the sugarcane biomasses. The optimum hydrolysis time was predetermined as 24 h. The Box–Behnken design for the optimization of the hydrolysis of hemicellulose from optimized SAA ptd SB and SCT by endo-1,4-β-xylanase (CtXyn11A) and α-L-arabinofuranosidase (PsGH43_12) and the response, TRS (XOS), yields are shown in Tables S1 and S2, respectively. The second-order quadratic equation for the hydrolysis process of ptd SB was generated by the Design-Expert 7.0 software and is as follows:

$$
\text{TRS yield (mg/g ptd biomass)} = 32.60236 + (14.826 \times \text{biomass loading}) \\
+ (0.154 \times \text{xylanase loading}) \\
+ (0.173 \times \text{arabinofuranosidase loading}) \\
- (0.0035 \times \text{biomass loading} \times \text{xylanase loading}) \\
- (0.0109 \times \text{biomass loading} \times \text{arabinofuranosidase loading}) \\
- (0.00015 \times \text{xylanase loading} \times \text{arabinofuranosidase loading}) \\
- (2.56 \times \text{biomass loading}^2) - (0.00014 \times \text{xylanase loading}^2) \\
- (0.00031 \times \text{arabinofuranosidase loading}^2)
$$

The suggested quadratic model fits well with the response data (Table S1). The model F value of 108.36 and p value of <0.0001 from ANOVA showed that the model was significant. Among the three factors studied, the highest F value of xylanase loading indicated that it is the most significant factor in SB hydrolysis. The p values of the model terms $A_1$, $B_1$, $C_1$, $B_1C_1$, $A_1C_1$, $A_1^2$, $B_1^2$, and $C_1^2$ were less than 0.05, which implied that all of these terms were significant. The model’s regression coefficient was found to be 0.9929, which showed high accuracy of the model (Table S3). The TRS(XOS) increased with an increase in the xylanase and arabinofuranosidase loading (Figure 4A–C). Initially, the TRS(XOS) also increased with an increase in biomass loading up to 3.5%, and then it started decreasing (Figure 4A,B). The predicted optimum biomass loading ($A_1$), xylanase loading ($B_1$), and arabinofuranosidase loading ($C_1$) for SB hydrolysis was 3.45% (w/v), 459 U/g ptd biomass, and 111 U/g ptd biomass, respectively. The predicted TRS(XOS) yield under the above-mentioned conditions was 92.1 mg/g ptd biomass. It was validated by carrying out an experiment (triplicate) in 1 mL reaction volume under the predicted optimum conditions, and the experimental TRS(XOS) yield was 93.2 ± 3.2 mg/g ptd biomass (3.2 mg/mL) (Table S5).

The second-order quadratic equation for the saccharification process of ptd SCT is as follows:

Figure 3. FESEM images of (A) untreated SB, (B) optimized SAA ptd SB, (C) untreated SCT, and (D) optimized SAA ptd SCT.
TRS yield (mg/g ptd biomass)  
= 32.15461 + (14.12 × biomass loading)  
+ (0.119 × xylanase loading)  
+ (0.1416 × arabinofuranosidase loading)  
− (0.0046 × biomass loading × xylanase loading)  
− (0.0122 × biomass loading × arabinofuranosidase loading)  
− (0.000095 × xylanase loading × arabinofuranosidase loading)  
− (2.30 × biomass loading²) − (0.000096 × xylanase loading²)  
− (0.00023 × arabinofuranosidase loading²)

The ANOVA test shows the model F value and p value as 90.33 and <0.0001, respectively. Hence, the quadratic model was significant with a regression coefficient (R²) of 0.9915 (Table S4). The p value being less than 0.05 implied that A², B², C², A₂B₂, B₂C₂, A₂C₂, A₂², B₂², and C₂² were significant model terms. The trend observed for all the three factors (with respect to TRS yield) was similar to the trend observed in the previous model of SB hydrolysis (Figure 4D–F). The predicted optimum biomass loading (A₂), xylanase loading (B₂), and arabinofuranosidase loading (C₂) for SCT hydrolysis were 2.9% (w/v), 447 U/g ptd biomass, and 145 U/g ptd biomass, respectively. The predicted TRS(XOS) yield from the above conditions was 85.8 mg/g ptd biomass, whereas the experimentally observed TRS(XOS) yield was 85.1 ± 2.9 mg/g ptd biomass (2.5 mg/mL) (Table S5).

2.8. Optimization of Hydrolysis of TRS(XOS) Produced in the First Step of Saccharification from ptd Sugarcane Biomass (SB and SCT) by BoGH43 in the Second Step. In the second step of saccharification, the TRS(XOS) produced in the first step was hydrolyzed to xylose by xylosidase, BoGH43, at 37 °C. The second step of saccharification was optimized by following one variable at a time optimization strategy. The
optimized conditions of XOS concentrations of 3 mg/mL TRS(XOS) (for SB) and 2.5 mg/mL TRS(XOS) (for SCT), 20 U/g BoGH43 loading, and the reaction time period of 1 h (Figure 5A–C) were used for the second step of saccharification.

These optimized conditions gave the maximum TRS yields of 1.77 mg/mg of TRS(XOS) and 1.73 mg/mg of TRS(XOS) from SB and SCT, respectively. Hence, after the second step of saccharification, the final TRS yields from SB and SCT were 164.7 mg/g ptd SB (5.31 mg/mL) and 147.2 mg/g ptd SCT (4.32 mg/mL), respectively, using the formula as follows:

\[
\text{Final TRS yield} = \frac{\text{TRS yield (first step saccharification)}}{\text{TRS yield (second step saccharification)}} \times \text{TRS yield (first step saccharification)}
\]

The NaOH ptd finger millet stalk after saccharification by xylanase and xylosidase gave 70 mg/g ptd biomass (1.16 mg/mL) TRS yield,\(^{19}\) whereas corn husk on enzymatic saccharification by xylanase, xylosidase, arabinofuranosidase, and acetyl esterase gave 2.2 mg/mL TRS yield.\(^{24}\) In another report, steam explosion and alkaline delignification were used for SB pretreatment, and hydrolysis by cocktail of cellulase, glucosidase, arabinofuranosidase, and pectinase resulted in a TRS concentration of 3.3 mg/mL.\(^{25}\)

2.9. Thin-Layer Chromatography Analysis of Hydrolyzed Products. The hydrolyzed products obtained after the first and second step of enzymatic saccharification were visualized on the thin-layer chromatography (TLC) plate (Figure 6). The lane 3 in Figure 6A and B showed xylo-oligosaccharides and arabinose produced after the first step of saccharification by endo-1,4-β-xylanase (CtXyn11A) and α-L-arabinofuranosidase (PsGH43_12) from SB and SCT, respectively (Figure 6). The TLC analysis confirmed the hydrolysis of the hemicellulosic part of SB and SCT to xylose and arabinose.

2.10. Monosaccharide Analysis after the Second Step of Saccharification by High-Performance Liquid Chromatography. The TFA hydrolysis of the optimized SAA ptd SB yielded 173 mg of xylose and 21 mg of arabinose/g of ptd SB, whereas that of SCT yielded 139 mg of xylose and 27 mg of arabinose/g of ptd SCT (Table S5). The high-performance liquid chromatography (HPLC) analysis of the hydrolysate

Figure 5. Optimization of the second-step hydrolysis of TRS(XOS) produced in the first-step saccharification by xylosidase, BoGH43: (A) TRS(XOS) loading, (B) BoGH43 loading, and (C) time optimization of the second-step saccharification.

Figure 6. TLC of the hydrolyzed products from ptd sugarcane biomasses by hemicellulases under optimized conditions. SB (A): Lanes: 1, standard xylose, xylobiose, xylotriose, and xylotetraose; 2, standard arabinose; 3, xylo-oligosaccharides and arabinose produced by endo-1,4-β-xylanase (CtXyn11A) and α-L-arabinofuranosidase (PsGH43_12) from SB; and 4, xylose production from SB xylo-oligosaccharides by the action of BoGH43. SCT (B): Lanes: 1, standard arabinose; 2, standard xylose, xylobiose, xylotriose, and xylotetraose; 3, xylo-oligosaccharides and arabinose produced by endo-1,4-β-xylanase (CtXyn11A) and α-L-arabinofuranosidase (PsGH43_12) SCT; and 4, xylose production from SCT xylo-oligosaccharides by the action of BoGH43.
Table 2. Comparison of Xylan-to-Xylose Conversion in This Study with Other Studies

| biomass          | pretreatment conditions                        | saccharification conditions                                      | xylan-to-xylose conversion (%) | reference |
|------------------|------------------------------------------------|---------------------------------------------------------------|-------------------------------|-----------|
| sugarcane bagasse| SAA (18.5 wt % ammonia, 70 °C, 14 h)          | (i) endo-1,4-β-xylanase (CtXyn11A) and α-L-arabinofuranosidase  | 69.6                          | this study|
|                  |                                                 | (PgGH34_12), 40 °C, pH 7.0, 24 h; (ii) exo-1,4-β-xylanosidase  |                               |           |
|                  |                                                 | (BoGH43A), 37 °C, pH 7.0, 1 h                                  |                               |           |
| sugarcane trash  | 1% (w/v) NaOH + autoclave, 120 °C, 60 min      | commercial cocktails (10 FPU/g biomass) supplemented with xylanase | 9.6                           | ref 26    |
|                  |                                                 | (XlnB and XlnC) (7.5 U/g), 50 °C, pH 5.0, 72 h                 |                               |           |
| sugarcane bagasse| ozonolysis (ozone 3.44%, moisture 80%, 1 h)    | 10 FPU/g cellulose and xylanase from Trichoderma reesei, 50 °C, | 52.4                          | ref 29    |
|                  |                                                 | pH 4.8, 24 h                                                   |                               |           |
| sugarcane bagasse| 1:1 mixture of 8.74 M acetic acid and 21.6 M | endo-1,4-xylanases from Penicillium funiculosum + feruloyl     | 65                           | ref 30    |
|                  | H₂O₂, solid–liquid ratio 1:20 m/v, 60 °C, 7 h | esterase in sodium phosphate buffer (pH 5.0), 50 °C, 48 h     |                               |           |
| finger millet straw| 1% (w/v) NaOH combined with oven heating, 120 °C, 20 min | (i) endo-1,4-β-xylanase (CtXyn11A), 55 °C; (ii) exo-1,4-β-xylanosidase | 24.7                          | ref 19    |
|                  |                                                 | (BoGH43A), 37 °C, both at pH 7.5                              |                               |           |
| brewers-spent grain| 5% (v/v) SAA, 70 °C, 22 h | enzyme cocktail comprising xylanase, xylodase, cellulase, and cellobiase, 30 °C, 72 h, pH 5.0 | 44                           | ref 28    |
| corn stover      | Steam explosion at 195–211 °C, 1.4 MPa, 4 min | Cellulase and hemicellulase from Caldibellulosiruptor owensensis, 70 °C, pH 6.0, 48 h | 59                           | ref 27    |
| corn cob         | 0.15 mol oxalic acid, 140 °C, 2.5 h           |                                                               | 85                           | ref 31    |
| sugarcane bagasse| 4 wt % sulfuric acid, 90 °C, 400 min           |                                                               | 80                           | ref 32    |
| sugarcane bagasse| 20 °C and 3.6% (v/v) sulfuric acid, 50 min    |                                                               | 90                           | ref 33    |

After the second step of saccharification of SB yielded 120.4 mg of xylose and 14 mg of arabinose/g of ptd SB and that of SCT yielded 89.2 mg of xylose and 18.9 mg of arabinose/g of ptd SCT. The HPLC results displayed 69.6% and 64.1% xylan-to-xylose conversion with respect to the total xylose present in the optimized SAA ptd SB and SCT biomass, respectively. The percentage of xylose conversion after the second-step hydrolysis was calculated using the formula:

\[
\text{% conversion} = \frac{\text{mg of xylose produced by saccharification} \times 100}{\text{mg of xylose produced by TFA hydrolysis}}
\]

In another report, for finger millet stalk, the xylan-to-xylose percent conversion obtained using endo-1,4-β-xylanase and xylodidase was 24.7%.\textsuperscript{19} Alkali ptd SB hydrolyzed by xylanase along with accellerase from Aspergillus nidulans gave 19.6% xylan-to-xylose conversion.\textsuperscript{26} From corn stover, 59% xylan-to-xylose conversion was reported using enzymes from Caldibellulosiruptor owensensis, with an initial 2% xylan concentration.\textsuperscript{25} Xylan-to-xylose conversion of 44% was reported from ammonia ptd brewers’ spent grain after hydrolysis with enzyme cocktail comprising xylanase, xylodidase, cellulase, and cellobiase.\textsuperscript{28} Pretreatment of SB by ozonolysis followed by enzymatic hydrolysis by the enzyme produced by Trichoderma reesei converted 52.4% of xylan to xylose.\textsuperscript{20} SB on pretreatment with glacial acetic acid and hydrogen peroxide and after enzymatic saccharification by endo-β,1,4-xylanases from Penicillium funiculosum and feruloyl esterase gave 65% conversion of xylan to xylose.\textsuperscript{30} Corn cob after acid hydrolysis with 150 mmol/L oxalic acid gave 85% xylan-to-xylose conversion,\textsuperscript{17} whereas acid hydrolysis of SB by sulfuric acid gave 80%\textsuperscript{32} and 90%\textsuperscript{33} xylan-to-xylose conversion. The higher percentage achieved by the enzymatic saccharification of xylan to xylose in this study was due to the synergistic behavior displayed by the two efficient enzymes, i.e., α-L-arabinofuranosidase (PgGH34_12) with endo-1,4-β-xylanase (CtXyn11A). A brief comparison of xylan-to-xylose conversion from different reports is listed in Table 2.

**2.11. Overall Mass Balance.** The mass balance was carried out for optimized SAA pretreatment and two-step enzymatic hydrolysis. After SAA pretreatment of raw biomass under optimized conditions, 78% and 75% of solid biomass was recovered for SB and SCT, respectively (Figure 7). Out of

![Figure 7. Mass balance for SAA ptd SB and SCT hemicellulose.](https://doi.org/10.1021/acsomega.1c01262)
3. MATERIALS AND METHODS

3.1. Reagents, Substrates, and Materials Used.
Kanamycin, ampicillin, and IPTG were purchased from Sigma-Aldrich Co. LLC., USA. The analytical-grade reagents and chemicals, namely, sodium chloride, sodium acetate, glucose, yeast, extract, peptone, tryptone, di-potassium phosphate, monopotassium phosphate, glycerol, sodium carbonate, sodium bicarbonate, sodium potassium tartrate, sodium sulfate, copper sulfate, ammonium molybdate, trifluoroacetic acid (TFA), Coomassie Brilliant Blue G-250, and sodium arsenate were purchased from Himedia Pvt. Ltd., India. The xylan substrate was purchased from Carbosynth, UK, and rye arabinobioxylan from Megazyme, Ireland.

3.2. Biomass Processing. Sugarcane wastes (SB and SCT) were collected from a local market of Kamrup district of Assam, India. Both biomasses were washed and dried in an oven at 75 °C for 16 h. The dried biomasses were crushed and sieved using an 850 μm sieve.

3.3. Pretreatment of SB and SCT. 3.3.1. Alkaline Pretreatment. Three grams (10%, w/v) of powdered SB or SCT and 30 mL of 0.6% (w/v) NaOH were added in a 100 mL reagent bottle and incubated in a boiling water bath at 100 °C for 1 h. After the pretreatment, the biomass was filtered through a muslin cloth and washed with distilled water to remove residual NaOH. The filtered biomass was kept for drying in a hot air oven at 75 °C for 12 h.

3.3.2. MAIS Pretreatment Using NaCl and FeCl3. Five grams (10%, w/v) of SB or SCT biomass was mixed with 50 mL of 1 M NaCl in 100 mL reagent bottles, followed by heating the samples in an microwave oven at 200 W for 5 min, as reported earlier.34 The ptd biomass was then filtered, washed, and dried at 75 °C for 12 h. Similarly, 5 g of biomass (SB or SCT) was mixed with 50 mL of 1 M FeCl3 in 100 mL reagent bottles and then subjected to heat treatment using a microwave oven at 200 W for 5 min.34

3.3.3. SAA Pretreatment. Three grams (10%, w/v) of SB or SCT biomass was mixed with 30 mL of 0.6% (w/v) NaOH were added in a 100 mL reagent bottle and incubated in a boiling water bath at 100 °C for 1 h. After the pretreatment, the biomass was filtered through a muslin cloth and washed with distilled water to remove residual NaOH. The filtered biomass was kept for drying in a hot air oven at 75 °C for 12 h. Then, the ptd biomass was washed, and dried at 75 °C for 12 h. Similarly, 5 g of biomass (SB or SCT) was mixed with 50 mL of 1 M FeCl3 in 100 mL reagent bottles and then subjected to heat treatment using a microwave oven at 200 W for 5 min.34

3.4. Purification and Assay of Recombinant Xylanolytic Enzymes. The enzymes, endo-1,4-β-xylanase, (CtXyn11A) from Hungateiclostridium thermocellum, α-L-arabinofuranosidase (PsGH43_12) from Pseudomonas stutzeri, and β-xylanase (BoGH43) from Bacteroides ovatus, were used in this study. The recombinant plasmids expressing CtXyn11A and BoGH43 were gifted by Prof. Carlos Fontes of NZT Tech Pvt. Ltd., Portugal. The cloning, expression, and biochemical characterization of CtXyn11A,35 PsGH43_12, and BoGH4336 were reported earlier. The enzymes were purified by immobilized metal-ion affinity chromatography (IMAC) using a Sepharose column (HiTrap Chelating, GE Healthcare, USA).37 The xylanase (CtXyn11A) activity was measured by estimating the liberated reducing sugar from xylan (Carbosynth). The reaction mixture (100 μL) contained 50 μL of 1% (w/v) xylan in 50 mM sodium phosphate buffer (pH 7.5), 40 μL of 50 mM sodium phosphate buffer (pH 7.5), and 10 μL of purified enzyme (5 μg/mL) solution. The reaction mixture was incubated at 65 °C for 1 min. The arabinofuranosidase (PsGH43_12) activity was measured by estimating the liberated reducing sugar (arabinose) from rye arabinbioxylan. The reaction mixture (100 μL) containing 90 μL of 1% (w/v) rye arabinbioxylan dissolved in 50 mM sodium phosphate buffer (pH 7.5) and 10 μL of enzyme solution (50 μg/mL) was incubated at 50 °C for 5 min. The xylosidase (BoGH43) activity was measured by estimating the liberated reducing sugar (xylose) from xylan (Carbosynth). The reaction mixture (100 μL) containing 90 μL of 1% (w/v) xylan dissolved in 50 mM sodium phosphate buffer (pH 7.5) and 10 μL of enzyme solution (50 μg/mL) was incubated with xylan at 40 °C for 5 min. The concentration of reducing sugars was determined by following the method of Nelson38 and Somogyi,39 using respective standards, xylose and arabinose. The absorbance at 500 nm was recorded using a spectrophotometer (Thermo Scientific Multiskan SKY, Singapore). One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of product per min, under their respective optimum conditions.

3.5. Selection of the Best Pretreatment Method. The biomass obtained from each pretreatment was hydrolyzed by endo-1,4-β-xylanase, CtXyn11A, and the resulting TRS yield was the basis for the selection of the best pretreatment method. In total, 1% (w/v) of each ptd biomass of SB or SCT was treated with xylanase with a total enzyme loading of 100 U/g of ptd biomass, dissolved in 50 mM sodium phosphate buffer (pH 7.5), and the total reaction volume used was 1 mL. Then, the reactions were incubated in a shaking water bath at 150 rpm, at 65 °C for 3 h. After the incubation, TRS concentration was determined by the method of Nelson38 and Somogyi.39

3.6. Optimization of SAA Pretreatment. The parameters of the pretreatment process to be optimized were percentage of aqueous ammonia used, temperature, time, and solid–liquid ratio. After the optimized pretreatment, the hemicellulose content of ptd SB and SCT was determined by the TAPPI40 method. The response factor chosen for the analysis of the experimental run was the TRS concentration. The TRS was estimated after the saccharification of ptd biomass by treating with 100 U/g xylanase (CtXyn11A). All the analysis was carried out in technical replicates.

3.7. FTIR Analysis of Untreated and ptd Biomass (SB and SCT). The untreated and ptd biomass (SB and SCT) samples by SAA (18.5 wt % + heat treatment at 70 °C for 14 h) were analyzed by Fourier transform infrared spectroscopy (FTIR). Potassium bromide (KBr) (200 mg) was mixed with each dried biomass sample in a ratio of 100:1 (KBr:biomass) and ground using a mortar and pestle, and pellets were made using a hydraulic press. The pellets were scanned using an FTIR spectroscope (Spectrum Two, Perkin-Elmer, Waltham, MA) within the wavenumber range of 4000–450 cm⁻¹.

3.8. FESEM Imaging of Untreated and ptd Biomass (SB and SCT). SB and SCT biomass, untreated and ptd (by SAA, 18.5 wt % combined with heat treatment at 70 °C for 14 h) samples, were separately (approx. 200 μg each) placed on a carbon tape and fixed to a stub surface. Each sample on the stub was gold coated (double) and kept in a vacuum chamber to remove the remaining moisture from samples. Then, the samples were scanned for surface imaging by FESEM (Sigma, Zeiss, Germany).

3.9. Optimization of Enzymatic Saccharification. The optimization of enzymatic saccharification of the optimized SAA ptd SB and SCT was carried out in two steps. In the first step, the optimization was performed using the Box–Behken
design. In the first step of saccharification, the biomass loading (1%-5%, w/v) and enzyme loading of xylanase CtXyn11A (50-500 U/g) and α-L-arabinofuranosidase and PsGH43_12 loading (20-200 U/g) were optimized. The reactions were carried out in 1 mL volume in 50 mM sodium phosphate buffer at pH 7.0 in a 2 mL microcentrifuge tube incubated at 40 °C and 150 rpm for 24 h. The TRS-containing XOS (TRS(XOS)) obtained in the first step of enzyme saccharification was used for the second step of enzyme saccharification. The second step of enzyme saccharification was optimized by one variable at a time optimization strategy. After the first step, TRS(XOS) concentration obtained was 3.2 mg/mL for SB and 2.5 mg/mL for SCT, as calculated and described in Section 3.7. Therefore, the TRS(XOS) loading was varied from 0.5 to 3 mg/mL for SB and it was varied from 0.5 to 2.5 mg/mL for SCT, keeping the constant enzyme loading at 100 U/g BoGH43 and the constant reaction time of 4 h. The enzyme, BoGH43, loading was varied from 4 U/g to 200 U/g using the optimized TRS(XOS) loading and the reaction time of 4 h. The time period of enzyme saccharification was varied from 10 min to 4 h using optimized TRS(XOS) concentration and optimized BoGH43 loading. All the reactions were carried out in 1.0 mL of 50 mM sodium phosphate buffer at pH 7.0 by incubating at 37 °C and 150 rpm.

3.10. TLC Analysis. The hydrolyzed products of the ptd biomasses obtained from the enzymatic hydrolysis using CtXyn11A and PsGH43_12 under the optimized conditions (Section 3.9) in the first step of saccharification and also the hydrolyzed product obtained by BoGH43 after the second step of saccharification were filtered through a 0.4 μm filter membrane (PVDF), separately. A sample mixture (0.5 μL) of standards containing xylose, xylobiose, xylotriose, xylotetraose, and arabinose (2 mg/mL) procured from Sigma-Aldrich, USA, were loaded on the TLC plate (4 x 11 cm, TLC silica gel 60 F254, Merck, Germany) and dried at 70 °C for 10 min. A mixture containing glacial acetic acid, chloroform, and water in a ratio of 6:7:1 (v/v) was used as the mobile phase. The TLC plate was kept inside the developing chamber and run for 90 min. After the run, the plate was dried and then immersed in the visualizing solution containing sulfuric acid/methanol (5:95, v/v) and 0.5% w/v α-naphthol and then dried at 80 °C to visualize the hydrolyzed products.

3.11. Determination of Released Products after the Second Step Saccharification by HPLC. One gram of SB or SCT was treated with 1 mL of 2 M TFA in a 2 mL microcentrifuge tube (MCT) and kept in a boiling water bath for 2 h. Both the tubes were centrifuged at 14 000g at 25 °C for 15 min. The supernatants were filtered through a syringe filter using 0.2 μm membrane and kept in a hot air oven at 75 °C for 12 h. One milliliter of degassed Milli-Q water was added to the tube and mixed well. The monosaccharides were estimated by HPLC. The total xylose and arabinose concentration in the TFA-treated sample was taken as the total hemicellulose content available for the enzymes (CtXyn11A, PsGH43_12, and BoGH43) during the saccharification process under optimized conditions. The percentage of xylan-to-xylose conversion during saccharification was calculated by determining the xylose in the TFA-treated sample (total xylan present) and the saccharified sample (hydrolyzed xylose). The xylose concentration determination was carried out by the HPLC method described earlier. The concentration of each sugar in the sample was calculated with respect to the area of the corresponding peaks of the standard sugars.

4. CONCLUSIONS

The lignocellulosic biomasses, SB and SCT, were ptd for delignification. The xylan portions of the hemicellulosic part of SB and SCT were saccharified by recombinant enzymes. The SAA pretreatment was found to be better than alkali or MAIS pretreatments. The SAA pretreatment method parameters were optimized for maximum delignification with least hemicellulose loss. The optimized conditions of SAA pretreatment by the Box–Behnken design were aqueous ammonia of 18.5 wt %, temperature of 70 °C, solid–liquid ratio of 1:9, and time period of 14 h. SAA pretreatment being less harsh causes comparatively lower loss of xylan. The first step of saccharification of ptd SB and SCT by CtXyn11A and PsGH43_12 gave TRS yields of 93.2 mg/g ptd SB and 85.1 mg/g ptd SCT. The XOS produced in first step are of immense importance as functional food prebiotic additives. The second-step saccharification gave final TRS yields of 164.7 mg/g for ptd SB and 147.2 mg/g for ptd SCT. The HPLC analysis displayed 69.6% xylose-to-xylose conversion for ptd SB and 64.1% for ptd SCT. The study showed an overall hemicellulose conversion of 73.5% from SB and 71.1% from SCT. This pretreatment and saccharification conditions can be used for the large-scale production of xylose and in turn xylitol or bioethanol production.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c01262.

Three-dimensional response surface plots for the interaction between the independent variables involved in the optimization of pretreatment. (A) Pretreatment optimization for SB, (B) pretreatment optimization for SCT. (i) Temperature and ammonia concentration, (ii) time and ammonia concentration, (iii) S/L ratio and ammonia concentration, (iv) time and temperature, (v) S/L ratio and temperature, and (vi) S/L ratio and time (Figure S1); Box–Behnken design and response for hemicellulose hydrolysis from pretreated SB by endo-1,4-β-xylanase (CtXyn11A) and α-L-arabinofuranosidase (PsGH43_12) (Table S1); Box–Behnken design and response for hemicellulose hydrolysis from pretreated SCT by endo-1,4-β-xylanase (CtXyn11A) and α-L-arabinofuranosidase (PsGH43_12) (Table S2); ANOVA for the quadratic model of hemicellulose hydrolysis from pretreated SB by endo-1,4-β-xylanase (CtXyn11A) and α-L-arabinofuranosidase (PsGH43_12) (Table S3); ANOVA for quadratic model of hemicellulose hydrolysis from pretreated SCT by endo-1,4-β-xylanase (CtXyn11A) and α-L-arabinofuranosidase (PsGH43_12) (Table S4); Hemicellulose content and TRS yield after each step (Table S5) (PDF)

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https://doi.org/10.1021/acsomega.1c01262
ACS Omega 2021, 6, 11772−11782
Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.1c01262

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research work on the saccharification of SCT was financially supported by a joint project (no. BT/PR20671/PBD/26/528/2016) with the Avantha Centre for Industrial Research and Development, Yamuna Nagar, Haryana, India. The work on α-1-arabinofuranosidase (PsGH43_12) and the related SB saccharification was supported by a trilateral project with IIT Bombay, Mumbai, India, and All India Institute of Medical Sciences, New Delhi, under the DBT-Twining project scheme (no. BT/PR24786/NER/95/853/2017) from the Department of Biotechnology (DBT), Ministry of Science and Technology, New Delhi, Government of India, to A.G. The authors acknowledge the use of an FTIR spectrophotometer procured from the India–Finland project (BT/IN/Finland/08/AG/2011) funded by the DBT, Government of India, to A.G. and FESEM facility at Central Instrumental Facility, IIT Guwahati, India.

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