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Impacts of Cellulase and Amylase on Enzymatic Hydrolysis and Methane Production in the Anaerobic Digestion of Corn Straw

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Abstract: The impacts of enzyme pre-treatments on anaerobic digestion of lignocellulosic biomass were explored by using corn straw as a substrate for enzyme pre-treatment and anaerobic digestion and by utilizing starch and microcrystalline cellulose as substrates for comparative analysis. The cellulase pre-treatment effectively improved the enzymatic hydrolysis of cellulose, decreased the crystallinity, and consequently showed 33.2% increase in methane yield. The methane yield of starch increased by 16.0% through amylase pre-treatment. However, when the substrate was corn straw, both the efficiencies of enzymes and methane production were markedly reduced by the lignocellulosic structure. The corn straw's methane yields were 277.6 and 242.4 mL·CH_4/g·VS with cellulase and amylase pre-treatment, respectively, which was 11.7% and 27.9% higher than that of the untreated corn straw. It may imply that the lignocellulose should be broken up firstly, enzyme pre-treatments could have great potentials when combined with other methods.

Keywords: anaerobic digestion; cellulase; amylase; methane yield; enzymatic hydrolysis; biogas

1. Introduction

Lignocellulosic biomass is the most abundant and renewable material on earth, the supply of which is approximately 200 billion tons per year [1]. However, lignocellulosic biomass is currently undervalued, considering that nearly 72.5% of crop straws are usually combusted, lost, or discarded, leaving only 0.5% for biogas production [2,3]. Anaerobic digestion is one of the highly promising methods of lignocellulosic biomass utilization for renewable energy biogas production [4].

Cellulose, hemicellulose, and lignin are the primary components of lignocellulosic biomass, whose interactions create a highly resistant and recalcitrant lignocellulosic structure [5]. However, the resulting contact between the enzyme and the substrate may be limited, and hydrolysis is the generally the rate-limiting step in anaerobic digestion of lignocellulosic biomass [6]. Therefore, improving the enzymatic hydrolysis process of anaerobic digestion is important in developing the application of lignocellulosic biomass for biogas production [7]. Sun et al. [8] claimed that all pre-treatment processes were performed to reduce the recalcitrance of lignocellulose to enzymes. However, the effects may vary because of the differences in the pre-treatment methods, including lignin removal or redistribution, in which the different effects may include increased porosity and disrupted linkages of lignin with the rest of the substrates [9–11]; hemicellulose removal or disruption that hamper the access of cellulase to cellulose [12,13]; reduced crystallinity or polymerization of cellulose [8,14]; increased content of reducing sugar due to cellulose or hemicellulose enzymatic

hydrolysis [15,16]; and reduced particle size [17]. Thus, two aspects should be emphasized: the first aspect is the pre-treatment of lignocellulose by using the physical or chemical method to extend the accessibility of an enzyme to the substrate, while the second aspect is supplying additional enzymes or microbes and subsequently enhance the enzymatic hydrolysis process.

Different from the physical and chemical pre-treatment methods, the biological method has many advantages, including its low-intensive, no-chemical, and mild-condition requirements [8,18]. Karray et al. [19] obtained the maximum reducing sugar concentration of *Ulva rigida* after pre-treating it with an enzyme (commercial pure β-glucosidase, a crude broth of *Aspergillus niger*) at 50 °C and 100 rpm for 2 h and found that the biogas yield can be increased to 626.5 mL/g·COD. Zhao et al. [16] obtained the maximum reducing sugar concentration of a corn straw by using enzyme T (extracted from a *Trichoderma harzianum* culture) that was pre-treated for six days and determined that methane yield can be improved to 273.75 mL/g·TS. In the above two studies, the enzyme pre-treatment was superior to the thermo-alkaline and acid catalytic methods. However, the research on the enzyme pre-treatments of the lignocellulosic substrate during anaerobic digestion remains far from ideal. The effects of enzyme pre-treatments on the anaerobic digestion of the lignocellulosic substrate need to be further clarified by amassing research on this topic.

Corn straw is the main lignocellulosic biomass in China, outputting 280 million tons yearly [20]. Corn straw has been proven to have great potential in producing methane in anaerobic digestion; however, the methane yield is low without pre-treatments because its lignocellulosic structure [21]. Thus, corn straw was used as a lignocellulosic substrate in this study. Corn straw is mainly consists of cellulose, hemicellulose, lignin, and starch. Corn straw mainly consists of cellulose, hemicellulose, lignin, and starch. Amylase is commonly used to improve the hydrolysis of starch, but it is neglected and seldom used in anaerobic digestion. Cellulose and starch are both important components of corn straw; thus, amylase and cellulase were used to explore their impacts on the hydrolysis and methanogenesis process. The effects of enzymes and the impact of lignocellulosic structure were also explored by using pure substances, such as soluble starch and microcrystalline cellulose, as the substrates for the anaerobic digestion. Then, the findings were comparatively analyzed to derive a quantitative analysis of the enzyme enhancement. Based on the analyzed hydrolysis process and methanogenesis process, the performance of enzyme pre-treatment to improve anaerobic digestion was further evaluated.

By studying the hydrolysis process and methanogenesis process of corn straw and the specific pure substances, the impacts of amylase and cellulase on corn straw anaerobic digestion can be explored. Thus, this study aimed at clarifying the performance of enzyme pre-treatment in anaerobic digestion of corn straw and find out how to improve the efficiency in future research. Low efficiency is one of the most important challenges which cuts off the application of enzyme treatment. A high-efficiency enzyme treatment method will promote the utilization of lignocellulosic biomass by anaerobic digestion, which generates renewable energy and achieve great environmental benefits.

2. Materials and Methods

2.1. Materials

The starch, cellulose, and urea (Sigma-Aldrich Ltd.) used in this study were all analytically pure. The starch specifically referred to the soluble starch, while the cellulose specifically referred to the microcrystalline cellulose. Two kinds of microcrystalline celluloses with different particle sizes and crystallinity indices (CrIs) were employed: Cellulose I, with an average particle size of approximately 90 um and a CrI of 56.3%, to test the activity of the inoculum, while Cellulose II, with an average particle size of approximately 120 um and a CrI of 73.8%, was used to test the hydrolysis performance of the cellulase (in Section 3, "cellulose" refers to microcrystalline Cellulose II). The corn straw, obtained from the stubble field in Daxing District, Beijing, was chopped in <5 mm lengths. Inoculums were sampled

from an anaerobic digester of cow manure. The amylase and the cellulase (Novozymes Co., Ltd.) were processed in their liquid form.

2.2. Enzyme Pre-Treatment

The corn straw used in this study presented high contents of cellulose and starch. The cellulase and the amylase were used to improve the hydrolysis in the test. The effects of amylase and cellulase were further explored by using the pure substances (soluble starch and microcrystalline Cellulose II) as the substrates in the pre-treatment and the anaerobic digestion. The working temperature of the amylase was set to 30–80 °C, while the working temperature of the cellulase was set to 50–55 °C following the manufacturer's instruction (Novozymes Co., Ltd.). Then, on the basis of an optimization study on the working conditions for the abovementioned substrates, the amylase pre-treatment and the cellulase pre-treatment were conducted at 38 °C and 55 °C in a water bath for 18 h, respectively.

2.3. Biochemical Methane Potential Tests

The effects of the enzymes on methane production during anaerobic digestion were analyzed by performing biochemical methane potential (BMP) tests using the Automatic Methane Potential Test System II (AMPTS II) (Bioprocess Control AB, Sweden). The methane productions were recorded automatically and continuously by the flow cell in AMPTS II. The BMP tests were conducted according to the VDI 4630 guidelines of the Association of German Engineers. The volatile solid (VS) ratio of the inoculum to the substrate was 2. The fermentation temperature was 37 ± 0.5 °C. The BMP tests were continued only until a small volume of biogas was produced, given that the daily methane production was less than 1% of the cumulative methane production. The BMP test feeding with Cellulose I (low CrI) was conducted to evaluate the quality of the inoculums. The methane yield of 342.8 mL·CH_4/g·VS, i.e., a value higher than 80% of the theoretical value, was used to confirm the positive activity of the employed inoculums. A blank treatment was set up to quantify the amount of methane production of inoculums, which were excluded in the methane production of the substrates. The volume of the methane production was corrected according to standard conditions. All trials were performed in triplicate.

2.4. Analytical Methods

TS and VS were measured according to standard methods [22]. The cellulose concentration of the corn straw was analyzed according to the Van Soest method [23]. The carbon and nitrogen contents were analyzed using the Vario EI Elemental analyzer (Germany). The amylase and the cellulase were used to pre-treat the starch, cellulose, and corn straw, which were then used to analyze the saccharide hydrolysis. The concentrations of total sugar, amylose, reducing sugar, and oligosaccharide were analyzed according to GB 5009.7–9-2016. Scanning electron microscopy (SEM; Zeiss EVO18, Germany) was employed to observe the microstructural variations of the corn straw under different conditions. X-ray diffraction (XRD) test was performed in an X-ray diffractometer (Bruker, Germany) and by following a method provided in the literature [24]. The CrI was determined according to the XRD curves as follows [25]:

$$\mathrm{CrI}(\%) = \frac{I_{002} - I_{am}}{I_{002}}, \quad (1)$$

where I_{002} is the maximum peak intensity at approximately 2θ = 22.5°, while I_{am} is the amorphous peak intensity at approximately 2θ = 18°.

2.5. Statistical Analysis

All statistical analyses of the experimental data were conducted by Microsoft Excel 2019 (Microsoft Corporation) and Origin 9 (OriginLab Corporation). The values of the collected data represent the averages of the measurements performed in triplicate.

3. Results

3.1. Enzymatic Hydrolysis

The initial characteristics of the amylose, celluloses, and corn straw are shown in Table 1. The cellulose and starch contents of corn straw are 34.10 ± 0.12% and 31.63 ± 0.22%. As the cellulose and starch contents are high in the corn straws, pure substances (soluble starch and microcrystalline Cellulose II) and corn straw were both employed as the substrates as a means to fully understand the effects of amylase and cellulase. Amylase was used to improve the hydrolysis of starch. Cellulase was used to improve the hydrolysis of cellulose. Amylase and Cellulase were both used to improve the hydrolysis of corn straw as it contains considerable amounts of starch and cellulose. The specific configurations of enzyme pre-treatment are shown in Table 2. The total sugar, reducing sugar, oligosaccharide concentrations and the CrI with or without enzyme pre-treatment were measured. The results are shown in Figure 1. In the succeeding discussions, "cellulose" denotes microcrystalline Cellulose II, while "starch" denotes soluble starch.

Table 1. Initial characteristics of starch, cellulose, and corn straw.

	TS [1] (g/100g)	VS [2] (% of TS)	CrI [3]	C (% of TS)	N (% of TS)	O (% of TS)	H (% of TS)
Starch	100	100	-	40.45	0	49.38	6.17
Cellulose I	100	100	56.3%	40.45	0	49.38	6.17
Cellulose II	100	100	73.8%	40.45	0	49.38	6.17
Corn Straw	92.15 ± 0.01	90.56 ± 0.01	49.8%	44.18 ± 0.05	0.95 ± 0.01	49.10 ± 0.10	6.67 ± 0.08

[1] TS, Total Solids; [2] VS, Volatile Solids; [3] CrI, Crystallinity Index.

Table 2. Configurations of the enzyme pre-treatment and the biochemical methane potential tests.

Name	Substrate	Enzyme Treatment
CSN	Corn Straw	None
CSC	Corn Straw	Cellulase (1% g·TS/g·TS), 55 °C for 18 h
CSA	Corn Straw	Amylase (0.6% g·TS/g·TS), 38 °C for 18 h
SN	Soluble Starch	None
SA	Soluble Starch	Amylase (0.6% g·TS/g·TS), 38 °C for 18 h
CN	Microcrystalline Cellulose II	None
CC	Microcrystalline Cellulose II	Cellulase (1% g·TS/g·TS), 55 °C for 18 h

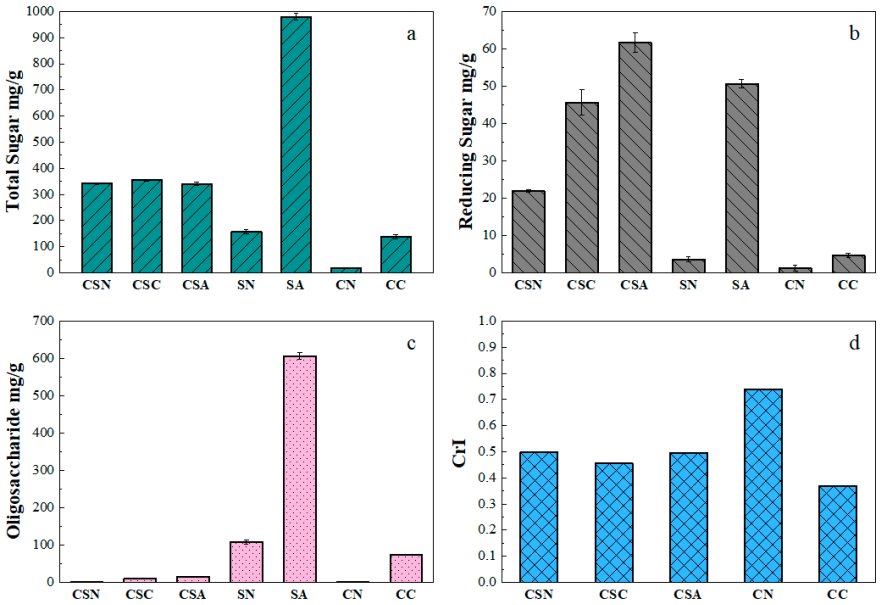

Figure 1. Variations in: total sugar (**a**); reducing sugar (**b**); oligosaccharide concentrations (**c**); and crystallinity indices (CrI) (**d**).

The cellulose in the CC test was treated with cellulase at 55 °C for 18 h. In the control named CN, the cellulose was treated with distilled water but without cellulase under the same condition. With the influence of cellulase, the reducing sugar concentration in the CC increased from 1.2 to 4.6 mg/g, the oligosaccharide concentration increased from 2.1 to 73.5 mg/g, and the total sugar concentration increased from 17.9 to 138.5 mg/g. The cellulase improved the hydrolysis of the cellulose and fragmented the microcrystalline structure. This finding was further proven by the decrease in CrI. The CrI of the cellulose decreased from 73.8% to 36.8%, which is a reduction of the rate in half.

The starch in the SA test was dissolved into distilled water and treated by amylase at 38 °C for 18 h under the anaerobic condition. In the control named SN, the starch was treated under the same condition, i.e., without adding amylase. After the amylase pre-treatment, the reducing sugar concentration increased from 3.6 to 50.7 mg/g, the oligosaccharide concentration increased from 108.3 to 607.1 mg/g, and the total sugar concentration increased from 125.7 to 978.5 mg/g. The amylase pre-treatment remarkably improved the hydrolysis of the starch under the mesophilic anaerobic circumstance. Thus, by taking advantage of the simplified process without any pre-treatment, the amylase can also be directly added to anaerobic digesters as a way to improve enzymatic hydrolysis.

In the CSC, CSA, and CSN tests, the corn straw was used as the substrate and treated with cellulase at 55 °C for 18 h, with amylase at 38 °C or 18 h, and with distilled water at 55 °C, respectively. The reducing sugar concentrations of corn straw increased from 21.9 to 45.5 mg/g after cellulase pre-treatment and from 21.9 to 61.6 mg/g after amylase pre-treatment, while the oligosaccharide concentrations increased from 21.9 to 45.5 mg/g after cellulase pre-treatment and from 21.9 to 61.6 mg/g after amylase pre-treatment. However, as shown in Figure 1, the total sugar concentration and the CrI was hardly changed. Only the cellulase pre-treatment slightly reduced the CrI of the corn straw, from 49.8% to 45.5%. Figure 2 shows the SEM of corn straws with different treatments. The surface of untreated corn straw was smooth and tight. As the enzymes were dissolved in water to pre-treat corn straw, the SEM morphology of corn straw treated by water was analyzed, showing no obvious change compared with that of the untreated corn straw. The surface of corn straw showed erosion traces after cellulase pre-treatment, while that was negligible after amylase pre-treatment. However, the SEM morphological changes caused by enzyme pre-treatments were insignificant compared with those caused by physical or chemical pre-treatment in previous studies [25,26]. The effects of amylase and cellulase were weakened in the hydrolysis of the corn straw.

3.2. Methane Production

After the enzyme pre-treatments, BMP tests were conducted to investigate the effects of these enzyme pre-treatments and the impact of the lignocellulosic structure on methane production during anaerobic digestion. According to the configurations in Table 2, corn straw, cellulose, and starch were used as substrates. Figure 3 shows the cumulative methane yields of different BMP tests. The SN stopped producing methane on the 27th day, whereas the SA stopped production on the 29th day. The methane yields were 332.1 mL·CH_4/g·VS in the SN and 385.2 mL·CH_4/g·VS in the SA. The methane yield of starch increased by 16.0% with the enhancement of amylase. The CC stopped producing methane on the 20th day, while the CN stopped production on the 21st day, which were much earlier than those of the starch and the corn straw. This finding may be due to the lack of cellulase in the digester and the death of hydrolytic bacteria in the latter stage of anaerobic digestion. In the end, the methane yield of cellulose was increased by 33.2%, from 281.1 to 374.3 mL·CH_4/g·VS. After 30 days of fermentation, the methane yields of corn straw were 217.1, 277.6, and 242.4 mL·CH_4/g·VS in the CSN, CSC, and CSA tests, respectively. The methane yield of corn straw increased by 11.7% due to the amylase pre-treatment and by 27.9% due to the cellulase pre-treatment.

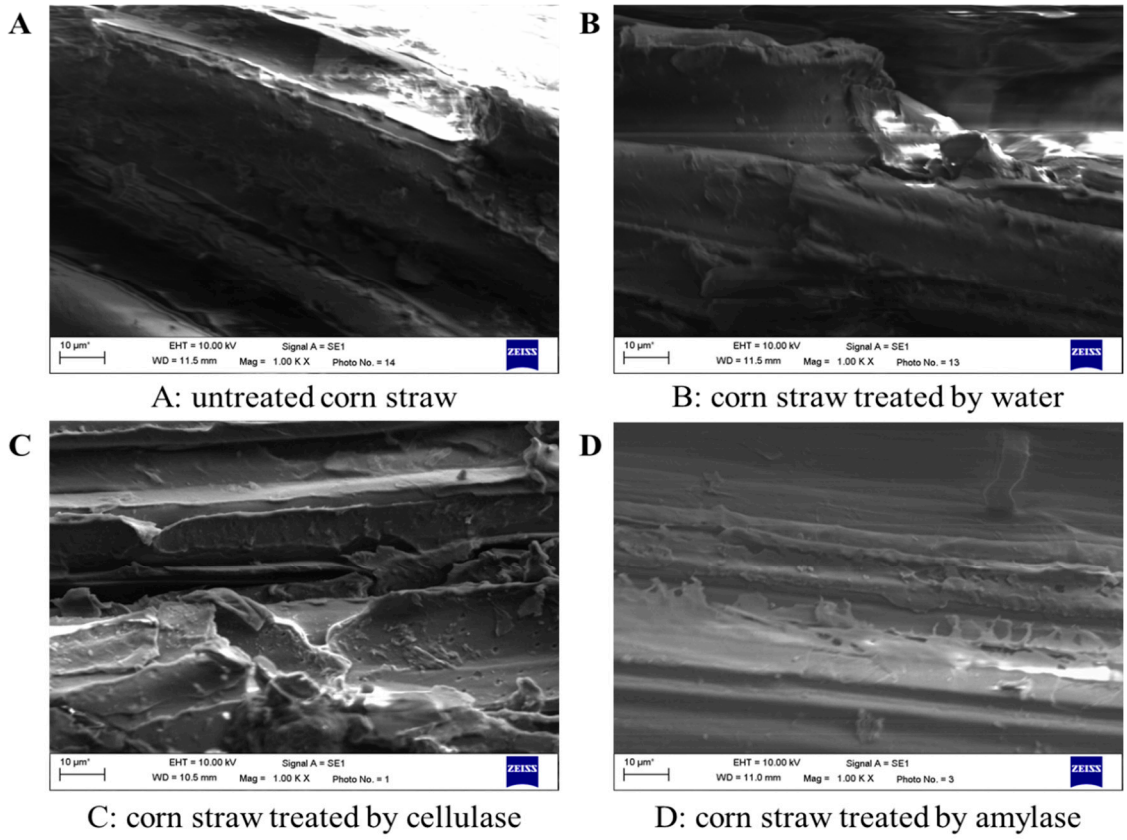

Figure 2. Scanning electron microscopy (SEM) of corn straws with different treatments.

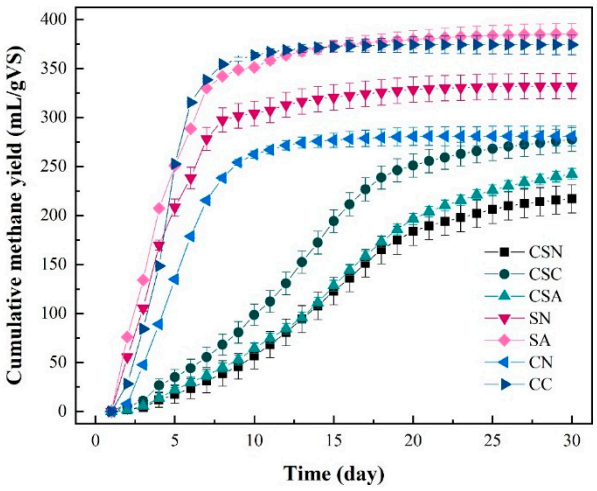

Figure 3. Cumulative methane yields of soluble starch, microcrystalline cellulose, and corn straw anaerobic digestions with or without enzyme enhancement.

The theoretic maximum methane yield of carbohydrates in a complete anaerobic digestion process can be calculated using Buswell's formula (Equation (2)) [27,28]. According to the elements analysis, the carbon, hydrogen, and oxygen contents of the corn straw (TS) were 44.18%, 6.32%, and 49.10%, respectively. The resulting corn straw formula is $C_6H_{11}O_5$. Based on Equation (2), the theoretic maximum methane yield of corn straw in this study was 429.4 mL·CH_4/g·VS. Meanwhile, given the

same chemical formula of $(C_6H_{10}O_5)_n$, the maximum methane yields of starch and cellulose were 414.8 mL·CH_4/g·VS.

$$C_nH_aO_b + \left(n - \frac{a}{4} - \frac{b}{2}\right)H_2O \rightarrow \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4}\right)CH_4 + \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4}\right)CO_2, \quad (2)$$

The conversion rate of an organic matter is defined as the ratio of actual methane yield to the maximum methane yield. Figure 4 shows the calculated conversion rate of the organic matter in this study over a period of 30 days. After 30 days of digestion, the conversion rates were as follows: the organic matter of starch was initially 80.1% and improved to 92.9% with the amylase pre-treatment, that of cellulose was initially 67.8% and improved to 90.2% with the cellulase pre-treatment, and that of the corn straw was initially 50.6% and improved slightly to 56.5% with the amylase pre-treatment and 64.7% with the cellulase pre-treatment.

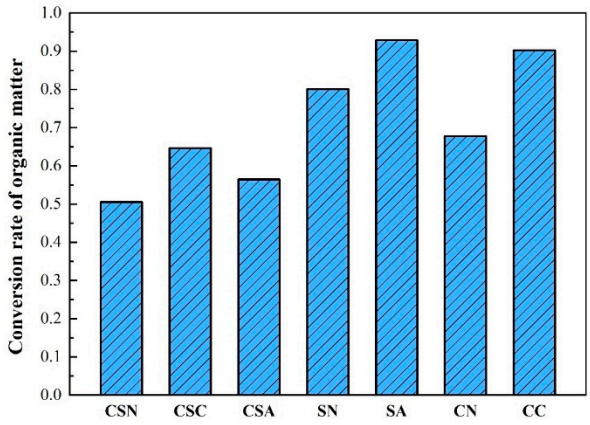

Figure 4. Conversion rates of organic matter in the anaerobic digestion of soluble starch, microcrystalline cellulose, and corn straw with or without enzyme pre-treatment.

4. Discussion

The results of enzymatic hydrolysis indicate that cellulose is more difficult to hydrolyze compared with starch. Cellulose and starch are both polymers of glucose, and they have the same chemical formula of $(C_6H_{10}O_5)_n$. The similarity indicates that chemical structure plays a significant role in hydrolysis process. The highly crystalline structure of cellulose, which is formed by the ordering of polymer chains, are water insoluble and resistant to depolymerization [29]. Cellulase hydrolyzes the glycoside bond, which is present between two or more carbohydrate molecules or between non-carbohydrate and carbohydrate molecules [30].

In terms of improving the hydrolysis of the corn straw, the results notably differ between the addition of amylase and cellulase. Corn straw mainly consists of cellulose, hemicellulose, and lignin and many other organic compounds, including starch and sugars [31,32]. The lignocellulosic structure of the corn straw in this study formed a strong native recalcitrance to the enzymes, resulting in a relatively low hydrolytic ability. The structures of cellulose and hemicellulose are chain polysaccharides. Lignin can be closely integrated with cellulose and hemicellulose as it is a heterogeneous cross-linked three-dimensional phenyl–propane polymer [8,33]. Here, our SEM obtained non-significant variations in the corn straw structure after amylase pre-treatment or soaking in water (Figure 2). After pre-treatment with cellulase only, the corn straw presented a relatively rough surface. Therefore, although the cellulase and the amylase could improve the hydrolysis of cellulose and starch, the lignocellulosic structure could not be destructed, the total sugar of the treated corn straw after pre-treatment with enzymes could not notably increase, and the efficiencies of the enzymes were weakened by the complex lignocellulosic structure of the corn straw, which differed from the effects on starch and cellulose. Furthermore, the cellulose and starch concentrations were high in the

corn straw; however, the methane yield of corn straw was much lower than that of cellulose or starch regardless of the applied enzyme pre-treatment. The improvement on methane production of enzymes was markedly weakened in the anaerobic digestion of corn straw.

The amylase and cellulase pre-treatments effectively improved the utilization of starch and cellulose for methane production. In particular, relatively short lag phases and high methane production were observed during anaerobic digestion. As amylase could improve the hydrolysis of starch under mesophilic anaerobic conditions, it could be used in the pre-treatment or as an additive for anaerobic digestion. When using amylase in the pre-treatment, the amylase attached to the substate would be fed in the anaerobic digester and be continuously active. With the enhancement of amylase, the reducing sugar and oligosaccharide contents were produced much more rapidly when the substrate contained large amounts of starch. The reducing sugar and oligosaccharide contents favored the action of the fermentative and acetogenic bacteria. Acetate, which was required by the methanogens, was further enriched [34]. As the multiplication of the methanogens accelerated, the lag phase shortened, and the methane production was initially increased. Zhao et al. [14] similarly proved that high concentrations of reducing sugar are favorable for a single fermentation undergoing methane production. Moreover, the contents of oligosaccharides, such as cellobiose, are usually used as prebiotics, which are defined as "non-digestible carbohydrates that beneficially affect the host by selectively simulating the growth and/or activity of microflora" [35]. The increase in oligosaccharide concentration could have also benefitted the hydrolysis process and the growth of anaerobic microbes. Subsequently, at the latter phase of batch anaerobic digestion, all the microbe multiplications slowed down along with the consumption of organic matter. The system with the amylase pre-treatment continued to show strong ability to produce methane during the last week of anaerobic digestion.

The cellulase pre-treatment effectively improved the methane production not only because of the increased reducing sugar and oligosaccharide concentrations resulting from cellulose hydrolysis but also due to the decrease in CrI and the destruction of the microcrystalline structure. As the cellulose was treated by cellulase, the reducing sugar content was already increased before cellulose was added to the digester. Thus, the methane yield increased initially. However, the cellulase eventually lost its activity in the digester, and the growth rate of methane production decreased accordingly along with the large consumption of available reducing sugars and oligosaccharides.

In terms of improving the methane production of corn straw, the cellulase performed better than the amylase; this phenomenon can be attributed not only on the increments of reducing sugar and oligosaccharide concentrations but also on the disrupted lignocellulosic barriers, indicating that the hydrolytic enzymes could penetrate the lignocellulosic structure and cause hydrolysis [29,36]. Kupski et al. [37] similarly found enhanced activities of the cellulolytic complex to achieve starch and protein digestibility. This phenomenon could have also benefitted the hydrolysis process in anaerobic digestion of corn straw.

As for the case of the corn straw's anaerobic digestion, the lignocellulosic structure prominently played a negative role in anaerobic digestion, as evidenced by slow growth of methane content, long lag phase, low methane production, and hampered enzymatic hydrolysis. Therefore, the impacts of cellulase or amylase pre-treatments were limited in terms of improving the anaerobic digestion of corn straw. Despite the enhancement of cellulase, the conversion rate of the organic matter of corn straw only reached 64.7%, while the grow rate of the methane yield was 27.9%. Zhao et al. [16] also found that the methane yield of corn straw could increase by 10.7% by using an enzyme extracted from the *T. harzianum* culture. Weide et al. [38] used enzyme mixtures to improve the methane production of agricultural wastes, but the derived increases (i.e., −2.7% to 9.4%) in the biogas yields were not detectable in most cases after 60 days of BMP tests. However, in other cases, the enzyme pre-treatment reportedly works effectively and can improve methane production. Ziemiński and Kowalska Wentel [39] obtained the maximum growth rate of 57.7% in methane yield by enhancing the anaerobic co-digestion of sugar beet pulp silage and vinasse through an enzyme pre-treatment. In our previous study [40], the cellulase and amylase treatments also significantly improved the methane

production in anaerobic co-digestion of corn straw and cow manure. In our present work, the effects of the enzyme pre-treatments could have been limited in the anaerobic digestion of lignocellulosic biomass. Nonetheless, enzyme pre-treatments have great potentials when combined with other methods, such as the adoption of C/N of feedstocks by anaerobic co-digestion, the addition of trace elements to encourage microbe multiplications, or the application of pre-treatment methods to destroy lignocellulosic structures. The research on "super-enzyme" development, which can overcome the barriers of lignocellulosic structure, is another potential option.

5. Conclusions

In this study, the impacts of cellulase and amylase pre-treatments on improving the anaerobic digestion of corn straw was investigated, and starch and microcrystalline cellulose were used as substrates for the comparative analysis. The cellulase pre-treatment decreased the CrI of cellulose by 50.1% and increased the methane yield of cellulose by 33.2%. The amylase pre-treatment increased the methane yield of starch by 16.0%. However, the effects of cellulase and amylase were both weakened from the viewpoint of improving the hydrolysis of corn straw because of the presence of a lignocellulosic structure. The methane yields of corn straw were 277.6 and 242.4 mL·CH_4/g·VS with cellulase and amylase pre-treatment respectively, which were 11.7% and 27.9% higher than that of the untreated corn straw. The efficiency of the enzyme pre-treatments as a means to improve the anaerobic digestion of lignocellulosic biomass remains limited at present. The enzyme pre-treatments could have great potentials when combined with other methods to break up the lignocellulose structure firstly.

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