Survival and Performance of Two Cellulose-Degrading Microbial Systems Inoculated into Wheat Straw-Amended Soil

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Received: February 16, 2011 / Revised: August 16, 2011 / Accepted: September 20, 2011

A cellulose-degrading composite microbial system containing a mixture of microbes was previously shown to demonstrate a high straw-degrading capacity. To estimate its potential utilization as an inoculant to accelerate straw biodegradation after returning straw to the field, two cellulose-degrading composite microbial systems named ADS3 and WSD5 were inoculated into wheat straw-amended soil in the laboratory. The microbial survival of the inoculant was confirmed by a denaturing gradient gel electrophoresis (DGGE) analysis, whereas the enhancement of straw degradation in soil was assessed by measuring the mineralization of the soil organic matter and the soil cellulase activity. The results indicated that most of the DGGE bands from ADS3 were detected after inoculation into straw-amended autoclaved soil, yet only certain bands from ADS3 and WSD5 were detected after inoculation into straw-amended non-autoclaved soil during five weeks of incubation; some bands were detected during the first two weeks after inoculation, and then disappeared in later stages. Organic matter mineralization was significantly higher in the soil inoculants ADS3 and WSD5 than in the uninoculated controls during the first week, yet the enhanced degradation did not persist during the subsequent incubation. Similar to the increase in soil organic matter, the cellulase activity also increased during the first week in the ADS3 and WSD5 treatments, yet decreased during the remainder of the incubation period. Thus, it was concluded that, although the survival and performance of the two inoculants did not persist in the soil, a significant enhancement of degradation was present during the early stage of incubation.

Keywords: Composite microbial system, soil inoculant, soil organic matter mineralization, cellulase activity, denaturing gradient gel electrophoresis (DGGE)

In China, approximately 20×10⁶ ha is sown with wheat annually, creating approximately 90×10⁶ tons of wheat straw residues that are returned to the fields [2]. Returning straw to the fields after harvest is a common method to improve the soil organic matter content [7, 20]. However, since straw residues have a high lignocellulose content and high carbon to nitrogen (C:N) ratios, they decompose slowly in the fields [24], creating difficulties for farming operations and dry soil. In some localities, Chinese farmers burn the straw after the wheat harvest to allow immediate sowing of the next crop; the pressure of highly efficient land use for growing multiple crops each year to produce enough food has increased substantially. Thus, accelerating straw degradation in soil will help discourage straw burning and increase the soil organic matter.

The addition of microorganisms to polluted soils to enhance the transformation rate of contaminants has already been reported in many studies [4, 12, 23], and the enhancement of straw composting by lignocellulolytic strains has also been confirmed [1, 26]. However, the direct introduction of cellulose-degrading strains into soil to enhance straw degradation has seldom been reported, essentially due to the challenges of biotic and abiotic stresses. The main limiting factor to soil inoculation is the general obstinacy of the soil ecosystem, which acts as a buffer against soil inoculants [23]. Halsall and Gibson [6] introduced two Cellulomonas strains into soil over 38 days of incubation in the laboratory to study the interaction with an Azotobacter in the degradation of wheat straw and associated nitrogen fixation, yet they did not monitor the introduced strains. The use of microbial inoculants to enhance straw degradation has recently been introduced in China, yet most inoculants have been produced and utilized blindly without examination or confirmation of activity through scientific experiments [15], so their ultimate success remains uncertain. Therefore, the development of highly efficient inoculants for promoting straw-return to soil is urgently required.
Two composite microbial systems with a high natural lignocellulose-degrading capacity have already been successfully constructed, ADS3 and WSD5 [15, 25], both of which contain several bacterial and fungal species. WSD5 was screened to obtain an efficient natural lignocellulolytic complex enzyme, whereas ADS3 was specifically selected to accelerate decomposition when wheat straw is directly returned to the field. Accordingly, in the present study, ADS3 and WSD5 were separately inoculated into straw-amended soils to estimate their potential for field application and obtain test parameters for future field experiments. As the survival and catabolic activity of introduced microorganisms are the key factors to successful inoculation [12], the survival of the microbial composition was checked using a PCR–DGGE analysis, while the enhancement of the straw degradation was monitored by measuring the organic matter mineralization and cellulase activity during 5 weeks of incubation.

**MATERIALS AND METHODS**

**Straw and Soil Preparation**
The original soil used in this study was sampled from the top layer (0–20 cm) of a field located at the experimental farm of China Agriculture University (Beijing, China). The soil was characterized as sandy Chao soil (water capacity 41.8%, total N 1.05 g/kg, organic carbon 18.3 g/kg, pH 7.8). The freshly collected soil samples were gently crushed and fully mixed. The wheat straw, also obtained from the experimental farm of China Agriculture University (Beijing), had no diseases or contaminating insects and was air-dried. The whole plants, including stalks and leaves, were then crushed into 2-mm-long pieces. The wheat straw had the following physical and chemical characteristics: total N was 4.35 g/kg, total C was 423.8 g/kg, hemicellulose was 28.8%, cellulose was 47.7%, lignin was 4.8%, and the C/N ratio was 97.4.

**Seeds of ADS3 and WSD5 and Their Characterization**
The previously characterized ADS3 and WSD5 were found to degrade up to 60% of wheat straw in flasks after 15 days of incubation. DGGE analyses of the V3 region and DNA sequencing of the 16S rRNA gene and D1 region of the fungal 26S rRNA gene indicated that ADS3 contained 12 bacterial species and 1 fungus, whereas WSD5 contained 6 bacterial species and 2 fungi. The seeds of the two inoculants were from a fermentation broth, as previously described [15, 25].

**Experimental Design and Sampling**
The experiment was performed indoors based on putting 50 g of treated soil into plates (15 cm diameter) with 0.5 g of wheat straw (2 mm) amendments. To monitor the survival and performance of the introduced ADS3 or WSD5, about 10^6 CFU of the inoculant was introduced to each plate to make four sets (of 15 plates each) consisting of inoculated/uninoculated and straw-amended/unamended treatments. The sampling was performed at weeks 0, 1, 2, 3, and 5, where three plates were taken for each treatment at each sampling time. The moisture content was adjusted to 80% of the field capacity using diluted inoculants or sterile deionized water (blank), and readjusted on day 7 based on drying the soil at 80°C to a constant weight. All the plates were covered with loosely sealed film to maintain the soil moisture content. The experiment was carried out at 30°C in the dark for 5 weeks. Furthermore, to compare the survival of ADS3 in autoclaved soil as a sterile substrate, prepared plates with or without straw amendment were autoclaved at 121°C for 20 min before the inoculation. The inoculation and sampling strategies then followed the same experimental design as that used for the non-autoclaved soil.

The mineralization of the soil organic matter was estimated by combustion in an oven at 550°C to a constant weight [19]. The soil cellulase activity was determined by measuring the release of reducing sugars from the substrate carboxymethyl cellulose (CMC). Triplicate amounts (10.0 g) of each soil sample were individually placed in 50 ml Erlenmeyer flasks and 1 ml of toluene was added. The contents of the flasks were mixed thoroughly, and then after 15 min, 10 ml of a 0.5 M (pH 5.9) sodium acetate buffer was added, followed by 10 ml of 1% CMC. After 30 min of incubation at 37°C, the suspension was filtered through Whatman No. 1 filter paper and the volume of the filtrate increased to 50 ml with distilled water. The resultant filtrate was used to determine the reducing sugars using the 3,5-dinitrosalicylic acid (DNS) method, as previously described [5]. All the assays included negative controls. The cellulase activity was defined as the microgram glucose equivalent per gram of soil per 30 min (µg GE/30 min/g soil). The soil samples used for DNA extraction were stored at −20°C. T-tests were performed using Microsoft Excel 2003 software (Microsoft Corporation, USA) to test for significant differences in the soil organic matter mineralization among the two inoculants and controls. Statistical significance was determined at the 0.05 level (p < 0.05).

**DNA Extraction and PCR–DGGE Analysis**
The DNA extraction was performed using a MoBio PowerSoil DNA Isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, USA), where 0.25 g soil samples were processed according to the protocol provided by the manufacturer. The same isolation kit was used to extract the DNA of the inoculant from the sediment of 5 ml of the seed fermentation broth after centrifugation at 8,000 rpm for 10 min.

Primer pairs 357f–GC and 517r [16] were used for the V3 region of the 16S rRNA gene sequence amplification. For the PCR program, the initial DNA denaturation was performed 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, with a final elongation step at 72°C for 6 min. Each 50 µl PCR reaction contained 1 µl (30 ng) of template DNA, 5 µl of 10× buffer, 4 µl of each dNTP (2.5 mM/l), 3 µl of MgCl2 (25 mM/l), 1 µl of each primer (45 µmol/l), and 0.25 µl of Takara Taq (5 U/µl) (Takara, Japan). Negative controls (without any template) were run in parallel for all the reactions.

Primers NL1-GC and LS2 [3] were used for amplification of the D1 region of the fungal 26S rRNA gene sequence. The PCR program was as follows: 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, with a final elongation step at 72°C for 6 min.
The PCR products were analyzed using a DCode system (Bio-Rad Laboratories, Hercules, CA, USA), as described by Muyzer et al. [16]. The samples were loaded onto a 6–12% (w/v) polyacrylamide gradient gel in a 0.5% TAE electrophoresis buffer (20 mM Tris-HCl, pH 8.3; 10 mM acetic acid; 0.5 mM EDTA) with a 25–50% denaturant gradient (where 100% is defined as 7 M urea with 40% formamide). The gels were run at a constant voltage of 200 V and temperature of 61°C for 5 h in the 0.5% TAE electrophoresis buffer, and then stained with SYBR Green I (Molecular Probes, Eugene, OR, U.S.A.) and photographed under UV (302 nm) using an Alpha Imager 2200 Imaging System (Alpha Innotech, USA). The individual DGGE bands were excised immediately, purified, and reamplified for a further sequencing analysis using a clone library approach [27], where 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 1.83 [10]. A phylogenetic tree was also constructed using the neighbor-joining method with the MEGA version 4.0 software program [22].

RESULTS

Survival of ADS3 in Autoclaved Soil

The microbial survival of the ADS3 inoculant in the autoclaved soil was examined using PCR–DGGE (Fig. 1A). Based on the migration of the 16S rRNA gene fragments, almost all the bands from ADS3 (bands a to l) were detected in both the straw-amended soil (ADS3+straw) and the soil with no straw (ADS3-straw). In the two uninoculated controls, several bands not corresponding to ADS3 were detected, mainly bands n, o, p, q, and r, while many more bands were detected in the uninoculated soil with straw amendment (unino+straw) than in the uninoculated soil without any straw amendment (unino-straw). The closest identified microbial relatives for each band migrating at different positions are shown in Fig. 1B.

Organic Matter Mineralization of ADS3 and WSD5 in Non-Autoclaved Soil

When comparing the inoculation treatments with the uninoculated controls, the former showed more initial mineralization of soil organic matter with estimates of 3.9% (ADS3), 3.1% (WSD5), and 1.2% (CK) during the first week. The T-tests also indicated significant differences between the ADS3 or WSD5 and the controls, yet no significant differences between the ADS3 and the WSD5. For the samples from subsequent weeks, no significant differences were detected, implying that the enhanced biodegradation declined with additional incubation (Fig. 2).

In the soils without straw amendments, the T-tests indicated no significant differences in the organic matter mineralization between the soil inoculants and no inoculation (p>0.05) during the entire incubation period (Fig. 2 histogram), with the soil organic matter stabilizing around
37 g/kg. This implied the absence of any enhanced biodegradation efficiency.

Cellulase Activity of ADS3 and WSD5 in Non-Autoclaved Soil

The effect of the inoculation on the soil cellulose enzyme activity was apparent (Fig. 3). When compared with the uninoculated controls, the ADS3 and WSD5 inoculants caused dramatic increases in the cellulase activity, which were estimated as 252.8 and 277.8 µg GE/30 min/g soil, respectively, during the first week. Thereafter, the cellulase activity gradually decreased, falling to 109.5 and 124.8 µg GE/30 min/g soil, respectively, by the end of the incubation period. The cellulase activity in the uninoculated control gradually increased and peaked after 3 weeks at 167.0 µg GE/30 min/g soil. The two inoculants and uninoculated controls did not differ significantly from each other during the final two weeks.

Survival of ADS3 and WSD5 Inoculants in Non-Autoclaved Soil

The microbial survival of each soil inoculant was analyzed based on the migration of the 16S rRNA gene fragments in the DGGE gels (Fig. 4). Bands a, b (Fig. 4) from ADS3, which matched Bacillus firmus (95%), Pseudomonas sp. (100%), respectively, and bands 1 and 2 from WSD5, which matched Cytophaga sp. (98%) and uncultured Alphaproteobacterium (99%), respectively, were detected in the first two weeks, and then disappeared during the subsequent incubation. However, some bands from the inoculants (Fig. 1A) were not detected during the subsequent incubation times (Fig. 4).

Based on the migration of the 26S rRNA gene fragments in the DGGE gels (Fig. 5), band f3 from ADS3, which matched an uncultured fungus (98%), was detected. However, bands f12 and f13 from WSD5, which matched Podospora fimbriata (97%) and Pseudallescheria boydii (99%), respectively, were not detected after inoculation.
into the soil. In addition, different band changes were noted among the two inoculations and controls at different sampling times. The closest identified relatives of all the isolated DNA sequences from the fungal DGGE gel bands are shown in Table 1.

**DISCUSSION**

The inoculants ADS3 and WSD5 were originally developed based on long-term directed acclimation [15, 25], and showed a high degradation capacity and microbial stability. Composite microbial systems as a new form of microbial utilization (e.g., XCD2 [5] and MC1 [8]), not simply being a mixture of culturable microbes, have demonstrated a high degradation capacity with significance for potential environmental improvement. Some reports have interpreted the great efficiency and stability of microbial composite systems by emphasizing the cooperation of the composite microbes [14, 13].

In this study, it was difficult to use culture-dependent approaches to monitor the strains of ADS3 and WSD5, as some of the microbial components were uncultured according to the analysis of the 16S rRNA gene sequences. From the results of the DGGE profiles, the 16S rRNA gene fragments corresponding to ADS3 survived well in the autoclaved soil, indicating that the soil nutritional conditions were suitable for the introduced microbes, and the theoretical possibility of successfully introducing those microorganisms into soil.

Crop straw as fresh organic matter returned to the field can dramatically enhance the soil organic matter content. In this study, when wheat straw powder was added to the experimental soil, the soil organic matter was significantly improved from about 37 g/kg to about 52 g/kg; therefore, the amount of soil organic matter mineralization was used to reflect the enhancement of straw degradation. According to the results, the ADS3 and WSD5 inoculants were able to accelerate the straw degradation, yet only showed a significant difference (p<0.05) in the sample assays during the first week, indicating that the initial effect from the soil inoculation did not persist. Similarly, the cellulase activity in the soil inoculant samples was relatively higher than that in the control samples during the first three weeks of incubation, yet not significantly different during the last two weeks. Cellulase activity is potentially correlated with the fungal and bacterial populations in soil [11]. Thus, the decline of the cellulase activity further indicated that the efficiency of the inoculum did not persist.

Corresponding to the performance of the inoculum, many of the DGGE bands linked to the introduced microorganisms, including bacteria and fungi, were not detected during the extended incubation times, indicating a low survival and that the accelerated performance was to some extent related to the survival of the introduced inoculants. Moreover, the variation in DGGE band changes among the different inoculations and controls indicated that the introduced microbes exerted some effect on the indigenous soil microorganisms. Thus, in agreement with other reports [9], the current results indicated that the population sizes of microbes decline more or less rapidly following introduction into a natural soil; the effect has been termed “soil microbiostasis.” In the absence of wheat straw, neither the soil inoculants nor the uninoculated controls had any
significant affect on the mineralization of the soil organic matter, indicating that the enhanced degradation only existed with straw amendment. Specific bacteria and fungi have been introduced into soil as an agricultural practice for decades [12, 17, 23]. As mentioned in the introduction, the survival and catabolic activity of such introduced microorganisms are the two key factors for successful inoculation. However, the present results support the frequently reported observation that prevailing soil conditions are unfavorable for maintaining microbial survival and/or catabolic activity. Therefore, the improvement due to an inoculum is transient, as the number of introduced microorganisms decreases shortly after biomass addition to a site [9, 23]. Nonetheless, despite these difficulties, successful cases have been reported for both non-vegetated and planted soils [12, 18, 21].

To summarize the present results, the two introduced cellulose-degrading microbial composite systems were effective in enhancing straw degradation, yet their effect did not persist, ascertaining that the introduced microorganisms were only abundant during the initial stage, plus the performance was enhanced by straw addition. Thus, the survival of the inoculants could be prolonged by improving the microbial adaptability to enhance straw degradation, or the soil agricultural management could be adjusted to focus on the early stage after inoculation in the field. However, even though the bands of the 16S rRNA or 26S rRNA gene fragments from the inoculants were not detected by DGGE after inoculation into the soil, this does not preclude their ability to survive in soil if it is assumed that there may have been a decline in the microbial population due to limitations of the detection method. Thus, DNA-based methods using a real-time quantitative PCR and terminal restriction fragment length polymorphisms (TRFLPs) will be used in future studies.

Acknowledgments

This work was supported by the National Key Technology Research and Development Program of China (No. 2008BADC4B01, 2008BADC4B17), and the National Commonwealth Scientific Research Fund (No. 200803033).

REFERENCES


