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Evaluation of Feasibility of Using the Bacteriophage T4 Lysozyme to Improve the Hydrolysis and Biochemical Methane Potential of Secondary Sludge

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Abstract: Anaerobic digestion (AD) of secondary sludge is a rate-limiting step due to the bacterial cell wall. In this study, experiments were performed to characterize secondary sludges from three wastewater treatment plants (WWTPs), and to investigate the feasibility of using bacteriophage lysozymes to speed up AD by accelerating the degradation of bacterial cell walls. Protein was the main organic material (67.7% of volatile solids in the sludge). The bacteriophage T4 lysozyme (T4L) was tested for hydrolysis and biochemical methane potential. Variations in the volatile suspended solid (VSS) concentration and biogas production were monitored. The VSS reduction efficiencies by hydrolysis using T4L for 72 h increased and ranged from 17.8% to 26.4%. Biogas production using T4L treated sludges increased and biogas production was increased by as much as 82.4%. Biogas production rate also increased, and the average reaction rate coefficient of first-order kinetics was 0.56 ± 0.02/d, which was up to 47.5% higher compared to the untreated samples at the maximum. *Alphaproteobacteria*, *Betaproteobacteria*, *Flavobacteriia*, *Gammaproteobacteria*, and *Sphingobacteriia* were major microbial classes in all sludges. The interpretation of the microbial community structure indicated that T4L treatment is likely to increase the rate of cell wall digestion.

Keywords: sludge hydrolysis; biochemical methane potential (BMP); bacterial community

1. Introduction

Anaerobic digestion (AD) is one of several process (e.g., incineration, landfill, composting, and anaerobic digestion) that have been used to degrade sludge that remains after the treatment of domestic wastewater [1]. AD degrades volatile solid (VS), decreases the chemical oxygen demand (COD), and produces biogas (primarily methane). The process leaves little residual sludge production, despite a high organic loading rate, and requires little energy [2,3]. Besides, AD can produce biogas as a fuel and a small amount of sludge residue [4]. AD is a four-stage process in which the complex organic components of the waste are solubilized, broken down, and fermented into intermediate products that are subsequently reduced to methane and carbon dioxide [5]. The stages are referred

to as hydrolysis or liquefaction, acidogenesis, and methanogenesis, respectively [6]. An AD process involves a syntrophic consortium of bacteria and archaea: organic acid-producing (acidogens) and methane-forming (methanogens) substances [7]. Acidogenic bacteria convert crude organics to volatile fatty acids and hydrogens, and methanogenic archaea then reduce these products to methane and carbon dioxide [8,9]. If the balance between two bacterial groups is broken, the AD process can become unstable, so VS/COD removal or biogas production declines [10]. Beside the major advantage of using biogas as sources of electricity and heat, the AD process also generates a low volume of sludge, despite a high organic loading rate (OLR) [11].

Secondary sludge particles are essentially concentrated aerobic microbial cells, so anaerobic bacteria must release exo-enzymes that solubilize microbial cell walls, which are composed of peptidoglycan, protein, and lipid bilayers [12]. Degradation of these components is difficult, so the conventional AD process has difficulty digesting more than 35% of the organics in secondary sludge [13]. Therefore, the biological hydrolysis of cells is considered to be a rate-limiting step in most AD of secondary sludge [14].

Various physico-chemical (thermal, ultrasonic, acidic, and alkaline) and biological pretreatment methods (enzymatic and biosurfactants) have been suggested to increase the hydrolysis efficiency and biogas yields in the anaerobic digestion of secondary sludge [15–17]. Although the thermochemical pretreatment of sludge results in an increased biodegradability of up to 70%, the process consumes a substantial amount of energy and exhibits a high level of chemical consumption [16]. Although thermal pretreatment can effectively hydrolyze the cell wall structure and improve the dewaterability of the secondary sludge, it produces harmful contaminants and does not increase the residual degradability [18,19]. Ultrasound technology breaks up the bacterial cell floc matrix and accelerates the degradation of sludge, but it only provides minimal solubilization [20,21]. Acid chemical treatment can be used for hemicellulose removal or cellulose solubilization [22]. Alkaline chemical pretreatments, such as the use of sodium hydroxide, have also been tried, but frequently create aggressive reaction conditions [23]. These physico-chemical methods, however, usually require a high energy and corrosive pretreatment equipment, result in an inhibiting effect, and need steps for purification from chemicals [24,25].

Enzymatic pretreatment is an ecologically benign method used to accelerate the hydrolysis of secondary sludge [26]. Lysozymes hydrolyze the β-(1,4)-glycosidic bonds between N-acetyl-D-glucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) of peptidoglycan of the cell wall [27,28]. Various living organisms (e.g., animals, plants, phages, bacteria, and viruses) excrete lysozymes as a defense mechanism. Lysozymes can be divided into six groups: goose, chicken, invertebrate, plant, bacterial, and bacteriophage lysozymes [29]. Bacteriophage lysozymes are categorized into three groups: glycosidase (i.e., bacteriophage T4 lysozyme), transglycosidase (i.e., bacteriophage lambda lysozyme), and amidase (i.e., bacteriophage T7 lysozyme) [30]. Both glycosidase and transglycosidase break β-(1,4)-glycosidic bonds of peptidoglycan, while transglycosidase conserves the bond [31,32]. Amidase hydrolyzes the bond between MurNAc and tetrapeptide [33]. Despite the potential advantages of using bacteriophage lysozymes to treat secondary sludge, the strategy has not been evaluated, possibly because of the difficulty in extracting lysozyme from bacteriophages.

In this study, we introduce a novel technique to pretreat sludge by using lysozyme that originates from bacteriophages. The main objective was to investigate the feasibility of using bacteriophage lysozymes to pretreat secondary sludge. The specific objectives were to evaluate (1) the effect on the hydrolysis of secondary sludge after bacteriophage lysozyme treatment, and (2) the variations in the biogas yield and reaction rates during biochemical methane potential (BMP) tests using different secondary sludges with and without the bacteriophage lysozyme.

2. Materials and Methods

2.1. Expression and Purification of Bacteriophage Lysozymes

The bacteriophage lysozyme recombinant gene was used because it is more easily combined with a plasmid than other lysozyme genes [34]. *Escherichia coli* strain DH5α was used for the construction and propagation of plasmids that express recombinant bacteriophage lysozymes. Lysogeny broth (LB), super optimal broth with catabolite repression (SOC), and LB solid medium containing 15 g/L agar were used for the cloning and cultivation of *E. coli* cells. RM medium was used for the overexpression of T4 lysozymes to reduce their leaky expression by the P_{BAD} promoter.

We used six plasmids and developed six oligonucleotide sequences (Table 1). To express the bacteriophage T4 lysozymes in *E. coli*, the pBAD expression system (Invitrogen) was used; hexa-histidine (His_6) was tagged to the N-terminus of T4. To construct the plasmids that express T4, the 492-bp (GenBank accession number: KJ477684) was retrieved. The His_6 tag sequence was added to each DNA sequence and these designed DNA sequences including the His_6 tag were synthesized by Macrogen (Seoul, Republic of Korea). To clone the synthesized T4 gene into the pBAD/Myc-His/lacZ plasmid (Invitrogen), the pBAD/Myc-His/lacZ plasmid was digested with NcoI and HindIII restriction enzymes. The PCR-amplified T4 gene was assembled with a digested pBAD/Myc-His/lacZ plasmid into the pBAD-T4L plasmid, using the Gibson Assembly Master Mix (New England Biolabs).

Table 1. Plasmids and oligonucleotide sequences used in this study.

Plasmids	**Description**	**Sources**
pBAD/Myc-His/lacZ	Expression vector, P_{BAD}:*lacZ*, pBR322 ori, Amp^R	Invitrogen
pBAD24	Expression vector, P_{BAD}, pBR322 ori, Amp^R	Invitrogen
pBAD-T7L	Plasmid expressing T7 lysozyme with C-terminal 6xHis tag, Amp^R	This study
pBAD-T4L, LaL	Plasmid expressing T4, λ lysozyme with N-terminal 6xHis tag, Amp^R	This study
pLysS	Plasmid containing T7 lysozyme gene	Novagen
pSyn-T4L, LaL	Plasmid containing synthesized T4, λ lysozyme gene	This study
Oligonucleotides	**5′ to 3′**	**Reference**
T4L-F	gctaacaggaggaattaacc	This study
T4L-R	tgagtttttgttcgggccca	This study
LaL-F	gctaacaggaggaattaacc	This study
LaL-R	tgagtttttgttcgggccca	This study
T7L-F	ggctaacaggaggaattaaccatggctcgtgtacagtttaa	This study
T7L-R	tgagtttttgttcgggcccaagctttccacggtcagaagtgacca	This study

To express and purify the recombinant bacteriophage lysozyme, SDS-PAGE and immobilized metal affinity chromatography (IMAC) using a Profinia^{TM} system (Bio-Rad, Hercules, CA) were performed. *E. coli* LMG194 were introduced to RM medium with 100 μg/mL ampicillin, and then cultured at 37 °C for 16 h at 200 rpm. Cultured *E. coli* were diluted with RM medium and grown at 37 °C until the optical density (OD) at the wavelength λ = 600 nm was 0.4–0.5. The expression of the recombinant bacteriophage lysozyme was induced by adding 0.2 g/L of L-arabinose, and then incubating it at 37 °C for 4 h. Protein concentrations were quantified by measuring the OD at λ = 595 nm.

The activity of lysozyme was measured using the EnzChek® Lysozyme Assay Kit (Invitrogen). Purified lysozyme was reacted at 37 °C for 30 min in darkness with *Micrococcus lysodeikticus* labeled with fluorescein. The specific lysozyme activity (1 unit) was defined as the amount of lysozyme required to hydrolyze 1 μg of *M. lysodeikticus* cell walls per concentration of lysozyme (mg). Three bacteriophage lysozymes were tested (i.e., T4, lambda, and T7) (Figure 1). The bacteriophage T4 lysozyme (T4L) showed the highest specific activity of 662.2 ± 18.5 U/mg, vs. 95.1 ± 6.5 for the lambda lysozyme and 294.1 ± 39.8 for the T7 lysozyme. Therefore, T4L was chosen for use in the trials.

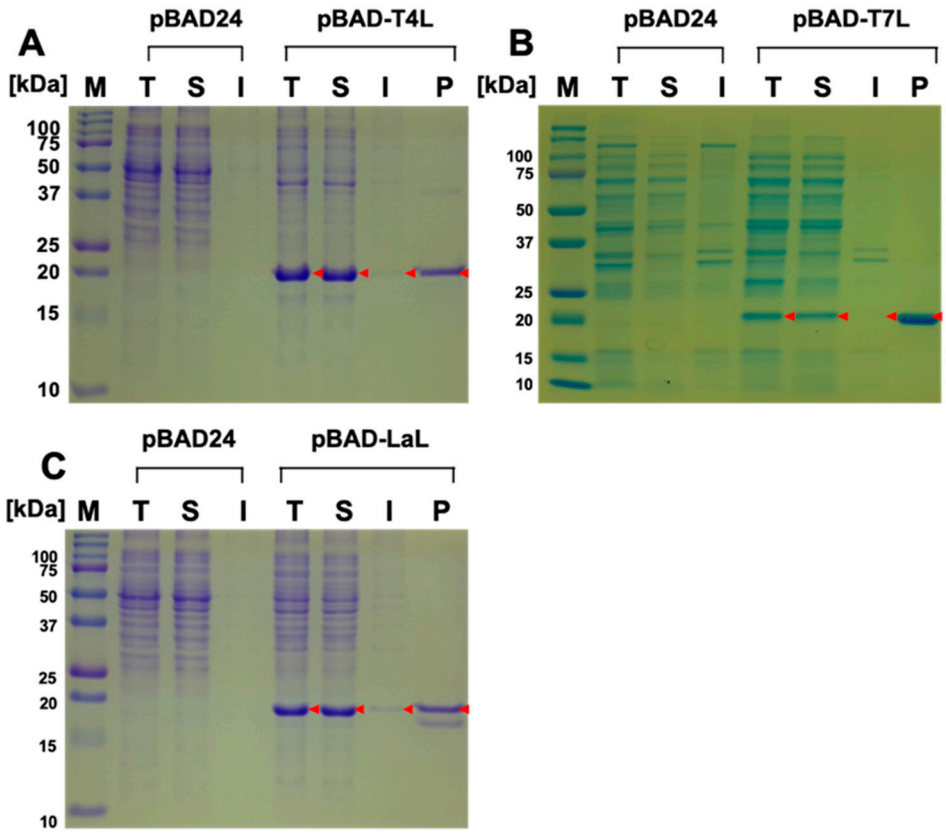


Figure 1. Expression and purification of bacteriophage T4 lysozymes. *Escherichia coli* LMG194 strain harboring the pBAD24 (negative control), (A) pBAD-T4L (T4 lysozyme), (B) pBAD-T7L, or (C) pBAD-LaL (lambda lysozyme) plasmid were cultivated at 37 ºC for 4 hours after the addition of 2 g/L of L-arabinose. Lane M: standard marker protein; T, total cell lysate; S, soluble fraction; I, insoluble fraction; P, purified proteins.

2.2. Secondary Sludge as Substrates

Secondary sludges, taken directly as aerobic reactors for the T4L treatment, were obtained from three municipal wastewater treatment plants (WWTPs) in Korea: one in Busan (Busan WWTP), one in Pohang (Pohang WWTP), and one in Ulsan (Ulsan WWTP). These WWTPs used different processes to treat the domestic wastewater. Busan WWTP used a modified Ludzack–Ettinger (MLE) method. Pohang WWTP used a membrane bioreactor (MBR). Ulsan WWTP used the anaerobic-anoxic/oxic (A2O) process (Table 2). All sludge samples were collected on the same date, using collection chambers that had first been flushed three times with the wastewater. All samples were immediately sealed in 500 mL sterile plastic bottles, transported to the laboratory within 12 h, and stored in a refrigerator at 4 °C until they were used.

Table 2. Experimental conditions of wastewater treatment plants (WWTPs).

Parameters	Busan	Pohang	Ulsan
Daily flow rates (m^3/day)	450,000	232,000	250,000
Treatment process	MLE	MBR	A2O
Operational modes	CSTR	SBR	SBR
Aerobic reactor size (m^3)	2821	3221	3593
pH in aerobic reactor	7.3	6.8	6.9
Alkalinity (mg $CaCO_3$ eq./L) in aerobic reactor	257	288	430

* CSTR: completely stirred tank reactor; SBR: sequencing batch reactor; MLE: modified Ludzack–Ettinger; MBR: membrane bioreactor; A2O: anaerobic-anoxic/oxic.

2.3. Investigation of the Hydrolysis Efficiency and Biochemical Methane Potential (BMP)

Secondary sludge was treated with T4L at the pH of 7.0 and temperature of 45.0 °C in working volumes of 50 mL. Concentrations of volatile suspended solid (VSS) were measured at 0, 24, 48, and 72 h (Table 3). The initial mixing ratio of secondary sludge to T4L was 1:1 (i.e., (g sludge VSS)/(g T4L VS)). Control groups for hydrolysis were performed with distilled water instead of T4L. BMP tests were conducted to monitor the variations in biogas formation with the T4L treatment. The initial F/M ratio was 1 g VS/g VSS. Tests were conducted in 100 mL serum bottles with a working volume of 70 mL. N_2 gas was used to flush the headspace of each serum bottle before incubation was initiated. All serum bottles were incubated at 37 °C and pH 7.5 for 28 d. Control groups for the BMP test were performed in the following: (a) inoculum with only sludge; (b) inoculum with only T4L; and (c) only inoculum.

Table 3. Experimental conditions of the hydrolysis efficiency and biochemical methane potential (BMP) test.

Hydrolysis experiment	
Parameters	**Description**
Substrate (S)	Secondary sludges from Busan, Pohang, Ulsan WWTPs
Enzyme (E)	Bacteriophage T4 lysozyme (T4L)
pH	7.0
Temperature (°C)	45.0
S-E ratio	1 g sludge VSS/g VS of T4L
Working Volume (mL)	50
BMP test	
Parameters	**Description**
pH	7.5
Temperature (°C)	37°C
F/M ratio	1 g VS/g VSS
Working Volume (mL)	70
Medium A (g/L)	NH_4Cl, 100; NaCl, 10; $MgCl_2$ $6H_2O$, 10; $CaCl_2$ $2H_2O$, 5
Medium B (g/L)	K_2HPO_4 $3H_2O$, 200
Medium C (g/L)	Resazurin, 0.5
Medium D (g/L)	$FeCl_2$ $4H_2O$, 2; H_3BO_3, 0.05; $ZnCl_2$, 0.05; $CuCl_2$ $2H_2O$, 0.038; $MnCl_2$ $4H_2O$, 0.05; $(NH_4)_6Mo_7O_{24}$ $4H_2O$, 0.05; $AlCl_3$, 0.05; $CoCl_2$ $6H_2O$, 0.05; $NiCl_2$ $6H_2O$, 0.092; ethylenediaminetetraacetate, 0.5; concentrated HCl, 1 mL; Na_2SeO_3 $5H_2O$, 0.1
Medium E (g/L)	Biotin, 2; folic acid, 2; pyridoxine acid, 10; ridoflavin, 5; thiamine hydrochloride, 5; cyanocobalamine, 0.1; nicotinic acid, 5; P-aminobenzoic acid, 5; lipoic acid, 5; DL-pantothenic acid

Microbes used for inoculum was taken from the lab reactor. The VSS of inoculum was 14.9 g VSS/L, COD was 37.6 g COD/L, and COD removal was 54.3%. Medium for the BMP test was prepared as reported earlier, and media A–E were added: (A) 10 mL, (B) 2 mL, (C) 1mL, (D) 1mL, and (E) 1mL, in 975 mL of distilled water (Table 3). A total of 0.5g of cysteine hydrochloride and 2.6 g of $NaHCO_3$ were added, and sodium sulfide nonahydrate was then added to a final concentration of 0.025% [35].

A rate coefficient of the biogas production was estimated using the following Equation (1) [36]:

$$Y_t = A\ (1 - \exp^{-k \cdot t}), \quad (1)$$

where, Y_1 is the cumulative biogas production (NmL/g VS_{in}) at time t; A is the biogas production (NmL/g VS_{in}) at the end point; k is the biogas production rate coefficient (/d); and t is the incubation time (d).

2.4. Analytical Protocols

The solid concentration and chemical oxygen demand (COD) were analyzed according to the Standard Methods [37]. Samples were passed through a 1.2 μm GF/C filter (Whatman, Little Chalfont,

Buckinghamshire, UK) to measure the collected suspended solid or sCOD. Carbohydrate was measured using the phenol-sulfuric acid method [38], and the protein concentration was measured using the Kjeldahl method [37]. The lipid content was analyzed by gravimetric analysis after the extraction of lipid using chloroform: methanol (1:2, v/v) [39]. Gas production was monitored daily, and gas contents were analyzed using a gas chromatograph (6890 plus, Agilent, Santa Clara, CA, USA) equipped with an HP-5 capillary column (Agilent) and a thermal conductivity detector.

The bacterial 16S rRNA gene was sequenced using the Ion PGM^{TM} System (Life Technologies, Carlsbad, CA, USA). The purified DNA was amplified and barcode-labeled with the Ion Plus fragment library kit (Life Technologies). Emulsion PCR was performed using an Ion PGM Hi-Q template kit (Life Technologies, Carlsbad, CA, USA). The template-positive ion sphere particles were loaded on an Ion 318™ chip (Life Technologies). Obtained valid sequences with ≥97% similarity were clustered as operational taxonomic units (OTUs,) using UPARSE (USEARCH version 7.0.1090, Sonoma, CA, USA). OTUs were classified using SILVA database version 132.

3. Results and Discussion

3.1. Characteristics of the Sludge and Bacteriophage T4 Lysozyme Treatment

The physicochemical characteristics (Table 4) of the secondary sludges showed different contents of solids and organic matter, with an average ratio of organic materials to total solid (i.e., VS/TS) of 83.7 ± 5.3%. The average ratio of VSS to VS was 82.4 ± 4.7%, which indicates that most of the organics in the sludges were in suspended form. Carbohydrates (67.7 ± 6.4 %), proteins (14.4 ± 1.0 %), and lipids (14.7 ± 4.2 %) were the major organic components (average total 96.8 ± 4.2% of VS).

Table 4. Characteristics of the secondary sludge taken from wastewater treatment plants in the cities of Busan, Pohang, and Ulsan.

Analytical Items (g/L)	Raw Secondary Sludges					
	Busan		Pohang		Ulsan	
	Average	Stdev. *	Average	Stdev.	Average	Stdev.
pH	7.3		6.8		6.9	
TS	12.3	0.1	6.1	0.0	4.3	0.2
VS	10.3	0.0	5.4	0.1	3.4	0.1
TSS	9.6	0.9	5.7	0.1	3.6	0.0
VSS	8.2	0.7	4.3	0.2	3.0	0.1
COD	14.7	1.0	7.3	0.4	5.5	0.0
sCOD	0.1	0.0	0.0	0.0	0.4	0.0
Total carbohydrate	1.5	0.2	0.8	0.0	0.5	0.0
Protein	7.7	0.3	3.5	0.2	2.1	0.0
Lipid	1.0	0.3	0.9	0.0	0.6	0.2
VS/TS (%)	83.5		89.1		78.5	
VSS/TS (%)	66.6		70.9		68.9	
VSS/VS (%)	79.7		79.6		87.8	

* Standard deviation.

The bacteriophage T4 lysozyme decreased the VSS concentrations of the sludges after 72 h of T4L treatment; the reduction efficiencies were 28.9% in the sample from Busan WWTP, 18.1% in the sample from Pohang WWTP, and 24.8% in the sample from Ulsan WWTP (Figure 2). These results are a strong indication of disintegration in the cellular structure of the substrate sludge. The VSS reduction efficiencies varied from 18.1% to 28.9%, but the ratios of the VSS and each crude organic molecule (i.e., protein, carbohydrate, and lipid) to the VS content were nearly identical, as presented above. Therefore, the difference in efficiencies suggests that different microbes comprised the secondary sludges.

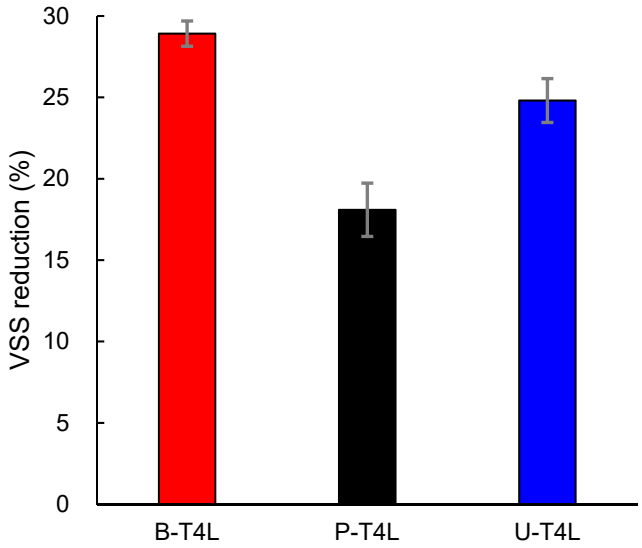


Figure 2. Volatile suspended solid reductions after 72 h treatment of the bacteriophage T4 lysozyme. B-T4L: Busan; P-T4L: Pohang; U-T4L: Ulsan.

3.2. *Effect of Bacteriophage T4 Lysozymes on Biogas Production*

Biochemical methane potential (BMP) tests using raw and T4L-treated sludges were conducted to investigate the variation in biogas production for 28 d (Table 5). T4L treatment increased the accumulated biogas production during the 28 days of incubation (Figure 3). The total amount of biogas production using raw sludge samples was Busan WWTP: 58.2 ± 3.3 NmL/g VS_{in}; Pohang WWTP: 106.2 ± 2.1 NmL/g VS_{in}; and Ulsan WWTP: 94.8 ± 2.4 NmL/g VS_{in}. The maximal values of biogas production using T4L-treated sludge were 111.0 ± 2.9, 130.3 ± 2.4, and 106.2 ± 2.4 NmL/g VS_{in}, correspondingly.

Table 5. Summary of biogas production and reaction rate coefficients (*k*) in the BMP test.

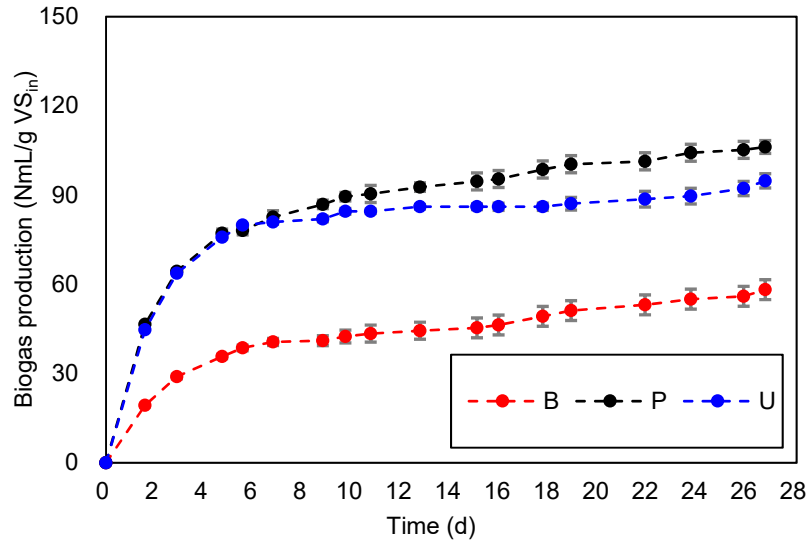
	Busan		**Pohang**		**Ulsan**	
	Raw	**T4L**	**Raw**	**T4L**	**Raw**	**T4L**
Biogas production (NmL/g VS_{in})	58.2	111.0	106.2	130.3	94.8	106.2
Methane content (%)	64.7	65.4	60.6	66.1	58.9	64.1
t_{75}(d) *	12.7.	6.8	6.8	5.5	4.7	4.7
k (d)	0.37	0.54	0.50	0.57	0.47	0.58

* Elapsed time to reach 75% of final biogas production.

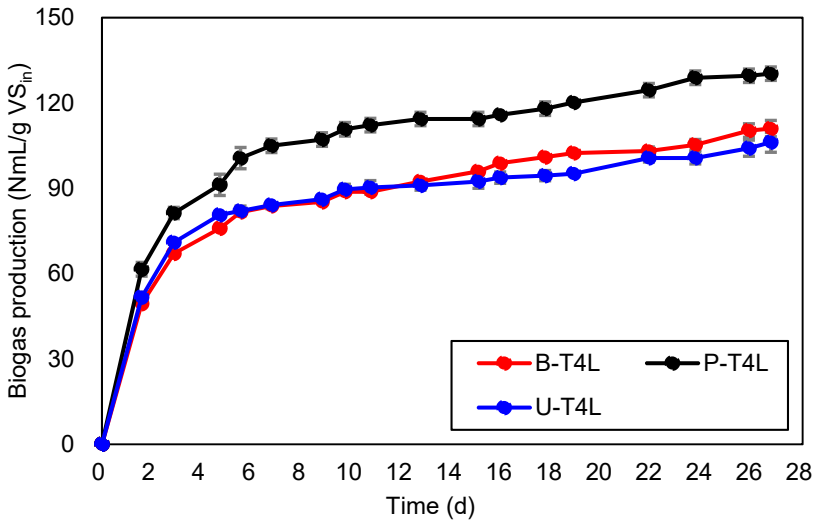
Therefore, the total biogas production from each WWTP sludge with the enzyme treatment increased by 82.4% in samples from Busan WWTP, 21.4% in samples from Pohang WWTP, and 13.0% in samples from Ulsan WWTP. In the sample from Busan WWTP, 76.3% of the total biogas was produced in the first 12.7 d of incubation, whereas, in the other samples, 77.3% of the total biogas was produced during the first 6.8 d (Figure 3). The gas production potential varied among samples, but the average methane content of the biogas was nearly the same (average 63.3 ± 2.9%). The VSS reduction efficiencies of T4L-treated sludge in the BMP tests were 22.8% in the sample from Busan WWTP, 19.1% in the sample from Pohang WWTP, and 23.3% in the sample from Ulsan WWTP. These results mean that the overall VSS removal efficiencies were 51.7% (Busan WWTP), 37.2% (Pohang WWTP), and 48.1% (Ulsan WWTP), and demonstrate that the use of T4L for sludge pretreatment can increase gas production, as well as VSS removal.

The first-order model (Equation (1)) was used to describe the cumulative biogas production (Table 5, Figure 3). All r^2 values were nearly 1.0, which indicates an excellent match of the curve to the data. The coefficients of biogas production were 0.37/d to 0.50/d in raw sludge and 0.54/d to 0.58/d in T4L-treated sludges. These results clearly demonstrate that T4L treatment increased the initial reaction

rates, which ranged from 13.9% (Pohang WWTP) to 47.5% (Busan WWTP). The coefficients when raw sludge was used varied among the sample origins, but were nearly identical when T4L-treated sludge was used (i.e., 0.56 ± 0.02/d).



(**a**)



(**b**)

Figure 3. Accumulated biogas production in the BMP tests using (a) raw secondary sludges, and (b) sludges treated with the bacteriophage T4 lysozyme (B: Busan sample, P: Pohang sample, U: Ulsan sample; T4L: treated with the bacteriophage T4 lysozyme).

Experimental data sets were fitted using an exponential and linear model of the reduction of secondary sludge without lysozyme treatment (Figure 3a). The values of (a) the biogas production potential (NmL/g VS_{in}) and (b) biogas production rate (k, d^{-1}) were in the following order: (a) Pohang WWTP (106.2) > Ulsan WWTP (94.8) > Busan WWTP (58.2), and (b) Pohang WWTP (0.50) > Ulsan WWTP (0.47) > Busan WWTP (0.37). The sample from Pohang WWTP showed the highest initial biogas production potential (106.2 NmL/g VS_{in}) and the highest maximum biogas production (0.50/d). Treatment with lysozyme increased every parameter for secondary sludge in comparison with parameters for raw sludge (Figure 3b). The k values of secondary sludge treating lysozyme were 48.7% (B-T4), 5.1% (P-T4), and 25.1% (U-T4) higher than in raw sludge. Treatment with lysozyme increased

the methane yield of secondary sludge (Figure 3) to 72.6 NmL CH_4/g VS_{in} (84.4 % increase) in the sample from Busan WWTP, to 86.1 NmL CH_4/g VS_{in} (42.4 % increasing) in the sample from Pohang WWTP, and to 68.0 NmL CH_4/g VS_{in} (103.0 % increase) in the sample from Ulsan WWTP. The methane yield was highest in the sample from Pohang WWTP.

Overall biogas productions and reaction rates in the BMP tests using the different raw sludges were more variable than in T4L-treated sludge (Table 5). With the T4L-treated sludges, the average biogas yield was 115.8 ± 12.8 Nml/g $VS_{in,}$ and the average reaction rate was 0.56 ± 0.02/d. Both biogas production and the reaction rate increased after T4L treatment of the raw sludges, but seemed to converge to similar values.

The biogas production potential using the raw sludges observed in this research (58.2–106.2 NmL/g VS_{in}) is in the range of values obtained previously [40]. The methane production potential also falls in the ranges of values obtained previously [41]. In contrast, the methane production potential of sludge after T4L treatment (106.2–130.3 NmL/g VS_{in}) was similar or higher than values from other studies that used thermal pretreatment at 55 °C [42].

The VS or VSS is widely used to express the microbial concentration in environmental research, and is a collective term that does not explain the characteristics of the sludge with respect to variations in the microbial community. Relatively high variations in the biogas yield and production rate using the raw sludges indicated that the activities of anaerobic microbes in the seed for the BMP tests were probably affected by the different characteristics of the substrate sludges, possibly by differences in the microbial diversity of the secondary sludges. Therefore, the same microbial community (i.e., the seed at start-up of the BMP) used the different substrates at different efficiencies. However, the biogas yield and production rate were similar in the T4L-treated sludges, which suggests that the T4L treatment homogenized the characteristics (i.e., microbial composition) of the raw sludges to a form that was readily utilized by the seed microbes in the BMP test.

3.3. Differences in Bacterial Communities

The microbial communities were broadly similar among the raw sludges (Figure 4). Microbes from classes *Alphaproteobacteria*, *Betaproteobacteria*, *Flavobacteriia*, *Gammaproteobacteria*, and *Sphingobacteriia* were major microbial groups in secondary sludges before T4L treatment [43]. These five classes comprised 85.5%, 82.9%, and 67.0% of the total identified microbial groups (i.e., 100%, 99.6%, and 98.8%) in the sludges of Busan WWTP, Pohang WWTP, and Ulsan WWTP, respectively.

All microbes in these classes are gram negative, so the enzyme activity of T4L was likely to be identical for the cell membrane hydrolysis, and thus to yield products that have similar characteristics. An investigation of the microbial characteristics or activities at the species level would be an idea, but the AD process involves mixed populations of microorganisms, so these mixes should be studied under controlled conditions in the laboratory. Such study would allow quantification of the interactions and of the collective properties of the mixture of species that was selected by the conditions imposed (i.e., T4L treatment in this research). Lysozyme digestion can disrupt the sludge flocs and disintegrate the sludge microbes [44], and can thus be regarded as a kind of selective pressure on the microorganisms that compose the sludge [45]. Due to the dissimilarity among the microbial communities (e.g., gram positive vs. gram negative), some species can survive the lysozyme treatment, whereas other species are subject to the selection of disintegration or eliminated [46].

The secondary sludge that is produced by domestic wastewater treatment operations and processes is usually a liquid or semisolid, containing from 0.25% to 5% solids, depending on the operations and processes used [47]. The sludge contains bacteria, viruses, protozoa, and other microorganisms, some of which are pathogens. The species and density of microorganisms present in the sludge produced from a particular municipality depend, to a large extent, on the activities of the local community, and vary substantially over time. Although microbial compositions of secondary sludge are increasingly being reported in the literature [48,49], these regional differences, water uses, seasonal and diurnal

variations, and other factors impede the accurate identification of the general characteristics of the secondary sludge.

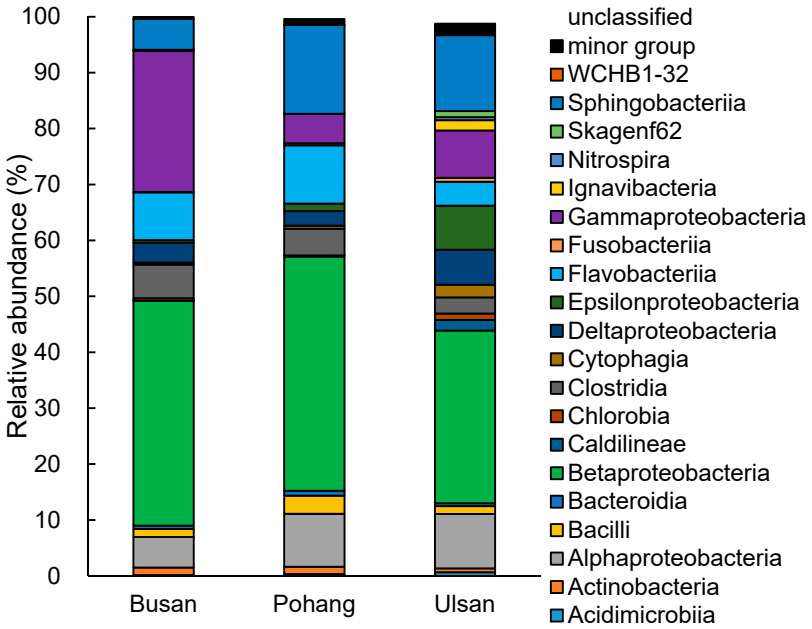


Figure 4. Relative abundance (%) of bacterial classes found in secondary sludge from WWTPs in Busan, Pohang, and Ulsan.

Biogas production and the initial rate in the BMP test of secondary sludge decreased in the order of Pohang WWTP, Ulsan WWTP, and Busan WWTP. The relative abundances of some bacterial groups were correlated with this trend. The relative abundances of classes (a) *Sphingobacteriia* and (b) *Alphaproteobacteria* decreased in the same order as biogas production and the initial rate: (a) Pohang WWTP (15.9%) > Ulsan WWTP (13.6%) > Busan WWTP (5.5%), and (b) Pohang WWTP = Ulsan WWTP (9.5%) > Busan WWTP (5.5%). These groups were also the main classes in the secondary sludge, so the bacteriophage T4 lysozyme would be a primary hydrolyzer of these classes. In contrast, *Gammaproteobacteria* was one of the main classes in the secondary sludge from all three WWTPs, but its relative abundance in the secondary sludge decreased in the order opposite to the trends of biogas production and the initial rate (Busan WWTP (25.2%) > Ulsan WWTP (8.5%) > Pohang WWTP (5.3%)). Most of the class *Gammaproteobacteria* (13.0 ± 10.7%) in these samples consisted of species from the order *Xanthomonadales* (11.1 ± 10.8%), which does not produce glycosidase [50]; this result suggests that the composition of microbial groups in secondary sludge could be considered a target for T4L treatment.

This work demonstrated the feasibility of using the bacteriophage T4 lysozyme to increase the hydrolysis of secondary sludge. This technology can be applied to sludge pretreatment and anaerobic digestion for WWTPs. However, this was just a lab-scale study; scaling the process to the scale of WWTPs would entail massive enzyme production, and would encounter environmental regulations because the process uses a genetically modified organism to produce the enzyme. The bacteriophage T4 lysozyme, known as the GlcNAc hydrolysis enzyme, also has the possibility of being used for crustacean waste, because most of the total weight of crustaceans consists of chitin, which combines a lot of GlcNAc linkage [51].

4. Conclusions

This study shows that secondary sludge and biogas production by the anaerobic digestion of secondary sludge can be increased by adding the bacteriophage T4 lysozyme to the sludge. VSS reduction was quantified, a BMP test was conducted, and next-generation genome sequencing was performed to investigate the effect of lysozyme treatment. VSS reduction efficiencies were 28.9%, 18.1%,

and 24.8% in the secondary sludge of WWTPs in Busan, Pohang, and Ulsan, respectively. The initial biogas production (k, /d) was 0.50, 0.47, and 0.37 in Pohang, Ulsan, and Busan WWTPs, respectively. The three dominant bacterial classes in the secondary sludge were *Betaproteobacteria* (37.7 ± 5.9%), *Gammaproteobacteria* (13.0 ± 10.7%), and *Sphingobacteriia* (11.6 ± 5.4%). Relative abundances of *Sphingobacteriia* and *Alphaproteobacteria* were positively correlated with biogas production and the initial rate of the BMP test, whereas those of *Gammaproteobacteria* were negatively correlated with biogas production. Further study to improve the hydrolysis efficiency of lysozyme should consider using different bacteriophage lysozymes, and the random mutagenesis and selection of bacteriophage lysozyme genes.

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Abbreviations

TS	Total solid
VS	Volatile solid
TSS	Total suspended solid
VSS	Volatile suspended solid
COD	Chemical oxygen demand
T4L	Bacteriophage T4 lysozyme
T7L	Bacteriophage T7 lysozyme
LaL	Bacteriophage lambda lysozyme
AD	Anaerobic digestion
OLR	Organic loading rate
BMP	Biochemical methane potential
WWTPs	Wastewater treatment plants
GlcNAc	N-acetyl-D-glucosamine
MurNAc	N-acetylmuramic acid
CSTR	Completely stirred tank reactor
SBR	Sequencing batch reactor
MLE	Modified Ludzack-Ettinger
MBR	Membrane bioreactor
A2O	Anaerobic-anoxic/oxic

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