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Microbial populations associated with fixed- and floating-bed reactors during a two-stage anaerobic process

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Microbial populations associated with methanogenic fixed- or floating-bed bioreactors used for anaerobic digestion of lignocellulosic waste were investigated. Fluorescent in situ hybridization (FISH) was used to characterize microorganisms in samples obtained from different heights in the reactors, which were operated in a semi-continuous manner (feeding and mixing once every 2 days). The FISH results showed that *Methanosaeta concilii* cells were most numerous at the bottom of both reactors. *M. concilii* cells were more abundant in the fixed-bed reactor (FXBR), which performed better than the floating-bed reactor (FLBR). Species of the *Methanosarcina* genera (mainly *M. barkeri* and *M. mazei*) were also observed in the FLBR but rarely in the FXBR. Methane production in each of the reactors ranged from 0.29 to 0.33 m^3 CH_4/kg COD_{rem} (chemical oxygen demand removed). The removal of volatile fatty acids (VFA; 70–75 h) in the FXBR was more efficient than in the FLBR. [**Int Microbiol** 2007; 10(4):245-251]

Key words: *Methanosaeta* · *Methanosarcina* · anaerobic digestion · methanogenesis · bed reactors

Introduction

To treat different types of wastes efficiently, anaerobic technology has evolved over the years from low- to high-rate reactors, such as upflow anaerobic sludge blanket (UASB), anaerobic filter, upflow fluidized bed, fixed- and floating-bed, expanded granular sludge bed (EGSB), hydrolysis upflow sludge bed (HUSB), and anaerobic hybrid (AH) reactors [9,18,21,29]. However, questions about the performance of different reactors treating the same waste and about the role of different support materials on the maintenance of microbial populations established in the reactors remain unanswered.

Of the many methanogenic genera, only two, *Methanosaeta* and *Methanosarcina*, are known to grow acetoclastically, producing methane from acetate [20,31]. At high acetate concentrations, the growth rates of *Methanosaeta* spp. are lower than those of *Methanosarcina* spp., but their affinity for acetate is five to ten times higher [27]. *Methanosaeta concilii* is solely acetoclastic and is the only mesophilic species of its genus [25]. *Methanosarcina barkeri* is metabolically the most versatile of all the mesophilic methanogenic archaea isolated in axenic culture, since it can form methane from H_2, and CO_2 from methanol, and methylamines and acetate [10].

Occasionally, an increased concentration of propionic acid (more than 1000 mg/l) can inhibit methanogenesis, with a concomitant decline in gas production and chemical oxygen demand (COD) removal efficiency [28]. The accumula-

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tion of volatile fatty acids (VFA) in the anaerobic digester reflects the kinetic uncoupling between acid producers and consumers, which may result in a shift of the metabolic pathway to a less favorable one [4,8,21]. Under these conditions of unstable operation, the accumulation of intermediates leads to process failure [6,30]. Anaerobic digestion processes also have some limitations, namely, the hydrolysis of wastes, which are polymers. The major biomass polymers cellulose, hemicellulose, and amylopectin are difficult to degrade for most anaerobes whereas lignin is recalcitrant to degradation by most bacteria [15,24]. Only rumen microbial consortia are known to have the capability to degrade these substances in a relatively short time (1–2 days).

Here, we report our observations on the ability of microbial populations associated with different support materials (fixed and floating) in laboratory-scale two-stage anaerobic fixed- and floating-bed bioreactors (FXBR and FLBR, respectively) to treat lignocellulosic waste (grass). In these experiments, rumen content was used as the inoculum source. Microbial populations at different heights in the reactors were investigated using fluorescent in situ hybridization (FISH) coupled to confocal laser scanning microscopy (CLSM). Chemical analyses were carried out to monitor digester performance.

Materials and methods

Feed material. Roadside grass was used as the lignocellulosic organic feed substrate [COD, 1200 g/g; total solids (TS), 35%; organic solids (OS), 70%; cellulose, 28%; hemicellulose, 14%; lignin, 18%] to compare the process efficiency of FXBR and FLBR. A slurry of the grass prepared with tap water at a ratio of 1:10, 1:5, and 3:10 (w/v) and 10% (v/v) rumen content (partially digested feed material in the rumen), as a source of inoculum for efficient hydrolysis, were added. The feed material was hydrolyzed in a 6-l glass reactor (first stage) under mesophilic (37°C) and semi-anaerobic conditions at a hydraulic retention time (HRT) of 2 days, as optimized in our earlier studies [Elucidation mechanism of organic acids production from organic matter (grass) using digested and partially digested cattle feed. Proc. 2nd IWA specialised conference on Environmental Biotechnology New Zealand, 2002, pp 403-408]. The characteristics of the feed after hydrolysis (hydrolyzed feed) were: COD, 20 g/l; TS, 12%; OS, 75%; cellulose, 6%; hemicellulose, 2%; lignin, 6%. The hydrolyzed feed was filtered through a sieve (1.4-mm pore size) and grass fibers were separated and removed. The filtrate was diluted with tap water to obtain final concentrations of 2, 4, 8, and 12 kg COD/m^3 day. The liquid slurry was then fed into the FXBR and FLBR for methanogenesis (second stage).

Methanogenesis (reactor set-up). For methanogenesis, 6-l glass reactors with 5-l working volumes were used (Fig. 1). The lids of the reactors had four openings, each fitted with a plastic pipe. The first two openings, used as feed inlet and outlet, were connected with a two-way pump. The other two openings were used as gas outlets and sampling ports/pH adjustment (if required). Gas counters were attached to the reactors to continuously monitor the volume of biogas production. One-third of the volume of the FLBR and the FXBR was filled with foam cubes (volume of 1×10^{-6} m^3) or clay beads (diameter 4.5–8 mm), respectively, as support material. The reactors were designed to ensure effective contact between the feed and the anaerobic biomass attached to the surface of the support material. To achieve this, the feed was introduced through the top in the FLBR and through the bottom of the FXBR. In addition, a metal sieve of 5-mm pore size was fitted in the FLBR (Fig. 1) to prevent the foam cubes from coming into contact with headspace gases. Second-stage reactors were fed at loading rates of 2, 4, 8, and 12 kg COD/m^3 day at different times and in a sequential manner.

Acclimatization of second-stage reactors and analyses. Four liters of effluent from an already-running reactor was mixed with 1 liter fresh feed and poured into the second-stage glass reactors. The reactors were sealed and flushed with nitrogen gas for 10 min (flow rate of 0.1 l/ min) to remove oxygen and to provide an anoxic atmosphere. The reactors were then kept at 37°C for 15–30 days for acclimatization and enrichment of the microbes.

Organic acids were estimated every second day by gas chromatography using a 30-m DB-FFAP column and helium as the carrier gas. COD, TS, and OS were analyzed by standard procedures (DIN 38414). Gases were analyzed after every 24 h by an infra-red gas analyzer (Model GA 94, Ansyco, Karlsruhe, Germany). The reactors were eventually fed in a semi-continuous manner by the provision of 2.5 l hydrolyzed feed on alternate days. The overall hydraulic retention time (HRT) of the process was maintained at 6 days (2 days for hydrolysis and 4 days for methanogenesis). The experiments were carried out twice and in duplicate for 10 cycles at each loading rate and the results are presented as the mean values.

Calculation of biodegradability. Although several kinetic models can be fitted to anaerobic digestion processes, the first-order model (Eq. 1), linearized as given in Eq. 2, is adequate and simple, and permits deduction of the biodegradation rate constant [19].

$$-\frac{dS}{dt} = kS \qquad (1)$$

$$\frac{S_o - S_t}{S_t} = kt \qquad (2)$$

$$t\,\frac{1}{2} = 1/k \qquad (3)$$

where S = substrate concentration (mg/l), t = time (days), k = biogasification rate constant and

$$t\,\frac{1}{2} = \text{time required for 50\% removal.}$$

The percentage of substrate converted to other forms during digestion time t (days) is given by Eq. 4.

$$X\,(\%) = [1 - 1/(1 + kt)] \times 100 \qquad (4)$$

where X = percentage of converted substrate.

Sample collection. Reactor samples was collected from the inlet and outlet (for effluent sampling). Samples were also collected from different heights of the reactor by using 10-ml sterile plastic syringes inserted into the sampling ports. Two ml of the collected sample were fixed in paraformaldehyde [1] and the rest was used for chemical analysis (VFA and COD). As the reactors were operated in a semi-continuous manner by feeding and mixing once every 2 days, different population could become established at different reactor heights.

Sample preparation. Fixed samples were homogenized for 8 min on ice with a homogenizer run at 2000 rpm (RW20 DZM Janke & Kunkel IKA Labortechnik, Staufen, Germany), and applied directly into wells on gelatin-coated slides [22] or diluted prior to their application on the slides. The dilution was obtained by adding 10 ml of sample to 490 ml of a 0.1% solution

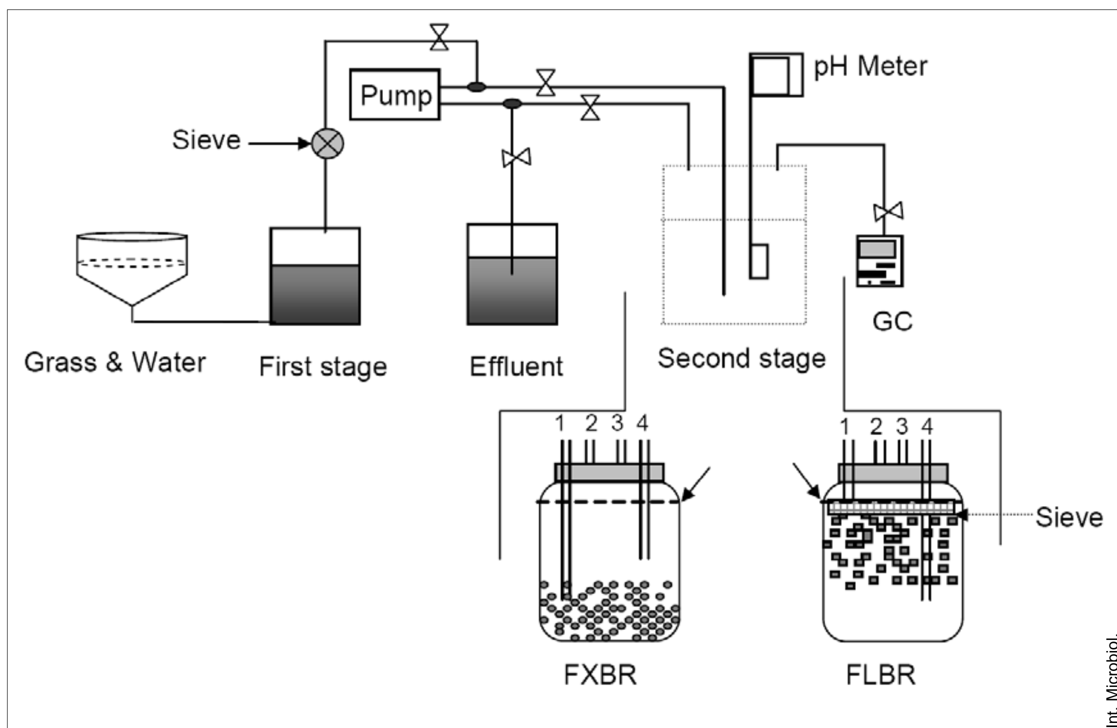

Fig. 1. Two-stage anaerobic reactor design used to study the effect of support material on process efficiency and microbial population. 1, Feed inlet; 2, sampling port/pH adjustment; 3, gas outlet; 4, effluent outlet.

of sodium pyrophosphate. Samples were immobilized on glass slides by air-drying, followed by serial ethanol dehydration (50, 80, and 100%), and used for hybridization.

Oligonucleotide probes and fluorescence in situ hybridization (FISH). Unless otherwise stated, information on the oligonucleotide probes and hybridization conditions used in this study were obtained from probeBase [17]. The following probes were used: ARCH915 and ARC344 [22], are specific for Archaea, EUB338 [1], EUB338 II and EUB338 III [5], specific for Bacteria, Planctomycetales, Verrucomicrobiales, respectively, KOP1 specific for propionate oxidizer strain KOPROP1, MPOB1 propionate oxidizer strain MPOB [12], MB310 specific for *Methanobacterium* spp., *Methanobrevibacter* spp., *Methanosphaera* spp. MC1109 for *Methanococcus* spp. MG1200 for *Methanomicrobium* spp., *Methanogenium* spp., *Methanoculleus* spp., *Methanospirillum* spp., *Methanocorpusculum* spp., *Methanoplanus* spp., MS821 for *Methanosarcina frisius*, *Methanosarcina* spp., *Methanosarcina acidovorans*, *Methanosarcina thermophila*, *Methanosarcina barkeri*, *Methanohalophilus mahii*, *Methanophilus* spp., MS1414 for *Methanosarcina* spp., *Methanococcoides* spp., *Methanolobus* spp., *Methanohalophilus* spp. *Methanosarcina mazei*, *Methanococcoides* spp., *Methanolobus* spp., *Methanohalophilus* spp., *Methanosaeta* spp., MSMX860 for *Methanosarcina* spp. and MX825 *Methanosaeta* spp., SRB385 specific for some sulfate reducing bacteria of the Deltaproteobacteria, other Deltaproteobacteria, and gram-positive bacteria [1]. All oligonucleotide probes labeled with the fluorescent cyanine dies Cy3 or Cy5 and were purchased from MWG Biotech (Ebersberg, Germany). FISH was performed as described by Raskin et al. [22] with some modifications in terms of temperature, incubation time [Fall, PAD (2002) PhD Thesis No.172; Technical University of Munich, Germany].

Microscopy and image processing. An Aristoplan epifluorescence microscope (Leica, Wetzlar, Germany) was used for the determination of total microscopic counts. For detailed FISH investigations, single optical sections or xy image series (z-stacks) were collected using a CLSM 410 confocal laser scanning microscope coupled to an Axiovert 135 M inverted microscope (Carl Zeiss, Jena, Germany) and operated with a Zeiss LSM software package (version 3.95).

Results

Reactor performance. After a 25-day acclimatization period, the second-stage reactors were initially fed with hydrolyzed feed (filtered effluent of the hydrolysis reactor) at a loading rate of 2 kg COD/m^3 day. After achieving steady state (10–15 days), the loading rate was increased sequentially (4, 8,12 COD/m^3 day). Feeding was stopped for three days under steady-state conditions at all loading rates to determine VFA removal and biogas production during that period. All results presented are for a loading rate of 8 kg COD/m^3 day.

VFA removal. The removal of VFA was determined for 72 h under steady-state conditions, at a loading rate of 8 kg COD/m^3 day, without in-between feeding. In the FLBR, there was a sharp decline in acetic acid production from 1550 to 1100 mg/l (Fig. 2), and in *n*-butyric acid production, from 1460 to 600 mg/l, in the first 2 h after feeding (data not shown). However, 2 h after the reactor had been fed, propi-

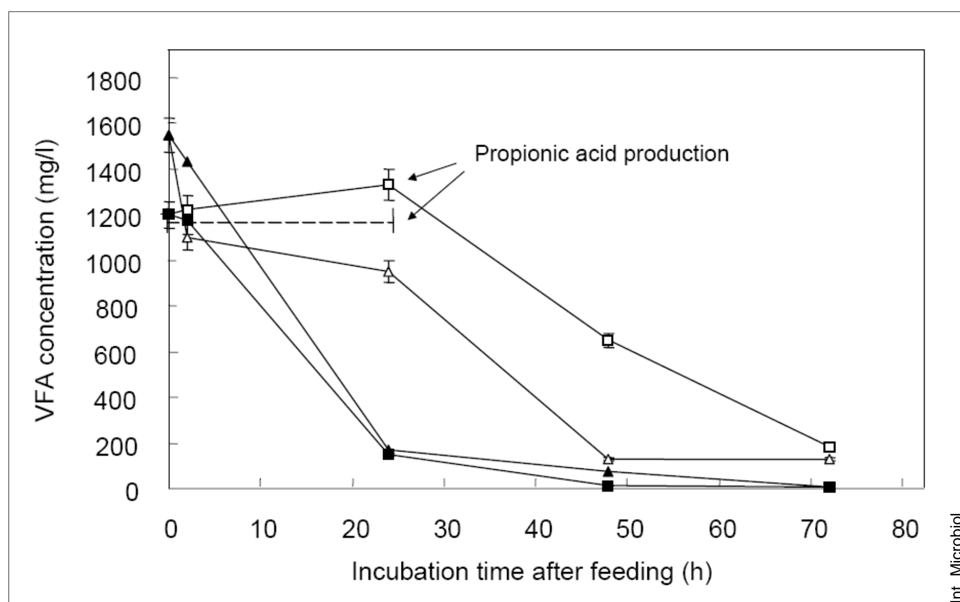

Fig. 2. VFA removal in FLBR and FXBR during steady-state conditions at a loading rate of 8 kg COD/m^3 day. Values are based on the mean of duplicate analyses. Key: Acetic acid (FLBR): open triangles. Propionic acid (FLBR): open squares. Acetic acid (FXBR): closed triangles. Propionic acid (FXBR): closed squares.

onic acid production reached 1330 mg/l (beyond the optimal range) and retarded the removal of acetic acid. This continued until 24 h after feeding, after which the total VFA concentration decreased to 850 mg/l in 48 h. In the FXBR, total VFA removal decreased from 4700 to 375 mg/l during the first 24 h. Within 48 h, the VFA concentration had decreased to 130 mg/l. VFA analysis of samples collected from different heights revealed that maximum degradation of VFA took place at the bottom of the reactor rather than at the top (data not shown). The removal of acetic acid, propionic acid, and butyric acid was faster in the FXBR, as indicated in Fig. 2. In this reactor, the presence of propionic acid was not observed after 48 h, in contrast to the FLBR. VFA removal was monitored at different heights in both reactors. These observations showed that up to 55% of VFA degradation took place at the bottom, 25–30% at the middle, and only 10% at the top of the FXBR. In the FLBR, 10–15%, 20–25%, and 40–50% VFA was degraded at the bottom, middle, and top of the reactor, respectively.

Biogas production. During the same period, the production of biogas was also monitored. In the first 10 h, the production rate was 0.7–0.8 l/h. Thereafter, in the FLBR, it was retarded until 24 h (for a period of 11–12 h). This may have been because propionic acid was being produced during that period, during which there was a change in the composition of the microbial population (data not shown). However, biogas production in the FLBR was subsequently revived and 4 l of biogas originated in the following 24 h. The revival of biogas production was corroborated by the higher rate of VFA removal (after 24 h).

Table 1. Biogas production and substrate conversion at different loading rates

	Units	FLBR			FXBR		
Loading rate	kg COD/m^3 day	4	8	12	4	8	12
Biogas	m^3/kg COD$_{rem}$[a]	0.45	0.45	0.41	0.48	0.47	0.42
Methane	m^3/kg COD$_{rem}$	0.31	0.31	0.29	0.34	0.32	0.30
Carbon dioxide	m^3/kg COD$_{rem}$	0.06	0.06	0.06	0.07	0.07	0.06
Biogasification rate (k)[b]	–	0.5	0.4	0.30	1.0	0.67	0.37
Conversion (X)[c]	%	79	91	80	88	93	97

[a]rem: removed.
[b]Calculated from Eq. 3.
[c]Calculated from Eq. 4.

The rate of biogas production in the FXBR was nearly 0.9 l/h in the first 12 h at a loading rate of 8 kg COD/m^3 day. However, over the following 36 h, only 5.5 l of biogas originated. The same trend of biogas production was observed when the reactor was fed at a loading rate of 4 and 12 kg COD/m^3 day (data not shown). Although biogas production was the same at 4 and 8 kg COD/m^3 day, it decreased by 20% when the reactors were fed at 12 kg COD/m^3 day. Table 1 shows CH_4 and CO_2 productions at different loading rates.

Detection of populations using FISH. Hybridization of the reactor samples with fluorescent probes resulted in strong signals, and demonstrated the presence of *Archaea* and *Bacteria*, including sulfate-reducing bacteria. Hydrogen-utilizing methanogens belonging to the families *Methanococcaceae* and *Methanobacteriaceae* were either not detected or detected in very small numbers, which suggested that the hydrogen concentration was low in all the reactors and that most of the biogas production resulted from the acetate route. Based on these findings, FISH assays were concentrated on the detection of species belonging to the families *Methanosarcinaceae* and *Methanosaetaceae*.

In the FXBR, cells resembling *Methanosaeta concilii* and hybridizing with probe MX825 were predominant in the form of clusters, whereas cells in the FLBR were randomly scattered. Most cells of this species concentrated at the bottom of the reactors. As a result, VFA degradation was faster at the bottoms of the reactors. *Methanosarcina mazei*, *M. barkeri*, as well as cells that hybridized with the MSMX860 and MS821 probes were also present in the FLBR and were mostly at the top of this reactor (Table 2). Our results showed that 1.5 times more *Methanosaeta-concilii*-resembling cells were present in the FXBR than in the FLBR. In addition, the microbial population that hybridized with MS821 was twice as abundant in the FLBR as in the FXBR. Total anaerobic microbial populations in the FXBR and FLBR reactors were nearly the same (their relative abundances are shown in Fig. 3) as were the abundances of cells hybridizing with probes SRB385 and EUB338. We also observed, from DAPI-stained effluent samples, that the washout of cells in the FLBR was nearly 1.5 times greater than that in the FXBR (Table 2).

Discussion

At low loading rates, the methane content of both reactors was low and was coupled to poor substrate removal rates. However, as the loading rate increased, the quality of biogas improved, resulting in higher CH_4 and lower CO_2 contents. The maximum substrate conversion rate in the FXBR was 0.34 (±0.002) m^3 CH_4/kg COD_{rem} (COD_{rem}: COD removed after methanogenesis) at a loading rate of 4 kg COD/m^3 day.

Table 2. Analysis of microbial populations, and their distribution in the reactor, using 16S rRNA oligonucleotide probes

				Floating-bed reactor				Fixed-bed reactor			
				T[a]	M	B	E	T	M	B	E
EUB338	MB310			5[b]	–[c]	–	5	5	–	–	5
EUB338	MC119			–	–	–		–	–	–	–
EUB338	MG1200			–	–	–	–	–	–	–	–
EUB338	MSMX860	MS1414	MS821	10	–	10	5	–	5	5	–
EUB338	MSMX860	MSMX860	MX825	5	10	15	5	10	15	25	5
EUB338	SRB385			5	10	10	5	10	10	10	5
EUB338	MOPB1 & KOP1			–	–	–	–	–	–	–	–

[a]T, top; M, medium; B, bottom; E, effluent.
[b]Abundance of probe-positive cells: approximately 5, 10, 15, and 25%, respectively (±1%), relative to DAPI-stained cel for probes in frames.
[c]No probe-positive microorganisms detected.

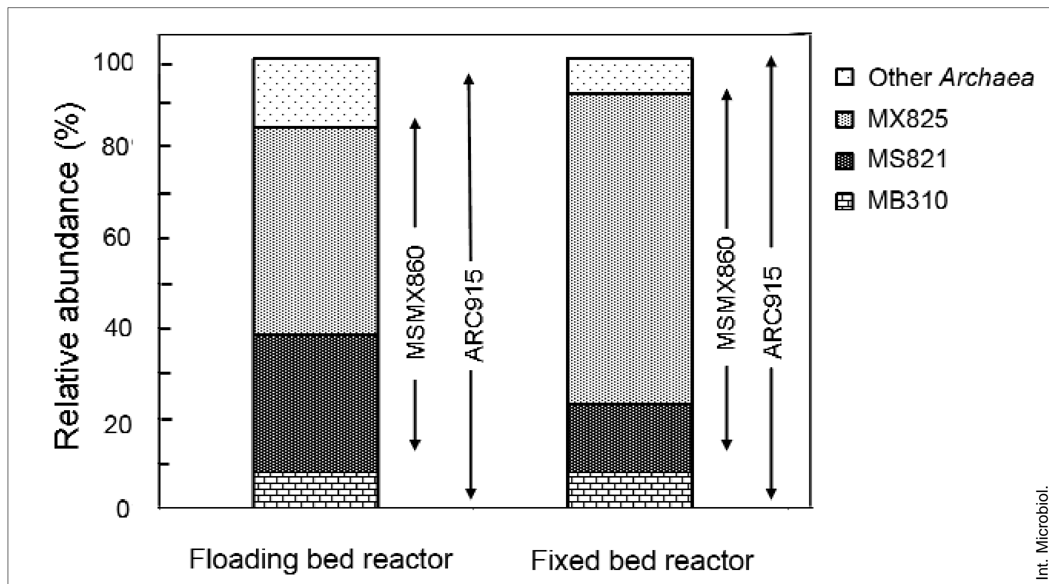

Fig. 3. Relative abundance of the total archaea population during steady-state conditions in methanogenic reactors.

In the FLBR, the conversion rate was 0.310 (± 0.005) m^3 CH_4/kg COD_{rem} at the same loading rate (Table 1). This difference in process efficiency and delay in biogas production was caused by the accumulation of propionic acid in the FLBR. The concentration of acetic and propionic acids are critical factors that regulate anaerobic digestion processes, since oxidation of propionic acid to acetic acid is the slowest among the VFA [7]. It has been reported that a propionic-acid concentration of more than 1000 mg/l inhibits bacterial populations [14]. Harper and Poland [13] reported that changes in acidogenic species alter the metabolism from acetic-acid formation to propionic-acid formation by inhibiting the reoxidation of NADH to NAD^+ during the dehydrogenation of glyceraldehyde-3-phosphate in the glycolysis pathway [15]. In the case of the FLBR, this could explain the excess production of propionic acid and the slow-down of the process.

In the mesophilic anaerobic reactor, the methanogens that are likely to be present belong to four of the five described orders of methanogens, i.e., *Methanomicrobiales*, *Methanobacteriales*, *Methanococcales*, and *Methanosarcinales*. Members of the fifth order of methanogens, *Methanopyrales*, are extremely thermophilic and are not likely to be present in mesophilic anaerobic bioreactors [3]; therefore, we did not use probes specific for *Methanopyrales* in this study. *Methanosaeta* species belonging to the Methanosarcinales order have a low threshold for acetate as compared to *Methanosarcina* of the same order and thus have a competitive advantage over *Methanosarcina* species at low acetate concentrations. At high levels of acetate, *Methanosarcina* species generally dominate [11,23,27]. This could explain the dominance of cells resembling *Methanosaeta* spp. in the FXBR and the presence of *Methanosarcina* sp. (*Methanosarcina barkeri* and cells resembling *M. mazei*) at the top of FLBR anaerobic reactors. In other studies, cells suggested to be *Methanosaeta concilii* were reported to comprise more than 90% of the archaeal population in an anaerobic bioreactor, with *Methanosarcina* accounting for less than 1%, as determined by FISH [11]. In our study, *Methanosaeta-concilii*-like cells formed clusters inside the FXBR; such clusters might be the early stage of granular formation. Both acetate- and hydrogen-utilizing methanogens have been detected in anaerobic granular sludge reactors, but *Methanosaeta* cells were the predominant group in the central cores of the granules [16]. Sulfate-reducing bacteria (SRB) were also seen when samples were hybridized with the SRB385 probe. The coexistence of methanogens and SRB was also reported in studies carried out in attached-growth reactors or in reactors with granules, i.e., systems in which factors such as mass-transfer limitations, microbial colonization, and adhesion are important phenomena that may affect the existence of these bacteria [23]. In conclusion, the predominance of *Methanosaeta concilii*-like cells in the FXBR coincided with higher reactor stability, led to faster (within 45–50 h) removal efficiencies than in the FLBR (within 70–75 h), and resulted in low effluent acetate concentrations.

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