INFLUENCE OF TEMPERATURE DECREASE ON THE PHYSICAL AND MICROBIAL CHARACTERISTICS OF RETAINED SLUDGE IN EGSB REACTOR FOR LOW-STRENGTH WASTEWATER TREATMENT

Wilasinee YOOCHATCHAVAL, Haruhiko SUMINO, Akiyoshi OHASHI, Hideki HARADA, Takashi YAMAGUCHI, Nobuo ARAKI and Kazuaki SYUTSUBO

1Dept. of Env. Sys. Eng., Nagaoka University of Technology (Research student, NIES)
   (1603-1 Kamitomioka, Nagaoka, Niigata, 940-2188, Japan)
   E-mail:wilasinee.y@nies.go.jp
2 Member of JSCE, Research assistant, Dept. of Civil Eng., Gifu National College of Technology
   (2236-2 Kamimakuwa, Motosu, Gifu, 501-0495, Japan)
   E-mail: sumino@gifu-nct.ac.jp
3Member of JSCE, Professor, Grad. Sch. of Eng., Hiroshima University
   (1-3-2 Kagamitayama, Higashihiroshima, 739-8511, Japan)
   E-mail: ecoakiyo@hiroshima-u.ac.jp
4Member of JSCE, Professor, Dept. of Civil Eng., Tohoku University
   (6-6 Aoba, Aramaki, Aoba, Sendai, 980-8579, Japan)
   E-mail: harada@epl1.civil.tohoku.ac.jp
5 Member of JSCE, Associate Professor, Dept. of Env. Sys. Eng., Nagaoka University of Technology
   (1603-1 Kamitomioka, Nagaoka, Niigata, 940-2188, Japan)
   E-mail: ecoya@vos.nagaokaut.ac.jp
6 Member of JSCE, Professor, Dept. of Civil Eng., Nagaoka National College of Technology
   (888 Nishikatakai, Nagaoka, Niigata, 940-8532, Japan)
   E-mail: araki@nagaoka-ct.ac.jp
7Member of JSCE, Senior researcher, Wat. and Soil Env. Div., Natl. Inst. for Env. Stud. (NIES)
   (16-2 Onogawa, Tsukuba, Ibaraki, 305-8506, Japan)
   E-mail: stubo@nies.go.jp

A laboratory-scale EGSB reactor was operated for 400 days to investigate the effect of a temperature decrease on the physical and microbial characteristics of retained granular sludge. The reactor was inoculated with 20°C-grown granular sludge and started up at 15°C. The influent COD of synthetic wastewater was set at 0.6-0.8 g COD/L. The process temperature was reduced stepwise from 15°C to 5°C during the experiment. Decreasing the temperature from 15°C to 10°C decreased COD removal efficiency. However, continuous operation of the EGSB reactor at 10°C led to an efficient treatment of wastewater (70% of COD removal, 50-60% of methane recovery). Unfortunately, at 5°C COD removal efficiency drastically decreased and the amount of removed COD dropped to half of that at 15°C. A decrease in sludge concentration and a major deterioration of the retained sludge’s settleability were observed while the reactor was operated at 5°C. We confirmed the remarkable increase of methanogenic activity of retained sludge at 15-20°C due to the low temperature operation of the reactor. The increment of activity of retained sludge as compared with seed sludge was higher at 20°C for acetate (3.9 times higher) and at 15°C for H2/CO2 (6.4 times higher). Changes in the microbial structure of retained sludge with respect to Archaea were investigated by 16S rDNA-targeted DGGE analysis and cloning. This revealed that the genus *Methanospirillum*, a hydrogen-utilizing methanogen, proliferated. An expected decrease in some *Methanobacterium* spp. due to low temperature operation of the reactor occurred. On the other hand, genus *Methanoseta* was abundant as an acetoclastic-methanogen throughout the experiment.

Key Words: EGSB, low strength wastewater, low temperature, methane fermentation, *Methanospirillum*, microbial structure
1. INTRODUCTION

As the need for sustainable development has increased, anaerobic wastewater treatment systems have emerged as a promising technology for both developed and developing countries. The advantages of these systems are their low operation and maintenance costs with small amounts of sludge produced\(^1\),\(^2\). Anaerobic biofilm processes such as those involving upflow anaerobic sludge blanket (UASB), fluidized bed and fixed bed have usually been used to treat high-concentration wastewater under mesophilic and thermophilic conditions. However, most wastewater is discharged at ambient temperatures and low organic concentrations\(^3\),\(^4\). When these systems were used on low-concentration wastewater, treatment did not meet an acceptable level and poor sludge properties were observed.

Recently, the Expanded Granular Sludge Bed (EGSB) system has been applied, in order to improve the anaerobic treatment technology of low concentration wastewater at low temperature. Effluent recirculation has been used to enhance the contactability between substrate and granular sludge. Also the reactor has to be inoculated with granular sludge, in order to maintain a sufficient sludge retention time\(^5\),\(^6\).

In our previous study, we investigated the reliability of an EGSB reactor for the treatment of low-concentration wastewater (0.6-0.8 g COD/L)\(^7\). We found that the EGSB reactor performed successfully (12 kg COD/m\(^3\)/day loading, 70% COD removal) at 20°C.

In order to verify that temperature was a limiting factor in the efficient treatment of wastewater by an EGSB reactor, an experiment using a laboratory-scale EGSB reactor was conducted. We investigated the effect of a temperature decrease (from 15°C to 5°C) on the performance of an EGSB reactor and on the physical and microbial properties (methanogenic activity, microbial community structure) of the retained sludge.

2. MATERIALS AND METHODS

(1) Experimental conditions

A 2-L EGSB reactor (column volume : 1.3 L, gas-solid separator volume : 0.7 L) was operated for over 400 days with 0.6-0.8 g COD/L of synthetic wastewater composed of sucrose, acetate, propionate and yeast extract in a COD ratio of 4.5: 2.25: 2.25: 1. The concentrations of basal minerals and trace elements of the feed were as reported in Syutsubo et al.\(^8\). The reactor was inoculated with a granular sludge grown at 20°C\(^7\) and started up at 15°C. The organic loading rate (OLR) was controlled by varying the hydraulic retention time (HRT) (Table 1). Effluent was recirculated to provide an up-flow velocity of 5 m/h in the column. The temperature was reduced stepwise from 15°C to 5°C.

(2) Analyses

The influent and effluent of the reactor were sampled for pH, COD, volatile fatty acid (VFA), suspended solid (SS) and sulfate analyses. The composition of biogas was analyzed by TCD gas chromatography.

(3) Measurement of the physical properties of retained sludge

The physical properties of the retained sludge, including sludge concentration (MLVSS) and sludge volume index (SVI), were analyzed periodically by collecting sludge from port No.2, 22.5 cm from the bottom of reactor (column height = 61 cm).

(4) Measurement of methanogenic activity

The methane producing activity of the retained sludge was determined in duplicate on days 0, 42, 132, 196 and 454. The test substrates were acetate, propionate and H\(_2\)/CO\(_2\) gas (80% : 20%, V/V). All vials were incubated on a reciprocal-shaker (120 rpm) at five different temperatures between 10°C and 45°C. Analysis procedures followed those of the previous study\(^9\).

(5) Analysis of microbial community structure

The microbial community structure of the retained sludge was investigated by 16S rDNA-targeted DGGE (Denaturing Gradient Gel Electrophoresis). DNA was extracted from sludge samples by using an Isoil beads beating kit (Nippon gene, Japan). PCR (Polymerase Chain Reaction) was performed using a specific primer for amplifying either Domain Bacteria (341F, 534R) or Domain Archaea (PARCH340F, 519R)\(^10\),\(^11\).

Table 1 Operating conditions for the EGSB reactor during the continuous flow experiment.

<table>
<thead>
<tr>
<th>Phase 1</th>
<th>Phase 2</th>
<th>Phase 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temp.</td>
<td>15°C</td>
<td>10°C</td>
</tr>
<tr>
<td>Day</td>
<td>0-20</td>
<td>21-139</td>
</tr>
<tr>
<td></td>
<td>146-165</td>
<td>166-242</td>
</tr>
<tr>
<td></td>
<td>251-291</td>
<td>292-453</td>
</tr>
<tr>
<td>OLR(^*)</td>
<td>4.0</td>
<td>5.5</td>
</tr>
<tr>
<td>HRT(^**)</td>
<td>4.0</td>
<td>3.6</td>
</tr>
</tbody>
</table>

\(^*\)OLR = Organic Loading Rate

\(^**\)HRT = Hydraulic Retention Time
DGGE analysis was conducted using a DCode™ gel electrophoresis system (Bio-Rad, USA), on a gradient gel (35% - 55% of denaturant for Bacteria and 40% - 60% for Archaea) at 60°C for 3.5 h. Major bands containing DNA were excised, and these nucleotide sequences were determined by a genetic analyzer.

In order to determine the 16S rRNA gene sequence of Methanospirillum spp., clone analysis was conducted. About 1100 bp of 16S rRNA gene were amplified by PCR using the primers A109f-m (5’T-AMDGCTCAgTAACAGtT-3’) and MG1193R [(5’TgTAgCCcggATAATTC-3’), a modification of MG120012], specific to the Order Methanomicrobiales. To capture and clone PCR fragments, TOPO-TA cloning kit (Invitrogen, USA) was used.

3. RESULTS AND DISCUSSION

(1) EGSB reactor performance

To investigate the influence of decreasing the temperature on the performance of the EGSB reactor, a laboratory-scale continuous-flow experiment was conducted. The performance of the EGSB reactor is shown in Fig. 1.

We started up the reactor at 15°C with an OLR of 4 kg COD/m^3/day and an HRT of 4 h. Then, the OLR was increased to 5.5 kg COD/m^3/day by reducing HRT to 3 h.

During the first half of the 15°C phase (Phase 1, Table 1), COD removal-efficiency tended to decrease. However, after 100 days operation at 15°C, the reactor achieved sufficient performance, with an 80% COD removal efficiency and a 53% methane conversion rate (based on removed COD) at an OLR of 5.5 kg COD/m^3/day.

On day 140, the temperature was reduced to 10°C (Phase 2). In order to avoid this temperature reduction triggering a major decrease in performance, OLR was reduced to the same as during the start-up period and then increased to 6 kg COD/m^3/day. As a result, the COD removal efficiency and the methane conversion rate decreased owing to inactivation of the bacteria. However, two months later, the methane conversion rate had recovered to 40-50% of removed COD, and the COD removal efficiency had also increased. The average removed COD during Phase 2 amounted to 93% of that removed during Phase 1 (temperature: 15°C). These results indicated that anaerobic bacteria might be capable of adapting to 10°C conditions.

During the final phase (from day 243, Phase 3), the temperature was reduced to 5°C. As a result, a sharp decrease in COD removal efficiency occurred. In this phase, we could not maintain the COD loading as in phases 1 and 2. A longer HRT than 6 hours was necessary to achieve a COD removal efficiency of 60-70%. Consequently, the average removed COD was only 44% (3.53 g COD/day) of that during the 15°C phase. However, during the 5°C phase, sucrose was not detected in the effluent. Therefore, acidification of sucrose is not a rate-limiting step even at low temperatures.

The concentrations of acetate and propionate in the effluent were almost identical (between 30 and 200 mg COD/L) throughout the experiment. Therefore, the main reason for the reduction in COD removal efficiency was inactivation of both acetogens and acetoclastic methanogens. On the other hand, no significant change of the hydrogen partial pressure of the biogas was observed. It is thought that the generated hydrogen was immediately consumed by hydrogen-utilizing bacteria (sulfate-reducing bacteria, hydrogenotrophic methanogens) in the reactor.
(2) Physical properties of retained sludge

Fig. 2 shows the changes in the physical properties of the retained sludge. At 15°C (Phase 1), the sludge concentration remained at 41-45 g VSS/L. The MLVSS decreased slightly, to 39 g VSS/L (including the influence of sampling loss), at 10°C (Phase 2). At 15°C and 10°C, sludge settleability (as described by SVI) was sufficiently maintained between 20 to 22 mL/g VSS. The good maintenance of these key physical properties of retained sludge contributed to the stable operation of the EGSB reactor at 15°C and 10°C.

Unfortunately, the sludge concentration drastically decreased in Phase 3 (5°C), reaching 22 g VSS/L at the end of experiment. We also observed a steady decrease of settleability during this phase (5°C), SVI reaching 32 mL/g VSS at the end of this phase.

Long-term operation of the reactor at 5°C resulted in a significant decrease in the methane producing activity of retained sludge (see Fig. 3). Inactivation and self-digestion of bacteria as a consequence of the lower temperature might have caused the observed deterioration of physical properties.

We confirmed the presence of acid-forming bacteria (filamentous bacteria and Lactococcus spp.) on the surface of the low-temperature adapted granular sludge by SEM observation and DGGE analysis. However, at 5°C, the activity of acid-forming bacteria persisted (as evidenced by the absence of sucrose in the effluent), in contrast with the inactivation of acetogens and methanogens. Therefore, a relative increase in the acid-forming bacteria is thought to be a cause of the decreased sludge settleability.

These phenomena indicated that operating temperatures for the methanogenic treatment of low-concentration wastewater by EGSB reactor are limited to 10°C.

(3) Methanogenic activity of retained sludge

In order to investigate the changes in the microbial properties of the retained sludge, its methanogenic activity was measured. Fig. 3 shows that methanogenesis was temperature dependent. Test temperatures ranged from 10°C to 45°C.

Continuous operation of the reactor led to increasing methanogenic activity of the retained sludge until the 10°C phase (Phase 2).

The highest methanogenic activity occurred at the mesophilic temperature range (35-45°C) throughout the experiment. Both acetate-fed and propionate-fed methanogenesis peaked at 35°C (day 196), while H2/CO2-fed methanogenesis showed the maximum value at 45°C. Microbial structure analysis of retained sludge showed the presence of acetoclastic Methanosaeta, hydrogenotrophic Methanobacterium, propionate-degrading Syntrophobacter (Fig. 5, 6). Temperature tendencies of methanogenic activity corresponded to the optimum growth temperature of the dominant acetoclastic methanogen (Methanosaeta concilii, optimum temperature : 37°C), a hydrogenotrophic

Fig. 2 Time course of MLVSS (a) and SVI (b) of retained sludge

Fig. 3 Temperature-dependent methanogenic activity of the retained sludge harvested at different temperatures; test substrates (a) acetate, (b) H2/CO2 and (c) propionate
methanogen (*Methanobacterium formicicum*, optimum temperature: 40-45 ºC) and a propionate-degrading acetogen (*Syntrophobacter fumaroxidans*, optimum temperature: 37 ºC).

During Phase 3 (5°C), methanogenic activity of the retained sludge significantly decreased, less than in the seed sludge (day 0). The decrease was especially marked in propionate-fed methane producing activity. Anaerobic degradation of propionate is carried out by an unstable syntrophic association between acetogens and hydrogen-utilizing bacteria (methanogens, sulfate reducing bacteria), therefore, the lower temperature of Phase 3 may have strongly influenced the activity of acetogen.

The decrease in methanogenic activity was also apparent in the reduction in total methane conversion (Fig. 1(b)). During this phase, we also observed a major deterioration of the retained sludge’s physical properties (i.e. settleability and concentration) due to the inactivation of the bacteria. These results indicate that operating the reactor at 5°C is not suitable for the methanogenic treatment of wastewater.

The methanogenic activity of the retained sludge on day 196 as compared with the activity of seed sludge is shown in Fig. 4. We confirmed the significant increase of methanogenic activity of retained sludge at 15°C and 20°C.

Specifically, the H₂/CO₂-fed methanogenic activity at 15°C and 20°C increased by 6-6.4 times and this is two times higher than activity increment at 35°C. Previous studies have also reported a significant increase of methanogenic activity (through increased acetate-fed and H₂/CO₂-fed methanogenesis) in anaerobic sludge at low temperatures (11-22°C)

These results are evidence of a low-temperature adaptation in hydrogenotrophic bacteria in the retained sludge due to low temperatures of the reactor. This tendency was also confirmed by an Arrhenius plot of hydrogen-fed methanogenic activity of retained sludge (data not shown).

The activity of the retained sludge attributable to acetoclastic-methanogens was 2.3 - 3.9 times higher than that of the seed sludge at temperatures ranging from 10°C to 45°C. The increment of acetate-fed activity was slightly higher at 15°C.

The propionate-fed activity (due to acetogenic bacteria) followed a consistent pattern for all test temperatures. This result indicates that no significant changes of dominant acetogenic bacteria during low temperature operation of the reactor. In addition, mesophilic propionate-degrading bacteria was able to grow at 20°C to 40°C (optimum temperature: 37°C). This wide range of temperature dependency caused the good activity to the low temperature condition.

![Figure 4](image-url) Increment of the methanogenic activity of the retained sludge on day 196 as compared with the activity of the seed sludge

![Figure 5](image-url) DGGE profiles of retained sludge with respect to 16S rDNA of the Domain Bacteria

### (4) Microbial community structure changes

Changes in the microbial community structure of the retained sludge were investigated by 16S rDNA-targeted DGGE analysis. The intensity of DGGE band shows a relative amount of DNA (population size) of bacterial group in samples (amplified DNA product).

In Fig. 5, the DGGE profiles of community structure of the Domain Bacteria are presented. No significant differences were observed between the community structures of Bacteria and the 20°C-grown seed sludge (Fig. 5). We confirmed the predominance of sugar-degrading *Lactococcus* (band 7) and *Aerovibrio* (band 9) in the retained sludge. A relative population size of these acid forming bacteria (bands 7, 8 and 9) tended to increase during low temperature operation (5°C to 10°C) of the reactor.

The propionate-degrading acetogen *Syntrophobacter fumaroxidans* was also detected (bands 4 and 5). Until the operational temperature of 10°C, propionate-fed activity kept high level. However, 5°C operation caused the significant decrease of propionate-degrading activity (Fig. 3). At this time, we confirmed the decreasing of relative population size of *Syntrophobacter* (bands 4 and 5).
DGGE profiles of the Domain Archaea are illustrated in Fig. 6. The acetoclastic methanogenetic genus *Methanosaeta*, corresponding to band 4 (99% homology to *Methanosaeta concilii* strain: H-3, AB212065), was abundant throughout the experiment. The species at DGGE-band 2 was closely related to *Methanobacterium formicicum* (99% homology to strain S1, DQ649309) and was a major hydrogen-utilizing methanogen in the retained sludge.

Decreasing the operational temperature caused the proliferation of *Methanospirillum* (band 5; 98% homology to Uncultured *Methanospirillum* sp. clone DI_E07, AY454786). On the other hand, populations of *Methanobacterium* spp. corresponding to bands 1 (96% homology to *Methanobacterium oryzae*, AF028690) and 3 (98% homology to *Methanobacterium aarhusense*, AY386124) tended to decrease during operation of the reactor at lower temperatures.

In order to determine the 16S rRNA gene sequence of *Methanospirillum* in the retained sludge, clone analysis was conducted for the order *Methanomicrobiales*. The DNA extracted from day 242 (Phase 2, 10°C)-sludge was used as the template for PCR amplification. Forty of 41 clones belonged to the family *Methanospirillaceae*. Fig. 7 shows the phylogenetic relationships between the clones of the family *Methanospirillaceae*.

Clone D242_16 was detected most frequently (21 of 41 clones). Furthermore, 38 of 41 clones were grouped in cluster 2 of Fig. 7. The cluster 2a includes the *Methanospirillum* sp. strain TM20-1 that had been isolated from paddy field soil by 20°C enrichment with H₂/CO₂. Most of clones in cluster 2a originated from ambient temperature treated samples, such as clone PL-21A5 (AY570675.1) obtained from a low-temperature oil reservoir, clone 103 (uncultured archaeon, AJ831041) obtained from landfill leachates, and clone LF-H2-A (AB236096.1) obtained from lotus field sediment.

On the other hand, only 2 of 41 clones were placed in cluster 1 (in Fig. 7). Cluster 1 includes *Methanospirillum hungatei*, a mesophilic H₂/CO₂-utilizing methanogen. This cluster is mainly composed of clones obtained from mesophilic UASB granular sludge.

From these results, we concluded that operating the EGSB reactor at low temperatures caused changes in the community structure of hydrogen-utilizing methanogens because of the associated retention time. On the other hand, no changes in the community structure of acetoclastic methanogens were detected.
4. CONCLUSIONS

The EGSB reactor performed well until the operating temperature reached 10°C (Phase 2). During Phase 1 (15°C) and Phase 2 (10°C), a significant increase in methanogenic activity of the retained sludge was observed, and the sludge’s physical properties persisted. Using granular sludge as seed material for the start-up of an EGSB reactor is an effective way of retaining the sludge long enough for the accumulation of anaerobic bacteria, including psychrotolerant *Methanospirillum*, even at low temperatures.

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