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Degradation of Lignocelluloses in Rice Straw by BMC-9, a Composite Microbial System

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To evaluate the potential utility of pretreatment of raw biomass with a complex microbial system, we investigated the degradation of rice straw by BMC-9, a lignocellulose decomposition strain obtained from a biogas slurry compost environment. The degradation characteristics and corresponding changes in the bacterial community were assessed. The results showed that rapid degradation occurred from day 0 to day 9, with a peak total biomass bacterium concentration of 3.3×10^8 copies/ml on day 1. The pH of the fermentation broth declined initially and then increased, and the mass of rice straw decreased steadily. The highest concentrations of volatile fatty acid contents (0.291 mg/l lactic acid, 0.31 mg/l formic acid, 1.93 mg/l acetic acid, and 0.73 mg/l propionic acid) as well as the highest xylanse activity (1.79 U/ml) and carboxymethyl cellulase activity (0.37 U/ml) occurred on day 9. The greatest diversity among the microbial community also occurred on day 9, with the presence of bacteria belonging to *Clostridium* sp., *Bacillus* sp., and *Geobacillus* sp. Together, our results indicate that BMC-9 has a strong ability to rapidly degrade the lignocelluloses of rice straw under relatively inexpensive conditions, and the optimum fermentation time is 9 days.

Keywords: Microbial community, rice straw degradation, qPCR, volatile fatty acids

Introduction

Ligonocellulosic biomass is one of the most abundant resources in the world; it is renewable and can be degraded by microorganisms. Agricultural waste products such as rice straw are important sources of lignocellulosic biomass, and more than 0.6 million tons of rice straw are produced annually in Jilin Province alone [18]. As an energy source, lignocellulosic biomass offers an important sustainable and environmentally friendly alternative to the current natural gas platform [16]. The biodegradation of lignocellulosic biomass through the use of complex microbial communities has been proposed as a highly efficient approach for biotechnological applications [4, 13].

Natural lignocellulosic biomass is difficult for microorganisms to degrade because it is composed of a combination of cellulose, hemicelluloses, and lignin [6, 11]. Although microbial decomposition of lignocelluloses has been studied extensively, most of these studies used pure cultures of microorganisms [20]. In our laboratory, we have tested a composite microbial system (MC1, XCD-2, and WDC-2) obtained from a straw and manure compost environment and found efficient and stable cellulose degradation [12, 13, 31]. Furthermore, we tested the performance of this system for biogas production, and the results showed that biogas production *via* anaerobic digestion offers significant advantages over other forms of agricultural waste treatment. However, the optimum duration of pretreatment with a composite microbial system has not been determined. Therefore, in the present study, we selected a composite microbial system, BMC-9, from a biogas slurry compost environment and analyzed the characteristics and microbial composition of this system for future application in the fermentation of straw to produce biogas *via* acidification.

The objective of this study was to characterize the degradation of rice straw by BMC-9 over a 12-day course,

including the changes in the microbial community and total bacterial biomass using the quantitative polymerase chain reaction (qPCR). Additional variables measured during the 12-day process were related to the state of degradation, such as loss of rice straw mass, cellulase activity (assayed using carboxymethyl cellulose, CMC), xylanase activity, and changes in pH and volatile fatty acid (VFA) content in the medium.

Materials and Methods

Lignocellulosic Material

Rice straw was obtained locally (Beijing, China) after harvest from the experimental fields at China Agricultural University Beijing, China, and oven-dried at 60°C for 24 h. It was cut into pieces approximately 3–5 cm in length for further use.

Alkaline Pretreatment

Fifty grams of dried rice straw was soaked in 1 L of 1% (w/v) NaOH solution under static conditions at room temperature, resulting in a concentration of 5% (w/v) dry straw solids. After 24 h, the rice straw was collected, washed with tap water to reach a neutral pH, and then oven-dried to a constant weight at 60°C.

Medium and Culture Conditions

The medium was peptone cellulose solution composed of 5 g peptone, 10 g cellulose (rice stalk), 1 g yeast extract, 3 g $CaCO_3$, and 5 g NaCl in 1 L of H_2O (pH 8.0). For sterilization, the medium was autoclaved at 121°C for 20 min.

The culture conditions were as follows. After inoculation (seed volume of 5%), the medium was cultured under static conditions at 60°C. Fermentation lasted 12 days, and samples were taken to obtain correlative numerical data on days 0 (immediately after inoculation), 1, 3, 6, 9, and 12.

Fermentation and Determination of Biomass Weight Loss and Composition

After inoculation (seeding volume of 5%) of the activation culture, the medium was cultured under static conditions at 60°C. Fermentation lasted for 12 days, and samples were taken on day 0 (immediately after inoculation) and then after 1, 3, 6, 9, and 12 days for analysis.

The complete fermentation materials (including the fermentation broth and residual lignocellulosic materials) were centrifuged at 12,000 ×*g* for 10 min. The precipitates were washed with an acetic acid/nitric acid solution and then with water to remove non-cellulosic materials. Non-inoculated medium served as a control. The weight loss of residual substrate was determined using a previously reported procedure [20]. For composition analysis, residual lignocellulosic material was passed through a 1-mm screen, and a 0.5 g sample was transferred into a special pocked (Model F57, USA). The components of residual lignocellulosic

material were analyzed using a fiber analyzer (Model ANKOM[220], USA) as described elsewhere [32].

The pH of the fermentation broth during the 12-day degradation process was also measured on days 0, 1, 3, 6, 9, and 12 using a Horiba Compact pH meter (Model B-212, Japan).

Crude Enzyme Extraction and Determination of Enzymatic Activity

Cellulose degradation was examined in terms of cellulase activity (assayed using CMC). First, 200 μl of culture supernatant (culture solution was centrifuged at 12,000 ×*g* for 10 min) and 200 μl of 1% (w/v) CMC solution were mixed and incubated for 10 min at 60°C. Next, 600 μl of DNS reagent solution (1% NaOH, 20% Rochelle salt, 2% phenol, 0.005% sodium sulfite, and 1% 3,5-dinitrosalicylic acid (DNS)) was added to the mixture, which was then incubated for 10 min at 100°C. The 1 ml mixtures were cooled on ice for 5 min and centrifuged, and the absorption at 540 nm of each mixture was measured. In addition, a standard curve was prepared using 5 mmol/l glucose, and a blank control was prepared for each sample. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 mmol of reducing sugars per minute under the above conditions [22, 30].

Xylanase activities were assayed according to the methods of Bailey *et al.* [1]. The substrate solution contained 1% oat spelt xylan (Sigma) dissolved in phosphate buffer (pH 6.0). The reaction mixture consisted of 200 μl of substrate solution and 200 μl of appropriately diluted enzyme. The reaction mixture was incubated at 60°C for 20 min prior to reducing-sugar estimation. Enzyme and reagent blanks were also simultaneously incubated with the test samples. Color development was measured at 540 nm using the DNS method. The enzyme activities (excluding cell-associated enzyme activity) were expressed per milliliter of original volume of fermentation broth. One unit (U) of enzymatic activity was defined as the amount of enzyme required to liberate 1 μmol of glucose or xylose in 1 min from carboxymethyl cellulose or xylan, respectively.

HPLC Analyses of Organic Acid Products

The measurement of short-chain organic acids was performed on a Shimadzu high performance liquid chromatography (HPLC) system equipped with an Aminex HPX-87H column (300 mm × 7.8 mm), a Shimadzu SPD-M20A HPLC detector, and a Finger-tight I PEEK Green fitting (Bio-Rad) prior to the HPX-87H column. Samples were analyzed at 35°C, with 5 mmol H_2SO_4 as the influent phase at a flow rate of 0.6 ml/min [26]. Data analyses were performed using the Shimadzu HPLC Software LCMS solution at a detection wavelength of 210 nm. The retention times were used for qualitative criteria, and the peak areas as quantitative criteria. Calibration was done using external standards of the respective components. Liquid samples for organic acid analyses were taken on days 0 1, 3, 6, 9, and 12 by removing 1 ml of the liquid. Samples were stored at –20°C until analysis. The samples were centrifuged for 10 min at 12,000 ×*g*, filtered through a

0.45 μm polytetrafluoroethylene filter, and injected (20 μl) directly into the HPLC system.

Analyses of the Microbial Community Using PCR-DGGE

Genomic DNA was extracted using an automated nucleic acid extractor (Bioteke Biotech 149 Co., Ltd., China) and was used as the PCR template. The DNA was eluted with 40 μl of Tris–HCl buffer (pH 8.0) and stored at –20℃. DNA extraction was carried out according to the benzyl chloride method [34].

16S rRNA PCR amplification was performed using the Gene Amp PCR System (Model 9700; Applied Biosystems, USA). The primers used for denaturing gradient gel electrophoresis (DGGE) were eubacterial universal primers 357F-GC (5'-CCTACGGGA GGCAGCAG-3') with a GC-clamp (5'-CGCCCGCCGCGCGCG GCGGGCGGGGCGGGGGCACGGGGG G-3') and 517R (5'-ATTACCGCGGCTGCTGG-3'), which was used to amplify the V3 region of the 16S rRNA gene [17]. An initial DNA denaturation step was performed at 95℃ for 10 min, followed by 30 cycles of denaturation at 93℃ for 1 min, annealing at 48℃ for 1 min, and elongation at 72℃ for 90 sec, which was followed by a final elongation step at 72℃ for 5 min. The products were examined by electrophoresis on 2% agarose gels [28].

DGGE analysis of the PCR products was performed using the DCode system (Bio-Rad Laboratories, Hercules, CA, USA) as previously described [7, 17]. Samples were applied to 1-mm-thick 6–12% (w/v) polyacrylamide gradient gels in a 0.5× Tris-acetate-EDTA (TAE) electrophoresis buffer (20 mmol/l Tris-HCl, pH 8.3, 10 mmol/l acetic acid, and 0.5 mmol/l EDTA), with a 30–60% denaturant gradient (where 100% was 7 mol/l urea with 40% formamide). Electrophoresis was performed at a constant voltage of 200 V and a temperature of 61℃ for 5 h. After electrophoresis, the gels were stained with SYBRs Green I (Molecular Probes, Eugene, OR, USA) and photographed as previously described [21]. The DGGE gel bands were observed under ultraviolet light at 302 nm using an Alpha Imager 2200 Imaging System (AlphaInnotech, USA). The individual DGGE bands were excised immediately, purified, and reamplified for further sequencing analysis using a clone library approach [33], in which one clone was randomly selected from each band for sequencing.

The resulting sequences were compared with those in the National Center for Biotechnology Information (NCBI) GenBank using the BLAST program and aligned using ClustalX ver. 1.83 [8].

Quantitative PCR for BMC-9 Characterization

Quantitative PCR (qPCR) was performed on the ABI 7500 system (Model 7500; Applied Biosystems). qPCR mixtures (20 μl) consisted of 10 μl of SYBR Green Super mix (Invitrogen, Life Technologies, USA), 7.8 μl of PCR-grade water, 0.4 μl of forward and reverse specific primers (final concentration, 10 μM), 0.4 μl of Rox, and 1 μl of template DNA. Two replicates were analyzed per sample. The forward primer was 63F (5'-GCAGGCCTAACACAT GCAAGTC-3'), and the reverse primer was 355R (5'-CTGCTG CCTCCCGTAGGAGT-3') [2]. The amplification protocol was as

follows: 2 min at 50℃, 2 min at 95℃, 40 cycles of 5 sec at 95℃, and combined annealing and extension for 45 sec at 68℃.

Results

Changes in pH During Degradation of Rice Straw

The pH range characteristic of the degradation of cellulose was reported to be pH 5.9–8.5 [6, 15]. In the present study, the pH of the fermentation broth declined rapidly from 7.4 to 6.2 during the first 2 days of fermentation. The pH value increased thereafter, reaching a value of 8.3 on day 12 (Fig. 1). Upon pH titration to 8.3, the anaerobic fermentation was successfully recovered from the diminished condition induced by acidification.

Degradation Capacity and Enzymatic Activity of BMC-9

As shown in Fig. 2, the mass of rice straw in the fermentation mixture had decreased by 75% after 12 days. Individually, the masses of the cellulose, hemicellulose, and lignin components decreased by 97.11%, 74.7%, and 48%, respectively. By comparison, with degradation by MC1, the overall dry weight of biomass decreased by 81%, with a 99% decrease in cellulose, a 74% decrease in hemicellulose, and a 24% decrease in lignin [27]. A comparison of these results suggests that BMC-9 and MC1 have a strong ability to degrade lignocelluloses of rice straw. The most readily used components of cellulose and hemicellulose were rapidly decomposed by microorganisms, and thus, the early fermentation times represented the main period, during which the rice straw was degraded the most
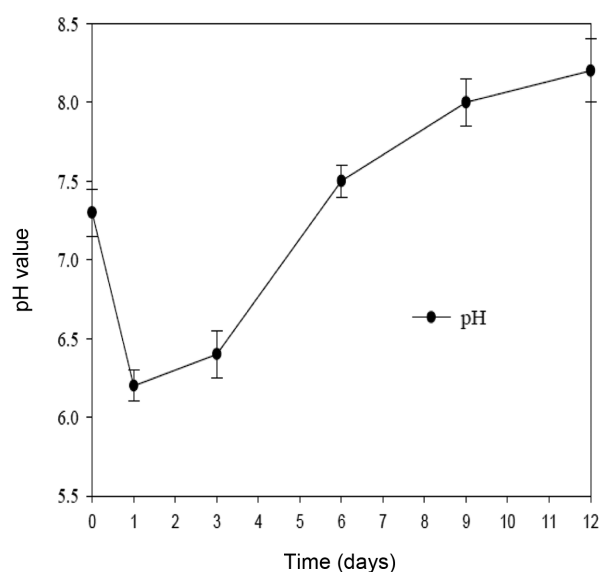

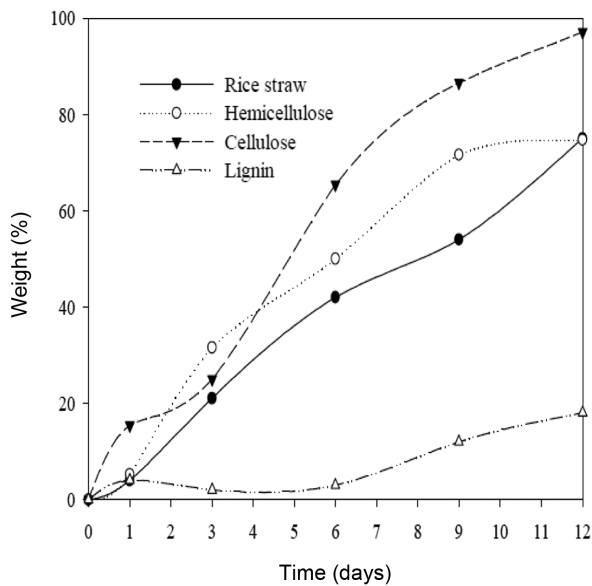**Fig. 1.** Changes in pH during the degradation of rice straw.

Fig. 2. Changes in rice straw, hemicellulose, cellulose, and ligin mass during the degradation of rice straw.

intensively. Further studies are necessary to determine the mechanisms by which BMC-9 degrades lignocelluloses within the acidification phase of biogas fermentation.

CMC and xylanase activities were determined over the 12-day course of rice straw degradation. Xylanse activity showed a rectilinear rise before peaking on day 9 with an activity of 1.79 U/ml. CMC activity reached a maximum of
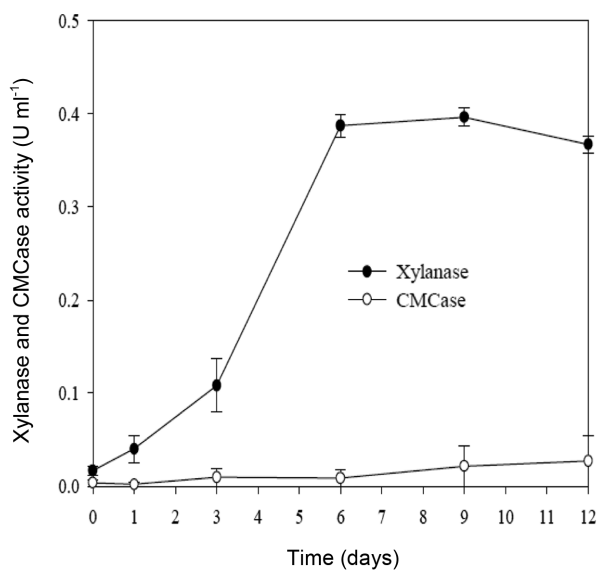
0.37 U/ml on day 9 and decreased thereafter (Fig. 3). The activities of these enzymes indicate that rice straw decomposition by BMC-9 was strongest on day 9 of fermentation.

Analyses of Rice Straw Degradation Products

The results of the qualitative analysis of the main volatile products resulting from rice straw degradation are shown in Fig. 4. The total levels of the volatile products peaked on day 9, similar to the results for enzyme activity. Four main volatile products were identified in the fermentation broth during rice straw degradation: formic acid, acetic acid, propionic acid, and lactic acid. The results of the quantitative analysis of the volatile products confirmed that the decrease in pH of the fermentation broth was due to the production of organic acids. The concentrations of the four main acids on day 9 were 0.291 mg/l lactic acid, 0.31 mg/l formic acid, 1.93 mg/l acetic acid, and 0.73 mg/l propionic acid. The rice stalks could produce organic compounds of low molecular weight through degradation by BMC-9. These compounds are all good materials in the anaerobic fermentation process; for example, acetic acid, which is a substrate for methane production, was produced in considerable amounts from days 3 to 12. If these compounds can be extracted and utilized in this period, the use of corn stalks would be valuable in reducing environmental pollution [24].

Bacterial DGGE Profiles

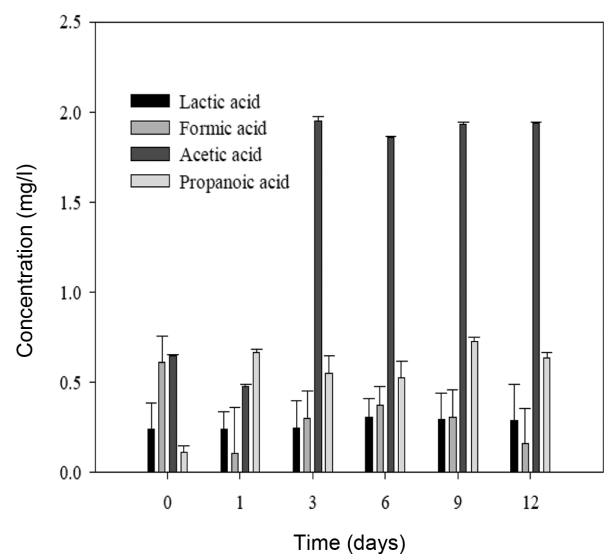Cells were collected on days 0, 1, 3, 6, 9, and 12 from the

Fig. 3. Changes in the enzyme activities of BMC-9 during the degradation of rice straw.

Fig. 4. Qualitative HPLC analysis of volatile products generated during the degradation of rice straw.
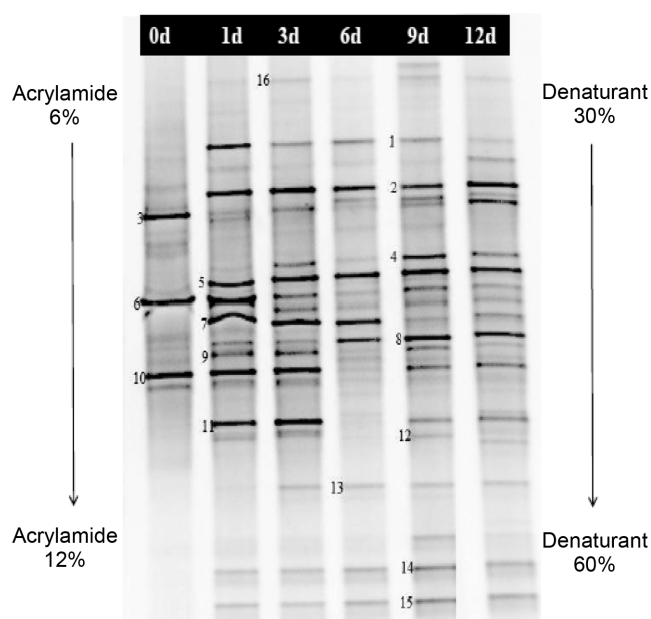
Fig. 5. Analysis of PCR-DGGE profiles of BMC-9 during the degradation of rice straw.

Table 1. Analysis of bacterial 16S rDNA sequences.

Band	Accession number	Closest relative	%
1	JN896752.1	*Magnetococcus marinus* MC-1	99%
2	AY466715.1	*Clostridiales bacterium*	98%
3	AB780862.1	*Clostridium* sp.	90%
4	EU178837.1	*Clostridiaceae bacterium*	98%
5	AY466716.1	*Clostridiales bacterium*	99%
6	AB260040.1	*Clostridiales bacterium*	91%
7	NR_041311.1	*Clostridium caenicola* strain	96%
8	AB089217.1	*Bacillus* sp.	98%
9	EF522948.1	*Clostridium* sp.	100%
10	NR_024829.1	*Clostridium straminisolvens* strain	85%
11	EF165015.1	*Clostridium* sp.	100%
12	FJ430056.1	*Geobacillus* sp.	98%
13	EU178827.1	*Clostridiaceae bacterium*	96%
14	AB436742.1	*Clostridium* sp.	85%
15	JX048197.1	*Bacterium* NLAE-zl-G140	91%
16	AB702935.1	*Clostridiales bacterium*	90%

38th generation. Fig. 5 shows the dynamics of the microbial population according to DGGE profiles. The appearance and disappearance of bands in the DGGE profiles indicate that the population shifted in the microbial community structure. Fig. 5 also shows that the bands changed greatly over time in the culture. Sixteen bands appeared in the profiles. Bands 1, 5, 14, 15, and 16 were visible from 1 to 12 days, and bands 1, 14, and 15 had the highest intensity on days 1, 9, and 9, respectively. Bands 2, 3, 5, 6, 8, and 10 were present throughout the entire monitoring period. Bands 2, 5, and 8 gradually increased in intensity, whereas the intensities of band 3, 6, and 10 gradually decreased. Bands 11 and 12 were visible on days 1, 3, 9, and 12. Band 13 was visible from 3 to 12 days with a constant intensity that predominated the microbial population. From the appearance, disappearance, and intensity of these bands, we know that the composition of the microbial population changed greatly during the fermentation process [14].

To investigate the relationships between individual microbes, bands on the profiles were excised and amplified by PCR. The most closely related bacteria based on BLAST matches to 16S rRNA sequences of purified DGGE bands are shown in Table 1. The 16 bands corresponded to three genera: *Clostridium* sp., *Bacillus* sp., and *Geobacillus* sp. Band 12 corresponded to *Geobacillus* sp., which was the most prevalent strain in the microbial community. This genus usually consists of aerobic or facultative anaerobic

bacteria and is grown in a 45–70°C thermophilic environment [5, 19, 23]. Bands 1, 8, and 15 corresponded to *Bacillus* sp. Previous studies have shown that the *Bacillus* sp. is a *Bacillus* genus that can decompose simple organic compounds into inorganic matter. It is capable of degrading lignin to produce a biological surface agent, promoting microbial decomposition of difficult-to-degrade organic matter, and improving the efficiency of composting and shortening the composting cycle [32]. Bands 2, 3, 4, 5, 6, 7, 9, 10, 11, 13, 14, and 16 corresponded to *Clostridium* sp., which is known to lead to a rapid rise in the compost temperature and to possess the ability to degrade cellulose [3, 25].

Quantitative Analysis of Total Bacterial Biomass

The relative abundance of each bacterium was determined by real-time PCR. Fig. 6 shows the relative abundance of each total biomass bacterium from day 0 to 12. The total abundance of bacteria in the biomass first increased and then decreased gradually with time in culture, with the peak value (3.3×10^8 copies/ml) measured on day 1 because the lignocelluloses were readily degraded by the bacteria on the first day.

Discussion

The goal of microbial pretreatment of raw biomass is to improve the production of target products during anaerobic
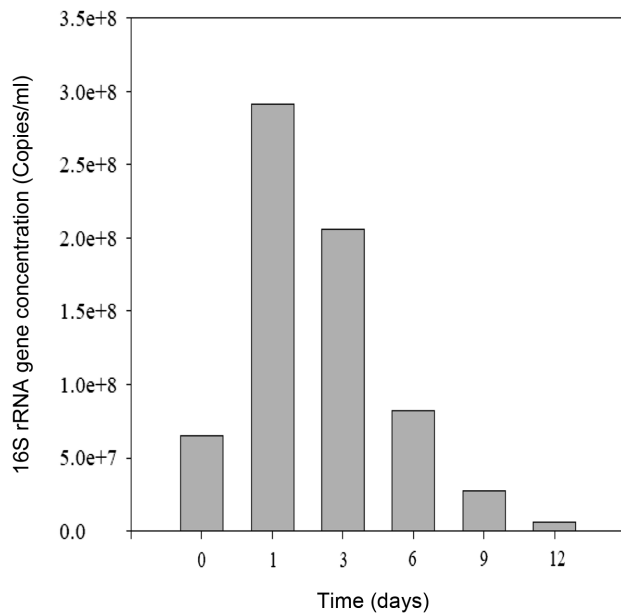
Fig. 6. Quantitative changes in abundance of bacteria during the degradation of rice straw based on 16S rRNA gene concentration in BMC-9.

fermentation. Yuan *et al.* [31] showed that pretreatment with a composite microbial system, XDC-2, was efficient for improving biomass biodegradability and enhancing biogas production from corn stalk. Compared with that from untreated corn stalk, the total biogas production and methane yield when using pretreated corn stalk were increased by 68.3% and 87.9%, respectively. In the present study, we investigated the ability of another composite microbial system, BMC-9, to degrade rice straw. Our results indicate that BMC-9 has a strong ability to degrade the lignocelluloses of rice straw (Figs. 2, 3, and 6), and the optimum fermentation time was 9 days. In addition, our results show that BMC-9 can be used to achieve rapid degradation of cellulosic matter in a relatively inexpensive PCS medium open to the air, which offers significant economic benefit. Furthermore, this degradation process produces considerable amounts of formic acid, acetic acid, and propionic acid in the fermentation liquid (Fig. 4), and these organic acids are substrates for methanogenesis in the production of methane [29].

To better understand the degradation ability of BMC-9, we investigated the bacterial composition of this complex microbial system. We identified three genuses, *Clostridium* sp., *Bacillus* sp., and *Geobacillus* sp., in BCM-9. Preliminary studies have shown that *Clostridium* sp. has a superior ability to decompose lignocelluloses and that *Clostridium*

thermosuccinogenes sp. nov. and other *Clostridium* spp. have the ability to degrade natural rice straw and office waste paper, obtained as a waste suspension and used as a main cellulosic constituent fed into the bioreactor [10]. Microorganisms of the genus *Bacterium* are the most commonly found organisms in naturally decomposing waste, organic dyes, and cellulose, and a *Bacillus* sp. has been shown to be able to degrade rice hull [9]. Therefore, these components of BMC-9 are responsible for the ability of BMC-9 to degrade lignocelluloses. Moreover, the increased bacterial abundance observed on day 1 of fermentation indicates that this is a rapid and efficient process for degrading straw resources available in rural districts. In the future, this system could provide significant economic benefit by increasing biogas production or increasing the treatment capacity of existing digesters by shortening the digestion time [31].

Acknowledgments

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