Methanol as electron donor for thermophilic biological sulfate and sulfite reduction

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Proefschrift

ter verkrijging van de graad van doctor

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Stellingen

1. Met methanol als electronendonor zijn hoge snelheden van sulfiet- en sulfaatreduktie haalbaar in thermofiele (65°C) reaktoren.
   dit proefschrift

2. In het temperatuurtraject van 30 tot 65°C bevindt zich een overgangstemperatuur waarbij de afbraak van methanol in niet-steriel bedreven anaërobe reactoren verschuift van methanogeen naar sulfidogeen.
   dit proefschrift

3. Gerapporteerde waarden voor de maximale groeisnelheid en groei-opbrengst van sulfaatreducerende bacteriën dienen met de nodige voorzichtigheid te worden betracht, aangezien deze groeikinetische parameters veelal bij niet-constante sulfideconcentratie worden bepaald.

4. Het benoemen van de door de Smul et al. gebruikte reactor als Expanded Granular Sludge Blanket reactor moet als 'wishful thinking' worden aangeduid.
   Smul, A. de, Dries, J. Goethals, L., Grootaerd, H., Verstraete, W. (1997). High rates of sulfate reduction in a mesophilic ethanol-fed expanded-granular-sludge-bed reactor. Appl.Microbiol.Biotechnol. 48, 297-303.

5. De export van afval naar landen met minder strenge milieuwetgeving remt de ontwikkeling van nieuwe technologiën voor milieusparende verwerking van dat afval.

6. Het regelmatig gebruik van het †-teken als index bij auteursnamen in wetenschappelijke tijdschriften kan bij nieuwkomers in de wetenschap tot de opvatting leiden dat ze een nogal ongezond beroep hebben gekozen.

7. De vrijwel totale afwezigheid van varkens in het Nederlandse landschap is bevreemdend als wordt bedacht dat Nederland bijna evenveel varkens als mensen telt.

8. Het kunnen uitlopen van een marathon is een betere indicatie voor een goede lichamelijke gezondheid dan het slagen voor menig medische keuring.

9. Het gebruik van de lift in het Biotechnion is een onvrijwillige vorm van onthaasting.

10. In Nederland wapperen 's zomers per vierkante kilometer meer Duitse vlaggen dan in Duitsland.

Stellingen behorende bij het proefschrift "Methanol as electron donor for thermophilic biological sulfate and sulfite reduction".
Jan Weijma, Wageningen, 20 oktober 2000.

Foar Heit en Mem

Cover: artist impression of anaerobic thermophilic sludge cultivated on methanol and sulfate

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Abstract- Weijma. J. (2000). Methanol as electron donor for thermophilic biological sulfate and sulfite reduction. Doctoral Thesis, Wageningen University, The Netherlands.

Sulfur oxyanions (e.g. sulfate, sulfite) can be removed from aqueous waste and process streams by means of biological reduction with a suitable electron donor to sulfide, followed by partial chemical or biological oxidation of sulfide to elemental sulfur. The aim of the research described in this thesis was to make this biological process more broadly applicable for desulfurization of flue-gases and ground- and wastewaters by using the cheap chemical methanol as electron donor for the reduction step. Besides determining the selectivity and rate of reduction of sulfur oxyanions with methanol in bioreactors, also insight was acquired into the microbiology of the process. It was found that at pH 7.5 and thermophilic (65°C) conditions (applicable for flue-gas desulfurization), sulfate-reducing microorganisms ultimately outcompete methanogenic consortia for methanol in anaerobic high-rate bioreactors. Methane formation from methanol was quickly inhibited by imposing slightly acidic pH-values (6.7 instead of 7.5). Acetate represented a side-product from methanol at 65°C, accounting for up to 13% of the methanol degraded. The rate of acetate formation was linearly correlated to the rate of sulfate and sulfite reduction with methanol. At a hydraulic retention time (HRT) of 10 h, maximum reduction rates of 6 $gSO_3^{2-}.L^{-1}.day^{-1}$ (100% elimination) and 4-7 $gSO_4^{2-}.L^{-1}.day^{-1}$ (40-70% elimination) were attained simultaneously in the reactors, equivalent to a sulfidogenic methanol-conversion rate of 6-8 $gCOD.L^{-1}.day^{-1}$ (COD:Chemical Oxygen Demand). The resulting sulfide concentration of about 1800 $mgS.L^{-1}$ (or the H_2S concentration of 200 $mgS.L^{-1}$ at pH 7.5) limited the rate of sulfate reduction at a HRT of 10 h. At a hydraulic retention time of 3-4 h, maximum reduction rates of 18 $gSO_3^{2-}.L^{-1}.day^{-1}$ (100% elimination) and about 12 $gSO_4^{2-}.L^{-1}.day^{-1}$ (50% elimination) were attained, equivalent to a sulfidogenic methanol-conversion rate of 19 $gCOD.L^{-1}.day^{-1}$. At this HRT, the sulfate reduction rate was limited by the biomass concentration of 9 to 10 $gVSS.L^{-1}$ that maximally was retained in the reactor. The time needed to reach maximum process performance amounted to 40-60 days. From one of the reactors a thermophilic sulfate-reducing bacterium, *Desulfotomaculum* strain WW1 was isolated, that probably represented the most abundant sulfate reducer. In the reactor, strain WW1 is not confined to the use of methanol, as it also grows on methanol degradation products like acetate, formate and H_2/CO_2. The presence of high numbers of methanol-oxidizing, hydrogen-producing bacteria in the sludge indicated that hydrogen may represent an important electron donor for sulfate reduction in the sludge. In the cultures in which the presence of these species was demonstrated, the formation of acetate (about 15% of the methanol degraded) seemed to be strictly coupled to growth of the methanol-oxidizing species. This might explain the coupling of sulfide and acetate formation from methanol in the reactors. Methanol was not a suitable electron donor for mesophilic (30°C) sulfate reduction, relevant for bio-desulfurization of cold or slightly heated ground- or wastewater. Under mesophilic conditions, methanol was primarily degraded to methane.

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General Introduction

1.1 Sulfur dioxide emission

Sulfur dioxide (SO_2) represents the main fraction of anthropogenic sulfur emissions worldwide. According to the U.S. Environmental Protection Agency (EPA), roughly 23 million tons of SO_2 are emitted annually in the United States. In the countries of the European Community, about 16.5 million tons of SO_2 were emitted in 1990. Anthropogenic sulfur dioxide emission is mainly caused by combustion of sulfur-containing fossil fuels like coal and oil. Power plants account for nearly 70% of all SO_2 emissions[1]. The second major source of sulfur dioxide originates from industrial combustion processes (boilers, process heaters, metallurgical operations such as roasting and sintering, coke oven plants, processing of titanium dioxide, pulp production, thermal treatment of municipal and industrial waste). Also some non-combustion processes add to sulfur dioxide emission, such as sulfuric acid production, specific organic synthesis processes, treatment of metallic surfaces and oil refining processes. Overall data (1990) for North America, Western, Central, Eastern Europe, and Central Asia indicate that 88% of total sulfur emissions originate from combustion processes, 5% from production processes and 7% from oil refineries. In terms of contribution by fuel type, coal-fired industrial and electricity-generating plants account for more than 90% of all SO_2 emitted by stationary fuel-combustion sources.

Sulfur dioxide (along with NO_x) has a number of environmental effects. First, acid rain is formed when sulfur dioxide mixes with and dissolves in the water in clouds, eventually forming dilute sulfuric acid. Acid rain causes lake and soil acidification, forest die-off and corrosion of stone and metalwork. Furthermore, SO_2 contributes to the formation of acid aerosols, which can cause a haze over large regions. It is believed that such haziness can substantially reduce average temperatures in affected areas[13]. In this way, SO_2 could affect the earth's climate. SO_2 and related pollutants have also been linked to a number of human diseases[4]. The need for sulfur dioxide removal from flue-gases is therefore evident and acknowledged by many countries in treaties like 'The protocol to the 1979 convention on long-range transboundary air pollution on further reduction of sulfur emissions' of the

United Nations Economic Commission for Europe and the '1990 Clean Air Act Amendments' of the United States government.

General options for reduction of sulfur emissions include energy management measures, increase of the proportion of non-combustion renewable energy sources (i.e. hydro, wind, etc.) to the total supply, fuel switching (e.g. from high- to low-sulfur coals and/or liquid fuels, or from coal to gas), fuel desulfurization and advanced combustion technologies (e.g. coal gasification combined with gas desulfurization). Another category of processes aims at removing already formed sulfur oxides, and is referred to as Flue-Gas Desulfurization (FGD) processes. FGD was already applied in the Battersea power plant in London in 1926, where it consisted of scrubbing the flue-gas with alkaline water[86]. The state-of-the-art technologies for flue-gas treatment processes are all based on the removal of sulfur dioxide by wet, dry or semi-dry (also referred to as wet and dry) absorption processes and catalytic chemical processes. In some cases, options for reducing sulfur emissions may also result in the reduction of emissions of CO_2, NO_x and other pollutants.

Flue-gas treatment processes currently applied include: lime/limestone wet scrubbing (LWS); spray dry absorption (SDA); Wellman Lord process (flue-gas scrubbing with sulfite); ammonia scrubbing; and combined NO_x/SO_x removal processes (activated carbon process and combined catalytic removal). In the power generating sector, LWS and SDA cover 85% and 10% of the installed flue-gas desulfurization capacity, respectively. In LWS, aqueous lime or limestone slurries are contacted with the flue-gas in a scrubber. The sulfur dioxide dissolves in the aqueous phase and then reacts with hydroxide ions to form bisulfite (HSO_3^-), which subsequently reacts with Ca^{2+} to form the poorly soluble $CaSO_3$. $CaSO_4$ (gypsum) is also a product, as part of the bisulfite is oxidized to sulfate, due to the presence of oxygen in flue-gas. The resulting $CaSO_3$ and $CaSO_4$ mixture can be used in construction materials. However, impurities such as fly-ash and dust originating from the flue-gas may limit this application. Disposal of the waste then becomes the only alternative, resulting in additional costs and environmental pollution. Since about a decade, efforts have been made to develop a biotechnological alternative for conventional physico-chemical processes for removal of sulfur dioxide from flue-gases. This process is called Biotechnological Flue-Gas Desulfurization (Bio-FGD). In Bio-FGD, bacteria are used to fix SO_2 as elemental sulfur. In paragraph 1.2 this process is described in more detail. Because besides SO_2 also heat is transferred from the flue-gas to the scrubbing solution, it is attractive to operate the desulfurization process at (moderate) thermophilic conditions, so that no cooling is required.

1.2 Biological desulfurization

In this paragraph, the biological desulfurization process is described in greater detail (paragraph 1.2.1) and the choice for methanol as electron donor for reduction of sulfur oxyanions is discussed (paragraph 1.2.2).

1.2.1 Process description

Biotechnological Flue-Gas Desulfurization makes use of the following conversions of the sulfur cycle:

$SO_2 + H_2O \Rightarrow HSO_3^- + H^+$ (1)

$HSO_3^- + \frac{1}{2} O_2 \Rightarrow SO_4^{2-} + H^+$ (2)

$HSO_3^- + 6\,[H] \Rightarrow HS^- + 3\,H_2O$ (3a)

$SO_4^{2-} + 8\,[H] \Rightarrow HS^- + 3\,H_2O + OH^-$ (3b)

$HS^- + \frac{1}{2} O_2 \Rightarrow S + OH^-$ (4)

Figure 1.1 shows the flow sheet for Bio-FGD. In the first step of biological flue-gas desulfurization, sulfur dioxide is scrubbed from the flue-gas with a bicarbonate solution (reaction 1). Presence of oxygen in the flue-gas results in oxidation of part of the sulfite into sulfate (2). In the subsequent step, sulfite and sulfate are reduced under anaerobic conditions with an added electron donor to sulfide by sulfate-reducing bacteria (3a and b). Thereafter, the produced sulfide is partially oxidized to elemental sulfur by autotrophic sulfur bacteria like *Thiobacillus* spp. in a micro-aerobic reactor with concomitant production of hydroxide (4). Separation of the solid sulfur particles from the medium enables the recovery of elemental sulfur as a valuable product. The remaining bicarbonate solution, with a pH of about 9, can be reused for scrubbing of sulfur dioxide. Because along with SO_2, also heat is transferred from the flue gas to the scrubbing solution, it is attractive to operate the desulfurization process at thermophilic conditions (50-65°C).

Comparison of the Bio-FGD process with conventional lime/limestone wet scrubbing technologies shows that the Bio-FGD requires less input of energy and chemicals. Most products of conventional and biological desulfurization processes are reusable such as gypsum, sulfuric acid or sulfur. Site-specific factors such as market conditions and quality standards determine the economic value of these products.

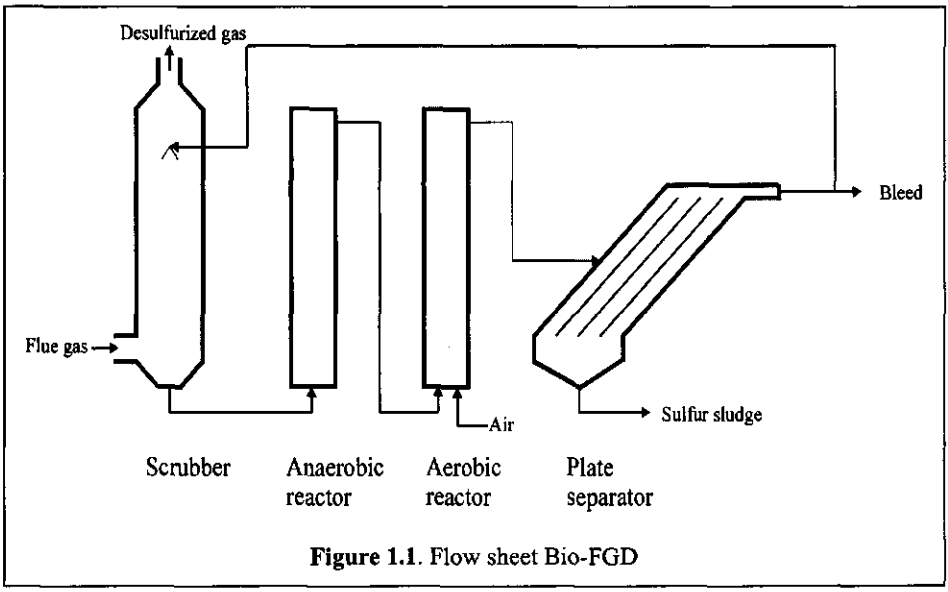

Figure 1.1. Flow sheet Bio-FGD

Apart from application in flue-gas desulfurization, sulfite- and sulfate-reducing bioreactors have potential application in other industrial desulfurization processes, including treatment of waste-gypsum[36,50] and in precipitation of heavy metals[32,116]. Also waste- and groundwater polluted with sulfate can be treated using the biological desulfurization process outlined above. In these cases, the process should preferably operate in the mesophilic temperature range due to the lower temperature of these wastestreams.

1.2.2 Choice for methanol as electron donor

An important factor determining the economic feasibility of biological desulfurization is the cost of the electron donor needed for sulfate reduction in the anaerobic step. Formation of undesirable side-products like methane and acetate needs to be minimized. Options for electron donors include organic waste materials such as primary sewage sludge, spent yeast from breweries, dairy whey, molasses and bulk chemicals like H_2, synthesis gas (a mixture of H_2, CO_2 and CO), ethanol and methanol (Table 1.1). Organic waste has the advantage of low costs, but adequate control of the process may be difficult because of its complex composition. For instance, intermediates formed during degradation of organic waste may promote undesirable growth of methanogens. Also, incomplete degradation of organic compounds may deteriorate the performance of the sulfide-oxidizing bioreactor of the desulfurization process[45].

The applicability of pure chemicals like lactate, ethanol, and acetate for sulfate reduction has been demonstrated in mesophilic laboratory-scale reactors (Table 1.1), but use of these chemicals on an industrial scale will probably be prohibitively expensive. Relatively cheap bulk chemicals synthesis gas or H_2/CO_2 are better options in this respect. Moreover, reasonable to good sulfate elimination rates can be achieved with these substrates in mesophilic gas-lift reactors (Table 1.1). However, under thermophilic conditions (preferable for Bio-FGD) elimination rates with H_2/CO_2 are lower, while it has been found that about half of the added hydrogen is used for methanogenesis, presumably due to good kinetic growth properties of thermophilic methanogens[40].

Table 1.1. Sulfate and sulfite elimination rates found in biological desulfurization processes with various electron donors.

Electron donor	T (°C)	bioreactor type	SO_4^{2-} removal ($g.L^{-1}.day^{-1}$)	SO_3^{2-} removal ($g.L^{-1}.day^{-1}$)	COD to H_2S/CH_4 (%/%)	ref.
molasses	31	packed bed	6.5	na[a]	nr[b]	66
m.s.d.[c]	30	packed bed	na	46	100/0	103
lactate	R.T[d]	plugflow	0.41	na	nr	32
acetate	35	packed bed	65	na	100/0	108
acetate	33	EGSB[e]	9.4	na	nr	22
ethanol	35	UASB[f]	6	na	nr	47
syngas	30	gas-lift	10	na	100/0	39
H_2/CO_2	30	packed bed	1.2	na	100/0	23
H_2/CO_2	30	gas-lift	30	na	100/0	38
H_2/CO_2	55	gas-lift	7.5	9.3	50/50	50
CO	30	packed bed	2.4	na	100/0	23

a) na = no sulfate or sulfite added; b) nr = not reported; c) m.s.d. = municipal sewage digest; d) R.T.= room temperature; e) EGSB = expanded granular sludge bed; f) UASB = upflow anaerobic sludge bed.

In the present study, the use of methanol as an alternative electron donor for thermophilic sulfate reduction was investigated. Methanol is a relatively cheap bulk chemical and therefore an attractive substrate for use in biotechnological processes[20]. Methanol is for instance successfully used as electron donor in denitrification[28] and it also has been proposed as electron donor in other sulfate-reducing processes[34]. Moreover, chemically synthesized

methanol contains little organic impurities, resulting in only a low extent of undesired biological side-reactions from these impurities. A low amount of impurities also makes additional treatment of biologically desulfurized wastewater redundant. Hence, methanol was selected as electron donor for reduction of sulfur oxyanions in our investigation.

1.3 Microbiology of thermophilic anaerobic methanol degradation

In this paragraph, the possible biological degradation routes of methanol are presented (paragraph 1.3.1). An overview of known sulfate reducers, methanogens and acetogens possibly involved in thermophilic methanol degradation is given in paragraphs 1.3.1, 1.3.2 and 1.3.3, respectively.

1.3.1 Anaerobic degradation of methanol

Possible degradation pathways for methanol under anaerobic conditions are shown in Figure 1.2. Reaction stoichiometries and Gibbs free energy changes are shown in Table 1.2. Three groups of microorganisms are involved in anaerobic methanol degradation, viz. sulfate-reducing bacteria (SRB), methanogenic archaea (MA) and homoacetogenic bacteria (AB). Methanol can be used directly as carbon and energy source by SRB (conversion 1)[74], MA (conversion 5a)[113] and AB (conversion 9)[97]. In addition, MA may reduce methanol to methane with H_2 (conversion 5b). Therefore, SRB, MA and AB will compete for the available methanol in mixed cultures. Thermophilic SRB (e.g. *Desulfotomaculum thermoacetoxidans*[71] and MA[77] may also compete for acetate (conversions 2 and 6), the product of methanol catabolism by AB (conversion 9). It has also been demonstrated that acetate can be oxidized to H_2/CO_2 (conversion 11) under mesophilic[101], as well as under thermophilic conditions[141]. Therefore, degradation of methanol to methane by a triculture consisting of a methylotrophic acetogen, acetate oxidizing species, and a hydrogenotrophic methanogen is theoretically possible. Furthermore, anaerobic bacteria may partially oxidize methanol to H_2/CO_2 (conversion 10), when the H_2 concentration is kept low by hydrogenotrophic sulfate reducers (conversion 3) or methanogens (conversion 7)[19]. In the mesophilic temperature range, even methanogens have been shown to produce H_2/CO_2 from methanol when grown in the presence of SRB[87]. Thus, competition for H_2 may take place as well.

At a high hydrogen partial pressure, H_2 may be consumed by homoacetogens[129]. As methanol oxidation to hydrogen is thermodynamically unfavourable at a high hydrogen

partial pressure, methanol oxidation followed by acetogenesis from H_2/CO_2 is not likely to occur. Therefore, this conversion is not included in Figure 1.2 and Table 1.2. Methanol conversion to formate (conversion 12) is thermodynamically unfavourable under standard conditions. To our knowledge, formate formation from methanol has not been reported in literature. However, besides hydrogen, formate can be important in methanogenic environments[8,21]. SRB (conversion 4)[74] and MA (conversion 8)[136] can subsequently use formate. Methanol degradation can even be more complex as formate conversion to hydrogen and acetate[31] and methanol degradation to butyrate[11] (not shown in Figure 1.2) may also occur. The above illustrates that mixed cultures may mineralize the relatively simple C_1-compound methanol in a complex way. As a consequence, SRB and MA may not only compete for methanol, but also for hydrogen, acetate, and formate.

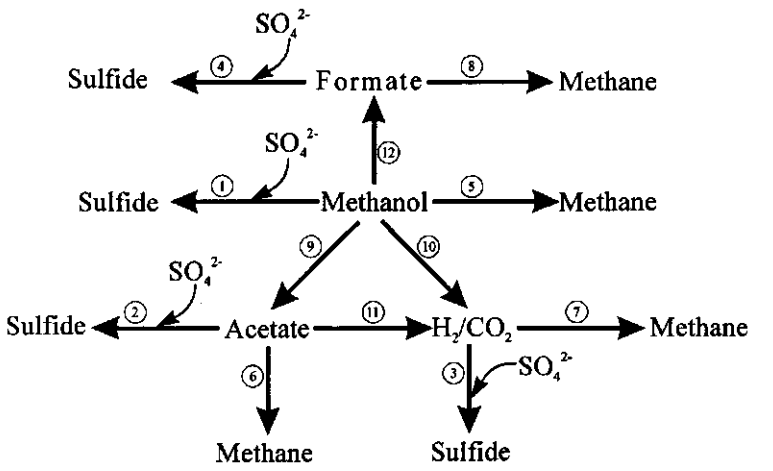

Figure 1.2. Anaerobic methanol mineralization. Conversion numbers correspond to the numbers of reaction equations in Table 1.2

Table 1.2. Stoichiometry and Gibbs free energy changes at standard conditions and pH 7 of reactions possibly involved in anaerobic methanol degradation. Calculated from Thauer et al. (1977)[112].

Reaction	$\Delta G^{0'}$ (kJ/reaction)
1) $4\ CH_3OH + 3\ SO_4^{2-} \Rightarrow 4\ HCO_3^- + 3\ HS^- + 4\ H_2O + H^+$	-364
2) $CH_3COO^- + SO_4^{2-} \Rightarrow 2\ HCO_3^- + HS^-$	-48
3) $4\ H_2 + SO_4^{2-} + H^+ \Rightarrow HS^- + 4\ H_2O$	-152
4) $4\ HCOO^- + SO_4^{2-} + H^+ \Rightarrow HS^- + 4\ HCO_3^-$	-172
5a) $4\ CH_3OH \Rightarrow 3\ CH_4 + HCO_3^- + H_2O + H^+$	-316
5b) $CH_3OH + H_2 \Rightarrow CH_4 + H_2O$	-113
6) $CH_3COO^- + H_2O \Rightarrow CH_4 + HCO_3^-$	-31
7) $4\ H_2 + HCO_3^- + H^+ \Rightarrow CH_4 + 3\ H_2O$	-136
8) $4\ CHOO^- + H_2O + H^+ \Rightarrow CH_4 + 3\ HCO_3^-$	-132
9) $4\ CH_3OH + 2\ HCO_3^- \Rightarrow 3\ CH_3COO^- + H^+ + 4\ H_2O$	-220
10) $CH_3OH + 2\ H_2O \Rightarrow 3\ H_2 + HCO_3^- + H^+$	+23
11) $CH_3COO^- + 4\ H_2O \Rightarrow 4H_2 + 2\ HCO_3^- + H^+$	+104
12) $CH_3OH + 2\ HCO_3^- \Rightarrow 3\ HCOO^- + H_2O + H^+$	+19

1.3.2 Thermophilic sulfate reducing bacteria

A common characteristic of sulfate-reducing bacteria is their ability to conserve energy by the reduction of sulfur oxyanions like sulfite, sulfate and thiosulfate. SRB vary widely in their morphological, physiological and phylogenetical characteristics. Table 1.3 summarizes some physiological characteristics of thermophilic SRB. The optimum pH for growth of known thermophilic sulfate-reducing eubacteria lies in the range of 6.5-7.5, and the optimum temperature in the range of 54-70°C. The upper temperature limit for growth of the known eubacterial SRB is 85°C. Most thermophilic SRB are able to grow at moderate or high NaCl-concentrations of up to 70 $g.L^{-1}$. The archaeon *Archaeglobus profundus* has different optima (optimum pH: 6.0, optimum temperature: 82°C). Most isolated thermophilic eubacterial SRB belong to the genus *Desulfotomaculum*, that includes some mesophilic species. Genera only consisting of thermophilic species are *Desulfacinum, Thermodesulfobacterium, Thermodesulforhabdus* and *Thermodesulfovibrio*. All species except *Thermodesulforhabdus norvegicus* use hydrogen as electron donor. Some species need acetate as carbon source for growth on hydrogen. *D. thermoacetoxidans* produces acetate and sulfide while growing on an excess of H_2/CO_2. Growth on formate with acetate as carbon source is common. Acetate is utilized only by a few species (Table 1.3). Methanol is utilization is even more rare, only a few *Desulfotomaculum* species grow on

this substrate, but utilization of methanol was not tested for some species (Table 1.3). Use of sulfite and thiosulfate as alternative electron acceptor is common among SRB, while some species additionally use elemental sulfur (e.g. *D. thermoacetoxidans*). *D. thermobenzoicum* is capable of nitrate reduction to ammonium. Under sulfate limiting conditions, some thermophilic SRB ferment pyruvate (e.g. *D. infernum, D. thermosapovorans, T. yellowstonii*).

Thermophilic *Desulfotomaculum* species have been isolated from various sources such as geothermal ground water[18,62,74], cold marine sediment[44], compost[25] and oil field waters[76,94]. Only D. *thermoacetoxidans*[71] and *D. thermobenzoicum*[110] were isolated from enrichment cultures that originated from methanogenic digesters. The importance of the latter two species in their original habitat seems rather minor, as enrichment procedures primarily select for SRB with a high maximum growth rate while in the methanogenic digesters (containing no or only low amounts of sulfate in the influent), the selection was mainly for sulfate reducers with a high affinity for sulfate or with the capacity of fermentative growth.

It was suggested by Widdel[124] that sulfate reduction in habitats with temperatures between 50 and 65°C is carried out by *Desulfotomaculum* species, while *Thermodesulfobacterium* species may also play a role above a temperature of 60 to 65°C. *Desulfotomaculum* species have a broad substrate range. Common substrates include alcohols, organic acids, hexoses and benzoate. *D.australicum, D.kuznetsovii, D.thermoacetoxidans* and *D. geothermicum* are capable of complete oxidation of organic substrates to CO_2. However, *D. geothermicum* does not grow on acetate. Appearance of spores is a clear indication that a sulfate-reducing isolate belongs to *Desulfotomaculum*, as this characteristic has not been observed for other SRB. Most thermophilic *Desulfotomaculum* species stain Gram-negative, although their cell wall has a typical Gram-positive structure. Phylogenetically, *Desulfotomaculum* species cluster with the branch of Gram-positive bacteria with DNA of low GC-content, and may be regarded as clostridia-like bacteria which have the additional capacity of dissimilatory sulfate reduction[124].

Two *Thermodesulfobacterium* species have been described to date. *T. commune* was isolated from sediment of a hot spring in Yellowstone National Park, USA[133] and *T. mobile* originated from warm oil field water[96]. Both species have a limited substrate range; besides H_2/CO_2 and formate, only lactate and pyruvate can serve as electron donor for sulfate reduction. The latter compounds are incompletely oxidized, to acetate. Phylogenetically, the genus branches near the root of the eubacterial tree and is therefore separate from other

Gram-negative SRB from the delta group of Proteobacteria or from Gram-positive *Desulfotomaculum* species[125].

Thermodesulfovibrio yellowstonii is the only species in this genus to date. It was isolated from a hydrothermal vent[35]. The only other growth substrates besides hydrogen and formate are lactate and pyruvate, which only are oxidized to acetate. *T. yellowstonii* represents a lineage that branches deeply within the Bacteria domain.

Like many other thermophilic SRB, *Thermodesulforhabdus norvegicus* and *Desulfacinum infernum* were isolated from samples collected from oil field water[7,90]. Both species belong to the sulfate-reducing bacteria of the delta subdivision of the Proteobacteria.

The capability of dissimilatory sulfate reduction is also found among hyperthermophilic archaea like *Archaeglobus profundus*[9]. Hyperthermophilic microorganismes are not able to grow below 60°C. Hyperthermophiles have mainly been isolated from submarine hydrothermal vents, oil field waters and continental solfataras, but never from anaerobic digesters. Sulfate reduction has only been reported for *A. profundus* and *A. fulgidus*. Sulfite and thiosulfate reduction has been reported for a number of other species[107].

Table 1.3. Selected physiological characteristics of thermophilic SRB.

Organism	C_1 and C_2 growth substrates	MeOH [a] tested	T-range[b] (°C)	T_{opt}[c] (°C)	pH_{opt}[d]	ref.
Archaeoglobus profundus	H_2 (+Ac)[e]	yes	65-90	82	6	9
Desulfacinum infernum	H_2+CO_2, formate, Ac[f], EtOH[g]	no	40-65	60	7.1-7.5	90
Desulfotomaculum australicum	H_2+CO_2, Ac, EtOH	yes	40-74	68	7-7.4	62
Desulfotomaculum geothermicum	H_2+CO_2, formate, EtOH	yes	37-57	54	7.3-7.5	18
Desulfotomaculum kuznetsovii	H_2+CO_2, MeOH, Ac, EtOH	yes	50-85	60-65	nr[h]	74
Desulfotomaculum nigrificans	H_2+CO_2(+Ac), EtOH formate(+Ac),	yes	30-70	55	nr	10,52
Desulfotomaculum thermoacetoxidans	H_2+CO_2, formate, Ac	no	45-65	55-60	6.5	71
Desulfotomaculum thermobenzoicum	H_2+CO_2, formate(+Ac), EtOH	yes	40-70	62	7.2	110
Desulfotomaculum thermocisternum	H_2+CO_2, EtOH	yes	41-75	62	6.7	76
Desulfotomaculum thermosapovorans	H_2+CO_2, formate, MeOH, EtOH	yes	35-60	50	7.2-7.5	25
Desulfotomaculum strain T93B/T90A	H_2+CO_2, formate, MeOH, EtOH	yes	43-78	65	7.0	94
Thermodesulfo-bacterium commune	H_2(+Ac)	no	45-85	70	7.0	133
Thermodesulfo-bacterium mobile	H_2(+Ac), formate(+Ac)	yes	45-85	65	nr	95,96
Thermodesulfor-habdus norvegicus	Ac, EtOH	no	44-74	60	69	7
Thermodesulfovibrio yellowstonii	H_2(+Ac), formate(+Ac)	no	40-70	65	6.8-7.0	35

a) MeOH: methanol; b) T-range: range of growth temperature; c) T_{opt}: optimum growth temperature; d) pH_{opt}: optimum pH for growth; e) +Ac: acetate needed as carbon source; f) Ac: acetate; g) EtOH: ethanol; h) nr: not reported.

1.3.3 Thermophilic methanogens

Thermophilic methanol degrading methanogens isolated to date all belong to the genus *Methanosarcina* (Table 1.4). These *Methanosarcina*-strains probably all are *M. thermophila*[15,137]. Accordingly, these strains have very similar physiological characteristics. The optimum temperature for growth lies in the range of 50 to 57°C while no growth is possible beyond 65°C. *M. thermophila* TM-1 is the only hydrogen-utilizing species, but acetate can be used by all *Methanosarcina* strains. Most *Methanosarcina* species were isolated from anaerobic digesters[80,113,138,142]. The only other acetate-utilizing methanogenic genus is *Methanothrix*, for which the upper temperature for growth is 70°C[137]. Thermophilic *Methanothrix* have been isolated mainly from anaerobic digesters. Hydrogenotrophic methanogenesis may occur at temperatures as high as 97°C[107]. A review on (hyper)thermophilic methanogenesis from H_2/CO_2 can be found elsewhere[64]. In this thesis work, research has been done with the moderately thermophilic genus *Methanobacterium*. Species belonging to this genus are commonly isolated from anaerobic digesters[114,135,136]. All *Methanobacterium* species grow autotrophically on H_2/CO_2. In addition, some species use formate. The optimum pH for most moderately thermophilic methanogens lies around neutral values.

1.3.4 Thermophilic acetogens growing on C_1-compounds

An overview of thermophilic homoacetogenic bacteria has been made by Lowe et al.[64]. For growth on methanol, CO_2 must be present as electron acceptor. The best characterized methanol degrading homoacetogens are *Moorella thermoautotrophicum* and *M. thermoaceticum* (Table 1.5). Some thermophilic acetogens are known to utilize sulfite or thiosulfate as electron acceptor[37,131]. However, none of the latter species use methanol as electron donor. About half of the known homoacetogenic clostridia produce some butyrate[11]. However, among the described methanol degrading thermophiles, butyrate is a less common product[54].

Table 1.4. Selected physiological characteristics of moderately thermophilic MA.

Organism	Growth substrates	T-range[a] (°C)	T_{opt}[b] (°C)	pH_{opt}[c]	ref.
Methanosarcina CHTI 55	MeOH[d]	35-63	57	6.8	113
Methanosarcina MP	MeOH	30-60	55	6.5-7.0	80
Methanosarcina MSTA-1	Ac[e], MeOH, methylamines	30-65	55	7	15
Methanosarcina CALS-1	MeOH, Ac	30-60	55-58	6.5	138
Methanosarcina thermophila TM-1	H_2/CO_2, Ac, MeOH, methylamines	35-60	±50	6-7	142
Methanothrix sp. CALS-1	Ac	45-65	60	6.5	139
Methanothrix thermoacetophila	Ac	nr[f]-70	65	nr	77
Methanobacterium thermoautotrophicum	H_2/CO_2	40-75	65	7.2-7.6	134
Methanobacterium thermoformicicum	H_2/CO_2, formate	nr	55	7-8	136

a) T-range: range of growth temperature; b) T_{opt}: optimum growth temperature; c) pH_{opt}: optimum pH for growth; d) MeOH: methanol; e) Ac: acetate; f) nr: not reported.

Table 1.5. Selected physiological characteristics of thermophilic methanol- and H_2/CO_2-utilizing homoacetogens.

Organism	C_1 growth substrates	T-range[a] (°C)	T_{opt}[b] (°C)	pH_{opt}[c]	ref.
Moorella thermoautotrophicum	H_2/CO_2, formate, CO, MeOH[d]/CO_2	36-70	56-60	5.8	61,129
Moorella thermoaceticum	H_2/CO_2, formate, CO, MeOH /CO_2	nr[e]	55-60	nr	51,130
Clostridium thermocellum	MeOH /CO_2, formate	nr	60-64	7	64
Acetogenium kivui	H_2/CO_2, formate	50-73	66	6.4	56
Strain AG	MeOH /CO_2	55-75	70	nr	19

a) T-range: range of growth temperature; b) T_{opt}: optimum growth temperature; c) pH_{opt}: optimum pH for growth; d) MeOH: methanol; e) nr: not reported.

1.4 Sulfate/sulfite reduction and methanogenesis in anaerobic reactors

Classic anaerobic treatment of wastewater primarily aims to convert organic substances to methane and carbon dioxide by methanogens, thereby reducing the COD-content of the wastewater. From this perspective, sulfate reduction is disadvantageous because it leads to unwanted formation of remaining COD in the form of sulfide in the effluent of the reactors. Apart from that, the build-up of hydrogen sulfide increases the risk of process failure as a result of inhibition by sulfide of bacteria involved in the degradation of organic waste. Other disadvantages of the presence of sulfide in methanogenic digesters are the malodor ('rotten eggs') of hydrogen sulfide, the lower amount and quality of biogas and corrosion of metal and stone, as reviewed by Hao et al.[33]. However, in biological desulfurization processes methanogenesis should be avoided as it decreases the selectivity of sulfate reduction with the added electron donor. In paragraph 1.4.1 the factors that determine whether sulfate reduction or methanogenesis prevails in bioreactors are discussed.

In order to attain high reduction rates of sulfur oxyanions in bioreactors, high biomass concentrations must be maintained, e.g. by self-immobilization of biomass as in upflow anaerobic sludge blanket reactors. Some examples from literature concerning self immobilization of sulfate-reducing biomass in reactors are presented in paragraph 1.4.2.

Sulfite is an important constituent of the scrubber liquor in biological desulfurization of flue-gases. Introduction of sulfite may affect the performance of sulfate-reducing bioreactors, as will be discussed in paragraph 1.4.3.

1.4.1 Competition between methanogens and sulfate reducers in bioreactors

Competition between mesophilic MA and SRB has been studied quite extensively. Reviews on this subject have been presented elsewhere[16,42,83]. Competition between methanogens and sulfate reducers in high-rate anaerobic reactors is not merely determined by growth kinetics, but also by immobilization properties of the various microorganisms, substrate diffusion limitations inside biofilms, environmental conditions such as hydrogen sulfide concentration, the composition of the medium, temperature and pH. In addition, the bacterial composition of the seed sludge and the applied hydraulic retention time[3] may also be important. In this paragraph, general aspects of competition between MA and SRB (identified mostly in mesophilic systems) are discussed. Special attention will be paid to competition between thermophilic MA and SRB for methanol, hydrogen and acetate. The latter two substrates are possible degradations products of methanol under anaerobic conditions.

Thermodynamics

A simple method to predict the outcome of competition between bacterial species for a common substrate is to calculate the Gibbs free energy change of the conversion the substrate. The species performing the conversion with the highest Gibbs free energy change presumably outcompete other bacteria. Based on such calculations, SRB should outcompete MA for substrates like methanol, acetate, hydrogen and formate (Table 1.2). However, this does not always correspond with findings from literature. For instance Gupta et al.[30] found that methanol was solely used by methanogens in mesophilic chemostats.

Growth kinetics

The rate at which bacteria grow can be described by the classical Monod equation:

$$\mu = \mu_{max} * \frac{S}{S + K_s}$$

in which: μ : specific growth rate
S: substrate concentration
μ_{max} : maximum specific growth rate
K_S: affinity constant for substrate.

For sulfate-reducing bacteria, the Monod-equation can be extended to:

$$\mu = \mu_{max} * \frac{S}{S + K_s} * \frac{SO_4^{2-}}{SO_4^{2-} + K_{SO4}^{2-}}$$

in which: SO_4^{2-}: sulfate concentration
K_{SO4}^{2-}: affinity constant for sulfate

According to Monod growth kinetics, growth only stops when all substrate is depleted. However, many bacteria, including MA and SRB, stop growing below a certain substrate 'threshold' concentration[17,63]. In addition, sulfate reducers may encounter a threshold concentration for sulfate as well[105]. The Monod equation can be adapted to account for threshold concentrations[85]:

$$\mu = \mu_{max} * \frac{(S - S_t)}{(S - S_t) + K_s}$$

in which: S_t = substrate threshold concentration.

For SRB, the equation becomes:

$$\mu = \mu_{max} * \frac{(S - S_t)}{(S - S_t + K_s)} * \frac{(SO_4^{2-} - SO_4^{2-}{}_t)}{(SO_4^{2-} - SO_4^{2-}{}_t) + K_{SO4}^{2-}}$$

in which: $SO_4^{2-}{}_t$: sulfate threshold concentration

The kinetic parameters of the Monod equation are conditional constants: they depend on environmental conditions such as pH and temperature. Growth kinetics may be used to explain the outcome of competition between microbial species in high-rate anaerobic reactors. For instance, *Methanothrix* species will dominate in thermophilic anaerobic sludge cultivated at low acetate concentrations because of their higher acetate affinity as compared to that of *Methanosarcina*[140]. It should however be kept in mind, that most reported values for kinetic growth properties were determined at optimal growth conditions in pure culture, and such optimal and well-defined conditions obviously do not prevail in bioreactors.

The ratio μ_{max}/K_S is a useful parameter for comparing growth properties of bacteria on a common substrate. At substrate concentrations around or below the K_S, bacteria with a high μ_{max}/K_S-ratio have better growth properties than bacteria with a low μ_{max}/K_S-ratio.

As outlined in paragraph 1.3.1, common substrates for which MA and SRB may compete in the anaerobic degradation of methanol comprise methanol and methanol degradation products like hydrogen, formate and acetate. Also homoacetogens may compete with the MA and SRB for methanol. The kinetics of acetate and hydrogen degradation by mesophilic MA and SRB has been studied rather extensively[16,83]. Some relevant information about the growth kinetics of hydrogen and acetate utilizing thermophilic MA is also available (Table 1.6 and 1.7), but so far this is hardly the case for thermophilic SRB. Unfortunately, to date not for a single thermophilic sulfate reducer both the K_s- and μ_{max}-are known. In general it can be stated that the K_s-values for hydrogen are about 40 times lower for SRB than for MA, while the values for μ_{max} of MA are maximally about 10 times higher compared to those of SRB. Therefore, it looks reasonable to expect a higher μ_{max}/K_S-ratio for hydrogen for thermophilic SRB than for MA. Consequently, SRB likely will outcompete MA at low hydrogen concentrations. At a high hydrogen concentration, the situation is reverse due to the high maximum specific growth rates of MA. For acetate the situation is much less clear, as no K_S values of thermophilic SRB have been reported to date. Growth kinetic data for thermophilic methanol-degrading sulfate reducers and homoacetogens are summarized Table 1.8. No data are available for methanogens. The limited amount of data does not allow to draw a conclusion on the outcome of the

competition for methanol. Because no growth kinetic data are available for growth on formate of thermophilic SRB and MA, a comparison of growth properties of these groups of bacteria is not possible.

Table 1.6. Selected growth kinetic properties of thermophilic MA and SRB on acetate.

Acetate degrading culture	μ_{max} (h^{-1})	K_S (mM)	threshold (mM)	yield[a]	μ_{max}/K_S ($h^{-1}.mM^{-1}$)	ref.
Methanogenic						
Methanosarcina thermophila TM-1	0.058	4.8	1	nr[b]	0.012	142
Methanosarcina CALS-1	0.058	nr	0.8-2.5	nr	nr	70,140
Methanosarcina MP	nr	nr	nr	nr	nr	80
Methanosarcina MSTA-1	0.052	11.4	4.1	3.1-4.6	0.0046	15
Methanosarcina CHTI 55	0.085	10	nr	1.4	0.0085	113
Methanothrix thermoacetophila	nr	nr	nr	nr	nr	77
Methanosaeta sp. P_T	0.020	nr	nr	nr	nr	48
Methanothrix sp. CALS-1	0.028	<1.1	0.012-0.021	nr	>0.025	139
TAM	0.012	0.85	0.025-0.075	nr	0.014	2
Sulfate-reducing						
Desulfotomaculum thermoacetoxidans	0.022	nr	nr	nr	nr	71

a) yield expressed in g dry cells/mol acetate; b) nr = not reported.

Table 1.7. Selected growth properties of thermophilic MA, SRB and AB on hydrogen.

Hydrogen degrading culture	μ_{max} (h^{-1})	K_S (μM)	threshold (Pa)	yield[a]	μ_{max}/K_S ($h^{-1}.mM^{-1}$)	ref.
Methanogenic						
Methanobacterium thermoautotrophicum	0.14-0.69	80-120	5	0.6-1.6 3[b]	0.0018-0.004	102,111, 134,98
Methanobacterium Strain THF	nr[c]	nr	14	nr	nr	63
Sulfate-reducing						
Desulfotomaculum thermoacetoxidans	0.077	nr	nr	nr	nr	71
Desulfotomaculum spp.	nr	2	0.01	nr	nr	98
Strain SR	0.052	nr	nr	nr	nr	19
Thermodesulfobacterium Strain JSP	nr	2.4[d]	1.2	nr	nr	105
Thermodesulfovibrio Strain R1Ha3	nr	1.9[d]	0.5	nr	nr	105
Homoacetogenic						
Moorella thermoautotrophicum	0.021	nr	nr	nr	nr	97
Acetogenium kivui	0.35	nr	1000	nr	nr	56,17

a) yield expressed in g dry cells/mol end product; b) under hydrogen limitation; c) nr: not reported; d) K_m.

Table 1.8. Selected growth properties of thermophilic SRB and AB on methanol.

Methanol degrading culture	μ_{max} (h^{-1})	yield (g dry wt/mol acetate)	ref.
Sulfate-reducing			
Desulfotomaculum kuznetsovii	0.03	nr[a]	29
Coculture acetogen AG and sulfate reducer SR	0.011	nr	19
Homoacetogenic			
Strain AG	0.07	nr	19
Moorella thermoautotrophicum	0.077	6-9	97

a) nr: not reported

Environmental conditions

In bioreactors fed with an influent containing methanol, sulfite, and sulfate, inhibition may result from high concentrations of substrates or possible intermediates and products such as acetate and sulfide. A different susceptibility of SRB and MA towards these compounds may act as a selection criterion in bioreactors. Also the pH, temperature and presence of trace elements may affect the competition. All these factors are discussed below.

Sulfide toxicity. Sulfate reduction results in production of hydrogen sulfide (H_2S), which, at higher concentrations, can become quite inhibitory for microbial growth. H_2S is a very weak acid (pK_a of 7.0 at 30°C) and therefore at neutral values, the optimal pH range for most anaerobic microorganisms, sulfide is mainly present as H_2S (hydrogen sulfide or free sulfide) and HS^- (bisulfide). The sulfide ion (S^{2-}) only occurs (>1% of total sulfide) as important sulfide species at pH > 10, because the pK_a of HS^- is about 12 [104]. Hydrogen sulfide is considered to be the most toxic form of sulfide[92], because of the neutrality of the H_2S-molecule, which allows its easy diffusion through the lipid cell membrane into cytoplasm, where it reacts with cell components. The reversibility of sulfide inhibition, as observed by Okabe et al.[78] and Reis et al., seems contradictory to this hypothesis as it may be expected that chemical reactions with cell components are irreversible. As sulfide is a characteristic end product of sulfate-reducing bacteria, it may be speculated that SRB have developed a high tolerance towards sulfide in order to prevent self-poisoning. However, this is not necessarily the case; hydrogen sulfide concentrations as low as 60 $mgS.L^{-1}$ are already inhibitory for a thermophilic *Desulfotomaculum*-species[71]. Moreover, bacteria not capable of dissimilatory sulfate reduction such as methanogens may have a higher tolerance to H_2S than SRB[67,115].

The presence of sulfide may affect SRB in several ways. For the mesophilic *Desulfovibrio desulfuricans* it was demonstrated that 250 $mgS.L^{-1}$ of total sulfide lowers the growth rate and growth yield by 50%[79]. By contrast, the substrate utilization rate increased at higher sulfide concentration, showing that growth and activity were uncoupled. Uncoupling of growth and activity at higher sulfide concentrations was also observed for anaerobic sulfate-reducing and methanogenic sludge granules[119]. By increasing the total sulfide concentration, the cell size may decrease, as was shown for *Desulfovibrio desulfuricans*[78]. This may partly explain the decreased cell yield at increasing sulfide concentrations.

Literature regarding H_2S inhibition levels at mesophilic conditions has been reviewed elsewhere[16,33,83]. The free H_2S levels which are inhibitory for mesophilic methanogenesis vary from 50-400 $mg.L^{-1}$. Complete inhibition of growth of mesophilic SRB has been observed at a H_2S concentration of 85 $mg.L^{-1}$ [123] to 547 $mg.L^{-1}$ [91]. No data are available on

sulfide inhibition of methanogens at thermophilic conditions. Complete inhibition of growth of thermophilic SRB may occur at total sulfide levels as low as 60 $mgS.L^{-1}$ [71] or as high as 400 $mgS.L^{-1}$ [74]. The variation in literature data regarding H_2S-toxicity reflects the complexity of the matter, i.e. the influence of several factors, such as the type of bacterial species studied, growth substrate[65] and time of exposure to sulfide. For undefined cultures, the discrepancies may also be a result of interference with competitive and mutualistic microbial interactions between individual species. Another cause of the discrepancies in literature may originate from neglecting pH and sulfide concentration gradients in biofilms[57].

The lack of uniformity in methods for quantifying sulfide inhibition, the many factors that affect sulfide inhibition and the possible interference with bacterial interactions and diffusion hardly justify comparison of literature data. In effect, based on literature data, it can not be predicted whether SRB or MA will be more affected by sulfide in a specific situation.

Sulfate and sulfite toxicity. Sulfate is generally not toxic for anaerobic bacteria at concentrations up to 10 $g.L^{-1}$ [49,72]. For most wastewaters, as well as for the scrubbing solution from a Bio-FGD plant, sulfate toxicity is not relevant, as the concentration generally remains below this value. On the other hand, sulfite is very toxic for microorganisms and it is for that reason used as anti-bacterial agent, for example in wine processing. The mechanism of sulfite inhibition is not exactly known[12].

In pure cultures of SRB, complete inhibition of growth at concentrations as low as 40 $mg.L^{-1}$ (0.5 mM) sulfite was observed[126]. Methane production by *Methanobacterium ruminantium* decreased by a factor 2 at 100 $mg.L^{-1}$ sulfite[88]. Sulfite may have two effects on the activity of methanogenic sludge. Puhakka et al.[89] found that sulfite toxicity leads to a prolonged lag phase in methane production by anaerobic sludge in batch reactors at concentrations exceeding 250 $mg.L^{-1}$. In addition, the rate of methane production decreased linearly to very low values in the range of 150 to 2500 $mg.L^{-1}$ sulfite. However, after repeated sulfite addition to sludge, the toxicity effect may decrease due to growth of sulfate-reducing bacteria or due to adaptation of the biomass.

Methanol toxicity. Alcohols are toxic for microorganisms at higher concentrations, presumably due to the fact that they damage the cell membrane and due to end product inhibition of glycolytic enzymes[24]. Most bacteria are able to withstand ethanol concentrations of at least 10 $g.L^{-1}$. As alcohol toxicity towards bacteria decreases with decreasing chain length, it may be speculated that methanol toxicity will not occur at

concentrations below 10 $g.L^{-1}$ (0.3 M). This was confirmed for *Moorella thermoautotrophicum* and *Moorella thermoaceticum* as these species tolerate methanol concentrations up to 16 $g.L^{-1}$ (0.5 M) [128,130]. With 10 $g.L^{-1}$ methanol, 22 $g.L^{-1}$ sulfate can be reduced to sulfide. As sulfate concentrations normally will be less than 6 $g.L^{-1}$ in biodesulfurization of flue-gases, added methanol concentrations will normally not exceed 3 $g.L^{-1}$, which likely does not result in toxicity effects.

Acetate toxicity. Methanol degradation by homoacetogens may result in accumulation of acetate, which is toxic for microorganisms at higher concentrations. Similar to sulfide, unionized acetate (acetic acid) is considered the most toxic form[73]. Van Lier[60] found 50% inhibition of methane formation by thermophilic sludge occurred at an acetic acid concentration of about 1 mM, while they observed a 10 times lower susceptibility of mesophilic methanogenic sludge towards acetic acid. For thermophilic methylotrophic *Methanosarcina* spp., complete inhibition of growth was found at 9 mM acetic acid[132]. Inhibition by acetic acid may manifest in weakly buffered bioreactors producing acetate. At pH 6 and a temperature of 55°C, a concentration of 1 mM of undissociated acetate already is present at a total acetate concentration of 17 mM. This can be calculated using a pK_a for acetic acid of 4.8 at 55°C [104].

pH. SRB and MA may have different pH-optima or pH ranges for growth on common substrates. As the speciation of compounds like acetate, H_2S and NH_4^+ is affected by the pH, the effect of a pH change on the growth of SRB and MA may partially also result from a change in the concentration of these compounds. Visser et al.[121] found for anaerobic sludge that thermophilic (55°C) SRB outcompete methanogens for acetate at pH 8.3-8.6, while the rates of methanogenesis and sulfate reduction at pH 7.6-7-9 were about equal. Minami et al.[72] suggested that pH may have a large effect on the occurrence of methanogenesis or sulfate reduction from methanol. They found that sulfate reduction prevailed at pH 7.0-7.5 in a moderate thermophilic (53°C) methanol-fed bioreactor. At pH values between 6.2 and 6.8, sulfate reduction was suppressed and methanogenesis prevailed. Inhibition of SRB in the lower pH range may however also have resulted from elevated H_2S concentrations.

Temperature. Differences in optimal growth temperatures and growth temperature ranges may cause shifts in the microbial composition of mixed cultures upon a temperature change. A shift from a methanogenic to a sulfate-reducing population or vice versa also alters the anaerobic mineralization profile, as exemplified by a study of Visser et al.[122]. They found a rapid shift from methanogenesis to sulfate reduction after elevating the temperature of an acetate and sulfate fed UASB reactor from 30 to 55°C. A temperature increase from 37 to

55°C had the same effect[93]. No acetoclastic methanogens have been isolated growing beyond a temperature of 70°C[137]. It may therefore be speculated that acetoclastic methanogenesis does not occur in reactors beyond this temperature. As acetotrophic sulfate reduction is still possible up to at least 85°C (Table 1.3), the electron flow in acetate-rich environments may therefore be diverted from methane to sulfide as a result of a temperature increase from below 70°C to 70-85°C. The situation is similar for methanol; no methylotrophic methanogens are known that grow at temperatures above 65°C (Table 1.4), while the methanol-utilizing sulfate reducer *Desulfotomaculum kuznetsovii* was reported to grow up to 85°C[74]. Temperature may also affect SRB and MA indirectly, as temperature decreases the concentration of inhibitory H_2S due to a lower pK_a of H_2S at increasing temperature.

Trace elements. As essential constituents of cell components, in particular proteins, trace elements need to be available to microorganisms in order to facilitate growth. Bacteria compete for trace elements when these are limiting, and it may be expected that species with a low (or no) requirement or a high affinity for limiting trace elements will eventually dominate. Iron, cobalt, nickel, zinc and copper were identified as trace elements that are necessary to maintain maximum growth of methanogens[106]. Growth of mesophilic methylotrophic methanogens and homoacetogens was found to be optimal at an added cobalt concentration of 0.1 $mg.L^{-1}$ [27]. In this case, cobalt requirement may be explained by the high content of corrinoids of methanol-grown methanogens and acetogens[43,55,109]. Corrinoids are cobalt-containing co-factors involved in the first step of methanol degradation in both trophic groups[68,69]. It is not known if corrinoids are involved in methanol degradation by SRB. As opposed to MA, little is known about trace element requirements of SRB. Under sulfate-reducing conditions, it may be speculated that trace metals like zinc and cobalt are growth-limiting as the concentration of these metals may be extremely low due to precipitation of insoluble metal sulfides. However, Parkin et al.[84] found that the concentration of trace metals in microbial cultures was independent of the sulfide concentration, which was explained by microbial production of chelating agents.

Other factors affecting competition

At a COD/sulfate-ratio of 1.7 of the influent of the anaerobic reactors SRB outcompete MA under mesophilic conditions, while the opposite is true for a ratio above 2.7 [14]. Time is another important factor in the competition between SRB and MA in anaerobic reactors with high sludge retention times, as the microbial composition of anaerobic sludge changes only slowly due to the relatively low growth rates of SRB and MA. Therefore, it may take

very long periods of time (years) until sulfate reduction dominates over methanogenesis when SRB are present in low numbers in the seed sludge of anaerobic reactors[82]. This demonstrates the need for patience when studying microbial competition in anaerobic reactors. The seemingly contradictory results in the literature concerning competition therefore can be attributed -at least partially- to a different time scale of experiments.

Better adhesion properties of MA compared to that of SRB were used to explain the decrease of sulfate reduction in favor of methanogenesis following an increase of the liquid upward velocity in an UASB reactor[81].

Diffusion limitation may also affect competition. Nielsen[75] showed that sulfate-reducing activity in biofilms that are only several hundred μm thick, is limited by sulfate diffusion into the biofilm at sulfate concentrations below 50 $mg.L^{-1}$. This observation might, in some cases, represent an explanation for the relatively poor capacity of the SRB to compete with methanogenic bacteria in anaerobic bioreactors with sludge retention based on immobilization.

1.4.2 Self-immobilization of sulfate-reducing biomass

Stable performance of high-rate anaerobic reactors, as used in this study, relies on maintaining a high biomass concentration in the reactor. This can be achieved by biomass retention within the reactor or by sedimentation and recycling of washed-out biomass. UASB (upflow anaerobic sludge bed) reactors rely on retention of well settleable biomass inside the reactor. Preferably, the biomass in such reactors should consist of sludge granules (macroscopically smooth, round to oval shaped spheres up to 5 mm in size). Such granules have good settling characteristics. The increased liquid upflow velocities typical for EGSB (expanded anaerobic sludge bed)-reactors provide a selection pressure for development of granules[58] as non-granular sludge particles with poor settling properties will wash out. The biomass must however have the intrinsic ability to form granules, otherwise granulation will not occur, irrespectively of the selection pressure. The mechanism of granulation has not been elucidated entirely, though many factors involved have been identified, as reviewed by Schmidt and Ahring[99]. Two factors presumably involved in granulation will not prevail in upflow reactors in which SRB are virtually the only metabolically active species. First, growth of methanogens of the genus *Methanosaeta*, which presumably initiates or at least enhances granulation[41], will not occur. This may explain the lack of granulation in a completely sulfidogenic UASB[120], while in reactors which produced methane in addition to sulfide, granulation proceeded satisfactorily. By contrast, Omil et al.[81] did find granulation in a UASB producing only sulfide, although the formed

sulfidogenic granules had a much lower strength compared to methanogenic granules. Apparently, *Methanosaeta* species are beneficial, but not strictly necessary for granulation. Secondly, acidifiers will also be absent in anaerobic reactors in which sulfate-reducing bacteria use the added electron donors directly for sulfate reduction. Vanderhaegen[118] indicated that acidifiers are also important in the granulation process. This is supported by findings of Uemura and Harada[117] and van Lier[59] who reported granulation of thermophilic methanogenic sludge when sucrose or glucose was added to the influent.

1.4.3 Sulfite reduction

Sulfur dioxide easily dissolves in water (at 15°C, 45 L of SO_2 dissolves in 1 L of water) to form sulfurous acid. The pK_a of HSO_3^- in water is 7.2 at 25°C[100]. Sulfite reduction is energetically more favorable than sulfate reduction. On biochemical level, this is manifested by the ATP demanding activation of sulfate to adenosine-5'-phosphosulfate (APS) by ATP-sulfurylase, which is followed by APS reduction to form sulfite and AMP. Sulfite is directly suitable as electron acceptor for the SRB. A review of the biochemistry of sulfate reduction can be found elsewhere[127]. Use of thiosulfate as terminal electron acceptor is energetically also more favorable than the use of sulfate, as thiosulfate reduction requires, similar to sulfite reduction, no ATP-dependent activation[53]. This may explain the preferential use of thiosulfate over sulfate as electron acceptor in fresh water sediment[46]. Thiosulfate reduction by SRB may lead to higher cell yields compared to sulfate reduction[5].

Disproportionation

Bak and Pfennig[6] were the first to describe the disproportionation of sulfite and thiosulfate to sulfide and sulfate by the sulfate-reducing bacterium *Desulfovibrio sulfodismutans* according to the following stoichiometry:

$$S_2O_3^{2-} + H_2O \Rightarrow SO_4^{2-} + HS^- + H^+ \quad (\Delta G^{o'} = -21.9 \text{ kJ/mol } S_2O_3^{2-})$$

$$4\ SO_3^{2-} + H^+ \Rightarrow 3\ SO_4^{2-} + HS^- \quad (\Delta G^{o'} = -58.9 \text{ kJ/mol } SO_3^{2-})$$

Later it was found that many SRB are able to disproportionate sulfite and thiosulfate[53]. Jørgensen and Bak[46] demonstrated that disproportionation and thiosulfate reduction may occur simultaneously. Even disproportionation of elemental sulfur by SRB has recently been demonstrated[26]. For growth of disproportionating SRB known thus far, acetate is needed as carbon source. Whether methanol can be used as carbon source by disproportionating bacteria is not known.

Sulfite reduction in anaerobic reactors

Although it is toxic, sulfite is readily reduced to sulfide and causes no toxicity related problems in anaerobic bioreactors when an active sulfate-reducing population is present. However, peaks in the sulfite loading rate or low activity of the SRB (e.g. at the start-up) may lead to accumulation of sulfite in the bioreactor, and accordingly, to inhibition of the biomass. The process performance may even be impaired for a long time when sulfite inhibition is irreversible. To prevent sulfite toxicity during start-up, one might introduce only sulfate until a substantial sulfate- (and sulfite) reducing population is present. Alternatively, aerating wastewater prior to anaerobic treatment might be applied to oxidize sulfite to the non-toxicant sulfate.

Sulfite addition to sulfidogenic bioreactors may lead to the chemical formation of thiosulfate[53]:

$4\ HSO_3^- + 2\ HS^- \Rightarrow 3\ S_2O_3^{2-} + 3\ H_2O$ ($\Delta G^{o'} = -167$ kJ/mol)

Van Houten et al.[40] detected about 15 $mg.L^{-1}$ (0.13 mM) thiosulfate in a thermophilic bioreactor fed with H_2/CO_2, sulfite and sulfate. The rather low thiosulfate concentration led to the conclusion that the rate of sulfite reduction was higher than the chemical conversion rate of sulfite with sulfide to thiosulfate. An alternative explanation could be that the rates of thiosulfate formation and reduction are about equally high.

1.5 Scope of this thesis

The aim of the research described in this thesis was to study the use of methanol as external electron donor for biological desulfurization of flue-gases and ground- and wastewaters. In such a process methanol is used as external electron donor for anaerobic biological reduction of sulfur oxyanions to hydrogen sulfide.

The main part of the thesis is dedicated to sulfate and sulfite reduction with methanol at thermophilic (65°C) conditions, applicable for biological desulfurization of hot flue-gases. In Chapter 2 the selectivity of thermophilic sulfate reduction with methanol in bioreactors is investigated, while in Chapter 5 experiments are described that aimed to increase the selectivity, i.e. to inhibit methane formation from methanol. Chapters 3 and 4 focus on the microbiology of the thermophilic process, giving insight in the way methanol is degraded by the sulfate-reducing community in the bioreactor. In Chapters 6 and 7 the maximum attainable rates of thermophilic sulfite and sulfate reduction in anaerobic bioreactors of the EGSB-type (EGSB: Expanded Granular Sludge Bed) are determined. Results on mesophilic (30°C) sulfate reduction with methanol, relevant for bio-desulfurization of cold or slightly heated ground- or wastewater, are presented in Chapter 8. Chapter 9 presents an overall discussion of the results dealing with thermophilic sulfate and sulfite reduction.

Expanded Sludge Bed reactors were used in this study for continuous reactor experiments, because these reactors provide a good contact between biomass and medium as a result of the imposed high upflow liquid velocity[58]. As a result, mass transfer limitations, which are expected because mixing due to biogas bubbles is preferably absent, may be overcome.

1.6 References

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Thermophilic sulfate reduction and methanogenesis with methanol in a high-rate anaerobic reactor

2.1 Abstract

Sulfate reduction outcompeted methanogenesis at 65°C and pH 7.5 in methanol and sulfate-fed Expanded Granular Sludge Bed reactors operated at hydraulic retention times (HRT) of 14 and 3.5 hr, both under methanol limiting and methanol overloading conditions. After 100 and 50 days for the reactors operated at 14 and 3.5 hr respectively, sulfide production accounted for 80% of the methanol-COD consumed by the sludge. The specific methanogenic activity on methanol of the sludge from a reactor operated at HRTs of down to 3.5 h for a period of 4 months gradually decreased from 0.83 $gCOD.gVSS^{-1}.day^{-1}$ at the start to a value of less than 0.05 $gCOD.gVSS^{-1}.day^{-1}$, showing that the relative number of methanogens decreased and eventually became very low. By contrast, the increase of the specific sulfidogenic activity of sludge from 0.22 $gCOD.gVSS^{-1}.day^{-1}$ to a final value of 1.05 $gCOD.gVSS^{-1}.day^{-1}$ showed that sulfate-reducing bacteria were enriched. Methanol degradation by a methanogenic culture obtained from a reactor by serial dilution of the sludge was inhibited in the presence of vancomycin, indicating that methanogenesis directly from methanol was not important. This methanogenic culture degraded H_2/CO_2 and formate, but not acetate, to methane in the presence of vancomycin. These results indicated that methanol degradation to methane occurs via the intermediates H_2/CO_2 and formate. The high and low specific methanogenic activity of sludge on H_2/CO_2 and formate, respectively, indicated that the former substrate probably acts as the main electron donor for the methanogens during methanol degradation. As sulfate reduction in the sludge was also strongly supported by hydrogen, competition between sulfate-reducing bacteria and methanogens in the sludge seemed to be mainly for this substrate. Sulfate elimination rates of up to 15 $gSO_4^{2-}.L^{-1}.day^{-1}$ were achieved in the reactors. Biomass retention limited the sulfate elimination rate.

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2.2 Introduction

Methanol can be used under thermophilic conditions as carbon and energy source by sulfate-reducing bacteria (SRB)[11] methanogenic archaea (MA)[19] and homoacetogenic bacteria (AB)[16]. As a result, these microorganisms will compete for the available methanol in mixed cultures. In anaerobic reactors with biomass retention, this competition is not only determined by growth kinetics, but also by immobilization properties, substrate diffusion limitations inside biofilms and environmental conditions such as hydrogen sulfide concentration, temperature and pH[2,14]. The bacterial composition of the seed sludge and the applied hydraulic retention time (HRT)[1] may also be important. To increase this complexity even more, thermophilic SRB (e.g. *Desulfotomaculum thermoacetoxidans*[9])and MA[12] may also compete for acetate, the product of methanol catabolism by AB. Furthermore, acetogenic bacteria may partially oxidize methanol to hydrogen and carbon dioxide, when the hydrogen concentration is kept low by hydrogenotrophic sulfate reducers or methanogens[3]. This implies that, in addition to competition for methanol and acetate, there may also be competition for hydrogen between SRB and MA in mixed microbial cultures growing on methanol.

So far, little information is available about methanol degradation with sulfate under thermophilic conditions in anaerobic high-rate reactors. With methanol as the only substrate, Minami et al.[10] found that, at moderate thermophilic (53°C) conditions, sulfate reduction with methanol was stimulated at pH 7.0-7.5 in a packed-bed reactor, while methanogenesis prevailed at pH 6.2-6.8. Addition of cobalt stimulated methanogenesis and acetogenesis from methanol under mesophilic conditions[4]. The present study was initiated to assess the use of methanol for sulfate reduction in a high-rate anaerobic reactor at the lowest possible production of methane and acetate. To suppress methane and acetate formation, cobalt was omitted from media in our experiments. An operational temperature of 65°C was chosen to prevent growth of methanol consuming *Methanosarcina* species, which have never been reported to grow at or beyond a temperature of 65°C [13,19,25].

The objective of the experiments described in this chapter was to determine the outcome of the competition between SRB, MA and AB in an EGSB-reactor operated at 65°C and at a pH of 7.5, using an influent containing methanol as the sole carbon and energy source and sulfate as the external electron acceptor. Special attention was paid to the degradation route of methanol.

2.3 Materials and Methods

Reactors. For the continuous experiments, glass EGSB-reactors with a working volume of 4 L were used. A schematic drawing is presented in Figure 2.1. The reactor was equipped with a double wall through which water, heated to 65°C in a Haake waterbath (Haake, Karlsruhe, Germany), was circulated. The pH in the reactor was maintained at 7.5±0.1 by automatic pH control, adding 0.1 N NaOH when necessary. The pH was measured with a sulfide-resistant pH-electrode (type Flushtrode, ThIS Scientific, Sliedrecht, The Netherlands) connected the pH control unit. The pH-electrode was checked every week and recalibrated when necessary.

Biogas was collected in a gas-solid-liquid separator and then led through a waterlock filled with a 3 N NaOH solution and a column filled with soda lime pellets to remove H_2S and CO_2 from the gas, before the gas flow was measured with a Mariotte flask or a wet-type precision gas meter (Schlumberger Industries, Dordrecht, The Netherlands). Effluent recycling was applied to increase the liquid upward velocity. For the medium flow and the recycling flow, peristaltic pumps (type 505S, Watson Marlow, Falmouth, UK) and Marprene (Watson Marlow) tubing were used. The lower 5 cm of the reactor was filled with glass Raschig rings (1 cm) to evenly distribute the influent over the sludge bed.

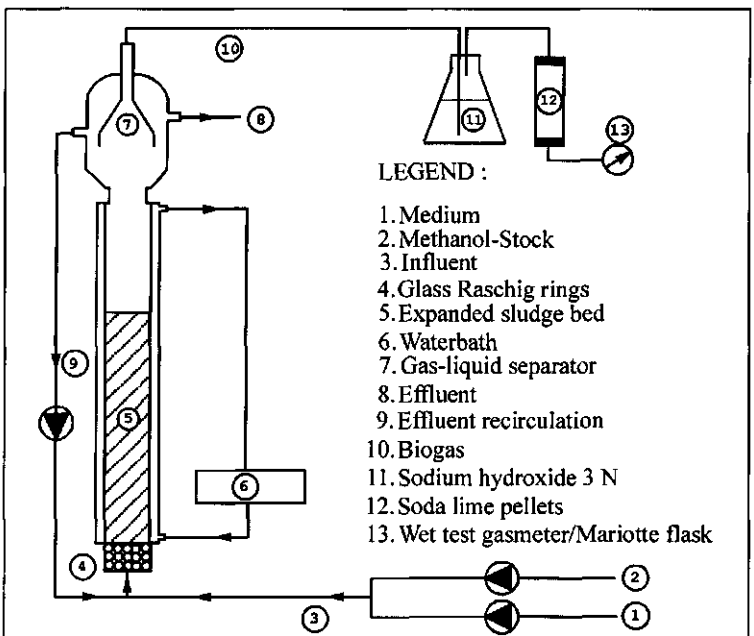

Figure 2.1. Expanded Granular Sludge Bed reactor

Two EGSB-reactors (I and II) were used for the continuous experiments. In all experiments, the ratio COD/sulfate in the influent was kept at about 0.67 (gCOD per gSO_4^{2-}), so theoretically 100% sulfate elimination can be achieved with all added methanol being utilized. The volumetric methane production rate, expressed as $gCOD.L^{-1}.day^{-1}$, was calculated from the flows and methane concentrations in the biogas and in the effluent. The methane concentration in the effluent was calculated from the methane concentration in the biogas, assuming equilibrium between biogas and medium in the reactor, using a gas-liquid distribution coefficient of 40.8 [24]. The sulfide production rate was calculated in a similar way. The hydrogen sulfide concentration in the biogas was calculated from the hydrogen sulfide concentration in the liquid (H_2S and HS^-), assuming equilibrium between gas and liquid hydrogen sulfide concentrations. A dissociation constant for H_2S of 6.6 [17] and a dimensionless gas-liquid distribution coefficient of 0.67 [24] were used for calculations. The acetate production rate was calculated from the effluent flow and acetate concentration.

EGSB-I was inoculated with about 40 gVSS elutriated sludge from a pilot plant for sulfate reduction of scrubber liquid from a flue-gas scrubber of a coal-fired power plant in Geertruidenberg, The Netherlands. This sludge was kindly provided by Biostar, Balk, The Netherlands and had been cultivated on an ethanol/methanol (95/5%) mixture and sulfate/sulfite at 55°C. The ash content of this sludge was 44% (percent of dry weight), the remainder was VSS. EGSB-I was started at a HRT of 14 h, an OLR of 2.3 $gCOD.L^{-1}.day^{-1}$ and a Sulfate Loading Rate (SLR) of 3.4 $gSO_4^{2-}.L^{-1}.day^{-1}$. Methanol and sulfate influent concentrations were 1.27 $gCOD.L^{-1}$ and 1.9 $gSO_4^{2-}.L^{-1}$, respectively. At day 135, the OLR and SLR were doubled to 4.5 $gCOD.L^{-1}.day^{-1}$ and 6.2 $gSO_4^{2-}.L^{-1}.day^{-1}$ respectively, by doubling the influent methanol and sulfate concentrations. The liquid upward velocity ranged from 3.0 to 4.2 $m.h^{-1}$.

EGSB-II was inoculated with about 20 gVSS of thermophilic sludge that had been cultivated on methanol and sulfate for 6 months, and 20 gVSS of the same sludge used for inoculation of EGSB-I. EGSB-II was started at a HRT of 11 h which was decreased to 6 h at day 25 and to 3.5 h at day 33. Influent methanol and sulfate concentrations were 2.6 $gCOD.L^{-1}$ and 3.84 $g.L^{-1}$, respectively. A liquid upward velocity (v_{up}) of 3 $m.h^{-1}$ was applied, which was increased to 6 $m.h^{-1}$ at day 32. From day 53 to 67 the v_{up} was temporarily increased to 8-10 $m.h^{-1}$. Acetate was added to the influent of EGSB-I during day 96-102 at a concentration of 0.3 $gCOD.L^{-1}$.

Media. Reactors were fed with a basal medium consisting of ($g.L^{-1}$): NaCl (7), $MgCl_2.6H_2O$ (1.2), KCl (0.5), NH_4Cl (0.3), $CaCl_2$ (0.15), Na_2SO_4 (2.8 or 5.6), KH_2PO_4 (0.2), and a trace element solution (1 $mL.L^{-1}$) containing ($mg.L^{-1}$): $FeCl_2.4H_2O$ (1500),

$MnCl_2.4H_2O$ (100), $ZnCl_2$ (70), H_3BO_3 (62), $Na_2MoO_4.2H_2O$ (36), $NiCl_2.6H_2O$ (24), $CuCl_2.2H_2O$ (17), EDTA (500) and HCl 37% (7 $mL.L^{-1}$). Cobalt was not present as trace element. Demineralized water was used to prepare media and stock solutions. A stock solution containing methanol (5 M) was pumped into the influent with a Gilson Minipuls3 Peristaltic Pump. After day 60 for EGSB-I, and during the whole experiment in EGSB-II, the methanol-stock also contained yeast extract (2 $g.L^{-1}$), resulting in an influent concentration of 20 $mg.L^{-1}$. All chemicals were of analytical grade and supplied by Merck (Darmstadt, Germany) except for yeast extract that was obtained from Life Technologies (Paisley, Scotland) and methanol that was obtained from Labscan Ltd. (Dublin, Ireland).

The anaerobic medium used for activity assays had a similar composition as the reactor influent. However, 1 $mL.L^{-1}$ resazurine solution (0.5 $g.L^{-1}$), 50 $mL.L^{-1}$ bicarbonate/sulfide solution, containing 80 $g.L^{-1}$ $NaHCO_3$ and 12 $g.L^{-1}$ $Na_2S.7\text{-}9H_2O$ and 30 $mL.L^{-1}$ phosphate solution (27.2 $g.L^{-1}$ KH_2PO_4 and 35.7 $g.L^{-1}$ $Na_2HPO_4.2H_2O$) were added to the medium, for redox potential control and pH-buffering. Na_2SO_4 was either omitted from the medium or added at a concentration of 2.8 $g.L^{-1}$. Methanol, acetate and formate were added from concentrated stock solutions, to give concentrations of 1.4, 1.3 and 0.25 $gCOD.L^{-1}$, respectively. The pH of the medium was adjusted to pH 7.5 by addition of a few drops of NaOH (0.1 N). When acetate, methanol, or formate were tested as electron donor, the headspace in the vials consisted of N_2/CO_2 (80/20 v/v). When hydrogen was the tested electron donor, nitrogen was replaced by H_2. Conversion factors for converting gCOD to mol are: methanol: 1/48 $mol.gCOD^{-1}$; acetate: 1/64 $mol.gCOD^{-1}$; formate: 1/16 $mol.gCOD^{-1}$; hydrogen: 1/16 $mol.gCOD^{-1}$; sulfide: 1/64 $mol.gCOD^{-1}$.

The medium for incubations with diluted sludge for enrichment of methanogens had the same composition as the medium used in activity assays except that sulfate was omitted. Basal media and stock solutions were autoclaved before use. Vancomycin (Sigma, St. Louis, USA) was filter-sterilized before use and added to a concentration of 2 $g.L^{-1}$ when inhibition of eubacteria (e.g. sulfate reducers and acetogens) was desired.

Activity assay. Activity assays were carried out in 120 mL-vials containing 50 mL anaerobic medium and 0.5-2 $gVSS.L^{-1}$ sludge. During the assay, the vials were placed in a waterbath with shaker (100 rpm) at 65°C. When H_2/CO_2 was the substrate, the vials were placed horizontally in the waterbath to optimize mass transfer of hydrogen from gas to liquid. After 30 to 60 min of acclimatisation at 65°C in anaerobic medium, the sludge was transferred to a vial with fresh anaerobic medium. The sludge was acclimatized because a short (<30 min) lag phase in methane and/or sulfide production was sometimes observed in the preincubations. Samples were taken from the liquid (0.5 mL) and gas (0.2 mL) phase

for analysis of methanol, acetate, formate, sulfide and methane. The specific acetogenic, methanogenic and sulfidogenic activities were calculated from the linear increase (taking at least three measurements) of the amount of acetate, methane and sulfide in the vials and the amount of VSS at the end of the assay. The length of the incubations was 4-6 hr. Growth was considered negligible in the assay. The sulfide concentration in the headspace was calculated from the total sulfide concentration in the liquid (unionized H_2S and HS^-), using a dissociation constant for H_2S of 6.6 [17] and a dimensionless gas-liquid distribution coefficient of 0.67 [24]. Equilibrium between gas and liquid hydrogen sulfide concentrations was assumed. Activity assays were carried out in duplicate.

Methanogenic enrichment culture. Fresh sludge was diluted in a dilution series in liquid medium with methanol (1.4 $gCOD.L^{-1}$), but without sulfate. The highest dilution showing methane formation (dilution 10^5) was transferred to fresh methanol containing medium with and without vancomycin (2 $g.L^{-1}$). Vancomycin inhibits eubacterial growth (SRB and MA) by blocking the polymerization of N-acetylmuramic acid and acetylglucose amine units to peptidoglycan (Nicklin et al. 1999), while archaea (MA) are not affected. The culture was also transferred to media with vancomycin (2 $g.L^{-1}$) and acetate (1.3 $gCOD.L^{-1}$), formate (0.25 $gCOD.L^{-1}$) or H_2/CO_2 (80/20 v/v, 1 atm.).

Analyses. The volatile suspended solids (VSS) concentration in reactors at the start and at the end of experiments was calculated from the total volume of the wet sludge and the VSS content of a sample from the sludge. During the experiments, the VSS concentration in reactors was estimated from the volume of the static sludge bed and the VSS content of a sample from the sludge with a known volume. VSS was determined according to Dutch Standard Methods (NEN 32355.3).

Samples for methanol and VFA analysis were centrifuged at 17000 g for 5 min., diluted with a 3% formic acid solution (final concentration formic acid 1-2%), and stored at 4°C. Methanol and acetate were determined by gas chromatography using a Hewlett Packard model 5890 equipped with a 6 m × 2 mm glass column packed with Supelco port, 100-120 mesh, coated with 10% Fluorad FC 431 (3M). The flow rate of the carrier gas (nitrogen saturated with formic acid) was 40 $mL.min^{-1}$, and the column pressure was ± 3 bar. The column temperature was 80°C, the injection port and the detector temperature were 200°C and 280°C, respectively. Propionate and butyrate were analysed in the same way as methanol except that the temperatures of the column, the injector port and the flame ionization detector were 130, 200 and 280°C, respectively.

Formate was determined by high performance liquid chromatography (LKB) with a Chrompack organic acid column (temperature, 60°C) at a flow rate of 0.60 mL.min^{-1} with 0.01 N H_2SO_4 as eluent.

Composition of the biogas from the reactor (CO_2, CH_4 and N_2) was determined with a gas chromatograph (Fisons Instruments GC 8000) equipped with two columns: 1.5 m x 1/8 inch Teflon packed with Chromosorb 108 (60-80 mesh) and 1.2 m x 1/8 inch stainless steel packed with molecular sieve 5A (60-80 mesh). The columns were connected in parallel with a split of 1:1. Helium was used as carrier gas. The total carrier gas flow rate was 45 mL.min^{-1}. The temperature of the columns, injection port and thermal conductivity detector were 40, 110 and 100°C, respectively.

Hydrogen was determined with a gas chromatograph (Hewlett Packard 5890), using a 1.5 m x 1/8 inch stainless steel column packed with molecular sieve 5A (60-80 mesh). The temperature of this column, injection port and thermal conductivity detector were 40, 125 and 110°C, respectively. Argon was used as carrier gas at a flow rate of 20 mL.min^{-1}.

In the activity assay, methane was measured on a 406 Packard gas chromatograph equipped with a thermal conductivity detector (TCD), 100 mA. The gases were separated with argon as the carrier gas on a molecular sieve column (13X, 1.8 m by 1/4 inch, 60-80 mesh) at 100°C.

The total sulfide concentration is defined as the sum of H_2S, HS^- and S^{2-} sulfide species. The total sulfide was determined colorimetrically using the methylene blue method[20].

The samples for sulfate analysis were diluted (1:40) with a 30 mM mannitol solution and after centrifugation they were stored at -18°C until analysis. Sulfate was analysed by high-pressure liquid chromatography. Ions were separated on a Dionex column (Ionpac AS9-SC) with an eluent consisting of 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ at a flow rate of 1 mL.min^{-1} at room temperature. The anions were detected with suppressed conductivity.

2.4 Results

Continuous experiments

The performance of EGSB-I is shown in Figure 2.2A-C. One week after the start-up, already a high rate of sulfate reduction and methanogenesis was observed. The methane production rate reached a maximum of 1.4 $gCOD.L^{-1}.day^{-1}$ at day 18, while the sulfide production rate at that day amounted to 0.46 $gCOD.L^{-1}.day^{-1}$ (Figure 2.2A). Methanogenesis still dominated at day 18, accounting for 75% of total COD-conversion, while SRB only used 24% of the COD (Figure 2.2C). After day 18, the sulfide production rate increased steadily at the expense of the methane production rate; at day 79 the MA and SRB used 34 and 65% of the consumed methanol, respectively. Temporary deviations from this trend during short periods subsequent to days 40, 53 and 71 very likely reflect a higher sensitivity of the sulfidogenic biomass as compared to the methanogenic biomass towards temperature drops to room temperature for 0.5-1 day as occurred at days 40 and 53, and towards a 5-hour methanol-starvation period at day 71. At day 80 the operation was interrupted and the sludge was removed from the reactor for determination of the wet volume and VSS-content of the sludge. The operation of the reactor was resumed at the same day, but with only 60 vol.% of the sludge. The results in Figure 2.2 reveal that it took 3 weeks until the sulfide production rate recovered to the level evident on day 79, whereas the methane production rate only recovered to about 50% of the level found at that day. The shift in methanol utilization from MA to SRB from then onwards continued until SRB and MA accounted for 82 and 16% of the electron flow at day 135, respectively. During most of the period between day 0-135, the reactor was underloaded. In order to assess the competition between SRB and MA at higher methanol concentrations, the OLR and SLR were doubled at day 135 to 4.5 $gCOD.L^{-1}.day^{-1}$ and 6.2 $SO_4^{2-}.L^{-1}.day^{-1}$, respectively, by doubling the influent methanol and sulfate concentrations. Table 2.1 shows that within two days, the methane and sulfide production rates increased from 0.3 $gCOD.L^{-1}.day^{-1}$ to 0.5 $gCOD.L^{-1}.day^{-1}$ and from 1.7 to 2.5 $gCOD.L^{-1}.day^{-1}$, respectively. The acetate production rate remained very low at a value of 0.04 $gCOD.L^{-1}.day^{-1}$. After elevation of the methanol load on day 135, the effluent methanol concentrations varied between 0.7 to 1.3 $gCOD.L^{-1}$. Under these overload conditions, the methane production remained stable at 0.5 $gCOD.L^{-1}.day^{-1}$, while the sulfide production varied between 2.0-2.9 $gCOD.L^{-1}.day^{-1}$, resulting in a total sulfide concentration in the effluent ranging from 568 to 829 $mgS.L^{-1}$ (Figure 2.2B). The relative electron flow towards sulfide and methane formation remained constant beyond day 137. During the experiment, the total of sulfide and sulfate (expressed as mol

$S.L^{-1}.day^{-1}$) leaving the reactor deviated less than 15% from the sulfate entering the reactor (data not shown). The acetate production rate increased slightly during the first 10 days of overloading, reaching a maximum of 0.15 $gCOD.L^{-1}.day^{-1}$ at day 145, but it then decreased to a value of around 0.10 $gCOD.L^{-1}.day^{-1}$ between day 149 and 155. The maximum acetate production calculated on the basis of the data contributed only 6% of the methanol conversion. Propionate and butyrate concentrations remained around or below the detection limit during the entire experiment, while formate could never be detected (data not shown). The hydrogen partial pressure in the biogas fluctuated between 20 and 75 Pa.

Table 2.1. Performance of EGSB-I before and after doubling the OLR and SLR on day 135. TS_{eff}: Total sulfide in effluent; $MeOH_{eff}$: methanol in effluent; Ac_{eff}: acetate in effluent VMC: volumetric methanogenic COD-conversion; VAC: volumetric acetogenic COD-conversion; VSC: volumetric sulfidogenic COD-conversion.

	TS_{eff}	$MeOH_{eff}$	Ac_{eff}	VMC	VAC	VSC	MeOH removal	SO_4^{2-} removal
day	($mgS.L^{-1}$)	($gCOD.L^{-1}$)	($gCOD.L^{-1}$	($gCOD.gVSS^{-1}.day^{-1}$)			(%)	(%)
135	494	0.07	0.02	0.3	0.04	1.7	95	78
137	729	0.96	0.02	0.5	0.04	2.5	65	58

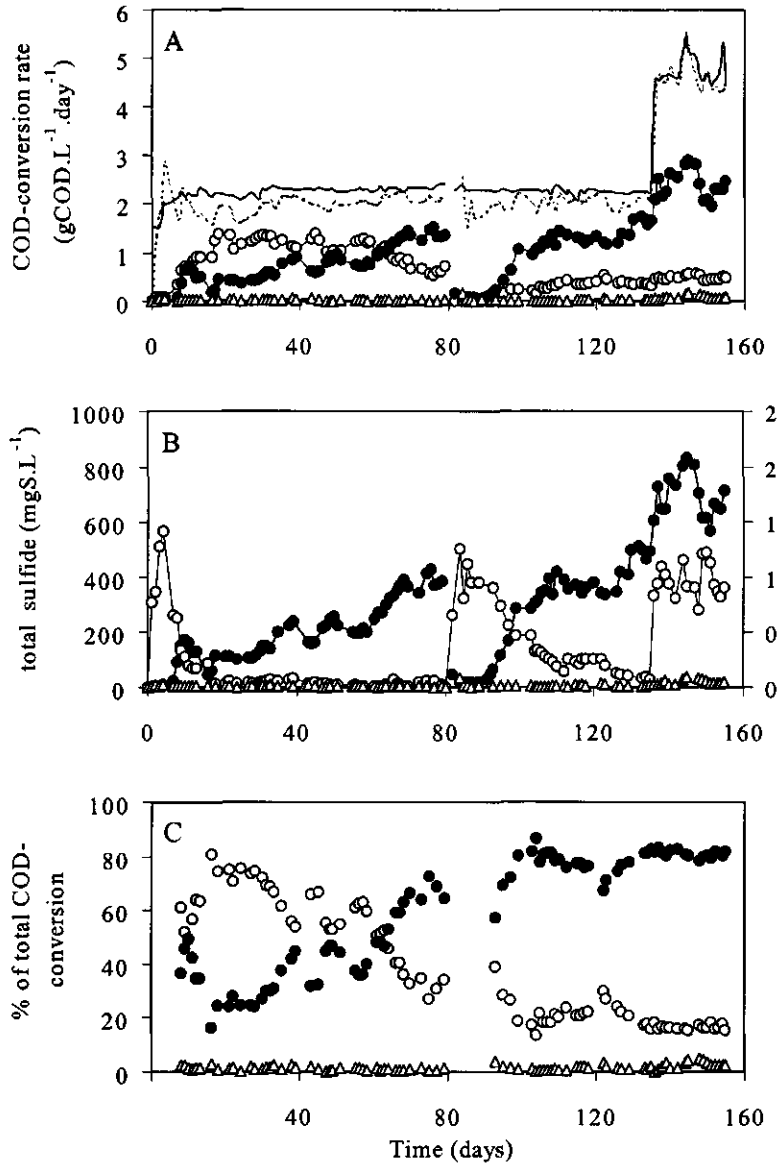

Figure 2.2. Performance of EGSB-I, operated at a HRT of 14 h. During day 1-135 an organic loading rate (OLR) of 2.3 $gCOD.L^{-1}.day^{-1}$ and a sulfate loading rate (SLR) of 3.4 g $SO_4^{2-}.L^{-1}.day^{-1}$ were applied, which were increased during day 135-155 to 4.5 $gCOD.L^{-1}.day^{-1}$ and 6.2 $SO_4^{2-}.L^{-1}.day^{-1}$ respectively.

A. Volumetric COD-conversion rate of sulfide (●), methane (○), acetate (Δ), imposed OLR (—) and sum of volumetric COD-formation rates of sulfide, methane and acetate (--).

B. Effluent concentrations of sulfide (●), methanol (○) and acetate (Δ).

C. Percentage COD-conversion to sulfide (●), methane (○), and acetate (Δ).

The competition between SRB and MA at lower HRTs was studied in EGSB-II. Within 10 days after start-up at a hydraulic retention time of 11 h and an OLR of 5 $gCOD.L^{-1}.day^{-1}$, the methane and sulfide production already attained a value of 2.9 and 0.6 $gCOD.L^{-1}.day^{-1}$, respectively (Figure 2.3A). A rapid decrease of the methane production rate, from 3.0 to 0.7 $gCOD.L^{-1}.day^{-1}$, was observed between day 11 and 19, while at the same time the sulfide production rate increased from 1.3 to 3.6 $gCOD.L^{-1}.day^{-1}$. The methane production rate recovered during the period from day 25 to 30 after the OLR was increased from 5.0 to 9.0 $gCOD.L^{-1}.day^{-1}$ by reducing the HRT from 11 to 6 h at day 25. The HRT was decreased further to 3.5 h at day 33, which resulted in an increase of the OLR to 16 $gCOD.L^{-1}.day^{-1}$. A 10-h interruption in the methanol feed occurring at day 37 caused a temporary inhibition of the sulfide production rate, but the system recovered within a few days, while the methane and acetate production rates remained unaffected. The acetate and methane production rate reached a maximum of 1.4 and 3.9 $gCOD.L^{-1}.day^{-1}$, respectively at day 38 and then both declined to less than 0.4 $gCOD.L^{-1}.day^{-1}$ after day 60, also at the relatively high methanol concentrations of 0.7 to 1.5 $gCOD.L^{-1}$ prevailing in the reactor after day 45. On the other hand, the sulfide production rate increased from 3.8 $gCOD.L^{-1}.day^{-1}$ at day 24 to 9.7 $gCOD.L^{-1}.day^{-1}$ at day 42. From day 60 onwards until the termination of the experiment at day 129, the electron flow was almost exclusively directed towards sulfide. In this period, only 5 to 16% of the consumed methanol was converted to methane and acetate, while the remainder was used for sulfate reduction, resulting in a total sulfide concentration of 239-651 $mgS.L^{-1}$ (Figure 2.3B). Acetate was added to the influent during day 96-102 at a concentration of 0.3 $gCOD.L^{-1}$, in order to assess a possible stimulatory effect of acetate as carbon or energy source for the MA and SRB. The results in Figure 2.3A show that adding acetate did not stimulate the methane production rate. The calculated acetate consumption in this period amounted only to about 0.06 $gCOD.L^{-1}.day^{-1}$, which means that the acetate production rate in fact was negative, as shown in Figure 2.3A. Due to the large fluctuations in the sulfide production rate, from 3.8 to 8.9 $gCOD.L^{-1}.day^{-1}$ in the pseudo steady state after day 60, no clear effect of the acetate addition on the sulfide production rate could be observed. The sludge concentration after termination of the experiment on day 129 was 7.1 $gVSS.L^{-1}$. Based on this value and the sulfide production in the reactor on day 129, a specific sulfidogenic activity of 0.95 $gCOD.gVSS^{-1}.day^{-1}$ can be calculated. This value corresponds well with the measured value of the specific activity of 1.05 ± 0.07 $gCOD.gVSS^{-1}.day^{-1}$ of the sludge on day 129.

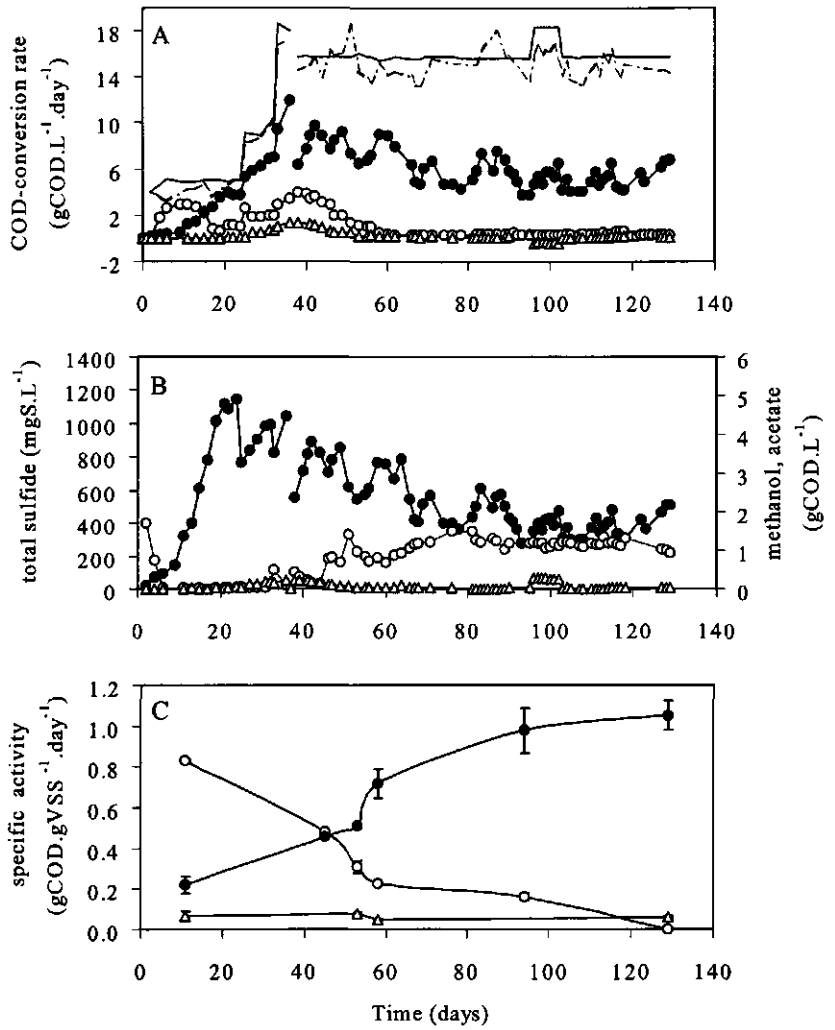

Figure 2.3. Performance of EGSB-II. The operation of EGSB-II was started at a HRT of 11 h, which was decreased to 6 h at day 25, and to 3.5 h at day 33. Influent methanol and sulfate concentrations were 2.6 $gCOD.L^{-1}$ and 3.84 $g.L^{-1}$, respectively.
A. Volumetric COD conversion rate of sulfide (●), methane (○), acetate (Δ), OLR (—) and sum of volumetric COD-conversion rate of sulfide, methane and acetate (---).
B. Effluent concentrations: (●) sulfide; (○) methanol; (Δ) acetate.
C. Specific activity of sludge as assessed in batch assays: (●) sulfidogenic; (○) methanogenic; (Δ) acetogenic. Bars represent standard deviation.

The competition between SRB and MA in EGSB-II could be followed more closely by regular assessment of the specific sulfidogenic activity (SSA), the specific methanogenic activity (SMA) and the specific acetogenic activity (SAA) of freshly sampled sludge using methanol (1.4 $gCOD.L^{-1}$) and sulfate (1.9 $g.L^{-1}$). The results, shown in Figure 2.3C, reveal a significant increase in the SSA from 0.22 to 1.05 $gCOD.gVSS^{-1}.day^{-1}$ between day 11 and 129, and a drop of the SMA from 0.83 $gCOD.gVSS^{-1}.day^{-1}$ at day 11 to values less than 0.03 $gCOD.gVSS^{-1}.day^{-1}$ at day 129. Also after crushing of the sludge, no methanogenic activity was measured at day 129. The specific acetogenic activity (SAA) remained low throughout the experiment with a value of about 0.05-0.10 $gCOD.gVSS^{-1}.day^{-1}$. The flow of electrons generated by methanol oxidation in activity assays differed from the electron flow found in the reactor. E.g. a calculation made on basis of the data in Figure 2.3A and 3B shows that in the activity assay performed at day 58 with sludge from EGSB-II, the production of acetate, methane and sulfide accounted for respectively 5, 23 and 72% of the amount of methanol converted, while at the same day in the reactor these values amounted to 2, 4 and 94%, respectively.

Specific methanogenic and sulfidogenic activities of sludge with acetate, H_2/CO_2 and formate

In order to assess whether or not the SRB and MA compete for degradation products from methanol like acetate, H_2/CO_2 and formate, the specific sulfidogenic and methanogenic activities with H_2/CO_2 (80/20 v/v, 1 atm.), acetate (1.3 $gCOD.L^{-1}$) and formate (0.25 $gCOD.L^{-1}$) in the presence of 1.9 $g.L^{-1}$ sulfate was determined regularly. Results are presented in Table 2.2. For comparison, the specific activities with methanol are also included in Table 2.2. Acetate was not degraded in the assays carried out at day 24 and 59, and no methanogenic activity was observed with acetate. Moreover, the measured SSA with acetate was also low on these days (0.06 $gCOD.gVSS^{-1}.day^{-1}$). It was confirmed experimentally that acetate at the applied concentration of 1.3 $gCOD.L^{-1}$ was not inhibitory for sulfate reduction or methanogenesis with methanol (data not shown).

High values for the SMA and SSA were found with hydrogen as electron donor. While the SMA with H_2/CO_2 gradually decreased from 1.4 to 0.2 $gCOD.gVSS^{-1}.day^{-1}$ between day 47 and day 129, the SSA increased from 0.78 to 1.48 $gCOD.gVSS^{-1}.day^{-1}$ in the same period. Using formate, the SMA at day 24 amounted to only 0.04 $gCOD.gVSS^{-1}.day^{-1}$, while at day 129 no methanogenic activity could be detected with this substrate. Without substrate and sulfate, the sludge still produced 0.05 $gCOD\text{-}CH_4.gVSS^{-1}.day^{-1}$ at day 53, which is close to the SMA found with formate at day 24. Contrary to the low SMA, the sludge exerted a high

specific sulfidogenic activity of 0.39 gCOD.gVSS^{-1}.day^{-1} on formate at day 24, which had increased to 0.87 gCOD.gVSS^{-1}.day^{-1} at day 129. Formate and sulfate were degraded in the expected stoichiometry of 4:1 (data not shown).

Table 2.2. Specific methanogenic and sulfidogenic activities (gCOD.gVSS^{-1}.day^{-1}) of fresh sludge from EGSB-II with sulfate (1.9 g.L^{-1}) and methanol (1.4 gCOD.L^{-1}), acetate (1.3 gCOD.L^{-1}), formate (0.25 gCOD.L^{-1}), H_2/CO_2 (80/20 v/v, 1 atm.).

	specific methanogenic activity (gCOD.gVSS^{-1}.day^{-1})					
	day					
substrate	**24**	**47**	**58**	**59**	**94**	**129**
methanol	.[a]	0.49±0.03	0.22±0.00	-	0.16±0.02	<0.03
acetate	0	-	-	0	-	-
formate	0.04±0.01	-	-	-	-	0
H_2/CO_2	-	1.39±0.04	0.79±0.03	-	0.42±0.04	0.20±0.04
none	-	0	0	-	-	-
	specific sulfidogenic activity (gCOD.gVSS^{-1}.day^{-1})					
	day					
substrate	**24**	**47**	**58**	**59**	**94**	**129**
methanol	-	0.46±0.00	0.72±0.07	-	0.98±0.11	1.05±0.07
acetate	0.06±0.00	-	-	0.06[b]	-	-
formate	0.39±0.01	-	-	-	-	0.87±0.03
H_2/CO_2	-	0.78±0.06	0.48±0.12	-	1.26±0.12	1.48±0.18
none	-	0.21±0.02	0.08±0.01	-	-	-

a) not determined; b) single measurement.

Substrates for methanogenic culture obtained from sludge

Substrate utilization by MA was investigated in more detail for a mixed methanogenic culture grown on methanol, which was obtained from the sludge cultivated in EGSB-II through serial dilution. The sludge was taken from the reactor at day 67. As follows from the results shown in Table 2.3, H_2/CO_2 and formate supported methanogenesis in the presence of vancomycin (a specific inhibitor of eubacteria), but no methanogenesis was found with methanol and acetate. During methanol degradation in the absence of

vancomycin, hydrogen was detected as an intermediate in the culture at a partial pressure of 50-100 Pa.

Table 2.3. Methane formation in methanogenic culture obtained through serial dilution (dilution factor 10^5) of a sludge sample taken from EGSBII at day 67, with methanol, formate and H_2/CO_2 in the presence of vancomycin.

Substrate (initial concentration)	**Vancomycin** ($2\ g.L^{-1}$)	**CH_4-production after 10 days** ($gCOD.L_{medium}^{-1}$)
methanol ($1.4\ gCOD.L^{-1}$)	no	0.84
methanol ($1.4\ gCOD.L^{-1}$)	yes	0
H_2/CO_2 (80/20 v/v, 1.7 atm.)	yes	1.3
acetate ($1.3\ gCOD.L^{-1}$)	yes	0
formate ($0.32\ gCOD.L^{-1}$)	yes	0.15

Inhibition of methanol degradation at high hydrogen partial pressure

The previous results indicate that methanol oxidation to H_2/CO_2 and the subsequent methanogenesis and sulfate reduction from H_2/CO_2 could play a significant role in methanol degradation in the sludge. It can be calculated that at hydrogen pressures exceeding 6500 Pa, oxidation of methanol to H_2/CO_2 at 65°C becomes thermodynamically unfavorable[17]. Applying a high hydrogen partial pressure to the sludge should therefore inhibit methanol oxidation to H_2/CO_2. The effect of high hydrogen partial pressures on the specific methanol degradation rate (SMDR) was assessed on day 94 in activity assays with fresh sludge removed from EGSB-II. In the presence of methanol and sulfate, the SMDR amounted to $0.92 \pm 0.05\ gCOD.gVSS^{-1}.day^{-1}$, but when the sludge was simultaneously exposed to methanol and H_2/CO_2 (1 atm., 80/20 vol./vol.), a SMDR of $0.41 \pm 0.04\ gCOD.gVSS^{-1}.day^{-1}$ was found (Table 2.4), which is 55% less. The specific sulfidogenic activity with merely methanol amounted to $0.98 \pm 0.11\ gCOD.gVSS^{-1}.day^{-1}$. As a SMDR of $0.41 \pm 0.04\ gCOD.gVSS^{-1}.day^{-1}$ was found at a high hydrogen partial pressure, it can be concluded that the methanol flow to sulfide decreased 60% in the presence of a high hydrogen partial pressure.

Table 2.4. Specific methanogenic (SMA), sulfidogenic (SSA) and acetogenic (SAA) activities ($gCOD.gVSS^{-1}.day^{-1}$) and specific methanol degradation rates (SMDR, $gCOD.gVSS^{-1}.day^{-1}$) of fresh sludge taken from EGSB-II at day 94 with methanol (1.4 $gCOD.L^{-1}$), and with methanol (1.4 $gCOD.L^{-1}$) and H_2/CO_2 (80/2 0 v/v, 1 atm.).

substrate	SMA	SSA	SAA	SMDR
		($gCOD.gVSS^{-1}.day^{-1}$)		
methanol	0.16± 0.02	0.98± 0.11	0.06± 0.01	0.92± 0.05
methanol + H_2/CO_2	0.55± 0.02	1.41± 0.12	0.10± 0.03	0.41± 0.04

Development of sludge

The seed sludge used for inoculation of EGSB-I and -II consisted of granules with a diameter of up to 3 mm and a large (about 30% of the wet volume) fraction of disperse biomass. As a result, in the first days of the continuous experiments, a significant fraction (20-30%) of the sludge washed out from the reactor. During the course of the experiments, the granules slowly disintegrated. Along with this disintegration, sludge particles became covered with a fluffy cotton-like material, presumably consisting of newly formed biomass. Sludge particles were loosely linked to each other forming aggregates, sometimes up to a length of 3 cm, where the fluffy sludge material seemed to act as a 'glue' (Figure 2.4). Surprisingly, these voluminous aggregates did not wash out from the reactor at the applied high liquid upward velocities of 3 to 6 $m.h^{-1}$. On the other hand, the rather fluffy nature of these biomass aggregates led to a high expansion of the biobed, even up to 300-400% at an upflow liquid velocity of 3 $m.h^{-1}$. Occasionally, the voluminous biomass aggregates clotted together in larger entities until ultimately the whole sludge bed seemed aggregated. This occurred especially in periods of low biogas production. Applying high upward liquid velocities of 6 to 10 $m.h^{-1}$, as exposed to EGSB-II during day 53-67 could not prevent such a sludge bed aggregation. Sludge bed aggregation led to channelling. Disruption of the sludge bed could be accomplished by applying a very high upward velocity (50 $m.h^{-1}$) for a few minutes, or by gently stirring, using a piece of rubber tubing.

Figure 2.4. Sludge aggregate from EGSB-II. The aggregate consists of loosely linked granules and flocculent sludge particles. Bar represents 2 mm.

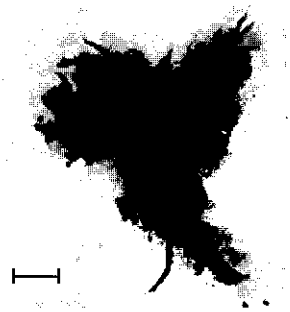

Figure 2.5. Filamentous sludge floc from EGSB-II. Bar represents 2 mm

Morphologically, the sludge remained heterogeneous during the whole period of the experiments; it consisted of (disintegrated) granules and small particles (separately or held together in aggregates) and filamentous and fluffy flocs. After removal of the sludge from the reactor at the termination of experiments, it turned out that it contained large filamentous flocs up to several cm in size, which were mechanically rather strong (Figure 2.5).

The height of the static sludge bed decreased only significantly during the first week of operation when there was severe sludge washout, and when sludge was discharged from the reactors. As the VSS content of the sludge bed remained fairly constant after the first week of operation at a value of 60 ± 10 $gVSS.L^{-1}$ of sludge, there appeared to be no net biomass accumulation in the reactors. Installation of an external settler during day 78-129 in EGSB-II, from which settled biomass particles were recycled to the reactor once per day, did not result in significantly higher biomass concentrations or sulfidogenic production rates mainly because recycled biomass particles washed out again within a few hours.

Methanosarcina and *Methanosaeta* morphotypes could not be observed in the sludge using phase contrast microscopy. Fluorescence microscopy of freshly sampled sludge taken from EGSB-II at day 51, when methane production in the reactor was still relatively high, revealed a dominance of rod-shaped methanogens resembling *Methanobacterium* species, estimated at >90% of the total number of methanogens. The remainder of the methanogens were almost exclusively coccoid in shape.

2.5 Discussion

The results obtained in EGSB-I and -II clearly reveal that methanol is efficiently used by the mixed microbial communities present in thermophilic anaerobic reactors as carbon and electron source for sulfate reduction and only for a minor extent for acetate and methane production. Ultimately, methanogenesis becomes almost completely suppressed, at methanol concentrations in the reactor ranging from less than 0.02 to 1.7 $gCOD.L^{-1}$, and at hydraulic retention times ranging from 3.5 to 14 hr. The remaining methanogenesis presumably mainly results from growing dispersed thermophilic methanogens, which still are present due to their low doubling time[6]. Acetogenesis accounted for maximally 8% of the total amount of methanol converted, only when conditions in the reactors were altered from underloading to overloading.

The rather pronounced drop of the methane production capacity in the continuous reactors probably results from the gradual decline of the fraction of methanogens in the sludge, because the specific methanogenic activity of the sludge from EGSB-II was found to deteriorate. The nearly complete loss of methanogenic activity of the sludge from EGSB-II on methanol indicates that the relative number of methanogens indeed became very low. On the other hand, the gradual increase of the specific sulfidogenic activity of sludge from EGSB-II on methanol to a final value of 1.05 $gCOD.gVSS^{-1}.day^{-1}$ (Table 2.2) reveals a clear enrichment of sulfate-reducing bacteria. The relative electron flow from methanol oxidation to sulfate reduction in activity assays was usually lower than the values deduced from the performance of the EGSB, while the relative electron flow to methane in activity assays was usually higher compared to that in the reactor. These discrepancies can be attributed to the lower methanol concentrations prevailing in EGSB-reactors as compared to the methanol concentrations applied in the activity assays, and to the different mixing regimes prevailing in both systems, resulting in different substrate transport rates. But unrepresentative sampling of sludge from the sludge bed for use in activity assays also could be a reason.

The rapid increase in sulfide production at the expense of methanogenesis that occurred during a period of methanol limitation in EGSB-II (day 11-19), and the recovery of the methane production after elevating the methanol load (day 25-30), indicates that the SRB have a higher affinity than the MA for their common substrate(s). Apparently, substrate kinetics play an important role in the fate of methanol in the reactor.

The inhibition of growth by vancomycin of the methanol degrading methanogenic culture obtained from EGSB-II, reveals that methanol did not serve as the direct precursor for

methane, which means that no competition for methanol exists between SRB and MA in the sludge. This was supported by the absence of *Methanosarcina* species in the sludge, because the only methanol degrading methanogens isolated from anaerobic digesters to date belong to the genus *Methanosarcina*[22]. The absence of any *Methanosarcina* morphotypes in the bioreactors operated at 65°C is in line with observations made by Zinder et al.[25]. They were not successful in growing *Methanosarcina* cultures at 65°C. In this work it was found in activity assays, with sludge from EGSB-II, that acetate was not degraded and that no methane and only little sulfide was formed from acetate, compared to the control assay conducted with methanol. The observed small sulfide production on acetate presumably results from endogenous electron donors present in the sludge. The presence of endogenous electron donors was confirmed by the results of activity assays without substrate, carried out at days 47 and 58. The assessed SSA at these days amounted to 0.21 and 0.08 $gCOD.gVSS^{-1}.day^{-1}$, respectively. The absence of any acetate degradation also means that oxidation of acetate to H_2/CO_2, as observed by other authors in thermophilic methanogenic sludge[15] and a thermophilic co-culture[26] did not occur. However, acetate may act as a vital carbon source to enable growth of SRB[3]. Apparently, methanogens in the EGSB-reactors use substrates other than acetate and methanol. These substrates could be H_2/CO_2 and/or formate, because the methanogenic culture obtained from EGSB-II used these substrates in the presence of vancomycin. This also confirmed that the methanogens were not affected by vancomycin. The abundance, in the sludge, of methanogens resembling *Methanobacterium*, which are known to grow only on H_2/CO_2 or formate[22], also indicates that methane is mainly formed from H_2/CO_2 or formate. Moreover, the detection of hydrogen in the headspace of the methanogenic culture also confirms the intermediary role of hydrogen during methanogenic methanol degradation. Hydrogenotrophic methanogens were indeed present in high numbers, as shown by the high specific methanogenic activity (SMA) on H_2/CO_2. Because of the relatively low SMA on formate, which was about 14 times lower than the SMA on methanol, formate is presumably not an important precursor for methane in the sludge.

The measured high specific sulfidogenic activity on hydrogen of sludge from EGSB-II shows that this substrate was also suitable as electron donor for sulfate reduction. Since hydrogen apparently is the main electron source for the methanogens in the sludge, very likely the competition between SRB and MA for hydrogen plays an important role in the fate of electron equivalents derived from methanol. This would explain why sulfate reduction ultimately dominates over methanogenesis in the reactors, because it is well known that SRB outcompete MA for hydrogen when sufficient sulfate is present[5,21]. This presumably can be attributed to more favourable kinetic parameters of SRB[14,23] or a lower

hydrogen threshold level[8] over MA. Although the results suggest a key role for hydrogen in the competition between SRB and MA in the reactor, it is still unclear to which extent methanol is directly consumed by sulfate reducers or first converted to hydrogen and formate before being used for sulfate reduction. However, the observed 60% inhibition of sulfidogenic methanol degradation in activity assays at a high hydrogen partial pressures suggests that hydrogen, formed through methanol oxidation, indeed may act as a major electron donor for sulfate reduction in the sludge. Methanol oxidation to H_2/CO_2 coupled to hydrogenotrophic methanogenesis and sulfate reduction has been demonstrated for a thermophilic co-culture of a methylotrophic acetogen and a hydrogenotrophic methanogen or sulfate reducer[3].

The observed high efficiency of methanol utilization for sulfate reduction, and the concomitant appreciable sulfide production rates of 5 to 10 $gCOD.L^{-1}.day^{-1}$ achieved in EGSB-II, show that thermophilic sulfate reduction with methanol is feasible. The maximum value of the sulfide production rate corresponds with a sulfate elimination rate of 15 $gSO_4^{2-}.L^{-1}.day^{-1}$, which is twice as high as the value found by van Houten et al.[6] who used H_2/CO_2 for thermophilic sulfate reduction in gas-lift reactors. The observed large fluctuation in the sulfide production most likely results from periodical substrate limitation prevailing in heavily aggregated zones of the sludge bed. Apparently, the hydraulic mixing in EGSB-reactors is insufficient for preventing such an extent of aggregation and thus for maintaining a good contact between biomass and medium, even at upflow liquid velocities of 10 $m.h^{-1}$. Furthermore, the results also clearly demonstrate that in fact the sludge retention in the EGSB-reactors was poor, because biomass concentrations never exceeded 4 and 7 $gVSS.L^{-1}$ in EGSB-I and -II respectively. As the EGSB-concept is based on the use and maintenance of well settleable (granular) sludge, which is easily retained in the reactor in an expanded, well-mixed sludge bed, EGSB-reactors apparently are not the proper system for thermophilic sulfate reduction with methanol. The biomass concentration rather than the sulfide concentration limits the sulfate elimination rate in EGSB-II at day 129, because the calculated specific sulfidogenic activity in the reactor amounted to 0.95 $gCOD.gVSS^{-1}.day^{-1}$, which is very close to the maximum specific sulfidogenic activity of the sludge of 1.05±0.07 $gCOD.gVSS^{-1}.day^{-1}$ assessed in activity assays on that day. Apparently, inhibition by the total sulfide concentration (512 $mgS.L^{-1}$) or by the free hydrogen sulfide concentration (57 $mgS.L^{-1}$) was not important on day 129.

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Isolation and characterization of a methanol-utilizing, sulfate-reducing bacterium isolated from a thermophilic sulfidogenic bioreactor

3.1 Abstract

From anaerobic sludge cultivated in a thermophilic (65°) sulfate-reducing bioreactor (EGSB-II, Chapter 2) fed with methanol as electron donor, a sulfate-reducing bacterium (strain WW1) was isolated with methanol as sole carbon and energy source. On most substrates, strain WW1 is rod shaped (2-5 μm x 0.8 μm), non-motile, gram-negative, and formes central spores that cause distension of cells. However, during growth on H_2/CO_2, lactate or pyruvate, cells are 8 to 12 times as long, and spores were never found. Optimum growth occurred around pH 7.5 and a temperature of 65°C. Strain WW1 was identified as a *Desulfotomaculum*-species by its morphology and 16SRNA-sequence. In the sludge, strain WW1 is not confined to the use of methanol, as the strain also grows on anaerobic degradation products of methanol, like acetate, formate and H_2/CO_2. Growth of strain WW1 on methanol and sulfate already stops at a total sulfide concentration of 220 $mgS.L^{-1}$, while growth on hydrogen and sulfate is possible up to 640 $mgS.L^{-1}$ of total sulfide. Thus, the high sulfide levels (up to 700 $mgS.L^{-1}$) produced in the bioreactor might be explained by assuming growth of strain WW1-like sulfate reducers on hydrogen rather than on methanol.

3.2 Introduction

As described in Chapter 2, a high rate of sulfate reduction with methanol as electron donor was found in thermophilic (65°C) high-rate bioreactors, while only a minor part of the methanol was converted to methane and acetate. In the bioreactor, the sulfate-reducing bacteria (SRB) may not only use methanol as electron donor for sulfate reduction, but also products of methanol degradation products, e.g acetate and hydrogen[1]. Only a few of the known thermophilic SRB are able to use methanol and acetate as electron donor, while all described thermophilic SRB except *Thermodesulforhabdus norvegicus* use hydrogen as electron donor (Chapter 1). However, some species need acetate as carbon source for growth on hydrogen.

In this chapter, the isolation is described of a sulfate reducer originating from anaerobic sludge that was cultivated in a thermophilic (65°C) upflow reactor fed with an influent containing methanol and sulfate (EGSB-II, Chapter 2). By characterizing the isolated sulfate reducer insight is gained into the microbiology of thermophilic sulfate reduction with methanol and accordingly, the microbial conversions of methanol in the process.

3.3 Materials and methods

Source of organisms. Strain WW1 originated from anaerobic sludge that was cultivated in a thermophilic (65°C) laboratory-scale expanded granular sludge bed (EGSB) reactor. The medium fed to the EGSB-reactor had a similar composition as the medium used for enrichment and cultivation of WW1, except for vitamins and the trace element cobalt. Methanol and sulfate concentrations of the reactor influent were 56 mM and 42 mM, respectively. The pH in the reactor was controlled at 7.5. The sludge sample was taken after day 79 of operation, when 92% of the consumed methanol was used for sulfate reduction, while only 6 and 2% of the methanol was used for methane and acetate formation, respectively. More details of the description and operation of the reactor can be found in Chapter 2 (EGSB-II). *Methanobacterium thermoautotrophicum* ΔH was from our own laboratory strain collection.

Media and cultivation. The basal liquid culture medium contained ($g.L^{-1}$): NaCl (7), $NaHCO_3$ (4), Na_2SO_4 (2.8), $MgCl_2.6H_2O$ (1.2), KCl (0.5), NH_4Cl (0.3), KH_2PO_4 (0.2), $CaCl_2$ (0.15), $Na_2S.7\text{-}9H_2O$ (0.3), yeast extract (0.02 $mg.L^{-1}$) and a trace element solution (1 $mL.L^{-1}$) containing ($mg.L^{-1}$): $FeCl_2.4H_2O$ (1500), $CoCl_2.2H_2O$ (190), $MnCl_2.4H_2O$ (100),

$ZnCl_2$ (70), H_3BO_3 (62), $Na_2MoO_4.2H_2O$ (36), $NiCl_2.6H_2O$ (24), $CuCl_2.2H_2O$ (17), EDTA (500) and HCl 37% (7 $mL.L^{-1}$). Additionally, vitamins were added from a concentrated stock solution[13]. Solid media were prepared by addition of 1.5% granulated agar to 20 mL of liquid medium in agar shake tubes. Liquid cultures were routinely grown at 65°C in 117-mL serum vials with butyl rubber stoppers and aluminum crimp seals. The vials contained 50 ml basal medium and a gas phase of 1.7 bar N_2/CO_2 (80:20, v/v). In case H_2/CO_2 was tested as growth substrate, nitrogen in the gas phase was replaced by hydrogen. Substrates were added from concentrated stock solutions that were sterilized by heat before use, except for pyruvate that was filter-sterilized. Routinely, the pH of the medium was 7. By varying the CO_2 concentration in the headspace and adding a few drops of 0.1 N HCl or NaOH, the pH of the medium could be adjusted within the range 5.5-8.5. In all growth experiments in liquid medium, the inoculum size was 10%. To test syntrophic growth on methanol, cells were inoculated in a dense culture of exponentially H_2-grown cultures of *Methanobacterium thermoautotrophicum* ΔH in the same liquid medium as described above, but without sulfate. Before inoculation, the gas phase was changed to N_2/CO_2 and methanol was added. Growth experiments were conducted at least in duplicate.

Isolation. Fresh sludge was taken from a thermophilic sulfate-reducing bioreactor fed with methanol as sole energy- and carbon source. Under a nitrogen atmosphere, 2 mL of sludge was disintegrated using a mortar. The sludge was brought in an anaerobic tube, and after 5-fold dilution with medium, the sludge was further disintegrated by pressing it repeatedly through a syringe needle (Microlance 3, 0.6 x 25 mm). Serial dilutions of the sludge were made in liquid media containing 30 mM methanol, 2 mM acetate, and 20 mM sulfate. The highest dilution showing growth at 65°C was designated SRC-11 (sulfate-reducing culture from 10^{11} times diluted sludge). Repeated serial dilution and subsequent incubation in liquid medium further purified the culture. The resulting purified culture was diluted in agar (1.5% Agar Noble) shake tubes. Colonies from the highest dilution were again diluted in agar shake tubes. This procedure was repeated two times until a pure culture was obtained. Purity was checked by inoculating the culture in medium containing 10 $g.L^{-1}$ yeast extract (BBL-Becton Dickinson, Cockeyesville, MD, USA) or 15 $g.L^{-1}$ Wilkins-Chalgren anaerobe broth (Oxoid, Basingstroke, UK) and incubation at 30 and 65°C under anaerobic and aerobic conditions. After incubation, the cultures were examined microscopically.

16S RNA Sequence analysis. For the genetic characterization of strain WW1, chromosomal DNA was isolated from a liquid culture as described previously[4]. The 16S rDNA gene was selectively amplified by PCR, using oligonucleotide primers complementary to conserved regions of the eubacterial 16S rDNA. The following primer

pair was used: 5' ACCTAATACGACTACTATAGGGAGAGTTTG-ATCCTGGCTCAG 3' (positions 8-27, *E. coli* numbering) and 5' ATTGTAAAACGACGGCCAGT-GGTTACCTTGTTACGACTT 3' (positions 1492-1510, *E. coli* numbering). The PCR amplification products were sequenced with an amplified Biosystems model 373A DNA sequencer by using the *Taq* DyeDeoxy terminator cycle sequencing method and custom primers based on conserved regions.

The assembled DNA sequence was aligned with the equivalent 16S rDNA sequences of closely related strains found in the GenBank database using CLUSTAL W. A phylogenetic tree was constructed from a distance matrix based on the neighbour-joining method[11] as implemented in the program TREECON[9]. No correction method was applied and tree topology was re-examined by using bootstrap analysis (100 trees).

Analysis of cell compounds. Isolation and purification of DNA was carried out according to standard protocol[5]. The G+C content of the genomic DNA was determined by thermal denaturation method[8]. Gram-staining was done according to standard procedures[2].

Analytical methods. Samples for methanol and VFA analysis were centrifuged at 17000 g for 5 min., diluted with a 3% formic acid solution (final concentration formic acid 1-2%), and stored at 4°C. Methanol and acetate were determined by gas chromatography using a Hewlett Packard model 5890 equipped with a 6 m × 2 mm glass column packed with Supelco port, 100-120 mesh, coated with 10% Fluorad FC 431 (3M). The flow rate of the carrier gas (nitrogen saturated with formic acid) was 40 $mL.min^{-1}$, and the column pressure was ± 3 bar. The temperature of the column, injection port and detector were 80, 200, and 280°C, respectively. Propionate and butyrate were analysed in the same way as methanol except that the temperatures of the column, the injector port and the flame ionization detectors were 130, 200 and 280, respectively.

Methane and hydrogen were measured on a 406 Packard gas chromatograph equipped with a thermal conductivity detector (TCD), 100 mA. The gases were separated with argon as the carrier gas on a molecular sieve column (13X, 1.8 m by 1/4 inch, 60-80 mesh) at 100°C. The total sulfide concentration is defined as the sum of H_2S, HS^-, and S^{2-} sulfide species. The total sulfide was determined colorimetrically using the methylene blue method[14].

3.4 Results

Isolation and morphological characterization

A sulfate-reducing culture was obtained from the 1 x 10^{11}-fold dilution of disintegrated anaerobic sludge, inoculated in medium containing methanol, sulfate and a low amount of acetate. The culture did not show methane formation, nor formation of acetate. By repeatedly using the agar shake method pure sulfate-reducing cultures were obtained. One of these, designated strain WW1, was used for further study. The morphology of WW1 depended on the substrate tested. During growth on sulfate and substrates other than H_2/CO_2, lactate or pyruvate (see below), cells were rod shaped (2-5 μm x 0.8 μm), and formed central spores, causing distension of the cells (Figure 3.1). During growth on sulfate and H_2/CO_2, lactate or pyruvate, cells were 8 to 12 times as long (width unchanged) and spores were never found (Figure 3.1). Cells stained Gram-negative, but electron microscopical examination the strain revealed a typical Gram-positive cell wall structure (not shown).

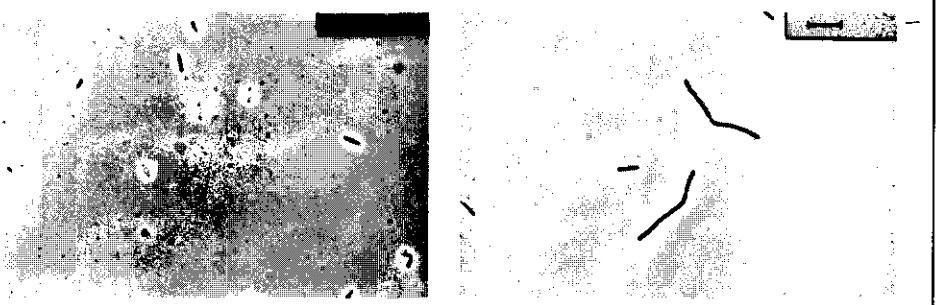

Figure 3.1. Phase contrast photomicrographs of strain WW1 growing on methanol plus sulfate (left graph) and H_2/CO_2 and sulfate. Bar = 10 μm.

Growth and substrate utilization

The optimum growth temperature for strain WW1 was 7.6, while growth still occurred at pH 6.3 and pH 8.3. Strain WW1 grew optimally between 62 and 68°C, but little growth occurred at 45 and 75°C. The substrate and electron acceptor range of strain WW1 is summarized in Table 3.1.

Table 3.1. Compounds tested as growth substrate or electron acceptor for strain WW1. The concentrations of all substrates was 20 mM, except methanol which was 50 mM.

Utilized in the presence of 20 mM sulfate:
Methanol, H_2/CO_2 (excess, 80/20 v/v), ethanol, propanol, butanol, isobutanol, formate, propionate, butyrate, succinate, fumarate, lactate and pyruvate
Tested, but not utilized in the presence of 20 mM sulfate:
Benzoate, isopropanol, fructose and glucose
Utilized as electron acceptor:
Sulfite (5 mM), thiosulfate (5 mM)
Tested, but not utilized as electron acceptor:
Nitrate (10 mM)

Although strain WW1 was isolated on sulfate, methanol and acetate, the strain was able to grow on sulfate and either methanol or acetate alone. No acetate was formed during growth on methanol. Both methanol (30 mM) and acetate (2 mM) were degraded when incubated together, but sulfide production was not higher than with methanol alone. WW1 could grow autotrophically on H_2/CO_2 and sulfate. Acetate was not formed during growth on H_2/CO_2. Acetate and methanol were not degraded in the presence of an excess of H_2/CO_2. On methanol, acetate, and H_2/CO_2, sulfide formation did not proceed exponentially. Therefore, calculation of the maximum specific growth rate on these substrates was not possible.

Incubation of the culture with methanol and *Methanobacterium thermoautotrophicum* strain ΔH (no sulfate) resulted in only a low methane formation. However, the original culture that was obtained by serial dilution of the anaerobic sludge, did form high amounts of methane after incubation with methanol and *M. thermoautotrophicum* strain ΔH. Results on this newly assembled culture are presented in Chapter 4.

Sulfide toxicity

Growth of strain WW1 on methanol or acetate was rather poor; in 20 days only 200 to 300 $mgS.L^{-1}$ (6 to 9 mM) of total sulfide was produced at an initial pH of 7, after which the sulfide concentration no longer increased. Sulfide formation in 5 other pure sulfate-reducing cultures (of which the morphology strongly resembled that of strain WW1) obtained from culture SRC-11 also proceeded up to maximum levels of around 300 $mgS.L^{-1}$.

In contrast with methanol, with H_2/CO_2 all added sulfate 640 $mgS.L^{-1}$ (20 mM) was reduced within 16 days by strain WW1. At initial concentrations of 220 $mgS.L^{-1}$ (7 mM) total sulfide, no growth occurred with methanol and sulfate. Elevating the initial pH from 7 to 7.5 and 8, did not result in formation of higher sulfide concentrations. Poor growth of strain WW1 was not caused by toxicity of impurities of the commercial sodium sulfide that was used for reduction of the medium, as suggested by Widdel[15], because replacing commercial sodium sulfide with sterile biogenic sulfide (obtained by filter-sterilizing effluent from reactor EGSB-II, Chapter 2) did not improve formation of sulfide. Also, addition of autoclaved sludge (1 $mL.L^{-1}$) from the reactor from which strain WW1 was isolated, or filter-sterilized sludge supernatant (20 $mL.L^{-1}$), did not stimulate sulfide formation.

G+C-content. The G+C-content of the DNA of strain WW1 amounted to 49.6 mol%.

Phylogenetic analysis of 16S rDNA sequences. Using 6 primers, a nearly complete sequence consisting of 1403 basepairs of the amplified 16S rDNA gene of strain WW1 was obtained. On the basis of 16S rDNA sequences, strain WW1 was most similar to *Desulfotomaculum thermocisternum* (99% similarity value) and *Desulfotomaculum australicum* (98% similarity value) (Figure 3.2). The result of the phylogenetic analysis based on the nucleotide sequence of the 16S rDNA gene clearly indicate that strain WW1 is a member of the genus *Desulfotomaculum*.

Figure 3.2: An unrooted phylogentic tree based on the results of 16S rDNA sequence comparisons showing the relationship of *Desulfotomaculum* strain WW1 with other *Desulfotomaculum* species. Bootstrap values (> 50) expressed as percentages are shown at the branch points. Roman numerals refer to the cluster and subclusters of the *Desulfotomaculum* species as described by Stackebrandt *et al.*[12].

3.5 Discussion

The isolation of strain WW1 from the highest methanol degrading, sulfate-reducing serial dilution of disintegrated anaerobic sludge strongly indicates that this microorganism is the most abundant sulfate reducer involved in methanol degradation in the sludge. In the sludge, strain WW1 may not be confined to the use of methanol, as the strain also grows on anaerobic degradation products of methanol, like acetate and H_2/CO_2. However, acetate did not serve as sole electron donor for sulfate reduction in the sludge from which strain WW1 was isolated (Chapter 2). Strain WW1 uses acetate in the presence of methanol, but not in

the presence of H_2. Therefore, the lack of acetate consumption by the sludge might be explained by assuming that hydrogen is the main electron donor for sulfate reduction in the sludge. The presence of methanol oxidizing, hydrogen-producing bacteria in the highest methanol degrading, sulfate-reducing serial dilution of the sludge supports this hypothesis. The methanol oxidizing species likely is not strain WW1, as the latter was not able to grow on methanol in co-culture with *M. thermoautotrophicum* ΔH. The methanol-oxidizing species is currently under study in our laboratory. The results support our hypothesis stated previously that methanol oxidation to H_2/CO_2, and subsequent sulfate reduction with H_2, plays an important role in the sludge (Chapter 2). In this scenario, strain WW1-like sulfate reducers act as the hydrogen scavengers.

With respect to pH and temperature, strain WW1 was well adapted to the environmental conditions prevailing in the reactor, as optimum pH (7.5) and temperature (62-68°C) of strain WW1 coincided with the imposed conditions in the reactor (pH 7.5, 65°C). Strain WW1 did not grow on methanol at initial total sulfide concentrations above 220 $mgS.L^{-1}$ (7 mM), indicating sulfide toxicity. Toxicity seemed to be related to the total sulfide concentration rather than the H_2S concentration, as elevating the pH of the culture to 7.5 and 8 did not relieve inhibition. The total sulfide concentration in the original habitat of WW1 amounted to values as high as 700 $mgS.L^{-1}$ (22 mM). The difference with the sulfide inhibition level of WW1 may be explained by assuming that hydrogen acts as the main electron donor in the sludge, as with this substrate growth of strain WW1 at 640 $mgS.L^{-1}$ (20 mM) was still possible. Apparently, the metabolism of methanol in strain WW1 is much more sensitive towards sulfide than the hydrogen metabolism, e.g. due to high sensitivity of enzymes involved in methanol conversion. In addition, factors may be present in the sludge that protect strain WW1 from sulfide toxicity. These factors must be heat labile and not permeable through sterilization filters, as autoclaved sludge or filter-sterilized sludge supernatant did not stimulate growth.

Taxonomy. The topology of the phylogenetic tree constructed was almost indentical with earlier data concerning *Desulfotomaculum* species[3,6,7,10,12]. Strain WW1 was located in subcluster Ic, consisting of closely related thermophilic *Desulfotomaculum* species with similarity values of more than 93%.

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Interspecies hydrogen transfer in a thermophilic methanol-degrading culture obtained from a sulfate-reducing bioreactor

4.1 Abstract

In the bioreactor-experiments described in Chapter 2, sulfate-reducing bacteria may either directly use the added methanol for reduction of sulfate, or they may use intermediates from methanol degradation like hydrogen, formate and acetate. In this chapter it is shown that bacteria able to oxidize methanol to hydrogen (and presumably CO_2) most likely are present in high numbers in the bioreactor. This indicates that hydrogen may represent an important intermediate in sulfidogenic methanol degradation at 65°C.

4.2 Introduction

To gain insight in the microbial conversions of methanol in a thermophilic (65°), sulfate-reducing bioreactor, the dominant microorganisms from this reactor are studied. A sulfate-reducing culture (culture SRC-11) growing on methanol, sulfate and a small amount of acetate was obtained from 10^{11}-fold diluted anaerobic sludge cultivated in the EGSB-reactor described in Chapter 2 (EGSB-II). From culture SRC-11, the thermophilic sulfate-reducing strain WW1, as described in Chapter 3, was isolated. Strain WW1 appeared to be a *Desulfotomaculum* spp. able to use methanol and possible anaerobic degradation products of methanol, viz. hydrogen, formate and acetate as electron donor for sulfate reduction. Therefore, methanol in culture SRC-11 may be either directly used as electron donor for sulfate reduction by strain WW1-like microorganisms or it may first be degraded to intermediates like hydrogen, formate and acetate by for instance homoacetogens, followed by sulfidogenic oxidation of such intermediates. The occurrence of such a methanol degradation route would imply the presence of species in culture SRC-11 that degrade methanol to such intermediates.

The objective of the work described in this chapter is to investigate the occurrence of organisms possibly involved in the conversion of methanol to hydrogen and acetate.

4.3 Materials and methods

Origin of the bacterial cultures. Culture SRC-11 originated from anaerobic sludge cultivated in a thermophilic sulfate-reducing bioreactor fed with methanol, as described in more detail in Chapter 3. *Methanobacterium thermoautotrophicum* ΔH was from our own laboratory strain collection. The thermophilic sulfate reducer strain SR was isolated in our laboratory[2].

Media and cultivation. Media and cultivation were largely as described in Chapter 3. Unless stated otherwise, media contained methanol (30 mM), acetate (2 mM), yeast extract (20 $mg.L^{-1}$) and sulfate (20 mM) as substrates. Liquid cultures were incubated at 62°C. When inhibition of sulfate reducers or methanogens was desired, molybdate and 2-bromoethanesulfonate (sodium salts), respectively, were added from concentrated stock solutions. The stock of 2-bromoethanesulfonate was filter-sterilized before use and the stock of molybdate was heat-sterilized. Routinely, the inoculation size of the cultures was 10%. Growth experiments were conducted at least in duplicate.

Solid media had the same composition as liquid media. In addition, 1.5% agar Noble (soft agar shake tubes) or 3% Bacto-agar or granulated agar (Hungate roll-tubes) was added. Solid media were incubated at 62°C. Agar media were sterilized at 115°C, 40 min.

Analytical methods. Analysis of methanol, volatile fatty acids (VFA) and sulfide in liquid samples and methane and hydrogen in the headspace of vials were conducted as described in Chapter 3.

Other methods. Gram-staining was done according to standard procedures[3].

Phylogenetic analysis

DNA extraction. Nucleic acids were extracted using a direct lysis protocol modified from Moré et al.[6] as described previously[4]. Cells (50 mL) were harvested by centrifugation and cell pellets were transferred into 2-mL screw cap tubes. Approximately 1 g of sterilized (170°C for 4 h) zirconia beads (0.1 mm diameter; Biospec products Inc., Bartlesville, Ok, USA), 800 µl Na-phosphate buffer (120 mM, pH 8), and 260 µL SDS-solution (10% SDS, 0.5 M Tris/HCl, pH 8.0, 0.1 M NaCl) were added to the cells, and resuspended homogeneously by vortexing. Cells were lysed for 45 s by shaking in a cell disruptor (FP120 FastPrep, Savant instruments Inc., Farmingdale, NY, USA) at a setting of 6.5 m s^{-1}. After centrifugation (3 min at 12,000 × g) the supernatant was collected, and the soil-beads mixture was extracted a second time by resuspension in 700 µL phosphate buffer. Proteins and debris were precipitated from the supernatant by adding 0.4 volumes of 7.5 M ammonium acetate, followed by incubation on ice for 5 min. After centrifugation at 12,000 × g for 3 min nucleic acids were precipitated by addition of 0.7 volumes of isopropanol, followed by centrifugation at 12,000 × g and 4°C for 45 min. Subsequently, the DNA pellet was washed with 70% ethanol at 4°C, and dried under vacuum. Finally, DNA was resuspended in 200 µL Tris-EDTA buffer (10 mM Tris-base, 1 mM EDTA, pH 8).

PCR amplification. For PCR amplification a SSU rRNA-based primer set was used, i.e. an "Universal" primer set targeting all life which was modified from Weisburg et al.[15] by using 533f (GTGCCAGCAGCCGCGGTAA) and 907r (AATTCCTTTGAGTTT) (*Escherichia coli* positions 515-533 and 907-922[2] as forward and reverse primers, respectively. Primers 907r had a GC-clamp (cgcccgccgcgccccgcgcccggcccgccgcccccgcccc) at its 5' end.

PCR buffer (20 mM Tris-HCl, pH 8.3, 50 mM KCl), 1 U *AmpliTaq* DNA polymerase (Perkin Elmer Applied Biosystems, Weiterstadt, Germany), 0.5 µM of each primer, 100 µM of each deoxynucleoside triphosphate (Amersham Life Science, Braunschweig, Germany), and 1 µL of template DNA were added to a total reaction volume of 50 µL at 4°C.

Amplifications were started by placing cooled (+4°C) PCR tubes immediately into the preheated (94°C) thermal block of a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). The thermal cycling profile consisted of an initial denaturation of 3 min at 94°C, followed by 28 to 32 cycles of 30 s at 94°C, 30 s at an annealing temperature of 60 to 50 °C (touch down in 20 0.5°C increments), and 45 s at 72°C (elongation), and 5 min at 72°C for the last cycle.

Aliquots (5 µL) of PCR products were analyzed by electrophoresis on 3% agarose gels, stained with ethidium bromide. Gels were photographed with an imaging system (MWG Biotech, Germany).

Denaturing gradient gel electrophoresis. DGGE was carried out as described previously in detail with minor modifications[13]. PCR-products were separated using a DCode System (Bio-Rad, Munich, Germany) on 1-mm thick polyacrylamide gels (6.5% w/v acrylamide:bis acrylamide (37.5:1); Bio-Rad) prepared with and electrophoresed in 0.5 × TAE, pH 7.4 (0.04 M Tris-base, 0.02 M sodium-acetate, 1 mM EDTA) at 60°C, and constant voltage. A denaturing gradient of 80% (vol/vol) denaturant corresponded to 6.5% acrylamide, 5.6 M urea and 32% deionized formamide. Gels were poured on GelBond PAG film (FMC Bioproducts, Rockland, ME, USA) to avoid gel distortion. A denaturant gradient of 35 to 70% was used, and gels were elctrophoresed at 150 V for 5h. Gels were stained with 1:50,000 (vol/vol) SYBR-Green I (Biozym, Hessisch-Oldendorf, Germany) for 30 min, and scanned with a Storm 860 phosphor imager (Molecular Dynamics, Sunnyvale, CA, USA).

Extraction of PCR products from DGGE gels. We visualized DGGE bands in SYBR Green I-stained gels with blue light ($\lambda >$ 400 nm) using a Dark Reader transilluminator (Clare Chemical Research, Ross on Wye, UK). Individual DGGE bands were sampled by excising a small core with a sterile 200 µL pipette tip, reamplified, and reanalyzed by DGGE to verify band purity.

Sequencing of DGGE bands. Reamplified PCR products of excised DGGE bands were purified using the EasyPure DNA purification kit (Biozym, Hessisch-Oldendorf, Germany). Concentration and purity of PCR products were determined by absorption at 260 nm and 280 nm of a 1:20 dilution in H_2O with a GeneQuant spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Sequencing reactions were performed using the ABI Dye-terminator cycle sequencing kit (Perkin Elmer Applied Biosystems,) with 30-180 ng template DNA as specified by the manufacturer. Cycle sequencing products were purified from excess dye terminators and primers using Microspin G-50 columns (Pharmacia Biotech, Freiburg,

Germany), and analyzed with an ABI 373 DNA sequencer (Perkin Elmer Applied Biosystems).

Sequences were analyzed using the Lasergene software package (DNASTAR, Madison, WI, USA). 16S rDNA sequences were aligned and phylogenetically placed with the ARB software package[14]. Evolutionary distances between pairs of sequences were calculated by using the Jukes-Cantor, and Felsenstein equations[16] implemented in the ARB package. Sequences were compared to those available in GenBank using the BLAST search algorithm[1]. Phylogenetic trees were constructed by using the neighbor joining algorithm supplied by the ARB software package[14].

4.4 Results

Origin of starting culture SRC-11

The starting culture SRC-11 originated from a thermophilic (65°C) sulfate-reducing bioreactor that was fed for several months with methanol as the sole carbon and energy source (EGSB-II, Chapter 2). Culture SRC-11 comprised the highest (1×10^{11}) liquid dilution of crushed, flocculent anaerobic sludge from this reactor that showed growth at 65°C in a medium containing methanol (30 mM), acetate (2 mM), yeast extract (20 $mg.L^{-1}$) and sulfate (20 mM). Culture SRC-11 did not degrade methanol in the absence of sulfate, indicating that methanol degrading homoacetogens and methanogens were not present in the culture.

Demonstration of interspecies hydrogen transfer in culture SRC-11

The ability of culture SRC-11 to oxidize methanol to H_2/CO_2 was tested by inoculation (10%) into an exponential-phase H_2-grown culture of *M. thermoautotrophicum* strain ΔH with methanol (30 mM), acetate (2 mM) and yeast extract (20 $mg.L^{-1}$). The resulting culture formed substantial amounts of methane. After several transfers in sulfate-free medium a stable culture was obtained that degraded methanol to methane and acetate (Figure 4.1). From the data in Figure 4.1 it can be calculated that approximately 70% and 30% of the degraded 25 mM methanol was used for formation of methane and acetate, respectively. Because the original culture SRC-11 did not form methane from methanol in the presence or absence of sulfate after prolonged incubation, it is clear that methane formation in the new culture indeed was due to the presence of *M. thermoautotrophicum* strain ΔH. Thus, in the new culture methane is formed from H_2/CO_2, as *M. thermoautotrophicum* strain ΔH can

only grow on this substrate. The culture did not degrade acetate (20 mM) and formate (20 mM) to methane. Furthermore, methanol was not degraded when the methanogen was inhibited by 10 mM 2-bromoethanesulfonate.

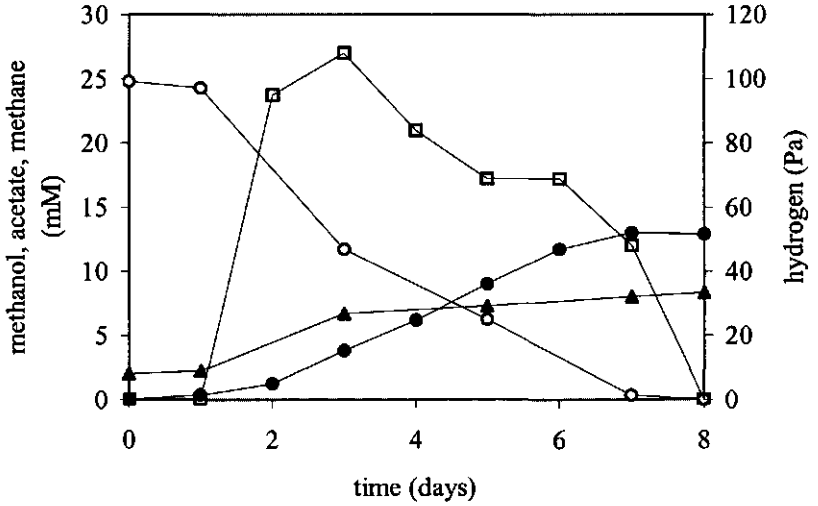

Figure 4.1. Methanol degradation in culture SRC-11 in the presence of *M. thermoautotrophicum* strain ΔH without sulfate. Symbols: (○) methanol, (●) methane, (▲) acetate, (□) hydrogen pressure headspace. The methane concentration is expressed as mmol methane produced per L of medium.

Attempts to obtain a methanol degrading methanogenic co-culture

The newly assembled methanol-degrading methanogenic culture was purified in several serial liquid dilutions. In the purified culture, besides *M. thermoautotrophicum* strain ΔH, two morphologies were distinguished in high numbers. Cells of morphotype 1 were Gram-negative, non-motile rods (2-5 μm x 0.8 μm) with or without central spores. Cells of morphotype 1 were obtained in pure culture from culture SRC-11, and one strain was designated WW1, as described in Chapter 3. Strain WW1 appeared to be a *Desulfotomaculum* spp. that could grow on methanol, acetate, hydrogen and formate in the presence of sulfate. Cells of morphotype 2 were non-motile rods, sometimes occurring in pairs, and were 1-2 μm x 0.2-0.5 μm in size. The relative number of morphotypes 1 and 2, and *M. thermoautotrophicum* strain ΔH in the culture amounted to approximately 10%,

30% and 60%, respectively. The culture was serially diluted in agar-shake tubes with methanol (30 mM), acetate (2 mM) and yeast extract (20 mg.L^{-1}), with the aim to obtain a defined co-culture of methanol-oxidizing species and the methanogen. Colonies only formed in tubes with 1 x 10^1-10^3-fold diluted culture. Also repeated dilution of the colonies failed to yield a co-culture as all three morphotypes remained present. Attempts to inhibit growth of cells resembling the sulfate-reducing strain WW1 by addition of 1 mM molybdate, a specific inhibitor of sulfate reduction, also failed as molybdate was found to completely inhibit growth of the culture. However, when the same amount of molybdate was added to a growing culture, methanol consumption and methane formation proceeded as in controls without addition of molybdate (data not shown). From these results we concluded that sulfate-reducing activity or presence of specific sulfate-reducing species is required to initiate methanogenic growth of the culture on methanol.

Transfer of methanol-oxidizing activity from methanogenic to a sulfate-reducing culture

In a subsequent effort to obtain a defined co-culture of a methanol-oxidizing species and a hydrogenotroph, the methanogen was replaced with hydrogenotrophic sulfate reducer. The heterotrophic sulfate-reducing strain SR, isolated in our laboratory a few years ago, was selected for this purpose. Strain SR is not able to grow on methanol or acetate plus sulfate, which makes strain SR suitable to replace *M. thermoautotrophicum* strain ΔH as hydrogenotroph in the methanol-degrading culture. Furthermore, strain SR is a small vibrio and can therefore easily be distinguished by means of light microscopy from morphotype 1 and *M. thermoautotrophicum* strain ΔH. The methanogenic methanol degrading culture was repeatedly diluted in dense cultures of strain SR with methanol (30 mM), acetate (2 mM), sulfate (20 mM) and 20 mg.L^{-1} yeast extract. Acetate was added as carbon source for strain SR. After 3 subsequent dilution series, morphotype 1 was no longer observed in the culture. In addition, the culture did not form any methane from methanol or H_2/CO_2 in the presence or absence of added sulfate, indicating that *M. thermoautotrophicum* strain ΔH had been diluted from the culture. Instead, methanol (28 mM) was used for sulfide formation (15 mM) and acetate formation (3 mM), as shown in Figure 4.2. Thus, 75% and 15% of the methanol-COD (COD: chemical oxygen demand) was used for sulfate reduction and acetate formation, respectively, while 10% of the degraded methanol remained unrecovered. Morphotype 2 was still present in the culture, although in relatively low numbers, while the growth of a large number of vibrio's indicated growth of strain SR. These results show that indeed strain SR had replaced the methanogen as hydrogenotroph in the methanol-oxidizing culture, and the sulfate-reducing culture was designated MOSC (methanol-oxidizing

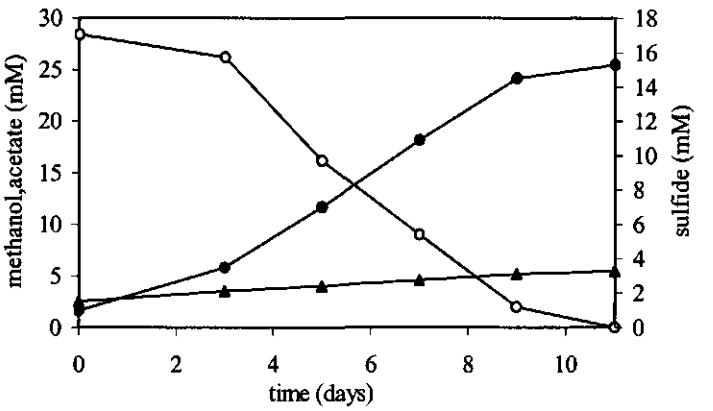

Figure 4.2. Methanol degradation in culture MOSC. Symbols: (o) methanol, (●) sulfide, (▲) acetate.

sulfate-reducing culture). Besides morphotype 2 and the vibrio-shape, a third morphology (morphotype 3) had appeared in the culture, viz. a thin rod clearly distinct from morphotype 2. This species presumably was a contaminant in the culture. It later appeared that this contaminant probably was already present in our culture of strain SR even though this culture seemed to be microscopically pure.

Growth of culture MOSC on methanol was not retarded when acetate and yeast extract were omitted (8 subsequent transfers). Clearly, strain SR used part of the acetate present in the inoculum or the acetate produced during growth of culture MOSC as carbon source. In roll-tubes with Bacto-agar and granulated agar that were inoculated with up to 1 x 10^5-fold diluted culture MOSC, disc-shaped colonies formed within one week. These colonies consisted of cells of Morphotype 3 that probably grew on agar constituents, as similar colonies were also observed in agar dilutions without methanol and acetate. In the dilution series with Bacto-agar medium two other colony types were observed after 2 weeks incubation in the 1 x 10^1-fold dilution: colony type 1 was dark coloured while type 2 appeared more brownish. It should be noted that the true colour was difficult to assess due to darkening of the agar at 62°C. The striking feature of these colonies was that type 1 was always overlapping with a type 2 colony. Microscopic examination of these overlapping colonies revealed that they consisted of vibrio-shaped cells (strain SR) and morphotype 2. As colonies consisting of cells of morphotype 3 were also present in high numbers in higher dilutions, it was not surprising that inoculation of overlapping colonies into liquid media

resulted in cultures that morphologically strongly resembled the original culture MOSC, with Morphotype 3 still present. Thus, a defined co-culture was not obtained.

Attempts to grow culture MOSC on methanol in the absence of sulfate

Several homoacetogens are known to produce hydrogen from methanol in the presence of hydrogenotrophs[3,4,8]. Therefore, it was tested if the methanol-oxidizing species in culture MOSC could be enriched as homoacetogens growing on methanol, which would facilitate its isolation. However, methanol was not degraded in culture MOSC in the absence of sulfate. Also in growing cultures of MOSC methanol degradation stopped as soon as sulfate (5 mM) was depleted. Under sulfate limitation, the hydrogen partial pressure in the headspace, which amounted to less than 50 Pa when sulfate was still present, accumulated to a final value of 1300 Pa. Culture MOSC also did not degrade methanol in the absence of sulfate at 55 and 70°C.

Phylogenetic analysis of culture MOSC

By assessment of the phylogenetic position of the microbial species in culture MOSC, it was aimed to gain more information on the type of species present in the culture. For this purpose, a partial phylogenetic characterization of the species in culture MOSC was conducted. First, 16S rDNA PCR products (fragments of approximately 400 bp) obtained from culture MOSC were separated by denaturing gradient gel electrophoresis (DGGE). The resulting three partial 16S rDNA-bands were purified by again subjecting these bands to DGGE (Figure 4.3), followed by sequencing the DNA. Comparing the partial 16S rDNA-sequence of the purified bands with known sequences revealed a strong (99%) homology of the DNA in the three bands with partial 16S rDNA-sequences of *Coprothermobacter proteolyticus*, *Thermodesulfovibrio* sp. TGE-P1 and *Clostridium* strain PB, respectively.

	Closest Genbank relative	Sequence difference (%)
MOSC		
MOSC-1	*Coprothermobacter proteolyticus*	1.3
MOSC-2	*Thermodesulfovibrio* sp. TGE-P1	1.3
MOSC-3	*"Clostridium* sp. PB"	1.3
MOSC		

Figure 4.3. Purification of DGGE-bands from enrichment culture MOSC and their phylogenetic affiliation as based on BLAST comparisons of partial 16S rDNA-sequences.

4.4 Discussion

The formation of high levels of methane from methanol in the culture assembled from culture SRC-11 and *M. thermoautotrophicum* strain ΔH obviously strongly supports the occurrence of interspecies hydrogen transfer. Presumably, methanol is oxidized directly to hydrogen and not first to acetate and formate, as the latter substrates did not support methane formation in the assembled culture. Methanol oxidation to hydrogen and CO_2 has been reported for several co-cultures consisting of a homoacetogen and a hydrogenotrophic methanogen or sulfate reducer[3,4,8]. In all these cases, the methanol-oxidizing species grows homo-acetogenically on methanol in pure culture, while methanol is degraded to both H_2/CO_2 and acetate in the presence of a hydrogenotroph. Also in our methanol-degrading methanogenic culture acetate was formed, but no acetogenic activity was detected in the culture when the methanogen was inhibited by BRES. Also when the methanogen was replaced by a hydrogenotrophic sulfate reducer strain SR, acetate was formed from methanol in the resulting culture MOSC, but again acetogenesis stopped when activity of the hydrogenotroph stopped due to sulfate depletion. From these results it appears that methanol oxidation coupled to hydrogen formation is coupled to the formation of acetate. The possibility that hydrogen consumption by *M. thermoautotrophicum* strain ΔH and strain SR is coupled to acetate formation can be ruled out as these species do not form acetate during growth on H_2/CO_2. The question arises whether the hydrogen forming species concomitantly produces some acetate, analogous to the previously described homoacetogens in co-culture with hydrogenotrophs, or that other species, depending on the methanol oxidizers for their growth, produce the acetate. As the methanol-oxidizing cultures were still undefined, this question remains to be solved.

The crude phylogenetic analysis of culture MOSC indicated that the culture consists of a *Thermodesulfovibrio*-like species, a species phylogenetically resembling the proteolytic *Coprothermobacter proteolyticus*[11], and a species closely related to the *Clostridium* strain PB[7]. Possibly, strain SR represents the species closely related to the undescribed *Thermodesulfovibrio* strain TGE-P1, because strain SR morphologically (vibrio-shape) and physiologically (growth on hydrogen and sulfate but not on methanol and sulfate) resembles other species of this genus. The physiological role of the species closely related to the *Coprothermobacter proteolyticus* in the culture is not clear. When it also represents a proteolytic species, it might not have any role in methanol oxidation or sulfate reduction in culture MOSC, but just grow on proteins released in the medium due to e.g. cell decay. Possibly, this species represents the contaminant growing on agar constituents that was found in culture MOSC. However, some physiological function, such as providing essential nutrients (e.g. vitamins) for the methanol oxidizer or sulfate reducer, can not be excluded. Thus, the molecular-biological analysis of culture MOSC suggests that it consists of three species, of which two are species closely related to the non-methanol degrading genera *Thermodesulfovibrio* and *Coprothermobacter*, respectively. When the MOSC culture indeed consists of 3 species, this would indicate that the remaining third species, closely related to *Clostridium* strain PB, represents the methanol-degrading bacterium. Interestingly, the thermophilic (optimum 58°C) *Clostridium* strain PB is able to grow homoacetogenically on methanol[4], but it is not known if this species is able to oxidize methanol to H_2 and CO_2. However, this bacterium has been shown to oxidize acetate to H_2 and CO_2 in co-culture with a hydrogenotrophic methanogen. It therefore seems that besides a phylogenetic relationship, there is also some physiological resemblance between the presumable methanol-oxidizing species in culture MOSC and *Clostridium* strain PB. Because we did not obtain the methanol-oxidizing species in culture MOSC in pure culture, it is not possible to make a more detailed comparison with that strain.

Unfortunately, the results obtained so far do not allow for a conclusive identification of the species present in culture MOSC and of their role in the culture. Further research is needed to elucidate these questions.

Acknowledgements

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Optimization of sulfate reduction in a methanol-fed thermophilic bioreactor

5.1 Abstract

Several methods were tested to optimize sulfate reduction and minimize methane formation in thermophilic (65°) Expanded Granular Sludge Bed (EGSB) reactors fed with a medium containing sulfate and methanol. Continuous addition of 2 $g.L^{-1}$ 2-bromoethanesulfonate (BRES) was ineffective as complete inhibition of methanogenesis was obtained for two days only. Lowering the pH from 7.5 to 6.75 resulted in a rapid irreversible decrease of methane formation, and a concomitant increase in sulfate reduction. Inhibition of methanogens by sulfide at pH 7.5 was only effective when the total sulfide concentration was above 1200 $mgS.L^{-1}$. Methanol limiting conditions favoured sulfate reduction over methanogenesis. High methane production at pH 7.5 only occurred when the amount of sulfate was limited or when the sulfate-reducing capacity of the reactor was low. For practical applications, a relatively short exposure to a slightly acidic pH alone, or in combination with operating the reactor at an organic loading rate (OLR) close to the maximum volumetric sulfidogenic production rate, seems to be the most effective strategy for a rapid development of sulfidogenic biomass with nearly complete inhibition of methanogenic activity.

A modified version of this chapter has been submitted to *Wat.Res.*

5.2 Introduction

In Chapter 2 it was shown that methanol can be efficiently used as electron donor and carbon source for thermophilic (65°C) sulfate reduction. However, a strong competition between sulfate-reducing bacteria (SRB) and methanogenic archaea (MA) may occur for a considerable period of time (up to 5 months) when unadapted sludge is used as inoculum for the anaerobic reactor. Formation of methane is undesirable as it adversely affects the process economics due to the inefficient use of methanol.

One method to minimize methanogenesis is the use of agents that selectively inhibit growth of methanogens. A widely used inhibiting agent of methanogens is 2-bromoethanesulfonate (BRES), which functions as a competitive inhibitor of the methyl-CoM reductase complex (CoM), which is a key enzyme of methanogens[5]. Chloroform is another inhibitor of methanogens[2], but this compound is less selective as it may also inhibit SRB[7].

Competition between SRB and MA can also be steered by making use of a differential response of their growth rates to changes in environmental conditions[1]. The tolerance of SRB and MA towards the toxic unionized (hydrogen) sulfide can, for example, be quite different[3]. Free hydrogen sulfide is considered the most toxic form of sulfide[6] presumably because it can easily diffuse through the lipid cell membrane into the bacterial cytoplasm, where it can react with cell components. SRB and MA may also have a different pH-optimum for growth on common substrates. As the speciation of the weak acid hydrogen sulfide into H_2S, HS^-, and S^{2-} is affected by the pH, the effect of a change in pH on the growth of SRB and MA may be the result of a change in the concentration of free hydrogen sulfide. In Chapter 2 it was found that at a temperature of 65°C and pH 7.5, SRB gradually outcompete MA for methanol in continuously operated anaerobic EGSB-reactors in which sulfide was produced up to a concentration of 1100 $mgS.L^{-1}$, corresponding to a free hydrogen sulfide concentration of about 120 $mgS.L^{-1}$. It was however not clarified if inhibition by hydrogen sulfide played a role in the competition between MA and SRB.

The objective of the research described in this chapter is to find the proper conditions to selectively inhibit or minimize methane formation in thermophilic anaerobic reactors fed with methanol and sulfate, so that SRB do not have to compete with MA for methanol, or products from methanol catabolism like H_2 and acetate.

5.3 Materials and methods

Reactors. Two 4-L EGSB-reactors (I and II) were used for continuous experiments. A detailed description of the reactor and mineral medium composition of the influent were presented in Chapter 2. The upward liquid flow was 3 $m.h^{-1}$ in all experiments. The EGSB-reactors were kept at a temperature of 65°C. Automatic pH control was applied, adding 0.1 N NaOH or 0.1 N HCl when necessary. The pH was controlled at a value of 7.5, unless stated otherwise.

A stock solution containing 5 M methanol (Labscan Ltd., Dublin, Ireland) was pumped into the influent with a Gilson Minipuls3 peristaltic pump at a flow rate necessary to obtain the desired influent concentration. Yeast extract (Life Technologies, Paisly, Scotland) was dissolved in the methanol stock solution to give a concentration of 20 $mg.L^{-1}$ in the influent.

In EGSB-I, experiments were carried out to assess the effect of BRES and pH variations on methane formation. EGSB-I was inoculated with 2.5 L elutriated sludge from a pilot plant for biological sulfate reduction of scrubber liquid from a flue-gas scrubber of a coal-fired power plant (Amercentrale, Geertruidenberg, The Netherlands). This sludge is further referred to as Amer sludge. Amer sludge was kindly provided by Paques Bio Systems (Balk, The Netherlands), and was developed at 55°C on a medium containing ethanol (accounting for about 95% of the chemical oxygen demand or COD), methanol (accounting for about 5% of the COD) and sulfate/sulfite. EGSB-I was started at an hydraulic retention time (HRT) of 13 h, an organic loading rate (OLR) of 2.2 $gCOD.L^{-1}.day^{-1}$ and a sulfate loading rate (SLR) of 3.4 $gSO_4^{2-}.L^{-1}.day^{-1}$. BRES (sodium salt of 2-bromoethanesulfonic acid, Acros Organics, New Jersey, USA) was added to the influent from day 9 to 18 at a concentration of 2 $g.L^{-1}$. The pH in the reactor was lowered from 7.5 to 7.15 on day 45, and further reduced to 6.75 on day 55. The pH was reset at 7.5 on day 83.

EGSB-II was used for assessment of the effect of the sulfide concentration and the COD/sulfate ratio on methane and sulfide production. EGSB-II was inoculated with 500 mL Amer sludge and 500 mL Amer sludge that had been pre-adapted to methanol and sulfate for several months at pH 7.5 and a temperature of 65°C. EGSB-II was operated at a HRT of 4 h. During the first week, the sulfate concentration in the influent amounted to 0.50 $g.L^{-1}$, while the methanol concentration in the influent was kept at a value of 1.6 $gCOD.L^{-1}$, resulting in an OLR of 8 $gCOD.L^{-1}.day^{-1}$ and a SLR of 2.6 $gSO_4^{2-}.L^{-1}.day^{-1}$. On day 8, the methanol concentration was increased to 2.8 $gCOD.L^{-1}$ in order to prevent substrate limitation, giving an OLR of 15 $gCOD.L^{-1}.day^{-1}$ from that day onwards. From day 21 to day 54, Na_2S (Merck, Darmstadt, Germany) was added to the influent from a

concentrated stock solution. By adjusting the flow of the sulfide stock solution, the sulfide concentration in the reactor could be controlled. The sodium concentration in the reactor was kept at 125 ± 10 mM during Na_2S-addition by adjusting the NaCl concentration of the influent. At day 62 the SLR was increased from 2.6 to 19.8 $gSO_4^{2-}.L^{-1}.day^{-1}$, while the OLR was kept at 15.4 $gCOD.L^{-1}.day^{-1}$. At day 68 the OLR was decreased to 9.2 $gCOD.L^{-1}.day^{-1}$, and further decreased to 6.6 $gCOD.L^{-1}.day^{-1}$ at day 72. At day 78, the OLR was reset at 15.4 $gCOD.L^{-1}.day^{-1}$.

The inoculation material that was used in batch assays for determination of sulfide toxicity (see below), was obtained from an EGSB-reactor which had been inoculated with 220 mL elutriated Amer sludge and 200 mL Amer sludge that already had been adapted to methanol and sulfate for 3 months in an EGSB-reactor. Operating conditions for this reactor were the same as for EGSB-II except that an OLR of 8.5 $gCOD.L^{-1}.day^{-1}$ and a SLR of 13 $gSO_4^{2-}.L^{-1}.day^{-1}$ were applied. Sludge was taken from this reactor at day 7 and day 31 for assessment of the effect of sulfide on the specific methanogenic and sulfidogenic activity, respectively. The volumetric sulfidogenic and methanogenic COD-conversion rates in the EGSB-on day 7 were 4.3 and 1.2 $gCOD.L^{-1}.day^{-1}$, respectively, while at day 31, these values amounted to about 2.6 and 1.2 $gCOD.L^{-1}.day^{-1}$, respectively.

Activity assay. Batch assays were carried out in 117-mL vials for determination of the specific sulfidogenic and methanogenic activity at various sulfide concentrations. The vials contained 50-mL medium and a gas phase of 67 mL. The mineral medium used for activity assays had a similar composition as the reactor influent and was described in Chapter 2. Na_2SO_4 was omitted from the medium when the specific methanogenic activity was measured or added to a concentration of 3.8 $g.L^{-1}$ in case the specific sulfidogenic activity was determined. Methanol and yeast extract were added from concentrated stock solutions, to give initial concentrations of 1.0 $gCOD.L^{-1}$ and 20 $mg.L^{-1}$ respectively. Prior to inoculation, sodium sulfide was added from a concentrated stock solution that was neutralized with HCl. Total sulfide concentrations ranged from 30 to 2500 $mgS.L^{-1}$. Depending on the applied Na_2S concentration, the NaCl-concentration in the medium was adjusted in order to keep the Na^+ concentration at the same value in all bottles. The pH of the medium was adjusted, if necessary, to a value of 7.4-7.6 by addition of a few drops of HCl or NaOH (0.1 N). Calculation of the specific methanogenic and sulfidogenic activities was done as described in Chapter 2.

Analyses. A detailed description of the analytical procedures for determination of methanol, acetate, sulfide, sulfate, biogas composition and VSS were described in Chapter 2. Bromide was analysed with the same method as sulfate. The H_2S concentration was

calculated from the measured total sulfide concentration and pH using a pK_a-value for H_2S of 6.6 at 65°C[8].

5.4 Results

Effect of BRES addition

One week after start-up of EGSB-I, the methane and sulfide production amounted to 1.7 and 0.4 $gCOD.L^{-1}.day^{-1}$, respectively (Figure 5.1). Methanogenesis ceased completely within two days after starting addition of BRES (2 $g.L^{-1}$) on day 9. Despite the continuous addition of BRES, already at day 11 some methane was formed again, and subsequently the methane production recovered to a maximum value of 0.9 $gCOD.L^{-1}.day^{-1}$ at day 15. Detection of bromide in the effluent from day 9 to 18 indicated breakdown of BRES. The sulfide and acetate production increased strongly by BRES addition to maximum values of 2.6 and 1.3 $gCOD.L^{-1}.day^{-1}$, respectively, on day 13. The organic and sulfate loading rates were temporarily increased to 4.8 $gCOD.L^{-1}.day^{-1}$ (day 12-14) and 6.8 $gSO_4^{2-}.L^{-1}.day^{-1}$ (day 12-18), respectively, on day 12 to prevent methanol and sulfate limitation. The acetate production decreased from day 13 onwards, and on day 18 acetate could no longer be detected in the effluent, while the sulfide production had decreased to 1.4 $gCOD.L^{-1}.day^{-1}$. Termination of BRES addition on day 18 had no immediate effect on methane, acetate and sulfide production, but from day 18 onwards the methane production increased gradually until a relatively constant level of about 1.1 $gCOD.L^{-1}.day^{-1}$ was reached at day 27. In a second experiment with the same seed sludge and using an identical experimental system, a pulse of chloroform was injected in the reactor to give a concentration of 10 μM. Surprisingly, chloroform selectively inhibited sulfate reduction in this experiment and therefore it seemed to be an ineffective agent for inhibition of methanogenesis (data not shown).

Effect of pH

The effect of lowering the pH from 7.5 to 7.15 on methane and sulfide production from methanol was studied in EGSB-I during day 45 to 54. The results in Figure 5.1 reveal that within one day after the pH drop, the methane production decreased by 25% as compared to methane production at pH 7.5 on day 44. Concomitantly, the free H_2S-concentration increased from 24 to 58 $mgS.L^{-1}$. The subsequent adjustment of the pH to 6.75 at day 55 resulted in a further increase of the free H_2S-concentration of 63 to 104 $mgS.L^{-1}$. Within 2

days, an additional 38% decrease of the methane production was observed, and the overall inhibition of the methane production from day 45 to 57 amounted to 76%. In case the inhibition merely would be caused by the increased free H_2S-concentration, a 50% inhibition value of 79 $mgS.L^{-1}$ can be estimated from these data. Following the quick drop in the methane production from day 55 to 57, it further decreased slowly from 0.3 $gCOD.L^{-1}.day^{-1}$ at day 57 to 0.05 $gCOD.L^{-1}.day^{-1}$ at day 82. The methane production did not recover when the pH was reset to 7.5 from day 83 to 87. The sulfide production increased gradually during the period of lowered pH from 0.9 $gCOD.L^{-1}.day^{-1}$ at day 45 to about 2.0 $gCOD.L^{-1}.day^{-1}$ at day 81. By the increase of the pH at day 83 the sulfide production further increased to 2.4 $gCOD.L^{-1}.day^{-1}$ at the end of the experiment on day 87, corresponding to a sulfate elimination efficiency of 90%.

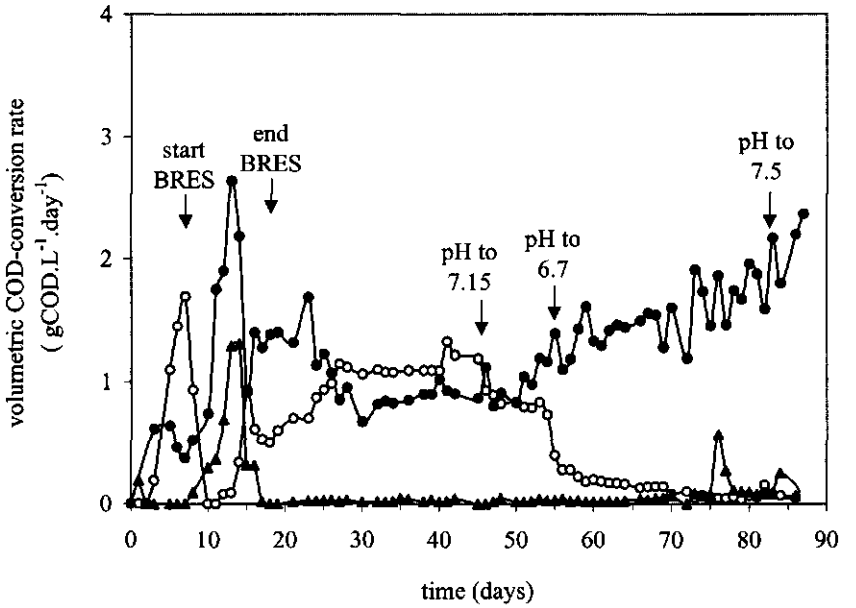

Figure 5.1. Volumetric sulfidogenic (●), methanogenic (○), and acetogenic (▲) COD-conversion rates in EGSB-I. EGSB-I was started at an HRT of 13 h, an OLR of 2.2 $gCOD.L^{-1}.day^{-1}$ and a SLR of 3.4 $gSO_4^{2-}.L^{-1}.day^{-1}$. From day 12 to day 14 the OLR was increased to 4.8 $gCOD.L^{-1}.day^{-1}$ and from day 12 to day 18 the SLR was increased to 6.8 $gSO_4^{2-}.L^{-1}.day^{-1}$. Arrows indicate start BRES 2 $g.L^{-1}$ addition (day 9); termination BRES addition (day 18); pH reduced from 7.5 to 7.15 (day 45); pH reduced from 7.15 to 6.75 (day 55); pH increased from 6.75 to 7.5 (day 83).

Effect of sulfide

Batch experiments were conducted to elucidate the effect of sulfide on the specific methanogenic and sulfidogenic activity of the sludge. The specific methanogenic activity decreased linearly with the total sulfide concentration in the range of 200-1600 $mgS.L^{-1}$ at pH 7.5 (Figure 5.2A). The results show that 50% inhibition occurs at a total sulfide concentration of 980 $mgS.L^{-1}$. On the other hand, sulfide had a positive effect on the specific sulfidogenic activity, because an increase of approximately 40% was found in the sulfide concentration range 50-900 $mgS.L^{-1}$ (Figure 5.2B). The effect of sulfide on the specific sulfidogenic activity in the range 900-2500 $mgS.L^{-1}$ is less clear due to the observed large variation in the specific sulfidogenic activity. However, the results of the assays indicate that the methanogenic activity is selectively at total sulfide concentrations ranging from 200 to at least 900 $mgS.L^{-1}$.

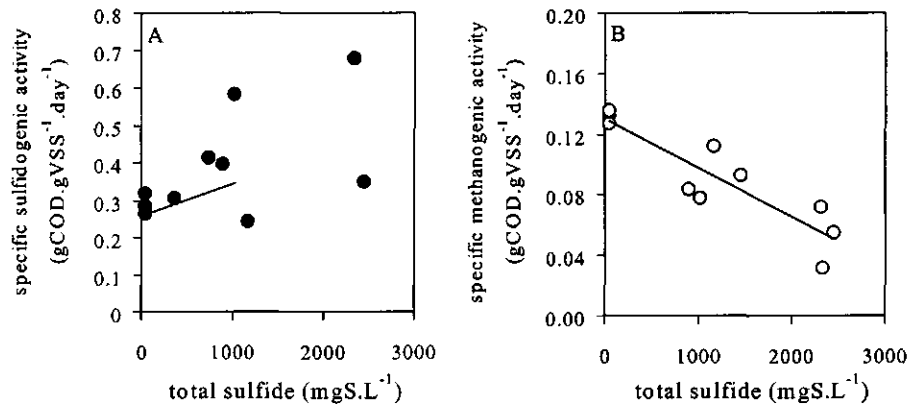

Figure 5.2. Effect of total sulfide concentration on specific sulfidogenic (A) and methanogenic (B) activity of anaerobic sludge with methanol as electron donor, as assessed in batch assays.

Complementary to the batch experiments, the effect of sulfide on the methanogenic activity of the sludge was investigated in EGSB-II, which was fed with an influent containing methanol and sulfate. Sulfate (0.5 $g.L^{-1}$ in the influent) was almost completely reduced in EGSB-II from day 3 onwards, accounting for a consumption of 0.34 $gCOD.L^{-1}$. For the remainder of the methanol, no substrate competition occurred between methanogens and sulfate reducers. This enabled us to assess the effect of external sulfide addition on the methane production independently from substrate competition effects. Furthermore, imposing organic overloading conditions to the system ensured that sulfide inhibition did

not interfere with effects of methanol limitation. Sulfide addition was started at day 21 when the methane production had been relatively stable for a week at 5 $gCOD.L^{-1}.day^{-1}$ (Figure 5.3). Inhibition of the methane production was not observed in the period from day 22 to 25, when sulfide addition led to a total sulfide concentration of 600-700 $mgS.L^{-1}$. On the contrary, even a clear increase in the methane production occurred from 5.2 $gCOD.L^{-1}.day^{-1}$ at day 22 to 6.1 $gCOD.L^{-1}.day^{-1}$ at day 25. A subsequent elevation of the sulfide load, resulting in a total sulfide concentration of about 1250 $mgS.L^{-1}$ between day 27 and 29, led to a steady decrease of the methane production from 5.6 to 4.9 $gCOD.L^{-1}.day^{-1}$. The methane production decreased further at approximately the same rate to a final value of 4.0 $gCOD.L^{-1}.day^{-1}$ upon elevating the total sulfide concentration in the reactor to 1550-1800 $mgS.L^{-1}$ between day 31 and 34. In the subsequent period, the total sulfide concentration was decreased to 1250 $mgS.L^{-1}$ on day 35 and to 900 $mgS.L^{-1}$ at day 36, and from then onwards until day 52 the sulfide concentration was kept between 800 and 1000 $mgS.L^{-1}$. Under these conditions, the methane production initially (day 36 to day 41) remained relatively stable, but from day 41 onwards it steadily recovered until a value of 6.3 $gCOD.L^{-1}.day^{-1}$ was reached at day 52. At day 54 the external addition of sulfide was stopped, leading to a drop of the sulfide concentration to the level resulting from sulfate reduction alone. As a result, from day 54 onwards the methane production increased further until a value of 8.4 $gCOD.L^{-1}.day^{-1}$ was reached at day 61.

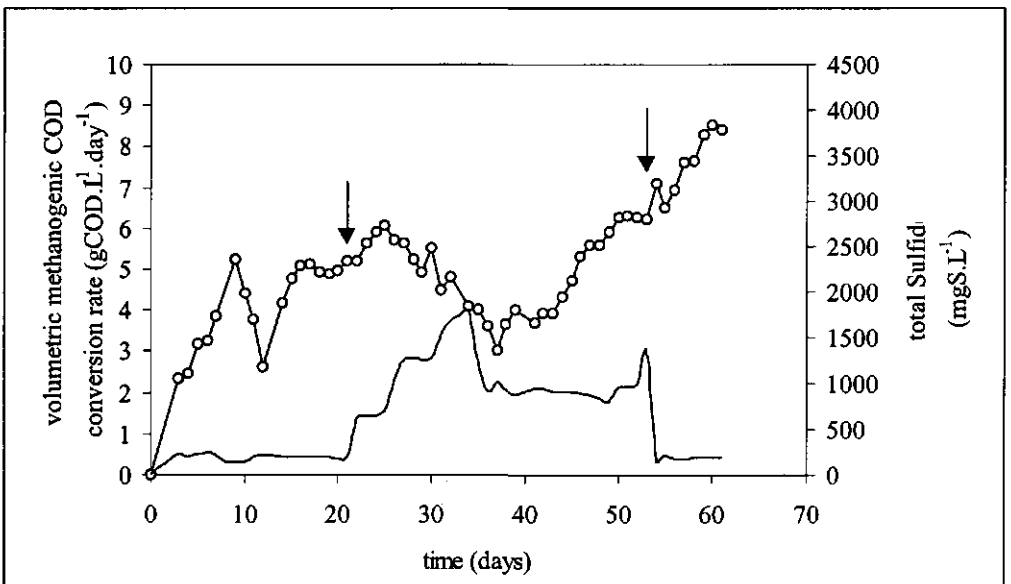

Figure 5.3: Effect of sulfide (—) on volumetric methanogenic COD-conversion rate (o) in EGSBII, operated at an OLR of 15.4 $gCOD.L^{-1}.day^{-1}$ and a SLR of 2.6 $gSO_4^{2-}.L^{-1}.day^{-1}$. Arrows indicate start (day 21) and termination (day 54) of Na_2S-addition.

Effect of COD/sulfate ratio

As mentioned previously, EGSB-II was operated under sulfate-limiting conditions from day 4 to 61 as indicated by the measured low sulfate concentrations of 0.05 $g.L^{-1}$ in the effluent. At day 62 the sulfate concentration in the influent was increased from 0.5 to 3.8 $g.L^{-1}$, and accordingly the COD/sulfate ratio dropped from 5.6 to 0.78 $gCOD/gSO_4^{2-}$. As shown in Table 5.1, a sulfate concentration of 2.2 $g.L^{-1}$ was present in the effluent from day 62 to 67, indicating that sulfate was no longer limiting. The elevated sulfate load had an instantaneous and profound effect on the sulfide and methane production (Figure 5.4). The average sulfide production increased from 2.2 $gCOD.L^{-1}.day^{-1}$ during day 59-61 to 6.4 $gCOD.L^{-1}.day^{-1}$ during day 62-67, whereas the average methane production decreased from 8.4 to 5.4 $gCOD.L^{-1}.day^{-1}$ (Figure 5.4 and Table 5.1).

Competition for reducing equivalents between sulfate-reducing bacteria and methanogens was even more profound by reducing the OLR at day 68 to 9.2 $gCOD.L^{-1}.day^{-1}$, resulting in a COD/sulfate ratio of 0.46. This decreased OLR had little effect on the sulfide pro[illegible]
By contrast, the methane production immediately dropped from 5.4 $gCOD.L^{-1}$
day 62-67 to 3.6 $gCOD.L^{-1}.day^{-1}$ during day 68 to 71. From day 68 onwar
production remained stable at an average value of 3.5 $gCOD.L^{-1}.day^{-1}$ (Figu. . day
72 when the OLR was further reduced to 6.6 $gCOD.L^{-1}.day^{-1}$, resulting in a C[illegible]/sulfate ratio of 0.30. Following the second lowering of the OLR, also the sulfide production became affected; it decreased from 7.3 $gCOD.L^{-1}.day^{-1}$ on day 71 to 5.8 $gCOD.L^{-1}.day^{-1}$ on day 72, while the methane production decreased further from 3.4 $gCOD.L^{-1}.day^{-1}$ on day 71 to 1.6 $gCOD.L^{-1}.day^{-1}$ on day 72. Methanol became limiting in the period from day 72 to day 77, as indicated by the low methanol concentration in the reactor of 0.03 $gCOD.L^{-1}$ in this period (Table 5.1).

The overall decrease of the methane production due to the two imposed drops in the OLR amounted to 70%. In order to assess whether this decrease was reversible, the OLR was increased to 15.4 $gCOD.L^{-1}.day^{-1}$ at day 78, restoring the methanol overloading conditions from this day onwards (Table 5.1). The methane production recovered only to 3.1 $gCOD.L^{-1}.day^{-1}$, which is 40% less than the 5.4 $gCOD.L^{-1}.day^{-1}$ that was achieved between day 62 and day 67, when the same OLR was applied. On the other hand, the sulfide production fully recovered. Thus, by imposing methanol limiting conditions for 10 days to the sludge, a partial irreversible and selective decrease of the methane production was achieved.

The acetate production remained relatively low compared to the sulfide and methane production throughout the operation of EGSB-II, as it amounted to a maximum of only 0.7

gCOD.L^{-1}.day^{-1}, and the acetate production became even less (0.2 gCOD.L^{-1}.day^{-1}) in the period from day 72-77, when methanol-limiting conditions prevailed (Figure 5.4). Surprisingly, acetate production did not recover during day 78 to 88, when the methanol limitation was ended.

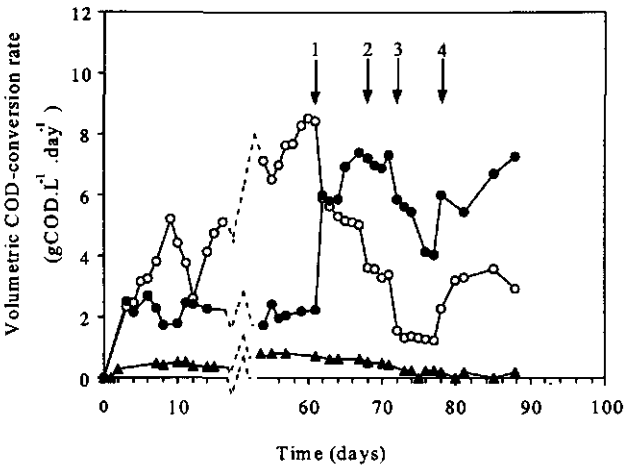

Figure 5.4. Effect of sulfide on volumetric sulfidogenic (●), methanogenic (○), and acetogenic (▲) COD-conversions in EGSB-II. EGSB-II was operated at a HRT of 4 h and was started at an OLR of 15.4 gCOD.L^{-1}.day^{-1} and a SLR of 2.6 gSO_4^{2-}.L^{-1}.day^{-1}. Arrows: (1) SLR increased to 19.8 gSO_4^{2-}.L^{-1}.day^{-1} at day 62; (2) OLR decreased to 9.2 gCOD.L^{-1}.day^{-1} at day 68; (3) OLR decreased to 6.6 gCOD.L^{-1}.day^{-1} at day 72; (4) OLR increased to 15.4 gCOD.L^{-1}.day^{-1} at day 78.

Table 5.1. Performance of EGSB-II under sulfate limiting and methanol limiting conditions. $MeOH_{eff}$, $Sulfate_{eff}$: methanol and sulfate concentration in effluent. VSC, VMC, VAC: volumetric sulfidogenic, methanogenic and acetogenic COD-conversion rates, respectively.

	day				
	59-61	**62-67**	**68-71**	**72-77**	**78-88**
OLR ($gCOD.L^{-1}.day^{-1}$)	15.4±0.1	15.4±0.0	9.2±0.1	6.6±0.0	15.4±0.2
SLR ($gSO_4^{2-}.L^{-1}.day^{-1}$)	2.6±0.0	19.8±0.0	19 .8±0.0	19.6±0.0	16.4±0.6
COD/SO_4^{2-} (g/g)	5.9	0.78	0.46	0.34	0.93
$MeOH_{eff}$ ($gCOD.L^{-1}$)	0.37	0.40±0.01	0.13±0.01	0.03±0.01	0.47±0.16
$SO_4^{2-}{}_{eff}$ ($g.L^{-1}$)	0.05	2.2±0.1	-[a]	-	-
VSC ($gCOD.gVSS^{-1}.day^{-1}$)	2.2±0.0	6.4±0.7	7.1±0.2	5.0±0.9	6.3±0.8
VMC ($gCOD.gVSS^{-1}.day^{-1}$)	8.4±0.1	5.4±0.0	3.5±0.2	1.6±0.8	3.1±0.5
VAC ($gCOD.gVSS^{-1}.day^{-1}$)	0.7	0.6±0.0	0.5±0.0	0.2±0.1	0.1±0.1

a) -: not determined.

5.5 Discussion

The experiment carried out in EGSB-I clearly revealed that BRES is not a suitable compound for a durable inhibition of methanogenesis. Although temporarily methane production ceased completely, methane production already started to recover within 3 days after continuous addition of BRES was started. This possibly may be attributed to degradation of BRES, as indicated by the appearance of bromide in the effluent. The data do not allow an unambiguous explanation for the observed temporary increase in both the sulfide and acetate production after start of BRES addition because interactions between SRB, MA and AB on substrate level were not resolved. Moreover, BRES contains additional COD-value and the sulfonate moiety may have been reduced to sulfide. The results obtained with EGSB-I also revealed that chloroform inhibits SRB instead of MA. Recently, also Scholten[7] found inhibition of SRB in a freshwater sediment by chloroform. Decreasing the pH from 7.5 to 7.15 and subsequently to 6.75 apparently is effective for a rapid and selective inhibition of methanogenesis. As a change in pH around neutral values strongly affects the free H_2S concentration, this inhibition may be caused by the increased free H_2S concentration, viz. from 24 to 104 $mgS.L^{-1}$, rather than from the drop of the pH.

On the other hand, results from EGSB-II showed that methane production remained unaffected by a H_2S concentration ranging from 90 to 110 mgS.L^{-1} at pH 7.5, despite the fact that this reactor had been seeded with a similar inoculum and had been operated under almost identical conditions. Based on this observation, the reduced methane production following the pH drop more likely is the result of inhibition by the low pH rather than of hydrogen sulfide inhibition. The sulfidogenic COD conversion rate doubled in the period of low pH, apparently benefiting from the reducing equivalents that no longer were consumed by MA. Minami et al.[4] found that sulfate reduction becomes stimulated between pH 6.2 and 6.8, while in pH range 7.0 to 7.5 methanogenesis prevailed under thermophilic (53°C) conditions in a packed-bed reactor fed with methanol and sulfate. These contrasting results show that even relatively small changes in the experimental system may have a strong impact on the anaerobic degradation of methanol. Returning the pH to 7.5 did not result in a recovery of methane production within a period of 5 days. Such a period is short compared to the long solid retention times typical for high-rate anaerobic reactors, and it may be speculated that methanogenesis may recover on the long term. On the other hand, SRB almost completely outcompeted MA at pH 7.5 using identical reactors, seed sludge and operating conditions (Chapter 2). Consequently, it can be expected that methane production remains low once sulfate reduction predominates at pH 7.5. The pH drop of 7.5 to 6.75 for accomplishing a 76% inhibition of methane production requires only a low amount of acid. Therefore, temporarily imposing slightly acidic pH-values represents a practical and feasible method for selective and durable suppression of methanogenesis in a full-scale process.

Under sulfate limiting conditions, a 35% decrease of the methane production was observed in EGSB-II over a 10-day period at a total sulfide concentration ranging from 1200 to as high as 1700 mgS.L^{-1} at pH 7.5. Subsequent lowering of the total sulfide concentration to values between 800-1000 mgS.L^{-1} resulted in a gradual and complete recovery of the methane production within 2 weeks. These results show that high (1200 to 1700 mgS.L^{-1}) total sulfide concentrations must be maintained for several weeks in order to achieve a considerable decrease of methanogenic activity. In reactors seeded with sludge exerting a low specific sulfidogenic activity, such high sulfide concentrations can only be achieved by applying very long hydraulic retention times for prolonged periods, which is not very practical. It still remains unclear why methanogenesis increases in a continuous reactor at a total sulfide concentration ranging from 800 to 1000 mgS.L^{-1}, while in batch reactors under similar environmental conditions a 40 to 50% inhibition of the specific methanogenic activity was observed in the same concentration range. It is rather unlikely that these large differences can be attributed exclusively to differences in hydraulic mixing conditions in

the batch and continuous reactor or to small differences in the applied environmental conditions.

Keeping the OLR close to the maximum volumetric sulfidogenic conversion rate in the reactor minimizes methane formation at pH 7.5. Upon the transition from sulfate limiting to non-sulfate limiting conditions and subsequently imposing a lower OLR under non-sulfate limiting conditions, a 90% decrease of methane production was found in EGSB-II. It is relevant to note that the reactor remained overloaded with methanol during the transition from sulfate limiting to non-sulfate limiting conditions, as the methanol concentration in the effluent remained at 0.4 $gCOD.L^{-1}$. Therefore, the decrease of the methane production following the transition cannot be attributed to kinetic limitations of methylotrophic MA. The sulfide production only decreased when the OLR was lower than the volumetric sulfidogenic COD-conversion rate under overloading conditions. This shows that SRB compete much better for available reducing equivalents than MA. Apparently, the major part of the reducing equivalents derived from methanol only becomes available for the MA when utilization of these electron equivalents by SRB is limited by the amount of sulfate or by the absence of a sufficient sulfate-reducing capacity of the reactor.

5.6 References

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Thermophilic sulfate and sulfite reduction with methanol in a high-rate anaerobic reactor

6.1 Abstract

Besides sulfate, sulfite is an important constituent in the influent of anaerobic reactors for biological flue-gas desulfurization. Reduction of sulfite and sulfate with methanol as the sole carbon and energy source for the sulfate-reducing bacteria was studied in thermophilic (65°C) high-rate anaerobic reactors operated at pH 7.5. At a hydraulic retention time (HRT) of 4 hr, sulfite and sulfate elimination rates of up to 18 $gSO_3^{2-}.L^{-1}.day^{-1}$ (100% elimination) and 14 $gSO_4^{2-}.L^{-1}.day^{-1}$ (80% elimination), respectively, were achieved. Sulfite and sulfate reduction accounted for 85-90% of the electrons released during degradation of methanol. In addition, 10-13% and 1-2% of the consumed methanol was converted to acetate and methane, respectively. Acetate was not utilized as electron donor for sulfate reduction. Acetate production seemed to be linearly correlated to the amount of sulfite and sulfate reduced. Sulfite disproportionating activity of the sludge was demonstrated by the simultaneous appearance of sulfide and sulfate in batch tests with sulfite. However, sulfite disproportionation rates were 4 times lower than sulfate reduction rates with methanol. The results clearly demonstrate that methanol can be efficiently used as electron and carbon source to obtain high sulfite and sulfate elimination rates in thermophilic bioreactors.

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6.2 Introduction

In Chapter 2 the use of methanol for thermophilic sulfate reduction was investigated. With this electron donor, a low production of methane and acetate was obtained. Besides sulfate, sulfite is an important constituent in the influent of anaerobic reactors for biological flue gas desulfurization (Bio-FGD). Introduction of sulfite into sulfate-reducing bioreactors may affect its performances in several ways. Firstly, using sulfite instead of sulfate may lead to higher growth yields of the SRB and higher conversion rates in bioreactors, because reduction of sulfite to sulfide with methanol has a higher Gibbs free energy change than sulfate reduction with methanol[6]:

$CH_3OH + HSO_3^- \Rightarrow HCO_3^- + HS^- + H_2O + H^+$ $\Delta G^{\circ\prime}$=-108.8kJ/mol CH_3OH

$4\ CH_3OH + 3\ SO_4^{2-} \Rightarrow 4\ HCO_3^- + 3\ HS^- + 4\ H_2O + H^+$ $\Delta G^{\circ\prime}$=-91.1 kJ/mol CH_3OH

Secondly, sulfite is inhibitory for microorganisms and is for that reason used as bactericidal in for example wines[1]. In pure cultures of SRB, sulfite toxicity at concentrations as low as 0.5 mM (40 $mg.L^{-1}$) has been observed[7].

Besides reduction of sulfite, many SRB are capable of disproportionation of sulfite[4]:

$4\ SO_3^{2-} + H^+ \Rightarrow 3\ SO_4^{2-} + HS^-$ $\Delta G^{\circ\prime} = -58.9$ kJ/mol SO_3^{2-}

In the experiments described in this chapter, the potential of methanol for use as electron donor in thermophilic sulfate and sulfite reduction in anaerobic high-rate reactors was assessed.

6.3 Materials and methods

Reactors. Two 4-L EGSB-reactors (I and II) were used for continuous experiments. A detailed description of the reactor set-up and composition of the influent were presented in Chapter 2. Effluent recycling was applied to increase the liquid upward velocity. The pH in the reactor was normally maintained at 7.5±0.1 by automatic pH control, adding 0.1 N NaOH when necessary. When sulfite was added, pH was controlled with 0.1 N HCl. The reactors were kept at a temperature of 65°C. Sulfate was added as the sodium salt to a concentration of 2.0 or 4.0 $gSO_4^{2-}.L^{-1}$, depending on the desired sulfate loading rate. Stock solutions, containing methanol (5 M) and Na_2SO_3 (1 M) were pumped into the influent with a Gilson Minipuls3 Peristaltic pump at a flow rate that was necessary to obtain the desired

influent concentrations. Yeast extract was dissolved in the methanol stock solution to give a concentration of 20 $mg.L^{-1}$ in the influent. Prior to use, the sulfite stock solution was brought to a pH of about 7 with HCl. The sulfite stock was continuously flushed with nitrogen (nitrogen 3.0, Hoek Loos, The Netherlands) to prevent exposure to oxygen. Chemicals were of analytical grade and supplied by Merck (Darmstadt, Germany) except for yeast extract which was obtained from Life Technologies (Paisly, Scotland) and methanol which was obtained from Labscan Ltd. (Dublin, Ireland). Volumetric methanogenic and acetogenic conversion rates were calculated as described in Chapter 2. The actual use of electron equivalents for sulfite and sulfate reduction (i.e. the sulfidogenic COD-conversion rate) was calculated by subtracting the sulfite-COD entering the reactor from the sulfide-COD leaving the reactor via the effluent and the biogas.

EGSB-I was inoculated with 1 L (approximately 60 g of VSS) of wet anaerobic sludge that originally had been cultivated on an ethanol/methanol (95/5%) mixture and sulfate/sulfite at 55°C in a pilot plant for sulfate reduction of scrubber liquid from a flue-gas scrubber of a coal fired power plant in Geertruidenberg, The Netherlands. This sludge was precultivated on methanol and sulfate for 2 months in an EGSB-reactor prior to inoculation of EGSB-I. EGSB-I was started at an HRT of 11 hr, an organic loading rate (OLR) of 2.3 $gCOD.L^{-1}.day^{-1}$ and a sulfate loading rate of 4.1 $gSO_4^{2-}.L^{-1}.day^{-1}$. At day 4, 350 mL sludge were withdrawn from the reactor. Until day 29 cobalt was omitted as a trace element. At day 21 the HRT was decreased to 4 hr, and the OLR and sulfate loading rate were increased to 7.4 $gCOD.l^{-1}.day^{-1}$ and 11.3 $gSO_4^{2-}.L^{-1}.day^{-1}$ respectively. At day 42 the OLR and sulfate loading rate were about doubled to 15 $gCOD.L^{-1}.day^{-1}$ and 22.6 $gSO_4^{2-}.L^{-1}.day^{-1}$. From day 45 onwards, the lower 10 cm of the sludge bed was intermittently (5 seconds per 50 seconds) stirred at 45 rpm with 3 two bladed turbines (dimension stirrer blades 1.5*2 cm). From day 49 onwards, the sulfate loading rate was decreased to 11.4 $gSO_4^{2-}.L^{-1}.day^{-1}$ and sulfite was introduced into the reactor at a loading rate of 10 $gSO_3^{2-}.L^{-1}.day^{-1}$, thereby keeping the sulfur loading rate at the same level. The operation of EGSB-I was terminated at day 54.

EGSB-II was inoculated with the 350 mL of wet sludge that had been sampled from EGSB-I at day 4, and that had been stored at 4°C for 2 months. EGSB-II was started at a HRT of 4 hr, an OLR of 14 $gCOD.L^{-1}.day^{-1}$ and a sulfate loading rate of 23 $gSO_4^{2-}.L^{-1}.day$. From the start, the lower part of the sludge bed was intermittently stirred as described for EGSB-I.

Activity assays. Batch assays were carried out in 120-mL vials for determination of disproportionating activity and thiosulfate-reducing activity of the sludge. Sludge

concentrations in the assays were 2.5-3.5 gVSS.L^{-1}. Assays were performed as described in Chapter 2.

Analyses. A detailed description of the analytical procedures for determination of methanol, acetate, sulfide, sulfate, biogas composition and VSS was presented in Chapter 2. Sulfite and thiosulfate were analysed with the same method as sulfate. Samples collected from EGSB-II after day 27 were analysed for sulfate and thiosulfate with an HPLC equipped with a VYDAC Ion Chromatography column (cat#302 IC, 250 x 4.6 mm). The temperature of the column and detector (Waters 431 conductivity detector) were 20 and 35°C, respectively. As eluent 0.018 M potassium biphthalate, at a flow rate of 1.2 mL.min^{-1}, was used. Samples were fixed by 2- to 4-fold dilution with a 0.1 M zinc acetate solution, centrifuged (3 min 10000 g) and diluted with demineralized water to a concentration below 500 mg.L^{-1}. The sulfite concentration in the samples collected from EGSB-II after day 27 was determined semi-quantitatively using test strips (Merckoquant cat. nr. 1.100-13).

6.4 Results

Continuous experiments

EGSB-I was started to determine the sulfite-reducing and sulfite-disproportionating potential of anaerobic biomass cultivated on sulfate and methanol. The course of sulfidogenic, methanogenic and acetogenic COD-conversion rates in this reactor is shown in Figure 6.1. Sulfate reduction started immediately, but methane and acetate production generally remained low (< 0.5 gCOD.L^{-1}.day^{-1}) throughout the experiment. Following the decrease of the HRT from 11 to 4 hr on day 21, the sulfide production increased gradually to 7-8 gCOD.L^{-1}.day^{-1} and remained at this level until day 41, which is in the same range as the organic loading rate (OLR) applied in this period. Increasing the OLR and sulfate loading rate (SLR) at day 42 resulted in a further increase of the sulfide production. Removal of 150 mL wet sludge from the reactor on day 44 resulted in an immediate drop in the sulfide production, but it recovered within a few days. Unstable pH-control during day 46-48 also caused a temporary drop in the sulfide production. The sulfate concentration in the influent was reduced to 2 g.L^{-1} on day 49, while sulfite was introduced at a concentration of 1.75 g.L^{-1}. Sulfite was not detected in the effluent during day 49-54, indicating the presence of an active sulfate-reducing population in the sludge. At day 50, sludge was sampled from the reactor for assessment of the sulfite disproportionating activity (see below).

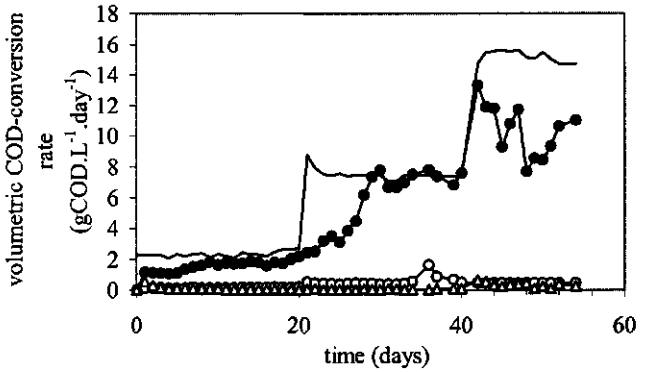

Figure 6.1. Volumetric COD-conversion rates of sulfide (●), methane (○), acetate (Δ) and imposed OLR (—) in EGSB-I.

Investigations were continued in EGSB-II for assessment of the maximum sulfate and sulfate-reducing capacity of the process. During the first 18 days only sulfate was added to the reactor medium (Figure 6.2A). The sulfidogenic COD-conversion rate increased gradually after start-up until it attained a stable level of 8-10 $gCOD.L^{-1}.day^{-1}$ between day 11 and 17 (Figure 6.2B). From day 18 onwards, sulfate in the influent was stepwise replaced with sulfite (on molar basis) until the ratio of the sulfite and sulfate loading rates amounted to about two at day 21. Introduction of sulfite did not strongly affect the sulfidogenic COD-conversion rate (Figure 6.2B). The sulfide concentration in the effluent even increased after sulfite addition (Figure 6.2C), as reduction of sulfite requires less electron donor compared to sulfate reduction. From day 35 onwards, the sulfate and sulfite loading rates were gradually increased (Figure 6.3A), keeping the ratio of sulfite loading rate to sulfate loading rate between 0.9 and 1.2, except during day 40 to 43 when it ranged from 1.4 to 1.8. Sulfite was completely eliminated during the entire experiment, as it was never detected in the effluent. From the data presented in Figure 6.2A it can be calculated that from day 45 to 49, high sulfite and sulfate elimination rates of 18 $gSO_3^{2-}.L^{-1}.day^{-1}$ (100% elimination) and 14 $gSO_4^{2-}.L^{-1}.day^{-1}$ (80% elimination), respectively, were achieved. Effluent sulfide concentrations amounted to values as high as 2000 $mg.L^{-1}$ (Figure 6.2C). Increasing the sulfite and sulfate loading rates to final values of $gSO_3^{2-}.L^{-1}.day^{-1}$ and 26 $gSO_4^{2-}.L^{-1}.day^{-1}$, respectively on day 52 did not further enhance sulfide production. Following a relatively long period of stable reactor operation, heavy sludge wash out occurred after day 59, causing a severe drop in the sulfide production from that day onwards. At the end of the experiment on day 64, the sulfide production only amounted to half of the maximum observed value.

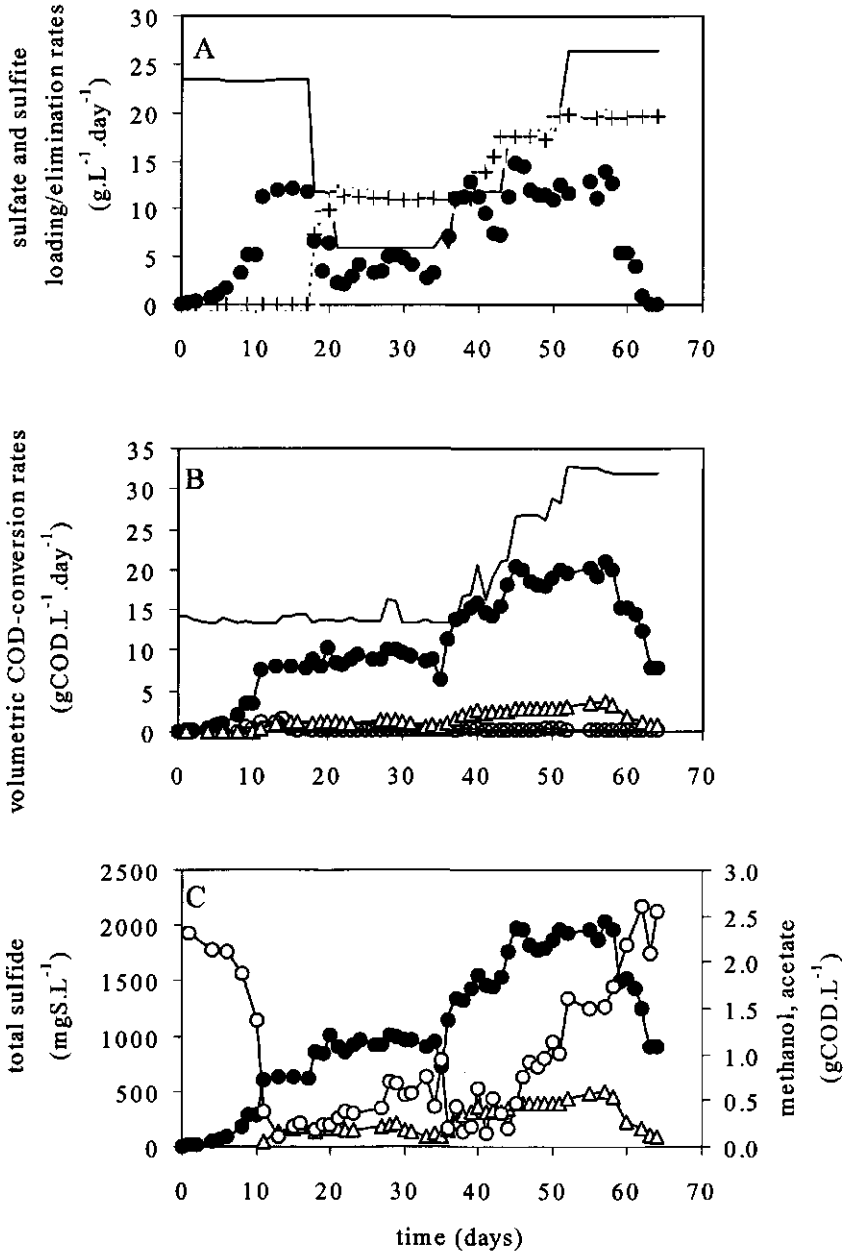

Figure 6.2. Performance EGSB-II.
A. Sulfate loading (——) and elimination (●) rate, sulfite loading (----) and elimination (+) rate. Note that sulfite loading rate equals sulfite elimination rate during entire experiment.
B. sulfidogenic (●), methanogenic (○), acetogenic (Δ) COD-conversion rates and imposed OLR (—).
C. Effluent concentrations: sulfide (●), methanol (○), and acetate (Δ).

Disproportionation

To investigate if sulfite disproportionation accounts for part of sulfite removal, batch tests were performed with fresh sludge taken from EGSB-I at day 50. Results are shown in Table 6.1.

Table 6.1. Disproportionation of sulfite by fresh sludge sampled from EGSB-I on day 10. SSA: specific sulfidogenic activity.
Assay A: disproportionation of sulfite in the presence of sludge;
Assay B: chemical formation of thiosulfate from sulfide and sulfite without sludge;
Assay C: control assay for determining sulfidogenic activity of sludge on acetate and sulfate;
Assay D: sulfidogenic activity of sludge on methanol and sulfate.
Changes in the concentration of SO_3^{2-}, TS, SO_4^{2-} and $S_2O_3^{2-}$ that lie within the range of the standard deviation of the measurement (0.3 mM) are considered 0. Duplicate assays gave a similar result.

	Initial concentrations (mM)					Formation after 7 hr (mM)			
Assay	SO_3^{2-}	TS[a]	SO_4^{2-}	MeOH[b]	Ac[c]	SO_3^{2-}	TS	SO_4^{2-}	$S_2O_3^{2-}$
A	3.5	0.9	1.6	0	2	-3.4	2.2	2.4	0
B	3.9	2.0	1.0	0	2	-3.1	-1.0	0	2.6
C	0	1.8	20	0	4	0	0.8	0	0
D	0	1	20	20	0	0	7.7	-6.9	0

a) TS: total sulfide; b) MeOH: methanol; c) Ac: acetate.

It was confirmed that acetate did not serve as electron donor for sulfate reduction as acetate was not degraded in the presence of sludge and sulfate (data not shown). The simultaneous disappearance of sulfite and appearance of both sulfate and sulfide in assay A indicates the occurrence of sulfite disproportionation. The ratio of ΔSO_3^{2-}:ΔTS:ΔSO_4^{2-} was around 4:2.6:2.8. Theoretically, 1 mol of sulfide and 3 mol of sulfate will be formed by disproportionation of 4 mol sulfite. The observed ratio ΔSO_3^{2-} to ΔSO_4^{2-} is rather close to the theoretical value, whereas the ΔSO_3^{2-} to ΔTS^- ratio is significantly lower than expected. This can be explained by additional sulfide formation from sulfur sources in the sludge (e.g. elemental sulfur), or by sulfate/sulfite reduction with electron donors originating from the sludge. Without sludge (assay B), the measured ratio ΔSO_3^{2-}:ΔTS:$\Delta S_2O_3^{2-}$ of 4:1.3:3.3 seems to indicate the following chemical reaction:

$$4\ HSO_3^- + 2\ HS^- \Rightarrow 3\ S_2O_3^{2-} + 3\ H_2O$$

This chemical reaction was described by Krämer and Cypionka[4]. Thus, it is possible that in the presence of sludge (assay A), 4 mol sulfite react with 2 mol sulfide to give 3 mol thiosulfate, and that subsequent biological disproportionation of 3 mol thiosulfate yields 3

mol sulfide and 3 mol sulfate. The overall stoichiometry of these reactions is the same as the stoichiometry of sulfite disproportionation. In any case, the direct or indirect (via thiosulfate) disproportionation of sulfite resulted in a specific sulfidogenic activity of the sludge of 0.13 $gCOD.gVSS^{-1}.day^{-1}$, which is considerably lower than the measured specific sulfidogenic activity of 0.68 $gCOD.gVSS^{-1}.day^{-1}$ on methanol and sulfate (assay D).

Acetate formation

Throughout the experiment in EGSB-II, acetate could be detected in the effluent at a concentration of up to 0.65 $gCOD.L^{-1}$ (Figure 6.2C). The acetogenic COD-conversion seemed to be correlated to the sulfidogenic COD-conversion (Figure 6.3). Acetate was not consumed in activity assays with sulfate and fresh sludge taken from EGSB-II at day 59. The coupling of acetate and sulfide production could not be studied on smaller scale in serum bottles, as the highest dilutions of a sludge dilution series showing sulfate reduction with methanol did not produce acetate, but even consumed acetate (results not shown).

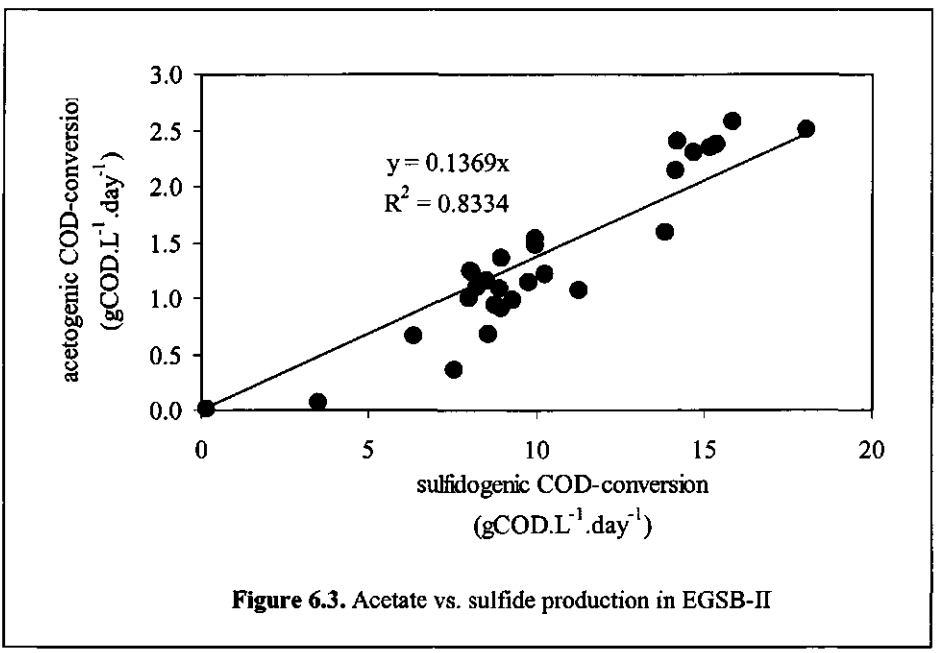

Figure 6.3. Acetate vs. sulfide production in EGSB-II

Thiosulfate reduction

Thiosulfate was sometimes detected in the effluent of EGSB-II, up to a concentration of 7 mmolS.L^{-1}. Most likely, thiosulfate was formed from the chemical reaction of sulfite with sulfide (see above). In order to assess the thiosulfate-reducing activity, sludge was taken from EGSB-II at day 10 and incubated with thiosulfate (15 mM) and methanol (25 mM). Thiosulfate was indeed reduced to sulfide, with a specific sulfidogenic activity of 0.47 gCOD.$gVSS^{-1}$.L^{-1}, while the sulfidogenic activity on methanol and sulfate amounted to 0.26 gCOD.$gVSS^{-1}$.L^{-1}.

6.5 Discussion

Thermophilic sulfate and sulfite reduction with methanol offers good potential for biotechnological flue-gas desulfurization. High sulfite and sulfate elimination rates of 18 gSO_3^{2-}.L^{-1}.day^{-1} (100% elimination) and 14 gSO_4^{2-}.L^{-1}.day^{-1} (80% elimination), respectively, were achieved. These elimination rates are twice as high as the elimination rates obtained in thermophilic gas-lift reactors with hydrogen as electron donor[3]. Moreover, a high efficiency of methanol utilization for sulfite and sulfate reduction was observed in the reactor as only 10-13% and 1-2% of the consumed methanol is converted to acetate and methane, respectively, while the remainder is used for sulfate reduction. The acetogenic COD-conversion seemed to be correlated to the sulfidogenic COD-conversion. It is known that some sulfate reducers produce acetate[5]. Another possible explanation for the observed coupling of acetate and sulfide formation is that methanol is mainly oxidized to H_2/CO_2 by homoacetogens while a minor fraction is converted to acetate, coupled to sulfate/sulfite reduction with hydrogen[2].

The results furthermore show that disproportionation is of minor importance for conversion of sulfite. Most, if not all, of the sulfite is reduced with methanol as electron donor.

6.6 References

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Performance of a thermophilic sulfate and sulfite reducing high-rate anaerobic reactor fed with methanol

7.1 Abstract

Thermophilic sulfate and sulfite reduction was studied in lab-scale Expanded Granular Sludge Bed (EGSB) reactors operated at 65°C and pH 7.5 with methanol as the sole carbon and energy source for the sulfate and sulfitereducing bacteria. At a hydraulic retention time (HRT) of 10 h, maximum sulfite and sulfate elimination rates of 5.5 $gSO_3^{2-}.L^{-1}.day^{-1}$ (100% elimination) and 5.7 $gSO_4^{2-}.L^{-1}.day^{-1}$ (55% elimination) were achieved, resulting in an effluent sulfide concentration of approximately 1800 $mgS.L^{-1}$. Sulfate elimination was limited by the sulfide concentration, as stripping of H_2S from the reactor with nitrogen gas was found to increase the sulfate elimination rate to 9.9 $gSO_4^{2-}.L^{-1}.day^{-1}$ (100% elimination). At a HRT of 3 h, maximum achievable sulfite and sulfate elimination rates were even 18 $gSO_3^{2-}.L^{-1}.day^{-1}$ (100% elimination) and 11 $gSO_4^{2-}.L^{-1}.day^{-1}$ (50% elimination). At a HRT of 3 h, the elimination rate was limited by the biomass retention of the system. 5.5±1.8% of the consumed methanol was converted to acetate, which was not further degraded by sulfate-reducing bacteria present in the sludge. The acetotrophic activity of the sludge could not be stimulated by cultivating the sludge for 30 days under methanol-limiting conditions. Omitting cobalt as trace element from the influent resulted in a lower acetate production rate, but it also led to a lower sulfate reduction rate. Sulfate degradation in the reactor could be described by zeroth order kinetics down to a threshold concentration of 0.05 $g.L^{-1}$, while methanol degradation followed Michaelis-Menten kinetics with a K_m of 0.037 $gCOD.L^{-1}$.

A modified version of this Chapter has been submitted to *Biodegradation.*

7.2 Introduction

In Chapter 6 high sulfate and sulfite elimination rates of 14 $gSO_4^{2-}.L^{-1}.day^{-1}$ and 18 $gSO_3^{2-}.L^{-1}.day^{-1}$ with methanol as electron donor were found in thermophilic anaerobic high-rate reactors at a hydraulic retention time (HRT) of 4 h. Methane production was low (<2% of methanol consumed), while 10-13% of the consumed methanol was converted to acetate. The formation of acetate is highly undesirable as it negatively affects the efficiency of methanol utilization for sulfate and sulfite reduction. A more serious problem is that the presence of acetate in the effluent of the anaerobic reactor of the biological desulfurization process will deteriorate the performance of the sulfide oxidizing reactor, due to growth of heterotrophic sulfur and sulfate-reducing bacteria[5]. In this chapter, investigations are aimed at assessing the maximum attainable sulfate and sulfite elimination rate at HRTs of 10 and 3-4 h and with the minimization of acetate formation as well.

7.3 Materials and Methods

Reactors. Three EGSB-reactors (IA, IB, and II) were used for continuous experiments. A detailed description of the experimental set-up including the reactor and medium composition, was presented in Chapter 2. The reactor was equipped with a screen (circle openings 1 mm) placed below the gas-solids separator device. In all EGSB-reactors, effluent recycling was applied to increase the liquid upflow velocity to 3 $m.h^{-1}$. The pH in the reactor was maintained at 7.5±0.1 by automatic pH control, adding 0.1 N NaOH when necessary. The temperature in the reactors was controlled at 65°C.

The basal medium composition was described in Chapter 2. Cobalt was omitted from the medium of EGSB-IA during day 0 to 12. Stock solutions containing methanol (5 M) and Na_2SO_3 (1 M) were pumped into the influent using a Gilson Minipuls3 Peristaltic Pump (Gilson, Villiers-le-Bel, France) at a flow rate needed to obtain the desired influent concentration. Yeast extract was dissolved in the methanol stock solution to give a concentration of 20 $mg.L^{-1}$ in the influent. All chemicals were analytical grade from Merck (Darmstadt, Germany) except for yeast extract that was obtained from Life Technologies (Paisly, UK), and methanol that was obtained from Labscan Ltd. (Dublin, Ireland).

The reactors EGSB-IA, IB and II were inoculated with 300, 300 and 430 mL, respectively, of a flocculent sludge that was precultivated for 3 months on a methanol/sulfate mixture at 65°C. The operational parameters (sulfate, sulfite and organic loading rates and the HRT)

were varied over the course of the experiment. These parameters were kept the same in EGSB-IA and IB during the first 12 days of operation of the reactors. From day 30 to 34, 15-18 $mL.min^{-1}$ nitrogen gas (nitrogen 3.0, Hoek Loos, The Netherlands) was bubbled into EGSB-IA, using a gas inlet positioned at 1-2 cm above the dynamic sludge bed. From day 70 onwards, the lower 10 cm of the sludge bed of EGSB-IA was intermittently (5 seconds per 50 seconds) stirred at 45 rpm with 3 two bladed turbines (dimension stirrer blade 1.5*2 cm) to prevent coagulation of the sludge bed that manifested around day 70. The operational conditions for reactor EGSB-II were: HRT: 12 h; sulfate loading rate: 7.6 $gSO_4^{2-}.L^{-1}.day^{-1}$; OLR: 5.2 $gCOD.L^{-1}.day^{-1}$. On day 13, the recycling flow was terminated for 1.5 h and a medium containing 2.4 $gCOD.L^{-1}$ methanol and 1.92 $gSO_4^{2-}.L^{-1}$ (ratio COD:sulfate = 1.25) was pumped into the reactor at an HRT of 10 min. During the following 12 h, operation of EGSB-II was continued in batch mode. At different time intervals, samples were taken for the determination of the total sulfide, methanol, acetate and sulfate concentration. The decrease in medium volume in the reactor due to sampling was less than 1% during batch operation. Subsequently, continuous operation was resumed, applying the same organic and sulfate loading rate as before the batch operation. At day 14 once again a batch experiment was performed in EGSB-II, but now at initial methanol and sulfate concentrations of 1.2 $gCOD.L^{-1}$ and 3.84 $gSO_4^{2-}.L^{-1}$, respectively (ratio COD:sulfate = 0.30).

Volumetric methanogenic and acetogenic conversion rates in the reactors were calculated as described in Chapter 6. The sulfidogenic COD-conversion rate was defined as the actual use of reducing equivalents for sulfite and sulfate reduction per unit of time per volume of reactor. Thus, the sulfidogenic COD-conversion rate was calculated by subtracting the amount of sulfite-COD converted in the reactor per unit of time per volume of reactor from the sulfide-COD leaving the reactor via the effluent and the biogas per unit of time per volume of reactor.

Activity assays. The specific sulfidogenic and methanogenic activity of sludge were assayed in 120-mL vials as described in Chapter 2.

Analyses. A detailed description of the analytical procedures for determination of methanol, acetate, sulfide, biogas composition, VSS and TSS have been described in Chapter 2. Sulfate and thiosulfate were analysed using a HPLC (Spectra Physics) equipped with a VYDAC Ion Chromatography column (nr. 302 IC, 250*4.6 mm). The temperature of the column and detector (Waters 431 conductivity detector) were 20°C and 35°C, respectively. As eluent 18 mM potassium biphthalate, at a flow rate of 1.2 $mL.min^{-1}$, was used. Samples were fixed by 2- to 4-fold dilution with a 0.1 M zinc acetate solution,

centrifuged (3 min, 10000 g) and diluted with demineralized water to a concentration below 500 mg.L^{-1}. Sulfite was determined semi-quantitatively using test strips (Merckoquant cat. nr. 1.100-13).

The sludge volume index (SVI) of sludge was determined according to Standard Methods[1]. The polysaccharide content of sludge was determined with the phenol-sulfuric acid method. 2 mL of sludge was vigorously mixed with 2 mL phenol solution (5 vol% in demineralized water), followed by addition of 10 mL of 18 M sulfuric acid. Then, the mixture was heated to 105°C for 10 min. After cooling, the extinction of the sample was measured at 480 nm. Calibration curves were made with glucose.

Methanol degradation kinetics. Methanol depletion data from the batch experiment in EGSB-II were fitted to an integrated solution of the Michaelis-Menten equation $V_{max}*t=S_0-S+K_m*\ln(S_0/S)$ using nonlinear regression analysis[11], where V_{max}: maximum consumption rate; t: time; S_0: initial substrate concentration; S:substrate concentration; K_m: half-saturation constant.

7.4 Results

Maximum sulfate and sulfite elimination rate at HRTs of 10 and 3-4 h

Reactor EGSB-IA was started to determine the maximum attainable sulfate and sulfite elimination rates at HRTs of 10 and 3-4 h. Results are presented in Figure 7.1A-D. In the start-up period, from day 0 to day 12, the organic, sulfate and sulfite loading rates were stepwise increased to 14 gCOD.L^{-1}.day^{-1}, 10 gSO_4^{2-}.L^{-1}.day^{-1} and 5.5 gSO_3^{2-}.L^{-1}.day^{-1}, respectively (Figure 7.1A and 7.1B). Methane production increased rapidly from the start, but following day 2 it decreased until, from day 15 onwards, only 0-3% of the consumed methanol was used for methanogenesis (Figure 7.1B). Acetate formation accounted for 5.5 ± 1.8% of methanol conversion during the entire experiment. Activity assays conducted at day 75 revealed that the sludge cultivated in EGSB-IA did not develop any acetotrophic sulfidogenic activity with sulfate (data not shown). Throughout the entire experiment, sulfide was the main product in the reactor, accounting for about 90% of the degraded methanol (Figure 7.1B).

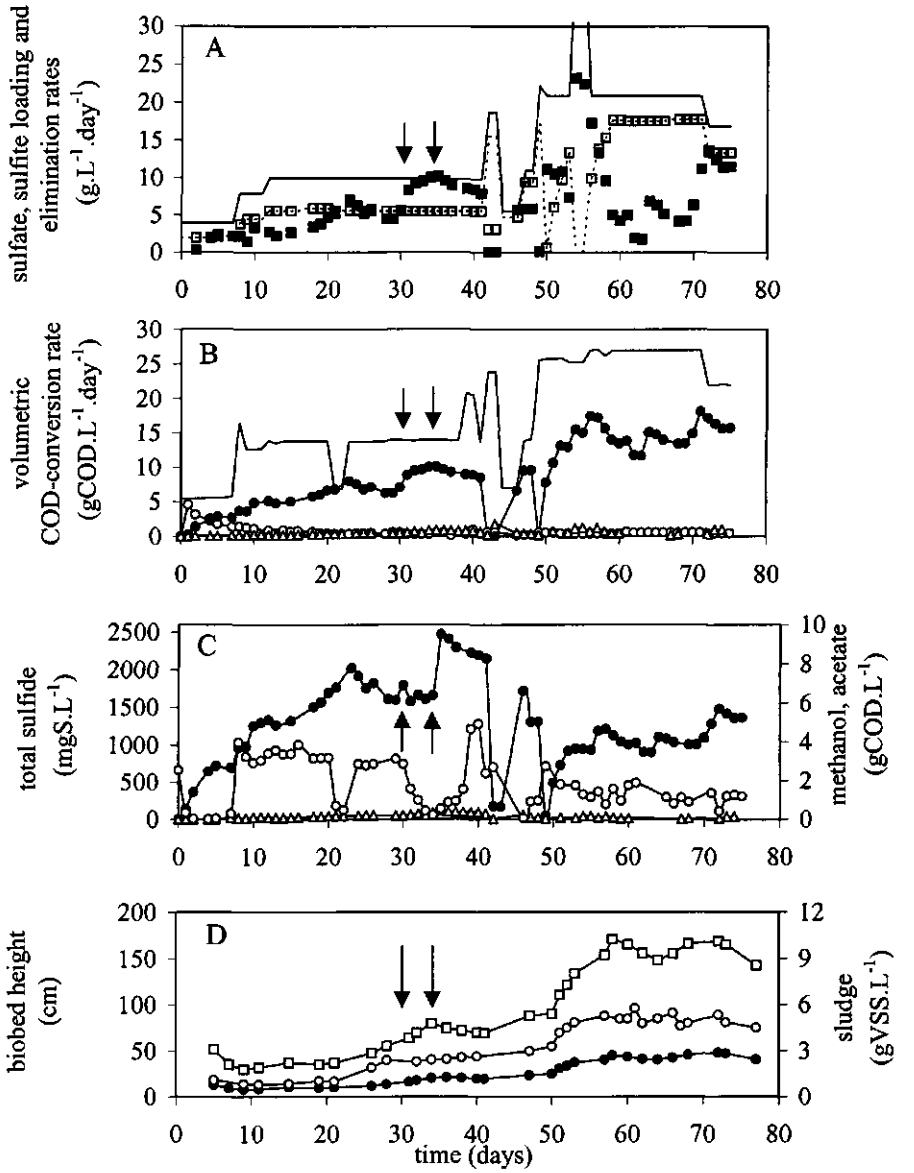

Figure 7.1. Performance EGSB-IA. Arrows indicate start (day 30) and termination (day 34) of H_2S-stripping with N_2.
A. Sulfate (—) and sulfite (---) loading rate, sulfate (●) and sulfite (+) elimination rate. On day 54 and 55, the sulfate loading rate was increased to 42 $gSO_4^{2-}.L^{-1}.day^{-1}$ (not shown).
B. Organic Loading Rate (—), Volumetric sulfidogenic (●), methanogenic (○) and acetogenic (Δ) COD-conversion rate
C. Sulfide (●), methanol (○) and acetate (Δ) effluent concentration.
D. Sludge concentration (□), static (○) and dynamic (○) biobed height

During the period from day 20 to day 30, the sulfidogenic COD-conversion rate remained stable at 7.0 ± 0.6 $gCOD.L^{-1}.day^{-1}$ (Figure 7.1B), at a total sulfide concentration of 1770 ± 140 $mgS.L^{-1}$ (Figure 71C). The imposed temporary decrease of the OLR to 6.9 $gCOD.L^{-1}.day^{-1}$ during day 21 and 22 resulted in a drop in the methanol concentration in the reactor from 3.2 to 0.67 $gCOD.L^{-1}$ (Figure 7.1C), but this did not strongly affect the calculated methanogenic, sulfidogenic and acetogenic COD-conversion rates. From day 20 to day 30, the calculated sulfite degradation rate amounted to 5.5-5.9 $gSO_3^{2-}.L^{-1}.day^{-1}$ (Figure 7.1A) (100% elimination), whereas sulfate was reduced at a rate of 4.4-7.0 $gSO_4^{2-}.L^{-1}.day^{-1}$ (45-70% elimination). H_2S was stripped from the bioreactor with nitrogen gas during day 30 to day 34. H_2S-stripping was effective, as the sulfide production steadily increased from 7.1 $gCOD.L^{-1}.day^{-1}$ on day 30 to 10 $gCOD.L^{-1}.day^{-1}$ on day 34 (Figure 7.1B), corresponding to 100% elimination of both sulfate and sulfite (Figure 7.1A). Termination of H_2S-stripping on day 34 did not immediately affect the sulfidogenic COD-conversion rate, which led to an increase of the sulfide concentration in the reactor from 1660 $mgS.L^{-1}$ on day 34 to 2470 $mgS.L^{-1}$ on day 35. However, the sulfide concentration gradually declined to 2150 $mgS.L^{-1}$ on day 41 and, accordingly, the sulfidogenic COD-conversion rate gradually decreased to 8.5 $gCOD.L^{-1}.day^{-1}$. In order to assess methanol toxicity, the OLR was temporarily increased to 21 $gCOD.L^{-1}.day^{-1}$ during the period day 38 to 40, resulting in elevated methanol levels of 4.8 $gCOD.L^{-1}$ (100 mM) in the reactor. This clearly did not affect the sulfidogenic COD-conversion rate (Figure 7.1B).

The HRT was decreased to 3 h on day 42, corresponding to an increase of the OLR to 24 $gCOD.L^{-1}.day^{-1}$ and of the sulfate and sulfite loading rates to 18.7 $gSO_4^{2-}.L^{-1}.day^{-1}$ and 15.5 $gSO_3^{2-}.L^{-1}.day^{-1}$. From the results shown in Figure 7.1B it appears that, within a few hours, the sulfidogenic activity of the biomass became severely inhibited, presumably due to toxicity of sulfite, which was detected in the effluent at a concentration above 400 $mg.L^{-1}$. As the sulfidogenic activity did not recover within two days, we decided to increase the HRT to 11 h, corresponding to a drop in the organic, sulfate and sulfite loading rates to 7 $gCOD.L^{-1}.day^{-1}$, 5.5 $gSO_4^{2-}.L^{-1}.day^{-1}$ and 4.7 $gSO_3^{2-}.L^{-1}.day^{-1}$ at day 44. The sulfide production recovered within 2 days, but subsequent stepwise decreases of the HRT to 3 h with concomitant increases of the loading rates to final values of 26 $gCOD.L^{-1}.day^{-1}$, 22 $gSO_4^{2-}.L^{-1}.day^{-1}$ and 17 $gSO_3^{2-}.L^{-1}.day^{-1}$ on day 49 once again resulted in a nearly complete inhibition of the sulfide production. However, after switching to an influent containing merely methanol and sulfate on day 50, the sulfide production recovered completely within one day, clearly showing that the inhibition was due to the presence of sulfite. During day 51-53, the sulfite loading rate was increased in small steps from 0 to 13.4 $gSO_3^{2-}.L^{-1}.day^{-1}$ without occurrence of inhibition. However, within hours following a moderate increase in

the sulfite loading rate at day 54, the sulfite concentration in the reactor increased to 40-80 $mg.L^{-1}$, and concomitantly, the sulfidogenic COD-conversion rate dropped dramatically (not shown). After ceasing the sulfite addition, the activity recovered almost immediately. The sulfidogenic COD-conversion rate with sulfate alone, as determined on day 54 and 55 at a sulfate loading rate of 42 $gSO_4^{2-}.L^{-1}.day^{-1}$, amounted to 15 $gCOD.L^{-1}.day^{-1}$. Based on this value, a maximum sulfite elimination rate of 25 $gSO_3^{2-}.L^{-1}.day^{-1}$ should be possible to achieve theoretically. In order to establish the sulfate-reducing potential of the system, the sulfate loading rate was decreased to 21 $gSO_4^{2-}.L^{-1}.day^{-1}$ on day 56, while the sulfite loading rate was stepwise increased from 0 to 18 $gSO_3^{2-}.L^{-1}.day^{-1}$ during days 56-59, well below the calculated maximum sulfite elimination rate. By following this procedure, sulfite inhibition could be prevented, viz. the sulfite was completely eliminated from day 56 onwards. Moreover, the results in Figure 7.1A reveal that the increase in the sulfite elimination rate during days 56-59 completely balanced the drop in the sulfate elimination rate (Figure 7.1A), indicating that sulfite is the preferred electron acceptor for the SRB. The sulfidogenic COD-conversion rate from day 59 to 71 remained relatively constant at a value of 17.5 ± 1.7 $gCOD.L^{-1}.day^{-1}$. Maximum sulfite and sulfate elimination rates of 18 $gSO_3^{2-}.L^{-1}.day^{-1}$ (100% elimination) and 11 $gSO_4^{2-}.L^{-1}.day^{-1}$ (50% elimination) were attained on day 71. The increase of the HRT to 4 h on day 72, corresponding to a lower organic, sulfate and sulfite loading rates of 22 $gCOD.L^{-1}.day^{-1}$, 17 $gSO_4^{2-}.L^{-1}.day^{-1}$ and 13 $gSO_3^{2-}.L^{-1}.day^{-1}$, improved the sulfate elimination efficiency from 50 to 70-80% from day 72 to 75, while sulfite elimination remained at 100%.

The VSS concentration increased from 4 $gVSS.L^{-1}$ on day 41 to about 10 $gVSS.L^{-1}$ on day 61 (Figure 7.1D). In order to prevent expansion of the sludge bed into the gas-solid separator, excess sludge was frequently removed from the reactor during the period from day 61 to day 75. As a result, the VSS concentration in the reactor remained in the range 9 to 10 $gVSS.L^{-1}$ from day 61 onwards. The calculated (from the data presented in Figure 7.1B and 7.1D) specific sulfidogenic activity of the sludge during this period amounted to 1.52 ± 0.19 $gCOD.gVSS^{-1}.day^{-1}$. The maximum specific sulfidogenic activity of the sludge, as determined in activity assays on day 75, was very similar, with a value of 1.47 ± 0.15 $gCOD.gVSS^{-1}.day^{-1}$.

Attempts to minimize acetate formation

As the formation of acetate is highly unwanted, attempts were made to minimize this. For this purpose we studied the effect of cobalt on the anaerobic conversion of methanol in EGSB-IA, by omitting the supply of cobalt in the feed of this reactor, while the feed of

reactor EGSB-IB contained 0.07 mg.L^{-1} cobalt. Cobalt may stimulate unwanted methane and acetate formation from methanol, analogous to the effect of cobalt on methanol degradation under mesophilic conditions[3]. A high methane production was found in EGSB-IA and IB merely during the first days (Figure 7.2A), but in both reactors these rapidly declined to a value of less than 1 gCOD.L^{-1}.day^{-1} on day 12. Apparently, cobalt does not strongly affect methane production. However, in contrast, cobalt strongly affects the formation of sulfide and acetate because omission of the cobalt supply led to a substantial lower production of these compounds after day 8 (Figure 7.2B and 7.2C). From day 12 onwards, cobalt was also added to the feed of EGSB-IA, and as can be seen from the results shown in Figure 7.1B, the sulfidogenic conversion rate in EGSB-IA indeed increased within the next 11 days to about the level found in EGSB-IB on day 12.

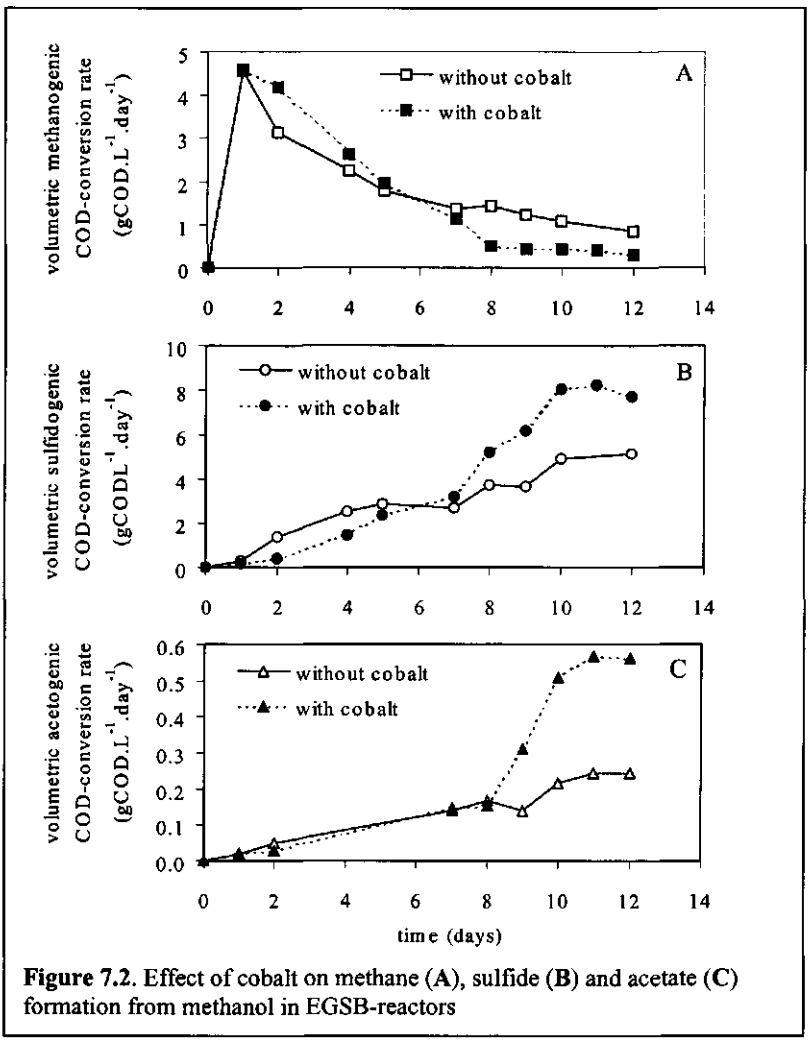

Figure 7.2. Effect of cobalt on methane (**A**), sulfide (**B**) and acetate (**C**) formation from methanol in EGSB-reactors

It was also attempted to stimulate acetotrophic sulfidogenic activity of the sludge by imposing methanol-limiting conditions to the sludge, keeping sulfate in excess. For this purpose, reactor EGSB-II was operated for 35 days under methanol-limiting conditions. The methanol concentration in the effluent of EGSB-II did not exceed 0.1 $gCOD.L^{-1}$ (Figure 7.3), whereas the sulfate concentration was always > 0.5 $g.L^{-1}$. The relative acetate production (the percentage acetogenic methanol conversion of total methanol conversion) increased from 5% on day 3 to 8.5% on day 17, but from then onwards until day 35 it remained in the range of 8 to 9% (Figure 7.3). The sludge did not develop any acetotrophic activity under the methanol limiting conditions applied in EGSB-II, as assessed in batch assays (data not shown).

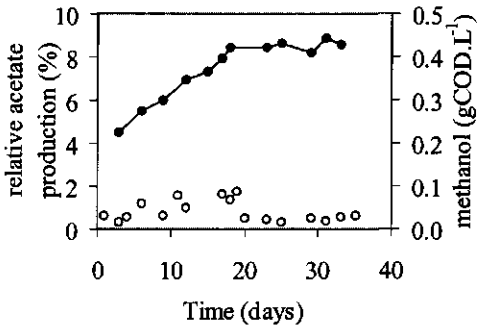

Figure 7.3. Acetate formation under methanol limiting conditions in EGSB-II. Relative acetate production (●) and methanol concentration effluent (○)

Methanol and sulfate depletion kinetics

On day 13, methanol, sulfate, sulfide and acetate concentrations of 2.4 $gCOD.L^{-1}$, 1.92 $g.L^{-1}$, 10 $mgS.L^{-1}$ and <0.02 $gCOD.L^{-1}$, respectively, were measured in EGSB-II. The course of the sulfate, methanol and acetate concentration during the subsequent 13 h batch-mode operation of EGSB-II are shown in Figure 7.4. During the period of sulfate reduction, about 14% of the consumed methanol was used for acetate formation (Figure 7.4A). However, once the residual sulfate concentration had dropped to 0.05 $g.L^{-1}$, the methanol degradation rate decreased by a factor 4, while the acetate formation remained almost the same. The sulfate depletion corresponds to zero-order kinetics down to a concentration of at least 0.1 $g.L^{-1}$ (Figure 7.4B). Sulfate depletion was not found at concentration < 0.05 $g.L^{-1}$. In the batch experiment carried out in EGSB-II at day 14, the initial methanol, sulfate, sulfide and acetate concentrations were 1.2 $gCOD.L^{-1}$, 3.84 $g.L^{-1}$, 30 $mgS.L^{-1}$ and <0.02 $gCOD.L^{-1}$, respectively. The results are depicted in Fig 7.5. From the data in Figure 7.5B an apparent K_m of 0.037 ± 0.012 $gCOD.L^{-1}$ (0.78 ± 0.25 $mmol.L^{-1}$) for methanol degradation was calculated. Here, about 12% of the consumed methanol was used for acetate formation.

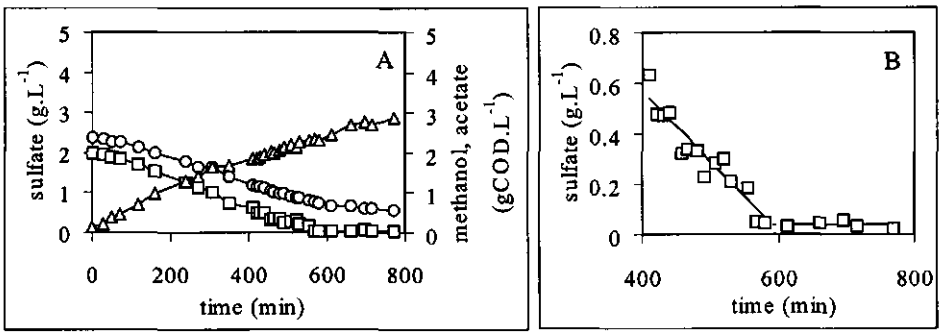

Figure 7.4. Batch-mode operation EGSB-II, with an initial COD/sulfate-ratio of 1.25 (excess methanol). Symbols: (□): sulfate; (○): methanol; (Δ): acetate.

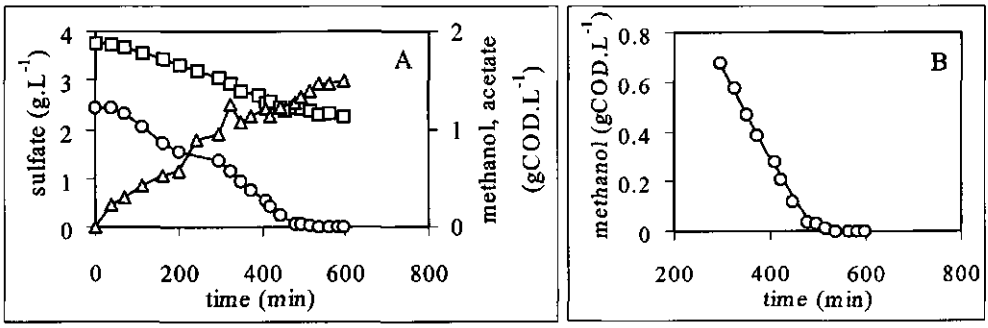

Figure 7.5. Batch-mode operation EGSB-II, with an initial COD/sulfate-ratio of 0.3 (excess methanol). Symbols: (□): sulfate; (○): methanol; (Δ): acetate

Biomass characteristics

The sludge developed in the EGSB-reactors consisted of small (<1 mm in diameter) flocs with a loose structure. Dense aggregates or granules did not develop. As can be expected for a flocculent type of sludge, the expansion of the sludge bed at the imposed superficial liquid velocities was high; at the applied upflow liquid velocity of 3 $m.h^{-1}$, the height of the dynamic sludge bed was twice as high as that of the static sludge bed (Figure 7.1D). The VSS content of the sludge from EGSB-IA gradually increased from 75% of TSS at

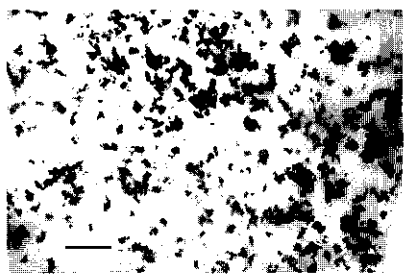

Figure 7.6. Sludge from EGSB-IA. Bar = 2 mm

the start, to 84% at the end of the experiment. At day 75, samples were taken from the sludge bed at a height of 10, 40 and 80 cm from the bottom of the sludge bed. The specific sulfidogenic and methanogenic activity, VSS-content, polysaccharide content and sludge volume index of these samples were determined. The results are summarized in Table 7.1.

Table 7.1. Characteristics of sludge taken from EGSB-IA at day 75. Samples were taken the sludge bed at a height of 10, 40 and 80 cm from the bottom of the sludge bed. SSA: specific sulfidogenic activity; SMA: specific methanogenic activity; VSS: volatile suspended solids; TSS: total suspended solids; PS: polysaccharide; SVI; sludge volume index.

Sample point	SSA ($gCOD.gVSS^{-1}.day^{-1}$)	SMA ($gCOD.gVSS^{-1}.day^{-1}$)	VSS-content $gVSS.gTSS^{-1}$	PS-content ($gPS.gVSS^{-1}$)	SVI ($mL.gTSS^{-1}$)
Upper	1.54 ± 0.24	0 ± 0.00	0.90 ± 0.01	0.040 ± 0.003	27 ± 4
Middle	1.48 ± 0.04	0.03 ± 0.02	0.86 ± 0.00	0.072 ± 0.001	21 ± 0
Lower	1.40 ± 0.10	0.05 ± 0.03	0.77 ± 0.01	0.076 ± 0.003	17 ± 0

7.5 Discussion

Our results show that methanol represents an attractive electron donor for thermophilic sulfate and sulfite reduction. High maximum sulfite and sulfate space elimination rates of 18 $gSO_3^{2-}.L^{-1}.day^{-1}$ (100% elimination) and 11 $gSO_4^{2-}.L^{-1}.day^{-1}$ (50% elimination) were found at an HRT of 3 h. In this situation, sulfate elimination was limited by the amount of biomass retained in reactor EGSB-IA. This can be concluded from the good agreement between the specific sulfidogenic activity of the reactor sludge (1.52 ± 0.19 $gCOD.gVSS^{-1}.day^{-1}$), calculated from the sulfidogenic COD-conversion rate and the average biomass concentration in the reactor, and the maximum specific sulfidogenic activity of the sludge as determined in batch assays (1.47 ± 0.15 $gCOD.gVSS^{-1}.day^{-1}$).

At an HRT of 10 h, it was furthermore found that the sulfate and sulfate-reducing biomass can tolerate high sulfide levels of up to 1600-1900 $mgS.L^{-1}$ at pH 7.5, resulting from reduction of approximately 6 $gSO_3^{2-}.L^{-1}.day^{-1}$ (100% elimination) and 6 $gSO_4^{2-}.L^{-1}.day^{-1}$ (50% elimination). The inhibiting effect of sulfide was revealed by the increase of the sulfate elimination to 100% once H_2S was stripped from the reactor. However, after ceasing the stripping, the sulfate elimination did not immediately revert to the level before stripping was applied, resulting in a high sulfide concentration of 2440 $mg.S^{-1}$. Apparently, the activity of the sulfate-reducing biomass is not instantaneously affected by such extremely high sulfide concentrations, but it was clearly detrimentally affected in the subsequent

week. The sulfidogenic COD-conversion rate then dropped with 10%. These results point to sulfide-induced uncoupling of growth and activity of the sulfate-reducing biomass. This can also be interpreted as a higher biomass decay rate at increasing sulfide concentrations. The net (i.e. growth minus decay) growth rate of the sulfate-reducing biomass presumably becomes too low at the high sulfide concentration of 2440 $mgS.L^{-1}$ to compensate for biomass washout, and as a result, a decrease of the biomass concentration will occur and accordingly, a decrease of the sulfidogenic COD-conversion rate. Uncoupling of growth and activity of sulfate-reducing biomass by sulfide was previously demonstrated by Okabe et al.[10] for the mesophilic sulfate reducer *Desulfovibrio desulfuricans*. Also Visser[14] found a similar uncoupling for mesophilic sulfate-reducing sludge cultivated on acetate, propionate and butyrate.

In addition to the high rates of sulfite and sulfate reduction that can be achieved in the process, also a high sulfate removal efficiency is possible with the reactor design used in this study. This follows from the sulfate depletion kinetics, that could be described with zero order kinetics down to a threshold concentration of 0.05 $g.L^{-1}$. This threshold value is so low that for practical applications the rate of sulfate reduction can be regarded as independent on the sulfate concentration in the reactor. On the other hand, the rate of methanol degradation coupled to sulfate reduction is dependent on the methanol concentration following classic Michaelis-Menten kinetics, with an *apparent* K_M-value of 0.037 $gCOD.L^{-1}$. However, still more than 80% of the maximum specific sulfidogenic activity of the sludge can be employed at a methanol concentration in the reactor exceeding 0.16 $gCOD.L^{-1}$.

A clear disadvantage of the use of methanol is the formation of acetate of up to 0.45 $gCOD.L^{-1}$ as by-product, which is highly undesirable as it negatively affects the efficiency of methanol utilization for sulfate and sulfite reduction. A more serious problem is that the presence of acetate in the effluent of the anaerobic reactor of the biological desulfurization process will deteriorate the performance of the sulfide oxidizing reactor, due to growth of heterotrophic sulfur and sulfate-reducing bacteria[5]. The lack of any acetotrophic sulfate-reducing activity in the sludge shows that either growth of acetotrophic SRB or MA is impeded under the prevailing conditions, or species capable of utilizing acetate as growth substrate were absent in the seed sludge in sufficiently high numbers. The reason for this cannot be the applied temperature of 65°C, because such a high temperature does not represent a barrier for growth of MA[9] and SRB[8] on acetate. The lack of methanogenic acetate degrading activity in the thermophilic sludge can be attributed to the low tolerance of thermophilic acetotrophic methanogens for hydrogen sulfide[2]. Likewise, the lack of

acetotrophic sulfidogenic activity may be explained by a high susceptibility of acetotrophic SRB for (hydrogen) sulfide[7], although the evidence for this was obtained with experiments conducted under mesophilic conditions.

Only the omission of the supply of cobalt from the influent lowered the acetogenic COD-conversion rate. However, the rates of sulfide formation decreased to the same extent, indicating that sulfide and acetate formation were coupled. This suggests that formation of acetate is an intrinsic feature of thermophilic methanol degradation under sulfate-reducing conditions. Thus, it appears that the formation of acetate from methanol cannot be reduced without diminishing the formation of sulfide as well. Therefore, for application of methanol in biological desulfurization of flue-gases, a compromise must be found between the formation of sulfide and acetate. Alternatively, measures can be taken to remove acetate at some stage in the process, e.g. by implementing, an additional anaerobic reactor prior to the methanol-fed reactor, in which acetate is used as electron donor for sulfite and sulfate reduction.

The results clearly reveal that sulfite overloading conditions cause a severe inhibition of the sulfidogenic biomass, which already will manifest at sulfite concentrations in the reactor as low as 40-80 $mg.L^{-1}$. This observation is in agreement with results of Widdel and Bak[15], who reported complete inhibition of growth of SRB at 40 $mg.L^{-1}$ sulfite. Inhibition by sulfite seems to be reversible, because the results show a rapid recovery of the sulfidogenic activity once the sulfite addition was stopped, even when the sludge had been exposed to high (>400 $mg.L^{-1}$) sulfite concentrations for more than 24 h. To our knowledge, such a reversibility of sulfite inhibition of SRB has so far not been reported. The reversible character of the inhibition shows that sulfite does not cause any serious damage to the sulfate and sulfate-reducing biomass. In order to prevent inhibition by sulfite, it is necessary to avoid any accumulation of sulfite in the process, which can easily be achieved by applying sulfite underloading to the reactor.

In view of the flocculent nature of the sludge, the retention of biomass in EGSB-IA was still relatively good, as indicated by the maximum value of the biomass concentration of 9-10 $gVSS.L^{-1}$. This can be attributed to the very low biogas production. Also the lower viscosity of aqueous media at increasing temperatures contributes to a better sludge retention in thermophilic high-rate reactors compared to mesophilic ones. The flocculent nature of the sludge resulted in a large expansion of the sludge bed of 100% at an upflow liquid velocity of 3 $m.h^{-1}$. Evidently, higher biomass concentrations than the maximum found value of 9 to 10 $gVSS.L^{-1}$ can be attained when a granular rather than a flocculent sludge had developed. Although high liquid upflow velocities were applied in the reactors to provide a selection

pressure for development of granules[6], such a granulation was not observed. Apparently, the thermophilic biomass cultivated on methanol does not have the intrinsic ability to form granules under the applied conditions in the reactor. The polysaccharide content of the sludge (4 to 8% of VSS) lies within the range of values (0.6 to 20%) that is normally found for the extracellular polymers content of granular sludge[12]. Therefore, a lack of extracellular polymers appears not to be the reason for the inability to form granules. The lack of growth of *Methanosaeta* species in the sludge, that presumably initiate granulation[4] or the lack of growth of acidifiers, which are believed to be involved in granulation as well[13], may explain why granules did not develop.

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Mesophilic sulfate reduction with methanol in high-rate anaerobic reactors

8.1 Abstract

In mesophilic (30°C) high-rate anaerobic reactors fed with an influent containing sulfate and methanol, more than 85% of the added methanol was degraded to methane, while maximally only 12% of the methanol was used for sulfate reduction. This methanol degradation pattern was independent of the pH in the range from 5 to 8; addition of acetate as co-substrate; addition of sulfite as alternative electron acceptor; and type of seed sludge. Applying temperature shocks of 65 or 80°C did not give sulfate-reducing consortia in the sludge a competitive advantage over the methanogens.

8.2 Introduction

Methanol is an attractive electron donor for biological desulfurization of wastewaters or groundwaters that contain insufficient electron donor to reduce sulfur oxyanions present. Methanol can also be used as external electron donor for the SRB in the biological treatment of acid mine water, i.e. reduction of sulfate by SRB in order to precipitate metals as metal sulfides. Biological desulfurization of these types of (waste)waters should be conducted at much lower temperatures than the high temperatures (>50°C) applied in the biological treatment of scrubber solutions used for the treatment of flue-gases, described in the previous chapters. Although methanol is used efficiently at 65°C as electron donor for sulfate reduction in anaerobic high-rate reactors with little or no formation of methane (Chapter 2, 5, 6 and 7), it is not clear if this is also the case at mesophilic conditions. The temperature may completely alter the anaerobic mineralization pattern of organic substrates. For instance, Visser and co-workers[7] found a rapid shift from methanogenesis to sulfate reduction after elevating the temperature of an acetate and sulfate fed UASB-reactor from 30 to 55°C. This result reflects the inability of bacteria to adapt to large temperature shifts. Consequently, significant alterations of the microbial population may occur due to such a temperature change. The occurrence of methanol-utilization by mesophilic sulfate-reducing bacteria (SRB) has been demonstrated[1,5], but acetate was always needed as carbon source.

It is well known that methane and acetate formation proceeds very well possible under mesophilic conditions, as methanol is a good substrate for mesophilic methanogenic archaea (MA)[8] and homoacetogenic bacteria (AB)[4]. Therefore, under these conditions, MA, AB and SRB will compete for methanol in anaerobic reactors fed with an influent containing methanol and sulfate. The competition between MA and SRB is not limited to methanol, because also possible anaerobic reaction products of methanol such as acetate and hydrogen are good substrates for mesophilic MA and SRB[6]. The ultimate outcome of the competition between microbial species for these common substrates in high-rate anaerobic reactors depends on a large number of factors[2] and can therefore in general not be predicted for a specific set of conditions. The aim of the work described in this chapter is to assess the applicability of methanol as electron donor for mesophilic sulfate reduction in high-rate anaerobic reactors.

8.3 Materials and methods

Reactors. One EGSB-reactor and two UASB (I and II) reactors were used in the continuous experiments. The experimental set-up for the three reactors was the same as for the thermophilic EGSB-reactor described in Chapter 2, but in the experimental set-up for UASB-I and -II, UASB-reactors replaced the EGSB-reactor. Effluent recycling was applied in all reactors. Operational parameters for the reactors are specified in Table 8.1 and 8.2. The pH in the reactors was controlled with automatic pH control. In the EGSB-reactor, the pH was kept at 7.5 during the entire operation and in UASB-I the initial pH was 6.7, which was stepwise lowered to a final value of 5 on day 87. UASB-II was started at pH 7.5, which was increased to 8 on day 21. All reactors were fed with a basal medium consisting of the following macro-nutrients ($g.L^{-1}$): NaCl (1.2), $MgCl_2.6H_2O$ (0.4), KCl (0.5), NH_4Cl (0.3), $CaCl_2$ (0.15), Na_2SO_4 (3.0), KH_2PO_4 (0.2), and 1 $mL.L^{-1}$ of the trace element solution described in Chapter 2. In addition, $NaHCO_3$ (1.26 $g.L^{-1}$) was added to the influent of the two UASB-reactors. Methanol was added to the influent of the reactors from a 5-M stock solution, at a flow rate to give the desired methanol organic loading rate. Yeast extract was added to the methanol stock solution to give an influent yeast extract concentration of 20 $mg.L^{-1}$. Acetate (sodium salt) was supplied to the methanol/yeast stock solution when added as co-substrate. The supply of sulfite to the EGSB-reactor was accomplished by addition of a 1-M sulfite stock solution, that was continuously flushed with nitrogen (nitrogen 3.0, Hoek Loos, The Netherlands) to avoid oxidation of sulfite by oxygen.

Table 8.1. Operating conditions for the continuous reactors.

		UASB-I	UASB-II	EGSB
working volume	(L)	5.5	6.0	4.05
temperature	(°C)	30	30	30
upward velocity	($m.h^{-1}$)	1.5 -2	1.5-2	2-5
hydraulic retention time	(h)	10	10	10
methanol loading rate	($gCOD.L^{-1}.day^{-1}$)	3.2	3.2	3.2
sulfate loading rate	($gSO_4^{2-}.L^{-1}.day^{-1}$)	4.8	[a]	4.8

a) The sulfate loading rate of UASB-II is shown in Table 8.2.

Table 8.2. The acetate and sulfate/sulfite loading rates of UASB-I and UASB-II.

		day			
UASB-I		**0-88**	**89-100**	**100-105**	**106-107**
acetate loading rate	($gCOD.L^{-1}.day^{-1}$)	0	0.1	0.4	0.7
		day			
UASB-II		**0-77**	**78-84**	**85-90**	
sulfate loading rate	($gSO_4^{2-}.L^{-1}.day^{-1}$)	4.8	2.4	0	
sulfite loading rate	($gSO_3^{2-}.L^{-1}.day^{-1}$)	0	2.4	4.8	

Biomass. The UASB-reactors were seeded with granular mesophilic methanogenic sludge from the full scale EGSB-plant treating the wastewater of the Heineken Brewery in Zoeterwoude, The Netherlands. The EGSB-reactor was seeded with granular mesophilic sulfidogenic sludge cultivated on sulfate and a mixture of acetate, propionate and butyrate in a laboratory-scale reactor. This sludge is further referred to as VFA-sludge. All reactors were inoculated with sludge up to 1/3 of the working volume of the reactor.

Activity assay. Activity assays were carried out in 120-mL vials for determination of the (specific) methanogenic and sulfidogenic activity of sludge at 30°C according to directions as described in Chapter 2. The preparation of the assay medium was also described in Chapter 2, except that the NaCl and $MgCl_2.6H_2O$ concentrations of the assay medium were lowered to 1.2 and 0.4 $g.L^{-1}$, respectively, to make the macro-nutrient composition of the assay medium similar to that of the medium fed to the reactors. Moreover, the $NaHCO_3$ concentration in the vials was lowered to 1.25 $g.L^{-1}$. Na_2SO_4 and methanol were added to the assay vials from concentrated stock solutions at a concentration of 2.8 $g.L^{-1}$ and 1.4 $gCOD.L^{-1}$, respectively. At the start of the assay, the medium had pH 7. The headspace in the vials consisted of 1.7 bar N_2/CO_2 (80/20 v/v). Inhibition of methanogens was accomplished by addition of 6 $g.L^{-1}$ BRES (sodium salt of 2-bromoethanesulfonate, Acros Organics, New Jersey, USA). The total amount of VSS in the vials was determined at the end of the assay when calculation of specific activities was desired.

Analyses. A detailed description of the analytical procedures for determination of methanol, acetate, sulfide, sulfite, biogas composition and VSS was presented in Chapter 2. In the activity assay, methane was measured on a 406 Packard gas chromatograph equipped with a thermal conductivity detector (TCD). Compounds were separated with argon as the

carrier gas on a molecular sieve column (13X, 1.8 m by 1/4 inch, 60-80 mesh) at 100°C. Sulfate and thiosulfate were measured with an HPLC equipped with a VYDAC Ion Chromatography column (cat. 302 IC, 250 x 4.6 mm). The temperature of the column and detector (Waters 431 conductivity detector) were 20°C and 35°C, respectively. As eluent, 0.018-M potassium biphthalate, at a flow rate of 1.2 $mL.min^{-1}$ was used. Samples were fixed by 2- to 4-fold dilution with a 0.1-M zinc acetate solution, centrifuged (3 min. 10000 g) and diluted with demineralized water to a concentration below 500 $mg.L^{-1}$. The sulfite concentration in the samples collected from UASB-II after day 77 was determined semi-quantitatively using test strips (Merckoquant cat. nr. 1.100-13).

8.4 Results

The effect of pH-variations, acetate addition, temperature shocks and sulfite addition on the methane and sulfide formation from methanol was studied in UASB-reactors inoculated with a methanogenic granular sludge. The effect of seed sludge on methane and sulfide formation was studied in an EGSB-reactor inoculated with a sulfidogenic granular sludge. The sludge loading rates in all reactors amounted to approximately 0.2 $gCOD.gVSS^{-1}.day^{-1}$.

Effect of pH

The results depicted in Figure 8.1A and 8.1B reveal that variation of the pH in the range from 5.0 to 8.0 did not strongly affect the fate of methanol. Methanogenesis was predominant at all tested pH-values, while the sulfidogenic and acetogenic COD-conversion remained low. Only after the pH drop from 5.5 to 5.0 in UASB-1 on day 87 the methane production temporarily decreased markedly, but it recovered within a few days. Already at day 2 and day 8 for UASB-I and UASB-II, respectively, the methanol removal efficiency reached a very high level (>97%), resulting in a low (< 0.05 $gCOD.L^{-1}$) effluent methanol concentration (data not shown). From the data presented in Figure 8.1A and 8.1B, it can be calculated that methanogenic, sulfidogenic and acetogenic COD conversion, accounted for 87-98%, 4-12% and about 2%, respectively, of the total COD conversion in the pH-range of 5 to 8.

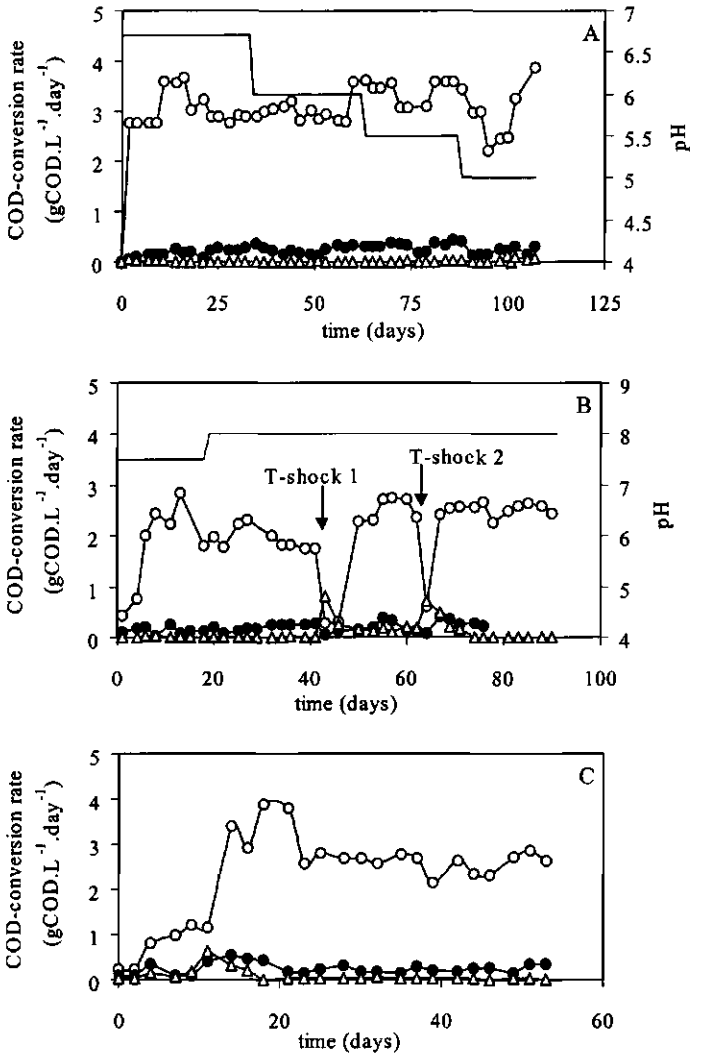

Figure 8.1. Performance of UASB-I (**A**), UASB-II (**B**) and the EGSB (**C**). Arrows in Figure 8.1B indicate start of a 24 h temperature shock of 65°C on day 42 (arrow 1), and of 80°C on day 63 (arrow 2). Arrow 3 indicates the start of sulfite addition on day 78. The sulfide production in the period following day 78 is depicted in Figure 8.2. Legend: pH (—), volumetric sulfidogenic (●), methanogenic (o) and acetogenic (Δ) COD-conversion rate.

Effect of acetate as co-substrate

The observed low sulfate reduction rate in UASB-I apparently cannot be attributed to a lack of carbon source for the SRB, because addition of 0.05 gCOD.L^{-1} of acetate as co-substrate to the influent of UASB-I from day 88 onwards did not result in stimulation of sulfate reduction (Figure 8.1A). Increasing the influent acetate concentration to 0.17 and 0.35 gCOD.L^{-1} on day 99 and 104, respectively, also did not affect sulfate reduction positively. Because acetate was almost completely (> 95%) degraded (data not shown), it is also possible that it never became available for the SRB.

Effect of temperature shock

Results of preliminary activity assays conducted with the seed sludge (2 gVSS.L^{-1}) of UASB-II revealed that temperature shocks imposed to the sludge of 65 and 80°C for 24 hr annihilated the methanogenic activity on methanol for at least 2 weeks, while 20 and 80% of the added methanol (1.3 gCOD.L^{-1}) was used for sulfate reduction and acetogenesis, respectively (data not shown). Based on these results, two temperature shocks lasting 24 h were imposed to UASB-II in order to minimize methane production: one shock of 65°C on day 42, and a second of 80°C on day 63. Both the methane and sulfide production decreased sharply due to the both temperature shocks (Figure 8.1B) but within 1 week the methanogenesis and sulfidogenesis recovered almost completely. The acetate production increased sharply during the temperature shocks, probably due to biomass lysis, but after both shocks it dropped to the same low level as before the shock.

Effect of sulfite

The effect of sulfite on the competition between SRB and MA was investigated in UASB-II. The transition from a merely sulfate-containing (2 g.L^{-1}) medium to a medium containing sulfite (1 g.L^{-1}) and sulfate (1 g.L^{-1}) on day 78 stimulated sulfide production. Sulfite was completely removed, but the sulfate was produced (Figure

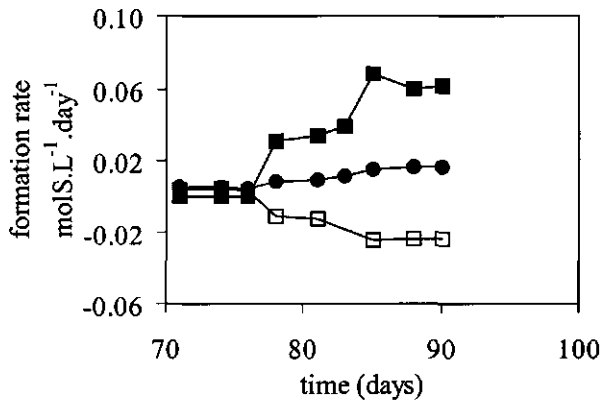

Figure 8.2. Disproportionation of sulfite (□) to sulfide (●) and sulfate (■) in UASB-II from day 78 to day 90.

8.2). Results of measurements of the sulfite concentration of the influent at the inlet of the reactor revealed no sulfite had oxidized to sulfate before entering the bioreactor. Increasing the sulfite concentration to 2 $g.L^{-1}$ from day 85 to day 91, while concomitantly omitting sulfate from the influent, led to a further increase of sulfide production (Figure 8.3). Sulfite was still completely removed during this period while concomitantly, the sulfate concentration increased. From the data presented in Figure 8.2 it can be calculated that for every 4 mol of sulfite removed in the reactor, about 1 mol of sulfide and 2 mol of sulfate appeared in the effluent. Although there is a deficit in the sulfur balance, the appearance of sulfate is a clearly indication for the occurrence of the sulfite disproportionation reaction:

$$4\ SO_3^{2-} + H^+ \Rightarrow 3\ SO_4^{2-} + HS^- \qquad \Delta G^{\circ\prime} = -58.9\ kJ/mol\ SO_3^{2-}$$

The results furthermore clearly demonstrate that the addition of sulfite did not affect the methane production (Figure 8.1B) from methanol, indicating that the SRB were unable to compete successfully with the methanogens for methanol to use it as electron donor.

Effect of seed sludge

In order to examine the effect of seed sludge on the competition between MA and SRB, an additional EGSB-experiment was conducted with a sulfidogenic sludge that was cultivated on a mixture of volatile fatty acids (VFA, i.e. acetate, propionate and butyrate). Results of previous activity assays with the VFA-adapted sludge revealed a high sulfate-reducing potential of this sludge with methanol: in vials with 2 $gVSS.L^{-1}$ of the seed sludge, sulfate (2 $g.L^{-1}$) was nearly completely reduced with methanol (1.4 $gCOD.L^{-1}$), while methane production remained negligible (Figure 8.3). In this assay the acetate formed from methanol constituted the sole electron donor for the SRB after day 3. An EGSB-reactor was chosen to ensure good mixing conditions. As shown in Figure 8.1C, the methane production in the

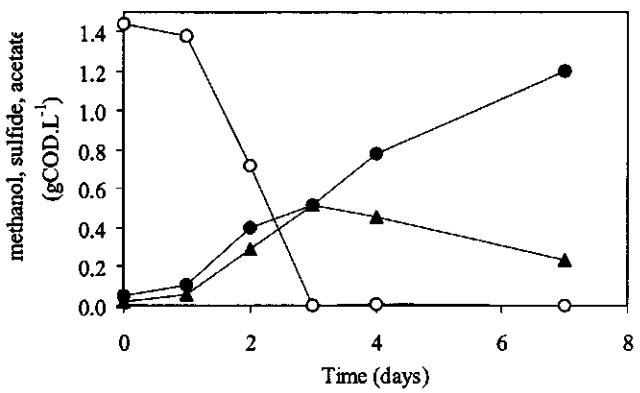

Figure 8.3. Sulfide (●), methanol (○) and acetate (▲) concentration in batch assay inoculated with seed sludge ("VFA-sludge") of the EGSB-reactor.

reactor rapidly increased from a very low initial level to a stable level of about 3 gCOD.L^{-1}.day^{-1} within two weeks (Figure 8.1C), corresponding to a high methanol removal efficiency exceeding 97% from day 14 onwards. The sulfide formation generally remained below 0.3 gCOD.L^{-1}.day^{-1}, except during the period from day 12 to day 18, when it attained slightly higher values of 0.3-0.6 gCOD.L^{-1}.day^{-1}. Moreover, also the acetate production reached a maximum in the same period, with a maximum value of 0.6 gCOD.L^{-1}.day^{-1} on day 11. However, following day 18 hardly any acetate was present in the effluent.

To assess the sulfate-reducing potential of the methanogenic sludge cultivated in the EGSB-reactor, the sulfidogenic, methanogenic and acetogenic activities with methanol and sulfate in the presence and absence of BRES were determined on day 54. The results show (Table 8.3) that the specific methanogenic activity on methanol is about 4 times as high as the specific sulfidogenic activity. Addition of BRES did not improve sulfate reduction. The specific acetogenic activity increased as a result of BRES addition, indicating that acetogens could benefit from the inhibition of methanogens. Alternatively, acetate accumulation may have resulted from the inhibition of acetate consumption by aceticlastic methanogens.

Table 8.3. Specific sulfidogenic and methanogenic activity of sludge taken from the EGSB-reactor at day 54. Sulfate (2 g.L^{-1}) was added to all assays.

	Specific sulfidogenic activity	Specific methanogenic activity	Specific acetogenic activity
	(gCOD.gVSS^{-1}.day^{-1})		
-BRES, +methanol	0.07±0.04	0.25±0.08	0.00±0.00
+BRES, +methanol	0.07±0.01	0.00±0.00	0.14±0.02
-BRES, -methanol	0.01±0.00	0.00±0.00	0.04±0.0

8.5 Discussion

The results indicate that methanol is not a suitable electron donor for sulfate reduction under mesophilic conditions using mesophilic seed sludges, because more than 85% of the added methanol was consumed by methanogenic consortia in high rate anaerobic reactors

fed with methanol and sulfate, while maximally only 12% of the methanol was used for sulfate reduction. This pattern was not dependent on: the pH of the reactor medium in the range from 5 to 8, the addition of acetate as co-substrate, the addition of sulfite as alternative electron acceptor, and the type of mesophilic seed sludge. The duration of the UASB/EGSB experiments was about 100 days, i.e. of the same order of magnitude as the sludge retention time of 0.5-1 year typical for high-rate anaerobic reactors[3]. Therefore, the observed low sulfate reduction rates in the reactors can not solely be attributed to the low growth rates typical for anaerobic microorganisms like SRB. Apparently, SRB are either very poor competitors for methanol in mesophilic high-rate reactors or are very poorly immobilized.

Even with mesophilic VFA-cultivated sulfidogenic sludge as seed material, the sulfate reduction rate remained low as the results of the EGSB-reactor demonstrate. Apparently, methanol is a poor electron donor for the VFA-utilizing sulfate reducers present in the seed sludge, despite the fact that the sludge showed considerable sulfate-reducing activity with methanol in the activity assay. These contrasting results most likely can be explained by the prevalence of substantial sulfate reduction capacity on acetate, which is formed from methanol in the assay, while this is not the case in the EGSB-reactor, except during the period from day 11 to 17. Probably, during this period, the reducing equivalents needed for sulfate reduction prevailing in this period originated mainly from acetate. Following day 17, methanogens outcompeted the homoacetogens and SRB rapidly. Apparently, under mesophilic conditions the methanogens have better growth properties on methanol than SRB and AB.

Methanol is also a poor electron donor for mesophilic sulfite reduction, because the sulfite removal in UASB-II was mainly due to disproportionation of sulfite to the products sulfide and sulfate, a reaction that does not result in a net reduction of sulfur species. Therefore, disproportionation of sulfite is not effective for desulfurization, as electrons are only redistributed among the sulfur species.

8.6 References

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Summary and Discussion

1 Introduction

The aim of the research described in this thesis was to study the use of methanol as external electron donor for biological desulfurization of flue-gases and ground- and wastewaters. In such a process methanol is used as external electron donor for biological anaerobic reduction of sulfur oxyanions to hydrogen sulfide.

Discharge of sulfur compounds by human activity via flue-gases and wastewaters causes serious environmental problems such as acid rain and anaerobiosis of surface waters. The removal of sulfur oxyanions so far mainly is achieved by fixing sulfur as poorly soluble $CaSO_4$ (gypsum). As alternative for the high chemical- and energy-requiring gypsum-processes, an ingenious biological process has been proposed[1-3]. This process accomplishes desulfurization with little consumption of chemicals and fixation of sulfur in its elemental, solid form. In the mainly biological process sulfur oxyanions (e.g. sulfate, sulfite, thiosulfate) dissolved in an aqueous phase first are reduced by sulfate-reducing bacteria (SRB) to sulfide with a suitable electron donor under anaerobic conditions. The generated sulfide is subsequently partially oxidized to elemental sulfur by Thiobaccilli spp. under micro-aerobic conditions[6]. The elementary sulfur produced possibly can be re-used e.g. for production of sulfuric acid. The large difference in redox potential between anaerobic reduction of sulfur oxyanions and micro-aerobic sulfide oxidation implies spatial separation of these conversions, e.g. in two separate bioreactors. This thesis focuses on the anaerobic stage, i.e. the reduction of sulfur oxyanions to sulfide.

Flue-gases and groundwater, and many wastewaters as well, do not contain sufficient electron donor for biological reduction of the large amount of sulfur oxyanions that can be present. This necessitates the addition of an external electron to supply the SRB with sufficient reducing equivalents. Such an external electron donor should be cheap as its price largely determines the operational costs of the treatment process. In this thesis, the suitability of the relatively cheap bulk chemical methanol as electron donor for reduction of sulfur oxyanions was investigated.

Decisive for the industrial applicability of methanol-based biological desulfurization processes is the selectivity and the rate of reduction of sulfur oxyanions that can be achieved in bioreactors. This thesis aims to find answers for these technological questions. In addition, this thesis intends to acquire insight in the microbiology of the process.

This chapter summarizes and discusses the results on sulfate and sulfite reduction with methanol at 65°C, and the feasibilityof this system for biological desulfurization of hot flue-gases. The results on mesophilic (30°C) sulfate reduction with methanol, which are of interest for bio-desulfurization of cold or slightly heated ground- or wastewater with a high content of sulfur oxyanions but with a low amount of electron donor, were already presented in detail in Chapter 8.

2 Thermophilic sulfate and sulfite reduction

In the biological flue-gas desulfurization process, SO_2 is scrubbed from the hot flue-gas using a bicarbonate solution. Herewith, a warm (50-65°C) sulfite and sulfate containing liquor is generated which subsequently is subjected to biological treatment. The composition of the synthetic influent used for the continuous sulfate reduction experiments closely resembled the composition of this liquor with respect to its salinity (7 $g.L^{-1}$ NaCl) and concentration of sulfate (1-4 $g.L^{-1}$) and sulfite (1-4 $g.L^{-1}$). The imposed temperature and pH in most experiments were 65°C and 7.5. The following aspects are discussed:

-the competition between sulfate-reducing, methanogenic and acetogenic communities for methanol in bioreactors (Chapters 2, 5, 6 and 7);

-the performance of the sulfate and sulfate-reducing bioreactors fed with methanol (Chapters 6 and 7);

-microorganisms involved in thermophilic sulfate reduction with methanol (Chapters 3 and 4).

2.1 Competition between thermophilic sulfate-reducing, methanogenic and acetogenic microorganisms for methanol in bioreactors

The selectivity of using methanol for biological sulfate reduction (equation 1) is determined by the ability of methanol-degrading sulfate-reducing bacteria to compete successfully with other methanol degrading microorganisms present in the reactor sludge, e.g. methanogens

and acetogens, that convert methanol to methane (equation 2) and acetate (equation 3), respectively.

$$3\ SO_4^{2-} + 4\ CH_3OH \Rightarrow 3\ HS^- + 4\ HCO_3^- + 4\ H_2O + H^+ \quad \text{(eq. 1)}$$

$$4\ CH_3OH \Rightarrow 3\ CH_4 + HCO_3^- + H_2O + H^+ \quad \text{(eq. 2)}$$

$$4\ CH_3OH + 2\ HCO_3^- \Rightarrow 3\ CH_3COO^- + H^+ + 4\ H_2O \quad \text{(eq. 3)}$$

The results presented in Chapter 2 clearly show that sulfate-reducing microorganisms ultimately outcompete methanogenic consortia in anaerobic high-rate bioreactors that are based on sludge retention via a mechanism of biomass self-immobilization. The reactors were fed with an influent containing methanol and sulfate (as model sulfur oxyanion) in a ratio of 0.67 gCOD/gSO_4^{2-} and they were operated at pH 7.5 and at a temperature of 65°C. Substantial methane formation persisted for several months using unadapted seed sludge. However, by imposing slightly acidic pH-values (6.7 instead of 7.5) it was possible to achieve selective and durable suppression of methanogenesis (Chapter 5). This might represent a practical method that also may be applied in a full-scale process.

Compared to methane and sulfide, acetate always was formed as a minor side-product (normally less than 1 mM) from methanol in the initial bioreactor-experiments described in Chapter 2. In later experiments, described in Chapters 6 and 7, acetate formation in sulfate- and sulfate-reducing bioreactors was substantially higher with effluent acetate concentrations of up to 9 mM, accounting for up to 13% of the degraded methanol. Batch tests showed that acetate did not serve as electron donor for the reduction of sulfate or carbon dioxide in any of the experiments, indicating that acetate was an end product from methanol degradation rather than an intermediate. A remarkable feature of the process is the observation in Chapter 6 that the rate of acetogenic COD-conversion is linearly correlated to the sulfidogenic COD-conversion rate. Although less evident, such a correlation also emerges from the data obtained in reactor experiments described in Chapter 2 and 7, conducted under very similar reactor conditions to those of the experiment in Chapter 6 (Figure 1). The data presented in Figure 1 point to a connection of sulfide and acetate formation, indicating that the microbial community responsible for sulfate reduction also directly or indirectly might be involved in the formation of acetate. Regression analysis of the data (forcing the regression line through the origin to facilitate comparison) reveals that in the experiment described in Chapter 6, an average value of 0.14 gCOD-methanol is used for acetate per gCOD-methanol consumed in sulfate/sulfite reduction (regression line B in Figure 1). In the other two experiments this ratio is only 0.05 to 0.06 (regression lines A and C, data from Chapters 2 and 7, respectively). The relatively small deviation between

regression lines A and C may be due to the natural scattering of the data but the strong deviation of line B from lines A and C indicates that the ratio between acetate and sulfide formed from methanol is not fixed, but it apparently depends on one or more unknown variable(s). A detailed analysis of the data and comparison of the experimental set-up and the operating conditions of the three experiments so far did not result in any clear evidence of such a variable. Therefore, more research still remains to be done.

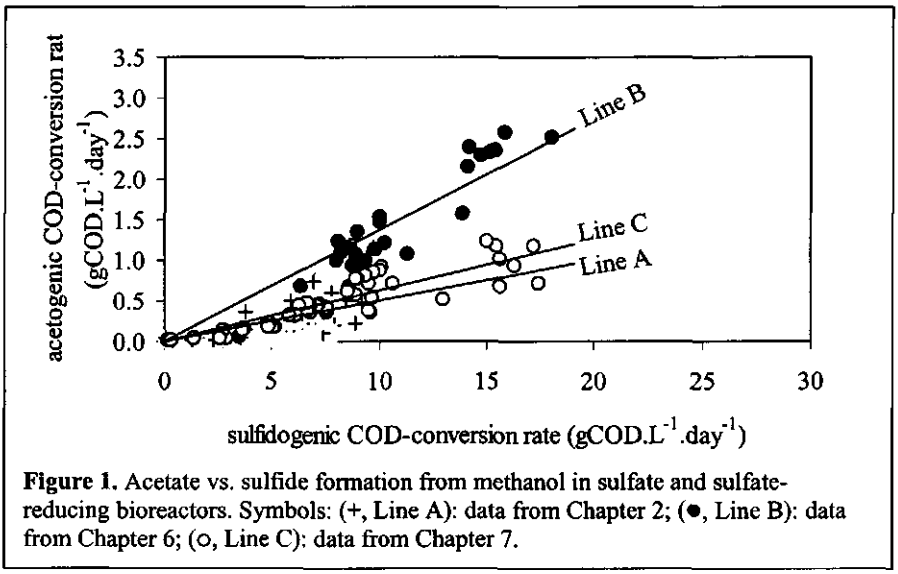

Figure 1. Acetate vs. sulfide formation from methanol in sulfate and sulfate-reducing bioreactors. Symbols: (+, Line A): data from Chapter 2; (•, Line B): data from Chapter 6; (o, Line C): data from Chapter 7.

The results indicate that the formation of acetate cannot be reduced without diminishing the formation of sulfide as well. Therefore, in case methanol would be applied for biological desulfurization of flue-gases, a compromise must be found between the formation of sulfide and acetate.

It is noteworthy that in the experiments presented in Chapter 2, no cobalt was added to the thermophilic reactors, because it was expected that cobalt might stimulate unwanted methane and acetate formation from methanol, analogous to its effect on methanol degradation under mesophilic conditions[4]. However, as demonstrated in Chapter 7, methane formation under thermophilic sulfate and sulfate-reducing conditions is suppressed just as rapidly with as without cobalt. On the other hand, omitting the supply of cobalt to the influent indeed resulted in a lower acetate formation rate, but at the same time the rate of sulfide formation from methanol decreased to the same extent. Therefore, applying cobalt-

limiting conditions does not improve the selectivity of sulfate/sulfite reduction with methanol.

2.2 Performance of methanol-fed sulfate- and sulfite-reducing bioreactors

The competitiveness of biological flue-gas desulfurization with other processes is largely determined by its loading potential. Low conversion rates due to poor retention of biomass in the reactor and inadequate mass transfer from bulk liquid to sludge should be avoided by selecting a proper reactor design. In this respect, the Expanded Granular Sludge Bed (EGSB) reactor looks a good choice because 1) good mixing of the bulk liquid is realized by the imposed high (> 2 $m.h^{-1}$) upflow liquid velocity and 2) at such high upflow liquid velocities sludge segregation will occur[8].

High rates of sulfate and sulfite reduction with methanol were found in the reactors. At a hydraulic retention time (HRT) of 10 h, maximum sulfite and sulfate reduction rates of 6 $gSO_3^{2-}.L^{-1}.day^{-1}$ (100% sulfite elimination) and 4-7 $gSO_4^{2-}.L^{-1}.day^{-1}$ (40-70% sulfate elimination) were attained simultaneously in EGSB-reactors. This is equivalent to a sulfidogenic methanol-conversion rate of 6-8 $gCOD.L^{-1}.day^{-1}$ (Chapter 7). However, under these conditions, the resulting sulfide concentration of about 1800 $mgS.L^{-1}$ (corresponding to a free hydrogen sulfide concentration of 200 $mgS.L^{-1}$ at the applied pH of 7.5) limited the rate of sulfate reduction. At a hydraulic retention time of 3-4 h, maximum sulfite and sulfate reduction rates of 18 $gSO_3^{2-}.L^{-1}.day^{-1}$ (100% elimination) and 11-14 $gSO_4^{2-}.L^{-1}.day^{-1}$ (about 50% elimination) were attained, which is equivalent to a sulfidogenic methanol-conversion rate of 19 $gCOD.L^{-1}.day^{-1}$ (Chapters 6 and 7). In this situation, the sulfate reduction rate was limited by the amount of biomass present in the system, i.e. a concentration of 9 to 10 $gVSS.L^{-1}$, which was the maximal amount that could be retained in the reactor. The time needed to reach the maximum sulfite and sulfate elimination rates in the reactors using adapted seed sludge amounted to 40-60 days (Chapters 6 and 7). For practical applications, in particular for anaerobic processes, such a period seems acceptable.

From the results it can be calculated that it is theoretically possible to achieve, at a HRT of 4 h, 100% elimination of 2.3 $gSO_3^{2-}.L^{-1}$ and 2.7 $gSO_4^{2-}.L^{-1}$ (molar ratio sulfite to sulfate = 1). The maximum sulfidogenic conversion rate of methanol then amounts to 19 $gCOD.L^{-1}.day^{-1}$ resulting in a maximum effluent sulfide concentration of 1800 $mgS.L^{-1}$. In this calculation it was assumed that no kinetic limitations exist for sulfite and sulfate reduction (zero order reaction rates). This assumption is justified for sulfate, as sulfate depletion kinetics could be described with zero order kinetics down to a low threshold concentration of 0.1 $g.L^{-1}$. This threshold value is so low that for practical applications the

rate of sulfate reduction can be regarded as independent of the sulfate concentration in the reactor. However, this maximum sulfate-reducing capacity can only be realized at methanol overloading conditions because the rate of methanol degradation coupled to sulfate reduction followed Michaelis-Menten kinetics, with an *apparent* K_M-value of 0.037 $gCOD.L^{-1}$ (Chapter 7). Still more than 80% of the maximum specific sulfidogenic activity of the sludge can be employed at a methanol concentration in the reactor exceeding 0.16 $gCOD.L^{-1}$.

With an influent containing 2.3 $gSO_3^{2-}.L^{-1}$ and 2.7 $gSO_4^{2-}.L^{-1}$ the (hydrogen) sulfide concentration becomes limiting at HRTs > 4 h and accordingly, the maximum sulfidogenic conversion rate of methanol cannot be maintained unless (hydrogen) sulfide is removed from the reactor, e.g. by means of stripping. At HRTs < 4 h, biomass retention limits the sulfite/sulfate reduction rate and although the maximum sulfidogenic conversion rate of methanol will be maintained, sulfate is no longer completely eliminated.

From the above it will be clear that the sulfidogenic conversion rate of methanol can be increased beyond 19 $gCOD.L^{-1}.day^{-1}$ provided that the biomass retention of the system is improved. The biomass retention of maximally 9 $gVSS.L^{-1}$ is low compared to the retained biomass concentrations of 20-30 $gVSS.L^{-1}$ as observed in other studies using EGSB-reactors[9]. The rather poor biomass retention in the reactors likely is due to the flocculent nature of the sludge developed in the reactors, as opposed to the very well settleable granular sludge present in methanogenic EGSB-reactors. Because the term Expanded *Granular* Sludge Bed reactor refers to the type of sludge developing in the reactor rather than to the reactor itself, this is in retrospect not an adequate name for the reactor-type used in this study. The system better can be indicated as 'High Upflow Anaerobic Sludge Bed (H-UASB) reactor. An improvement of biomass retention in the reactor possibly could be accomplished by lowering the upflow liquid velocity (v_{up}) below the applied value of 3-6 $m.h^{-1}$, because this would result in less expansion of the sludge bed and accordingly, a higher sludge hold-up. However, the contact between medium and biomass might become too poor below a v_{up} of 2.5 $m.h^{-1}$ even when some biogas is produced[7]. In this connection it also should be noted that already at a v_{up} of 3 $m.h^{-1}$ the lower part, or even the whole sludge bed occasionally aggregated completely, which caused channelling and sludge piston formation. Sludge bed aggregation is likely to aggravate at even lower upflow liquid velocities, although this may in part be overcome by moderate agitation of the sludge bed. Considering the above, a substantial improvement of biomass retention in the H-UASB reactors looks difficult to achieve under the applied conditions. Better sludge hold-up might

be obtained by increasing the density of sludge particles by means of adding a carrier material as in fluidized bed reactors.

2.3 Microorganisms involved in thermophilic methanol degradation under sulfate-reducing conditions

In order to improve the insight in the process, the dominant microorganisms prevailing in the sludge cultivated in a thermophilic, methanol-fed sulfate-reducing reactor were studied. The formation of sulfide, methane and acetate in bioreactors inoculated with unadapted sludge (Chapter 2), revealed the presence of sulfate-reducing bacteria in the sludge (SRB), as well as methanogenic archaea (MA) and (homo)acetogenic bacteria (AB).

It was shown in Chapter 2 that the dominant methane-producing species in the sludge were hydrogenotrophic methanogens instead of methanol-degrading methanogens. Because hydrogen likely represents the main electron donor for methanogenesis and hydrogen also supported high rates of sulfate reduction in the sludge, it follows that MA and SRB mainly compete for hydrogen. This may explain why sulfate reduction ultimately dominates over methanogenesis in the reactor. From literature it is well known that SRB outcompete MA for hydrogen when sufficient sulfate is present[5,10].

A sulfate-reducing bacterium, strain WW1, was isolated from the highest methanol degrading, sulfate-reducing serial dilution of the sludge, strongly indicating that it is the most abundant sulfate reducer involved in sulfidogenic methanol degradation (Chapter 3). Strain WW1 was identified as a *Desulfotomaculum*-species by its morphology and 16SRNA-sequence. In the sludge, strain WW1 is not confined to the use of methanol, as the strain also grows on anaerobic degradation products of methanol, like acetate, formate and H_2/CO_2. Therefore, methanol in the sludge may be either directly used as electron donor for sulfate reduction by strain WW1-like microorganisms or it may first be degraded to intermediates like hydrogen, formate and acetate by for instance (homo)acetogens, followed by sulfidogenic oxidation of such intermediates.

Two observations indicate that hydrogen indeed might be an important electron donor for sulfate reduction by sludge cultivated on methanol. Firstly, the strain WW1 does not use acetate in the presence of H_2. Therefore, the lack of acetate degradation by this sludge might be explained by assuming that hydrogen is the main electron donor for sulfate reduction. Secondly, growth of strain WW1 on methanol and sulfate already stops at a total sulfide concentration of 220 $mgS.L^{-1}$, due to toxicity of the produced sulfide, while on the other hand, growth of strain WW1 on hydrogen and sulfate up to 640 $mgS.L^{-1}$ of total

sulfide is possible. The latter sulfide concentration is in the same range as the total sulfide concentration in the bioreactor from which the strain was isolated. Thus, the high sulfide levels produced in the bioreactor might be explained by assuming growth of strain WW1-like sulfate reducers on hydrogen rather than on methanol.

The hypothesis that hydrogen is an important electron donor for sulfate reduction in the sludge implies that at least part of the added methanol is converted to hydrogen (and presumably CO_2). The presence of high numbers of methanol-oxidizing, hydrogen-producing bacteria in the sludge indeed was demonstrated (Chapter 4). Thus, methanol oxidation to hydrogen followed by sulfate reduction with hydrogen may represent an important methanol degradation route in the sludge. However, it is still unclear to which extent methanol is directly consumed by sulfate reducers in the sludge or first converted to hydrogen before being used for sulfate reduction.

It is noteworthy that in the undefined cultures in which the presence of methanol-oxidizing, hydrogen producing species was demonstrated, also acetate was formed from methanol (Chapter 4). In the cultures with the methanol-oxidizing, hydrogen producing species and an added hydrogenotrophic sulfate reducer, about 15% of the methanol was degraded to acetate. Moreover, the formation of acetate seemed to be strictly coupled to growth of the unidentified methanol-oxidizing species. The apparent coupling of methanol degradation to hydrogen and formation of acetate from methanol in the cultures might explain the coupling of sulfide and acetate formation from methanol in the reactors, assuming that indeed interspecies hydrogen transfer from methanol-oxidizing species to SRB plays a major role in the sludge.

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Samenvatting en discussie

1 Inleiding

Het doel van het in dit proefschrift beschreven onderzoek was het bestuderen van het gebruik van methanol als elektronendonor voor biologische ontzwaveling van rookgassen en grond- en afvalwater. In zo'n proces wordt methanol als externe elektronendonor gebruikt voor biologische reductie van geoxideerde zwavelverbindingen naar waterstofsulfide.

Uitstoot van zwavelverbindingen door menselijk handelen via rookgassen en afvalwater veroorzaakt ernstige milieuproblemen zoals zure regen en zuurstofdeficiëntie van oppervlaktewater. Verwijdering van geoxideerde zwavelverbindingen wordt tot nog toe vooral bewerkstelligd door het zwavel vast te leggen als slecht oplosbaar calciumsulfaat (gips). Dit 'gips'-proces heeft een hoog verbruik aan energie en chemicaliën. Voor het gips-proces is een biologisch alternatief[1-3], dat weinig chemicaliën vergt en het zwavel vastlegt als de vaste stof elementair zwavel. In het grotendeels biologische proces worden opgeloste geoxideerde zwavelverbindingen (zoals sulfaat, sulfiet en thiosulfaat) eerst onder anaërobe condities gereduceerd naar sulfide met een geschikte elektronendonor door sulfaatreducerende bacteriën (SRB). Het gevormde sulfide wordt vervolgens gedeeltelijk geoxideerd naar elementair zwavel door Thiobaccilli-bacteriën onder zuurstoflimiterende condities[6]. Het geproduceerde elementaire zwavel is te gebruiken voor bijvoorbeeld de productie van zwavelzuur. Het grote verschil in redoxpotentiaal tussen anaërobe reductie van geoxideerde zwavelverbindingen en oxidatie van sulfide naar elementair zwavel onder zuurstoflimiterende condities houdt in dat deze twee stappen altijd ruimtelijk gescheiden plaatsvinden, bijvoorbeeld in twee afzonderlijke bioreactoren. Dit proefschrift concentreert zich op de anaërobe stap, de omzetting van geoxideerde zwavelverbindingen naar sulfide.

Rookgassen en grondwater, alsmede vele afvalwaterstromen bevatten onvoldoende elektronendonor voor biologische reductie van het hoge gehalte aan geoxideerde zwavelverbindingen dat hierin aanwezig kan zijn. Om de sulfaatreducerende bacteriën van voldoende reductie-equivalenten te voorzien, is toevoeging van een externe elektronendonor nodig. Zo'n externe elektronendonor moet goedkoop zijn, omdat de kosten

hiervan grotendeels de operationele kosten van het zuiveringsproces bepalen. In dit proefschrift is de geschiktheid van de relatief goedkope bulkchemicalie methanol als elektronendonor voor reductie van geoxideerde zwavelverbindingen onderzocht.

Doorslaggevend voor de industriële toepasbaarheid van methanol voor biologische ontzwaveling zijn de selectiviteit en snelheid van reductie van geoxideerde zwavelverbindingen welke haalbaar zijn in bioreactoren. Dit proefschrift probeert deze technologische vragen te beantwoorden. Bovendien wordt gepoogd inzicht te verkrijgen in de microbiologie van het proces.

Dit hoofdstuk vat de resultaten betreffende sulfaat- en sulfietreductie met methanol bij 65°C samen, en bediscussieerd de toepasbaarheid van dit proces voor biologische ontzwaveling van hete rookgassen. De resultaten betreffende mesofiele (30°C) sulfaatreductie met methanol, van belang voor ontzwaveling van enigszins opgewarmd grond- van afvalwater met een hoog gehalte aan geoxideerde zwavelverbindingen maar met weinig elektronendonor, werden in detail in hoofdstuk 8 besproken.

2 Thermofiele sulfaat- en sulfietreductie

In het biologische proces voor rookgasontzwaveling wordt zwaveldioxide (SO_2) uit het rookgas gewassen met een bicarbonaat-oplossing. Daarbij wordt een warm (50-65°C) sulfiet- en sulfaat-houdende oplossing verkregen welke vervolgens biologisch wordt behandeld. De samenstelling van het synthetische influent gebruikt voor continu-experimenten kwam ongeveer overeen met de samenstelling van deze oplossing ten aanzien van het zoutgehalte (7 $g.L^{-1}$ NaCl) en de concentratie sulfaat (1-4 $g.L^{-1}$) en sulfiet (1-4 $g.L^{-1}$). De temperature en pH was in de meeste experimenten 65°C en 7.5. De volgende aspecten worden bediscussieerd:

-de competitie tussen sulfaatreducerende, methanogene en acetogene micro-organismen voor methanol in bioreactoren (hoofdstukken 2, 5, 6 en 7);

-het functioneren van sulfaat- en sulfietreducerende bioreactoren gevoed met methanol (hoofdstuk 6 en 7);

-micro-organismen betrokken bij thermofiele sulfaatreductie met methanol (hoofdstuk 3 en 4).

2.1 Competitie tussen sulfaatreducerende, methanogene en acetogene micro-organismen voor methanol in bioreactoren

De selectiviteit van biologische sulfaatreductie met methanol (vergelijking 1) wordt bepaald door de mate waarin methanol-afbrekende sulfaatreducerende bacteriën succesvol competeren met andere methanolafbrekende micro-organismen, met name methanogenen en acetogenen, welke methanol omzetten naar respectievelijk methaan (vergelijking 2) en acetaat (vergelijking 3).

$$3\ SO_4^{2-} + 4\ CH_3OH \Rightarrow 3\ HS^- + 4\ HCO_3^- + 4\ H_2O + H^+ \qquad \text{(verg. 1)}$$

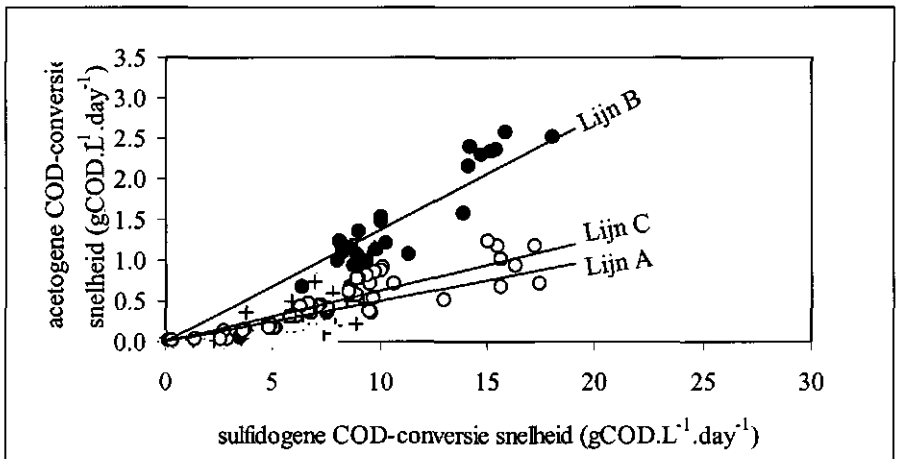
$$4\ CH_3OH \Rightarrow 3\ CH_4 + HCO_3^- + H_2O + H^+ \qquad \text{(verg. 2)}$$

$$4\ CH_3OH + 2\ HCO_3^- \Rightarrow 3\ CH_3COO^- + H^+ + 4\ H_2O \qquad \text{(verg. 3)}$$

De resultaten uit hoofdstuk 2 tonen duidelijk aan dat sulfaatreducerende micro-organismen uiteindelijk de competitie voor methanol winnen van methanogene consortia in anaerobe bioreactoren waarin auto-immobilisatie van de biomassa plaatsvindt. De reactoren werden gevoed met een influent dat methanol en sulfaat (als model geoxideerde zwavelverbinding) bevatte in een verhouding 0.67 $gCOD/gSO_4^{2-}$. Bij deze verhouding wordt juist genoeg methanol gedoseerd om al het aanwezige sulfaat te reduceren. De pH en temperatuur in de reactoren was respectievelijk 7.5 en 65°C. Een aanzienlijke methaanvorming bleef gedurende enkele maanden bestaan wanneer de reactor werd opgestart met niet-geadapteerd slib. Door echter de pH in de reactor op een waarde van 6.7 te brengen, kon de methaanvorming selectief en duurzaam onderdrukt worden (hoofdstuk 5). Dit is een vrij eenvoudige manier om methaanvorming te onderdrukken, welke mogelijk ook bruikbaar is in praktijk installaties op grotere schaal.

In vergelijking met methaan en sulfide werd acetaat altijd slechts als minder belangrijk nevenprodukt (meestal minder dan 1 mM in het effluent van de reactor) uit methanol gevormd in de eerste bioreactor-experimenten welke beschreven zijn in hoofdstuk 2. In latere experimenten, beschreven in hoofdstuk 6 en 7, werd duidelijk meer acetaat gevormd in sulfaat- sulfietreducerende bioreactoren, met acetaatconcentraties in het effluent tot 9 mM, waarbij 13% van het afgebroken methanol naar acetaat werd omgezet. Batch-experimenten toonden aan dat acetaat niet als elektronendonor voor reductie van sulfaat en koolzuur diende, wat er op wijst dat acetaat een eindproduct is van methanolafbraak en geen tussenprodukt. Een opmerkelijk kenmerk van het proces is dat de acetogene COD-omzettingssnelheid lineair gecorreleerd is aan de sulfidogene COD-omzettingssnelheid (hoofdstuk 6). Hoewel minder duidelijk, geldt een dergelijk correlatie ook voor de gegevens van reactorexperimenten beschreven in hoofdstuk 2 en 7

(Figure1). Deze eperimenten werden bij zeer vergelijkbare reactorcondities uitgevoerd. Het verband tussen de vorming van sulfide en acetaat wijst er op dat de micro-organismen welke betrokken zijn bij sulfaatreductie, ook direct of indirect betrokken zijn bij de vorming van acetaat. Regressie-analyse van de gegevens (waarbij de regressielijn door de oorsprong loopt om vergelijking te vergemakkelijken) laat zien dat in het experiment in hoofdstuk 6, gemiddeld 0.14 gCOD-methanol wordt omgezet naar acetaat per gCOD-methanol gebruikt voor sulfaat/sulfietreductie (regressielijn B in Figure1). In de andere twee experimenten is deze verhouding slechts 0.05 to 0.06 (regressielijnen A en C, gegevens uit respectievelijk hoofdstuk 2 en 7). De relatieve kleine afwijking tussen regressielijnen A en C zijn mogelijk toe te schrijven aan de natuurlijke spreiding van de gegevens, maar de sterke afwijking tussen lijn B en de lijnen A en C wijst er op dat de verhouding tussen de hoeveelheid gevormd acetaat en sulfide uit methanol niet vastligt maar blijkbaar afhangt van een of meer onbekende variabelen. Een gedetailleerde analyse van de gegevens en vergelijking van de experimentele opzet en de condities van de drie experimenten heeft echter geen duidelijk aanwijzingen voor een dergelijke variabele opgeleverd. Hiervoor is meer onderzoek nodig.



Figuur 1. Acetaat vs. sulfidevorming uit methanol in sulfaat- en sulfietreducerende bioreactoren. Symbolen: (+): gegevens uit hoofdstuk 2; (●):gegevens uit hoofdstuk 6; (○):gegevens uit hoofdstuk 7.

De resultaten geven aan op dat de vorming van acetaat niet gereduceerd kan worden zonder vermindering van sulfidevorming. Indien methanol wordt toegepast voor biologische ontzwaveling van rookgassen, moet derhalve een compromis gevonden worden tussen de vorming van sulfide en acetaat.

Het is noemenswaardig dat in de experimenten beschreven in hoofdstuk 2, geen kobalt werd toegevoegd aan de thermofiele reactoren, omdat verwacht werd dat kobalt mogelijk de ongewenste vorming van methaan en acetaat uit methanol zou stimuleren, analoog aan het effect van kobalt onder mesofiele condities[4]. Echter, zoals werd aangetoond in hoofdstuk 7, wordt methaanvorming onder thermofiele sulfaat- en sulfietreducerende condities net zo snel onderdrukt met als zonder kobalt. Anderszijds wordt, door kobalt weg te laten uit het influent weliswaar een lagere acetaatvorming gevonden, maar wordt ook de sulfidevorming uit methanol in gelijke mate verminderd. Hieruit blijkt dat de selectiviteit van het proces niet verbetert door het aanleggen van kobalt-limiterende condities.

2.2 Het functioneren van de sulfaat- en sulfiet reducerende bioreactoren gevoed met methanol

De concurrentiepositie van biologische rookgasontzwaveling ten opzichte van andere processen wordt grotendeels bepaald door de omzettingsnelheden welke bereikt kunnen worden. Lage omzettingssnelheden door matige retentie van biomassa in de reactor en niet-toereikende massa-overdracht uit de bulkvloeistof naar slib moet zoveel als mogelijk worden voorkomen door het juiste reactorontwerp te kiezen. In dit opzicht lijkt de 'geëxpandeerde granulair slib bed' (EGSB) reactor een goed keuze omdat ten eerste een goede menging van de bulk vloeistof wordt bereikt door een hoge (> 2 $m.h^{-1}$) opwaartse stroomsnelheid van de vloeistof aan te leggen en ten tweede bij een dergelijke hoge stroomsnelheid alleen goed bezinkbare deeltjes in de reactor worden gehouden[8].

Hoge reductiesnelheden van sulfaat en sulfiet met methanol werden gevonden in de reactoren. Bij een hydraulische verblijftijd (HVT) van 10 h, werden maximum omzettingssnelheden van sulfiet en sulfaat van respectievelijk 6 $gSO_3^{2-}.L^{-1}.day^{-1}$ (100% sulfietverwijdering) en 4-7 $gSO_4^{2-}.L^{-1}.day^{-1}$ (40-70% sulfaatverwijdering) tegelijkertijd bereikt in EGSB-reactoren. Dit komt overeen met een sulfidogene omzettingssnelheid van methanol van 6-8 $gCOD.L^{-1}.day^{-1}$ (hoofdstuk 7). Onder deze condities was de resulterende sulfideconcentratie van ongeveer 1800 $mgS.L^{-1}$ (overeenkomend met een concentratie 'vrije'waterstofsulfide van 200 $mgS.L^{-1}$ bij een pH van 7.5) limiterend voor de snelheid van sulfaatreductie. Bij een hydraulische verblijftijd van 3-4 h werden maximale sulfiet- en sulfaatreductiesnelheden van 18 $gSO_3^{2-}.L^{-1}.day^{-1}$ (100% verwijdering) en 11-14 $gSO_4^{2-}.L^{-1}.day^{-1}$ (ongeveer 50% verwijdering) gevonden. Dit is equivalent aan een sulfidogene omzettingssnelheid van methanol van 19 $gCOD.L^{-1}.day^{-1}$ (hoofdstuk 6 en 7). In deze situatie werd de snelheid van sulfaatreductie gelimiteerd door de hoeveelheid biomassa in de reactor, welke een maximale concentratie bereikte van 9 tot 10 $gVSS.L^{-1}$.

De tijd welke nodig was om de maximale sulfiet- en sulfaatomzettingsnelheden in de reactoren te bereiken met geadapteerd opstartslib begroeg 40 tot 60 dagen (hoofdstuk 6 en 7). Voor praktische toepassingen lijkt zo'n periode acceptabel.

Uit de resultaten kan worden berekend dat het theoretisch mogelijk is om bij een HVT van 4 h, 100% verwijdering van 2.3 $gSO_3^{2-}.L^{-1}$ en 2.7 $gSO_4^{2-}.L^{-1}$ uit het influent (molaire verhouding sulfiet/sulfaat=1) te bereiken. De maximale sulfidogene omzettingssnelheid van methanol bedraagt dan 19 $gCOD.L^{-1}.day^{-1}$, resulterend in een maximale sulfideconcentratie in het effluent van 1800 $mgS.L^{-1}$. In deze berekening is aangenomen dat er geen kinetische limitaties zijn voor sulfiet en sulfaatreductie (nulde orde reactiesnelheid). Deze aanname is geldig voor sulfaatomzetting, omdat de kinetiek van sulfaatreductie beschreven kon worden met nulde orde kinetiek tot een lage 'drempel'-concentratie van 0.1 $g.L^{-1}$. Deze drempelwaarde is dermate laag dat voor praktische toepassingen de snelheid van sulfaatreductie als onafhankelijk van de sulfaatconcentratie in de reactor kan worden beschouwd. Echter, deze maximale sulfaatreducerende capaciteit kan alleen bereikt worden wanneer de reactor wordt overbelast met methanol, omdat de afbraak van methanol gekoppeld aan sulfaatreductie de Michaelis-Menten kinetiek volgt, met een *schijnbare* K_M-waarde van 0.037 $gCOD.L^{-1}$ (hoofdstuk 7). Maar bij een methanolconcentratie in de reactor boven 0.16 $gCOD.L^{-1}$ wordt nog steeds meer dan 80% van de maximale specifieke sulfidogene activiteit van het slib benut.

Met een influent dat 2.3 $gSO_3^{2-}.L^{-1}$ en 2.7 $gSO_4^{2-}.L^{-1}$ bevat, wordt de (waterstof)sulfideconcentratie limiterend bij HVTs > 4 h en dus kan de maximale sulfidogene omzettingssnelheid van methanol niet worden gehandhaafd tenzij (waterstof)sulfide uit de reactor wordt verwijderd, bijvoorbeeld door strippen. Bij HVTs < 4 h, limiteert biomassaretentie de sulfiet/sulfaatreductiesnelheid en hoewel de maximale sulfidogene omzettingssnelheid van methanol gehandhaafd blijft, wordt sulfaat niet meer volledig verwijderd.

Uit het bovenstaande wordt duidelijk dat een hogere sulfidogene omzettingssnelheid van methanol dan 19 $gCOD.L^{-1}.day^{-1}$ alleen bereikt kan worden als de biomassaretentie van het proces wordt verbeterd. De biomassaretentie van maximaal 9 $gVSS.L^{-1}$ is laag in vergelijking met de biomassaconcentraties van 20-30 $gVSS.L^{-1}$ welke in andere studies met EGSB-reactoren bereikt werden[9]. De relatief slechte biomassaretentie in de reactoren is toe te schrijven aan het vlokkige karakter van het slib, in tegenstelling tot het zeer goed bezinkbare slib in methanogene EGSB-reactoren. Omdat de uitdrukking geëxpandeerd *granulair* slib bed reactor verwijst naar het type slib dat zich ontwikkelt in de reactor in plaats van naar de reactor zelf, is dit niet een geschikte naam voor het gebruikte reactor-

type. De reactor kan beter aangeduid worden met 'hoge-opstroom anaërobe slib bed (H-UASB) reactor. Een verbetering van de biomassaretentie in de reactor kan mogelijk worden bereikt door verlaging van de opstroomsnelheid (v_{up}) naar waarden lager dan de toegpaste waarde van 3-6 $m.h^{-1}$, omdat dit resulteert in minder expansie van het slibbed waardoor meer slib in de reactor wordt gehouden. Echter het contact tussen medium en biomassa wordt mogelijk ontoereikend bij een v_{up} lager dan 2.5 $m.h^{-1}$, zelfs wanneer enig biogas wordt gevormd[7]. In verband hiermee moet ook worden opgemerkt dat al beneden een v_{up} van 3 $m.h^{-1}$ het onderste gedeelte van het slibbed, of soms zelfs het gehele slibbed af en toe volledig aggregeerde, resulterend in kanaalvorming en 'zuiger'vorming van het slibbed. Aggregatie van het bed verergert waarschijnlijk bij nog lagere opstroomsnelheden, hoewel dit gedeeltelijk voorkomen kan worden door lichte menging van het slibbed. Gezien het bovenstaande lijkt een aanzienlijke verbetering van biomassaretentie in de H-UASB reactoren moelijk te realiseren onder de aangelegde condities. Betere biomassaretentie wordt mogelijk wel verkregen door de dichtheid van slibdeeltjes te verhogen door bijvoorbeeld een dragermateriaal aan de reactoren toe te voegen, zoals in gefluïdiseerd-bed reactoren.

2.3 Microorganismen betrokken bij thermofiele sulfaatreductie met methanol

Om het inzicht in het proces te verbeteren zijn de dominante micro-organismen in het slib uit een thermofiele, methanol-gevoede, sulfaat-reducerende reactor bestudeerd. De vorming van sulfide, methaan en acetaat in bioreactoren opgestart met niet-geadapteerd slib (hoofdstuk 2), verried de aanwezigheid in het slib van sulfaatreducerende bacteriën (SRB), methanogene archaea (MA) en (homo)acetogene bacteriën (AB).

In hoofdstuk 2 werd aangetoond dat de dominante methaan-producerende micro-organismen in het slib waterstofafbrekende methanogenen zijn in plaats van methanol-afbrekende methanogenen. Aangezien waterstof waarschijnlijk de belangrijkste elektronendonor voor methanogenese is en omdat het slib ook snel sulfaat reduceerde met waterstof, kan geconcludeerd worden dat MA en SRB voornamelijk competeren voor waterstof. Dit kan verklaren waarom sulfaatreductie uiteindelijk domineert ten opzichte van methanogenese in de reactor, omdat uit de literatuur bekend is dat SRB de competitie voor waterstof winnen van MA indien voldoende sulfaat aanwezig is[5,10].

Een sulfaatreducerende bacterie, stam WW1, werd geïsoleerd uit de hoogste methanol-afbrekende, sulfaatreducerende seriële verdunning van het slib. Dit duidt er sterk op dat het de meest voorkomende sulfaatreduceerder is welke betrokken is bij sulfidogene methanolafbraak (hoofdstuk 3). Stam WW1 werd geïdentificeerd als een

Desulfotomaculum-species door zijn morfologie en 16SRNA-sequentie. In het slib is stam WW1 niet beperkt tot het gebruik van methanol, omdat de stam ook groeit op afbraakprodukten van methanol, zoals acetaat, formiaat en H_2/CO_2. Daarom kan methanol in het slib direct gebruikt worden als elektronendonor voor sulfaatreductie door op stam WW1-gelijkende bacteriën, maar het is ook mogelijk dat methanol eerst wordt afgebroken naar tussenproducten als waterstof, formiaat en acetaat door bijvoorbeeld (homo)acetogenen, gevolgd door sulfidogene oxidatie van deze tussenprodukten.

Twee waarnemingen wijzen erop dat waterstof inderdaad een belangrijke elektronendonor voor sulfaatreductie is in het op methanol gekweekte slib. Ten eerste gebruikt stam WW1 geen acetaat in aanwezigheid van H_2. Het niet afgebroken worden van acetaat door reactorslib is daarom mogelijk te verklaren door aan te nemen dat waterstof de belangrijkste elektronendonor is voor sulfaatreductie. Ten tweede stopt groei van stam WW1 op methanol en sulfaat al bij een totale sulfideconcentratie van 220 $mgS.L^{-1}$, door toxiciteit van het gevormde sulfide, terwijl groei van stam WW1 op waterstof en sulfaat tot 640 $mgS.L^{-1}$ mogelijk is. Deze laaste sulfide concentratie is ongeveer even hoog als de maximale totale sulfideconcentratie in de bioreactor van waaruit de stam werd geïsoleerd. Indien stam WW1-achtige sulfaatreduceerders inderdaad de meest belangrijke sulfaatreduceerders in de reactor zijn, dan is de hoge sulfideconcentratie in de bioreactor mogelijk het gevolg van sulfaatreductie met waterstof in plaats van methanol.

De hypothese dat waterstof een belangrijke elektronendonor voor sulfaatreductie is in het slib houdt in dat tenminste een deel van het afgebroken methanol wordt omgezet naar waterstof (en CO_2). De aanwezigheid van hoge aantallen methanol-oxiderende, waterstof-producerende bacteriën in het slib werd inderdaad aangetoond (hoofdstuk 4). Methanoloxidatie naar waterstof gevolgd door sulfaatreductie met waterstof vormt dus mogelijk een belangrijke afbraakroute van methanol in het slib. Het is echter niet duidelijk in welke mate methanol direct wordt gebruikt door sulfaatreduceerders in het slib of eerst wordt omgezet naar waterstof gevolgd door omzetting van waterstof voor sulfaatreductie.

Het is noemenswaardig dat in de niet-gedefinieerde cultures waarin de aanwezigheid van methanoloxiderende, waterstofproducerende bacteriën werd aangetoond, ook acetaat werd gevormd uit methanol (hoofdstuk 4). In de cultures met de methanol-oxiderende, waterstof-producerende species en een toegevoegde waterstofafbrekende sulfaatreduceerder, ongeveer 15% van het ethanol naar acetaate werd omgezet. Bovendien scheen de vorming van acetaat strikt gekoppeld te zijn aan groei van de methanol-oxiderende bacteriën. De koppeling van methanolafbraak naar waterstof en vorming van acetaat uit methanol in de cultures is verklaart mogelijk de koppeling van sulfide en acetaatvorming uit methanol in de reactoren.

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Abbreviations

AB: acetogenic bacteria

Ac: acetate

Bio-FGD: biological flue gas desulfurization

BRES: 2-bromoethanesulfonate

COD: chemical oxygen demand

EGSB: expanded granular sludge bed

HRT: hydraulic retention time (h^{-1})

MA: methanogenic archaea

MeOH: methanol

OLR: organic loading rate ($gCOD.L^{-1}.day^{-1}$)

PS: polysaccharide

SAA: specific acetogenic activity ($gCOD.gVSS^{-1}.day^{-1}$)

SLR: sulfate loading rate ($gSO_4^{2-}.L^{-1}.day^{-1}$.)

SMA: specific methanogenic activity ($gCOD.gVSS^{-1}.day^{-1}$)

SMDR: specific methanol degradation rate ($gCOD.gVSS^{-1}.day^{-1}$)

SRB: sulfate-reducing bacteria

SSA: specific sulfidogenic activity ($gCOD.gVSS^{-1}.day^{-1}$)

SVI: sludge volume index (mL sludge per gTSS)

TS: total sulfide = the sum of the H_2S, HS^- and S^{2-} species

TSS: total suspended solids

UASB: upflow anaerobic sludge blanket

VAC: volumetric acetogenic COD-conversion ($gCOD.L^{-1}.day^{-1}$)

VFA: volatile fatty acids

VMC: volumetric methanogenic COD-conversion (gCOD.L-1.day-1)

VSC: volumetric sulfidogenic COD-conversion ($gCOD.L^{-1}.day^{-1}$)

VSS: volatile suspended solids

Dank- en nawoord

Op een niet meer te herinneren moment wordt het dan toch concreet. De sub-directory (folder volgens nieuwlichters) 'proefschrift' wordt gemaakt. Veel meer dan een wat vaag idee hoe het boekje eruit zou moeten zien is er nog niet. En ik had nog geen idee hoeveel uren het zou gaan kosten, wat maar goed is ook. Maar de berg is dan toch, eindelijk, beklommen. Hoewel een promotie-onderzoek toch al gauw een twintigste deel van een mensenleven omvat, lijkt het nog maar kort geleden dat ik Wageningen voor de eerste keer bezocht vanuit het 'hoge noorden' voor het sollicitatiegesprek. Het verblijf in Wageningen heeft me in velerlei opzicht meer gebracht dan ik toen had kunnen vermoeden. Het was wel even wennen in het begin. Maar na een tijdje keek ik niet meer verbaasd op van anaëroob korrelslib met een specifieke methanogene aktiviteit van 1 gCOD.gVSS-1.dag-1. Daarna kwam het gezwoeg met de reactoren waarbij menig storing het onderzoekershumeur danig bedierf. Voorvallen zoals het volledig overstromen van het 'modulair lab' op oudejaarsdag 1996 hielpen daar niet bepaald bij. Hoogtepunten waren er ook, zoals het bezoek aan het IWA-congres in Merida, Mexico. Het werken op de twee vakgroepen Milieutechnologie en Microbiologie gaf het voorrecht van het hebben van veel collega's, waarvan velen mijn verblijf in Wageningen hebben veraangenaamd. Niet alleen binnen de gebouwen van de universiteit, maar ook op het (zaal)voetbalveld (een blik op de ranglijst van de zaalvoetbalcompetitie bracht een feestelijk humeur), op de squashbaan, op de bospaden van de Veluwe, in de kroeg enzovoort. De ruimte ontbreekt hier om jullie allemaal te noemen, maar mijn dank daarvoor is daarom niet minder groot. De reis naar Berlijn samen met Ans, Alette, Caroline, John, Hauke en Fons voor het lopen van de plaatselijke marathon is voor mij onvergetelijk.
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Curriculum vitae

De auteur van dit proefschrift werd geboren op 10 juli 1967 te Leeuwarden. Na het behalen van het VWO-diploma in 1986 werd de studie Chemische Technologie aan de HTS in Leeuwarden begonnen. Hier werd de belangstelling gewekt voor bio(proces)technologie. Daarna volgde in 1990 de studie Technische Scheikunde aan de Rijksuniversiteit Groningen, met als specialisatie biotechnologie. Gedurende deze laatste studie werd een stageperiode van 4 maanden doorgebracht aan de Technische Universiteit van Lyngby, Denemarken. Hier werkte de auteur mee aan een onderzoek naar biologische aërobe afbraak van chlooralkenen. Na het behalen van de bul volgde een onvrijwillig maar nuttig uitstapje naar de Koninklijke Landmacht als operatiekamer-assistent, 'gelegerd' in ziekenhuis de Tjongerschans in Heerenveen. In maart 1995 werd de auteur aangesteld als STW-onderzoeker in opleiding bij de vakgroepen Microbiologie en Milieutechnologie van de Landbouwuniversiteit Wageningen. Het onderzoek betrof het gebruik van methanol als elektronendonor voor sulfaatreductie in anaërobe bioreactoren, hetgeen leidde tot het onderhavige proefschrift. Sinds oktober 1999 is de auteur als post-doc onderzoeker werkzaam bij de sectie Milieutechnologie van Wageningen Universiteit, in het kader van een EET-onderzoek naar gecombineerde biologische sulfaat-reductie en zinksulfide precipitatie.