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Sulfate Reduction at pH 5 in a High-Rate Membrane Bioreactor: Reactor Performance and Microbial Community Analyses

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High rate sulfate reduction under acidic conditions opens possibilities for new process flow sheets that allow the selective recovery of metals from mining and metallurgical waste and process water. However, knowledge about high-rate sulfate reduction under acidic conditions is limited. This paper investigates sulfate reduction in a membrane bioreactor at a controlled pH of 5. Sulfate and formate were dosed using a pH-auxostat system while formate was converted into hydrogen, which was used for sulfate reduction. Sulfide was removed from the gas phase to prevent sulfide inhibition. This study shows a high-rate sulfate-reducing bioreactor system for the first time at pH 5, with a volumetric activity of 188 mmol SO_4^{2-}/l/d and a specific activity of 81 mmol SO_4^{2-} volatile suspended solids/d. The microbial community at the end of the reactor run consisted of a diverse mixed population including sulfate-reducing bacteria.

Keywords: Sulfate reduction, membrane bioreactor, DGGE, community analysis, formate, thermodynamics

Waste and process water from the mining and metallurgical industry typically contains dissolved metal-ions and sulfuric acid [1]. Although these acidic waste streams are a major environmental problem, valuable metals can be recovered by microbiological reduction of the sulfate to sulfide, followed by metal-sulfide precipitation [2–4]. Sulfate reduction at neutral pH has been well studied [5–8], but by expanding the pH range of high-rate sulfate reduction to acidic conditions, the metal-sulfide recovery potential would be increased because metals can be selectively precipitated and recovered as metal-sulfides by varying the pH and sulfide concentration [3, 4]. Another advantage of sulfate reduction under acidic conditions is the reduced requirement for a neutralizing agent to increase the pH of the acidic waste stream. In addition, sulfide could be recovered more easily from the waste stream, as more of the sulfide is in the gaseous form (H_2S) at low pH and, consequently, decreases the cost of sulfide conversion to elemental sulfur by partial oxidization with oxygen [9, 10].

Several reports describe sulfate reduction in column experiments with acidic influent [11, 12]; however, the sulfate reduction process itself occurs at non-acidic conditions as indicated by the neutral effluent pH. Little is known about sulfate reduction under acidic conditions, and to our knowledge, no literature exists on bioreactor runs under a controlled pH below 5.5. Sulfate reduction at a pH as low as 3.8 is possible [13], but its use in industrial applications seems limited owing to the low conversion rate of 0.2 mmol/l SO_4^{2-} achieved.

In sulfidogenic systems, the major biological conversion processes are sulfate reduction, acetogenesis, and methanogenesis [14]. Even though these processes are well understood, the microbial populations responsible for these processes are not well described [15]. Combining molecular microbial community analyses with reactor performance data can substantially enhance the understanding of sulfidogenic systems. One method to characterize microbial communities is using denaturing gradient gel electrophoresis (DGGE) that separates DNA fragments based on the sequence rather than size, followed by sequence analysis of the DNA fragment. This approach has been used to analyze microbial communities in a full-scale sulfidogenic system at neutral pH treating wastewater from a zinc refinery [16], an upflow anaerobic sludge bed (UASB) treating papermill wastewater [15], and a laboratory-scale sulfidogenic fluidized bed reactor treating acidic metal-rich influent [17]. One disadvantage of DGGE is that it does not identify microorganisms that are less than 1% of the total population [18].

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This paper describes the effect of the lower operational pH of 5.0 on the performance and composition of the microbial community of a formate-fed sulfate-reducing high-rate membrane bioreactor (MBR). Sulfate (electron acceptor) and formate (electron donor and carbon source) were dosed using a pH-auxostat that has been previously applied for sulfate-reducing systems at pH 7 [19] and pH 6 [20]. This system doses influent based on the activity of the microorganisms and thus prevents formic acid inhibition and sulfate limitation. Sulfide was removed from the gas phase to prevent potential sulfide inhibition, while biomass washout was prevented using membrane separation.

MATERIAL AND METHODS

Experimental Set-Up

A 6-l submerged MBR was operated at pH 5.0 and a temperature of 30°C, of which a schematic representation is presented in Bijmans *et al.* [20]. A modified polyethylene membrane (Triqua, Wageningen, The Netherlands) was submerged in the reactor liquid of a gas-lift bioreactor. The membrane pore size of 0.2 μm enables biomass separation from the effluent. The temperature of the reactor liquor was controlled with a Tamson T1000 waterbath that recirculated the water through a water jacket. The pH was measured with a Schott H63 electrode (Schott A.G., Mainz, Germany) and controlled by an Endress+Hauser Liquisys P control unit (Endress+Hauser Holding A.G). The tubing and connectors were made from polytetrafluoroethylene (PTFE; Schott A.G., and Serto A.G.). The gas sparger was made from teflon (with 0.4 mm holes). The effluent gas of the reactor was recirculated to prevent loss of electron donor. A KNF-Verder N840.3FT.18 vacuum pump was used to recirculate the gas at a rate of 4 l min^{-1}, which was measured with a 100-9 McMillan (McMillan).

Experimental Design

A pH-auxostat system was used in this study according to Bijmans *et al.* [20], which was based on a proton equilibrium in which new influent was added when the $[H^+]$ decreases through neutralizing processes such as sulfate reduction. The decrease of the $[H^+]$ was measured *via* a pH electrode. Sulfide was removed from the gas phase before the gas was recycled into the reactor to be able to operate below the inhibiting concentration of sulfide for SRB. The recycle gas was passed over a $ZnCl_2$ solution in which sulfide was precipitated as zinc sulfide (Fig. 1). CO_2 was removed from the gas phase by bubbling the recycle gas through a NaOH solution (Fig. 1). The build-up of sodium was prevented by using sulfuric and formic acids instead of sodium sulfate and sodium formate.

Formate was used as electron donor as replacement for H_2 [19, 20]. The experiment was divided into 2 periods, where period I (days 0–17) provided the inoculum for period II (days 17–33). In period I, the reactor was filled with mineral medium A (Table 1), whereas medium B with higher concentrations of sulfate and formate was dosed by the pH-auxostat. From days 29–33 (period II), medium C was dosed by the pH-auxostat with double concentrations of sulfate and formate.

Inoculum

The inoculum contained 60 g of wet Eerbeek sludge (Industrie water Eerbeek, Eerbeek, The Netherlands), 30 g of wet Nedalco sludge (Royal Nedalco, Bergen op Zoom, The Netherlands), and 100 ml of supernatant of Zinifex sludge (Zinifex Budel Zinc, Budel, The Netherlands). The inoculum was crushed with a household blender for 3 min. Period II was started with 0.5 l of reactor liquid from period I.

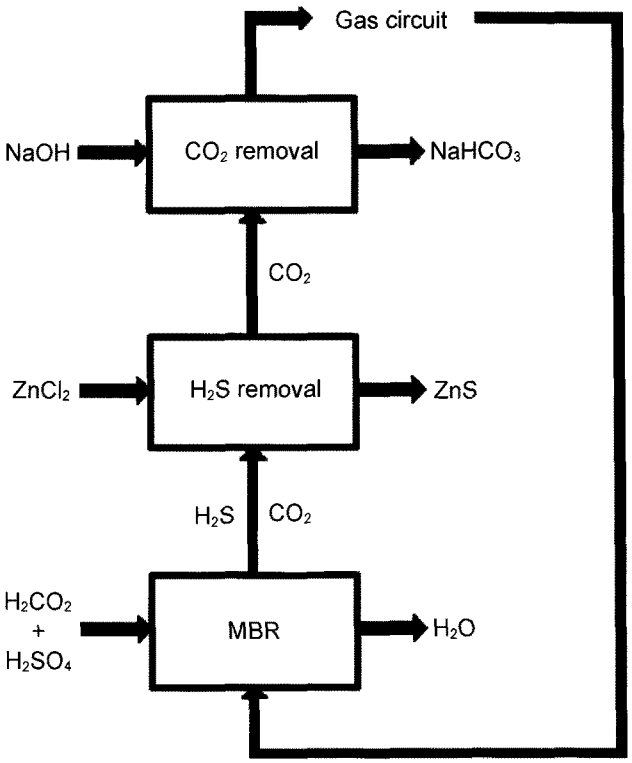

Fig. 1. Flow sheet of the 6-l membrane bioreactor and H_2S and CO_2 removal units.

Physicochemical Analyses

Sulfate and formate were analyzed with ion-chromatography as described by Sipma *et al.* [21]. Gas composition in the headspace

Table 1. Chemical composition of media A, B, and C.

Chemical	Medium A	Medium B	Medium C
Na_2SO_4 [g/l]	1.48	-	-
$NaHCO_2$ [g/l]	2.83	-	-
H_2SO_4 [M]	-	0.5	1.0
H_2CO_2 [M]	-	2.81	5.61
KH_2PO_4 [g/l]	0.41	2.05	2.05
NH_4Cl [g/l]	0.3	1.5	1.5
KCl [g/l]	0.37	1.85	1.85
$MgCl_2 6H_2O$ [g/l]	0.1	0.5	0.5
$CaCl_2 2H_2O$ [g/l]	0.11	0.5	0.5
$NaHCO_3$ [g/l]	1	1	1
Yeast extract [g/l]	0.1	0.5	0.5
Acid trace elements[a] [ml/l]	1	5	5
Alkaline trace elements[a] [ml/l]	1	5	5

[a]Described in Stams *et al.* [34].

Table 2. 16S rRNA gene and sequencing primers used in this study.

Primer	Position[a]	Sequences (5'-3')	Reference
ARC344F-GC[b]	344–363	ACG GGG YGC AGC AGG CGC GA	[35]
ARC915R	915–934	GTG CTC CCC CGC CAA TTC CT	[36]
GM5F-GC	341–357	CCT ACG GGA GGC AGC AG	[37]
DS907R	907–927	CCG TCA ATT CCT TTR AGT TT	[37]
M13 Universal	NA[c]	GTAAAACGACGGAGT	[38]
M13R	NA[c]	CAGGAAACAGCTATGACCATG	[38]

[a]Base position numbers correspond to *Escherichia coli* 16S rRNA gene.
[b]GC-clamp (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3') is attached to the 5' end of the primer [39].
[c]NA, not applicable.

was measured by gas chromatography, whereby H_2, N_2, O_2, and CH_4 were analyzed on a Hewlett Packard 5890 and CO_2 on a Fisons Instruments GC8000 according to Weijma *et al.* [22]. Volatile fatty acids (VFA) were analyzed on a Hewlett Packard series II GC [22]. Sulfide was analyzed using the Dr. Lange sulfide kit LCK-653 and a Xion 500 spectrophotometer (Hach Lange GMBH, Düsseldorf, Germany). Total suspended solids (TSS) and volatile suspended solids (VSS) were analyzed following standard methods [23]. The particle size distribution of the sludge was analyzed with laser scattering image analysis (Coulter laser LS 230, Beckman Coulter, U.S.A.).

Molecular Phylogenetic Analysis of the Microbial Community

The microbial community present at the end of period II was identified by DGGE and DNA sequencing. Duplicate samples (10 ml) from the bioreactor sludge were taken at the end of the experiment. DNA was isolated from one of the duplicate samples by bead beating followed by purification using the Wizard DNA Clean-Up System (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions [24]. DNA was isolated from the second duplicate sample by pelleting the cells (10,000 $\times g$ for 10 min) and resuspending in 500 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7) containing 0.12% (w/v) sodium dodecyl sulfate and 0.06 mg/ml proteinase K. The mixture was incubated for 1 h at 37°C and then DNA isolated by phenol chloroform extraction [25]. Both DNA preparations were PCR amplified [24] using the archaeal (ARC344F-GC and ARC915R) and bacterial (GM5F-GC and DS907R) specific primers (Table 2). The amplified DNA fragments were analyzed by DGGE using a denaturing gradient of 30–70% denaturant [24]. The DGGE bands were excised, cloned into the pGEM-T Easy Vector System (Promega, Madison, WI, U.S.A.), transformed into *Escherichia coli*, and sequenced (primers M13 Universal and M13R; Table 2) as described in Dopson and Lindström [24]. The obtained DNA sequences were checked for chimeras at the Ribosome Database Project II site (http://rdp.cme.msu.edu/html [26]) and aligned in the ARB program package [27] as described in Morales *et al.* [28]. One chimeric artifact (chimera) was identified and was not included in the phylogenetic analyses. The number of base pairs used for alignment and phylogenetic analysis for each of the clones is given in Table 3.

Gibbs Free Energy Calculations

Gibbs free energies of the reactions were calculated using thermodynamic data from Amend and Shock [29]. At the start of the experiment, 0.1% was used as hydrogen concentration to make thermodynamic calculations possible. The same concentration was used for CO_2 of which the concentration was low owing to stripping with a NaOH solution. For sulfide, a constant concentration of 0.4 mM was used, which was found to be an average value in the reactor liquor.

Mass Balance Sulfate Calculations

Nomenclature

Φ	Flow rate	[l/d]

Table 3. Cloned 16S rRNA gene fragments from the bioreactors and their closest related named gene sequences in the NCBI database.

Clone	Closest relative named species in database	Accession No[a]	% Similarity[b]	No. of bases[c]
MBR5-1	*Methanosaeta concilii* H-3	AB212065	97	423
MBR5-2	No named species in first 100 identified		<93	422
MBR5-3	*Petrimonas sulfuriphila* BN3	AY570690	99	577
MBR5-4	*Clostridium bowmanii* DSM 14206	AJ506120	99	555
MBR5-5	*Parabacteroides goldsteinii*	AY974070	94	563
MBR5-6	*Enterococcus durans* CECT411^T	AJ420801	98	433
MBR5-7	*Desulfovibrio fructosovorans*	AF050101	95	309
MBR5-8	*Desulfovibrio aerotolerans* DvO5	AY746987	93	583
MBR5-9	*Spirochaeta bajacaliforniensis* DSM 16054^T	AJ698859	92	580
MBR5-10	*Eggerthella lenta*	AF292375	91	550
MBR5-11	*Eggerthella lenta*	AF292375	90	511

[a]Accession number of the closest related named gene sequence obtained by BLAST comparison in the NCBI database (http://www.ncbi.nlm.nih.gov/).
[b]Percentage sequence similarity to the closest relative in the NCBI database.
[c]Number of base pairs used for the BLAST comparison and alignment in the ARB program.

ρ_m	Density of run medium	[kg/l]
C_f	formate concentration	[mmol/l]
C_s	Sulfate concentration	[mmol/l]
C_x	Biomass concentration	[gVSS/l]
M	Mass of influent	[kg]
r_v	Volumetric activity	[mmol/l/d]
V_r	Reactor volume	[l]
r_s	Specific activity	[mmol/gVSS/d]
t	Time	[d]

Subscript

in	entering the reactor
out	leaving the reactor
r	in the reactor

Assumptions:

1. The liquid volume of the reactor was considered constant during the experiment, which also means that the liquid flow going into the reactor was equal to the liquid flow going out the reactor ($\phi_{l,in}=\phi_{l,out}$) and is called ϕ_l.
2. The liquid flow was assumed to be the difference in weight of the medium divided by the density of the medium multiplied by the difference in time ($\phi_l=\Delta M/(\rho_m\cdot\Delta t)$). The time interval between the data points was 15 min of which an average of 19 data points was used.
3. Sulfate accumulation in the reactor as well as present in the effluent was neglected. This was possible because of the high sulfate concentration in the influent compared with the reactor concentration and owing to the small hydraulic retention time, which was dependent on the volumetric activity and concentration (that was never lower than 27 d).
4. To prevent false high specific activities, the highest $C_{x,r}$ value of the interval was taken. The biomass concentration in the liquid phase was used because there was no visible biofilm formation on the membrane or bioreactor wall. Thus, these can be neglected compared with the high biomass concentrations in the mixed reactor liquor.

With assumptions 1–4, the sulfate mass balance ($\frac{dS}{dt}$) was

$$\frac{dS}{dt}=\phi_l\cdot C_{s,in}-r_v\cdot V_r \quad (1)$$

For steady-state conditions, the volumetric activity (r_v) becomes

$$r_v=\frac{\phi_l\cdot C_{s,in}}{V_r} \quad (2)$$

The formate dosing rate was calculated by replacing the $C_{s,in}$ in formula 2 with $C_{f,in}$.
The specific activity (r_s) becomes

$$r_s=\frac{\phi_l\cdot C_{s,in}}{C_{x,r}\cdot V_r} \quad (3)$$

RESULTS AND DISCUSSION

Period I Reactor Performance

Sulfate reduction did not occur during the first 14 days despite an initial decrease in formate concentration (electron donor) during the first 6 days of the experiment. This initial decrease in formate was followed by a decrease in formate concentration, resulting in formate depletion at day 12 (Fig. 2A). Formate was converted into hydrogen (Table 4, Eq. 5) and acetate (Table 4, Eqs. 3 and 8) as indicated by the increase in the hydrogen and acetate concentrations from day 6 onwards (Figs. 2B and 2C). The hydrogen concentration further increased and reached a maximum concentration of 72% of the gas phase on day 17 (Fig. 2B) and acetate reached a maximum concentration of 17 mM on day 13 (Fig. 2C).

Interactions between formate and hydrogen in anaerobic systems have been well described and the preferred

Table 4. Overview of possible reactions and their Gibbs free energy (ΔG) values calculated in kJ per mole of transferred electron under various conditions.

No.	Formula	$\Delta G_r^{0'}$ [a] [kJ/e-mol]	ΔG_{pH7} [b] [kJ/e-mol]	ΔG_{pH5} [c] [kJ/e-mol]
1	$4HCO_{2(aq)}^- + SO_{4(aq)}^{2-} + 6H_{(aq)}^+ \rightarrow H_2S_{(aq)} + 4CO_{2(g)} + 4H_2O_{(l)}$	-17[d]	-17[d]	-25[d]
2	$4HCO_{2(aq)}^- + SO_{4(aq)}^{2-} + 5H_{(aq)}^+ \rightarrow HS_{(aq)}^- + 4CO_{2(g)} + 4H_2O_{(l)}$			
3	$4HCO_{2(aq)}^- + 3H_{(aq)}^+ \rightarrow Ac_{(aq)}^- + 2CO_{2(g)} + 2H_2O_{(l)}$	-14	-14	-18
4	$4HCO_{2(aq)}^- + 4H_{(aq)}^+ \rightarrow CH_{4(aq)} + 3CO_{2(g)} + 2H_2O_{(l)}$	-18	-18	-24
5	$HCO_{2(l)}^- + H_{(aq)}^+ \rightarrow H_{2(g)} + CO_{2(g)}$	-2	-2	-8
6	$4H_{2(g)} + SO_{4(aq)}^{2-} + 2H_{(aq)}^+ \rightarrow H_2S_{(aq)} + 4H_2O_{(l)}$	-15[e]	-15[e]	-18[e]
7	$4H_{2(g)} + SO_{4(aq)}^{2-} + H_{(aq)}^+ \rightarrow HS_{(aq)}^- + 4H_2O_{(l)}$			
8	$4H_{2(g)} + 2CO_{2(g)} \rightarrow Ac_{(aq)}^- + H_{(aq)}^+ + 2H_2O_{(aq)}$	-12	-11	-10
9	$4H_{2(g)} + CO_{2(g)} \rightarrow CH_{4(g)} + 2H_2O_{(l)}$	-16	-16	-16

[a]25°C, pH 7, and standard conditions of 1 M and 1 bar.
[b]30°C, pH 7, and concentrations of 1 M and 1 bar.
[c]30°C, pH 6, and concentrations of 1 M and 1 bar.
[d]Based on reactions 1 and 2 and pKa of 6.96 [40].
[e]Based on reactions 6 and 7 and pKa of 6.96 [40].

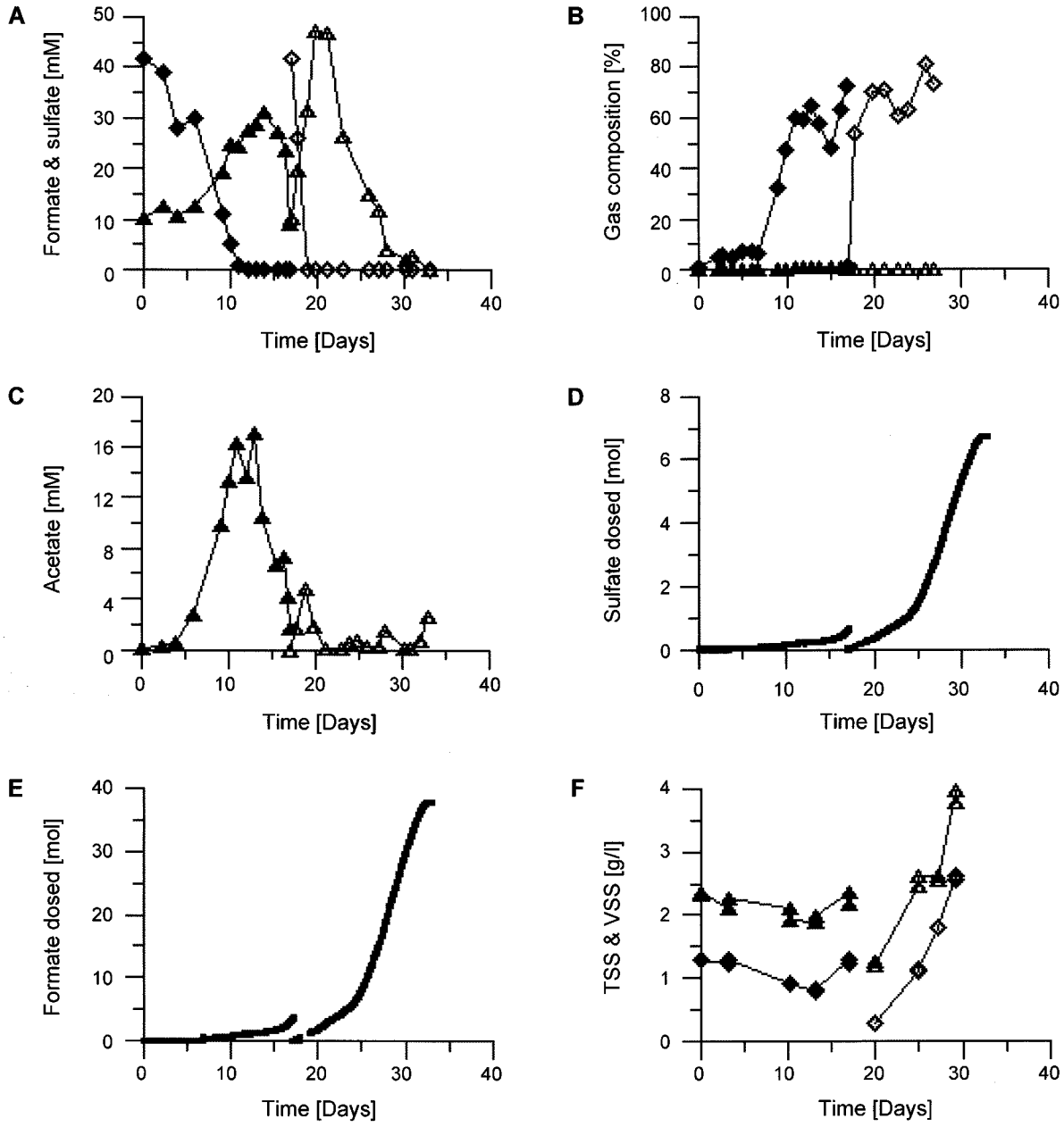

Fig. 2. Performance of the sulfate-reducing MBR at pH 5 with closed symbols for period I and open symbols for period II.
A. Formate (◆) and sulfate (▲) concentration in the reactor expressed as mM sulfate/formate. **B.** Gas-phase composition with hydrogen (◆) and methane (▲) in percentage of total gas phase. **C.** Acetate (▲) concentration in the reactor expressed as mM acetate. **D.** Accumulative sulfate dosed to the reactor by the pH-auxostat. **E.** Accumulative formate dosed to the reactor by the pH-auxostat. **F.** Total suspended solids (TSS, ▲) and volatile suspended solids (VSS, ◆) of the reactor expressed as gram TSS/VSS per liter of reactor liquor.

compound depends on the concentrations of the chemical species in the thermodynamic equation [20, 30–32]. The removal of CO_2 from the headspace of the present study resulted in an equilibrium shift to the production of hydrogen. Hydrogen production from formate is a proton consuming reaction (Table 4, Eq. 5) and, thus, pH increasing. The pH increase was compensated by dosage of sulfuric acid- and formic acid- containing medium by the pH-auxostat (Figs. 2D and 2E). Until day 14, only formate was converted from the medium, resulting in sulfate accumulation (Fig. 2A), indicating that no sulfate was reduced during the first 14 days (Table 4, Eqs. 1 and 2). Sulfate reduction started after day 14 as indicated by the decrease in sulfate concentration (Fig. 2A). In similar experiments at pH 6, sulfate reduction started within 7 to 9 days [20], suggesting that development of an active population at pH 5 demands more time. A small amount of methane was produced but the concentration did not rise above 1% of the gas phase (Fig. 2B).

No significant biomass growth was observed (Fig. 2F), as expected when only a small amount of electron donor was converted. Period I ended because of technical problems, and therefore run II was started in the same bioreactor with 0.5 l of reactor liquor from period I.

Period II Reactor Performance

After the restart on day 17, formate was converted to hydrogen and was depleted in 2 days (day 19; Fig. 2A). Hydrogen accumulated to 50% of the gas phase after 1 day (day 18) and 70% by day 20 (Fig. 2B). Despite the conversion of formate into hydrogen in the beginning of period II, sulfate accumulated suggesting that sulfate reduction was absent, as was observed during the first 14 days of period I. Acetate was the only VFA that exceeded 1 mM, with a peak concentration of 5 mM on day 19, followed by a rapid decrease and full depletion on day 21 (Fig. 2C). Unlike experiments at pH 6 [20], methane was absent during period II (Fig. 2B).

Sulfate reduction took place from day 21 onwards, leading to medium dosage by the pH auxostat and a lower HRT (Fig. 3D). The rate of medium dosage increased from day 24 onwards (Fig. 2D), suggesting an increase in volumetric activity (Fig. 3A) that fluctuated between 99 mmol L^{-1} SO_4^{2-} d^{-1} and 188 mmol L^{-1} d^{-1} between day 26 and 32, with a maximum specific activity of 81 mmol SO_4^{2-} $gVSS^{-1}$ d^{-1} (Fig. 3B). These rates were considerably higher than from other reported bioreactors operated close to pH 5 (Table 5). Van Houten *et al.* [6] reported a volumetric activity of 52 mM SO_4^{2-} d^{-1} at pH 5.5 in a hydrogen fed gas-lift bioreactor, while at pH 5.0 no activity was obtained. The sulfate conversion efficiency at the maximum volumetric activity in the present experiment was 97%, which was higher than most previously reported high-rate experiments, even at neutral pH (Table 5). The specific activity of the sludge in this study shows that the biomass retained by the membrane was active and only comparable to results from other MBR [20] and gas-lift bioreactors [6, 7] operated at a higher pH.

Despite increased medium dosage from day 21 onwards, formate remained depleted in the reactor liquor, indicating that it was directly converted and all remaining and incoming sulfate had been reduced (Fig. 2A). The hydrogen concentration

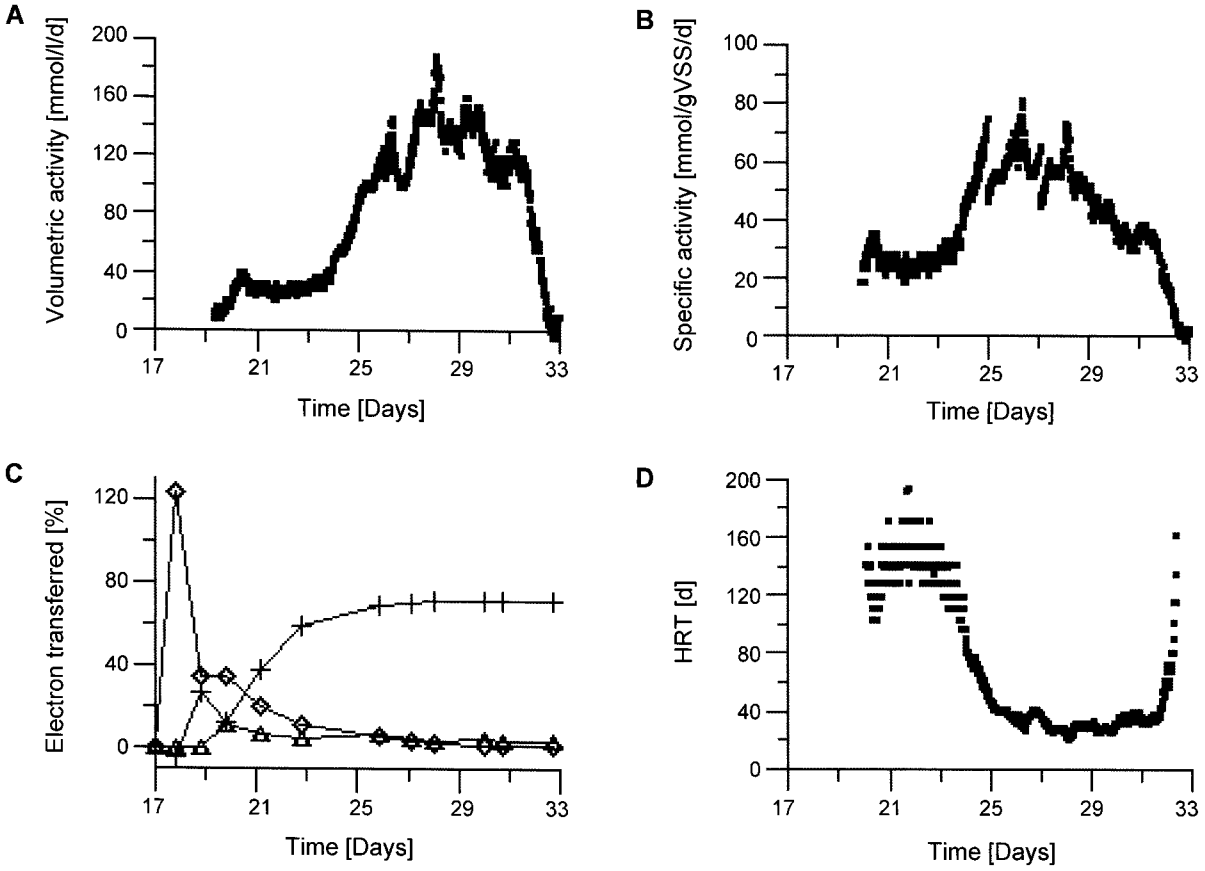

Fig. 3. Performance of the sulfate reducing MBR at pH 5 during period II with (**A**) volumetric activity of the reactor (millimole of sulfate reduced per liter of reactor liquor per day); (**B**) specific activity of the reactor (millimole of sulfate reduced per gram of VSS per day); (**C**) accumulative electrons transferred from formate to hydrogen (◇), sulfide (+), and biomass (△) (percentage of total electrons transferred from formate); and (**D**) hydraulic retention time of the bioreactor (day).

Table 5. The reactor concept, pH, temperature, substrate, volumetric activity, specific activity, and conversion rate from this study and the literature.

Reactor concept	pH	Temp [°C]	Substrate	Volumetric activity [mmol SO_4^{2-}/l/day]	Specific activity [mmol SO_4/gVSS/d]	Conversion efficiency [%]	References
MBR	5.0	30	Formate	188	81	97	This study
Gaslift[a]	7.0	30	Hydrogen	288	-[b]	59	[7]
Gaslift[a]	7.0	30	Hydrogen	156	-[b]	59	[6]
MBR	6.0	30	Formate	302	110	100	[20]
Gaslift[a]	6.0	30	Hydrogen	130	-[b]	49	[6]
Gaslift[a]	5.5	30	Hydrogen	52	-[b]	19	[6]

[a]With pumice as carrier material.
[b]No VSS concentration measured.

in the gas phase also increased to a maximum of 81% at day 26, from the remaining formate after all sulfate had been reduced, while methane was still absent in the gas phase (Fig. 2B). During period II, biomass gradually increased from 0.3 g VSS/l at day 20, to 2.5 g VSS/l at day 29 (Fig. 2F). The culture had a mean particle size of 29 μm at start-up, which decreased to 21 μm at day 27, which was similar to results at pH 6 [20].

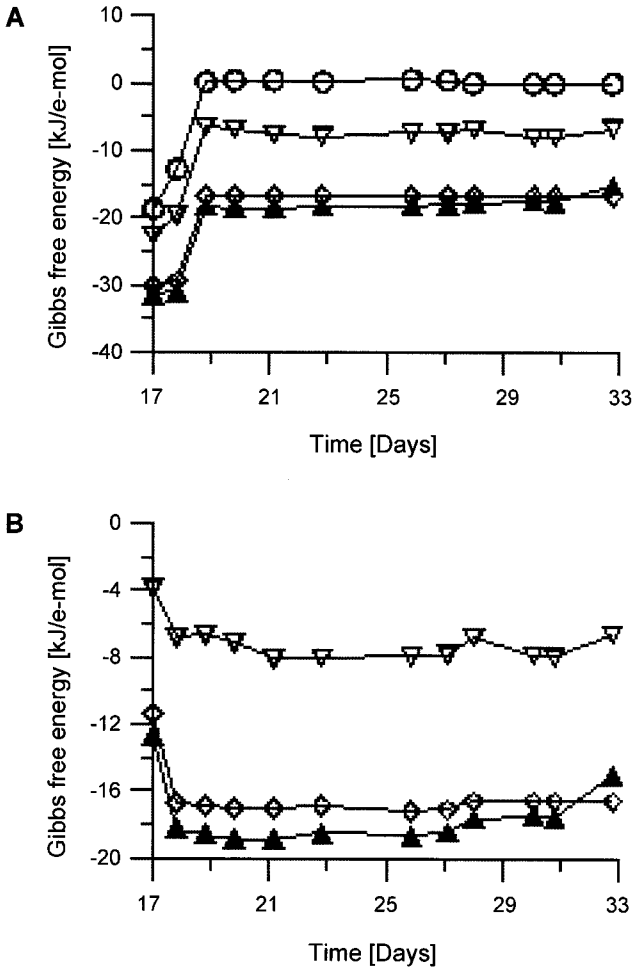

Fig. 4. Gibbs free energy of reaction expressed in kJ/e-mol for (**A**) reactions with formate as electron donor and sulfide as product (▲; Table 4, Eqs. 1–2), acetate (△; Table 4, Eq. 3), methane (◆; Table 4, Eq. 4), and hydrogen (○; Table 4, Eq. 5); and (**B**) reactions with hydrogen as electron donor and sulfate as product (▲; Table 4, Eqs. 6–7), acetate (△; Table 4, Eq. 8), and methane (◆; Table 4, Eq. 9).

After day 32, the volumetric activity dropped and sulfate reduction stopped at day 33 (Fig 2A). At this point, sulfate and formate were depleted while the reactor liquor remained at pH 5, indicating that the acidity of the bioreactor liquor was not based on formic or sulfuric acid. As the pH of the medium without formic and sulfuric acid was below the set-point, the alkalinity originally present in the start-up medium was washed out during reactor operation. This resulted in depletion of both formic and sulfuric acids causing the pH-auxostat to stop dosing and, thus, for the run to terminate. Addition of an alkaline compound to media B and C would prevent depletion of the formic and sulfuric acids by the pH-auxostat. Even though a MBR with a pH-auxostat has advantages in fundamental studies, applications in full scale systems could be unstable owing to the delicate chemical balance needed to operate a pH-auxostat, especially in the case when multiple acids are present.

Molecular Microbial Phylogeny of Bioreactor in Period II

The 16S rRNA gene similarities of the microorganisms present at the end of the bioreactor run in period II shows the presence of a diverse population of sulfate reducers (*Proteobacteria*), acetogens (*Firmicutes*), and methanogens (*Eukarchaeota*) (Table 3 and Fig. 5) as expected from a sulfidogenic bioreactor [15–17, 33]. Bacteria from the phyla *Actinobacteria*, *Spirochaetes*, and *Bacteroidetes* were also present, which are not uncommon in laboratory- [15, 17, 33] and full-scale [16] sulfidogenic bioreactors. These bacteria most likely live on organic matter from decayed biomass retained in the system by the membrane.

In total, 11 clones were identified by DGGE (Fig. 5) with between 90% and 99% 16S rRNA gene similarities with known species, indicating that there were both novel and previously characterized species present in the sludge. Of these 11 clones, MBR5-1 aligned within the archaeal

domain and the other clones with the bacteria (Table 3 and Fig. 6). Two clones were found in the bacterial δ-proteobacteria clade (Fig. 6), belonging to the sulfate-reducing *Desulfovibrio* family (Table 3). According to the NCBI database, the named species most closely related to MBR5-7 was *D. fructosovorans* (95% 16S rRNA gene similarity; Table 3), whereas MBR5-8 was most closely related to *D. aerotolerans* (93% 16S rRNA gene similarity; Table 3), suggesting that they might be novel sulfate-reducing species. In addition, MBR5-4 and MBR5-6 aligned within the *Firmicutes* clade (Fig. 6). MBR5-4 was most closely related to the named acetogenic species *Clostridium bowmanii* (99% similarity) and MBR5-6 to *Enterococcus durans* (98% similarity). The most closely related named species to the archaeon (MBR5-1) was the acetate-utilizing methane producer *Methanosaeta concilii* H-3 (97% similarity). MBR5-2, MBR5-3, and MBR5-5 all aligned within the *Bacteroidetes* clade. MBR5-9 was most closely related to the named species *Spirochaeta bajacaliforniensis*. MBR5-10 and 11 were both closely related to the named species *Eggerthella lenta*, but with different alignments in the phylogenetic tree and 91% similarity for MBR5-10 and 90% similarity for MBR5-11.

The archaeon and sulfate reducers found in the present study were closely related to uncultured archaea (strains 1B7, 1A3, 1E4, 1H10, and 1A7) and bacteria (strains E14 and E21) present in the papermill wastewater treatment sludge used in the inoculum ([15]; Fig. 6). Although no exact match was found, it is likely that these strains originate from the papermill sludge, indicating that the identified microorganisms were more closely related to microorganisms found in bioreactors than in acidic waste streams. This is in agreement with the fact that the inoculum originated from three near-neutral-pH full-scale bioreactors. Lowering the reactor liquid pH to 5 still resulted in a diverse population of probably acid tolerant microorganisms during the bioreactor operation.

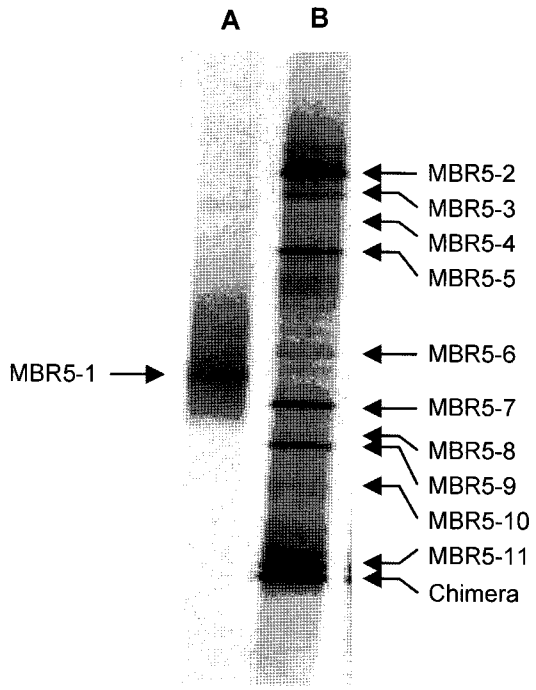

Fig. 5. DGGE gel of archaea (**A**) and bacteria (**B**) amplified from the pH 5 membrane bioreactor mixed culture.
Duplicate DNA samples were prepared by the Wizard DNA Clean Up System (Wiz) and phenol chloroform extraction with phase lock gel tubes (PLG). One chimeric artifact was identified that was not included in the phylogenetic analysis.

Electron Transfer Characteristics

During the experiment, formate was converted into hydrogen (Table 4, Eq. 5), which was the most likely electron donor for sulfate reduction (Table 4, Eqs. 6–7). The Gibbs free energy of formation (ΔG) of H_2 production from formate at pH 7 was −2 kJ/e-mol compared with −8 kJ/e-mol at pH 5 (Table 4, Eq. 5), indicating that at a lower pH, the production of hydrogen from formate generates more energy. In the first 2 days of period II (day 19), hydrogen production accounted for the major electron flow whereas sulfate reduction was absent (Fig. 3C). At the end of period II, the major electron flow was to sulfide (71%) and no other major electron flows could be identified (Fig 3C). The ΔG of hydrogen from formate (Table 4, Eq. 5) increased from −19 kJ/e-mol to around 0 kJ/e-mol on day 19 and then stabilized (Fig. 4A).

The ΔG of sulfate reduction with hydrogen decreased to −18 kJ/e-mol on day 1 (Fig. 4B), which made this reaction more thermodynamically favorable. The ΔG of methane production was close to that of sulfide production (Fig. 4), whereas methane was not produced in period II. Even though methane was not found in the headspace, archaeal specific primers amplified a single 16S rRNA gene sequence that aligned with the methanogenic archaea. This indicated that the archaeon was transferred with the inoculum and persisted in the MBR without significant activity. Although these methanogenic archaea were not thermodynamically limited, their activity did not proliferate owing to other factors such as non-optimal pH.

This study shows for the first time that a high-rate sulfate-reducing bioreactor system with high volumetric and specific activities can be reached at a controlled pH; for example, 5. This opens possibilities in new process flow schemes that require sulfate reduction at low pH, e.g. selective recovery of metals from waste and process water from mining and metallurgical industries.

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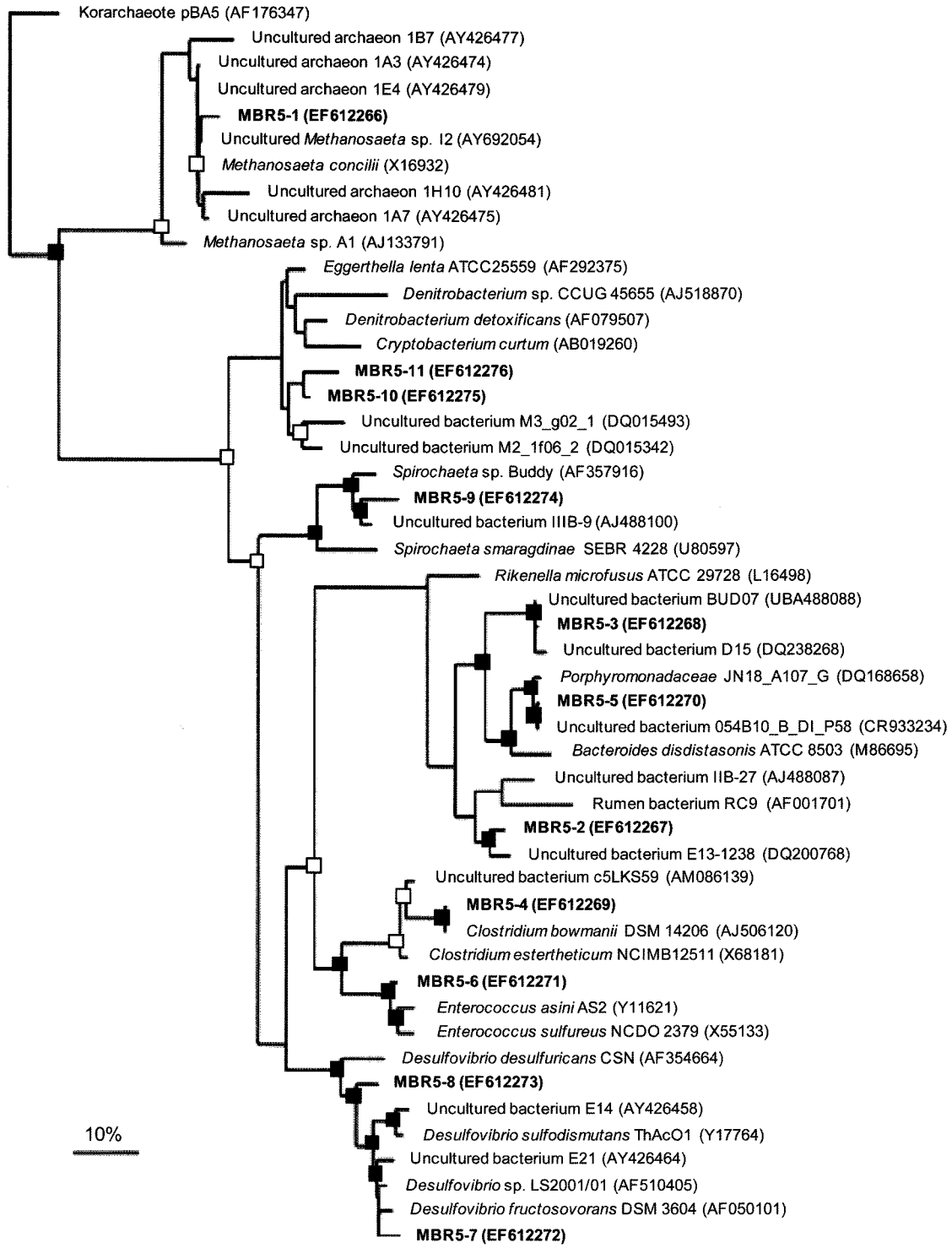

Fig. 6. Maximum likelihood phylogenetic tree based on partial 16S rRNA gene sequences of clones isolated from the pH 5 membrane bioreactor (designated MBR).

Phylogenetic analysis was carried out by the maximum likelihood, distance neighbor-joining, and DNA parsimony methods and the nodes supported by all 3 trees (■) and 2 trees (□) have been marked. Accession numbers are given in parenthesies. The scale bar corresponds to 10% sequence similarity.

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