Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–8, 2016

www.elsevier.com/locate/jbiosc

Effect of freeze–thaw process on physical properties, microbial activities and population structures of anaerobic sludge

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Received 6 August 2016; accepted 14 November 2016
Available online xxx

Effects of the freeze–thaw process on physical properties, cell viability, microbial activities and population structures of anaerobic sludge were investigated. It was found that the sludge volume index was greatly reduced from 16.4 mL/g in the original sludge to 4.0 mL/g in the solid fraction of the frozen–thawed sludge. Even though the freeze–thaw process decreased cell viability in the solid fraction of the frozen–thawed sludge, microbial activity tests showed that the freeze–thaw process enhanced acidogenic activity approximately 20%. The enhanced acidogenic activity of the solid fraction was in good agreement with the enrichment of Clostridiaceae, Porphyromonadaceae and Propionibacteriaceae found in the solid fraction. The relative abundances of Proteobacteria families Oxalobacteraceae, Moraxellaceae, and Pseudomonadaceae were found to be highest in the liquid fraction where they form a substantial proportion of the bacterial community (a total of 59%).

[**Key words:** Freeze–thaw; Anaerobic sludge; Microbial activity; Biohydrogen; Tagged 16S rRNA gene pyrosequencing]

Biological H_2 (biohydrogen) is considered to be an environmentally friendly energy source. Efficient production of biohydrogen depends on many factors including operating conditions, substrate compositions, and microbial community. Mixed cultures are sources of microorganisms that work in synergy in converting complex substrates to hydrogen. However, methanogens and homoacetogens, which are hydrogen-consuming bacteria, are present in most mixed cultures. Various pretreatment methods have been reported to eliminate hydrogen-consuming bacteria and thus to enrich hydrogen-producing bacteria. At present, the pretreatment methods include heat-shock, ultrasonic wave, acid, base, aeration, chloroform, sodium 2-bromoethanesulfonate and freezing and thawing (1–4). The best pretreatment method for enriching hydrogen-producing bacteria from seed sludge varies from case to case and seems to depend on the microbial community originally present in the seed sludge (3).

Among the pretreatment methods, the freeze–thaw process is one that has been successfully used as a pretreatment method for some types of anaerobic sludge. Cheong and Hansen (1) used this process to enrich hydrogen-producing strains from mixed bacterial populations in the preparation of effective inoculums for hydrogen production from glucose. They showed that the hydrogen production from glucose using frozen–thawed cattle manure sludge was about two-times higher than that obtained using untreated control sludge.

Up to authors' best knowledge, there is no literature data of any in-depth taxonomic-level investigation that could explain the effectiveness of freeze–thaw pretreatment for a variety of sludge types. The aim of the present study was to carry out this type of in-depth investigation for anaerobically digested sludge obtained from a domestic wastewater treatment plant near Bangkok. The quantity of this sludge is expected to increase to 300 tons per day by the year 2020, and the sludge can be a potential source of inoculums. Changes caused by the freeze–thaw process in physical characteristics and microbial activities of sludge samples were investigated using measurements of amount of settleable solids, sludge volume index and microbial activity tests. Modern genetic analysis tools, such as tagged 16S rRNA gene pyrosequencing, were used to help identify unique bacterial phylotypes and estimate their richness in the original and the frozen–thawed sludge samples.

MATERIALS AND METHODS

Materials Anaerobically digested sludge was used as the inoculum for anaerobic biogas production. This sludge was obtained from Ngaung-Khaem Water

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2016.11.005

Please cite this article in press as: Phalakornkule, C., et al., Effect of freeze–thaw process on physical properties, microbial activities and population structures of anaerobic sludge, J. Biosci. Bioeng., (2016), http://dx.doi.org/10.1016/j.jbiosc.2016.11.005

Quality Control Plant, a domestic wastewater treatment plant in Bangkok, Thailand. Based on three sludge samples from the same sludge source, the sludge had 68.3 ± 13.1 g/L of total solid (TS) and 27.3 ± 11.2 g/L of volatile suspended solid (VSS). The sludge is referred to as original sludge (OS) in this paper.

The original sludge was frozen at −20°C for 24 h and then thawed for 12 h at room temperatures between 30°C and 35°C (5). The sludge samples were then separated into a liquid portion and a solid portion by natural sedimentation for approximately 8 h. Meanwhile, the OS (the control) was allowed to stand still at the same room temperature. The solid portion was used as the inoculum and will be referred to as the frozen−thawed sludge (S-FS).

Cell viability assay Measurement of cell viability was performed using Live/DeadBacLight (L13152) kits (Invitrogen, USA), according to the manufacture's instruction (6). One gram of each sludge sample (OS and S-FS) was resuspended in 9 mL of sterilized normal saline solution. To obtain cell numbers appropriate for microscopic examination, each sludge suspension was diluted 100-fold. The Live/DeadBacLight component A and B were dissolved separately in 2.5 mL of filter-sterilized deionized water. Thereafter, equal volumes of the two components were mixed prior to use. The reagent mixture was combined with an equal volume of each sludge suspension and incubated at room temperature in the dark for 15−20 min. Microscopic count was performed using a Neubauer counting chamber (Baeco, Germany). The chamber was placed under a fluorescent microscope (Leica DMLS) equipped with a filter cube comprising of an excitation filter (BP 420−490) and a suppression filter (LP 515). Cells appearing green were counted as viable cells, while those appearing red were counted as non-viable cells.

Microbial activity tests Microbial activity tests were performed with three independent replications (different sludge samples of the same sludge source). The inoculum activities of the sludge samples were tested in 100 mL serum bottles at 35°C. Each serum bottle contained 70 mL of mineral stock solution mixed with the substrate specific for each activity test (7). The mineral stock solution contained per liter; 2.5 g KH_2PO_4, 1 g K_2HPO_4, 1 g NH_4Cl, 0.213 g $MgCl_2 \cdot 6H_2O$, 0.118 g $Na_2S \cdot 9H_2O$, 3.0 g $NaHCO_3$ and 0.2 g yeast extract (8). Thirty milliliters of sludge sample (OS or S-FS) were inoculated into the bottle, corresponding to 6 g VSS sludge per liter reactor. The pH was adjusted to 6.7 with 3 N NaOH or 1 N HCl. Anaerobic conditions were initiated by flushing the headspace of each serum bottle for 1 min with argon. The substrate utilization rate (or the product production rate) was determined from the slope of the initial linear part of each plot between substrate (or product) concentration versus time.

In the hydrolytic activity test, 0.21 g cellulose in the 70 mL of the mineral stock solution was used as the substrate, and concentration of reducing sugar was monitored every 2 d. In the acidogenic activity test, 3 g/L glucose was used as the substrate. The concentration of reducing sugar was then monitored every 2 h, and volatile fatty acids (VFA) concentration were monitored every 4 h. In the acetogenic activity test, 3 g/L butyrate or 3 g/L propionate was used as the substrate, and their concentrations were measured every 2 d. In the acetoclastic activity test, 3 g/L acetate was used as the substrate. Concentrations of acetate, the amount of gas and gas composition were monitored every 2 d. In the hydrogenotrophic activity test, 80:20 H_2:CO_2 (v/v) was used as the substrate. Each serum bottle was subjected to an initial overpressure of 1.2 atm which created a height of water of 2 m. Pressure reduction was monitored every hour by measuring the reduction in the height of water. For the liquid samples, 1.5 mL of liquid was sampled without opening the serum bottles.

Batch fermentative production of hydrogen The batch hydrogen fermentations were carried out in serum bottles, each having a total capacity of 116 mL and a working volume of 100 mL. Each bottle contained 6 g COD/L of canned fruit syrup (Malee Co. Ltd., Thailand) and 6 g VSS/L of sludge inoculums (either OS or S-FS). Initial pH was adjusted to 5, 6 and 7 with 3 N NaOH or 1 N HCl. The bottles were purged with argon gas for 1 min in order to ensure anaerobic conditions, subsequently sealed and placed at room temperature (33°C). The volume of biogas and gas composition were monitored every 4−6 h by hospital syringe and gas chromatography, respectively. Fermentations were terminated when no further gas production could be observed. All the measurements were carried out in triplicates.

Physical and chemical analysis Total chemical oxygen demand (tCOD), soluble chemical oxygen demand (sCOD), total nitrogen (TN), total phosphate (TP-PO_4^{3-}), TS, VSS, total volatile solid (TVS), mixed liquor suspended solid (MLSS), mixed liquor volatile suspended solid (MLVSS), the amount of settleable solids and the sludge volume index were determined according to standard methods (9). Concentrations of reducing sugars were determined by the dinitrosalicylic acid method (DNS method).

The biogas generated was collected using either 25 mL- or 50 mL-hospital needle syringes (10). Biogas content (H_2, CH_4, and CO_2) was measured periodically using a gas chromatograph (Shimadzu GC-2014, Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector (TCD) with a Unibeads C 60/80 column (GL Sciences, USA). Argon was used as a carrier gas. The temperatures of the injection port, the column oven and the TCD were 115, 115, and 180°C, respectively. VFAs and ethanol were analyzed by a gas chromatograph (Shimadzu GC-2010) equipped with a flame ionization detector and a Stabilwax DA capillary column (Restek Corporation, USA). The temperatures of the injection port and the detector were maintained at 230 and 250°C, respectively.

Tagged 16S rRNA gene sequencing Total genomic DNA was extracted from 250 mg of samples taken from the sludge samples using the benzyl chloride method (11). The concentration of extracted DNA was quantified using a NanoDrop ND-1000 spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany). The purified DNA (25 ng) was used as a template for amplification of the partial 16S rRNA gene fragment using E785F forward primer (5′-GGATTAGATACCCTGGTAGTCC-3′) and E1081R reverse primer (5′-CTCACGRCACGAGCTGACG-3′) encompassing the V5−V6 hypervariable regions in prokaryotic 16S rRNA gene (12). Before amplification, tag sequences (8-base) were attached at the 5′ terminus of each primer to identify the DNA samples as being from OS, solid fraction FS or liquid fraction FS sludge samples (13). The amplification resulted in an expected amplicon size of 296 bps. Polymerase chain reactions (PCR) were performed using DyNAzyme EXT DNA polymerase (Finnzyme, Espoo, Finland) on a MyCycler thermo cycler (Bio-Rad, Hercules, CA, USA). The temperature profile consisted of 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min 45 s, followed by a final extension step at 72°C for 10 min. The amplicons were purified using a GeneJETTM Gel Extraction kit (Fermentas, Vilnius, Lithuania). The purified amplicons were quantified using DNA chips on a Bio-Rad Experion Automated Electrophoresis Station (Bio-Rad Laboratories, CA, USA) and a TBS-380 Fluorometer (Turner BioSystems, Sunnyvale, CA, USA). The sequences were determined using an Ion PGM sequencer (Life Technologies, Grand Island, NY, USA) following the manufacturer's recommended protocols. The sequencing dataset was deposited in the NCBI SRA database with the accession ID SRP057674.

Diversity and statistical analyses The sequencing dataset was initially processed by removing low quality score readings with cutoff of 20 for Phred quality score. The sequencing dataset from a sample were then processed using QIIME (Quantitative Insights into Microbial Ecology) (14) to remove the tag sequences and obtain raw data sequences. Chimera (hybrid) sequences resulting from merger of DNA sequences from different parent sequences due to errors during PCR amplification, were then identified and removed by UCHIME (15) using a reference dataset from the Ribosomal Database Project (RDP) (16). Sequences that were 200 bps or longer in length were selected for further analysis. Taxonomic classifications were assigned using both homology and composition-based methods. Sequence homology search was performed using BLASTN against the 16S microbial rRNA database of NCBI to determine the approximate phylogeny with an E-value cutoff of 1e-10 and DUST filter option defined to "m D". The BLASTN best hit with the lowest E-value cutoff for each sequence was taken for taxonomic assignment. The composition based RDP-classifier (17) was used to assign approximate phylogeny by naïve Bayesian classification method with 80% confidence threshold. The difference of assigned taxons between the two methods was tested by a *t*-test. Operational taxonomic units (OTUs) were determined by the furthest-neighbor method of MOTHUR (18) at sequence dissimilarity levels of 0.03, 0.05, and 0.15. In order to reduce the bias resulting from unequal numbers of datasets for the three sludge types, normalization was performed by a subsampling method using cutoff of 10,000 sequences per sample.

The statistical analysis and visualization of microbial community were performed through STAMP (Statistical analysis of taxonomic and functional profiles) (19) version 2.0.9. This program computed the following statistics. The nonparametric diversity indexes including Shannon-Weaver index, Simpson's diversity index, the Chao1 richness estimator, and the abundance-based coverage estimator (ACE) were calculated at the genetic distance of 0.03 to measure and compare the diversity of bacterial species among the dataset. Good's coverage (G), an estimator for sampling completeness, was calculated as $G = 1-(n/N)$, where n is the number of OTUs that have been sampled once and N is the total number of individuals in the sample.

RESULTS

Physical characteristics of the original sludge and the frozen−thawed sludge The original sludge was voluminous. After the freeze−thaw process, the sludge was dewatered and became compact. The liquid portion could then be easily poured off to be analyzed separately. Physical characteristics of the solid and the liquid portions of the frozen−thawed sludge are reported in Table 1 in terms of weight per volume and weight per total dry weight of the original sludge. The TS, VS, MLSS and MLVSS expressed in g/L indicated that for the same volume occupied by the sludge the solid portion of the frozen−thawed sludge had more than double the solid content of the original sludge. The TS, VS, MLSS and MLVSS expressed in mg per g dry weight of the original sludge indicated that >90% of the total solid in the original sludge ended up in the solid portion after the freeze−thaw process. The amount of settleable solids expressed in mL/L and the sludge volume index in mL/g indicated that the frozen−thawed sludge had improved settling characteristics which are beneficial for its use as inoculums. The sludge volume

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