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Microbial and Physicochemical Monitoring of Granular Sludge During Start-up of Thermophilic UASB Reactor

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Abstract Mesophilically-grown granular sludge seeded in thermophilic UASB reactor was monitored to better understand the start-up process of the reactor. The reactor was fed with a synthetic wastewater containing glucose. As COD loading rate increased stepwise, methane production rate increased. Maximum values of COD removal efficiency (95%) and methane production rate (5.3 l/day) were achieved by approximately day-80 and remained constant afterward. However, physicochemical and microbial properties of granules kept changing even after day-80. Specific methanogenic activity (SMA) was initially negligible, and increased continuously until day-153 and remained constant afterward, showing the maximum value of 1.51±0.13 g CH_4-COD/g VSS/day. Deteriorated settling ability of granules recovered the initial value by day-98 and was maintained afterward, as determined by sludge volume index. Initially reduced granule size increased until day-126, reaching a plateau of 1.1 mm. Combined use of fluorescence *in situ* hybridization and confocal laser scanning microscopy (CLSM) allowed to localize families of *Methanosaetaceae* and *Methanosarcinaceae* in granules with time. Quantitative analyses of CLSM images of granule sections showed abundance patterns of the methanogens and numerical dominance of *Methanosaeta* spp. throughout the start-up period. The trend of SMA agreed well with abundance patterns of the methanogens.

Key words: Confocal laser scanning microscopy, fluorescence *in situ* hybridization, granular sludge, methanogens, thermophilic UASB reactor

Upflow Anaerobic Sludge Blanket (UASB) system has been widely applied to treat various wastewaters [16, 25]. Unlike other wastewater treatment systems [12, 13, 15, 30], the UASB system employs mixed microorganisms spatially self-organized in granular sludges that convert organic wastes to methane under anaerobic conditions. Methane production is generally indicative of the performance of a UASB reactor.

Thermophilic (45-65°C) UASB sludge usually shows much higher metabolic rates than a mesophilic (30-40°C) one, allowing shorter retention time of wastewater and better performance of the reactor, as determined by higher rate of methanogenesis [25]. Besides, the thermophilic anaerobic process can take advantage of high temperature of wastewaters coming from industries such as canneries, dyeing, distillery, and paper mill.

The keys to successful start-up of the UASB reactors are development and maintenance of the granular sludge and its gas production. The start-up of a thermophilic UASB reactor generally employs anaerobic digester sludge or mesophilically-grown granular sludge as inoculum. The latter has been reported to be more advantageous on the basis of start-up time, chemical oxygen demand (COD) removal efficiency, settling ability, and specific methanogenic activity (SMA) [8, 19, 28].

Most full- or pilot-scale UASB reactors are being operated at mesophilic or ambient temperatures despite better performance of thermophilic reactors [16, 18, 26]. Difficulty in thermophilic granule development during the start-up period is one of the reasons why few thermophilic UASB reactors are under operation in full- or pilot-scale. The mechanism of granular sludge development is not yet fully understood. While studies on the development of mesophilic granules have been conducted extensively so far, basic information on thermophilic granule development is still very limited [5, 25]. The lack of detailed information on the UASB sludge hampers successful start-up, which is a prerequisite for successful operation of a UASB reactor.

Since growth rates of fermentative bacteria are 5-10 times faster than methanogens [25], slow growth of methanogens is rate limiting for the granule development and anaerobic process. In particular, growth rates of acetoclastic methanogens are important, because acetate is not only the main intermediate in the anaerobic digestion process

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but also the major precursor for methanogenesis [25]. Approximately two-thirds of the produced methane is derived from acetate. Methanogens belonging to only two genera, *Methanosaeta* and *Methanosarcina*, are known to grow in acetoclastic mode [4]. *Methanosaeta* spp. produce methane solely from acetate, while *Methanosarcina* spp. form methane from acetate and from other substrates such as H_2/CO_2, methanol, and methylamines.

16S rRNA-targeted fluorescence *in situ* hybridization (FISH) combined with confocal laser scanning microscopy (CLSM) revealed layered structures of thermophilic and mesophilic granules. The outer layer consisted mainly of bacterial cells, while the inner layer contained mainly archaeal cells. The genus *Methanosaeta* was dominant among the methanogens localized inside the granules at steady state [24, 27]. However, no information is available on the relevant abundance of methanogens and development of the layered structure during the start-up period.

In this study, inoculated mesophilic granular sludge was monitored during the start-up of a thermophilic UASB reactor, and its dynamic changes in physicochemical and microbial aspects were revealed. Physicochemical characteristics of the granules were analyzed by measuring SMA, settling ability, and granule size, while microorganisms in granular sludge were observed by 16S rRNA-targeted FISH combined with CLSM. FISH combined with CLSM allowed monitoring developments of layers with acetoclastic methanogens. The results obtained in this study will help to understand development of thermophilic UASB granules during start-up of a UASB reactor.

MATERIALS AND METHODS

Start-up and Operation of UASB Reactor

This study employed a laboratory-scale UASB reactor (4.5 l; inner diameter, 9 cm) as shown in Fig. 1. The reactor was operated at 55°C and fed with a synthetic medium containing glucose and inorganic minerals [7]. COD load was increased stepwise from 1.3 to 4 g COD/l/day by changing hydraulic retention time (HRT), while keeping influent COD (2 g/l) constant. The inoculum (70 g VSS) was mesophilically (35°C) grown granular sludge obtained from another UASB reactor which was fed with the same synthetic wastewater. Sludge granule samples were taken from a port at a height of 19.5 cm from the bottom of the reactor and used for microbial and physicochemical tests. Sludge granules were stored in the synthetic medium containing glycerol (20%, v/v) at -80°C until used for *in situ* hybridization with fluorescently labeled probes.

Analytical Methods

SMA was measured in duplicate with 40-ml serum bottles sealed with butyl rubber stoppers and aluminum crimps, as

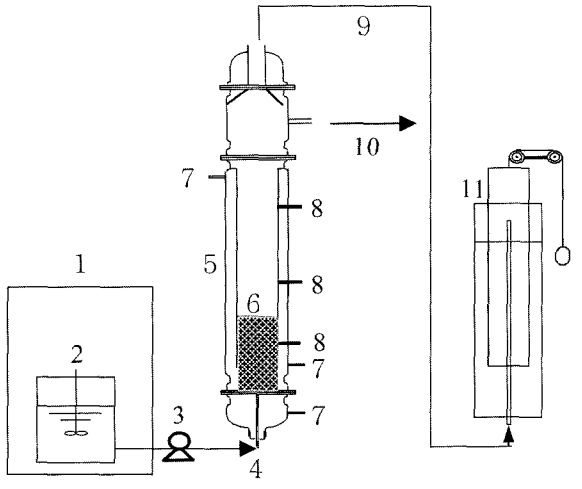

Fig. 1. Schematic diagram of the UASB reactor used in this study. 1, Cold room (4°C); 2, medium; 3, peristaltic pump; 4, influent; 5, UASB reactor; 6, sludge granules; 7, ports of water jacket (55°C); 8, sampling ports; 9, biogas outlet; 10, effluent; 11, gas collector.

described by Ahn *et al.* [1]. The methane content in the biogas was measured by a gas chromatograph (Model 680D; Young-In, Seoul, Korea) equipped with a thermal conductivity detector and a stainless-steel column (1.8 m× 0.3 cm) packed with Propack Q (80/100 mesh, Altech, Deerfield, IL, U.S.A.). The temperatures of the column, the injection port, and the detector were 70, 100, and 80°C, respectively. Helium was the carrier gas at a flow rate of 30 ml min^{-1}. The equation proposed by Yukselen [31] was used to calculate SMA.

Liquid samples were filtered through a 0.45 μm membrane filter and analyzed for soluble COD (sCOD) by a colorimetric method according to the manufacturer's instruction (DR-2000; Hach, Loveland, CO, U.S.A.). VSS, TSS (total suspended solid), and alkalinity were measured, according to the analytical procedures of the Standard Methods [3]. Settling ability of granular sludge was determined using sludge volume index (SVI), as described by the Standard Methods. Granule size was measured as described by Ahn *et al.* [2], and the median diameter was used to represent the size of sludge granules using the equation [20].

In Situ Hybridization of Sludge Granules with Fluorescently Labeled Probes

Granules thawed in ice were gently washed three times with two volumes of phosphate-buffered saline (PBS; 0.13 M NaCl, 10 mM Na_2HPO_4, pH 7.2) and fixed with 4% paraformaldehyde in PBS for overnight at 4°C. Fixed granules were incubated in 10 mM EDTA for 30 min to enhance probe penetration. The granules were then rinsed twice with PBS. The rinsed granules were serially dehydrated by 50, 80, and 100% ethanol, 50:50 (v/v) ethanol-xylene, and 100% xylene. Dehydrated granules were embedded in melted paraffin wax. Granule sections of 10-μm thickness

were obtained using a rotary microtome (Model HM 325; Microm, Walldorf, Germany) and mounted on poly-L-lysine coated glass slides. The sections were dewaxed through 100% xylene (2 times) and 100% ethanol (2 times), and dried at room temperature. Dried samples were used for FISH.

FISH was performed with middle sections of average-sized granules obtained at each time point in Fig. 5. The 16S rRNA targeted oligonucleotide probes used in this study were as follows: EUB338 for domain *Bacteria*; ARC915 for domain *Archaea*; MX825 for family *Methanosaetaceae*; MS1414 for family *Methanosarcinaceae* [23]. Probes 5'-end labeled with either Cy5 or TAMRA (tetramethyl rhodamine) and purified by reverse-phase high-pressure liquid chromatography were obtained from ThermoHybaid GmbH (Ulm, Germany). Specificity of each probe was confirmed by whole cell hybridization using the following reference organisms (data not shown): *Methanosarcina barkeri* DSM 800; *Methanosaeta concilii* DSM 3671; *Methanobacterium formicicum* DSM 1312; *Escherichia coli* DH5α. For *in situ* hybridization, the labeled probes were employed separately for single staining or simultaneously for double staining of the sections. For double staining, the probe requiring a higher stringency was employed for the first hybridization and washing, followed by the second hybridization with the other probe. *In situ* hybridization was performed at 46°C for 10 h with hybridization buffer [0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.01% sodium dodecyl sulfate] containing 2.5 ng each of probe per μl of hybridization buffer [27]. Formamide was added to the hybridization buffer to control stringency in the hybridization and washing steps: 5% for EUB338, 20% for MX825, and 35% for ARC915 and MS1414. Unhybridized probes were washed at 48°C for 30 min with washing buffer containing the same components as hybridization buffer, except for the probes. Washed sections were air dried and examined under a microscope.

Confocal Laser Scanning Microscopy, Image Processing, and Analysis

A confocal laser scanning microscope (Model LSM 510; Carl Zeiss, Jena, Germany) equipped with two HeNe lasers (543 nm and 633 nm) was used to visualize the sections hybridized with the probes. Fluorescence and transmitted-light images were collected simultaneously by 2- and 3-channel imaging procedures. Imaging was achieved with a 10×/0.3 objective lens in combination with laser intensity of 100%, pinhole setting of 1.82, and detector gain of 826. Images of granule sections hybridized with TAMRA-labeled probes were visualized by excitation at 543 nm and emission at 560-615 nm (filter BP560-615), whereas Cy5-labeled probes were visualized by excitation at 633 nm and emission at 650 nm afterward (filter LP650). Scanned images of granule sections were acquired by setting frame size of 1,024×1,024 pixels, where one pixel area was $1.27 \times 1.27\ \mu m^2$.

Digital images obtained by CLSM were processed and analyzed with the standard software package (Version 2.01, Carl Zeiss) installed in the instrument to determine size of the fluorescence area (number of pixels) in granule section image. Pixel number was used to determine the area of specific cells revealed by binding of 16S rRNA-targeted probe to target cells. Each pixel of digitized image was assigned a value between 0 and 255 in proportion to brightness (the intensity of light) at the point in the image. CLSM images were thresholded to define the object prior to the measurement of fluorescence area [14]. The lowest- and highest-intensity thresholds of fluorescent images were 35 and 254, respectively. The lowest threshold set at 35 was to exclude background signals. Quantitative analysis was performed using CLSM images obtained under the same microscopic magnification. Microsoft Excel was used for statistical analysis.

Results and Discussion

Monitoring of Reactor Performance

Methane production and COD removal efficiency are the most commonly used parameters to determine performance of a UASB reactor. The UASB reactor employed in this study was monitored using the parameters for more than 7 months of operation (Fig. 2). As changing HRT increased COD load stepwise, COD removal efficiency increased gradually up to 88% within the first 40 days. COD removal efficiency eventually reached a plateau of 95% from day-80, with COD loading rate of 4 g COD/l/day and HRT of 12 h. As COD loading rate increased, methane production rate increased as well. Maximum values of COD removal

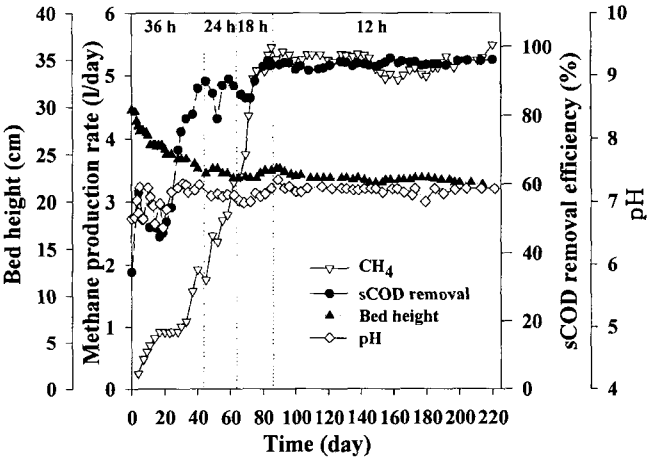

Fig. 2. Performance of thermophilic UASB reactor during the start-up.
Hydraulic retention times were indicated on the top of the graph.

efficiency (95%) and methane production rate (5.3 l/day) achieved by approximately day-80 were maintained constant afterward. Mass balance based on COD removal and methane production rate suggested that the produced amount of methane was 85% of theoretical production, calculated on the basis of removed COD. The remaining 15% could be converted to CO_2 and biomass.

The amount of inoculum was 70 g VSS that resulted in initial bed height of 29.5 cm (Fig. 2). The initial bed height decreased to 23 cm within the first 60 days and remained constant afterward. Effluent pH ranged from 6.8 to 7.3 throughout the experiment.

Monitoring of Granule Properties

Although the reactor showed steady performance in terms of COD removal efficiency and methane production rate from day-80, physicochemical and microbial properties of sludge granules kept changing even after the day, suggesting that COD removal efficiency and/or methane production rate are not fully representative of the status of the UASB reactor during the start-up period. Therefore, physicochemical and microbial monitoring of granular sludge could provide more information on start-up of the thermophilic UASB reactor.

Physicochemical characteristics of the granules were monitored by measuring SVI, granule size, and SMA (Fig. 3). Mesophilically grown granular sludge started to disintegrate after inoculation into the thermophilic reactor, as observed by a microscope (data not shown). Disintegrated granules then gradually recovered their structural integrity and density, suggesting development of thermophilic sludge granules. The observed integrity and density were correlated to settling ability of the granules. Settling ability of granules gradually deteriorated during initial operation of the reactor, as determined by SVI. Initial SVI (25 ml/g VSS) increased to 104 ml/g VSS during the first 50 days. Settling ability improved since then and eventually recovered initial settling ability by day-98, maintaining constant SVI afterward. Improved settling ability could be the basis of maintaining constant bed height despite of shortened HRT (Fig. 2). Since anaerobic process occurs in the sludge bed of the UASB reactor, developing granules with good settling ability is essential to establish a dense sludge bed and to maintain biomass in the reactor.

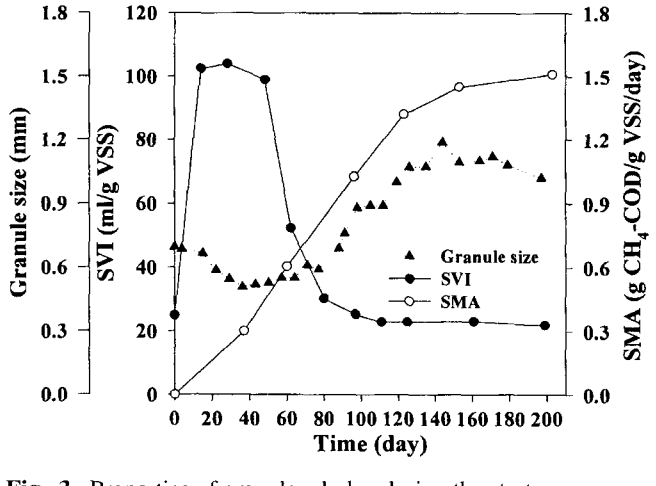

Fig. 3. Properties of granular sludge during the start-up.

The mean diameter of granular sludge gradually decreased until approximately day-50 (Fig. 3) due to partial disintegration of the inoculated granules, as observed by a light microscope (data not shown). Disintegrated granules recovered their integrity gradually afterward and the mean diameter increased until day-126, reaching a plateau of 1.1 mm.

SMA was initially negligible, probably due to shock caused by increased temperature. SMA increased continuously until day-153 and remained constant afterward, showing the maximum value of 1.51±0.13 g CH_4-COD/g VSS/day. This suggested a continuous increase in the number and/or activity of thermophilic methanogens until approaching steady state as thermophilic granules developed. Our previous study [1] showed that both coenzyme F_{420} contents and F_{420}-based autofluorescence values correlated well with SMA during the start-up of a thermophilic UASB reactor seeded with mesophilic granules. F_{420} is a two-electron carrier and plays as an electron donor in methanogenesis [25].

Monitoring of Microorganisms in Sludge Granules

FISH technique is shown to be useful to identify, visualize, localize, and enumerate microorganisms of interest [10, 23, 27]. Microorganisms in sludge granules were monitored by FISH combined with CLSM in this study. Simultaneous *in situ* hybridization with bacterial domain and *Methanosaetaceae* specific probes revealed layered structures of granules which were sampled on day-143 (Fig. 4A). Methanogens were present mainly in the inner layer, while bacterial cells were found mostly in the outer layer. Such layered architecture was also proposed or observed in anaerobic sludge granules mainly fed with carbohydrates [9, 10, 17, 22].

Specific methanogens in a sludge granule were monitored and quantified by *in situ* hybridization with a single probe specific to either family *Methanosaetaceae* or *Methanosarcinaceae* to avoid ambiguous quantification that could be caused by overlapping of cells probed by simultaneous *in situ* hybridization with two different probes (Figs. 4B to 4F). *Methanosaeta* is the only genus that is defined so far within the family of *Methanosaetaceae*, while genus *Methanosarcina* and other five genera (*Methanococcoides*, *Methanohalobium*, *Methanohalophilus*, *Methanolobus*, and *Methanosalsum*) belong to the family of *Methanosarcinaceae* [4]. The five genera in the family of *Methanosarcinaceae* are known to grow only on methyl

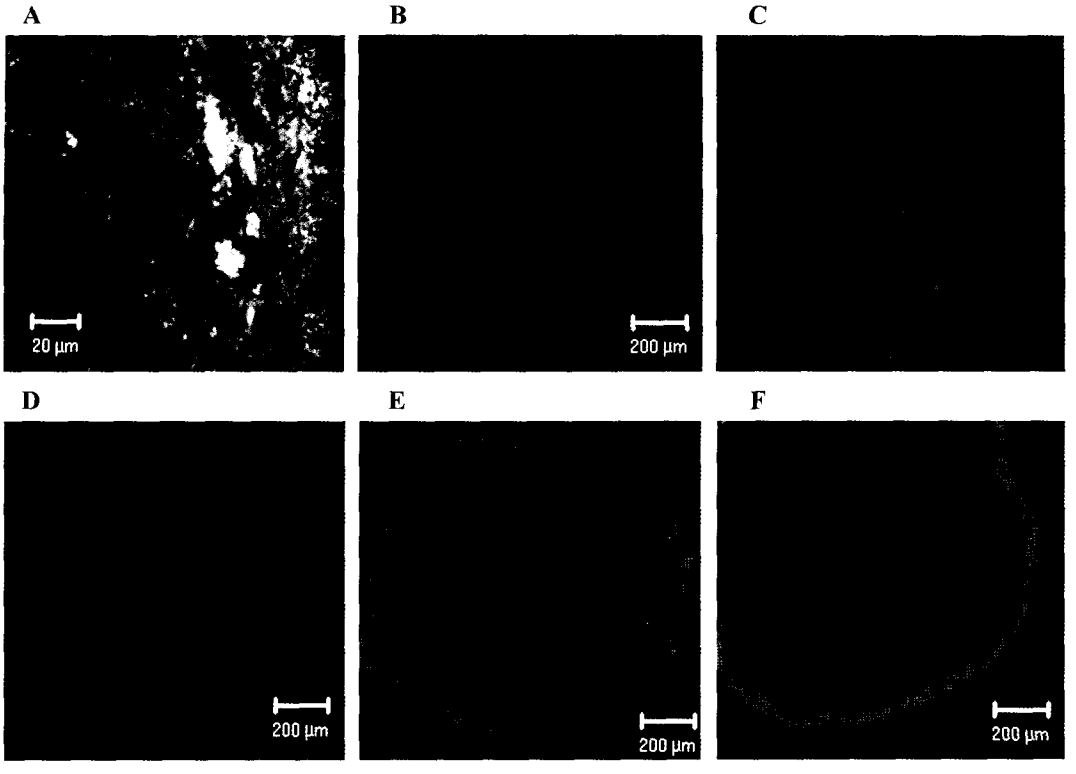

Fig. 4. Fluorescence *in situ* hybridization of granular sludge during the start-up.
A, Simultaneous *in situ* hybridization with Cy5-labeled EUB338 probe for bacterial domain (green) and TAMRA-labeled MX825 probe for the family *Methanosaetaceae* (red). B to F, Granule sections were hybridized with TAMRA-labeled MS1414 probe (B and C) or MX825 probe (D to F). Granule samples were taken on days 34 (B and D), 143 (A, C, and E), and 170 (F), respectively. Except for A, all pictures were obtained using the same magnification. Scale bars indicate 20 μm (A) and 200 μm (B to F).

compounds such as methanol, methylamines, and methyl sulfides. Genera *Methanosaeta* and *Methanosarcina* are acetoclastic methanogens. *Methanosaeta* spp. produce methane solely from acetate while *Methanosarcina* spp. form methane from acetate and from other substrates such as H_2/CO_2 and methyl compounds.

Layered structure of the probed methanogens shown in inoculated granules became undefined during the initial operation of the reactor (data not shown), due to partial disintegration of inoculated granules and probably death of mesophilic methanogens. Considering the fact that rRNA (target of probes used in this study) content is correlated directly with the degree of detection signal (intensity of fluorescence), 16S rRNA below detection level suggested that the methanogens belonging to the two families were moribund or stressed by high temperature (55°C), as determined by FISH [6, 21, 29].

Samples taken on day-34 (Figs. 4B and 4D) started to show faint layer and small clusters of *Methanosaetaceae* or *Methanosarcinaceae* inside the granule. The hint of layer became thicker and better defined afterward (Figs. 4C, 4E, and 4F). This result is in good agreement with the results of SMA and F_{420} content that started to increase at around the time mentioned above [1]. The observed faint layer suggested that the target microorganisms started to grow, but they were still inactive and/or acclimating at the time. The layer of *Methanosaetaceae* or *Methanosarcinaceae* became thicker and better defined with time, suggesting a continuous increase in the number and/or activity of thermophilic acetoclastic methanogens until reaching steady state. Overall, *Methanosaetaceae* was dominant compared to *Methanosarcinaceae*, as determined by visual observation of thickness and density of their layers revealed by FISH. Previous report [27] also showed dominance of *Methanosaetaceae* over *Methanosarcinaceae* in thermophilic UASB granules at steady state.

Quantitative Analysis of Specific Methanogens in Granule Section Using CLSM Image Analysis

Abundance specific methanogens in sludge granule section were quantified by analyzing digitized image of probe-targeted methanogens. It is based on the assumptions that an increase in cell number results in an increase in pixel number in the threshold range and that probes used in this study do not have penetration problem in FISH, if any, being equal to all target microorganisms.

Figure 5 shows abundance (based on increase in pixel density) of methanogens, belonging to *Methanosaetaceae*

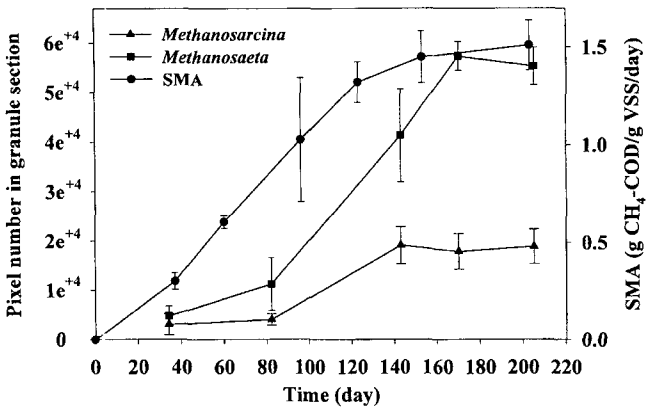

Fig. 5. Abundance of families *Methanosaetaceae* and *Methanosarcinaceae* in sludge granule as determined by quantitative analysis of CLSM image of the granule section.
Granule sections were hybridized with either MS1414 probe or MX825 probe to produce CLSM images. Pixel number indicates number of pixels in the threshold range as described in Materials and Methods. Error bars represent standard deviations of the mean; n=2 experimental sets for SMA while n=9 (3 sections each from 3 granules) for quantitative analysis of digital images.

and *Methanosarcinaceae*, in granule sections during the start-up period of the reactor. The trend of SMA agreed well with abundance patterns of the methanogens. Number of methanogens initially decreased by day-34 (data not shown) and thereafter increased continuously until reaching a plateau. Initially decreased number of cells was probably caused by death of mesophilic cells due to increased temperature, whereas increased cell number observed later on was probably caused by enrichment of thermophilic methanogens. *Methanosarcinaceae* increased by day-143 and remained constant afterward, while *Methanosaetaceae* grew continuously even after day-143 and reached a plateau by day-170. Decreased microbial growth rate could result from competition among mixed microbial species for substrate and space in biofilm [32]. Although the specific growth rates (μ) of *Methanosaetaceae* and *Methanosarcinaceae* could not be measured in this study, it is obvious that *Methanosaeta* spp. were dominant in number throughout the start-up period based on quantitative analysis of CLSM images.

To the best of our knowledge, the nonculture-based method used in this study is the first attempt to measure relative abundance of the two important phylogenetic groups of methanogens in granular sludge during the start-up of a thermophilic UASB reactor. Growth rates of thermophilic *Methanosarcina* and *Methanosaeta* spp. were reported using their suspended cultures [11, 33]. However, their growth rates in sludge granule are unknown. Nonculture-based methods such as slot blot hybridization and real time polymerase chain reaction are under study to obtain more information on the growth rates of the methanogens during the start-up.

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