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ORIGINAL ARTICLE

The removal characteristics and diversity of a microbial community capable of ammonia removal from compost

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Abstract In order to reduce the pollution and nitrogen loss resulting from ammonia emission during the composting process, a genetically stable microbial community, CC-E, capable of ammonia removal was enriched from cow feces using restrictive culture. The microbial diversity of CC-E was also investigated by 16S rRNA gene clone technology. Moreover, the effect of CC-E on composting inoculation was investigated by detecting the amount of ammonia produced. The results showed that the amount of ammonia produced from the treatments with the microbial community CC-E was 63 % lower (151.11 mg/kg) relative to the control (447.87 mg/kg) over the course of the 20-day culture period. The bacterial groups represented in the CC-E clone library were the gammaproteobacteria (16.7 %), betaproteobacteria (54.4 %), alphaproteobacteria (21.1 %), and uncultured compost bacterium (7.8 %). Furthermore, *Sphingomonas* sp., *Serratia* sp. and *Alcaligenes* sp. were isolated from the microbial community CC-E with ammonia removal capacity. It was found that ammonia emission was reduced to 60.7 % following CC-E inoculation during the first 20 days of composting. Nitrogen conservation was also influenced by inoculation. Results indicate that microbial community CC-E can be a useful resource for biological deodorization.

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Introduction

According to the collected data of the statistical bureau of the People's Republic of China in 2010 (Wang et al. 2010), there were 12.2 million cows in stock up to 2009, which account for 86.8 % of the total number of livestock across the whole nation. The daily outcome of cow feces reaches up to 1.4 to 2.1 million tons, especially in the regulated pasture field. The hazard produced by accumulation of feces is becoming a serious problem all over the world. Among these pollutants, stinky gases have become a more serious environmental hazard (Ye et al. 2009). One of the most common methods to deal with these wastes is through composting technology. However, a huge amount of hazardous gas is generated during the process (Pattey et al. 2005). Previous studies showed that ammonia is a major part of these gases; thus, the amount of ammonia directly determines the occurrence of the rest of stinky gases (Pagans et al. 2006). During the recycling process, ammonia removal by microbes has become a hot spot in the research field, since it combines several advantages, such as low cost, no secondary pollution, elevating nitrogen content, and so on (Ho et al. 2008) compared to the physical chemistry method and the biological physics method, in the effectiveness of ammonia removal. Zhang et al. (2004) found an ammonia elimination ratio of 64 % and 89 %, respectively, which demonstrated that addition of a microbe increased the ammonia removal ratio. Burgess et al. (2003) performed ammonia removal experiments, and among the methods of inoculations of ocean microbe, additive of zeolite and excessive exposure, results showed that both inoculation of *Ulva lactuca* and zeolite methods are more effective in ammonia removal

than the exposure method. Presently, biological technology is gradually becoming the major method in removing hazardous gases (Lee et al. 2001).

The purpose of this study was to find out innovative methods and exploit the most effective and stable microbial community in the ammonia removal process. The composites in a microbial community as well as an individual's role in the whole consortium were also investigated. The ammonia removal capacity of the whole microbial community was determined. This study will provide the theoretical bases for the application of microbial research.

Materials and methods

Experiment materials

Cow feces and corn straw were used as samples to construct the microbial community with ammonia removal capacity. Samples were collected from the dairy farm at Heilongjiang Bayi Agricultural University. Table 1 shows the materials as well as their physical and chemical properties. Other materials were of analytical reagent grade, and distilled water was used throughout the experiments.

Enrichment of microbial community from cow feces using restrictive culture

Samples of warm cow feces (20 g) were mixed with 100 ml ddH_2O in a 250-ml flask containing several glass beads, and shaken at room temperature for 20 min. After static placement for 10 min, 10 ml of the supernatant was inoculated to 50 ml of ammonia selective medium containing 50 g sucrose, 10 ml ammonia, 2.0 g KH_2PO_4, 0.5 g $MgSO_4$, 0.1 g $FeSO_4$, 5 ml 1 % $ZnSO_4$, 2.0 g NaCl in 1000 ml, which was sterilized at 121 °C for 20 min. The inoculum was cultured at 30 °C 160 rpm for 72 h. Odor was ranked using the sensory analysis detected qualitatively by human volunteers (20 female students of College of Life Science and Technology at Heilongjiang Bayi Agricultural University) in order to preliminarily abandon those bacteria with lower ammonia-removing efficiencies. Malodor strength (MS) was defined on a 0 to 5 scale according to Ohta and Ikeda (1978). The MS of normal raw and fresh cow feces was MS 3, and the MS below detection of the characteristic odor of fresh feces (sometimes different odors such as microbial odors were given by samples designated MS 0) was MS 0; stronger odors were given higher values, and the weaker odors were given lower values. The enrichment with higher ammonia-removing ability was inoculated into fresh ammonia selective medium with 10 % inoculum.

Bacterial enrichments were cultured through 20 generations, and the most effective enrichment with capacity for ammonia removal was selected as the microbial community that was used in subsequent analyses.

pH and removal characteristics of the microbial community capable of ammonia removal

The microbial community was inoculated and cultured on 10 ml of sterilized ammonia selective medium at 30 °C using a 160 rpm shaker. The pH of the microbial community was determined by compact pH meter B-212 (NORIBA) at the following time points: 0, 24, 48, 72, 96, 120 and 144 h. Each treatment was repeated three times.

The microbial community was inoculated into 200 g cow feces according to 10 % (v/w), mixed until homogenous, and then placed in a 1000 ml beaker. A 50-ml beaker containing 20 ml of 0.05 % of boric acid was placed and sealed within the 1000 ml beaker. The entire apparatus was cultured at 30 °C for 20 d. The ammonia released was determined by acid-base titration (Zhu et al. 1993) at days 1, 2, 3, 4, 5, 7, 10, 15, and 20.

Amplified ribosomal DNA restriction analysis (ARDRA), sequencing and phylogenetic analysis

DNA of the microbial community was extracted using the benzyl chloride method as described by Zhu et al. (1993). The16S rRNA gene was amplified by PCR from DNA samples of the microbial community with the forward primer 27 F (5′-AGAGTTTGATCCTGGCTCAG-3′) and the reverse primer 1492R (5′ -GGTTACCTTGTTACGACTT-3′). The PCR reaction mixture contained the following: 10×buffer 5 μl, 10 mM dNTP 1 μl, primer 27 F (50 μM) 0.5 μl, primer1492R (50 μM) 0.5 μl, template DNA 1 μl, Taq DNA polymerase (5 units/μl) 0.5 μl, fill up to 50 μl with DI

Table 1 Basic properties of compost materials

Materials	Moisture content (%)	pH	Carbon (‰)	Nitrogen (‰)	C/N
Cow feces	71.5	7.7	360.0	15.7	22.9
Corn straw	9.4	8.1	419.8	8.2	51.2

Table 2 Sensory analysis of odor qualities

Microbial community	CC-H	CC-C	CC-E	CC-F	CC-L	CC-N	CC-D	CC-M	CC-Q	CC-W	CK
Odor qualities	1	4	0	2	3	1	2	1	1	2	3

CK: Control, no microbial community added

water. The PCR reaction conditions were as follows: (1) 94 °C denaturing for 5 min; (2) 30 cycles of 94 °C denaturing for 1 min, 50 °C annealing for 1 min, 72 °C elongation for 2 min; and (3) elongation at 72 °C for 10 min. The amplification products were analyzed on a 1 % agarose gel.

PCR products were purified using a QIAquick PCR purification Kit (Qiagen, UK), and ligated into the pGEM-T easy vector (Promega, Madison, WI), then cloned into *Escherichia coli* TOP10. The positive clones were randomly picked by blue-white selection from overnight LB plates containing 20 mg/ml X-gal and 200 mg/ml IPTG. The recombinant plasmids were amplified as described above using the vector universal primers M13-47 (5′-CGC CAG GGT TTT CCC AGT CAC GAC-3′) and RV-M (5′-GAG CGG ATA ACA ATT TCA CAC AGG-3′). PCR products were digested by *Msp* I and *Hinf* I, analyzed on 2.0 % agarose gels, and grouped according to DNA fingerprinting.

The representative cloned fragments were sequenced with the vector primers M13-47 and RV-M at HUADA Genomics Company (China). 16S rRNA sequences were analyzed by the National Center for Biotechnology Information BLASTn (http://www.ncbi.nlm.nih.gov/blast/). Multiple alignments of sequences were performed using the CLUSTALX program, and the trees were constructed with MEGAR 4.0 software using the neighbor-joining method. The robustness of the phylogeny was tested by bootstrap analysis with 1000 iterations. The nucleotide sequences determined in this study have been deposited in Genbank under accession numbers JF776356, JF776357, JN650299, JN650300.

Isolation and ammonia removal characteristics of strains from the microbial community

The strain with capacity for ammonia removal was isolated from the microbial community by plate isolation method using ammonia selective medium. The microbial community was diluted by 10^{-4}, 10^{-5}, 10^{-6} times, and cultured at 30 °C, and the single colony was transferred to ammonia selective medium for three generations of consecutive isolation.

The 16S rRNA gene of each isolate was amplified by 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). All PCR amplification products of isolated were sequenced, and sequences were submitted to the BLAST network service of NCBI (http://www.ncbi.nlm.nih.gov/blast/) to identify the species.

The capacity of ammonia removal of each isolate obtained was investigated by the method described above.

Effects of inoculation of ammonia-removal microbial on temperature, nitrogen and NH_3 release during windrow composting

Cow feces and corn straw (1:1) were mixed and arranged in trapezoid-shaped piles (with an upper base 1.0 m, a lower base 2.0 m, a height 1.0 m, and a total length of 10 m). The water content of these mixtures ranged from 60–65 %. One pile was inoculated with 0.25 % of bacterial solution (v/v), and the second was inoculated with 0.25 % of DI water (v/v) as the control (CK). Each pile was topped with a stationary sample-collection case (0.2 m dia. × 0.8 m h) for collecting ammonia. An ET-100 blender (Frontier Co. of USA) was used to mix

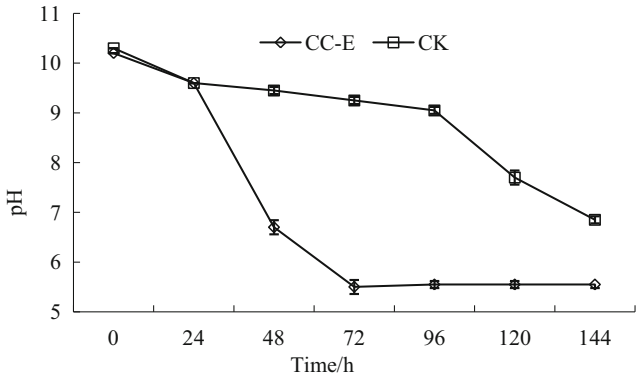

Fig. 1 Change of pH of microbial community in the medium

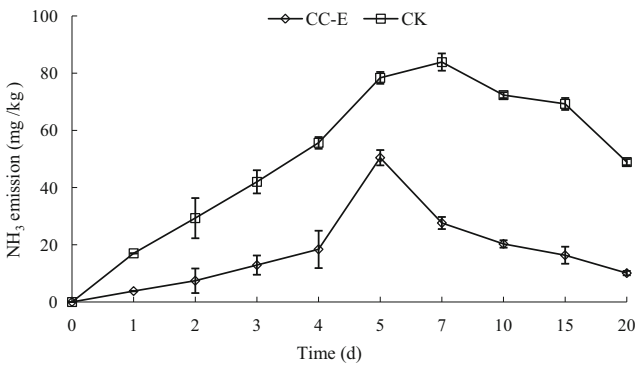

Fig. 2 Change of NH_3 emission of microbial community

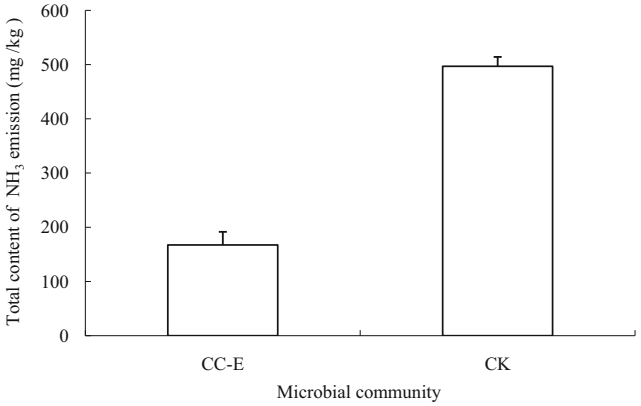

Fig. 3 Total content of NH_3 emission of microbial community

piles every 3 days (Wang et al. 2007). The experimental period was from 23 October 2010 to 21 December 2010.

An UNI-T UT320 digital thermometer was used each day at 10:00 a.m. to record pile temperatures at 50 cm. Ammonia production was determined using the Ammonia Meter Z-800 (Environmental Sensor Co.).

Results

Ammonia-removal capacity of microbial community

A restrictive culture was adopted for constructing a microbial community with ammonia removal capacity from cow feces in Daqing, China. Ten microbial communities were preliminarily screened from warm cow feces, and the results of the sensory analysis are shown in Table 2. Among these microbial communities, malodor strength of CC-E indicated the most effective ammonia removal, and it was adopted for the experiment that followed.

Characterization of ammonia removal of the microbial community CC-E was investigated. As seen in Fig. 1, the pH of the microbial community CC-E decreased significantly at 48 h compared to the control, then settled at 5.5. The microbial community CC-E was then inoculated into fresh cow feces, and the amount of ammonia released during 20-day culture period was monitored. It can be seen that the released amount of ammonia increased with culturing time (Figs. 2 and 3) until it reached a peak. However, a further increase led to a decrease in the amount of ammonia. Meanwhile, the one treated with CC-E reached its peak concentration of 50.46 mg/kg at day 5, while the one under CK treatment got its peak at day 7, with a concentration of 83.90 mg/kg. At any time during the 20 days of culture, the amount of ammonia was always detected as lower than that of the blank one, and the ammonia release amount was 151.11 mg/kg and 447.87 mg/kg by inoculation and CK treatment, respectively, while ammonia released from microbial-treated waste was 66.3 % less than CK-treated waste. These indicated that ammonia release amount can be effectively restrained by CC-E.

Diversity of microbial community CC-E

The diversity of the stable microbial community CC-E was analyzed by the ARDRA method. A total of 120 clones from bacterial 16S rRNA gene clone libraries were further analyzed, and four different RFLP patterns were detected and designated as operational taxonomic units (OTU, 97.0 % similarity). The adequacy of the sample size for the determination of diversity within the 16S rRNA clone library was evaluated by rarefaction analysis (Lee et al. 2001). As shown in Fig. 4, the calculated rarefaction curve did reach a clear saturation,

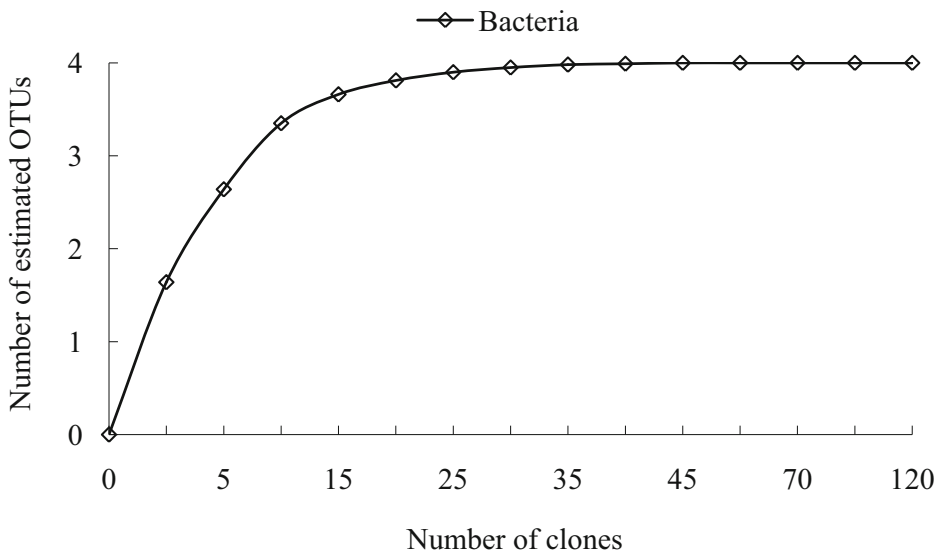

Fig. 4 Rarefaction curves of CC-E 16S rRNA gene sequences

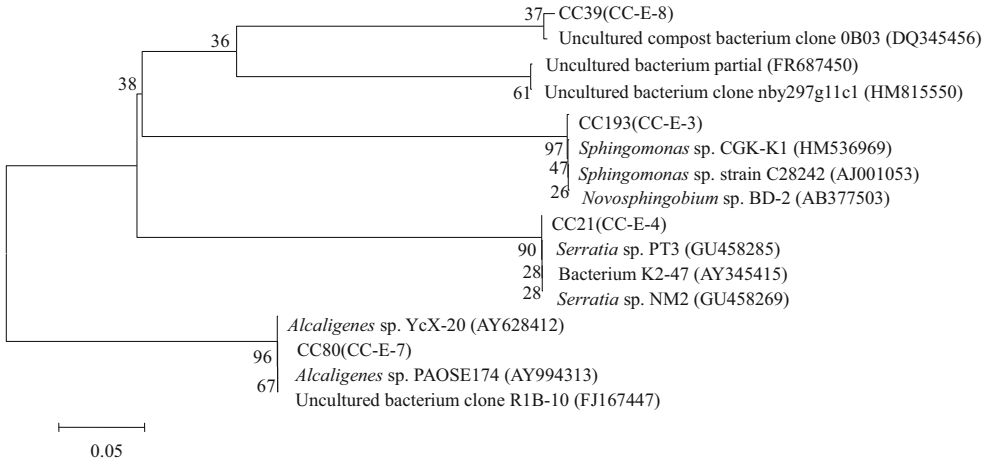

Fig. 5 Phylogenetic tree based on the 16S rRNA gene sequences of CC-E

suggesting that the analysis of the 120 clones had covered the diversity.

Phylogenetic analysis (Fig. 5) was performed to affiliate the estimated clone sequences to hitherto-determined groups. Results indicated that the major groups in the 16S rRNA clone library were *Serratia* (16.7 %), which belongs to Gammaproteobacteria, and *Alcaligenes faecalis* (54.4 %), which belongs to Betaproteobacteria, and *Sphingomonas paucimobilis* (21.1 %), which belongs to Alphaproteobacteria. The OTU CC39 represented the remaining clones (7.8 %), which were assigned to uncultured bacterium. CC39 has 99 % similarity to uncultured compost bacterium clone 0B03, which was found by the cloning of a feces sample, and its function remains unclear. No clonal sequences were detected as the chimeric artifact. In addition, *Alcaligenes faecalis* was an advantageous strain in the microbial community CC-E.

Separation of different strains of microbial community

Complex strains have exhibited dramatic differences in their phenotypic characteristics, so they must repeatedly go through streak culture, and we successfully obtained three pure bacterial strains (Table 3). These bacterial strains were named CC-E-3, CC-E-4 and CC-E-7 and identified by 16S rRNA sequence analysis as Sphingomonas sp., Serratia sp., and Alcaligenes sp., respectively.

Reported ammonia removal microbes are classified into following species: *Bacillus*, *Alcaligenes* sp, *Pseudomonas*, *Zoogloea*, *Acinetobacter*, *Thiobacillus*, *Saccharomyces*, *Candida*, *Aspergillus*, *Penicillium*, *Rhizopus*, and so on (Chen et al. 2004; Zhang et al. 2004; Pattey et al. 2005; Jian et al. 2006; Hort et al. 2009; Xue et al. 2010).

Characterization of ammonia removal of the three strains

In order to further confirm the capacity of ammonia removal of these strains, the characterization of ammonia removal of the three strains was investigated according to the method used for the microbial community CC-E. CC-E-3, CC-E-4, and CC-E-7, were inoculated into fresh cow feces respectively, and the amount of ammonia released during the 20-day culture period was monitored. The results showed that treatment with the three strains always played a role in ammonia removal during the 20-day culture period. The peak of ammonia emission appeared at the fifth day for all isolate strains (CC-E-3, CC-E-4, and CC-E-7), and reached 61.60 mg/kg , 71.60 mg/kg, and 55.60 mg/kg, respectively. However, the peak of ammonia emission appeared at the seventh day for the control, and reached 88.40 mg/kg (Fig. 6). During the 20-day culture period, the amount of ammonia produced from the

Table 3 Colony morphology characteristics of the three bacterial isolates

Isolate	Similar species	Colony Characteristics				
		Shape	Elevation	Margin	Surface	Pigment
CC-E-3	*Sphingomonas* sp. CGK-K1 (HM536969)	Circular	Flat	Entire	Smooth	Yellow
CC-E-4	*Serratia* sp. PT3 (GU458285)	Circular	Flat	Entire	Glistening	Milky
CC-E-7	*Alcaligenes* sp. YcX-20 (AY628412)	Circular	Convex	Entire	Smooth	Incarnadine

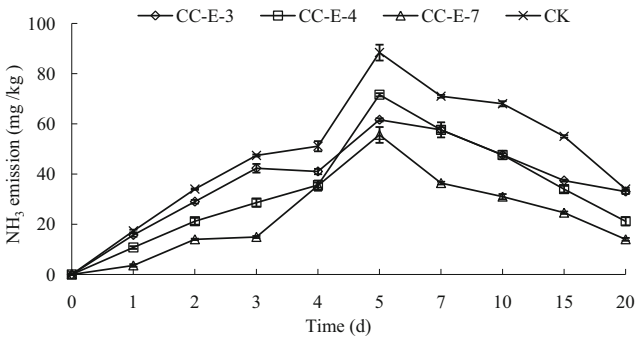

Fig. 6 Change in NH_3 emission with inoculation of different isolates of CC-E

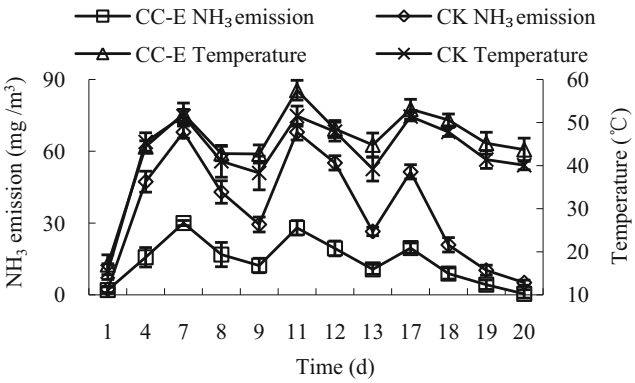

Fig. 8 Change of temperature and NH_3 emission during windrow composting process

treatments with CC-E-3, CC-E-4, CC-E-7, and CK were 332.19 mg/kg, 306.93 mg/kg, 197.93 mg/kg, and 432.23 mg/kg, respectively (Fig. 7). The percentages of ammonia removal of CC-E-3, CC-E-4, and CC-E-7 were 23.1 %, 29.0 %, and 54.2 %. Results showed that the percentage of ammonia removal by isolates was lower than that by the microbial community.

Effects of inoculum on ammonia removal during windrow composting

Figure 8 shows that the amount of released ammonia changed with temperature and it reached one peak amount as temperature increased, then it dropped as temperature decreased, and didn't stop until the last day of the 20-day period. During the pile-up of feces, temperature didn't show much difference; however, ammonia released from the CC-E treated sample was lower than for CK-treated all the time; at day 7, the gas release amount from those two sample had the largest differences of 30.0 mg/m^3 and 68.0 mg/m^3, and this was correlated with temperature change, and the final ammonia release amount for the CC-E treated sample was less than 60.7 % (Fig. 9). These data implied that ammonia release was constrained effectively by the inoculating microbes. Meanwhile, the content of nitrogen offered an initial upgrade, then a descending latter tendency during the pile-up of feces (Fig. 10). The final content of nitrogen for the CC-E treated sample was increased by 9.7 % and 5.8 % compared to initial and CK, respectively. These data indicated that the content of nitrogen was effectively conserved by the inoculated microbes.

Discussion

Based on previously successful cases in the construction of complex bacterial communities functionalized in disintegrating cellulose (Wang et al. 2007, 2011b), we have successfully obtained a microbial community capable of ammonia removal. A sample was taken from cow feces, and utilizing ammonia-selective medium, we put all those microbes with ammonia removal ability together, and through selection we acquired a complex bacterial community named

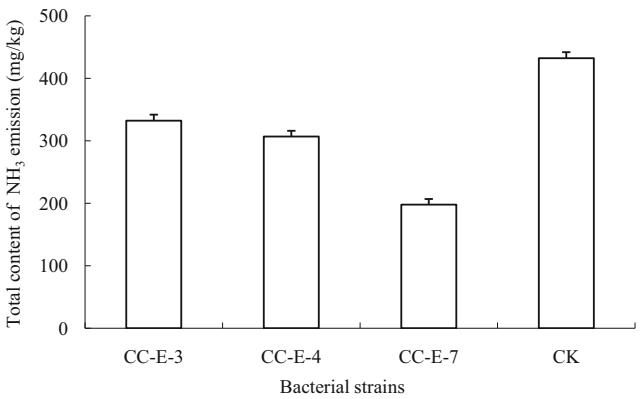

Fig. 7 Total content of NH_3 emission with inoculation of different isolates of CC-E

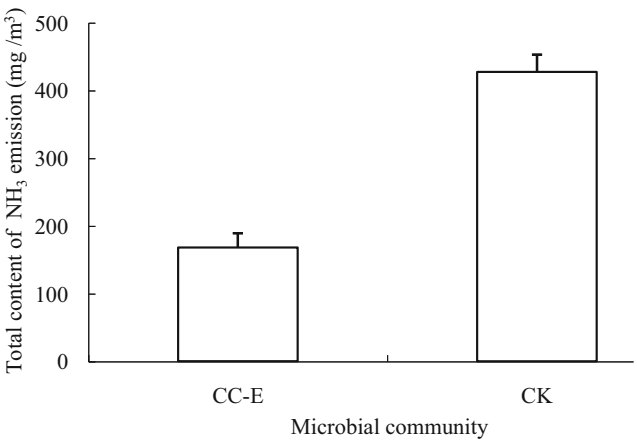

Fig. 9 Total content of NH_3 emission during windrow composting process

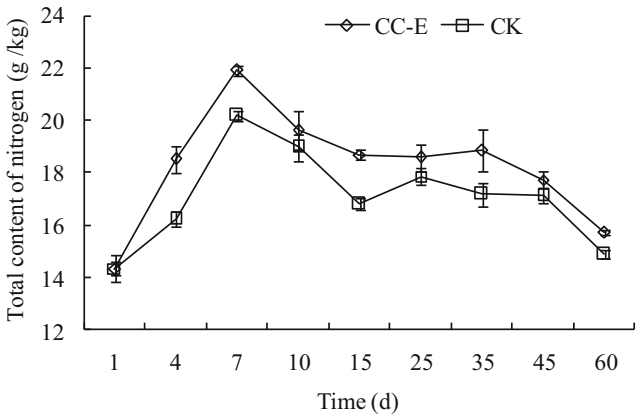

Fig. 10 Total content of nitrogen during windrow composting process

CC-E, which is stable in both ammonia removal effectiveness and genetic reproductiveness. This complex bacterial community is not merely a recombination of different strains, but is a cooperative group with ammonia absorption and transformation ability under natural circumstance. This complex consists of aerobic components that exhibit strong ammonia-removal ability at a culturing temperature of 30 to 50 °C. It can reduce ammonia in cow feces by 65 % or more when consecutively cultured for 20 generations. The result was stable and the pH value of the ammonia selective medium was decreased. We aimed to eliminate ammonia in cow feces, and found that CC-E has best effectiveness when initial pH is between 7.0 and 9.0, which corresponds with the Guštin and Marinšek-Logar (2011) theory that apH value close to 10 has the best ammonia removal effectiveness. Phenotypic characterization of isolates and sequence analysis of 16S rRNA indicate the existence of three cultivable strains and one temporarily uncultivable strain in CC-E. Results also suggest that they are mainly classified into following categories: *Serratia* sp., *Alcaligenes* sp., *Sphingomonas* sp. and uncultured compost bacterium, the complex mainly consisting of cultivable ones and uncultured ones, and it showing complicated bio-varieties. It has been reported that the above-mentionede strains are all used in the treatment of sewage. Gong et al. (2008) utilized one *Serratia* strain isolated from the soil as bio-flocculant in sewage treatment, the ammonia removal ratio of our *Serratia* strain was 29 %. Lin and You (1987) found that *Alcaligenes* sp. had a preference for utilizing NH_4^+ to NO_3^- under aerobic situation, the ammonia removal ratio of our *Alcaligenes* strain was 54 %. Patureau et al. (2000) isolated *Sphingomonas* sp. that had anti-nitrification function out of sewage under alternative aerobic and anaerobic conditions, the ammonia removal ratio of our *Sphingomonas* strain was 23 %. Overall, the point at which the ammonia removal capacity of the microbial community was more than for different strains has been acknowledged by most scholars (Wang et al. 2011a). This is probably because the synergistic effect of the microbial community may promote ammonia removal ratio of the key strains.

When we use biological ways to get rid of ammonia, we can either inoculate microbes into the originating place of ammonia generation or use a bio-reactor to eliminate collected ammonia. The former combines advantages of easy operation and low investment; however, the latter method involves building up a bio-reactor in advance, as well as taming these microbes in the reactor, which costs a lot. Ye et al. (2008) separated three strains with ammonia removal ability out of soil: *Bacillus*, *Streptomyces griseus* and *Sphaeropsidales*, combined and conjugated with certain carriers and then hung over both a pigsty and feces piling places. It was found that ammonia was reduced by 78.4 % and 84.4 %. Kim et al. (2000) inoculated one *Vibrio* strain isolated from the ocean into organic packing material, and placed it in a bio-filter where generated ammonia gas and the average elimination rate of ammonia was above 85 %. However, direct application of ammonia removal microbes into the ammonia generation origin has not yet been reported, so we came up with the innovative method of putting an ammonia removal microbial community in direct contact with ammonia generation origin by inoculating CC-E directly into the cow feces piling place. This can increase the content of nitrogen by 9.7 %, because of reducing 60.7 % of the ammonia release ratio. Thus, complex microbial communities have excellent ammonia removal and nitrogen conservation abilities, and can be widely applied in the recycling of cow feces.

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