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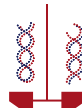

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Dynamic bacterial community changes in the autothermal thermophilic aerobic digestion process with cell lysis activities, shaking and temperature increase

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Autothermal thermophilic aerobic digestion (ATAD) is conducted for stabilization of sludge waste and is driven by the action of various microorganisms under aerobic conditions. However, the mechanism controlling bacterial community changes during ATAD via three (initial, middle and final) phases is currently unclear. To investigate this mechanism, activity analysis and a microcosm assay with shaking were performed on a bacterial community during the initial, middle, and final phases of incubation. Cell lysis activities toward gram-negative bacteria, but not gram-positive bacteria, were detected in the ATAD samples in the middle and final phases. During shaking incubation in initial-phase samples at 30 °C, major operational taxonomic units (OTUs) related to *Acinetobacter indicus* and *Arcobacter cibarius* dramatically increased along with decreases in several major OTUs. In middle-phase samples at 45 °C, we observed a major alteration of OTUs related to *Caldicellulosiruptor bescii* and *Aciditerrimonas ferrireducens*, together with distinct decreases in several other OTUs. Final-phase samples maintained a stable bacterial community with major OTUs showing limited similarities to *Heliorestis baculata*, *Caldicellulosiruptor bescii*, and *Ornatilinea apprima*. In conclusion, the changes in the bacterial community observed during ATAD could be partially attributed to the cell lysis activity toward gram-negative bacteria in the middle and final phases. The microcosm assay suggested that certain physical factors, such as a high oxygen supply and shearing forces, also might contribute to bacterial community changes in the initial and middle phases, and to the stable bacterial community in the final phase of ATAD.

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[**Key words:** Autothermal thermophilic aerobic digestion; Bacterial community changes; Cell lysis activity; Shaking; Temperature increase]

Autothermal thermophilic aerobic digestion (ATAD) is a sludge waste stabilization process driven by the action of a wide variety of microorganisms with air or pure oxygen supply. During ATAD, the temperature of the system gradually rises to a thermophilic range (45–65 °C) owing to the heat generated by microbial metabolism and the jacket that surrounds the bioreactor to prevent heat loss without any external heat supply (1). ATAD converts sludge wastes such as domestic waste (2,3), pharmaceutical sludge (4), and brewery wastewater (5) to soil conditioners or sanitary and stable fertilizers. ATAD has been widely applied in the transformation of animal waste; sewage sludge; and wastewater derived from households, agriculture, food processing, and other industries (6–9). The process has been shown to have many advantages: control of the process is simple, volatile solids are broken down with a short hydraulic retention time (saving space and cost), the temperature is self-regulating, and food-borne pathogens are eliminated at these high temperatures (10). Batch-mode ATAD processes can be divided into the following two distinct phases: mesophilic and thermophilic (10–12). To date, only a few studies have performed an analysis of the physicochemical and bacterial community structure in both full- and lab-scale bioreactors (13,14).

In our previous study, we analyzed dynamic changes in the bacterial community structure and physicochemical characteristics (temperature, dissolved oxygen [DO], oxidation-reduction potential [ORP], pH, ammonia, total carbon, total nitrogen, and other characteristics) of ATAD in a full-scale facility (Fukuoka, Japan) and found that the changes in bacterial community structure and physicochemical characteristics were unique compared to other ATAD bioreactors (15). We proposed that the ATAD process could be clearly divided into three phases (initial, middle, and final) according to differences in the above parameters. In particular, the bacterial community structures changed dynamically (at the phylum level) in relation to the temperature as follows: initial phase, *Proteobacteria* at 27–45 °C; middle phase, *Bacteroidetes* at 45–52 °C; final phase, *Firmicutes* and *Actinobacteria* at >52 °C. These results suggested that the bacteria more adaptable to changes in the environment would proliferate actively, while cell death or lysis would occur in less adaptive bacteria during the initial and middle phases. In the ATAD facility

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of Chikujo, Japan, 300 ppm (according to the instruction from full-scale bioreactor company) enzymatic powder (EP) containing lipase, cellulase, and protease is added to human excreta (HE) samples for stimulating digestion (16). Our previous study revealed that for approximately 3 weeks during ATAD, dynamic changes occurred in the bacterial community along with a destruction of several pathogenic bacteria and a decrease in the organic acid content (15). Notably, these changes in the bacterial community structure were unique when compared to the published studies, and the uniqueness was likely due to the use of a self-inducing aerator with complete aeration (15).

Several mechanisms, including cell lysis, enzymatic reactions and shaking, have been investigated as factors affecting cell proliferation, lysis, and death. It has been reported that activities of cell lysis and enzymes (such as glucanases, proteases, and lipases) play a role in digesting certain bacteria (17–21). On the other hand, lab-scale shaking exhibits two effects: increasing oxygen supply and the generation of shearing forces, which stimulate the proliferation of aerobic bacteria and damage bacterial cells, respectively. Thus, we hypothesized that certain activities and shaking may be key factors affecting the unique and dynamic changes in bacterial community structures during the ATAD process of treating HE.

In this study, we aimed to elucidate the mechanism of bacterial community changes in this unique ATAD system by analyzing activities of cell lysis and enzymes, and performing a microcosm assay on bacteria performing ATAD with shaking in three phases.

MATERIALS AND METHODS

ATAD samples from the full-scale bioreactor All the ATAD samples used in this study were collected from the liquid fertilizer manufacturing facility in Chikujo, Fukuoka Prefecture, Japan. Briefly, collected HE samples were filtered to remove foreign materials such as paper, stone, plastic, and metal; thereafter, enzymatic powder (EP; Asahi Kasei Clean Chemical Co. Ltd., Tokyo, Japan), which is listed as a digestive stimulator at 300 ppm by the manufacturer, was added to the raw HE. Next, the raw HE ($\sim$150 m^3) was fed into a semi-underground empty bioreactor that is covered with soil and 300-mm thick concrete, and has a working volume of 180 m^3 (3). Digestion was stimulated through simultaneous mixing and aeration using a self-inducing aerator in batch mode without any control of the temperature, pH, and DO parameters for approximately 3 weeks. For the activity assay, ATAD samples (0–22 d) were collected from the full-scale bioreactor from 28 October to 19 November 2014. For the microcosm assay, three phases of ATAD samples (initial, middle and final) were collected on day 0, 3, and 6 from the bioreactor between 17 June and 9 July 2014.

Preparation of crude enzyme solutions and activity assay of the full-scale ATAD process The ATAD samples (10 ml) were centrifuged at 20,000 $\times g$ for 10 min at 4 °C, and the supernatant fractions were used as extracellular enzyme solutions. The precipitate fractions were washed in 10 ml buffer including 0.05 M KH_2PO_4–NaOH (pH 7.0, pH 7.5, pH 8.4 and pH 9.0) or 0.05 M Tris–HCl (pH 7.5, pH 8.4 and pH 9.0), and centrifuged at 10,000 $\times g$ for 10 min 4 °C. The precipitates were re-suspended in 5 ml buffer, ultra-sonicated in an ice bath for 5 min, and centrifuged at 10,000 $\times g$ for 10 min at 4 °C. The resultant supernatant fractions were used as intracellular enzyme solutions. Thereafter, 1.0 g EP (Asahi Kasei Clean Chemical Co. Ltd.) was suspended in 9 ml 0.05 M KH_2PO_4–NaOH (pH 7.0) buffer, mixed thoroughly, and left for 10 min in an ice bath. The EP suspension was centrifuged at 10,000 $\times g$ for 15 min at 4 °C and the supernatant fraction was used as the extracellular enzyme solution. For the activity of β-1,4-glucanase and β-1,3-glucanase, 2 ml of the EP supernatant was dialyzed with a dialysis tube (No. 1-138-218, type SS-5, $\sim$10,000–14,000 molecular cutoff; Kenis, Osaka, Japan) in 2 L 0.05 M KH_2PO_4–NaOH buffer (pH 7.0) at 4 °C overnight with gentle stirring to remove reducing sugars. To prepare the intracellular enzyme solution from EP, the precipitated fraction was treated according to the same procedure for ATAD samples described above. All activities were expressed as unit of each assay per milliliter of HE sample (U/ml-HE).

Cell lysis activity assay The cell lysis activity assay for gram-positive bacteria was performed with a lysosome activity kit (Sigma Aldrich, St. Louis, MO, USA) using *Micrococcus lysodeikticus* cells as the substrate, according to the manufacturer's instructions (22). The reaction mixture was composed of 2.85 ml *Micrococcus lysodeikticus* cell suspension in 0.05 M Tris–HCl (pH 7.5, pH 8.4 and pH 9.0) with an optical density at 450 nm (OD_{450}) of 0.7 and 0.15 ml enzyme solution. The reaction was initiated by adding enzyme solution, incubating in a shaking water bath at 140 rpm at 30 °C, 45 °C and 53 °C for 5 h, respectively, and the OD_{450} was monitored. *Escherichia coli* JM 109 was used to analyze cell lysis activity for gram-negative bacteria (23). The procedures employed were identical to those for *Micrococcus lysodeikticus* as described above, with the exception of the OD value, which was measured at 540 nm. Egg-white lysozyme was used as a positive control. One unit was defined as the amount of enzyme required to reduce the OD_{450} or OD_{540} by 0.001 per minute under the assay conditions.

Protease activity assay Total protease activity was assayed as described previously (24). The reaction was started by adding enzyme solution and incubating at 30 °C and pH 7.5, 45 °C and pH 8.4, and 53 °C and pH 9.0, respectively for 20 min, and was stopped by adding 0.5 ml 10% (v/v) trichloroacetic acid. After storing the solution at room temperature for 30 min, the precipitate was removed by centrifugation at 19,200 $\times g$ for 30 min at 4 °C, and the absorbance of the supernatant at 400 nm (A_{400}) was measured with a UV–Vis spectrophotometer (V-630, JASCO Corporation, Tokyo, Japan). Trypsin was used as a positive control. One unit was defined as the amount of enzyme required to increase the A_{400} by 1.0×10^{-3} per minute.

β-1,4- and β-1,3-glucanase activity assay The activity of β-1,4-glucanase and β-1,3-glucanase were measured as the rate of production of reducing sugars from carboxymethyl cellulose sodium (CMC-Na) and laminarin, respectively, as reported previously with slight modifications (25–28). For the blank reaction, reaction mixtures without substrate or enzyme solutions were used. The reactions were started by adding enzyme solution and incubating at 30 °C and pH 7.5, 45 °C and pH 8.4, and 53 °C and pH 9.0, respectively, for 30 min, and were stopped by adding 1 ml Somogyi's Copper reagent (Sigma Aldrich). The concentration of reducing sugars liberated from the substrates was determined using D(+)-glucose as the standard substance according to the Nelson-Somogyi method (29,30). One unit of enzyme was defined as the amount of enzyme required to produce 1 μM glucose equivalent per minute under the above conditions.

Lipase activity assay Lipase activity was measured using *p*-nitrophenylpalmitate (*p*-NPP, No. 356–23,991, Wako Pure Chemical Industries, Osaka, Japan) as the substrate, as reported previously with slight modifications (31,32). The reaction was initiated by adding enzyme solution to a mixture of substrate solution and buffer pre-incubated at 30 °C and pH 7.5, 45 °C and pH 8.4, and 53 °C and pH 9.0, respectively, for 1 min; the reaction mixture was incubated at the respective temperatures and pHs for 3 h with shaking at 140 rpm, and the reaction was stopped by adding 1 ml 0.25 M Na_2CO_3. The blank reaction was performed by adding buffer instead of enzyme solution. The concentration of *p*-nitrophenol (*p*-NP) liberated by the hydrolysis of *p*-NPP was determined by measuring the absorbance at 410 nm (A_{410}) of the mixture. The molar extinction coefficient of *p*-NP at 410 nm was 5.5×10^3 cm^2 mol^{-1}. One enzyme unit (U) was defined as the amount of enzyme required to release 1 μmol equivalent of *p*-NP per milliliter per minute under the assay conditions.

Incubation conditions and microcosm assay in lab-scale incubation with shaking In a lab-scale 500-ml Erlenmeyer flask, 50 ml of fresh samples were collected at the initial, middle, and final phases of ATAD and incubated with shaking at 140 rpm (maximum amplitude and speed: 4 cm and 160 rpm) in a water bath for 24–33 h at 30 °C, 45 °C, and 54 °C, respectively, as the representative temperatures of each phase in the full-scale bioreactor. Next, 4-ml samples were taken at different time intervals for analysis.

Spectrophotometric assay The OD_{610} of microcosm samples was measured using a spectrophotometer (V-630, JASCO) to monitor the cell concentration. Next, collected samples were centrifuged at 10,000 $\times g$ for 10 min at 4 °C, and the A_{280} and A_{260} were measured as indicators of protein and DNA contents, respectively (33,34).

Total/live and dead cell counts Live and dead cells in the microcosm samples were counted using the LIVE/DEAD BacLight Bacterial Viability Kit L-7012 (Molecular Probes, Eugene, OR, USA). First, 3 ml of fresh microcosm samples were pretreated by ultrasonication (UD-200, Tomy Seiko Co., Ltd, Tokyo, Japan) with an output power of 80 W for 30 s (every 10 s with a 1-min interval) in an ice bath, and then diluted in phosphate-buffered saline (PBS) (+) buffer containing 10 mM EDTA and 0.01% (v/v) Tween 80 at a dilution factor of 10^{-1}–10^{-4}-fold. The cells in diluted suspensions were stained with premixed dyes according to the manufacturer's instructions, and were observed and counted using an HS All-in-one fluorescence microscope (BZ-9000, Keyence, Osaka, Japan) at an excitation wavelength of 490 nm.

PCR and denaturing gradient gel electrophoresis Genomic DNA extraction from the microcosm samples was conducted using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA). This kit could effectively extract DNA from both gram-negative (*Proteobacteria*, *Bacteroidetes*, *Spirochaetes*) and gram-positive (*Firmicutes*) bacteria, including actinomycetes (*Actinobacteria*), from ATAD samples (15). A partial 16S rRNA gene (V3 region) was amplified from the extracted DNA with a universal primer set: 357f-GC (5′-CGCCCGCCGCGCGCGGCGGGCGGGGC-GGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′) and 518r (5′-ATTACCGCGGCTGC-TGG-3′) (35). The PCR reaction mixture was composed of 25 μl Premix Taq (EX Taq version 2.0, Takara Bio, Shiga, Japan), 20 pmol of each primer, 2.0 μl DNA

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