Short communication

Succession of bacterial community and enzymatic activities of activated sludge by heat-treatment for reduction of excess sludge

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Abstract

Heat-treatment has been used for the reduction of excess sludge as a simple process. To clarify the relationship between reduction efficiency and biological response of sludge matrix during heat-treatment, microbial population and hydrolytic enzyme (protease) activity of a municipal activated sludge were studied. Culture-dependent analysis showed the rapid increase in the population of thermophilic bacteria at the early stage of heat-treatment and the emergence of protease-secreting bacteria. Culture-independent analysis by denaturing gradient gel electrophoresis (DGGE) revealed that the \textit{Bacilli}, which include most of thermophiles, became the dominant class in the community by the treatment. The protease activity in supernatant of the sludge increased instantly after 1 h heat-treatment, which was considered to be released from microbial cells by lysis. The protease activity succession was correlated with the microbial succession and also with the change in MLSS and TOC concentrations during heat-treatment, suggesting that the protease activity plays an important role in the lysis-cryptic growth induced by heat-treatment.

Keywords: Sludge reduction; Lysis-cryptic growth; Bacterial community; Protease; DGGE

1. Introduction

The activated sludge process has long been employed to treat a wide variety of wastewater. However, its main by-product, excess sludge, is one of the drawbacks of the activated sludge process. The treatment of excess sludge may account for between 25 and 60\% of the total cost of wastewater treatment operation \cite{1}. For this reason, reduction of excess sludge in an economic, environmental and practical way is a rising challenge.

Heat-treatment is considered to be simple to operate compared with other treatments such as ozonation, chlorination, capable of being applied separately or being combined with other methods, such as alkaline or acid treatment \cite{2}, membrane methods \cite{3}. Heat-treatment combined with thermophilic protease treatment has already been applied to engineering processes in Japan \cite{4}. However, the biological response of the sludge matrix induced by heat-treatment was poorly understood.

The reduction of excess sludge by heat-treatment is induced by sludge lysis and further cryptic growth (lysis-cryptic growth) \cite{2}. In the lysis-cryptic growth, sludge reduction is achieved because some portions of lysates are consumed for the catabolism and finally emitted as CO\textsubscript{2}. Consequently, the microbial community succession in the sludge should occur during heat-treatment. With the development of molecular microbiological techniques, denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rDNA has been used as a useful tool to analyze the diversity of a microbial community \cite{5}. Furthermore, sequence information determined from the analysis of DNA in each band of DGGE can be used to estimate the bacterial species present in the community.

In sludge, the protein content is reportedly as high as 20–60\% (w/w) \cite{6}. Proteolytic cleavage of peptide bonds by protease is considered to be the main enzymatic reaction in the digestion or lysis of excess sludge \cite{7}. Therefore, protease activity in the sludge should be an important factor for the sludge reduction efficiency during heat-treatment.

In this study, in order to elucidate the biological response induced by heat-treatment, we investigated the
microbial population and protease activity successions during heat-treatment.

2. Materials and methods

2.1. Collection of samples and conditions of heat-treatment

Excess sludge was sampled from a municipal wastewater treatment plant and protein content of this sludge was 34.7% (Tokyo, Japan). Sludge of 700 ml was incubated at 60°C, 120 rpm for 24 h in a 1 l Erlenmeyer flask. The samples were taken periodically during heat-treatment: 0 h (before treatment), and 1, 3, 5, 10, 22 and 24 h. Mixed liquor suspended solids (MLSS) concentration was measured according to the standard method [8], and the total organic carbon (TOC) concentration was analyzed by a TOC analyzer (TOC-V, Shimadzu Co., Kyoto, Japan).

2.2. Culture conditions and restriction fragment length polymorphism (RFLP)

The sludge samples were plated on a modified R2A agar medium [9], containing of 1% (w/v) skim milk, which allows isolation of the protease-secreting bacteria, since it can form a clear zone on the plate. Plates were incubated at 28°C (48 h) or 60°C (24 h). PCR primers 27f and 1492r (Table 1) were used to amplify 16S rDNA from culturable bacteria. The amplicons were digested by MspI (Roche) and HhaI (TaKaRa) to estimate the origins of the amplicons from the electrophoresis patterns of the digested fragments [10].

2.3. DNA extraction and PCR-DGGE

DNA was extracted from sludge suspensions of 1 ml using a DNA extraction kit (ISOFECAL, Nippon Gene Co., Ltd., Tokyo, Japan). PCR was conducted with the primers of GC-341f and 907r (Table 1). DGGE was performed on a DCode universal mutation detection system (Bio-Rad Laboratories, Inc., CA). PCR-amplified mixture of 6 μl (20 ng/μl) was loaded onto 6% (w/v) polyacrylamide gels (37.5:1, acrylamide:bisacrylamide) in 1× Tris–acetate–EDTA (TAE) buffer with a gradient ranging from 30 to 65% denaturant (100% denaturant contains 7 M urea and 40% (v/v) formamide in 1× TAE). Electrophoresis was performed at 60°C for 12 h at 110 V.

2.4. Sequencing of the DGGE bands

Gel slices containing a DNA in the bands of DGGE were excised and 20 μl sterile water was added, and kept at −80°C for 30 min and then transferred to room temperature. Freeze and dissolution was repeated three times. PCR was performed using 3 μl mixtures of extracted DNA solutions as templates with the primers of 341f and 907r (Table 1). PCR amplicons were purified and ligated into the pGEM-T (Promega Co., WI). Inserted DNA fragments were amplified with primers pGEM-T seq+ and pGEM-T seq− (Table 1) and subjected to RFLP analysis, and then to the sequence analysis using the DNA Analysis System (CEQ8000, Beckman Coulter Inc., CA).

2.5. Data analysis and nucleotide sequence accession numbers

Nucleotide sequences were compared with sequences in the GenBank. CLUSTAL X 1.83 was employed for the phylogenetic analysis [11]. Reference sequences used in tree construction were obtained from the GenBank. The sequences obtained in this study were deposited in GenBank under accession no. EF636472 to EF636488.

2.6. Detection of protease activities

The protease activity of 1.5 ml sludge suspension or supernatant (collected by centrifugation at 3000 × g for 10 min) of sludge was measured according to Kim’s methods [7]. One unit of the enzyme activity was defined as the amount of the enzyme which degraded 1 mg azocasein in 60 min at 28 or 60°C.

3. Results and discussion

3.1. Lysis of the sludge during the heat-treatment process

The time-dependent variations of MLSS and TOC concentrations during the heat-treatment of the excess sludge were shown in Fig. 1. Before heat-treatment, the MLSS was 6200 mg/l. After 5 h treatment, it decreased to 4380 mg/l, reaching a reduction...
ratio of nearly 30%. Then, the MLSS gradually decreased to 3790 mg/l at 24 h, with a final reduction ratio of about 39.5%. The TOC increased rapidly to the maximum value (355 mg/l) after 3 h, and then decreased gradually to 146 mg/l at the end of treatment.

3.2. Population analysis by culture-dependent method

Culture-dependent methods cannot detect all the bacteria in wastewater [12]. However, it can be applied to analyze the viable, active bacteria or the bacteria with some special features [13]. In this study, population of thermoduric and thermophilic bacteria, and protease-secreting bacteria was examined. The colony forming unit (CFU) numbers of the culturable bacteria at the respective 28°C (mesophilic bacteria) and 60°C (thermophilic bacteria) are shown in Fig. 2(a). Before heat-treatment (0 h), the number of the mesophilic bacteria was 1000 times higher than that of the thermophilic bacteria. During the initial 1 h heat-treatment, CFU at 28°C decreased from $4.6 \times 10^6$ to $1.2 \times 10^5$, indicating that nearly 98% of the mesophilic bacteria died, leaving 2% of thermoduric bacteria. On the other hand, the numbers of thermophilic bacteria kept increasing during the initial 7 h. Decrease in the number of mesophilic bacteria and increase in the number of thermophilic bacteria at the initial 7 h suggested the occurrence of lysis-cryptic growth during the heat-treatment. After 7 h, the numbers of ther moduric and thermophilic bacteria stayed constant, indicating the limitation of sludge reduction by the heat-treatment.

Fig. 2(b) shows the ratio of protease-secreting bacteria over the culturable bacteria. After 1 h heat-treatment, most of the thermoduric bacteria secreted protease. RFLP of ten colonies of protease-secreting bacteria showed the same pattern (data not shown) and sequencing result showed this bacterium was *Bacillus subtilis*. At 60°C, the ratio of protease-secreting bac-

![Fig. 2. Changes in CFU (a) and ratio of protease-secreting bacteria (b) at mesophilic condition (28°C) and thermophilic condition (60°C) during heat-treatment.](image)

Table 2

<table>
<thead>
<tr>
<th>No.</th>
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<th>Sequence length (bases)</th>
<th>Phylogenetic relationship</th>
<th>Accession no.</th>
<th>% Similarity</th>
</tr>
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<td>88</td>
</tr>
<tr>
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</tr>
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<tr>
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</table>

*a* The same numbers are used in Fig. 3(a).

*b* The same numbers are used in Fig. 3(b).
Fig. 3. (a) DGGE fingerprints of sludge at different period during heat-treatment. Samples were taken before treatment and heat-treatment for 1, 3, 5, 7, 10, 22 and 24 h. Band (B) represented *B. subtilis* and band (G) represented *G. stearothermophilus* which were isolated from the excess sludge at 28 °C after 24 h and at 60 °C after 24 h, respectively. The numbers 1–18 indicated that the band was excised from the DGGE. (b) The phylogenetic relationship of the bacteria in the excess sludge during heat-treatment based on 16s rDNA sequences. The scale bar length of 0.1 denotes the number of amino acid replacements per site validated with 10,000 bootstraps.
teria was not constant during heat-treatment. RFLP of the protease-secreting thermophilic bacteria was the same (data not shown), and sequencing result showed that this bacterium was *Geobacillus stearothermophilus*. It was anticipated that protease-secreting ability of these two strains made them to utilize the ingredients in the sludge matrix and grow advantageously during heat-treatment.

### 3.3. Microbial succession analyzed by DGGE

The bacterial community of excess sludge during heat-treatment was analyzed by DGGE of the PCR-amplified 16S rDNA (Fig. 3(a)). The nucleotide sequences of 18 bands from the DGGE were determined and their closest phylogenetic affiliations were summarized in Table 2. Phylogenetic relation based on the sequence results was shown in Fig. 3 (b). Bands 1–3, bands 4–10 and bands 11–18 were sliced from the DGGE fingerprints of the 1, 7 and 24 h heat-treatment samples, respectively. Bands 14 and 18 in the community belonged to *B. subtilis* and *G. stearothermophilus*, respectively, which were also isolated by the cultivation method. The bands derived from the bacteria which belonged to other class than *Bacilli* became weak at late stage of heat-treatment (e.g. bands 2–3 from *Anaeroliceae*, bands 5–6 from *Sphingobacterium*). On the other hand, bands 11, 12, 14, 17 and 18 from the *Bacilli* became more discernable, especially band 7 denoted *Anoxybacillus flavithermus* (the same as band 12) and band 11 representing *Anoxybacillus voynovskii*. Although DGGE is not a quantitative method due to the bias introduced by PCR, the density change of each band can be explained as a consequence of a change in the relative abundance of the microbes in the microbial community [14]. After 24 h heat-treatment, the *Bacilli* became the dominant class in the treated sludge, which was consistent with the observation that thermophiles predominantly belong to the *Bacilli* [15]. Incidentally, several bands in DGGE did not change during heat-treatment, which might have resulted from not only living cells but also from dead cells. However, in this paper, the community alteration mainly according to the increase or decrease in the bands number during heat-treatment were focused, which must have resulted from the death or growth of the cells.

The sludge reduction efficiency was high in the first 5 h. On the other hand, the results of the DGGE analysis showed that after 5 h some bacteria such as *Bacilli* (bands 11, 12, 14, 17 and 18) apparently grew. TOC alternation after 3 h began to decrease, after 5 h some bacteria such as *B. subtilis* became the dominant class in the treated sludge, which was consistent with the observation that thermophiles predominantly belong to the *Bacilli* [15]. Incidentally, several bands in DGGE did not change during heat-treatment, which might have resulted from not only living cells but also from dead cells. However, in this paper, the community alteration mainly according to the increase or decrease in the bands number during heat-treatment were focused, which must have resulted from the death or growth of the cells.

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### 3.4. Protease activities

The alteration of protease activities through heat-treatment was shown in Fig. 4. The overall activity measured at 60 °C was significantly higher than that at 28 °C, suggesting that the proteases secreted into the sludge were thermophile proteases. The maximum value of the total activities in the sludge was attained before heat-treatment. Then, the total protease activities decreased gradually through the heat-treatment. The activity of the supernatant was nearly zero before the treatment. After 1 h treatment, however, the activity of the supernatant increased instantly, implying that protease was released from inside the cells by the cell-lysis. Usually protease contained inside the cells of sludge is considered to be important for the endogenous respiration of the sludge [16] and these intracellular proteases would not contribute to the sludge reduction. However, once they are released outside the cells, these proteases could be considered to contribute to the hydrolysis of proteins in the sludge, providing the substrates for the microbial cryptic growth.

### 4. Conclusions

In order to elucidate the biological response of the sludge matrix during heat-treatment, the succession of microbial population and protease activity in excess sludge were examined by PCR-DGGE, cultivation method and enzymatic activity measurement. At the beginning of heat-treatment, cell-lysis was induced by the treatment, leading to protease release from the cells, which could contribute to the sludge lysis. Then, protease-secreting bacteria emerged in the sludge, suggesting protease-secretion facilitated these microbes to utilize the lysates for growth. These observations revealed that the protease activity in the sludge play an important role for the lysis-cryptic growth induced by heat-treatment. It was also suggested that the protease emerged during the heat-treatment were the thermophilic protease. The *Bacilli* class bacteria which include most of thermophiles become dominant in the microbial population at the late stages, suggesting the limitation of heat-treatment.

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References