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

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DGGE

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Abstract

Heat treatment has been used for the reduction of excess sludge. To clarify the

characteristics of lysis process during the heat treatment, bacterial population and h
hydrolytic enzyme (protease) activity succession of a municipal activated sludge have
been studied. Culture-dependent method and culture-independent method by
denaturing gradient gel electrophoresis (DGGE) were applied to analyze the change in
bacterial population. The cultivation method showed the thermophilic
protease-secreting bacterium of *Bacillus subtilis* and thermophilic protease-secreting
bacterium of *Geobacillus stearothermophilus* were the dominant bacteria during the
heat treatment. While the PCR-DGGE fingerprint showed that some bands disappeared
and some bands increased in density during the heat treatment, which could be
pertaining to the lysis-cryptic growth of microbial community in the sludge. After the
heat treatment, the *Bacilli* became dominant class in the community. The protease
activity in the supernatant of the sludge before the heat treatment was nearly zero,
however after 1 h heat treatment, the activity increased apparently, and the alternation
in protease activity indicated that the lysis process was induced by the heat treatment.

1. Introduction

The activated sludge process has long been employed to treat a wide variety of wastewater, and over 90% of municipal wastewater treatment plants use it as the core part of the treatment process[1]. 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 [2]. For this reason, reduction of excess sludge in an economic, environmental and practical way is a rising challenge. Until now, four kinds of strategy have been developed: (1) lysis-cryptic growth, (2) uncoupling metabolism, (3) maintenance metabolism, and (4) predation on bacteria [1, 3, 4]. Among these techniques, the lysis-cryptic growth method is considered to be the most feasible in achieving effective reduction of excess sludge generation [5].

Lysis-cryptic growth of microorganisms in activated sludge can be depicted as cell lysis of the excess sludge and the subsequent microbial growth by utilizing the lysates as the carbon energy and nutrient sources [1, 6]. The lysis-cryptic growth method can thus be divided into two stages, cell lysis and biodegradation of the lysates (cryptic growth) [7]. Since the rate-limiting step is the lysis step [4], the main objective of excess sludge reduction is how to increase the sludge lysis efficiency. Physical and

chemical approaches have been used to accelerate the cell lysis, such as heat treatment or comparing with thermophilic bacteria secreting thermophilic protease to solubilization of excess sludge [7-9], bead-mill disruption [10], chemical treatment such as acid, alkali, ozone and chlorine [1], or treatment by thermal alkaline, acid or the combination [11]. Among these methods, heat treatment is considered to be a relatively simple method, capable of being applied separately or being combined with alkaline or acid treatment [4]. Heat treatment combined with thermophilic protease has already been applied in engineering process in Japan [8]. In a sludge lysis by the heat treatment, succession of the bacterial community can indicate the behavior of each bacterium in the excess sludge, which will affect the cell lysis and biodegradation efficiencies. However, so far, the relation between the bacterial community succession and features of the heat treatment process has not been investigated. 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 [12]. 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 excess sludge, the protein content percentage is reportedly as high as 20-60%

(w/w) [13]. Proteolytic cleavage of peptide bonds by protease is considered to be the main enzymatic reaction in the digestion or lysis of excess sludge [14]. Thus, during the heat treatment, the enzymatic activity of protease in the excess sludge can be considered to be an important factor for understanding the sludge lysis process. Since there are a number of culturable bacteria with the ability of secreting protease [14, 15] during the heat treatment, variation in the protease-secreting bacterial population in a mixed community also needs to be monitored.

In this study, in order to make full use of the physicochemical method for excess sludge reduction in a wastewater treatment plant, we investigated the bacterial population and protease activity succession during the heat treatment process.

2, Materials and methods

2.1 Collection of samples and conditions of heat treatment

Excess sludge were sampled from a municipal wastewater treatment plant (Tokyo, Japan). 700 mL of the sludge were transferred to a 1L Erlenmeyer flask and incubated at 60°C, 120rpm for 24 hours in a reciprocal shaker. The samples were obtained at different time periods after the heat treatment: 0h (before treatment), and 1 h, 3 h, 5 h, 10 h, 22 h and 24 h . Mixed liquor suspended solids (MLSS) concentration was measured according to the standard methods [16], 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 [17], consisting of 1% (wt/vol) skim milk, and the plates were incubated at 28°C (48h) or 60°C (24h). PCR primers, 27f and 1492r (Table 1), were used to amplify the 16S rDNA from the culturable bacteria isolated from the sludge at different heat treatment periods. The amplicons were digested by *Msp*I (Roche) and *Hha*I (TaKaRa) in RFLP.

2.3 DNA extraction and PCR-DGGE

DNA was extracted from 1mL of mixed liquor obtained from the sludge samples using a DNA extraction kit (ISOFEAL, Nippon Gene Co., Ltd, Tokyo, Japan). PCR primers of GC-341f and 907r (Table 1) were used [18]. A modified form of the touchdown thermal profile technique [12] was used. DGGE was performed on a DCode universal mutation detection system (Bio-Rad Laboratories, Inc., CA). 6μL ~8μL (20ng/ul) of a PCR-amplified mixture was loaded onto 6% (wt/vol) polyacrylamide gels (37.5:1, acrylamide:bisacrylamide) in 1×Tris-acetate-EDTA (TAE) buffer [19] with a denaturing gradient ranging from 30 to 65% denaturant (100% denaturant contains 7M urea and 40%(vol/vol) formamide in 1×TAE).

Electrophoresis was performed at 60°C for 12 h at 110 V. The gel was stained with SYBR Gold ((Molecular ProbesTM, Invitrogen Co., CA), visualized on an UV transilluminator table, and photographed.

2.4 Sequencing of the DGGE bands

Gel slices containing a DNA band were excised and transferred into a sterile 1.5mL-micro tube and 20μL sterile water was added. The target DNA bands were extracted from the DGGE gel by a freeze-thaw method. The tube was kept at -80°C for 30 min, and then transferred to room temperature to help the mixture become liquid form. The same operation was carried out three times, and then the 3μL mixtures were used as the PCR templates. A PCR under the same condition as the pre-DGGE PCR was performed using polymerase (Ex TaqTM, TaKaRa Bio Inc., Otsu, Japan), except the primers were changed to 341f and 907r (Table 1). PCR amplicons were purified and ligated into the pGEM-T cloning vector (Promega Co., WI). Nearly 30 transformants were used as template, primers pGEM-T seq+ and pGEM-T seq- (Table 1) were used to amplify the DNA, and the amplicons were subjected to RFLP. Nucleotide sequences were determined using the DNA Analysis System (CEQ8000, Beckman Coulter Inc., CA), and were determined at least twice.

2.5 Data analysis

Nucleotide sequences were compared with sequences in the GenBank database [20] using the blastn program [21] and the SEQUENCE_MATCH program on the Ribosomal Database Project II (RDP) database [22]. Phylogenetic analysis employed CLUSTAL X 1.83 software [23]. Reference nucleotide sequences used in tree construction were obtained from the GenBank database.

2.6 Nucleotide sequence accession numbers

The sequences obtained in this study have been deposited in GenBank under accession no. EF636472 to EF636488.

2.7 Detection of protease activities

1.5 mL of the sludge suspension or 1.5 mL of the supernatant (collected by centrifugation at 900×g for 10 min) of sludge was added to a tube containing 0.5 mL of 0.5% (wt/vol) azocasein solution and incubated at 60°C and 28°C, respectively, in a shaking incubator. After 90 min, 1 mL 10% (wt/vol) trichloroacetic acid (TCA) solution was added to stop the proteolysis. The supernatant was collected by centrifugation at 900×g for 15 min, from which 1 mL was added to 1 mL 2 M NaOH and then the absorbance of the mixture was measured at 440 nm. One unit of the enzyme activity was defined as the amount of the enzyme which could degrade 1 mg azocasein in 60 min at 28°C 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 process of the activated sludge are shown in Fig.1. In the initial stage of the heat treatment (the first 5 hours), the MLSS decreased rapidly. Prior to heat treatment, the MLSS was 6200 mg/l, but after 5 hours it was decreased to 4380 mg/l, reaching a reduction ratio of nearly 30%. After 24 hours the MLSS gradually decreased to 3790 mg/l with a final reduction ratio of about 39.5%. While the TOC reached the maximum value (355 mg/l) after the heat treatment for 3h, and then decreased gradually.

3.2 Culturable bacteria and protease-secreting bacteria

The culturable bacteria population variation during the heat treatment was examined using modified R2A agar medium containing 1% (wt/vol) skim milk that could isolate the protease-secreting bacteria, since the protease-secreting bacteria can form a clear zone on the plate. The colony forming unit (CFU) numbers of the culturable bacteria at the respective 28°C and 60°C are shown in Fig.2 (a). The CFU at 28°C and 60°C represent the respective mesophilic and thermophilic bacteria. The figure showed that before heat treatment (0h), the CFU of the mesophilic bacteria was 1000 times higher than that of the thermophilic bacteria, while after 1 h heat treatment, nearly 99% of the

mesophilic bacteria died; the remaining 1% was thermophilic bacteria. On the contrary, the numbers of thermophilic bacteria increased during the heat treatment. From 1 to 24 h after the heat treatment, the number of the thermophilic bacteria became constant, while there was a remarkable increase in the number of thermophilic bacteria, especially in the first 7 h.

Fig.2 (b) indicates the ratio of protease-secreting bacteria over the culturable bacteria. At 28°C, numerous kinds of mesophilic bacteria were isolated from the initial sludge, whereas after 1 h heat treatment, most of the thermophilic bacteria were capable of secreting protease. RFLPs of ten protease-secreting bacteria showed the same pattern (data not shown) and the sequencing result showed this bacterium was *Bacillus subtilis*. At 60°C, the ratio of protease-secreting bacteria kept changing during the heat treatment process. RFLP confirmed that the pattern of the protease-secreting bacteria was the same (data not shown), and the number of protease-secreting bacteria increased 10000 times compared with the non-treatment sludge (Fig.2 (a)). The sequencing result showed that this bacterium was *Geobacillus stearothermophilus*.

3.3 Microbial succession analyzed by DGGE

The bacterial community structures of excess sludge during the heat treatment were analyzed by DGGE of the PCR-amplified 16S rDNA (Fig.3 (a)). From the fingerprint,

it could be seen that the bacterial community experienced sequential shift as the MLSS decreased during the heat treatment. The nucleotide sequences of 18 bands from the DGGE gel were determined and their closest phylogenetic affiliations were summarized in Table 2. Bands 1-3, bands 4-10 and bands 11-18 were sliced from the DGGE fingerprints after the heat treatment for 1, 7 and 24 h, respectively. With the BLAST and SEQUENCE_MATCH algorithms, the majority of the 16S rDNA sequences showed >93% similarity to the reference strains found in the GenBank except for band 2 (88%). Bands 7 and 12 were at the same position, indicating that they were the same bacteria. Bands 14 and 18 in the community belonged to *B. subtilis* and *G. stearothermophilus*, respectively, which were also isolated by the cultivation method as mentioned above. Fig.3 (b) shows the phylogenetic relationships of the bacteria which were sequenced. During the heat treatment, bacterial community changed from *Bacilli*, *Betaproteobacteria*, to *Sphingobacteria* classes; in particular, after 24 h treatment, the majority class was *Bacilli*. The bands excised from the 7-h lane indicated that the bacteria of *Deltaproteobacteria* and *Actinobacteria* classes in the sludge disappeared after the heat treatment for 24 h. Bands 2 and 3, representing the bacteria of *Anaerolineae* class disappeared in the later stage of the treatment. The DGGE fingerprint (Fig.3 (a)) indicated the successive alteration in the bacterial

population probably due to the cryptic growth process. Heat treatment increased the cell lysis, thus bacteria which could not survive at the high temperature would die. On the contrary, some thermophilic bacteria could use the sludge lysates to grow [6], which would have led to the alteration in community structure. Phylogenetic analysis based on the sequence results gave detailed information about which kinds of bacteria were viable, and which bacteria disappeared (Fig.3 (b)). In the DGGE fingerprint, bands 4-9 from the 7-h lane became more discernable, especially band 7 (the same as band 12) which denoted *Anoxybacillus flavithermus*, belonging to the anaerobic bacteria [24]. The density of band 11 excised from the 24-h lane apparently increased from the 10-h treatment. This band represented *Anoxybacillus voinovskiensis*, belonging to the aerobic bacteria [25]. Although DGGE is not a quantitative method due to the bias introduced by the PCR, the density change can be explained as a consequence of a change in the relative abundance of the microbes in the microbial community [26, 27]. After 24 h heat treatment at 60°C, the *Bacilli* became the dominant class in the activated sludge. It has been observed that thermophiles predominantly belong to the *Bacilli* [28], which can adapt to the high temperature environments. The results of the cultivation method depicted that 99% of active mesophilic bacteria lost their bioactivity after 1 h heat treatment, and *B. subtilis* and *G. stearothermophilus* became

the major bacteria in the mixed community. However, from the DGGE fingerprint these two bacteria were not dominant in the population. The difference between the culture-dependent and culture-independent methods was consistent with the well-known observation that the culture-dependent methods can only isolate the bacteria less than 15% of all viable cells in wastewater treatment process [29]. In addition, the bacterial cultivation in nutrient-rich medium may also change the community structure of activated sludge [30]. Although the culture-dependent method has this drawback, it can be applied to analyze the viable, active bacteria [6] or the bacteria with some special features in the environment [31]. During the heat treatment process, the protease-secreting bacteria of *B. subtilis* and *G. stearothermophilus*, which are Gram-positive bacteria and have the ability to form tough, protective endospores, were isolated by the cultivation method. Furthermore, the secretion of protease has also been observed during the disruption of excess sludge by bead-mill method [10].

3.4 Protease activities

The alteration of the protease activities through heat treatment was shown in Fig.4. The maximum values of the total activities in the sludge appeared before the heat treatment at the respective 28°C and 60°C. While after the treatment, the total protease activities decreased gradually. The activity of the supernatant was nearly zero before

the heat treatment, however after 1 h treatment, the activity of the supernatant apparently increased at both 28°C and 60°C. The overall activity measured at 60°C was significantly higher than that at 28°C. Since protein accounts for a relatively large portion of excess sludge, the protein digestion process in the lysis-cryptic process induced by the heat treatment needs to be studied. The results indicated that the protein hydrolysis had played an important role in the reduction of excess sludge during heat treatment. Before heat treatment, protease activities were detected in the sludge, rather than in supernatant. Usually protease contained inside the cells of sludge is considered to be important to the endogenous respiration of the sludge [10]; however these intracellular proteases may not be used for the sludge reduction. After 1 h heat treatment, the protease activities in supernatant of the sludge increased rapidly, which pertained to the cell-lysis process. Proteases released from the inside cells were considered to be able to accelerate the hydrolysis of proteins in the excess sludge, which could provide the substrates for the microbial cryptic growth and lead to the reduction of excess sludge.

Nearly 30% of the MLSS was reduced in the first 5 h of heat treatment. On the other hand, the results of the bacterial population succession showed that after 5 h treatment some bacteria such as *Bacilli* apparently grew. TOC alternation indicated that soluble

organic carbons increased at the initial stage of the cell-lysis. After 3 h, however, the TOC began to decrease, which could be caused by the cryptic growth of thermophilic bacteria in the sludge by using the lysates as the substrates. After 5 h, the cell-lysis might have become rate-limiting step, because the thermophilic *Bacilli* changed into the major populations in the sludge. Protease activities at 60°C were higher than those at 28°C, showing that the proteases in this excess sludge included thermophilic protease(s), which caused the protein to be hydrolyzed more quickly at the high temperature.

4. Conclusions

In order to understand the mechanism of heat treatment process for reduction of excess sludge, the succession of bacterial population and protease activity in a municipal activated sludge was studied by using different analytical methods. The results indicated that at the beginning of the heat treatment, cell lysis was accelerated, leading to the protease release from the cells, then the soluble protease helped to degrade the excess sludge, resulting the quick decrease in the MLSS; the sludge lysates were then used as the substrates for the cryptic growth, and the thermophilic bacteria such as the *Bacilli* class become the dominant species in the microbial population during the heat treatment. However, after the heat treatment for 5 h, because the

dominant thermophilic bacteria could not be lysed, the extent of reduction in MLSS decreased. From this study, it can be concluded that the time control for the heat treatment is needed to optimize the reduction efficiency of excess sludge.

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Table 1 Oligonucleotide primers used for PCR

Primer	Sequence(5'→3')
27f	AGAGTTTGATCCTGGCTCAG
1492r	GGTTACCTTGTTACGACTT
GC-341f	(GC clamp) *-CCTACGGGAGGCAGCAG
907r	CCGTCAATTCCTTT[A/G]AGTTT
pGEM-T seq+	ATTGGGCCCCGACGTCGCATG
pGEM-T seq-	GGAGCTCTCCCATATGGTCG

*GC-clamp: CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG

Table 2 Sequence length and closest phylogenetic affiliation of the bacteria in the activated sludge after heat treatment for different time

No. ^a	Clone no. ^b	Sequence length (bases)	Phylogenetic relationship		
			Closet relatives	Accession no.	% Similarity
1	1h-(1)	593	<i>Propionibacterium sp.</i>	EF636472	98
2	1h-(2)	592	<i>Uncultured antarctic bacterium</i>	EF636473	88
3	1h-(3)	581	<i>Uncultured anaerolineae bacterium</i>	EF636474	97
4	7h-(4)	610	<i>Uncultured bacterium</i>	EF636475	93
5	7h-(5)	608	<i>Uncultured sphingobacterium</i>	EF636476	93
6	7h-(6)	593	<i>Uncultured sphingobacterium</i>	EF636477	99
7	7h-(7)	577	<i>Anoxybacillus flavithermus</i>	EF636478	99
8	7h-(8)	589	<i>Uncultured betaproteobacterium</i>	EF636479	95
9	7h-(9)	608	<i>Uncultured deltaproteobacterium</i>	EF636480	94
10	7h-(10)	596	<i>Uncultured actinobacteria bacterium</i>	EF636481	97
11	24h-(11)	612	<i>Anoxybacillus voinovskiensis</i>	EF636482	99
12	24h-(12)	610	<i>Anoxybacillus flavithermus</i>	EF636478	99
13	24h-(13)	611	<i>Uncultured bacilli</i>	EF636483	98
14	24h-(14)	542	<i>Bacillus subtilis</i>	EF636484	98
15	24h-(15)	607	<i>Uncultured betaproteobacteria bacterium</i>	EF636485	96
16	24h-(16)	615	<i>Uncultured betaproteobacteria bacterium</i>	EF636486	95
17	24h(17)	624	<i>Anoxybacillus amylolyticus</i>	EF636487	96
18	24h-(18)	602	<i>Geobacillus stearothermophilus</i>	EF636488	99

^a The same numbers in the Fig.3(a)

^b The same numbers in the Fig.3(b)

Figure Captions

Fig.1. Changes in MLSS and TOC of the activated sludge during the heat treatment process. The experiments were performed in triplicate and the data were expressed by Mean \pm SD.

Fig.2. Changes in CFU (a) and ratio of protease secreting bacteria (b) at mesophilic condition (28°C) and thermophilic condition (60°C) during the heat treatment. All experiments were performed in triplicate and the data were expressed by Mean \pm SD.

Fig.3. (a) DGGE fingerprints of excess sludge at different time during the heat treatment process. Samples were taken before treatment and after heat treatment for 1, 3, 5, 7, 10, 22 and 24h. Band (B) represented *Bacillus subtilis*, which was isolated from the excess sludge at 28°C after 24h, and band (G) represented *Geobacillus stearothermophilus*, which was isolated from the excess sludge at 60°C after 24h. The numbers 1-18 indicated that the band was excised from the DGGE gel. (b) The phylogenetic relationship of the bacteria in the excess sludge during the heat treatment process based on 16s rDNA sequences. The scale bar length of 0.1 denotes the number of amino acid replacements per site validated with 10000 bootstraps.

Fig.4. Changes in enzymatic activities of protease of the excess sludge during the heat treatment process. All experiments were performed in triplicate and the data were expressed by Mean \pm SD.

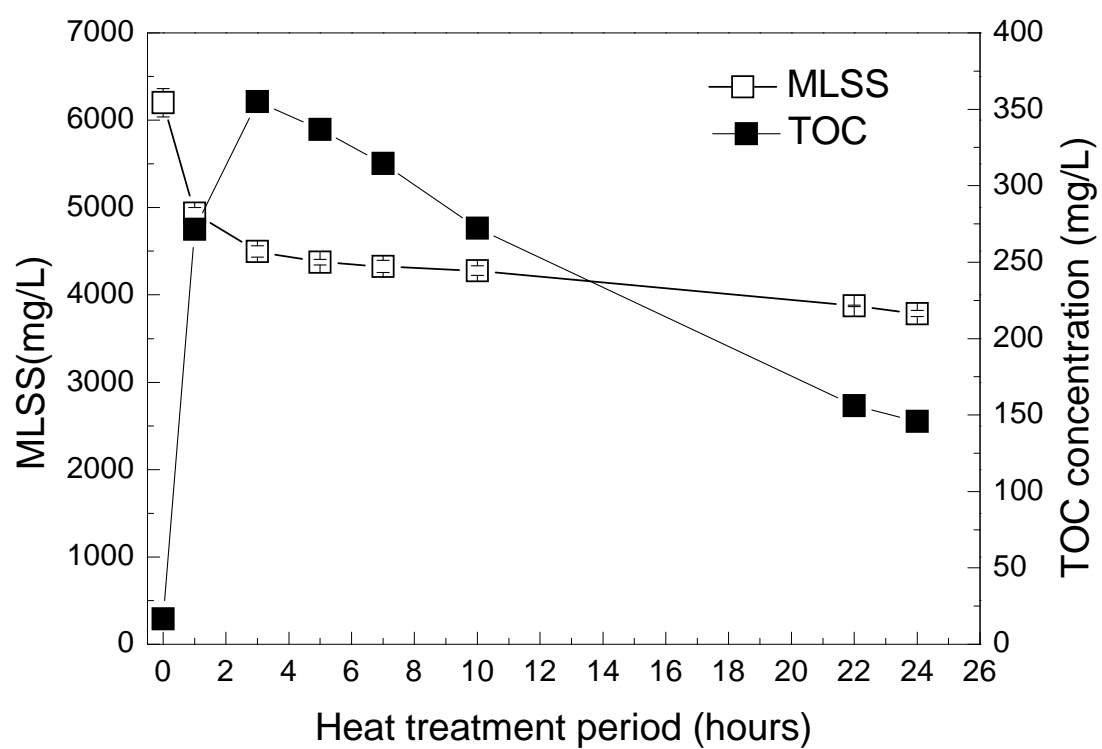


Fig.1 Yan *et al.*

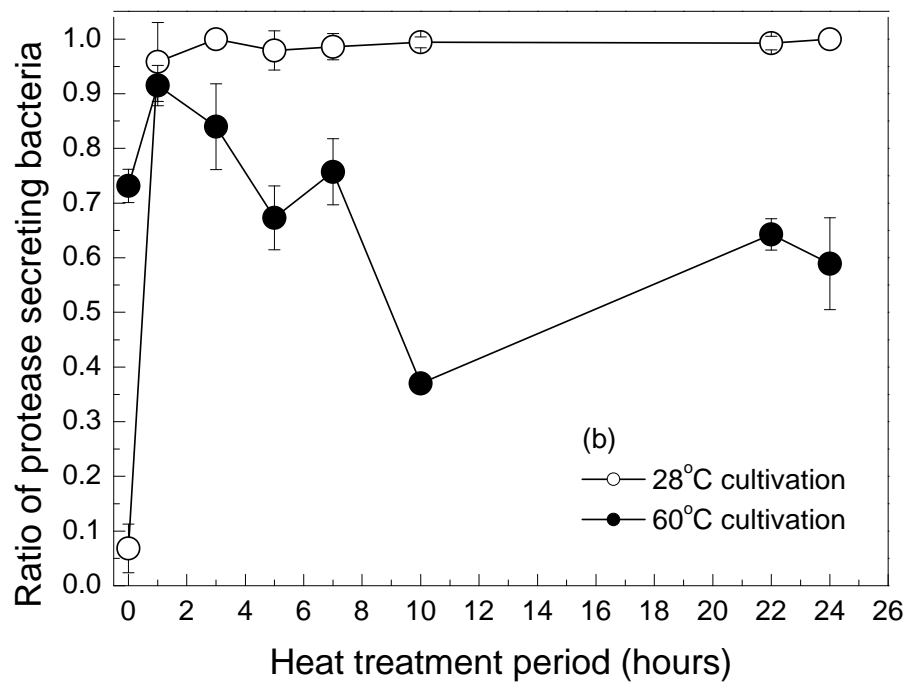
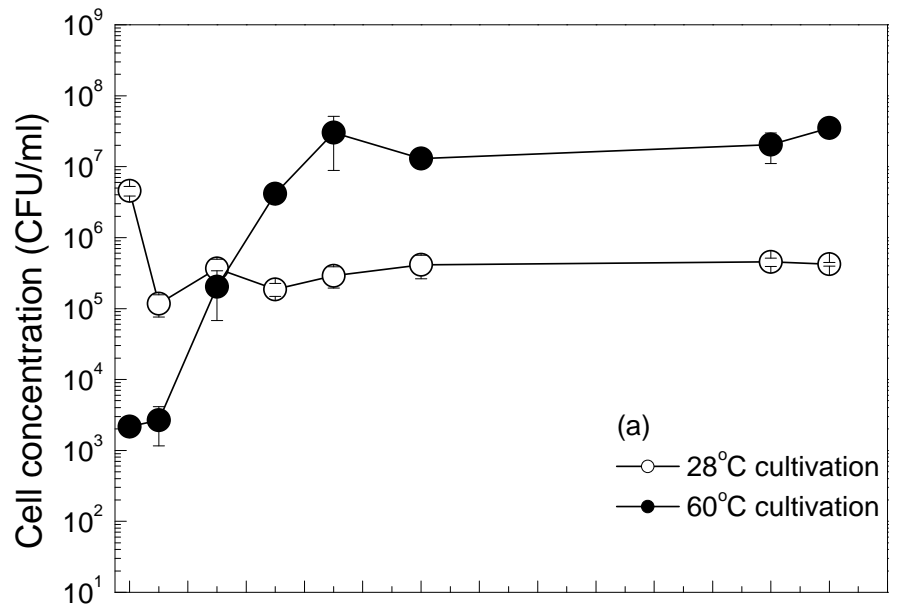
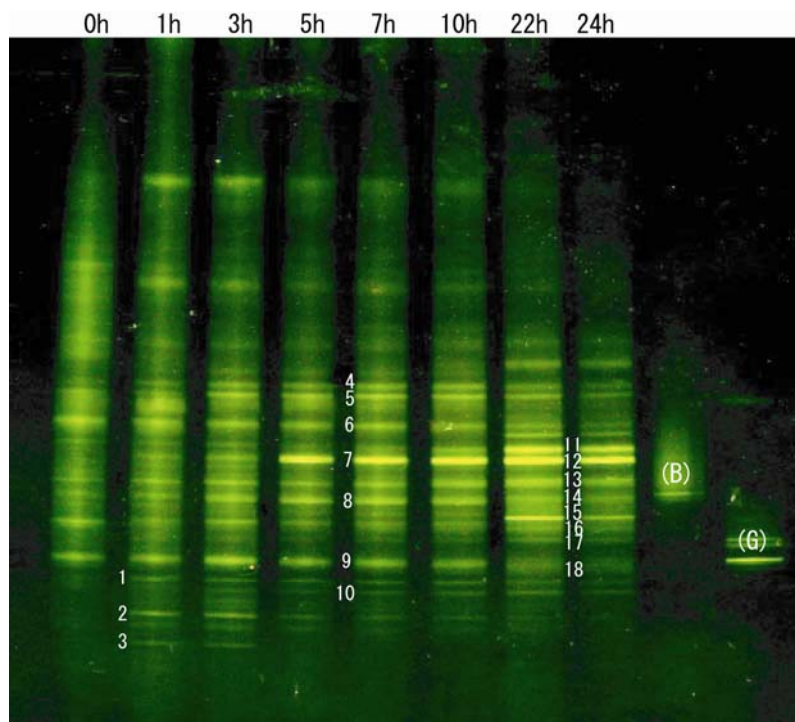
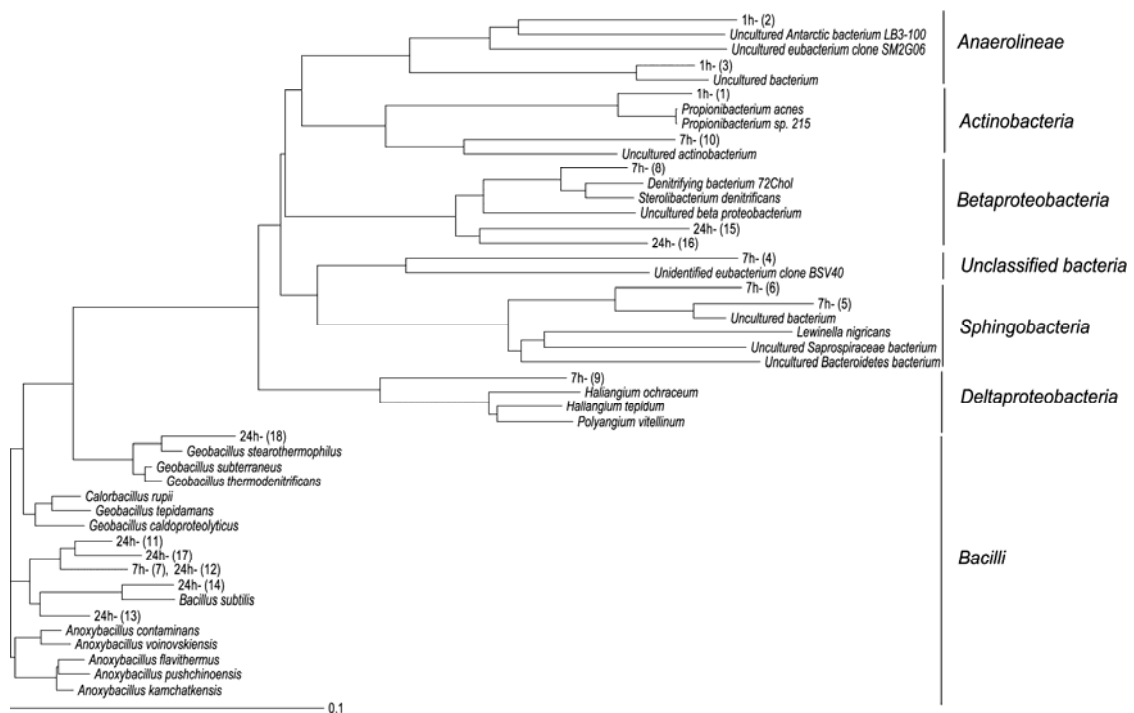


Fig.2 Yan *et al.*



(a)



(b)

Fig.3 Yan *et al.*

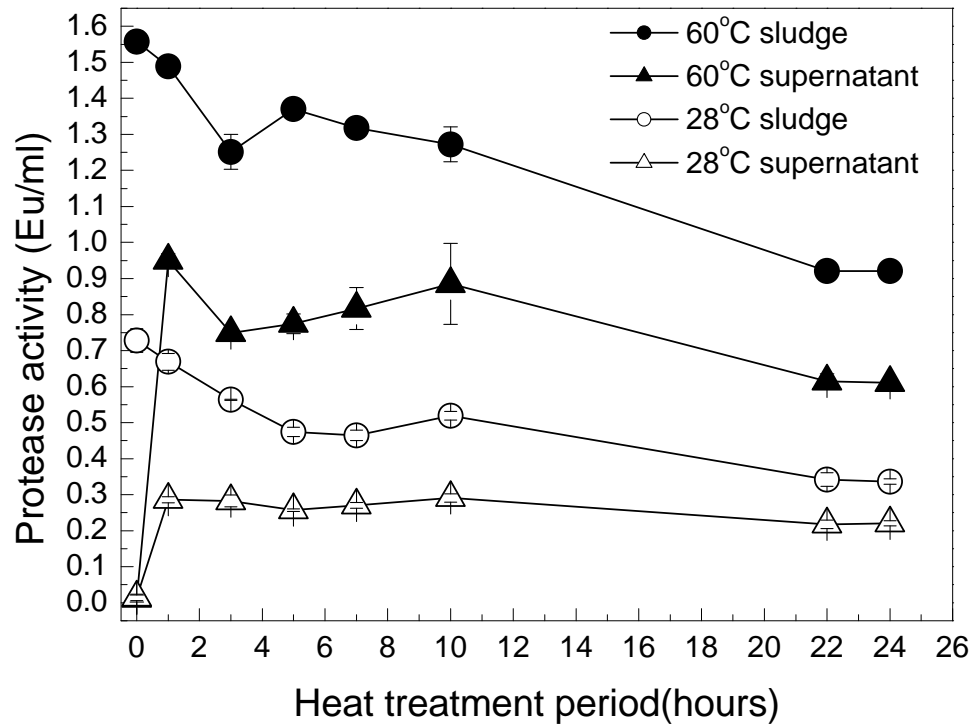


Fig.4 Yan *et al.*