Functional Characteristics and Diversity of a Novel Lignocelluloses Degrading Composite Microbial System with High Xylanase Activity

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To obtain an efficient natural lignocellulolytic complex enzyme, we screened an efficient lignocellulose-degrading composite microbial system (XDC-2) from composted agricultural and animal wastes amended soil following a long-term directed acclimation. Not only could the XDC-2 degrade natural lignocelluloses, but it could also secrete extracellular xylanase efficiently in liquid culture under static conditions at room temperature. The XDC-2 degraded rice straw by 60.3% after fermentation for 15 days. Hemicelluloses were decomposed effectively, whereas the extracellular xylanase activity was dominant with an activity of 8.357 U/ml on day 6 of the fermentation period. The extracellular crude enzyme noticeably hydrolyzed natural lignocelluloses. The optimum temperature and pH for the xylanase activity were 40°C and 6.0. However, the xylanase was activated in a wide pH range of 3.0–10.0, and retained more than 80% of its activity at 25–35°C and pH 5.0–8.0 after three days of incubation in liquid culture under static conditions. PCR–DGGE analysis of successive subcultures indicated that the XDC-2 was structurally stable over long-term restricted and directed cultivation. Analysis of the 16S rRNA gene clone library showed that the XDC-2 was mainly composed of mesophilic bacteria related to the genera *Clostridium, Bacteroides, Alcaligenes, Pseudomonas*, etc. Our results offer a new approach to exploring efficient lignocellulolytic enzymes by constructing a high-performance composite microbial system with synergistic complex enzymes.

Keywords: Lignocellulose degradation, composite microbial system, xylanase activity, composition diversity

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Lignocelluloses are found in nature almost exclusively in plant cell walls, and are some of the most abundant and renewable biomass resources in the world [44]. Agro-residues such as rice straws, wheat straws, and corn stalks are important lignocellulosic biomass, and China is one of the countries with the highest field crop straw production in the world. More than 600 million tons of crop straws are produced yearly in China [28]. Recently, field crop straws have become a focus of several studies for their expectant uses in bioconversion to biofuels [19] and forage [45]. Microbes play a critical role in these conversions. Microbial degradation of lignocellulosic materials is characterized by the biosynthesis of multicomponent enzymes (e.g., cellulase, xylanase, and peroxidase) that have been well studied, and these lignocellulolytic enzymes are either free or cell-associated [15, 18, 23]. Alternatively, cell-free lignocellulolytic enzymes are more available because of their ease of extraction.

However, it is difficult for microorganisms to catabolize natural lignocellulosic materials because of their combination of cellulose, hemicellulose, and lignin. Although microbial decomposition of lignocelluloses has been studied extensively, most of these studies focused on the pure culture of microorganisms [44]. Microorganisms isolated with pure culture are regularly characterised by unsatisfactory lignocellulolytic activities. A few of them had high lignocellulolytic activites and could degrade substrates with relatively simple composition and structure, such as xylan, carboxymethylcellulose, or other substrates pretreated under very rigorous conditions, but could not break down the natural lignocellulose with complex composition and structure, such as field crop straws and other plant fibers [11, 39]. Recent reports showed that a mixture of several enzymes could improve the lignocellulolytic activities of enzymes [40], but there are no reports about enzymes that could effectively hydrolyze natural lignocelluloses that were not treated under rigorous

conditions. Therefore, the absence of microbes and enzymes with efficient degrading capacities is still the major limitation to the conversion of lignocellulosic materials into energy.

Great attention has been paid to the lignocellulose-degrading capabilities of mixed microorganisms, and many reports indicated that mixing several isolated microorganisms yielded microfloras that were more effective than any single isolate [25, 33]. In fact, lignocellulosic materials are degraded by the joint activity of many naturally occurring microorganisms and it is difficult to achieve the degrading capability of such microorganisms through an isolate or simple mixture of isolates. In our laboratory, a stable thermophilic cellulose-degrading composite microbial system, MC1, was developed for effectively degrading natural cellulose under artificial conditions [8]. Previous investigations of the MC1 showed that celluloses were degraded only by viable organisms; extracellular enzyme activities were barely detected, and the metabolic products were very complicated, so it was not appropriate for saccharification of lignocelluloses.

Therefore, in the present study, to develop a lignocellulose-degrading composite microbial system able to degrade natural lignocelluloses and effectively secrete extracellular lignocellulases, we screened and acclimated a novel lignocellulose-degrading composite microbial system, XDC-2, by restricted cultivation at room temperature. The composite microbial system could degrade lignocelluloses such as corn stalk and rice straw as well as hemicelluloses; it also secreted extracellular xylanase effectively. The screening process, characteristics of enzyme activities, degradation capability, and composition diversity of the XDC-2 are described to provide the basis for exploration and development of highly efficient enzymes, and selectively extract hemicelluloses from natural lignocelluloses, thereby improving the accessibility of cellulose to microbes and enzymes for subsequent utilization. Ultimately, it would be possible to convert natural lignocellulosic resources such as agricultural residues to biofuels, livestock feeds, or other chemical products depending on depolymerization by this composite microbial system and its enzymes.

MATERIALS AND METHODS

Preparation of Lignocellulosic Materials and Medium for Screening and Fermentation

Lignocellulosic materials (rice straw or corn stalk) obtained locally from Beijing, China, were air-dried. For delignification to increase the exposure of polysaccharides to microbes and hydrolytic enzymes, the materials were submerged in 1% (w/v) sodium hydroxide at room temperature for 24 h, washed with tap water to neutral pH, and then oven-dried at 80°C. For submerged fermentation, the dried straws were cut into scraps approximately 2 cm in length and mixed thoroughly with the medium. In experiments of enzymatic hydrolysis, after delignification, some rice straws and decorticated corn stalks were milled to pass 1-mm screens, respectively.

The basal medium used in this study contained 5 g peptone, 1 g yeast extract, 2 g $CaCO_3$, 5 g NaCl, 0.35 g $MgSO_4 \cdot 7H_2O$, 1 g K_2HPO_4, a 1% (w/v) carbon source as rice straw, and 1,000 ml of distilled water (unadjusted pH=7.2±0.1). The medium was autoclaved at 121°C for 20 min. In some experiments, rice straws were replaced by other lignocellulosic materials, such as corn stalks and Whatman No. 1 filter paper.

Acronyms (or abbreviations) of the four different raw materials mentioned above are CS (corn stalk), CCS (core of decorticated corn stalk), RS (rice straw) and WF (Whatman No.1 filter paper), which will be mentioned below.

Screening and Acclimation of the Lignocellulose-Degrading Composite Microbial System

Microbial sources were collected from local composted agricultural and animal wastes amended soil in Beijing, China. A 20-g mixture of the soil was sampled into 500-ml Erlenmeyer flasks (stoppered with aluminum foils) containing 350 ml of autoclaved basal medium containing 1% (w/v) rice straw as a single carbon source, and then incubated under static conditions in the dark at room temperature (about 30°C) for enrichment cultivation. After 72 h cultivation, 20 ml of well-blended culture fluids was used to inoculate a fresh 500-ml Erlenmeyer flask that contained the same medium. For further acclimation, 5% (v/v) of inoculums derived from the previous subculture were inoculated into a fresh flask, and incubated under the conditions described above. In the process, subcultures were repeated many times every 3–6 days, and duplications lacking an effective degradation capacity were eliminated whereas efficacious inoculums were used for inoculation and stored at −80°C with 80% glycerol.

Fermentation and Determination of Weight Loss and Lignocelluloses Components of Lignocellulosic Substrates

After inoculation (seed volume of 5%) of the activation culture of the preserved inoculums, the medium was cultured under static conditions at 35°C. Fermentation lasted for 15 days, and samples were taken to obtain correlative numerical data on days 0 (immediately after inoculation), 1, 3, 6, 9, 12, and 15.

The whole fermentation materials (including fermentation broth and residual lignocellulosic materials) were centrifuged at 12,000 ×g for 10 min; the precipitates were washed with acetic acid/nitric acid reagent and then with water to remove non-cellulosic materials. An uninoculated medium served as control. The weight loss of residual substrates was determined using a procedure reported earlier [14]. Residual lignocellulosic materials were passed through 1 mm screens, and a 0.5-g sample was transferred into a special pocket (Model F57, U.S.A.). Components of residual lignocellulosic materials were analyzed using a fiber analyzer (Model ANKOM^{220}, U.S.A.) as described elsewhere [14].

The pH of the fermentation broth during the degradation was also determined on days 0, 1, 3, 6, 9, 12, and 15 using the HORIBA Compact pH meter (Model B-212, Japan).

Crude Enzyme Extraction and Determination of Enzyme Activities

To estimate enzyme activities, 7-ml culture samples were centrifuged at 12,000 ×g for 10 min at 4°C and the supernatants and pellets were separated. The supernatants were used as extracellular crude enzyme samples. The pelleted cells were washed twice by centrifugation in

phosphate-buffered saline solution (pH 7.4) as described previously [4]. The washed cells were suspended in 4 ml of 20 mM Tris hydrochloride buffer (pH 8.0). Then, 0.5 ml of a 1% (w/v) lysozyme (Sigma) solution was added to 4 ml of the cell suspension, and the mixture was incubated at 37°C for 30 min. The reaction system was blended with pipettes at 10-min intervals during the incubation. The supernatants, which were collected again at 12,000 ×g for 10 min at 4°C, were assayed as cell-associated enzyme activity.

Xylanase activities were assayed according to Bailey *et al.* [2]. The substrate solution contained 1% oat spelt xylan (Sigma) dissolved in phosphate buffer (pH 6.0). The reaction mixture consisted of 2.0 ml of substrate solution and 0.5 ml of appropriately diluted enzyme. The reaction mixture was incubated at 40°C for 30 min prior to reducing-sugar estimation. Enzyme and reagent blanks were also simultaneously incubated with the test samples. Color development was measured at 520 nm using the 3,5-dinitrosalicylic acid (DNS) method [27]. Similarly, the activity of carboxymethyl cellulase (CMCase) was assayed using 1% (w/v) CMC (Sigma) as substrate solution in the same conditions. The enzyme activities (including 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.

Optimization of Cultivation Temperature for Degradation and Enzyme Production

For estimating the optimum cultivation temperature for degradation and xylanase production, varying temperatures, 25, 30, 35, 40, 45, and 50°C, were studied under the incubation conditions described above, and the degradation ratio and enzyme activities were assessed by the respective assay procedures.

Effects of Temperature and pH on Xylanase Activity and Stability

The effect of temperature on enzymatic reaction was determined by incubating the enzyme with (1%) oat spelt xylan substrate (50 mM phosphate buffer, pH 6.0) for 30 min at various temperatures (*viz.,* 30, 35, 40, 45, 50, 55, 60, and 70°C). For thermostability determination, the crude xylanase in 50 mM phosphate buffer (pH 6.0) was incubated at different temperatures (30, 35, 40, 45, 50, 55, 60, 70, and 80°C) for 120 min. After cooling the treated enzymes on ice for 30 min, the residual xylanase activities were measured as described above and compared with the untreated enzyme activity.

The effect of pH on enzymatic reaction was assessed by incubating the enzyme with 1% oat spelt xylan substrate prepared in different buffers (50 mM) [*viz.,* glycine-HCl (pH 2–3), acetate buffer (pH 3–5), phosphate buffer (pH 5–8), Tris-HCl buffer (pH 8–9), or glycine-NaOH buffer (pH 9–10)] and incubated at 40°C for 30 min. To determine the pH stability of the enzyme, the crude xylanase was incubated in different buffers, as mentioned above, at room temperature for 2 h. The remaining activities of these treated enzymes were measured as described previously and compared with the untreated enzyme activity.

Determination of the Hydrolytic Capacity of the Crude Enzyme

To assess the hydrolytic capacity of the extracellular crude enzyme, the crude enzyme (enzyme was extracted after 6 days of cultivation according to the method described above) was incubated with 1% (w/v) sterilized treated corn stalk (stripped of the thick hardened horny layers on the surface and crushed to pieces by passing through

1-mm screens) and rice straw (passing through 1-mm screens) at 30°C. After 48 h, the solid residues of the straw substrates were collected by filtration, washed, and then dried at 80°C to constant mass and weighed. The loss of dry mass was calculated as the difference in weight against the substrates that were treated by the heat-inactivated (incubated at 70°C for 30 min) enzyme. The reducing sugars produced by the hydrolytic process were also determined according to the method [27]. The contents of reducing sugars were calculated as the difference against the content of the heat-inactivated enzyme solution.

PCR–DGGE Analysis of Microbial Composition Stability During Successive Subculture

To investigate the component stability of the XDC-2 during successive subcultivation, 1 ml of -80°C preserved inoculums of successive subcultures (from the 35ᵗʰ to 45ᵗʰ subcultures) were inoculated, respectively, into 25-ml Erlenmeyer flasks containing 15 ml of autoclaved basal medium and 1% (w/v) rice straw, and then incubated under static conditions at 35°C. After 6-day cultivation, 7 ml of the fermentation broth was centrifuged at 12,000 ×g for 10 min, and the supernatant was decanted carefully to obtain the sediment. An extraction buffer was used to suspend the sediment. Extraction of total genomic DNA was carried out using the benzyl chloride method [46].

Bacterial 16S rRNA gene PCR amplification was performed in a Mastercycler gradient (Eppendorf, Germany). The primers used for DGGE were 357F-GC (forward, 5'-CCTACGGGAGGCAGCAG-3'; *E. coli* positions, 341–357, attached to a GC-clamp (5'-CGCCCGCC GCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3') at the 5'-terminus, and 517R (reverse, 5'-ATTACCGCGGCTGCTGG-3'; *E. coli* positions, 517–534) [31, 45]. Primers were purchased from Sangon Biotech Co., Ltd., Beijing, China. Initial DNA denaturation was performed at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and elongation at 72°C for 1 min 10 s, followed by a final elongation step at 72°C for 3 min 50 s. Fungal 26S rRNA gene D1–D2 region PCR amplification was performed using the same PCR amplifier. Primer sequences for the amplification of a 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) [7]. Primers were purchased from Sangon Biotech Co., Ltd., Beijing, China. The PCR performed 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 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, U.S.A.) [31, 45] using polyacrylamide gels with a 30%–55% denaturing gradient (where 100% is defined as 7 M urea with 40% formamide). 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, U.S.A.) and photographed under UV (302 nm) using the Alpha Imager 2200 Imaging System (Alpha Innotech, U.S.A.). Individual DGGE bands were excised under UV illumination and the DNA fragments were recovered, and, with the same primers without GC-

clamp, were re-amplified for 25 amplification cycles under the same conditions. The amplified fragments were purified using the high purity PCR product purification kit (Tiangen Biotech Co., Ltd., China) and sequenced using the ABI 3730XL DNA Sequencer (Perkin Elmer) at SunBiotech Developing Center. The resulting DNA sequences were compared with the reference sequences available in the GenBank by BLAST search [1].

Cloning and Sequence Analysis of 16S rRNA Gene

For cloning of 16S rRNA gene amplicons, the total DNA of the composite microbial system, which was preincubated in the basal medium with rice straw as the sole carbon source under static conditions at 35°C for 6 days, was used as template for the construction of the 16S rRNA gene clone library, using universal bacterial primers 8F (5'-AGAGTTTGATCMTGGCTCAG-3')/907R (5'-CCGTCAATT CMTTTRAGTT-3'), as described previously [10,22]. The PCR reaction began at 94°C for 5 min, followed by 25 cycles of denaturation at 94°C for 1 min 30 s, annealing at 52°C for 1 min, and elongation at 72°C for 1 min 30 s, and then a final elongation step at 72°C for 5 min. The PCR products were then ligated into pGEM-T Easy Vector (Promega, Chiba, Japan) according to the manufacturer's protocol after purification by using the high purity PCR product purification kit (Tiangen Biotech Co., Ltd., China). A total of 200 white colonies were randomly picked and screened by DGGE profile as described above. Clones that produced unique bands with different melting positions were selected for sequence analysis. The insert DNA fragments were sequenced at SunBiotech Developing Center (Beijing, China). The resulting 16S rRNA gene sequences (about 920 bp) were compared with those in the GenBank database using the BLAST program [1] and were aligned by ClustalX 1.83 [38]. A phylogenetic tree was constructed using the neighbor-joining method in MEGA program version 4.0 [41].

Nucleotide Sequence Accession Numbers

Sequences obtained in this study have been deposited in the GenBank database under accession numbers EU635482 to EU635500 (16S rRNA gene PCR–DGGE), and FJ938122 to FJ938147 (16S rRNA gene clones).

RESULTS

Screening of the Lignocelluloses-Degrading Composite Microbial System

Following restrictive cultivation and several subcultures, a novel lignocelluloses-degrading composite microbial system (named XDC-2) was successfully screened and acclimated. The XDC-2 could degrade natural lignocelluloses and subcultured stably in liquid culture under static condition at room temperature. The inoculums of the XDC-2 could be conserved at a low temperature of −80°C with 80% (v/v) glycerin for later use.

Degradation Capacity and Characteristics of the Enzyme Activities of XDC-2

The weight loss of the rice straw, cellulose, and hemicellulose were determined during the 15 days of degradation. The
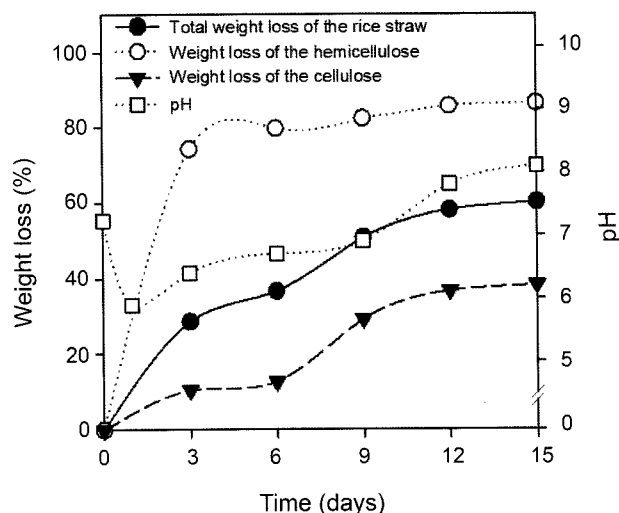

Fig. 1. Dynamics of pH and weight loss of the rice straw, hemicellulose, and cellulose during degradation.

rice straw was degraded most expeditiously before the first 3 days (Fig. 1). The weight loss of dry mass of rice straw was 28.6% after 3 days, in which hemicellulose drastically lost 77.1% of its weight and cellulose only lost 11.2% of its weight. The loss of dry mass of rice straw was 50.9% after 9 days and 58.2% after 12 days. As shown in the curve related to the loss of dry mass of cellulose, the cellulose degraded more strongly during days 6 to 12 than during the first 6 days. The cellulose degradation ratios were 29.2% (after 9 days) and 36.7% (after 12 days). The pH of the fermentation broth was also determined during degradation. One day after inoculation, the pH decreased rapidly from the initial value of 7.2 to 5.9, and then began to rise slowly (Fig. 1). The pH was 6.9 on the 9th day. During the period (<9 days), the degradation of rice straw was most visible, and the fermentation broth was always slightly acidic. After the 9th day, the rice straw was degraded more weakly and the pH became alkaline, reaching more than 8.0 after 15 days.

The extracellular activities and cell-associated enzymes were determined during 15 days of degradation of rice straw. The extracellular xylanase activities showed a rectilinear rise before peaking on the 6th day with an activity of 8.357 U/ml (Fig. 2). The cell-associated xylanase activity also peaked on day 6, but the activity was only 0.097 U/ml. The extracellular CMCase activity reached a maximum of 0.108 U/ml on day 6, and decreased thereafter, but the cell-associated CMCase increased after the 6th day, and peaked on day 9 with an activity of 0.318 U/ml. Enzyme activities indicated that the xylanases were overwhelmingly released into the culture solution, whereas CMCases were mainly cell-bound over the whole degradation process. Therefore, our analysis and discussion would focus on the extracellular xylanase activities in what follows.
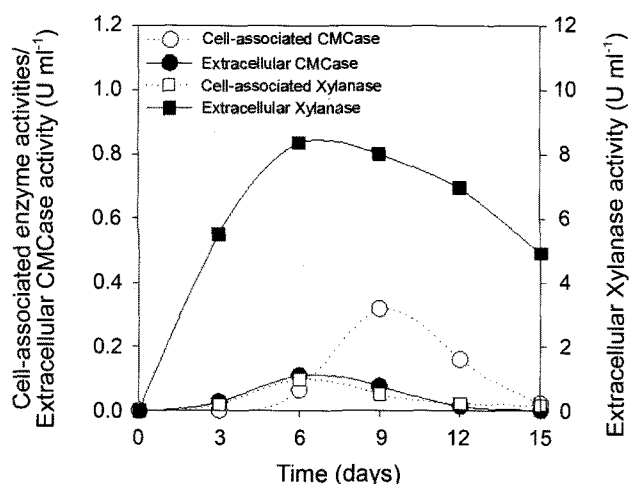
Fig. 2. Changes in the enzyme activities of XDC-2 during the degradation of rice straw.

The extracellular and cell-associated enzymes activities assays were carried out at pH 6.0 and 40°C.

Degradation of Different Lignocellulosic Materials by XDC-2 and Extracellular Xylanase Activities

The content of hemicellulose in the four different materials varied in the order CCS (28.2%) >CS (18.6%) >RS (13.1%) >WF (0); weight loss varied in the order CCS (89.5%) >CS (77.1%) >RS (72.3%) >WF (0), and total degradation ratio after 3 days varied in the order CCS (46.7%) >CS (31.9%) >RS (28.6%) >WF (10.2%) (Fig. 3). The total degradation ratio of different materials corresponded with their contents and weight loss of hemicellulose. XDC-2 could strongly degrade lignocellulosic materials in which the content of hemicellulose was relatively high. The weight loss of WF was only 10.2% on day 3, suggesting
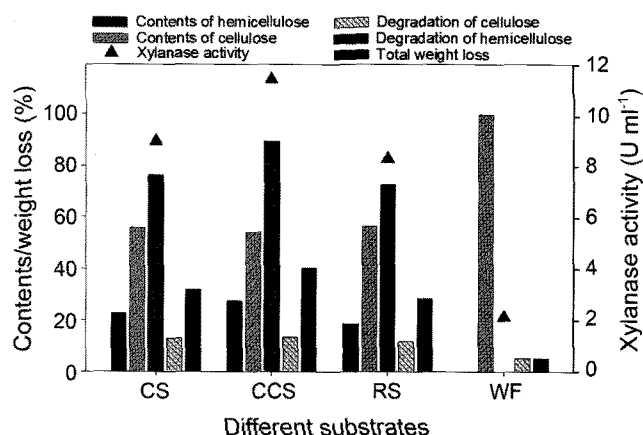
Fig. 3. Contents and weight loss of lignocellulosic components of different materials and xylanase activites.

CS: corn stalk; CCS: core of decorticated corn stalk; RS: rice straw; WF: Whatman No.1 filter paper. The weight loss of the different materials and components were determined after 3 days, and xylanase avtivities were determined after 6 days of fermentation.

that the XDC-2 degraded hemicelluloses more strongly than cellulose over the short term under our experimental conditions. The extracellular xylanase activities were determined on day 6. The xylanase activities reached a maximum of 11.415 U/ml with CCS as the sole carbon source, of which the weight loss was 40.1%. The xylanase activities (U/ml) were 9.013, 8.357, and 2.138 when CS, RS, and WF were used as the carbon sources and the weight losses were 31.9%, 28.4%, and 5.2%, respectively.

On the 6th day of rice straw degradation, the crude enzymes were extracted by centrifugation, and the hydrolytic capacity of the crude enzyme was determined. The loss of dry mass of the rice straw was 16.7%, in which the hemicellulose lost its weight from 18.7% to 7.1%; the loss of dry mass of corn stalk was 23.6%, in which hemicellulose lost its weight from 27.4% to 6.5% against the dry mass treated with heat-inactivated enzyme in the same conditions. The contents of reducing sugar produced from the hydrolysis of rice straw and corn stalk were 1.3 g/l and 2.4 g/l, respectively.

Effects of Initial pH and Cultivation Temperature on Degradation and Extracellular Xylanase Production

With rice straw as the sole carbon source, the degradation ratio and extracellular xylanase activities were determined at different cultivation temperatures (25–50°C). The xylanase activity and rice straw degradation ratio were relatively high at the cultivation temperature of 30–40°C, and the xylanase activity (relative activity 100%) peaked at 35°C (Fig. 4A). The degradation ratio of rice straw was highest at 33.4%. When the cultivation temperature was up to 45°C, the xylanase activity and degrading capability decreased substantially. The xylanase activity and degradation ratio were more visible when the cultivation temperature was less than 40°C than at higher temperatures. When the temperature was 25°C, the xylanase activity and degradation ratio were all visible. The results indicated that XDC-2 was a mesophilic lignocellulose-degrading microbial system, whose optimum cultivation temperature was 35°C.

With rice straw as the sole carbon source, the degradation ratio and extracellular xylanase activities were determined at different pHs of the cultivation media. The extracellular xylanase activity peaked with the relative activity of 100% at the pH of 8.0 (Fig. 4B). When the pH was 6.0–9.0, the relative activities were over 80%. The results indicated that the XCD-2 was stable under acidic or alkaline conditions. The weight loss of rice straw was 24.3% at pH 6.0, 33.1% at pH 7.0, 35.6% at pH 8.0, and 26.5% at pH 9.0. The weight loss and relative xylanase activities varied directly with changes in cultivation temperature and pH.

Optimization of pH and Temperature of Xylanase Enzyme Reaction

The activity of crude xylanase was determined within pH 3–10 at 40°C. The xylanase activity remained high at pH
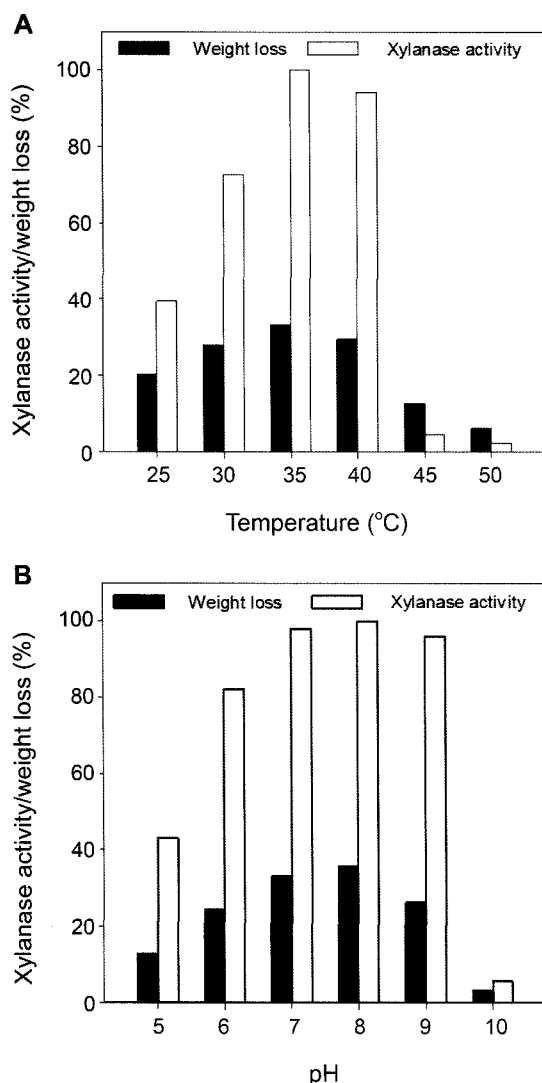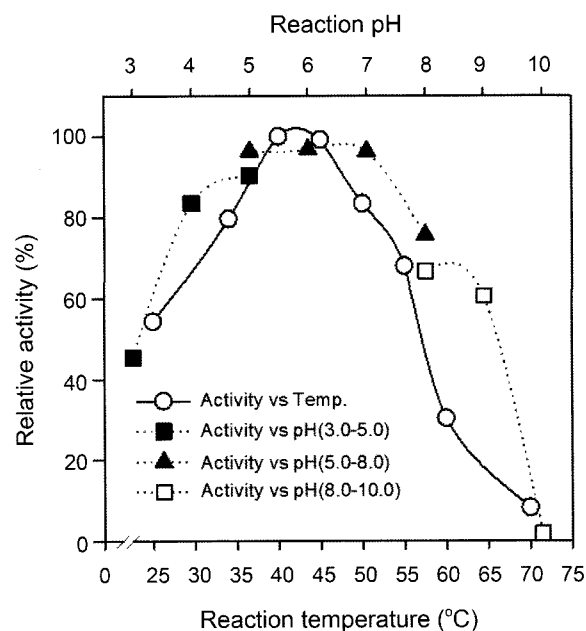
Fig. 4. Effects of incubation temperature (**A**) and pH (**B**) on xylanase production and rice straw degradation.

Xylanase activity and weight loss of rice straws were determined on the 3rd day of degradation. The maximum activity was defined as 100%.


Fig. 5. Effects of pH and temperature on xylanase enzyme reaction.

The pH of the enzyme reaction was determined at 40°C in different buffers of pH 3.0–10.0 for 30 min. The reaction temperature was determined in an enzyme reaction with 50 mM phosphate buffer (pH 6.0) at 25–70°C for 30 min. In each experiment, the highest xylanase activity was defined as 100%.

when the temperature rose to 50°C, the residual activity was 75.7% (Fig. 6A). An incubation at up to 55°C resulted in a 27.9% residual xylanase activity. When the crude xylanase solutions were incubated in pH 7.0 at 25–35°C over an extended period of 3 days, the crude xylanase activities retained were more than 90% (Fig. 6B).

5.0–7.0 (Fig. 5) and peaked at pH 6.0. At pH 6.0, the xylanase activity remained high at 40–45°C and peaked at 40°C.

Effects of pH and Temperature on Xylanase Stability

The xylanase activity was stable over a pH range of 3.0–10.0, and retained more than 90% activity after incubation with buffers (pH 5.0–8.0) at 30°C for 2 h (Fig. 6A). When the crude xylanase solutions were incubated at 30°C at pH 5.0–8.0 over an extended period of 3 days, the crude xylanase activities retained were over 90% at pH 6.0–7.0, 83% at pH 5.0, and 86% at pH 8.0 (Fig. 6C). The thermal stability of xylanase was determined at pH 6.0 by incubating the enzyme at each temperature for 2 h. Less loss of activity was observed after incubation at 30–45°C and

DGGE Analysis of Microbial Composition Stability During Successive Subculture

The primers 357F/517R and NL1/LS2 were used for bacterial 16S rRNA gene V3 region PCR amplification and fungal 26S rDNA gene D1–D2 region PCR amplification. No fungal 26S rDNA gene PCR products were examined by electrophoresis on agarose gel. Only 16S rRNA gene PCR–DGGE was performed. The PCR–DGGE profiles showed the different generations (from the 35th to 45th subcultures) of the XDC-2, which were cultured in the same media for 3 days, respectively. There were 19 visible bands on the gel (Fig. 7), and the strain of genetic relationship represented by each DGGE band was (A) *Alcaligenes* sp. (98%) (the value indicating similarity between the sequence of band and the closely related reference sequence); (B) *Alcaligenes faecalis* (91%); (C) Beta proteobacterium (94%); (D) Pseudomonadales bacterium (98%); (E) *Alcaligenes* sp. (95%); (F) Uncultured bacterium (95%); (G) Bacteroidales bacterium (95%); (H) Uncultured bacterium (99%); (I) Uncultured bacterium (97%); (J) Butyrate-producing bacterium

A

B   **C**
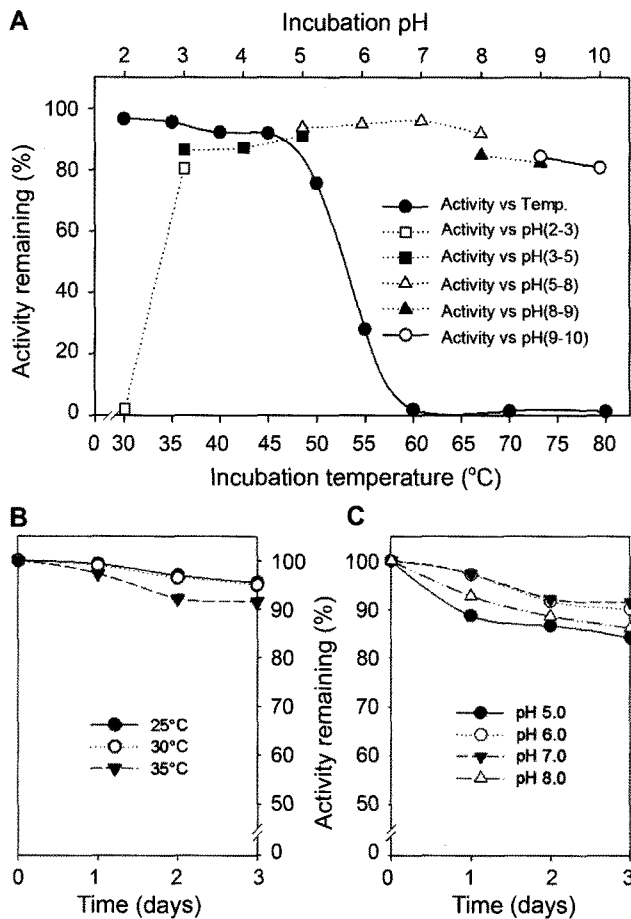
Fig. 6. Effects of incubation pH and temperature on enzyme activity. A. Residual activity was measured after incubating with different buffers at pH 2.0–10.0 at 30°C for 2 h, and with phosphate buffer (pH 6.0) at various temperatures (30–80°C) for 2 h. B. Residual activity was measured after storing with buffers (pH 7.0) at 25, 30, and 35°C for 3 days. C. Residual activity was measured after storing with different buffers (pH 7.0, 6.0, and 5.0) at 30°C for 3 days. For each experiment, the highest xylanase activity or untreated xylanase activity was defined as 100%.

(97%); (K) Uncultured bacterium (99%); (L) Uncultured bacterium (94%); (M) *Escherichia coli* (94%); (N) Uncultured *Tistrella* sp. (92%); (O) *Bacteroides* sp. (93%) (P) Uncultured bacterium (97%); Q. Uncultured *Clostridium* sp. (98%); (R) *Desulfovibrio intestinalis* (98%); and (S) Uncultured soil bacterium (92%). The relative positions of these bands remained constant, respectively, which indicated that the composition of XDC-2 was stable over more than 35 times of subculturing.

Analysis of 16S rRNA Gene Clone Library and Phylogenesis

A total of 200 recombinant clones were randomly selected, and 26 different clones were selected using the PCR–DGGE method. Plasmid DNAs of these different clones were extracted and sequenced. The sequence results indicated that the 26 clones assembled into three phyla: Clostridiales, Proteobacteria, and Bacteriodetes (Fig. 8). CloneXDC01–
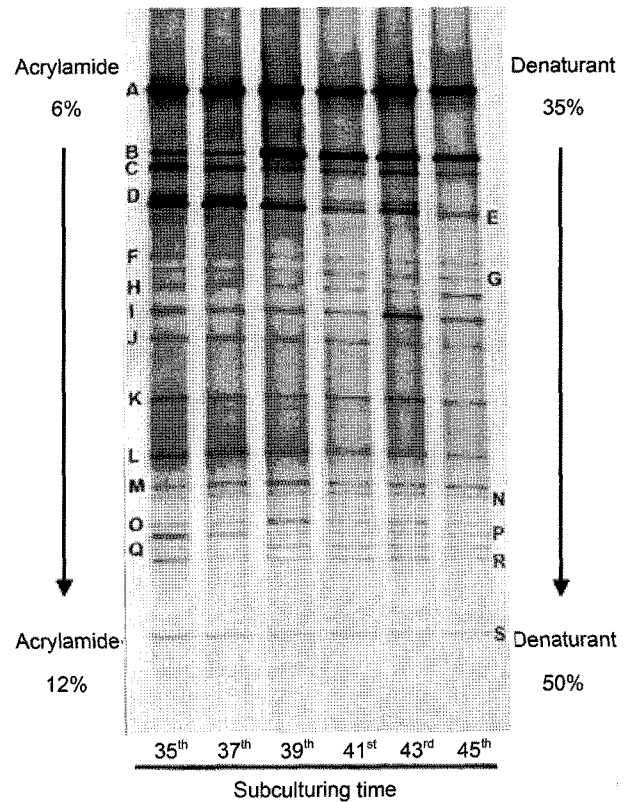
Fig. 7. DGGE profiles of the successive subcultures of XDC-2. The long arrows represent the direction of the denaturants (35–50%) and the polyacrylamide (6–12%) gradients. The bands that migrated to a similar position in the gel were presumed to have similar sequences. Bands (A–S) were excised from the gel and the DNAs were extracted, amplified, sequenced, and compared with the reference microorganisms available in the GenBank database.

CloneXDC12 accounted for 46.2% (12/26) of the 26 clones. Belonging to the cluster Clostridiales, they were related to the genera *Clostridium*, *Sporomusa*, *Oscillibacter*, and *Psychrosinus*. Clones XDC13–XDC16 belonged to the cluster Bacteroides, and account for 15.4% (4/26). They were mainly related to the genera *Dysgonomonas* and *Bacteroides*. Clones XDC17–XDC26 clustered to the Proteobacteria, and accounted for 38.4 % (10/26). They were related to the genera *Devosia*, *Pseudomonas*, *Escherichia*, and *Alcaligenes*.

DISCUSSION

The lignocelluloses-degrading composite microbial system (XDC-2) was successfully screened from composted agricultural and animal wastes amended soil and acclimated in our laboratory following restrictive cultivation. The results for the PCR–DGGE (Fig. 7) of the different generations indicated that the existence of the predominant strains of XDC-2 was very stable over long-term subcultivation. The stability of the microbial composition was the prerequisite

Clostridium from anoxic bulk soil XB90 (AJ229234)_97%
Uncultured Clostridium sp. clone AC036 (AY330126)_98%
● Clone XDC01
Clostridium sp. IMSNU 40011 (AY534872)_99%
● Clone XDC02
Clostridiaceae bacterium SN021 (AB298755)_94%
● Clone XDC03
Clostridium algidixylanolyticum (AF092549)_97%
Clostridium xylanolyticum DSM 6555^T (X76739)_98%
● Clone XDC04
Clostridium lentocellum (X71851)_97%
Clostridium lentocellum DSM 5427 (X76162)_98%
● Clone XDC05
Clostridium irregulare SN8 (EU887817)_99%
Clostridium irregulare DSM 2635 (X73447)_99%
● Clone XDC06
Clostridium tunisiense E1^T (AY187622)_94%
Clostridium nitrophenolicum 1D^T (AM261414)_94%
● Clone XDC07
Clostridium butyricum ATCC 43755 (X68176)_96%
Clostridium butyricum (AY442812)_96%
Clostridium corinoforum DSM 5906 (X76742)_97%
● Clone XDC08
Psychrosinus fermentans FCF9 (DQ767881)_92%
● Clone XDC09
Sporomusa sphaeroides DSM 2875 (AJ279801)_98%
● Clone XDC10
● Clone XDC11
Clostridiales bacterium JN18_V41_S (DQ168656)_98%
Oscillibacter valericigenes (AB238598)_96%
● Clone XDC12
Oscillospiraceae bacterium NML 061048 (EU149939)_100%

Clostridiales

Devosia sp. BD-c194 (EF575560)_95%
Devosia insulae DS-56 (EF012357)_95%
Uncultured soil bacterium clone PK_X (EF540440)_96%
● Clone XDC13
Hoeflea alexandrii AM1V30^T (AJ786600)_94%
Defluvibacter lusatiae DSM 11099^T (AJ132378)_96%
● Clone XDC14
Brevundimonas diminuta (X87274)_99%
● Clone XDC15
Pseudomonas stutzeri ATTC 17641 (AJ006106)_99%
● Clone XDC16
Pseudomonas aeruginosa (AF193514)_99%
● Clone XDC17
Uncultured bacterium clone RP_3aaa04f05 (EU778511)_99%
Escherichia sp. B4 (EU722735)_99%
● Clone XDC18
Alcaligenes faecalis (AF155147)_99%
● Clone XDC19
Alcaligenes sp. IS-18 (AY346137)_99%
● Clone XDC20
Alcaligenes sp. H (AJ412685)_95%
Alcaligenes sp. C4M17 (DQ089749)_95%
● Clone XDC21
Beta proteobacterium PII_GH1.2.A7 (AY162060)_98%
Alcaligenes sp. mp-2 (AY331576)_100%
● Clone XDC22

Proteobacteria

Uncultured compost bacterium clone 1B34 (DQ346466)_92%
Uncultured Bacteroidetes bacterium clone M113 (EU029290)_92%
● Clone XDC23
Uncultured Bacteroidetes bacterium clone Blci17 (AJ318110)_99%
Dysgonomonas mossii CCUG 43457^T (AJ319867)_99%
● Clone XDC24
Bacteroides intestinalis JCM 13266 (AB214329)_100%
● Clone XDC25
Bacteroides sp. XDT-1 (AB363973)_99%
● Clone XDC26

Bacteroidetes

0.02

Fig. 8. Phylogenetic tree showing relationships between representative clones in our study and reference organisms.
Representative clones in the library are indicated by the solid circle (●), reference sequences derived from the GenBank database are shown with their accession number in parentheses, and the similarity values between the clones and the closely related reference sequences are also shown after the underline (_). Cloned sequences were grouped into three phyla. The tree was constructed by the neighbor-joining method. Bootstrap values were based on 1,000 replications shown at branch points and the bar represents 2% sequence divergence. Only values of 50% or greater were included.

for successive subcultivation and degradation. The XDC-2 maintained an effective capability to degrade natural lignocellulosic materials (such as rice straw and corn stalk) after 35 cycles of subculturing.

The XDC-2 degraded rice straw by 60.3% within 15 days (Fig. 1), and mainly degraded the hemicelluloses, and secreted an extracellular xylanase more effectively in the early stage of the degradation process (Fig. 2); in the late stage (>6 days), cellulose degradation capability and cellulase activity that was mainly expressed as cell-associated were noticed. We ascribed this phenomenon of enzyme activities to the cellulolytic microbes adhering to the gradually exposed cellulose by prior degradation of hemicellulose. Further studies of the characteristics and patterns of

expression of the enzyme activity would be very helpful in elucidating this further. The XDC-2 degraded the lignocellulosic materials more effectively when contents of hemicellulose were higher. The crude extracellular enzyme of the XDC-2 had appreciable ability to degrade natural lignocelluloses, such as corn stalk and rice straw. The crude enzyme mainly expressed extracellular xylanase activity. Thus, it might be feasible to make an effective xylanase preparation from XDC-2 with a capability to hydrolyze natural lignocelluloses.

Most studies on microbial degradation of celluloses and hemicelluloses focused on the pure culture of fungi and bacteria [26, 44]. The decomposition activity and ability of pure culture microorganisms (especially of bacteria) were generally limited. The pure-culture isolates, so-called high activity, only degraded the substrates with relatively simple structure and composition, such as the artificial xylan and CMC, but were unable to use natural lignocelluloses. Scholten-Koerselman *et al.* [39] reported that a strain of *Bacteroides xylanolyticus* grew well on xylan, and degraded xylan with a high ratio of 75% under a strictly anaerobic condition, but did not grow with natural plant fibers. Recently, Chassard *et al.* [5] isolated several strains of *Bacteroides* sp. from human feces, of which the highest xylanase activities were 6–14 μmol of xylose per ml of protein per hr, but there are no reports about these isolates degrading natural lignocelluloses. Rogers and Baecker [36] isolated an anaerobic xylanolytic bacterium from decayed *Pinus patula* wood chips, the *Clostridium xylanolyticum* sp. nov. ATCC 49623 could degrade xylan at 35°C, but there are also no reports about these isolates degrading natural lignocelluloses. Kosugi *et al.* [20] determined the xylanase activity of *Clostridium cellulovorans* and the maximum activity was 0.5 μmol of xylose per ml of broth per min. Broda *et al.* [3] isolated an anaerobic *Clostridium algidixylanolyticum* from vacuum-packed lamb, which could degrade xylan and the maxium xylanase activity was 0.11 μmol of xylose per ml of broth per min with oat spelt xylan as the sole carbon source. The xylanase activity (8–12 μmol of xylose per ml of broth per min) and degrading capability of XDC-2 were much higher than those reported for pure cultured microbes.

Much attention has also been devoted to research on mixing different microorganisms to improve the capability to degrade natural lignocelluloses. Lewis *et al.* [25] mixed rumen microorganisms to degrade rice straw with a weight loss of 55.8%, but the extracellular xylanase activity of this mixture was not mentioned. Not only did XDC-2 degrade rice straw more efficiently (Fig. 1) than the mixed microorganisms reported by Lewis *et al.* [25], but it also secreted extracellular xylanase efficiently. We previously screened a microbial community, MC1 [8], which had strong capability to degrade cellulose, but was unable to secrets an extracellular enzyme. Until now, we are not aware of any report on microbial communities with strong capability to degrade natural lignocelluloses and secrete extracellular enzyme effectively.

The extracellular xylanase of the XDC-2 system was stable over a wide range of pH (3.0–10.0) at temperatures of 25–35°C (Fig. 6). The test duration in other studies on stability (pH stability or thermal stability) of xylanase generally were controlled for 1–2 h [9, 35], and only a few lasted up to 24 h. The extracellular xylanase of XDC-2 could maintain high activity at pH 5.0–9.0 and temperature of 25–35°C over 3 days (Fig. 6B and 6C). This characteristic stability of XDC-2 differs considerably from pure-culture microorganisms. We tentatively put forward that such stability of the extracellular xylanase of the XDC-2 at normal temperatures could provide a convenient option to explore effective xylanase preparations.

No colony of fungi was found by the plating method, and molecular analysis also showed that no fungal 26S rRNA gene was examined in this study, so the community was probably composed only of bacteria. The restrictive screening conditions might be the immediate causes of the elimination of fungi. Results of the 16S rRNA gene clone library and phylogenetic analysis showed no shortage of related species with abilities for decomposition of cellulose and xylan. Clone XDC05 was closely related to *Clostridium lentocellum* DSM 5427, with 98% similarity, which was isolated from river sediment containing paper-mill waste as an anaerobic cellulolytic organism [30]. Clone XDC04 was closely related to *Clostridium xylanolyticum* DSM 6555[T], with 98% similarity, which was an anaerobic, mesophilic, xylanolytic bacterium, isolated from decayed *Pinus patula* wood chips [36]. Clone XDC26 was closely related to *Bacteroides* sp. XDT-1, with 99% similarity, which was a strictly anaerobic, mesophilic, xylanolytic bacterium, isolated from a methanogenic reactor of cattle waste [32]. In addition, there were many bacteria using saccharide to produce acids. For instance, Clone XDC01 was closely related to *Clostridium* sp. XB90, with 97% similarity, which was first isolated from soil and could ferment glucose to acetic and propionic acids [6]. Clone XDC08 was closely related to the species *Clostridium butyricum*, with 96% similarity, which was anaerobic and widely distributed in soil and in the intestines of mammals. This could ferment saccharide to acetic and propionic acids [9, 42]. Clone XDC12 was closely related to the species *Oscillibacter valericigenes* Sjm18-20[T], with 96% similarity, which was an anaerobic, mesophilic bacterium, first isolated from Japanese corbicula clam, and could ferment glucose to organic acids [16]. The decrease in pH (Fig. 1) of the fermentation broth during rice straw degradation by XDC-2 might be related to these acid formers. Besides, this there were acidophilic bacteria; for example, Clone XDC17 was closely related to *Pseudomonas aeruginosa* NB1, with 99% similarity. This strain could grow with

organic acids, such as acetic and propionic acids, as sole carbon sources under aerobic conditions [12]. Clones XDC19–XDC22 were closely related to the genus *Alcaligenes*, most of which were studied extensively and found to utilize nitrogen sources such as urea to release ammonia, and also can ferment lactic acid. The recovery of the pH of the fermentation broth might be involved with these acidophilus strains. Phylogenetic analysis also showed that the Clostridiales cluster, which was closely related to Clones XDC01–XDC12, were mainly related to anaerobic bacteria [3, 6, 16, 30, 34, 36, 43]. The Proteobacteria cluster, which was closely related to Clones XDC13–XDC22, was mainly related to aerobic bacteria [13, 29, 37].

In conclusion, the present study showed that the composite microbial system (XDC-2) was structurally complicated. XDC-2 secreted enzymes and degraded lignocelluloses effectively and stably, which might be ascribed to the very complex and diverse composition of the microbial community. Mechanisms of synergistic interaction, mutual coordination, and restraint might conduce to constituting a relatively uniform and stable microbial system for effective degradation of lignocelluloses. The known bacteria frequently needed for strict anaerobic decomposition of cellulose and hemicellulose included *Clostridium* sp. [3, 30, 36, 43] and *Bacteroides* sp. [11, 32, 39]. However, XDC-2 could effectively degrade lignocelluloses in ordinary aerobic static conditions. This might be so because of (1) the presence of aerobic non-lignocellulolytic bacteria in the complex system that created favorable anaerobic conditions for anaerobic lignocellulolytic bacteria to degrade lignocelluloses; (2) The autorecovery of pH of the fermentation broth. Degrading activites were frequently inhibited by the imbalance in pH late in the degradation process [18]. The presence of acidophilus strains prevented the pH from dropping too rapidly to inhibit the degrading activities of the bacteria and enzymes; and (3) Mutual consumption of inhibiting substances of metabolites. Inhibiting substances of metabolites, such as organic acids, cellobiose, and glucose, produced from the degradation of polysaccharides, inhibited the degrading activity of the organism and enzymes [21, 24]. The inhibitors were unlikely to accumulate becuase of the presence of these saccharophilous or acidophilous non-lignocellulolytic bacteria, which removed the obstacles and made the lignocellulolytic bacteria degrade lignocelluloses and secrete enzymes continuously. Further study of the microbial associations and coordinated enzyme synthesis, composition, and characteristics should be a challenging and interesting work.

Acknowledgements

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