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Enhancing the Anaerobic Digestion of Corn Stalks Using Composite Microbial Pretreatment

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A composite microbial system (XDC-2) was used to pretreat and hydrolyze corn stalk to enhance anaerobic digestion. The results of pretreatment indicated that sCOD concentrations of hydrolysate were highest (8,233 mg/l) at the fifth day. XDC-2 efficiently degraded the corn stalk by nearly 45%, decreasing the cellulose content by 22.7% and the hemicellulose content by 74.1%. Total levels of volatile products peaked on the fifth day. The six major compounds present were ethanol (0.29 g/l), acetic acid (0.55 g/l), 1,2-ethanediol (0.49 g/l), propionic acid (0.15 g/l), butyric acid (0.22 g/l), and glycerine (2.48 g/l). The results of anaerobic digestion showed that corn stalks treated by XDC-2 produced 68.3% more total biogas and 87.9% more total methane than untreated controls. The technical digestion time for the treated corn stalks was 35.7% shorter than without treatment. The composite microbial system pretreatment could be a cost-effective and environmentally friendly microbial method for efficient biological conversion of corn stalk into bioenergy.

Keywords: Composite microbial system, biogas, pretreatment, hydrolysate, anaerobic digestion

In recent years, energy resources and environmental protection have become important global concerns. In developing countries, there has been increasing interest in developing technologies to harness and utilize renewable energy resources such as biomass [21]. Lignocellulosic biomass is one of the most abundant resources in the world; it is renewable and can be degraded by microorganisms [13]. Agricultural wastes such as rice straw, wheat straw, and corn stalks are important sources of lignocellulosic biomass, and China is among the countries with the highest field crop straw production in the world. More than 600 million tons of crop straw are produced annually in

China [14]. The production of biogas through anaerobic digestion offers significant advantages over other forms of agricultural waste treatment [22].

A lot of literature is written about different pretreatment methods to enhance the digestibility of lignocellulosic biomass, because pretreatment is the rate-limiting step. Physical (mechanical comminution and hydrothermolysis) and chemical (acid pretreatment, alkaline pretreatment, and oxidative delignification) pretreatments have been proven to effectively enhance anaerobic digestion. However, physical and chemical pretreatment methods require significant energy use and are not environmentally friendly. Biological pretreatment offers some conceptually important advantages, such as low chemical and energy uses, but very few controllable and sufficiently rapid systems have been found [2].

Natural lignocellulosic biomass is difficult for microorganisms to degrade because of the combination of cellulose, hemicellulose, and lignin. Although microbial decomposition of lignocelluloses has been studied extensively, most of these studies used a pure culture of microorganisms [7]. In our laboratory, some composite microbial systems (MC1, XDC-2, and SD-Y) with efficient and stable cellulose degradation characteristics have been developed. MC1 can degrade rice straw by 60% within four days at 50ºC [4, 9]; XDC-2 can degrade rice straw by 50% within nine days at 35°C [7]; SD-Y can degrade switchgrass by nearly 70% within four days, decreasing the cellulose content by 67.3% and the hemicellulose content by 73.5% [25]. However, these systems have not yet been tested for performance in biogas production. Therefore, in order to develop a novel microbial pretreatment method for biogas production, we used XDC-2 to pretreat and hydrolyze corn stalks in this study and analyzed the effect on biogas production.

The objectives of this study were: (1) to investigate the effect of microbial pretreatment on biogas production, and determine the optimal time of pretreatment; and (2) to analyze the changes in the main chemical components and microbial community during pretreatment.

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MATERIALS AND METHODS

Preparation of Materials and Medium for Pretreatment by Composite Microbial System XDC-2

Corn stalk: Corn stalks were harvested and dried naturally at the China Agricultural University (Haidian District, Beijing City, China). The characteristics of the corn stalk tissue used in this study are shown in Table 1. Before use, corn stalks were chopped into 10–20 mm pieces using pruning shears and dried again at 80°C for 48 h prior to use.

Bacterial community: XDC-2, which is capable of effectively degrading cellulose, was developed and characterized in our laboratory [7]. The system was cultured in peptone cellulose solution (PCS) containing 1% (w/v) corn stalk for three days at 5°C and stored at –20°C in 20% glycerol. The previous microbial composition results, which were analyzed by clone library and sequencing, indicated that the 26 different clones assembled into three phyla: Clostridiales, Proteobacteria, and Bacteriodetes. Among the 26 clones, 46.2% clones belonged to the cluster Clostridiales, and were related to the genera *Clostridium*, *Sporomusa*, *Oscillibacter*, and *Psychrosinus*; 38.4% belonged to the cluster Proteobacteria, and were related to the genera *Devosia*, *Pseudomonas*, *Escherichia*, and *Alcaligenes* [7].

Medium: The peptone cellulose solution (PCS) was composed of 2 g of peptone, 1 g of yeast extract, 2 g of $CaCO_3$, 5 g of NaCl, and 1 l of H_2O (pH 8.0). The medium was autoclaved at 121°C for 20 min.

Pretreatment Assays by Composite Microbial System XDC-2

The main purpose of pretreatment with the composite microbial system XDC-2 is to make cellulose and hemicellulose available and digestible for downstream processes. several 1,000 ml Erlenmeyer flasks (stoppered with aluminum foil) containing 500 ml of autoclaved PCS medium and 1% (w/v) corn stalk as the single carbon source were prepared. After inoculation with the activation culture of the preserved inoculums (seed volume of 5%), the medium was cultured under static conditions at 35°C. To determine the optimal length of time for pretreatment, fermentation was allowed to continue for 16 days, and samples were taken to obtain correlative numerical data on days 0 (immediately after inoculation), 1, 2, 3, 5, 7, 10, 13, and 16.

For weight loss tests, the hydrolysate (including both the fermentation broth and residual corn stalks) was centrifuged at 3,000 ×*g* for 10 min; the precipitate was washed with acetic acid/nitric acid reagent and then with water to remove noncellulosic materials. An uninoculated medium served as the control. The weight loss of residual substrates was determined using a procedure reported earlier [8]. Residual corn stalks were passed through 1 mm screens, and a 0.5 g sample was transferred into a special pocket (Model F57, USA). Components of residual corn stalks were analyzed using a fiber analyzer (Model ANKOM220, USA) as described elsewhere [24].

The pH of the hydrolysate during the pretreatment was also determined on days 0, 1, 2, 3, 5, 7, 10, 13, and 16 using a pH meter (Model B-212, Horiba, Inc., Japan).

The optimal length of time for pretreatment was determined by analyzing the changes in the main chemical components of the corn stalk.

Anaerobic Digestion

The results of pretreatment indicated that the optimal length of time for pretreatment is five days. Therefore, the samples (untreated corn stalks and 500 ml of hydrolysate treated for five days by XDC-2 with corn stalks) were digested in batch anaerobic digesters. The volume of each anaerobic digester was 1 l, with a working volume of 750 ml. Each digester was seeded with the anaerobic sludge taken from a mesophilic anaerobic digester from the Deqinyuan Biogas Plant (Beijing, China). The sludge contained 57.2 g/l total solids (TS), 31.5 g/l volatile solids (VS), and 39.6 g/l mixed liquor suspended solids (MLSS). The anaerobic sludge MLSS was seeded in each digester at 15 g/l [27]. Ammonia chloride (NH_4Cl) was added to each digester to adjust the carbon/nitrogen ratio (C/N) to 25, which is believed to be optimal for anaerobic bacteria growth [27]. The anaerobic digestion experiment was repeated three times at mesophilic temperature (35°C).

Analyses of Volatile Products of the Hydrolysate by GC–MS

On days 2, 5, 7, 10, and 16, samples obtained from the hydrolysate were filtered through an aperture of 0.22 μm and analyzed using GC–MS (model QP-2010, Shimadzu, Japan) on-line with a capillary column, CP-Chirasil-Dex CB (25×0.25 mm). The analytical conditions were as follows: column temperatures: 60°C (for 1 min) → 100°C, 7°C/min → 195°C (for 2 min), 18°C/min; injector temperature: 190°C; ion source temperature: 200°C; carrier gas: He (60 kPa); rate of flow: 34 ml/min; splitter ratio: 1/20; voltage of detector: 0.7 kV; sample volume: 1 μl.

The GC–MS peaks were qualitatively analyzed using the NIST database.

Biogas Analyses

Biogas volume was monitored every day using the water displacement method, and the corresponding cumulative biogas volume was calculated. The measured volume was then converted to a volume of gas at standard temperature and pressure using the ideal gas law. The methane content of the biogas was analyzed every day using a biogas analyzer (Biogas Check, Geotech, Britain).

Chemical Composition Analyses

The total carbon (TC) and total nitrogen (TN) of the corn stalks were determined by the TC analyzer (Skalar Primacsslc, The Netherlands). The TS, VS, and MLSS of the corn stalks, anaerobic sludge, and

Table 1. Characteristics of corn stalk used in the experiments.

Characteristics	Values[a]	Characteristics	Values[a]
TS (%)	94.91 ± 0.8	C/N	38.04
VS (TS%)	95.08 ± 1.2	Lignin (TS%)	7.70 ± 0.8
TC (TS%)	41.84 ± 1.9	Cellulose (TS%)	33.35 ± 1.6
TN (TS%)	1.10 ± 0.2	Hemicellulose (TS%)	28.74 ± 2.1

[a]Values are the means ± SD (n ≥ 3).

their mixture were measured according to APHA standard methods [1]. For soluble chemical oxygen demand (sCOD) tests, the hydrolysate was centrifuged at 3,000 ×g for 10 min, and then the supernate was tested by a COD analyzer (Model ET99731, Lovibond, Germany).

Microbial Community Analyses Using PCR–DGGE

DNA extraction was carried out on days 0, 2, 5, 10, and 16. Seven ml of hydrolysate was centrifuged at 15,000 ×g for 20 min. Extraction of total genomic DNA was carried out using the benzyl chloride method [29].

PCR amplification of the bacterial 16S rRNA gene and the fungal 26S rRNA gene region D1–D2 was performed using the GeneAmp PCR System (Model 9700, Applied Biosystems, USA). The primers for bacterial 16S rRNA gene PCR amplification were 357F-GC, 5'-CCTACGGGAGGCAG CAG-3' (*Escherichia coli* positions, 341–357), which was attached to a GC-clamp (5'-CGCC CGCCGCGCG CGGCGGGCGGGGCGGGGGCACGGGGGG-3') at the 5'-terminus, and 517R, 5'-ATTACCGCGGCTGCTGG-3' (*E. coli* positions, 517–534) [16]. Initial DNA denaturation was performed at 95°C for 10 min, followed by 30 cycles of denaturation at 93°C for 1 min, annealing at 48°C for 1 min, and elongation at 72°C for 1 min 30 s, followed by a final elongation step at 72°C for 5 min. Primer sequences for amplification of the fungal DNA fragment coding for the 26S rRNA were NL1 (forward, 5'-GCCATATCAATAAG CGGAGGAAAAG-3', attached to a GC-clamp at the 5'-terminus) and LS2 (reverse, 5'-ATTCCCAAACAACTCGACTC-3'; *S. cerevisiae* positions, 266–285). Primers were purchased from Sangon Biotech Co., Ltd. (Beijing, China). The PCR protocol was as follows: an initial denaturation at 94°C for 5 min, followed by 35 cycles with a temperature profile of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min 30 s. An extension period of 5 min at 72°C was carried out at the end of the 35 cycles. The products were examined by electrophoresis on a 2% agarose gel.

DGGE (denaturing gradient gel electrophoresis) analysis of PCR products was carried out using the DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using polyacrylamide gels with a 30%–55% denaturing gradient (where 100% is defined as 7 M urea with 40% formamide) [10]. Gels were run at a constant voltage of 200 V and temperature of 61°C for 5 h in 0.5×TAE electrophoresis buffer. Following electrophoresis, gels were stained with SYBR Green I (Molecular Probes, Eugene, OR, USA) and photographed under UV (302 nm) using the Alpha Imager 2200 Imaging System (Alpha Innotech, USA). The images and UPGMA cluster were analyzed using Quantity One Software (Bio-Rad, USA).

RESULTS AND DISCUSSION

Changes in pH of Hydrolysate During Pretreatment by XDC-2

The pH of the hydrolysate declined rapidly from the initial values of 7.7 to 6.4 during the first two days. The pH increased thereafter, reaching a value of 8.9 on the tenth day and remained relatively stable at about 9.1 on days 13 and 16 (Fig. 1). The observed change in pH is characteristic of cellulose degradation by XDC-2 [7].
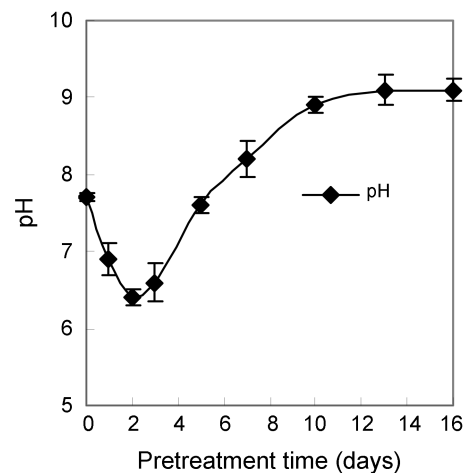

Fig. 1. Changes in pH during pretreatment of corn stalk.
The pH of the hydrolysates was determined during pretreatment by microbial system XDC-2.

Most anaerobic bacteria, including methanogens, perform well within a pH range of 6.8 to 7.2. The growth rate of methanogens is greatly reduced below pH 6.6 [15], whereas an excessively alkaline pH can lead to disintegration of microbial granules and subsequent failure of the process [20]. Therefore, the pH of the hydrolysate between days 3 and 5 was suitable for anaerobic fermentation.

Changes in Weight Loss of the Corn Stalk, Hemicelluloses, and Cellulose During Pretreatment by XDC-2

Weight losses of the corn stalk, cellulose, and hemicellulose were determined during the 16-day process of pretreatment. The corn stalk was degraded most expeditiously in the first 5 days (Fig. 2). The weight loss of dry matter from corn stalks was 20.0% at day 2 and 36.2% at day 5, with hemicellulose drastically losing 47.6% and 67.2% of its weight and cellulose losing only 9.7% and 13.4% of its weight. Dry matter loss by the corn stalk was 39.9% after 7 days and 44.0% after 16 days. The cellulose degradation ratios were 18.4% (after 7 days) and 22.7% (after 16 days) (Fig. 2). Corn stalk and hemicellulose dry matter weight decreased most sharply during the first 5 days: the weight of corn stalk and hemicellulose decreased from 5.00 g and 1.44 g to 3.19 g and 0.47 g, respectively. Lignin content did not change significantly (data not shown). During the first five days of pretreatment, the degradation of the corn stalks was most visible, and the hydrolysate was always slightly acidic and neutral. After the 7th day, the corn stalk was degraded more slowly and the pH became alkaline, reaching more than 9.0 after 13 days (Fig. 1).

Changes in sCOD During Pretreatment by XDC-2

sCOD concentration of the hydrolysate is depicted in Fig. 3. sCOD increased rapidly from the initial values of
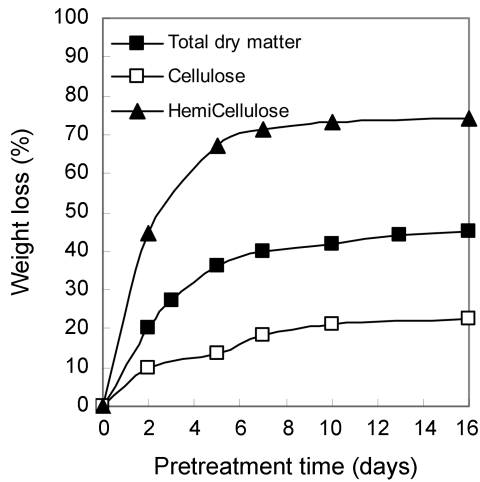
Fig. 2. Dynamics of the weight losses of corn stalk, hemicellulose, and cellulose during pretreatment.

2,850 to 8,233 mg/l during the first five days, and then decreased thereafter, reaching a value of 3,310 mg/l on the 16th day. It can be seen from Fig. 3 and Fig. 4 that there is a direct relationship between volatile products and sCOD concentrations. Since pretreatment of soluble organics occurs simultaneously during the pretreatment of corn stalks by XDC-2, sCOD concentrations were highest (8,233 mg/l) at the fifth day when the concentrations of volatile products were highest.

Analyses of Volatile Products During Pretreatment by XDC-2

Qualitative analysis of the main volatile products is shown in Fig. 4. The total levels of the volatile products peaked on the fifth day. Six volatile products were present in the hydrolysate: ethanol, acetic acid, 1,2-ethanediol, propionic acid, butyric acid, and glycerine. The levels of these six
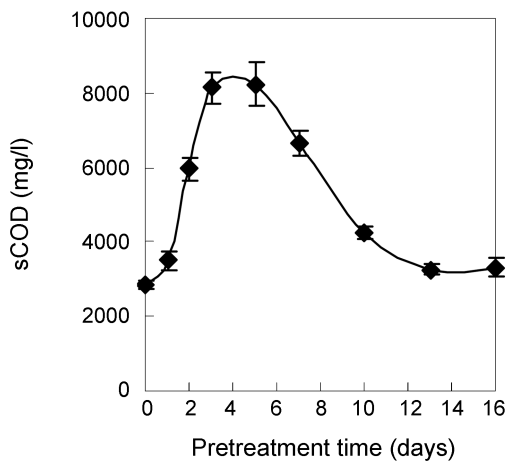

Fig. 3. Changes in sCOD concentration during pretreatment of corn stalk.
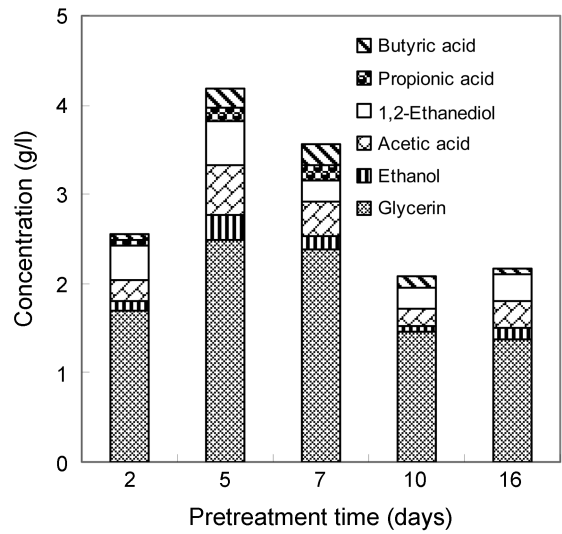

Fig. 4. Quantitative analysis of major volatile products by GC–MS during pretreatment.

compounds increased rapidly during the first five days. The levels on day 5 were as follows: ethanol, 0.29 g/l; acetic acid, 0.55 g/l; 1,2-ethanediol, 0.49 g/l; propionic acid, 0.15 g/l; butyric acid, 0.22 g/l, and glycerine, 2.48 g/l. After 10 days, the types and amounts of volatile products decreased gradually. This analysis of volatile products confirmed that the decrease in the pH of the hydrolysate could be due to production of organic acids (acetic acid, propionic acid, and butyric acid).

Short-chain volatile products are a key intermediate in the process of anaerobic digestion. Some research has shown that the formation of organic acids and ethanol plays an important role in enhancing the pretreatment of lignocellulose [26, 30]. In addition, mild acids can loosen the structure of lignocelluloses, resulting in an improved overall rate of pretreatment due to the increased accessibility to enzymes [3, 26]. Obviously, the corn stalk tissue can release short-chain organic acids through pretreatment by XDC-2. These compounds are all good materials for the subsequent anaerobic fermentation stage and were produced in considerable amounts from the fifth day to the seventh day.

In previous studies, mechanical pretreatments have proven effective in reducing the particle size of lignocelluloses without producing microbial inhibitors and usually leading to increased methane production. Chemical pretreatments are also considered very effective methods to change the chemical composition, chemical structure, and physical characteristics of lignocellulose. These changes work together to contribute to improve biodegradability and biogas production [28]. In this study, the composite microbial system XDC-2 was not only able to change the chemical composition and chemical structure of corn stalks, but it also increased the sCOD concentration of the hydrolysate and produced short-chain volatile products that can be directly

used in the subsequent anaerobic fermentation stage. The results of anaerobic digestion also confirm this view.

DGGE Analysis of Microbial Composition During Pretreatment by XDC-2

The primers 357F/517R and NL1/LS2 were used to amplify the bacterial 16S rRNA gene region V3 and the fungal 26S rDNA gene region D1–D2 in PCR amplification. No fungal 26S rDNA gene PCR products were detected by agarose gel electrophoresis. Only 16S rRNA gene PCR–DGGE was performed. A lot of research has been conducted on microbial pretreatment of natural lignocellulosic materials, but most of them focused generally on fungi, especially those with simple strains and pure cultured [19]. In this paper, XDC-2 was composed of bacteria, and there was no fungus.

During pretreatment, the changes in the population and composition of XDC-2 were reflected by the changes in the banding pattern (Fig. 5A). The results of the PCR–DGGE analysis revealed that the diversity of bacterial populations increased with time during pretreatment and decreased at the end of pretreatment. Certain bacterial strains, represented by bands A, B, C, and D, were present in all samples. The bacterial diversity at days 5 and 10 was higher than days 0, 2, and 16. The DGGE gel profile was also analyzed statistically using UPGMA cluster analysis. The relationships among the different time point samples are shown in Fig. 5B. The results show that the microbial populations on days 5, 10, and 16 are the most similar.

From day 0 to day 5, rapid bacterial growth and increased bacterial population may be an important reason for pretreatment of corn stalk tissue. Moreover, from day 5 to day 10, the abundant bacterial population may be the reason for the reduction in short-chain volatile products.

Anaerobic Digestion

The results of pretreatment indicated that the optimal length of time for pretreatment is five days, since sCOD concentration and short-chain volatile products of the hydrolysate are highest. Therefore, the untreated corn stalks and 500 ml hydrolysates treated for five days by XDC-2 with corn stalks were digested in batch anaerobic digesters. The daily biogas production is shown in Fig. 6A. The general pattern across samples was for biogas production to begin after seeding and to increase to a peak before subsequently declining. The main differences among the untreated and treated corn stalks were the peak values of daily biogas production and the duration of biogas production. The corn stalks treated by XDC-2 had the greatest daily biogas production (520 ml) on the 2nd day, in contrast to a peak of 225 ml on the 12th day for the untreated samples.

The total biogas production (TBP) and the total methane productions (TMP) are shown in Fig. 6B. The treated corn stalks obtained significantly higher TBP and TMP than the
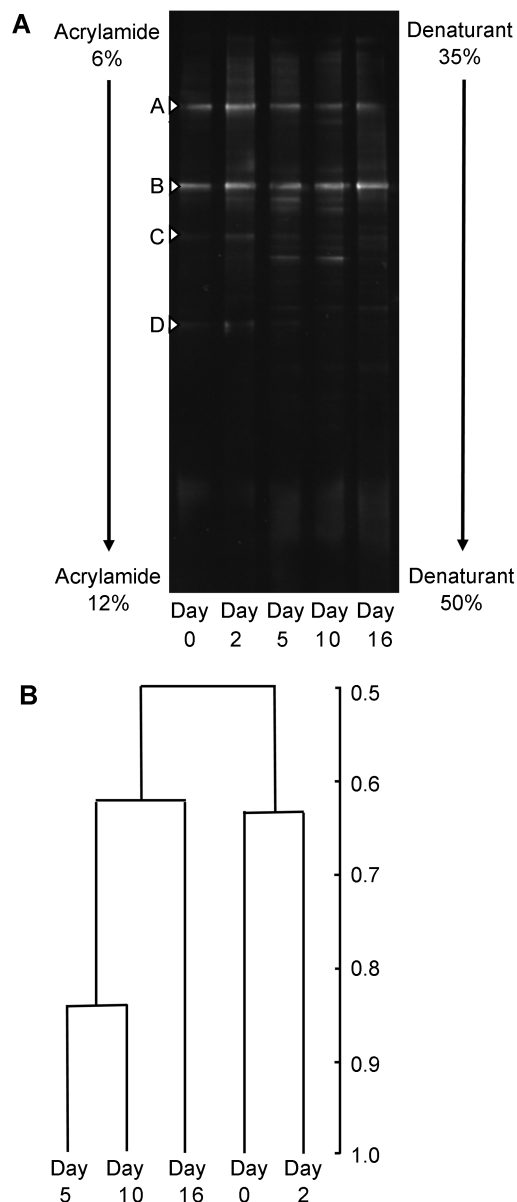

Fig. 5. PCR–DGGE profiles of XDC-2 and UPGMA cluster analysis during pretreatment.
A. Bacterial dynamics based on the V3 region of 16S rRNA genes, which were sampled at days 0, 2, 5, 10, and 16 during pretreatment of corn stalk by XDC-2. The long arrows represent the direction of the denaturants (35–50%) and the polyacrylamide (6–12%) gradients. **B**. UPGMA cluster analysis of PCR–DGGE profiles.

untreated controls. For the treated corn stalks, the TBP and TMP were 2,450 and 1,330 ml, respectively, 68.3% and 87.9% greater than the untreated samples.

The composition of the biogas produced from the untreated and treated corn stalks is shown in Fig. 7. The methane content increased significantly during the first 5 days of anaerobic digestion. However, the methane content of the biogas produced from the treated corn stalks increased faster than that produced from untreated corn stalks alone.
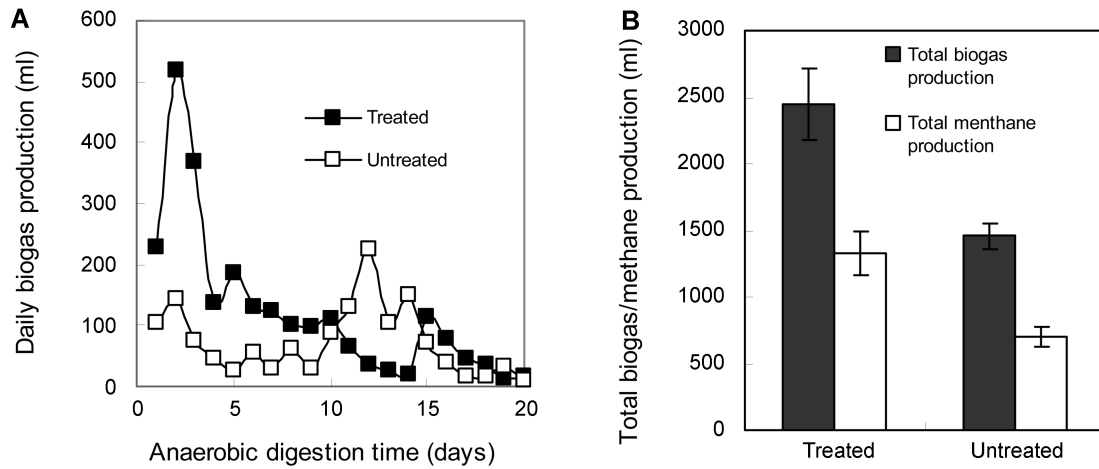
Fig. 6. Biogas production of untreated and treated corn stalks during anaerobic digestion.
A. Daily biogas production assay was carried out at 35ºC. **B**. Total biogas production (TBP) and total methane production (TMP) of untreated and treated corn stalks. Untreated, 5 g of untreated corn stalks and 500 ml of PCS medium. Treated, 500 ml of hydrolysate treated five days by XDC-2 with corn stalks.

The peak value of methane content for the treated corn stalks was 63.3% on the 5th day, compared with 58.2% on the 8th day for the untreated ones. These results indicate that the accumulation of intermediates in the XDC-treated corn stalk hydrolysate during the pretreatment period is beneficial to the subsequent anaerobic digestion.

Digestion time is another important indicator of substrate biodegradability and the effect of pretreatment. Technical digestion time (T80) is defined as the time needed to produce 80% of the maximal digester gas production [17]. The anaerobic digestion process in this study lasted up to 20 days until the biogas production neared zero. The total biogas production finally obtained was considered as maximal digester gas production and used to calculate T80 [28]. From Fig. 6A, we found that T80 was 9 days for the treated corn stalks, and 14 days for the untreated ones. T80 of the treated corn stalks was 35.7% shorter than the untreated ones. The significant reduction of digestion time
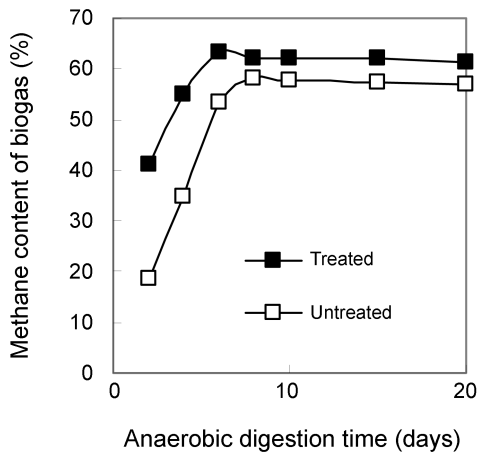
further indicated that the corn stalks had become more accessible and more readily biodegradable after pretreatment, thus taking less time to be digested. Moreover, this could bring significant economical benefit for increasing the production efficiency or treatment capacity of one existing digester by using shortened digestion time [28].

Previous studies on microbial pretreatment of celluloses mostly focused on the pure culture of fungi and bacteria [12, 23]. However, the decomposition activity and ability of pure culture bacteria were generally limited. The pure culture isolates only degraded the substrates with relatively simple structure and composition, such as the artificial xylan and pure cellulose [5, 6, 11, 18], but were unable to use natural lignocelluloses. XDC-2 can effectively degrade the core of decorticated corn stalk, rice straw, filter paper, [7] and corn stalk, respectively [this paper]. The significant increase in total biogas production and the shorter digestion time indicated that the corn stalks had become more accessible and more readily biodegradable after pretreatment by XDC-2. Moreover, this system could provide significant economic benefit by increasing biogas production or increasing the treatment capacity of existing digesters by using a shortened digestion time.

In conclusion, pretreatment with composite microbial system XDC-2 proved to be efficient in improving biodegradability and enhancing biogas production from corn stalks. XDC-2 increased the sCOD concentration of the hydrolysate and produced short-chain volatile products, which can be directly used in the subsequent anaerobic fermentation stage. It also changed the chemical structures through decreasing the cellulose content by 22.7% and hemicellulose by 74.1%. Compared with the untreated corn stalks, the total biogas production and methane yield were increased by 68.3% and 87.9%, respectively, and the technical digestion time (T80) was shortened by 35.7%.

Fig. 7. Methane content during anaerobic digestion.

Further studies of lignocellulosic biomass pretreatment by XDC-2 on different solid contents and co-digestion with anaerobic inoculum will be necessary.

Acknowledgments

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