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Synergistically Enhanced Enzymatic Hydrolysis of Sugarcane Bagasse Mediated by a Recombinant Endo-Xylanase from *Streptomyces ipomoeae*

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Abstract: Xylanase is commonly thought to effectively cooperate with cellulase to promote the bioconversion of lignocellulose. In this study, a novel xylanase, SipoEnXyn10A (Xyn10A), previously identified from *Streptomyces ipomoeae*, was employed to investigate its synergetic effects on sugarcane bagasse (SCB) transformation. It was shown that the relative increase in reducing sugars reached up to 65%, with enhanced yields of glucose and xylose by 78% and 50%, respectively, in the case of the replacement of cellulase with an equivalent amount of Xyn10A at an enzyme loading of 12.5%. The highest degrees of synergy (DS) for glucose and xylose could reach 2.57 and 1.84. Moreover, the hydrolysis rate increased evidently, and the reaction time to reach the same yield of glucose and xylose was shortened by 72 h and 96 h, respectively. This study on synergistic mechanisms demonstrated that the addition of Xyn10A could cause the destruction of substrates' morphology and the dissolution of lignin components but could not change the accessibility and crystallinity of substrate cellulose. The joint effect of cellulase and xylanase during the hydrolysis process was thought to result in a synergistic mechanism.

Keywords: endo-xylanase; hydrolysis; sugarcane bagasse; synergistic mechanism

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1. Introduction

Agricultural and forestry waste, including sugarcane bagasse, rice straw, wheat straw, white poplar, spruce, switchgrass, etc. [1,2], is known as a cheap, abundant, and innoxious lignocellulosic biomass. The global yield of bagasse has reached 5.6 billion tons annually [3] and China alone produces 33 million tons of bagasse annually [4], resulting in a residual accumulation problem that urgently needs solving. At present, a recognized effective way to degrade lignocellulosic materials for producing sugars has appeared [5], further converting these into biofuels, fine chemicals, etc. In this way, this environmental problem would be addressed through the high value-added utilization of the lignocellulosic materials.

The enzymatic hydrolysis of structural polysaccharides into fermentable sugars is seen as a key step in developing an economic and effective saccharification process. As the lignocellulosic matrix composed of cellulose, hemicellulose, and lignin biopolymers [6] exhibits a complex three-dimensional framework structure [7], a single degrading enzyme is notoriously unable to achieve the sufficient hydrolysis of lignocellulose [8]. The strategy of employing a multi-enzyme system with synergistic action [9–12] has been recognized as effective for achieving maximal hydrolysis efficiency. Particularly, those enzymes that give green high-value-added by-products are a possibility worth looking into, from the perspective of improving the comprehensive economic benefits of the process [13,14].

Being one of the hemicellulases, xylanases can degrade xylan into xylose and xylooligosaccharides (XOS) by randomly cleaving β-1,4-linked D-xylopyranose units from

the homopolymeric backbone structure [15]. The role of xylanase in advancing the hydrolysis of lignocellulose has been acknowledged [16], and more importantly, the by-product XOS is of great economic value, as it has immunomodulatory, anti-cancer, antioxidant, and hypolipidemic effects [17–19], as well as better biological properties than other oligosaccharides such as a higher resistance to digestion and the ability to stimulate the growth of bifidobacteria [20]. Therefore, the addition of xylanase into the hydrolysis process is promisingly valuable due to improving not only the utilization of lignocellulose substrate but also the process's economic benefits. Shibata et al. [21] reported that adding xylanase caused an increase in the glucose and xylose yields from 74% and 73% to 97% and 95%, respectively, when using SCB as the raw material. Accordingly, the synergistic improvement achieved 30% for these two products. Similar synergistic effects could be observed when various other substrates were employed, including wheat straw [22], rice straw [23], corn stover [24], and poplar [25].

To date, xylanase has been reported which is from different sources and possesses some unique properties, offering many choices for developing the mixed enzyme system to hydrolyze lignocellulose [26–28]. The overall synergistic efficiency of the hydrolysis process has been found to be related to reaction conditions besides the properties of the employed xylanase [29]. Many examples [30] have shown that the ratio between xylanase and cellulase influences the sugar yields. Wang et al. [31] reported a 61.6% increase in hydrolysis efficiency through their pretreatment investigation. Lan et al. [32] optimized the pH so as to improve the hydrolysis efficiency by 80%. Consequently, to meet the different requirement with various raw materials and enzymes, the optimization of the reaction conditions cannot be ignored. More importantly, although many studies have been carried out on the synergism of xylanase against cellulase, the mechanism behind this synergistic effect during the hydrolysis of lignocellulosic substrates is still unclear.

The xylanase Xyn10A from *Streptomyces ipomoeae*, identified in our previous work, has proven to efficiently hydrolyze the xylan of agricultural and forestry residues [33]. In this study, SCB, one of the agricultural and forestry wastes, was chosen as a model to explore the synergistic effect of xylanase and cellulose, as it is derived from sugarcane, which is widely cultivated worldwide [34]. The results showed that the enzyme mixture of Xyn10A and cellulase had good synergy in the SCB hydrolysis. The yields of both glucose and xylose were significantly increased and the reaction period was shortened, indicating that Xyn10A is a promising candidate enzyme for the lignocellulose hydrolysis process. The mechanism of the synergistic effect of multiple enzymes was also discussed.

2. Materials and Methods

2.1. Materials and Enzymes

The SCB was generously provided by Guangxi Bosco Environmental Protection Technology Co., Ltd. (Nanning, China) and was washed at least five times with tap water to remove the major soluble sugars. Afterwards, the SCB was dried and then mechanically ground to pass through a 60-mesh sieve for subsequent pretreatment. Commercial cellulase C2730 and β-glucosidase S10048 were purchased from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). The details of the expression and purification of the Xyn10A were described in a previous work [33]. Unless otherwise noted, the chemicals used in this study were all of analytical grade.

2.2. Pretreatment of SCB

The mechanical milling (MM) SCB was selected as a control. The pretreatment methods used were extracted from the previous literature [35–37]. For hydrogen peroxide (HP) or sodium hydroxide (SH) pretreatment, SCB was pretreated with 2% (w/v) H_2O_2 or NaOH in an autoclave at 121 °C for 20 min with a solid-to-liquid ratio of 1:10 (g/mL). For alkaline hydrogen peroxide (AHP I) pretreatment, the SCB was first treated according to the same procedure as the SH, and then 5% (w/v) H_2O_2 was mixed into the pretreated slurry after cooling to room temperature. The mixture was kept in an airtight container in a dark place

for 24 h. For AHP II pretreatment, the SCB was pretreated with a mixture of 2% (*w*/*v*) H_2O_2 and 0.5% (*w*/*v*) $MgSO_4$ with pH adjusted to 11.6 with NaOH at a solid to liquid ratio of 1:20 (g/ mL) for 4 h at 60 °C in a constant-temperature water bath. At the end of the reaction, the pretreated solids of SH, AHP I, and AHP II were washed with deionized water until neutral pH was achieved, while the HP-pretreated solids were washed with deionized water equal to 10 times the volume of the pretreated liquid to remove as many undesirable derivatives as possible. All of the rinsed solids were dried at 50 °C and collected for subsequent experiments.

2.3. Selection of Pretreatment Methods

The enzymatic hydrolysis was carried out on the different pretreated SCB samples, which were loaded at 2% (*w*/*v*) and placed in 2 mL of 0.1 mol/L citric/Na_2HPO_4 buffer (pH5.5) containing C2730 (2 mg protein/g substrate) and S10048 (1 mg protein/g substrate) with (for the mixed enzyme group) or without (for cellulase only) xylanase (1 mg protein/g substrate). The hydrolysis process was carried out in a water bath oscillator at 50 °C and at 150 r/min for 24 h. To establish the experimental control, the enzyme group was inactivated by boiling water for 20 min. The samples (1 mL) were taken from the reaction mixture for the analysis of reducing sugars using the dinitrosalicylic acid method [38]. Glucose was used as the standard for the mixed enzyme group and the monocellulase group, while xylose was taken for the monoxylanase group. The optimum pretreatment method was determined by the yield of reduced sugar obtained through hydrolysis. All experiments were performed at least in triplicate.

2.4. Effect of pH and Temperature on Synergistic Characteristics

The influence effects were characterized by measuring the reducing sugar yield on the AHP I SCB substrate at 2% (*w*/*v*) in 2 mL of 0.1 mol/L citric/Na_2HPO_4 buffer for 24 h. The reaction mixtures were placed in a water bath oscillator at 150 r/min. A series of reactions were carried out under the conditions of pH 4.5–6.5, temperature 50 °C; pH 5.5, temperature 45–65 °C. The total protein loading was 4 mg protein/g substrate with a ratio of C2730, S10048, and Xyn10A of 2:1:1. The experimental control was the group of enzymes that were inactivated by boiling water for 20 min. Following the reaction, the samples (1 mL) were extracted for the analysis of reducing sugars. All experiments were performed at least in triplicate.

2.5. Enzymatic Hydrolysis

The AHP I SCB in the proportion of solid to liquid of 2% (*w*/*v*) was weighed into 2 mL of 0.1 M citric/Na_2HPO_4 buffer (pH 5.5) in a 10 mL Eppendorf tube with a cover. The hydrolysis process was executed in a water bath oscillator set at 50 °C and 150 r/min. To investigate the impact of enzyme loading, the cellulase (C2730 and S10048) enzyme loading was replaced with various ratios of Xyn10A while maintaining a consistent enzyme concentration of 6 or 12 mg protein/g substrate, with a steady ratio of 3:1 for both C2730 and S10048. When measuring the hydrolysis process, a total enzyme concentration of 12 mg protein/g substrate with a ratio of 12.5% for Xyn10A was employed for the hydrolysis for from 3 to 120 h. Samples were withdrawn at specific times and inactivated by incubation at 100 °C for 10 min. The supernatant was collected for the analysis of glucose and xylose. Experimental control was performed as described above.

2.6. Xyn10A Pre-Adsorption Experiments and Enzymatic Hydrolysis

To prevent the 2% (*w*/*v*) substrate concentration of AHP I SCB from being hydrolyzed by Xyn10A in 2 mL of 0.1 mol/L citrate/Na_2HPO_4 buffer (pH 5.5), 12.5% of Xyn10A was added first, and the pre-adsorption experiments were performed at 4 °C [39,40]. The mixtures were then allowed to stand for 5 h and stirred every hour to achieve equilibrium for Xyn10A adsorption. After standing, 87.5% of the cellulase (3:1 for C2730:S10048) loading was added to the mixture as a mixed enzyme group containing Xyn10A pre-

sorption. For comparison, another mixed enzyme group started with the addition of 12.5% of Xyn10A only when the corresponding cellulase was added, which did not perform the pre-adsorption of Xyn10A. The enzyme group that was inactivated by boiling water for 20 min was set as the experimental control. The reaction mixtures were placed in a water bath oscillator at 50 °C and 150 r/min, and the reaction times were set at 24 h, 48 h, and 72 h. After the reaction, the samples were withdrawn at specific times and tested for the analysis of glucose and xylose.

2.7. Analytical Methods for SCB Chemical Composition

The chemical composition of SCB was analyzed following the standard procedure for biomass analysis provided by the National Renewable Energy Laboratory (NREL), Golden, CO, USA [41]. To begin with, 0.3 g of the samples were hydrolyzed by 3 mL of 72% (w/w) sulfuric acid for 1 h in a water bath at 30 °C. After this, 84 mL of deionized water was added to dilute the sulfuric acid to a concentration of 4% (w/w). The mixture was then autoclaved at 121 °C for 1 h. Once cooled, the slurry was filtered using a filtering crucible, and the solid component was dried at 105 °C for 6 h until a constant weight was obtained. The dried solid was then heated at 575 °C for 24 ± 6 h and weighed again to calculate the amount of acid-insoluble lignin (AIL). The liquid obtained after filtration was adjusted to neutral pH, and the concentrations of monosaccharides, such as glucose and xylose, in the neutral liquid were measured by the HPLC system.

The HPLC (ULTIMATE 3000, Thermo scientific, Waltham, MA, USA) system was set up with an ELSD detector (SEDEX 85, Cedex, France) and a Hypersil NH_2 column (250 × 4.6 mm, particle size of 5 μm) for the purpose of detecting the concentrations of glucose and xylose. The mobile phase was 85:15 (v/v) acetonitrile and water at a flow rate of 2 mL/min. The detector operated at a carrier-gas pressure of 3.5 bar and at 40 °C, while the column was maintained at 30 °C. Prior to analysis, all samples were filtered through a 0.22 μm micro-filter before being subjected to the HPLC system.

2.8. Calculation Method of the Hydrolysis Efficiency and the Degree of Synergy

Glucose and xylose yields were calculated according to the method described previously, and as follows [21].

$$\textit{Glucose yield}\ (\%) = \frac{m_1 \times \frac{162}{180}}{m_2} \times 100\% \quad (1)$$

$$\textit{Xylose yield}\ (\%) = \frac{m_3 \times \frac{132}{150}}{m_4} \times 100\% \quad (2)$$

where m_1 is the mass of glucose released, and m_2 is the mass of cellulose in the substrate; m_3 is the mass of Xylose released, and m_4 is the mass of xylan in the substrate.

The degree of synergy (DS) is defined as the ratio of products formed from the enzymes mixture to the sum of the products of the individual enzymes [42]:

$$\mathrm{DS} = \frac{Y_{1+2}}{Y_1 + Y_2} \quad (3)$$

where Y_{1+2} is the sugar released during hydrolysis when cellulase and xylanase are both added. Y_1 and Y_2 are the sugars, respectively, when cellulase and xylanase are added individually during hydrolysis.

2.9. Field Emission Scanning Electron Microscope (FE-SEM) Analysis

The effect of enzymatic hydrolysis on the SCB was examined using an emission scanning electron microscope (ZEISS SIGMA 500, Bruker, Cambridge, UK) operating at 3 kV. SCB was enzymatically hydrolyzed for 72 h and then dried at 50 °C. Before imaging, SCB samples were coated with a thin layer of gold to render them electrically

conductive, preventing charge accumulation and sample damage. Images were captured at magnifications ranging from approximately 600× to 20,000×.

2.10. Crystallinity Analysis

The crystallinity of the SCB samples was determined by X-ray diffraction (XRD) (Rigaku D/MAX 2500V, Rigaku, Tokyo, Japan). The X-ray diffractograms were recorded from diffraction angles (2θ) ranging from 5° to 60° at a scanning speed of 5°/min. The crystallinity index (CrI) was calculated using Segal's method [43] and the following expression:

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \times 100\% \quad (4)$$

where I_{002} is the intensity of the peak at a 2θ angle close to 22.5° and I_{am} is the scattering intensity of the amorphous fraction at a 2θ angle close to 18°.

2.11. Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR spectra were utilized for the analysis of various SCB samples. A mixture of SCB and KBr at a ratio of 1:100 was thoroughly ground until evenly mixed and then compressed into slices for detection and analysis using FT-IR (NICOLET IS 50, Thermo scientific, Waltham, MA, USA) with a scan resolution of 4 cm^{-1} and a wave number range of 4000–400 cm^{-1}.

3. Results and Discussion

3.1. Determination of Pretreatment Methods

In view of the fact that it is difficult to make natural lignocellulose an enzymatic hydrolysis reaction directly [44], pretreatment is useful as it can destroy the lignocellulose structure, remove lignin, and make the substrate much looser and easier to hydrolyze [45]. The main chemical compositions of the different pretreated SCB are shown in Table 1. The MM SCB contained a cellulose content of 43.0%, the xylan 20.35%, and the AIL 22.47%. This basically accorded with the reported content distribution law of the three components of SCB [46]. After HP pretreatment, the composition of each component was roughly unchanged as well as the reducing sugar yield (Figure 1), meaning this chemical procedure did not work in this case. An approximately 50%-higher content of cellulose was observed for the other pretreatment methods, which could be seen as the function of alkali causing the intermolecular ester bonds cross-linking the carbohydrates and lignin to be destroyed and the lignin to be partly removed [47]. In addition, the AHP pretreatments seemed to remove nearly 75% of the lignin. Ho et al. [48] pointed out that AHP pretreatment has the ability to selectively attack carbonyl and ethylene, thus enhancing the delignification of lignocellulosic biomass.

Table 1. The chemical composition of SCB with different pretreatments.

Pretreatment Methods of SCB [a]	Cellulose (%) [b]	Xylan (%)	AIL (%) [c]
Mechanical Milling (MM)	43.00 ± 1.42	20.35 ± 0.83	22.47 ± 1.46
Hydrogen Peroxide (HP)	47.14 ± 0.15	17.02 ± 0.35	22.24 ± 0.33
Sodium Hydroxide (SH)	61.37 ± 0.54	23.83 ± 1.29	7.02 ± 0.26
Alkaline Hydrogen Peroxide I (AHP I)	63.66 ± 0.09	22.39 ± 0.03	5.17 ± 0.19
Alkaline Hydrogen Peroxide II (AHP II)	60.02 ± 1.70	25.05 ± 0.31	5.02 ± 0.16

[a] SCB, sugarcane bagasse; [b] cellulose content of different pretreated SCBs was significantly different (p-value < 0.01); [c] AIL, acid insoluble lignin.

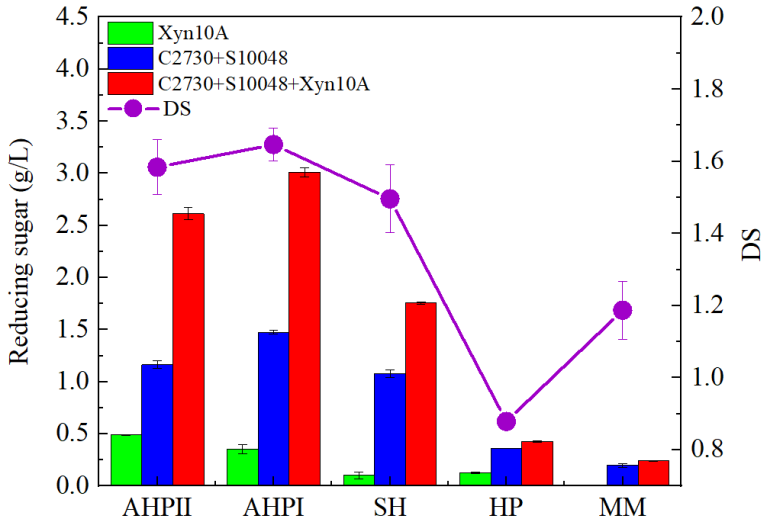


Figure 1. Degradation of five different pretreated SCBs by synergizing of Xyn10A with C2730 and S10048. Values are the mean ± SD of three parallel experiments.

Figure 1 shows the reducing sugar yield obtained by the enzymatic hydrolysis of different pretreated SCBs. Apparently, the pretreatment method greatly influenced the sugar yield. The MM and HP SCBs had the lowest sugar yield, a result that was predictable since there was no substantial change in the major components. AHPII pretreatment gave the highest sugar yield if Xyn10A was employed to hydrolyze the SCB, whereas AHP I became the best if cellulase was used. This phenomenon corresponded to the highest xylan content of AHP II and the highest cellulose content of AHP I. Nevertheless, comparing other pretreatment methods, it can be found that the order of xylan content in the different pretreated SCBs was AHP II > SH > AHP I > MM > HP, while the order of sugar yield from Xyn10A enzymatic hydrolysis was AHP II > AHP I > HP > SH > MM; there was no one-to-one correspondence between the two. Similarly, the order of the cellulose content and the order of the sugar yield obtained by enzymatic hydrolysis of only cellulase were not completely corresponding. This showed that the structure of the SCB was changed after pretreatment, and the polysaccharide content in the substrate was not the only factor that affected the sugar yield. As for the synergistic enzyme group, the change trend for sugar production was exactly the same as that of the monocellulase group, indicating that the AHP pretreatments, especially the AHP I, appear to be superior to the other pretreatments (Figure 1), which was consistent with previous studies. For example, Xu et al. [49], thought that the pretreatment of SCB with alkaline hydrogen peroxide, was able to avoid the non-productive adsorption of lignin on cellulase, reducing the inhibitory effect caused by lignin. Zhang et al. [50] proved that pretreatment with alkaline hydrogen peroxide can enhance the enzymatic digestibility of SCB due to the degradation of hemicellulose and lignin. Such a result could be attributed to the fact that the lignin content of the AHPs decreased greatly, possible because lignin and lignin-derived phenolic compounds inhibit or even inactivate the enzymes [51]. On the other hand, Monte et al. [37] stated that the porosity was very important for the enzymatic hydrolysis of SCB, as the increased porosity promoted the accessibility of the pretreated SCB to enzymes, which in turn increased the yield of reducing sugars for enzymatic hydrolysis. Reports by Cao et al. [36] and Huang et al. [52] also addressed that higher porosity was obtained using AHP SCB. In summary, AHP I, the pretreatment with the highest sugar yield and synergy, was selected as the pretreatment method for later research.

3.2. Effect of pH and Temperature on Synergistic Characteristics

It was generally agreed that the optimum pH and temperature for the cellulase produced by *Trichoderma reesei* were near 5 and 45 °C [53], while for Xyn10A, they were 6.5 and 75 °C [33]. Since these two enzymes had different catalytic properties, it is necessary to

explore the effect of pH and temperature on the hydrolysis when using the mixed enzymes. As shown in Figure 2, the mixed system displayed synergistic action within the whole selected range.

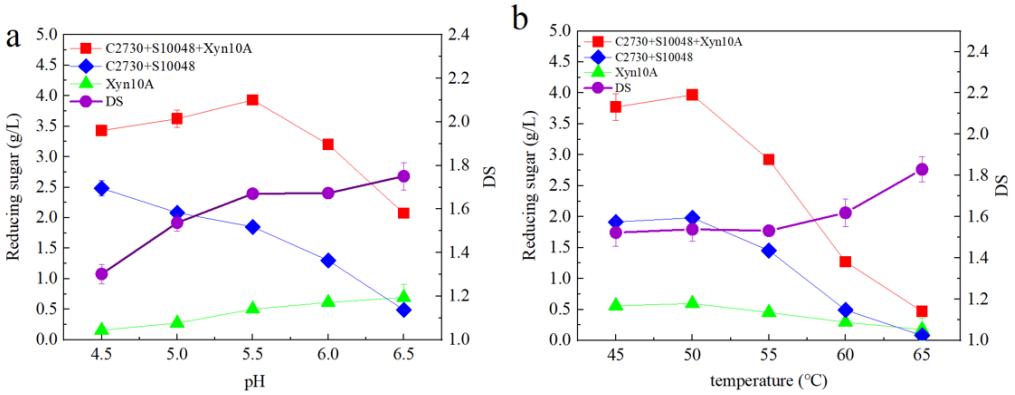


Figure 2. Effects of (**a**) pH and (**b**) temperature on reducing sugar yield and DS of each system. (■) mixed enzyme group, (♦) single cellulase group, (▲) single Xyn10A group, (●) degree of synergy. Values are the mean ± SD of three parallel experiments.

As shown in Figure 2, under the conditions of pH 5.5 and temperature 50 °C, the mixed enzyme group (xyn10A and cellulase) had the best reducing sugar yield and synergism. Unlike the case with only the cellulase, the sugar production mediated by the enzyme mixture first increased and then decreased with the pH (Figure 2a). The maximum production occurred at pH 5.5, higher than the max at pH 4.5 when only using the cellulase. This should be taken as a joint result of the monotonical increase in Xyn10A activity and the decrease in cellulase activity with the increase in pH. And the production at pH 6.0 (3.20 g/L) was similar to that at pH 4.5 (3.43 g/L), showing that the addition of Xyn10A enabled the synergy enzyme group to maintain the sugar yield in a wider pH reaction system, which is undoubtedly beneficial to the industrial application of enzymes. In terms of the synergy degree, it kept increasing with the increase in pH, showing the same trend as that of the Xyn10A. The DS could achieve 1.75 at pH 6.5, but it is probably not applicable for higher pH levels because the sugar production decreased significantly after this. This suggests that the degree of the DS may be mainly due to the action of xylanase, whereas the rate of sugar production is more related to the catalytic efficiency of cellulase. Shibata et al. proved that the xylanase was the main contributing factor of the synergistic effect in the case of SCB degradation by xylanase and cellulase [21]. Jia et al. [54] even found that synergy between different families of xylanases was responsible for the increased efficiency of bagasse hydrolysis.

As shown in Figure 2b, the changes in the sugar productions with the temperature were very similar for the cellulase and cellulase–xylanase systems. The sugar production began to plummet from 50 °C and, especially in the case of the synergistic enzyme group, only 11.8% of the yield was retained at 65 °C relative to that at 50 °C. It was found that about 50 °C was the optimum temperature as it agreed well with most of the reports on the cellulase–xylanase synergistic system [55]. Figure 2b shows that the sugar production of Xyn10A was found not to change much with the temperature, conforming to the previous conclusion that Xyn10A has a good thermal stability [33]. Thus, it is suggested that the relationship between the sugar production and temperature was mainly affected by the characteristics of the cellulase in the mixed enzyme system. The presence of Xyn10A obviously greatly enhanced the sugar production, so that the maximum sugar yield went from 1.98 g/L to 3.97 g/L at 50 °C with the addition of Xyn10A. In addition, with cellulase being more sensitive to temperature than Xyn10A, the DS may be dominated by the Xyn10A at high temperatures, as its inclusion achieved 1.83 at 65 °C. However, a higher temperature was found to not suitable because of the decreasing sugar production.

3.3. Effect of Enzyme Loading on Synergistic Behavior

Figures 3 and 4 reflected the effect of the Xyn10A ratio under different total enzyme loadings on the glucose yield, xylose yield, and DS. Obviously, a similar pattern was shown: the optimal ratio of Xyn10A and cellulase did not change with the different total enzyme loadings. The yields of glucose and xylose increased first and then decreased with the increase in the ratio of Xyn10A, which was similar to the report by Pavón-Orozco et al. [56]. For example, at a total enzyme loading of 12 mg/g substrate, even if only 6.25% of the Xyn10A replaced the cellulase, the glucose and xylose yields of the synergistic enzyme group attained 22.27% and 38.52%, increases of 55% and 62% compared to the yields of the monocellulase group. However, with the ratio of Xyn10A exceededing 12.5%, the monosaccharide yields began to decrease. This demonstrated that the synergistic effect of Xyn10A was not enough to make up for the decline in cellulase hydrolysis. Alternatively, it was the cellulase that played a major role in degrading the substrate. As for the DS value, the highest occurred at the rate of 50% of Xyn10A. The highest DS_{glu} of 2.57 under the total enzyme loading of 6 mg/g substrate was higher than that at 12 mg/g substrate, which is in line with the general rule of the DS_{glu} decreasing with an increase in enzyme loading [57]. The highest DS_{xyl} of 1.84 was found under 12 mg/g substrate rather than 6 mg/g substrate, suggesting that the xylan layer cross-linking with cellulose showed better synergistic degradation with the increase in enzyme loading. The results here seemed slightly different from what has been reported in the literature. The highest DS_{glu} occurred at a ratio of 75% of xylanase in the case of enzymatic hydrolysis pretreated rice straw [58], while, for the pretreated corn straw it was at a ratio of 86% [59]. Sanhueza et al. [60] even reported that the glucose yield continued to decrease with an increasing xylanase ratio, with the DS_{glu} value remaining less than one when the pretreated wheat straw was employed. It could be seen that the change pattern of the DS with the ratio of xylanase was not completely consistent, indicating that the DS was related to the specific substrate and the use of different enzymes.

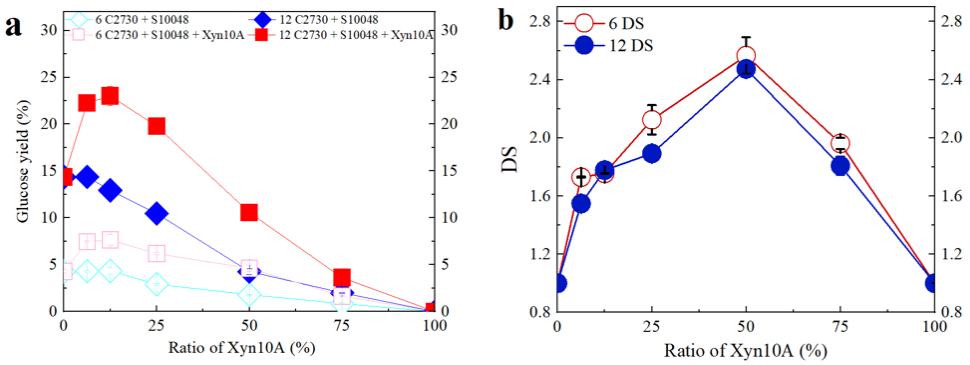


Figure 3. Hydrolysis of pretreated SCB using different ratios of cellulase and xylanase. Effect of Xyn10A ratio on (**a**) glucose yield and (**b**) DS_{glu} of each system were determined under different total enzyme loadings: (hollow) 6 mg/g substrates and (solid) 12 mg/g substrates. For each system, (square) mixed enzyme group, (diamond) single cellulase group, and (round) DS_{glu}. Values are the mean ± SD of two parallel experiments.

In addition, comparing Figures 3 and 4, it can be seen that the ratio value of Xyn10A where the highest DS was obtained did not match that for the highest sugar yield. Similar phenomena have been reported in other studies. In the enzymatic hydrolysis of pressed and pretreated sugarcane bagasse, synergistic effects between cellulase (Cellic® CTec2) and endo-xylanase (Cellic® HTec2) were observed only at a low cellulase dosage or at the beginning of the cultivation period [57]. Generally, the calculation of the DS is seen as an effective method to quantify the ability of synergy when different enzymes act on the substrate [61]. Another opinion was that the monosaccharide yield was primary and the DS might be of secondary importance [62]. A comprehensive consideration of the DS

and yield and the relationship between them would help to deepen the understanding of enzymatic lignocellulose and enzyme combination optimization.

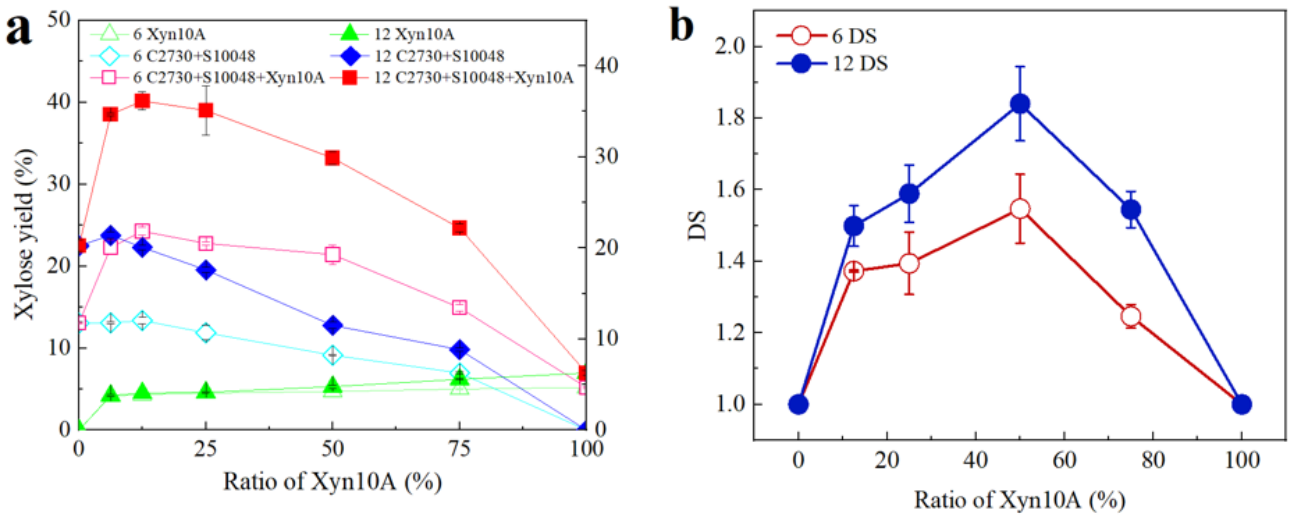


Figure 4. Hydrolysis of pretreated SCB using different ratios of cellulase and xylanase. Effects of Xyn10A ratio on (**a**) xylose yield and (**b**) DS_{xyl} of each system were determined under different total enzyme loadings: 6 mg/g substrates (hollow) and 12 mg/g substrates (solid). For each system, (square) mixed enzyme group, (diamond) single cellulase group, (triangle) single Xyn10A group, and (round) DS_{xyl}. Values are the mean ± SD of two parallel experiments.

3.4. Synergistic Degradation Process

The dynamic changes in the glucose yield, xylose yield, and DS were explored with the process of synergistic degradation (Figure 5). In terms of monosaccharide yield, the yields of both glucose and xylose increased the fastest in the first 24 h, and reached the reaction plateau phase at 72 h. Moreover, the addition of Xyn10A could not only improve the yield of monosaccharides but also shorten the reaction period. The glucose yield of the synergistic enzyme group at 48 h was almost the same as that of the monocellulase group at 120 h, and the xylose yield at 24 h was close to that of the monocellulase group at 120 h. This highlighted the potential application value of Xyn10A as a new xylanase in the synergistic enzymatic hydrolysis of lignocellulosic substrates. We found that the synergistic effect of this dual enzyme system was correlated with factors such as the substrate type, pretreatment method, and enzyme amount. Song reported that the hydrolysis yields caused by mixed cellulase (200mg/g substrate) and xylanase (200mg/g substrate) were improved by 133%, 164%, and 545% for corncob, corn stover, and rice straw, respectively. Recently, a novel and more efficient bifunctional xylanase/feruloyl esterase, XynII-Fae, could increase the reducing sugars yield from wheat straw and sugarcane bagasse by 81.9% and 56.7%, respectively [63]. In our case, at the total enzyme loading of 72 mg/g substrate, the reducing sugar yield of the synergistic enzyme group reached 17.41 g/L rather than 11.98 g/L of the single cellulase. Therefore, a more comprehensive optimization of the factors from an economic point of view is required to achieve the application of such dual-enzyme systems [63].

As far as the DS, the values for both the glucose and xylose remained greater than 1, implying that the synergistic behavior always existed through the entire reaction process under the currently optimization conditions. Specifically, the variation in the DS_{glu} was basically consistent with what was reported in the literature [52,64], that the value decreased steadily from the initial 1.88 to 1.35 as the reaction progressed. The usual explanation for this was that a decrease in the number of enzyme active sites during the hydrolysis process might render the activity synergy approach less effective [65,66]. With regard to the DSxyl, these appeared to be changed with the different sources of the enzyme, natures of the substrate, and so on [52,67]. The opposite trends would be observed even if using the same

substrate just because of the different pretreatment methods. In our case, the DS_{xyl} value increased rapidly at the initial stage of the reaction, reaching a maximum of 1.48 at 24 h, and then fell slightly to 1.3.

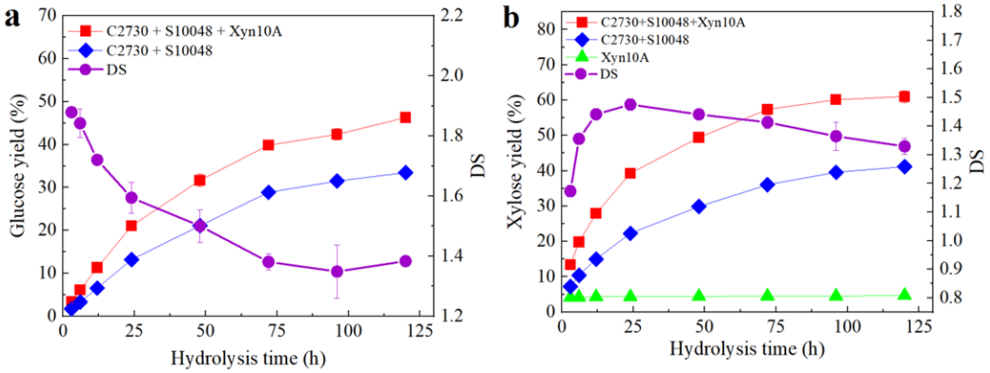


Figure 5. Monosaccharide yield and its DS during the synergistic degradation process, (**a**) glucose and (**b**) xylose. For each system: (■) mixed enzyme group, (♦) single cellulase group, (▲) single Xyn10A group, (●) DS. Values are the mean ± SD of two parallel experiments.

3.5. Synergy Mechanism

In spite of the many studies on the synergism of xylanase against cellulase, there are divided views on the mechanism behind the synergistic effect [67]. Based on the recognition that lignin has the ability of adsorbing hydrolytic enzymes non-productively and irreversibly [68], some researchers attributed the synergistic effect to the adsorption behavior between xylanase and the substrate (lignin), thus spatially blocking the non-productive contact between the cellulase and carbohydrates, believing that the equivalent of the increased ratio of cellulase functioned as a catalyst [69,70]. Given this, the effect of Xyn10A pre-adsorbed by SCB on the monosaccharide yield was carried out in our case, as shown in Figure 6. Unlike what was expected, the monosaccharide yield appeared unchanged under the condition that Xyn10A was firstly added to the reaction system. Meanwhile, some previous studies found that the simultaneous addition of xylanase at the beginning was surprisingly more advantageous than the sequence additions [9,59,71]. These findings, as well as ours, implied that the synergistic effect might result from the hydrolysis of Xyn10A on certain sites of the substrate rather than the non-productive adsorption of Xyn10A by the substrate.

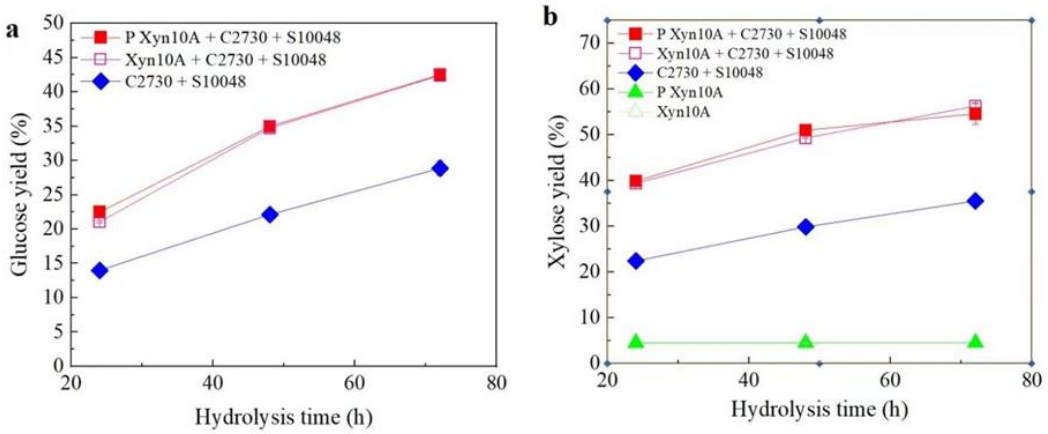


Figure 6. Effect of Xyn10A pre-adsorption on (**a**) glucose and (**b**) xylose yield. For each system:(square) mixed enzyme group, (diamond) single cellulase group, (triangle) single Xyn10A group. P Xyn10A represents the system with Xyn10A pre-adsorption, while Xyn10A represents the system without Xyn10A pre-adsorption. Values are the mean ± SD of two parallel experiments.

Many reports have described the interaction between xylan hydrolysis and cellulose degradation. Xylan in lignocellulosic biomass is loosely or tightly bound to cellulose, forming a three-dimensional structure [72]. The loosely bound part was thought to easily degraded while the tightly bound one would remain after enzymatic hydrolysis [73]. Thus, it thus made sense that the improved accessibility of cellulose to cellulase was observed in the presence of xylanase in many studies [52,68,74–76]. However, the addition of Xyn10A alone did not change the accessibility of cellulose in the hydrolysis process, and it only caused the formation of xylo-oligosaccharides. Actually, Long et al. presumed that the accessibility of cellulose should not be simply assessed by a single variable, i.e., the changes in the pores, according to the findings that the increase in pores in the substrate did not significantly improve the accessibility of cellulose [77]. Wu et al. also pointed out that the difference in substrate accessibility was related to the degree of xylan removal [78]. Vallejos et al. found that the significant removal of xylan would raise the accessibility [79]. Since lignocellulose is a complex network structure consisting of interwoven xylan and cellulose, and it was hypothesized that the hydrolysis of Xyn10A alone in this case would not be sufficient to open up this network intertwining structure, and that the hidden cellulose could not be exposed, implying that the cellulose accessibility could not be improved. On the other hand, with the crystallinity being a token parameter, the higher the crystallinity, the more stable the cellulose structure and the weaker the accessibility. We determined and calculated the crystallinity of the SCB in different groups. The intense peak at 22.5° in Figure 7 was attributed to crystalline cellulose, and the peak close to 18° represented the amorphous part of the SCB. Combining these results with those shown in Table 2 it is clear that the crystallinity of SCB in single the Xyn10A group did not change while that in the monocellulase group and synergistic enzyme group increased successively. This might be attributed to the further hydrolysis of amorphous cellulose by the cellulase, promoted by the addition of Xyn10A. As for the lack of crystallinity changes in the single Xyn10A group, on the one hand, it might be due to the fact that Xyn10A, as a xylanase, did not possess cellulose-degrading activity, thus showing no contribution to the destruction of crystalline cellulose. On the other hand, the low degradation efficiency of xylan in the single Xyn10A group (Figure 6b) implied that the single Xyn10A group had a limited ability to degrade, or "open", the complex lignocellulo-sic structure, and was not able to alter the crystallinity. Therefore, we supposed that the hydrolysis of xylanase alone cannot remove the xylan layer cross-linked with cellulose to the maximum (limited) extent, so that the change in accessibility or crystallinity could not be observed with Xyn10A alone. That is to say, for cellulose accessibility or crystallinity, when the degree of xylan removal is exceeded to a certain extent, a change in accessibility might be observed. Otherwise, it will depend on the joint action of other variables.

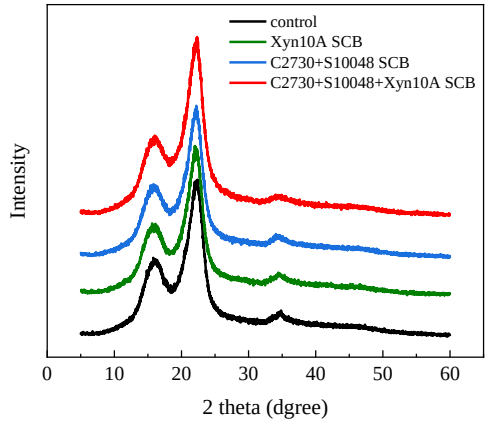


Figure 7. X-ray diffraction patterns of different SCB samples. (Red) mixed enzyme group, (blue) commercial cellulase group, (green) single Xyn10A group, (black) control.

Table 2. Cellulose crystallinity index of different SCB samples.

Enzymatic Hydrolysis Method	Crystallinity Index (CrI)
No enzyme	42.8%
Single Xyn10A	42.6%
single cellulase group	44.5%
Mixed enzyme	48.3%

Finally, scanning electron microscope images (Table 3) clearly indicated a change in the surface morphology of SCB. We observed that the presence of Xyn10A made the SCB produce more pores than the control. The SCB in the synergistic group was accompanied by cracking besides the observed cellulose layer stacking in any group. Correspondingly, clear changes in the FTIR spectra (Figure 8) were observed with the synergistic group despite no new group being found. The increased O-H stretching peaks at 3450 cm^{-1} might be an indicator of the broken inter- and intra-molecular hydrogen bonds in the cellulose to some extent, due to which the content of free hydroxyl groups was increased [49,80]. Also, the peaks at 1602 cm^{-1} and 1360 cm^{-1}, related to the aromatic skeletal vibrations and the deformation vibration of C-O and C-H in the aromatic ring, demonstrated that the main structure of lignin was changed [81,82]. The change in these peaks implies an increased disruption of the bagasse structure, which is consistent with the collapse of the bagasse structure observed by electron microscopy (Table 3). In summary, these investigations meant that Xyn10A should have the ability to destroy the network structure of lignocellulose substrate, thus promoting the synergistic disintegration of the substrate near the channel while providing an enzymatic hydrolysis channel for cellulase in the cooperative process. It should be the joint effect of cellulase and xylanase that promotes the hydrolysis and releases the substrates of each in time and space, which is more likely to be the origin of the synergistic mechanism.

Table 3. Scanning electron micrographs (SEM) of different SCB samples.

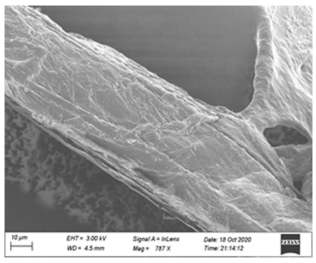
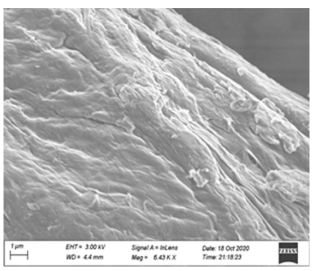
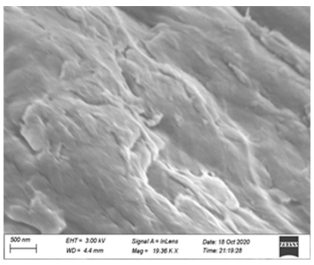
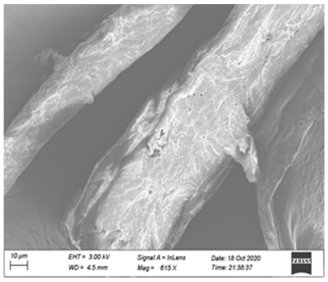
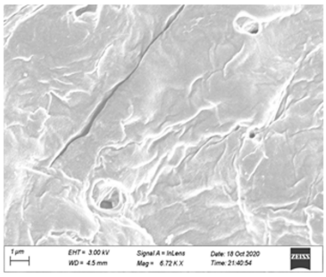
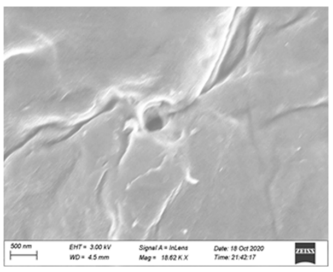
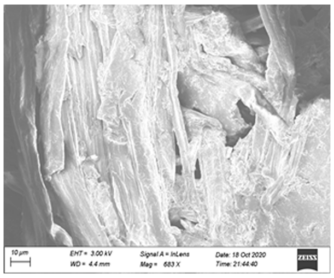
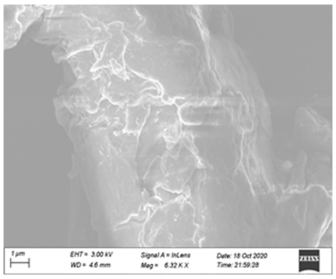
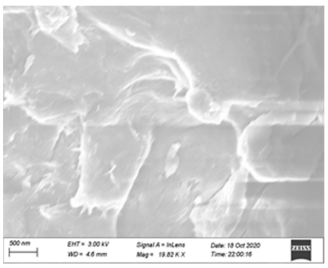
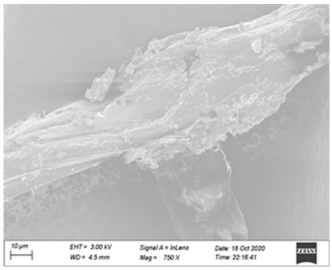
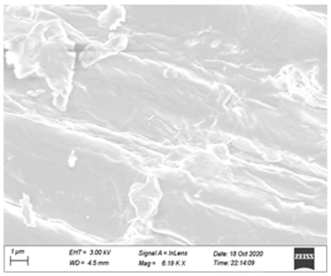
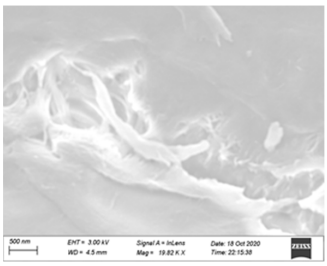
Enzymatic Hydrolysis Method	Scale Bar		
	10 μm	1 μm	500 nm
Control (No enzyme)			
Single Xyn10A			

Table 3. *Cont.*

Enzymatic Hydrolysis Method	Scale Bar		
	10 μm	1 μm	500 nm
single cellulase group			
Mixed enzyme			

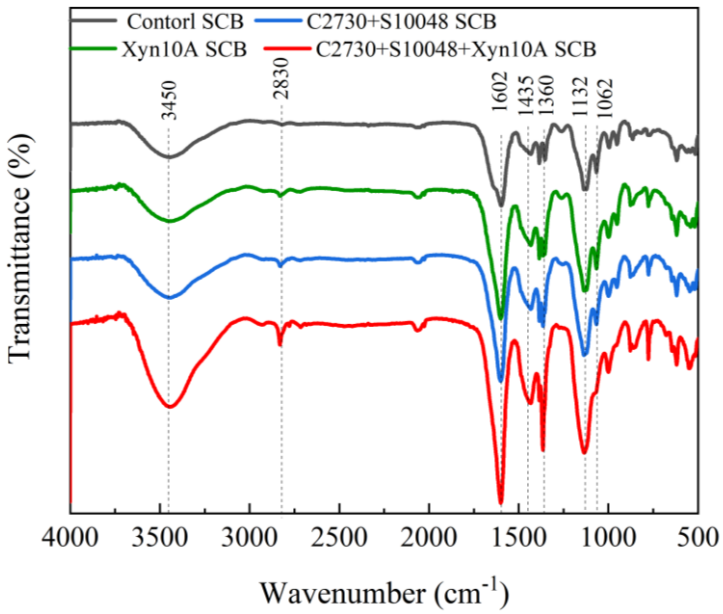


Figure 8. FTIR spectra of different SCB samples. (Red) mixed enzyme group, (blue) commercial cellulase group, (green) single Xyn10A group, (black) control.

4. Conclusions

Herein, we introduced a recombinant xylanase (Xyn10A) from streptomyces ipomoeae into the synergistic enzymatic hydrolysis of SCB and demonstrated its good performance in carrying out the synergistic enzymatic hydrolysis effect. Particularly, the loading of a small amount of Xyn10A caused the yields of glucose and xylose to increase by 78% and 80%, and shortened the reaction times to 72 h and 96 h. The synergistic effect may be related to the reduction in lignin fractions and non-acting adsorption; at the same time, the incorporation of cellulase and Xyn10A promoted the breaking of inter- and intra-molecular hydrogen bonds in cellulose molecules. To conclude, this work provides a new source of enzymes for

the simultaneous preparation of xylose and glucose from biomass feedstocks, and further enriches the research on the synergy mechanism of xylanase and cellulase.

Author Contributions: Conceptualization, Y.L.; Methodology, Z.L. and Y.L.; Validation, Y.D. and L.X.; Investigation, Y.D., J.L. and L.X.; Data curation, Z.L.; Writing—original draft, Z.L.; Writing—review & editing, Z.L. and Y.L.; Visualization, Y.D. and J.L.; Supervision, A.T., Q.L. (Qingyun Li), Q.L. (Qunliang Li) and Y.L.; Project administration, Q.L. (Qunliang Li); Funding acquisition, A.T., Q.L. (Qingyun Li) and Y.L. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare that there are no conflicts of interest.

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