Journal of Bioscience and Bioengineering
VOL. 110 No. 4, 419–425, 2010

www.elsevier.com/locate/jbiosc

Development and analysis of microbial characteristics of an acidulocomposting system for the treatment of garbage and cattle manure

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Received 30 October 2009; accepted 19 April 2010
Available online 23 May 2010

An acidulocomposting system for the treatment of cattle manure with little emission of ammonia gas was developed, and the structure of its microbial community was investigated by denaturing gradient gel electrophoresis (DGGE) and clone library construction. An acidulocomposting apparatus (BC20, 20 L) was operated for 79 days to treat 2 kg (wet wt) of garbage per 1 or 2 days. On day 80 of operation, the substrate was changed from garbage to cattle manure (1 kg of beef cattle manure was added to the apparatus every 2 or 3 days), and the system continued operating from days 80 to 158. The compost in the vessel was under acidic conditions at pH 5.2–5.8, and ammonia emission was below the detectable level (<5 ppm) throughout the period of cattle manure feeding. Total nitrogen and total carbon in the compost were 26–29 and 439–466 mg/g of dry weight, respectively, which are higher than those in general cattle manure compost. The main acids accumulated during operation were lactic and acetic. Sequencing analysis targeting the 16S rRNA gene revealed the stable dominance of the bacterial phylum *Firmicutes*, with a high proportion of the isolates belonging to the genus *Bacillus*. Using a culturing method with MRS agar, we isolated lactic acid bacteria (LAB) related to *Pediococcus acidilactici*, *Weissella paramesenteroides*, and *Lactobacillus salivarius*, indicating the existence of LAB in the system. These results indicate that acidulocomposting treatment of cattle manure is not accompanied by ammonia emission and that *Bacillus* and LAB may be the key components in the system.

[**Key words:** Composting process; Microbial community; PCR-DGGE; Isolation; Animal manure]

The management of the large volumes of excreta produced by the livestock industry is a major problem globally because they cause environmental damage, such as water pollution, eutrophication, and odor if they are not properly treated. Therefore, their treatment by an effective and environmentally safe method is strongly required.

Composting is a widely used technique to degrade organic wastes, such as animal waste, excess sludge, and garbage, into stable matter by passage through a thermophilic stage (1–4). Composting has received attention because it is useful for treating animal waste and fully utilizing organic resources, especially in Japan, which lacks many natural resources.

However, ammonia (NH_3) gas emission is a major problem of the composting process because evaporated NH_3 causes odor problems, and the loss of NH_3 from the organic matter represents a decrease in the fertilizer value of the final compost (5,6). General composting of animal manure emits several tens to several thousand milligrams per cubic meter of NH_3 (7,8). NH_3 gas has an irritating odor, and it is designated as a malodorous substance by Japanese odor regulations. High amounts of NH_3 emissions easily lead to odor problems for residents living in the vicinity of the source (1,2,9–13). The loss of NH_3 also represents a decrease in the fertilizer value of the compost. Of the initial total nitrogen, 24–33% in garbage (10), 14–42.5% in swine manure (7,14), 1.0–8.8% in cattle manure, and 39.9% in poultry manure could be lost in NH_3 emission (15). Additionally, NH_3 loss influences atmospheric chemistry and subsequent acid deposition on the land (16). NH_3 evaporated from manure forms ammonium sulfate upon interaction with sulfur dioxide (from fossil fuels), and after leaching by rainwater, the ammonium sulfate oxidizes to nitric and sulfuric acids in soil, resulting in reductions in pH (17,18). The resultant soil acidification has caused increasing concern, especially in developed countries.

The key factors affecting NH_3 emission from animal manure are temperature, initial concentration of ammonium ions (NH_4^+), and pH (19). NH_3 emission depends strongly on temperature and is restricted by low temperature in the compost (2,4). The initial NH_4^+ concentration is also related to emission during composting; a low initial concentration of NH_4^+ due to reduction in the initial input of raw manure restricts NH_3 emission. However, these modifications are difficult because their parameters are the characteristics of the compost itself.

On the other hand, pH modification is an appropriate method for restricting NH_3 emission during the process of composting. In general, the pH of the material influences the equilibrium between NH_3 and NH_4^+ in solution (12) and subsequent NH_3 emission (20) because pH changes shift the balance of the equilibrium between NH_3 and NH_4^+ (21). Previous studies have shown that NH_3 emission can be reduced by acidification of the organic matter, such as animal slurry (22,23).

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doi:10.1016/j.jbiosc.2010.04.006

NH_3 emission from cattle slurry can be reduced by a maximum of 15% by lowering the initial pH to 6.2. Setting a low pH in animal waste manure composts might be a useful approach to restrict NH_3 emission.

Acidulocomposting is a garbage treatment process that requires a heating and agitating apparatus that keeps materials under thermophilic conditions (40–65 °C) (24). This system emits little NH_3 gas, probably because of its low pH (3.5–6.5). The major microorganisms in the acidulocomposting system are lactic acid bacteria (LAB), such as *Lactobacillus* and *Pediococcus* spp., and they are considered to be metabolically active in the development of the thermoacidophilic conditions of the process (25). Therefore, this system might be an ideal method to treat animal wastes without large quantities of NH_3 gas emission. However, no studies have applied the acidulocomposting system to animal waste manure treatment at the laboratory scale or field scale. Monitoring the microbial structure in animal manure treatment systems is important for the effective management of acidulocomposting for various organic matters.

The objectives of this work were as follows. (i) To develop an acidulocomposting system to treat animal manure. For this, we first operated an acidulocomposting apparatus having a volume of 20 L with garbage for 78 days to develop and stabilize the acidulocomposting operation, and then we changed the substrate to cattle manure and operated the facility for another 80 days. (ii) To analyze the microflora in the product of the acidulocomposting process of garbage/cattle manure treatment by multiple approaches. Molecular techniques, including denaturing gradient gel electrophoresis (DGGE) and clone library and culturing techniques, were applied to the analysis because the microflora were expected to include several kinds of cultivable and uncultivable microorganisms. The data from these analyses allowed us to characterize the microflora of the acidulocomposting system used to treat garbage and cattle manure.

MATERIALS AND METHODS

Composting A BC-02 composting apparatus (Star Engineering, Hitachi, Japan) was used for acidulocomposting in this study. The structure of the apparatus has been described previously (24). It has a vessel capacity of 20 L and a maximum treatment capacity of 2 kg (wet wt) of garbage per 1 or 2 days. The temperature of the compost in the vessel was set at 55 °C. The first addition of the substrate (mixed garbage) was performed with the base material (cedar wood saw dust, 16 L). During operation from days 1 to 78, we added 0.5 kg of garbage per day, which was discarded from school lunch facilities and included rice, meat, fish, fish bones, vegetables, fruit rinds, and eggshells. Typically added substrates included food refuse and raw residual materials. Excess water in the fresh garbage was drained by using a basket. On day 79, the type of substrate was changed from garbage to animal manure, and from day 79 to the end of the study period (day 158), 1 kg of beef cattle manure, including cattle food residue and silage, was added to the apparatus every 2 or 3 days. Throughout the operation, tap water was added if required to maintain the water content in the vessel at approximately 30–40%. The volume of the product compost in the vessel gradually increased, and excess product was removed to maintain the volume of the compost in the vessel at less than 18 L.

Sampling and measurements Compost samples were routinely taken from the apparatus vessel every 2–4 days. The NH_3 emission from the system was measured inside the compost in the vessel with the cover of the apparatus open on every sampling day by using NH_3 gas detector tubes (GASTEC, Kanagawa, Japan). Water moisture was measured by the moisture determination balance FD-600 (Kett Electric Laboratory, Tokyo, Japan). Total carbon (T-C) and total nitrogen (T-N) were determined using an NC analyzer (Sumigraph NC-80 S, Sumica Chem. Anal. Service, Osaka, Japan). One gram of the compost sample was vigorously suspended in 10 mL distilled water and vortexed for 5 min. The resultant suspension was allowed to stand for 5 min at room temperature, and the pH of the supernatant was measured by a D-55 pH meter (HORIBA, Kyoto, Japan). The crude extract was centrifuged at 690×*g* for 5 min, filtered with a 0.45-μm membrane filter (Millipore, Bedford, MA, USA), and subjected to ICS-90 ion chromatography (DIONEX, Sunnyvale, CA, USA) to determine the concentrations of cations and anions in the sample. The filtrate was injected into a PU-980 high-performance liquid chromatography (HPLC) apparatus (JASCO, Tokyo, Japan) using an 870-UV detector (JASCO) to determine the concentrations of organic acids (lactic, acetic, formic, propionic, isobutyric, butyric, isovaleric, and valeric). The conditions for HPLC were as follows: mobile phase, 3 mM $HClO_4$; column, RSpak KC-811 (Showa Denko, Tokyo, Japan); column temperature, 50 °C; flow rate, 1.5 mL/min.

Extraction of DNA from compost Total DNA was extracted using the MoBio PowerSoil™ DNA isolation kit (Carlsbad, CA, USA) with the following modifications: 0.1 g soil was used, and the samples were homogenized using a Micro Smash (TOMY, Tokyo, Japan). The concentration of DNA extracts was determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

PCR amplification Fragments of the 16S rRNA gene were enzymatically amplified from total DNA samples using an iCycler system (Japan Bio-Rad Laboratories, Tokyo, Japan) with the primers GC-27f (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGG-GGCACGGGGGGCCCTGGCCGCAGCTAAAGCAGCCAAGTTCG-3′) and 518R 5′-GTAT-TACCGCGGCTGCTGG-3′) (26,27). A GC clamp was attached to the forward primer for DGGE-PCR as described by Muyzer and colleagues (26). The amplification mixture consisted of 1×Ex Taq buffer (Takara, Shiga, Japan), 0.2 mM of each dNTP, 0.25 μM of each primer, 1 μL of template DNA, and 1.25 units of Ex Taq polymerase (Takara) in a final volume of 50 μL. The amplification conditions were as follows: 95 °C for 3 min; 30 cycles of 95 °C for 30 ss, 55 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 7 min. A universal 16S/18S rDNA primer set, 520f (5′-GTGCCAGCMGCCGCGG-3′) and GC1400r (5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGG-GACGGGCGGTGTGTRC-3′) (25,28), was also used to evaluate the microbial community of the compost sample. The mixture for the amplification consisted of 1×Ex Taq buffer, 0.2 mM of each dNTP, 0.25 μM of each primer, 1 μL of template DNA, and 1.25 units of Ex Taq polymerase in a final volume of 50 μL. The amplification conditions were as follows: 95 °C for 3 min; 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 7 min. Five microliters of each PCR product was analyzed by electrophoresis in 1.2% agarose and visualized by ethidium bromide staining.

Analysis of PCR products by DGGE DGGE of the PCR products was performed using a DCode universal mutation detection system (Bio-Rad Laboratories, Richmond, CA, USA). A polyacrylamide gel [8% (w/v) of a 37.5:1 acrylamide:bisacrylamide mixture in 1×TAE buffer (0.04 M Tris base, 0.02 M acetate, and 1.0 mM EDTA)] was used for the 27f–518r primer set. For the 520f–1400r primer set, the same conditions were used except that the gel contained a gradient of polyacrylamide from 5% to 8% (w/v) in the direction of electrophoresis. Denaturant gradients from 40% to 70% (for the 27f–518r primer set) and from 15% to 55% (for the 520f–1400r primer set) [100% denaturant was 7 M urea plus 40% (w/v) formamide] in the direction of electrophoresis were used. The gels were subjected to a constant voltage of 100 V for 8 h at 60 °C, and after electrophoresis, they were stained for 10 min in GelStar® stain (Cambrex, Baltimore, MD, USA) and photographed using a UV transilluminator (ATTO, Tokyo, Japan). Digital images of the gels were acquired with a CCD video camera module (ATTO).

From the gel for primer set 27f–1492r, small pieces of selected DGGE bands were excised using sterilized cutter blades, transferred into 50 μL of sterile water, and incubated overnight at 4 °C. One microliter of the eluted DNA was used for reamplification without the GC-clamped primer, and the PCR products were checked by electrophoresis for their specificity. Amplified products were treated with ExoSAP-IT (Amersham Biosciences, Amersham, UK) to remove excess primers, and dNTPs and were sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing kit v1.1 (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's instructions, on an ABI 3130×l Genetic Analyzer (Applied Biosystems). Sequence accuracy was confirmed by bi-directional sequencing. The GenBank database was searched using the BLAST program for the most similar sequence type.

16S rRNA gene libraries Clone libraries were constructed for the compost samples obtained on days 46 and 137. Universal primers (27f: AGAGTTTGATCCTGGCT-CAG and 1492r: GGTTACCTTGTTACGACTT) (29) were used to amplify the 16S rRNA gene from DNA extracts obtained as described above. The PCR product was cloned in a pT7Blue vector according to the manufacturer's instructions (Perfectly Blunt Cloning kit; Novagen, Madison, WI, USA). Clones were screened by colony PCR using the vector-encoded primers and treated with ExoSAP-IT (Amersham Biosciences). Amplified DNA fragments were sequenced using an ABI PRISM Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) on an ABI 3130×l Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. The 27f primer was used in the sequencing reaction. The GenBank database was searched using the BLAST program for the most similar sequence type.

Phylogenetic analysis for the determined sequences was conducted using MEGA software (Version 4) by adding homologous 16S rRNA gene sequences registered in GenBank. Distance-based analyses were conducted using the formula for Kimura's distance (30). Phylogenetic trees were constructed using the neighbor-joining method in the MEGA software.

Isolation of LAB To confirm the existence of LAB in the composting process, we tried to isolate LAB from the sample obtained on day 158 of operation. MRS agar medium containing calcium carbonate at a final concentration of 1.0% was used. Colonies surrounded by a clear halo were picked with a sterile loop and streaked on the medium several times to separate into single species. The partial 16 S rRNA gene was amplified using a universal primer set, 27f–1492r (31), and the gene was sequenced as described above to identify the species of the isolates.

Nucleotide sequence accession numbers The nucleotide sequence data of the 16S rRNA genes obtained from DGGE analysis, clone libraries, and isolates were deposited in DDBJ/EMBL/GenBank under accession numbers AB441552–AB441560, AB441565–AB441619, and AB441678–AB441686, respectively.

RESULTS

Change in chemical characteristics During the first 79 days of operation for garbage treatment, two phases were observed: an acidic