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Full Length Research Paper

Characteristics and community diversity of a wheat straw-colonizing microbial community

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A microbial community named WSD-5 was successfully selected from plant litter and soil after long-term directed acclimation at normal temperature. After 15 days of cultivation at 30°C, the degradation rate of wheat straw by WSD-5 was 75.6%. For cellulose, hemicellulose and lignin, the degradation rates were 94.2, 81.9 and 21.3%, respectively. The optimal pH for filter paper, CMCase, avicelase, β-glucosidase and xylanse activities was 6.24, 6.24, 5.91, 5.91 and 6.24, respectively. The optimal reaction temperature for all enzyme activity was 55°C. Filter paper enzyme, cellulase and xylanase were secreted from the degradation of wheat straw by WSD-5. The highest filter paper, cellulose endonuclease (CMCase), cellulose exonuclease (avicelase), β-glucosidase and xylanase activities were 1.30, 4.35, 0.60, 0.43 and 15.16 U/ml, respectively. A library of bacterial and fungal ribosomal gene sequences obtained from the community showed the presence of *Ochrobactrum* sp*., Coprinus cinereus* and *Pseudallescheria boydii*. To our knowledge, this was the first report of a microbial community which consisted of bacteria and fungi and was selected in the presence of wheat straw to produce extracellular filter paper enzyme, cellulase and xylanase.

Key words: Microbial community, lignocellulose degradation, filter paper activity, CMCase, avicelase, β-glucosidase, xylanase.

INTRODUCTION

Lignocellulosic biomass is composed of cellulose (insoluble fibers of ß-1,4-glucan), hemicellulose (non-cellulosic polysaccharides, including xylans, mannans and glucans) and lignin (a complex polyphenolic structure) and is considered a potential resource for biofuel production (Mabee et al., 2005; Demain et al., 2005). The degradation of lignocellulose requires a complex set of extracelluar enzymes. Efficient cellulose hydrolysis requires the cooperative actions of endoglucanases (E.C.3.2.1.4), which hydrolyze the cellulose polymer internally, exposing reducing and non-reducing ends and exoglucanases (E.C.3.2.1.91) or cellobiohydrolases, which act on the reducing and non-reducing ends, releasing cellobiose and cellooligosaccharides (Lynd et al., 2002). The hydrolysis of cellulose is finalized through the action of β-glucosidase (E.C.3.2.1.21), which cleaves cellobiose, liberating two molecules of glucose- the end product (Lynd et al., 2002; Zhang and Lynd, 2004). In addition, xylanases (EC 3.2.1.8; endo-β-1, 4-d-xylanase) are mainly responsible for the hydrolysis of xylan with β-1, 4-xylanolytic linkages to produce xylooligosaccharides (Wong et al., 1988). Extracellular hydrolases are involved in the breakdown of lignocellulose and are produced by many of the known ligninolytic fungi (Valášková et al., 2007). Xylanase properties have been observed in many fungal species (Haltrich et al., 1996; Singh et al., 2003). From an industrial point of view, filamentous fungi are particularly interesting as producers of xylanases because they excrete substantially greater amounts of xylanolytic enzymes into an extracellular culture medium than do bacterial or yeast cultures (Haltrich et al., 1996). In recent years, lignocellulose-degrading basidiomycetes and their enzymes (for example, celluloase, xylanase, peroxidase) have been well studied (Baldrian, 2006; Baldrian and Valášková, 2008; Valášková et al., 2007).

However, it is difficult to utilize these microorganisms and enzymes to process natural cellulosic materials

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without pretreatment. It has been reported in a previous study that the co-cultivation of microbes in fermentation can increase the quantity of the desirable components of the cellulase complex (Brijwani et al., 2010; Kumar et al., 2008a). However, it is not easy for the pure culture of microorganisms to decompose natural lignocellulose. In our laboratory, a stable microbial community named 'MC1', that effectively degraded various cellulosic materials (for example, filter, cotton, rice straw, corn stalk and cassava residue) was constructed through a succession of enrichment cultures (Cui et al., 2002; Guo et al., 2008a, b; Haruta et al., 2002). However, MC1 was a high temperature microbial community cultured at 50°C with the low enzyme activity of extracellar (Piao et al., 2003) and the community was composed only of bacteria (Haruta et al., 2002). Hence, it is very necessary to cultivate a microbial community with efficient degradation ability and effective enzyme production. The objective of this study was to obtain a microbial community composed of bacteria and fungi in order to further study the complementary relationship between bacteria and fungi during the lignocellulose-degradation process.

In this study, we obtained a microbial community named 'WSD-5' with an effective enzyme system and efficient degradation of wheat straw at normal temperatures. Wheat straw, cellulose, hemicellulose and lignin degradation, as well as the production of the hydrolytic enzymes, carboxymethylcellulase (CMCase; endo-1,4-β-D-glucanohydrolase, E.C. 3.2.1.4), avicelase (exo-1,4-β-D-cellobiohydrolase, E.C. 3.2.1.91), β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) and the xylan-degrading enzymes (xylanase, EC 3.2.1.8), were all estimated during the wheat straw-decay process of WSD-5.The composition of the fungal and bacterial community was investigated with sequence analysis of cloned libraries of PCR-amplified bacterial 16S rRNA sequences and fungal 26S rRNA sequences.

MATERIALS AND METHODS

Pretreatment of wheat straw

Mature wheat straw was air-dried, then submerged in 1.5% (w/v) sodium hydroxide at room temperature for 48 h. After washing with tap water to reach a neutral pH, the preparation of material was finished by oven drying at 105°C for 48 h.

Establishment of WSD-5 microbial community for wheat straw degradation

Modified Mandels medium, which is based on Mandels medium (Gaunt et al., 1984), was used for cultivation. The solid medium contained $(NH_4)_2SO_4$ 1.4 g/l; $MgSO_4.7H_2O$ 0.3 g/l; KH_2PO_4 2 g/l; peptone 2.5 g/l; $CaCO_3$ 2 g/l; $FeSO_4.7H_2O$ 5 mg/l; $MnSO_4$1.6 mg/l; $ZnCl_2$ 1.7 mg/l; $CoCl_2$ 1.7 mg/l; agar 12 g; pH 7.0. Modified Mandels solid medium (40 ml) was poured into 100-ml flask, 0.5 g of wheat straw (9 cm in length) to be used as carbon source was added into the flask. All medium was autoclaved at 121°C for 15 min. The litter and soil obtained from Beijing medical botanical garden were spread into the straw surface after solidification of the medium. Static cultivation was conducted at 30°C. After 7 days of incubation, about 2 g (wet weight) wheat straw covered with microorganisms was transferred into fresh solid medium. Finally, WSD-5, a stable microbial community highly-efficient in wheat straw degradation, was successfully established after 25 to 30 generations of transfer and selection. In this study, the total number of transfers was 45 generations.

Wheat straw degradation assay

For the degradation assay, 40 ml medium and 0.5 g (dry weight) wheat straw was inoculated with about 2 g (wet weight) microorganism-covered wheat straw in a set of 100-ml flasks. A second set of solid Mandels medium was prepared using glucose 120 mg/l as the sole carbon source and inoculated by the same procedure. Flasks underwent static cultivation at 30°C. Flasks were sampled at 0, 15 days (three flasks each day); oven-dried at 60°C for 48 h and weighed, then decrease in weight was calculated. In all cases, the wheat straw treated in the same conditions without inoculation was used as the control and the group with glucose was used as a control to remove the impacts from the microbes.

Composition of wheat straw

Wheat straw residue was milled after drying, screened through a 1 mm sieve and a 0.5 g sample was transferred into a filter bag (Model F57, ANKOM Technology, USA). Components of soluble matter in rice straw, cellulose, hemicellulose, lignin and ash were analyzed using a fiber analyzer (Model ANKOM200, ANKOM Technology, USA), according to the method described by Goering and Van Soest (1970). Three replicates were carried out.

Preparation of crude enzyme

In this study, solid medium was available for the cultivation of WSD-5. The method of crude enzyme preparation was as follows: weighted 2 g (wet weight) wheat straw covered with microorganisms was initially suspended with 7.5 ml deionized water and the sample was re-suspended with 7.5 ml deionized water after vortexing of 1 min and centrifugation at 6000×*g* for 10 min at 4°C. Finally, about 15 ml of supernatant obtained was used as extracellular source of enzyme. The crude enzyme liquid was used to determine the filter paper enzyme, cellulase and xylanase activity.

Effect of temperature and pH on activity

Filter paper (Whatman, 1× 6 cm), carboxymethyl cellulose (CMC), avicel, salicin and oat xylan were used as substrates for filter paper enzyme (the total enzyme), cellulose endonuclease (CMCase), cellulose exonuclease (avicelase), β glucosidase (cellobiase) and xylanase activities. These substrates suspended in 1/15 mol/l disodium hydrogen phosphate-potassium dihydrogen phosphate buffer (from pH 4.92 to 8.67) were used for the measurement of enzyme activities and for the testing of optimal pH. The optimal temperature was evaluated by measuring the enzyme activity at the optimal pH at different temperatures (40 to 70°C). All dynamic enzyme activities in this study were measured under optimal pH and temperature.

Enzyme activity assay

Dynamic enzyme activities were determined for the WSD-5 cultures, including the dynamic trends of filter paper enzyme (the

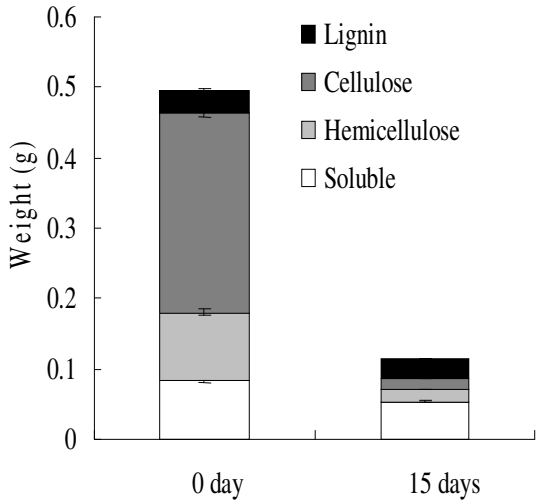

Figure 1. Composition of wheat straw at the start and end of wheat straw degradation by WSD-5.

total enzyme), cellulose endonuclease (CMCase), cellulose exo-nuclease (avicelase), β-glucosidase (cellobiase) and xylanase activities. The substrates for enzyme activity determination were as follows: 50 mg filter paper (Whatman, 1× 6 cm), 2% (w/v) carboxymethyl cellulose, 2% (w/v) avicel, 0.5% (w/v) salicin and 1% (w/v) oat xylan. These substrates were suspended in 1/15 mol/l disodium hydrogen phosphate-potassium dihydrogen phosphate buffer.

The 3,5-dinitrosalycylic acid method (Ghose, 1987) was used for the measurement of enzyme activity. After the reaction, the release of reducing glucose was measured at 530 nm. For avicelase, the test-tube was shaken every 10 min, and centrifuged at 6000×*g* for 1 min after incubation. 3 ml DNS was added into the supernatant (Beukes and Pletschke, 2006). For enzyme activity measurement, every assay included controls for comparison. 3 ml DNS was added into the control sample before incubation to inactivate enzyme activity. This removed the impact for the enzyme activity measurement from glucose in the sample. All samples, controls, glucose standard solutions and samples for adjusting the spectrum to zero were boiled before measurement. Enzyme activity was calculated based on standard curves. Glucose (for filter paper enzyme and cellulase activity) and xylose (for xylanase) were used as the standards for enzyme measurements, respectively. One unit of enzyme activity is defined as the amount of enzyme that released 1 µmol of glucose per min.

DNA extraction

About 0.5 g (wet weight) cultures of WSD-5 were transferred to centrifuge tubes and 500 µl extraction buffer (100 mmol/l Tris-HCl, pH 9.0), 40 mmol/l EDTA (pH 8.0) was added into the tube. After vortexing, samples were reserved at -20°C for later use (Wang et al., 2006b). Genomic DNA of WSD-5 was extracted according to the benzyl chloride method (Zhu et al., 1993).

Establishment of cloning libraries

The cloning libraries of bacterial 16S rDNA and fungal 26S rRNA was present in reference (Wang et al., 2006a). WSD-5 DNA was used as template for PCR amplification. Each 50 µl PCR mixture contained 10 ng of template DNA, 5 µl 10×PCR buffer, 4 µl dNTPs (2.5 mmol/l each), 3 µl $MgCl_2$ (25 mmol/l), 0.5 µl each primer (45 µmol/l) and 0.2 µl TaKaRa rTaq DNA polymerase (5 U/µl). The primers used for the amplification reaction of 16S rDNA of WSD-5 were 27f (5′-A GA GTT TGA TCC TGG CTC A G-3′) and 1492r (5′-GGC TAC CTT GTT ACG ACT T-3′) (Martin-Laurent et al., 2001). The primers for amplification of the D1/D2 region of the fungal 26S rRNA gene were NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAG ACG G -3′) (Poláková et al., 2009). The thermocycle program consisted of initial DNA denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 45 s and elongation at 72°C for 1 min 30 s, with a final elongation at 72°C for 6 min. PCR products were visualized with 2% agarose gel electrophoresis.

The PCR products were ligated into pGEM-T easy vector (Promega, Japan) according to the manufacturer's protocol. A total of 121 and 120 white colonies of bacterial and fungal clones were randomly picked. Colonies containing 16S rRNA gene or 26S rRNA gene inserts were screened by DGGE analysis (Wang et al., 2006a). The plasmid DNA from the corresponding clones was extracted by using SDS alkaline methodology (Sambrook et al., 1989). The plasmid inserts were sequenced with primers T7 and Sp6 (Randazzo et al., 2002). Sequence similarity searches were performed in GenBank using the BLAST algorithm. Software package Clustal X was used to analyze sequences (Thompson et al., 1997). The phylogenetic trees were established by the software MEGA4 (Kumar et al., 2008b).

Nucleotide sequence accession numbers

In this study, the obtained sequences were deposited in GenBank under accession numbers HQ154647 through HQ154666.

Statistical analysis

Each trial was performed in triplicates including appropriate control. The data were statistically analyzed as average values of different trials for each experiment. All data was subjected to ANOVA for significant differences by the general linear models procedure of statistic analysis system (Ver.6.12; SAS Inst., Cary, NC, USA).

RESULTS

Degradation capability of wheat straw by WSD-5

To analyze the ability of the community to degrade wheat straw, the community was inoculated into the modified Mandels solid medium containing no carbon source other than wheat straw. During the culture process, the wheat straw became soft at 8 days and proportion of degradation reached 75.6% after 15 days.

Degradation capability of WSD-5 for cellulose, hemicellulose and lignin

Composition of wheat straw was assayed at the start and the end of wheat straw degradation by WSD-5 (Figure 1). The analysis included water soluble substances, cellu lose, hemicellulose, lignin and ash. Initially, the contents of cellulose, hemicellulose and lignin were 282.252,

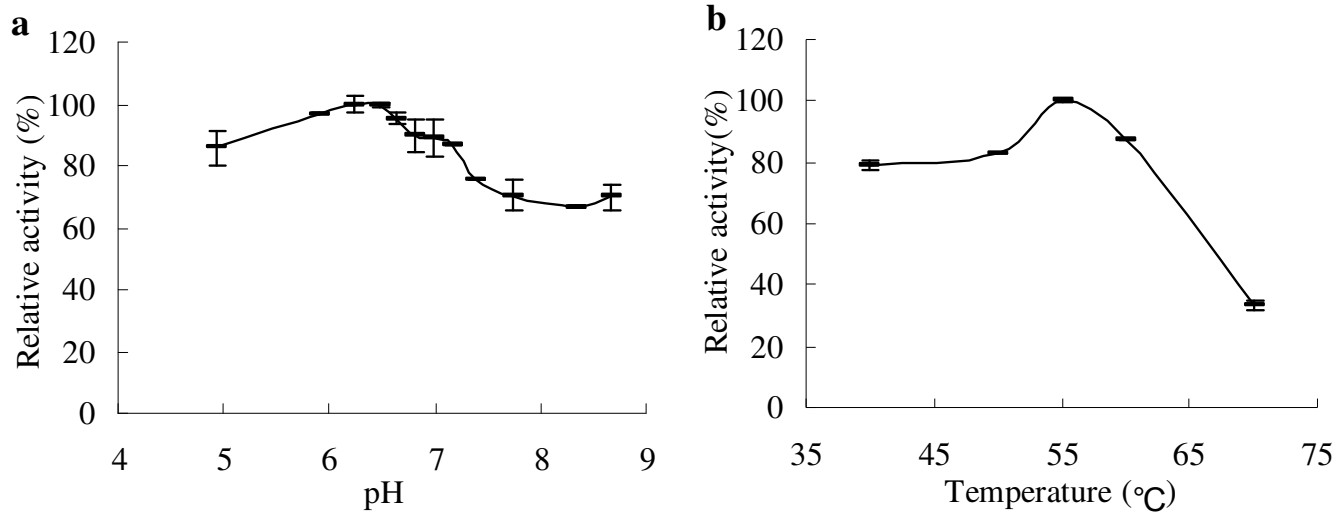

Figure 2. Relative enzyme activity of CMCase of the WSD-5 culture at different pH levels (a) and temperatures (b).

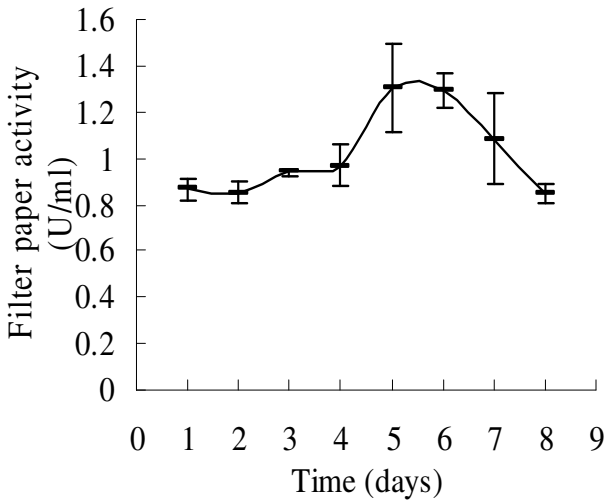

Figure 3. Dynamics of filter paper activity during decomposition.

97.889 and 33.324 mg, respectively. After 15 days degradation by WSD-5, the residues of cellulose, hemicellulose and lignin were 16.258, 17.670 and 26.216 mg, respectively. Therefore, the degradation rates of WSD-5 for cellulose, hemicellulose and lignin were 94.2, 81.9 and 21.3% after 15 days, respectively.

Effect of temperature and pH on activity

Enzyme activity was assayed at pH ranging from 4.92 to 8.67 so as to identify the impact of pH. The relative enzyme activity of CMCase reached 100% at pH 6.24 (Figure 2a). Enzyme activities at pH ranging from 4.92 to 8.67 reached more than 65% of the maximum activity. The result showed that the optimal pH for filter paper, CMCase, avicelase, β-glucosidase and xylanse activities was 6.24, 6.24, 5.91, 5.91 and 6.24, respectively.

The relative crude enzyme activity from 40 to 70℃ was assayed at the optimal pH 6.24 so as to identify the influence of temperature. The highest value of enzyme activity for CMCase was seen at 55℃ and the enzyme activities of WSD-5 remained around 80% of the maximum value under temperatures of 40 to 60℃ (Figure 2b). However, activity obviously declined at 70℃. The optimal reaction temperature for other enzyme activity was also determined to be 55℃.

Enzyme activities in degradation of wheat straw by WSD-5

Filter paper enzyme, cellulase and xylanase were produced during the degradation of wheat straw by WSD-5. Dynamic changes of all enzyme activities showed patterns of initially increasing and later declining (Figures 3 to 5). Filter paper enzyme activity was stable during the first 4 days and the peak activity was 1.3 U/ml on the 5th day, thereafter declined at a steady peace and at the 8th day reached the level identical to the 4th day (Figure 3). CMCase showed patterns similar to filter paper enzyme. During the first 4 days, it remained at low levels, then rapidly increased during days 4 to 6 and reached a peak of 4.35 U/ml at day 6. Thereafter, it slowly declined to 83.9% of the maximum after 8 days (Figure 4a). Avi-celase and β-glucosidase reached their peaks of 0.6 U/ml (Figure 4b) and 0.43 U/ml (Figure 4c), respectively, at day 5. At day 8 both were lower than their initial values. For xylanase, enzyme activity increased slowly during the be-ginning of cultivation, rapidly at days 4 to 6 and reached a peak of 15.16 U/ml on day 6. It thereafter slowly declined and reached 68.4% of the peak activity after 8

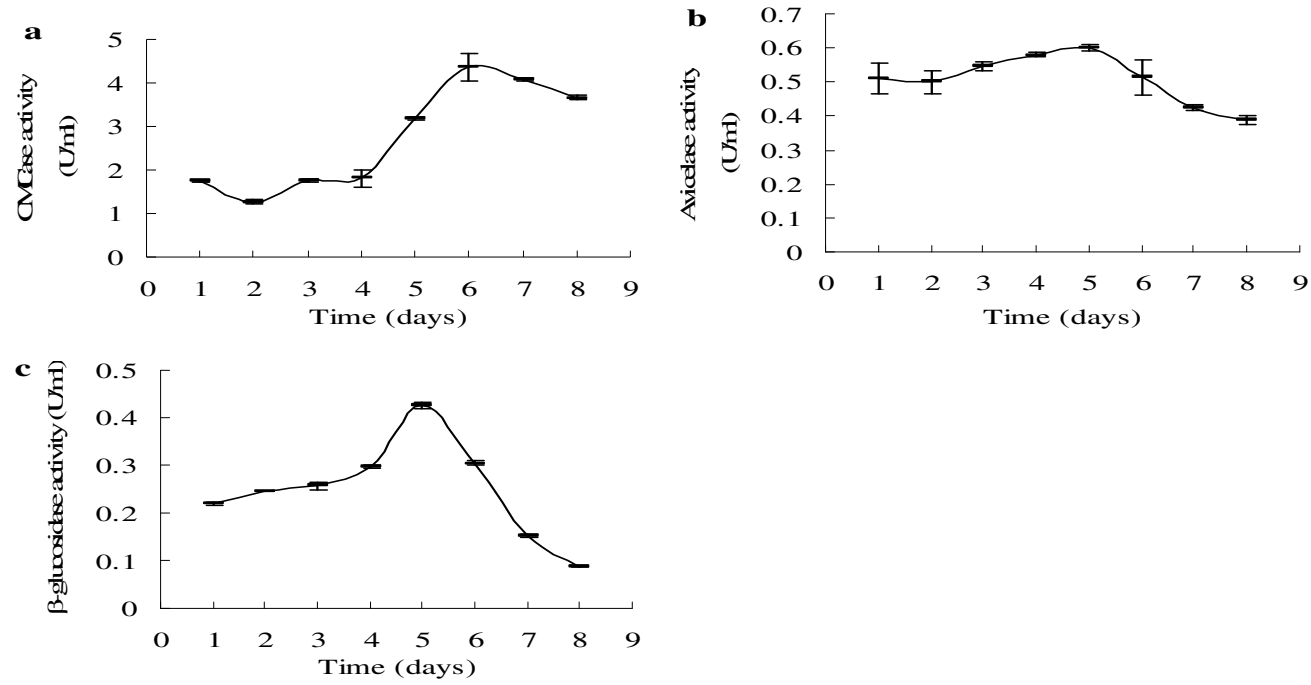

Figure 4. Dynamics of cellulose activity during decomposition (a) CMCase; (b) avicelase; (c) β-glucosidase.

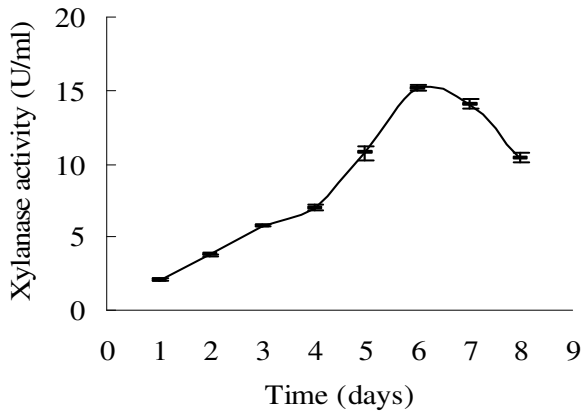

Figure 5. Dynamics of xylanase activity during decomposition.

days (Figure 5).

Diversity of the community

Based on the composition of the ribosomal gene sequences library, phylogenetic trees of the bacteria and fungi of the microbial community were constructed (Figures 6 and 7).

For the bacteria, 121 clones were obtained. The sequences of the clones were classified into three different groups. Of these sequences, 41.3% of the sequences were identical and shared 100% sequence similarity with *Ochrobactrum* sp. (DQ305290). Another 17.4% had 99.5% similarity with uncultured *alpha proteobacterium* (AY728070). For the fungi, a total of 120 clones were obtained. The sequences of the clones were classified into two different groups. Of these sequences, 68.3% of the sequences were identical and shared 99.8% sequence similarity with *Coprinus cinereus* (AF041494). Another 17.5% had 99.8% similarity with *Pseudallescheria boydii* gene (AB363764).

DISCUSSION

In nature, lignocellulosic materials are degraded with the cooperation of many microorganisms. In our previous study, an effective microbial community named MC1 with high lignocellulose-degradation ability was initially constructed (Cui et al., 2002; Haruta et al., 2002) and some research on the composition and application of microbial community was investigated (Feng et al., 2011; Guo et al., 2010; Lv et al., 2008; Wongwilaiwalin et al., 2010). These studies demonstrated potential industrial applications of the microbial community. However, close examination of these microbial communities showed that no colony of fungi were detected (Guo et al., 2010; Haruta et al., 2002; Lv et al., 2008). Furthermore, there were no reports of extracellular enzymes evidently secreted by a wheat straw-degradation microbial community obtained in a laboratory. Thus, the characteristics and community diversity of the constructed wheat straw-colonizing microbial community which can degrade wheat

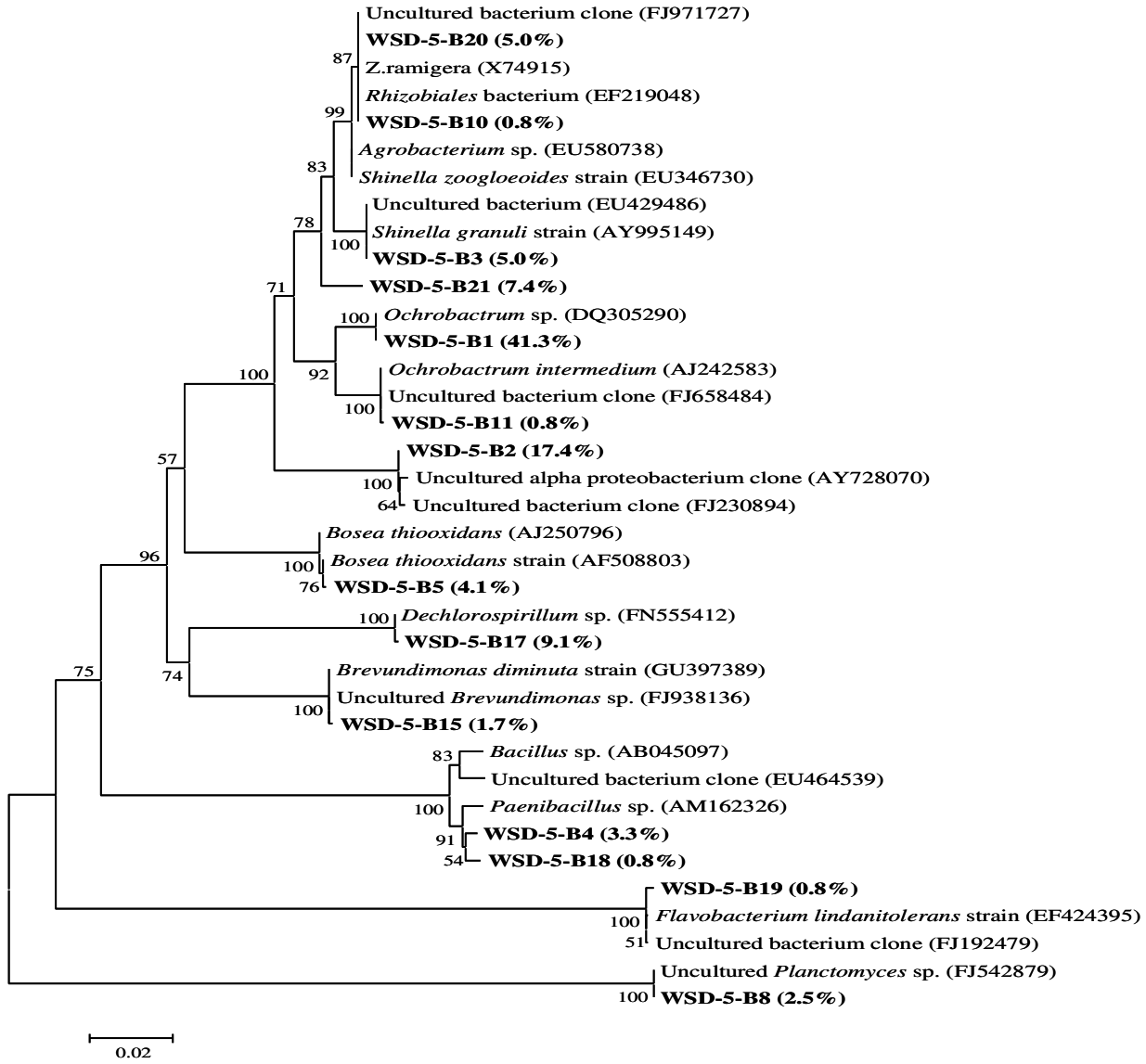

Figure 6. Phylogenetic analysis of the WSD-5 bacterial 16S rDNA sequences using the neighbor-joining method.

straw and effectively produce extracellular enzymes, reported herein, is the first report of such a community.

Degradation rates obtained by a reduction in weight can accurately indicate the degradation capability of a microbial community (Cui et al., 2002) In a previous study, for the microbial community WDC2 with PCS medium and wheat straw as a sole carbon source, degradation rate reached about 64.5% after 15 days cultivation at 60°C (Lv et al., 2009). Compared with WDC2, the degradation rate increased to 11.1% by using WSD-5. It was reported that the degradation activity of cellulolytic microorganisms varies depending on different characteristics of the substrate (Zhang et al., 2006). When it is used as a carbon source for the growth of several microorganisms, wheat straw has the drawback of low accessibility because of the presence of lignin in the straw. The degradation rates of WSD-5 for cellulose, hemicellulose and lignin were 94.2, 81.9 and 21.3%, respectively, after 15 days. This result clearly showed that WSD-5 has a strongly effective degradation capability for cellulose and hemicellulose in wheat straw. In other words, the degradation of wheat straw by WSD-5 was as a result of the effect of the process of degrading cellulose and hemicellulose inside wheat straw. Furthermore, WSD-5 has a potential greater range of applications because the microbial community flourished at normal temperature. Currently, it is being applied in experiments involving the return of straw to soil (unpublished date). The WSD-5 can also grow on other agricultural residues such as rice straw, corn stover and other similar substrates, as its sole nutritive source and shows high degradation rates with them (data not shown). Thus, it could be used in recycling

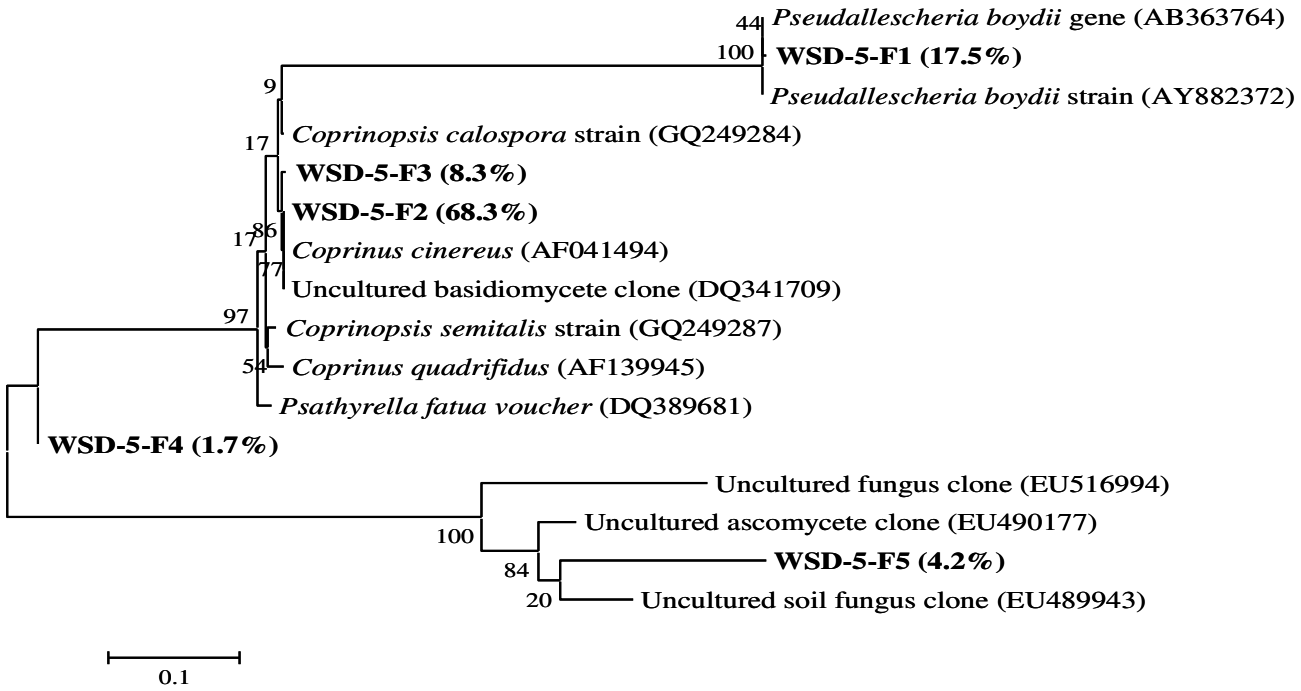

Figure 7. Phylogenetic analysis of the sequences of the D1/D2 region of the fungal 26S rDNA from WSD-5 on solid medium using the neighbor-joining method.

of these lignocelluloses residues.

Microbial enzyme production was influenced by a number of factors including the type of strain used, reaction conditions (for example, temperature and pH) and substrate type. Likewise the pH profile of CMCase activity, the xylanase, β glucosidase, avicelase and filter paper enzyme were stable over a wide pH range (4.92 to 8.67). The CMCase activity of the WSD-5 retained 70% of the maximal level at pH 8.67. The ability to retain high activity at elevated pH is a potentially useful property in the processes employing alkaline delignification (Saratale and Oh, 2011). When the effect of temperature was studied, all the enzyme activity of the WSD-5 remained high at 40 to 60℃ and reached a maximum at 55℃. It was similar to CMCase exhibited by *Streptomyces reticule* with optimum at 55℃ (Schrempf and Walter, 1995) and higher than xylanase produced by the micro-bial community EMSD5 at 50℃ (Lv et al., 2008). All the enzyme activity showed a maximum at 55℃ based on a range of various temperatures (40 to 70℃). The optimum temperature was even higher than the optimum temperatures (30℃) for the growth of the microbial community WSD-5. However, the reasons for accordance of optimum temperatures for all of the enzyme activities need to be further studied.

Extracellular hydrolases are involved in the breakdown of lignocellulose and are produced by many of the known ligninolytic fungi (Valášková et al., 2007). It was not unexpected to find white hyphae growth on wheat straw during cultivation of WSD-5. The enzyme profile revealed that the microbial community WSD-5 had the ability to produce extracellular enzymes during the degradation of wheat straw. In the whole process, all the enzyme activities peaked at day 5 or 6 and then decreased. The maximal levels of extracellular filter paper enzyme and cellulose produced by WSD-5 were lower than those produced by *Nocardiopsis* sp. KNU using rice straw (Saratale and Oh, 2011). For xylanase, the peak of enzyme activity reached 15.16 U/ml which was much greater than cellulase produced by WSD-5, but lower than xylanase produced by *Bacillus pumilus* ASH under submerged fermentation (Battan et al., 2007). Whether the microbial community WSD-5 produces oxidative enzymes or not during wheat straw, biodegradation needs to be further studied. Compared with many well-characterized isolated strains used in current studies (Battan et al., 2007; Romero et al., 1999), the enzyme activity in this microbial community was relatively low, but it produced the highest level of lignocellulose degradation.

The results of composition of the ribosomal gene sequence library showed that the microbial community WSD-5 was composed of bacteria and fungi. According to the frequency of the abundance in the cloning library, the most dominant bacteria were related to *Ochrobactrum* sp. According to previous reports, some bacteria belonging to the genus *Ochrobactrum* are capa-ble of opening the C-loop of some aromatic compounds (El-Sayed et al., 2003; Lechner et al., 1995; Song et al., 2000). This study was the first report to identify *Ochrobactrum* sp. in a lignocellulose degrading microbial

community. Whether these bacteria contribute to the cellulose or hemicellulose degradation needs further investigation. There is also the possibility that these bacteria utilize only the sugars released from wheat straw degradation. The predominant fungi were found to be related to *C. cinereus*. Generally, habitats of *C. cinereus* were in fields mown, cow dung and grassplots after rain. It has been used as a type strain in the field of genetics and cytology (Wälti et al., 2006; Ziemys and Kulys, 2007). *C. cinereus* caused a 37% decrease in nitrocellulose when provided with amino acids and starch as co-substrates (Auer et al., 2005). Moreover, there is a U.S. patent (Publication number: US 2003/0134406 A1) which indicates that *P. boydii* exhibits activity on cellulose.

The bacteria and fungi detected by 16S rDNA and 26S rDNA library did not exhaustively represent all members, thus, other minor microorganisms may play an important role in this community. However, the use of a microbial community for extracellular enzyme production has been demonstrated in our work and this microbial community and the associated enzyme production system appears to have potential applications.

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