Microbial hydrogenogenic CO conversions: applications in synthesis gas purification and biodesulfurization

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ABSTRACT

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Hydrogen gas attracts great interest as a potential clean future fuel and it is an excellent electron donor in biotechnological reductive processes, e.g. in biodesulfurization. Bulk production of H_2 relies on the conversion of organic matter into synthesis gas, a mixture of H_2, CO and CO_2. The relative abundance of CO restricts its applicability, due to toxicity to hydrogenotrophic microorganisms and poisoning of chemical catalysts in low temperature fuel cells. Currently, synthesis gas purification, i.e. CO conversion to H_2, is performed in chemical catalytic systems. A recently discovered group of thermophilic anaerobic bacteria is able to grow by converting CO with water to H_2 and CO_2. This feature makes these hydrogenogens interesting for cost effective hydrogen production.

Several anaerobic wastewater treating sludges harbor CO utilizing moderately thermophilic (55°C) hydrogenogenic microorganisms. CO conversion at 30°C resulted in the production of acetate, whereas at 55°C it proceeded via H_2/CO_2. One of the tested sludge samples could even reduce sulfate with the CO-derived H_2, tolerating and using high CO concentrations (P_{CO}>160 kPa). From this sludge a moderately thermophilic, anaerobic, sulfate-reducing bacterium was isolated, i.e. *Desulfotomaculum carboxydivorans*, capable of growth on CO as sole energy and carbon source both in the presence and absence of sulfate as electron acceptor. *D. carboxydivorans* grows rapidly at 200 kPa CO, pH 7.0 and 55°C (t_d of 100 minutes), producing nearly equimolar amounts of H_2 and CO_2 from CO revealing a high specific CO conversion rate of 0.8 mol CO.(g protein)$^{-1}$.hour^{-1}. Furthermore, *D. carboxydivorans* is capable of hydrogenotrophic sulfate reduction at partial CO pressures exceeding 100 kPa, at a maximal specific sulfate reduction rate of 32 mmol.(g protein)$^{-1}$.hour^{-1}. These characteristics make it an interesting candidate for synthesis gas purification as well as for the direct use of synthesis gas in biodesulfurization at elevated temperatures. Although in the latter case, the low sulfide tolerance of *D. carboxydivorans*, i.e. total inhibition at 5 mM and 9 mM at pH 6.5 and 7.2, respectively, may require special features to maintain sufficient low sulfide concentrations.

Thermophilic sulfate reduction using CO as electron donor with anaerobic granular sludge, from which *D. carboxydivorans* originated, showed that despite the high CO conversion capacity of the biomass present, the sulfate reduction capacity was limited due to strong competition for the produced H_2. Operation at HRT >9 hours resulted in a predominant consumption of the CO-derived H_2 by methanogens (up to 90%) and thus in a poor sulfate reduction efficiency (<15%). Although, the methanogens appeared to be more sensitive to pH and temperature shocks imposed to the reactor, they were not eliminated by these treatments. The high growth rates of the methanogens (t_d of 4.5 hours) resulted in fast recovery and domination of the

consumption of CO-derived H_2 by methanogens. At HRT <4 hours, the consumption of CO-derived H_2 was dominated by the sulfate reducing bacteria (up to 95%). The highest sulfate reduction rates achieved were 17 mmol.L^{-1}.day^{-1} at a HRT of 3 hours (87% of the H_2 used by sulfate reducers). These rates were limited by the amount of CO supplied and the CO conversion efficiency (85%) at higher CO loads (106 mmol.L^{-1}.day^{-1}), probably as a result of limited biomass retention in the reactor.

Elimination of methanogenesis is a prerequisite for practical application of both synthesis gas utilization as electron donor for thermophilic sulfate reduction processes and synthesis gas purification. Short-term (90 minutes) pretreatment of the sludge at 95°C eliminates methanogenesis, but not homoacetogenesis. Although, homoacetogens did not seem to reduce the electron flow towards sulfate reduction much, their activity represents an unwanted loss of H_2. For practical applications a complete heat-treatment of the sludge at temperatures exceeding at least 85ºC and treatment of the (empty) reactor system using steam as well as additional measures to prevent introduction of a methanogenic population could be considered.

Contents

1

Introduction

Synthesis gas, a mixture of H_2, carbon monoxide (CO) and carbon dioxide (CO_2), is produced by thermal gasification of a wide range of types of organic matter, including fossil fuels and biomass. Synthesis gas is the main source for bulk H_2 production. The current interest in H_2 as energy carrier ensures the need for cost effective production processes of high purity H_2. Besides its potential as a future energy carrier, H_2 is a potent electron donor in various reductive processes, both in chemical and biotechnological applications. However, the presence of CO limits its direct application, because of its toxic and poisoning effects, and extensive purification of the H_2 is required prior to its utilization. These purification processes are nowadays performed in so-called water-gas-shift reactors employing chemical catalysts at high temperatures and pressures. In these catalytic processes CO is converted with water into CO_2 and H_2, and thus increases the total H_2 yield as well as removes the unwanted CO.

The discovery of fast growing anaerobic thermophilic bacteria capable of performing the same CO conversion to H_2, presents an interesting alternative for the chemical catalytic process. These bacteria have been designated as **hydrogenogens** due to their capacity to metabolically convert CO coinciding with the formation of H_2 (Svetlichnyi et al., 2001), analogue to the use of the term methanogens for methane producing microorganisms. Furthermore, the terms **hydrogenogenic** and

hydrogenogenesis were proposed to refer to the type of metabolism and process of hydrogen formation by these microorganisms (Svetlichnyi et al., 2001).

In this thesis a study on microbial hydrogenogenic CO conversions with applications in synthesis gas purification and biotechnological desulfurization is presented.

Structure of the thesis

Chapter 2 presents an overview of anaerobic microorganisms able to convert CO present in synthesis gas. This chapter specially focuses on anaerobic microorganisms capable of hydrogenogenic CO conversion and their potential applications in biotechnology. **Chapter 3** describes the occurrence of moderately thermophilic hydrogenogenic bacteria in readily available anaerobic bioreactor sludges. **Chapter 4** presents the results of the effects of the CO concentration and H_2 on the hydrogenogenic CO conversion by two selected bioreactor sludges. Furthermore, results are presented in which the fate of sulfate is elucidated under an atmosphere of pure CO. From one of the investigated sludges the first sulfate reducer with apparent uninhibited growth on CO was isolated, which is described in **Chapter 5**. A detailed physiological study of this moderately thermophilic sulfate reducer focusing on parameters with direct relevance for biotechnological applications in both sulfate reduction with synthesis gas and synthesis gas purification is presented in **Chapter 6**. The unique property of sulfate reduction with CO derived H_2 using granular sludge was investigated, with special emphasis on competition for H_2 is presented in **Chapter 7**. In **Chapter 8**, the effect of decreased hydraulic retention times (HRT) on CO conversion and sulfate reduction in a gas lift reactor is presented. Finally, in **Chapter 9** the results of the work presented in this thesis are discussed and summarized, in English, and in Dutch.

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Biological hydrogenogenic CO conversions and their potential biotechnological applications

Abstract

A recently discovered group of thermophilic anaerobic bacteria is able to grow by converting CO with water to H_2 and CO_2. This feature makes these carboxydotrophic bacteria interesting for cost effective hydrogen production. Hydrogen gas attracts great interest as a potential clean future fuel for use in fuel cells and it is an excellent electron donor in biotechnological processes, especially in bio-desulfurization. Bulk production of H_2 relies on the conversion of fossil fuels, biomass or hydrocarbon rich wastes into synthesis gas by gasification, partial oxidation or steam reforming. These processes result in synthesis gas mainly composed of H_2, CO and CO_2. The relative abundance of carbon monoxide restricts its applicability in energy production by fuel cells and in biotechnological processes. CO is toxic to the catalyst in low temperature fuel cells as well as to many hydrogenotrophic microorganisms. Conversion of CO to H_2 will result in a greater utilization potential of synthesis gas. Currently, this CO conversion is performed in chemical catalytic systems. Application of H_2-evolving carboxydotrophic thermophiles in a biotechnological process allows more cost effective production of H_2 from synthesis gas. Furthermore, integration of CO conversion to H_2 in existing biotechnological processes enables the use of less pure H_2 streams and is expected to result in a significant reduction of operational costs.

2.1 Introduction

Hydrogen gas has great potential as a future fuel, as the greenhouse gas CO_2 is not produced upon its combustion. The transit from the current unsustainable fossil fuel based economy into a hydrogen economy may be realized gradually, in which fuel cells are the key energy providers both for stationary and mobile applications (Gosselink, 2002). Fuel cells, such as the low temperature proton exchange membrane fuel cell (PEMFC) and the high temperature solid oxide fuel cell (SOFC), hold the promise to develop into the ultimate universal, efficient, clean and silent devices for converting chemical energy directly into electricity. Progress on both fuel cells (Appleby, 1995) and H_2 storage alloys (Sandrock, 1999) was made and their applications are close to market penetration. These issues are, however, out of the scope of this paper.

Besides the use as a fuel, hydrogen is a potent electron donor for various reductive reactions in biotechnology and in chemical process industry. Processes in which H_2 was shown to be an excellent electron donor include mesophilic and thermophilic sulfate reduction (Van Houten et al., 1997; 1994), denitrification of drinking water (Szekeres et al., 2001; Kurt et al., 1987), reductive dehalogenation (Ferguson and Pietari, 2000) and catalytic hydrogenation of biomass as green alternative for the production of high demand commodity chemicals, e.g. 1,2-propanediol (Cortright et al., 2002).

Hydrogen is only a true "green" alternative for fossil fuels in case of hydrogen production from biomass fermentation or by extraction of H_2 from water utilizing renewable energy sources like wind, photovoltaics, geothermal energy and biomass. Increasing energy demands can be expected as a result of world population growth and their increased level of development. However, complete transformation from the current fossil fuel based to sustainable energy markets will take considerable time, if even possible at the present mineral fuel consumption in the prosperous world. In the meantime the fossil fuel based energy markets have to be transformed to mitigate the impact of CO_2 released by the use of fossil fuels on the earths climate. The production of H_2 gas from fossil fuels will be important in transition to a more sustainable energy production (Gosselink, 2002). Gasification of fossil fuels and subsequent purification

of the produced synthesis gas may yield a highly purified H_2 gas and could prevent the scattered production of CO_2, although the total amount of CO_2 produced will not decrease.

A potential way to decrease overall CO_2 emissions to the earths atmosphere is CO_2 fixation by carbon sequestration processes. Carbon sequestration would be feasible in case of centralized or large scale H_2 production. The use of fuel cells for generation of electricity with combined CO_2 capturing, concentration and sequestering is in line with current decarbonization trends (Gosselink, 2002; Anonymous, 1997). Several options for sequestration in geological formations have been evaluated, all with different ratings on capacity, costs, storage integrity and technical feasibility. In this respect carbon sequestration in active oil wells, coal beds, depleted oil/gas wells, deep aquifers, mined caverns/salt domes is considered (Gosselink, 2002). In addition, chemical sequestration could attain more social acceptance for the abatement of CO_2 emissions. However, chemical CO_2 sequestration options are limited. A very promising option of carbon sequestration is mineral carbonation, in which a (pressurized) CO_2 stream is reacted with magnesium or calcium silicate mineral deposits in an exothermic reaction to form geologically stable mineral carbonates. The reaction is part of the natural geological carbon cycle, and can be accelerated through technology. The mineral end products are naturally occurring and harmless. Furthermore, this technology offers a more permanent solution than gas storage, thereby eliminating legacy issues for future (Gosselink, 2002).

2.2 Synthesis gas

2.2.1 Synthesis gas production and composition

Synthesis gas can be produced by gasification of organic sources, like coal, cokes, oil and natural gas (Armor, 1999; Bartish and Drissel, 1978) as well as from biomass (Van der Drift et al., 2001) or industrial and municipal solid (organic) wastes (Belgiorno et al., 2003). Synthesis gas consists mainly of a mixture of H_2, CO and CO_2 and minor amounts of other gasses, e.g. methane, nitrogen and hydrogen sulfide (Perry et al., 1997). Besides the use of synthesis gas as fuel, it is used as a major

building block in the synthesis of various chemicals, e.g. in the synthesis of methanol and acetic acid (Armor, 1999; Bartish and Drissel, 1978).

Synthesis gas can be obtained by direct gasification, i.e. processes in which oxygen or air is used as the oxidant, as in partial oxidation of coal. These partial oxidation reactions with oxygen are exothermic (Armor, 1999). Indirect gasification employs steam as the oxidant, resulting in an endothermic and often heat transfer limited, but thermodynamically more efficient process (Armor, 1999). The composition of synthesis gas varies greatly depending on its source and the gasification conditions employed. The CO concentration in synthesis gas increases with increased C to H ratio in the hydrocarbon feed, as is illustrated for steam reforming of coal and natural gas (methane) by equations 1 and 2.

$$C_{(s)} + H_2O \rightarrow CO + H_2 \qquad \Delta G^0 = 100 \text{ kJ.mol}^{-1\#} \quad (1)$$

$$CH_4 + H_2O \rightarrow CO + 3 H_2 \qquad \Delta G^0 = 151 \text{ kJ.mol}^{-1\#} \quad (2)$$

Therefore, steam reforming of natural gas theoretically results in lower CO levels than coal gasification. Process conditions influence synthesis gas composition as well, e.g. by synthesis gas exit temperatures or by the steam-to-feed carbon ratio (Armor, 1999; Bartish and Drissel, 1978). Table 2.1 presents characteristic compositions of synthesis gas produced from different fossil sources.

[#] $\Delta G°$ value was calculated using the data set of Amend and Shock (2001).

Table 2.1 Typical composition of synthesis gas derived from various fossil fuel sources.

Fossil source	Composition vol%, dry basis						Ref.
	CO	CO_2	H_2	N_2	CH_4	Other	
Coke oven gas	5.6	1.4	55.4	4.3	28.4	4.9	1
Water gas	30.0	3.4	31.7	13.1	12.2	9.6	1
Natural gas, steam reforming	15.5	8.1	75.7	0.2	0.5	0	1
Naphta, steam reforming	6.7	15.8	65.9	2.6	6.3	2.7	1
Partial oxidation of heavy fuel oil	47.0	4.3	46.0	1.4	0.3	1.0	2
Coal gasification	59.4	10.0	29.4	0.6	0	0.6	1

References: [1] Bartish and Dissel. 1978; [2] Van Houten and Lettinga, 1996.

The gasification of solid waste and biomass is generally more complex due to heterogeneity of the carbon-based materials. For an efficient gasification process certain homogeneity is required (Belgiorno et al, 2003). Therefore, not all kinds of waste can be gasified as the required pre-treatment makes the process uneconomical. Waste types that are promising for production of synthesis gas include paper mill waste, mixed plastic waste, forest industry waste and agricultural residues (Belgiorno et al, 2003). A major difference between fossil fuel gasification and biomass gasification is the method usually adopted. With fossil fuels indirect gasification, with steam is the preferred method. While with biomass usually a gasification method is employed that uses air as the oxidant (Belgiorno et al, 2003). The high content of nitrogen in the produced synthesis gas results in relative low heating values and classifies such synthesis gas as a low-grade gas. The use of pure oxygen improves the quality of the produced synthesis gas considerably, but it will also increase the costs for production of the synthesis gas. Maschio et al. (1994) investigated a combined pyrolysis with indirect steam gasification (up to 950°C) of mixed woody biomass. This resulted in a synthesis gas with high concentrations of H_2 (20 – 50%) and CO (15

– 30%), which demonstrates the potential of biomass in the production of high quality synthesis gas. Another interesting method to convert biomass into a gaseous energy carrier is supercritical water gasification. This process directly deals with wet biomass without prior drying and is characterized by high gasification efficiencies at lower temperatures (Matsumura, 2002). A supercritical fluid is defined as a substance at conditions of temperature and pressure above its vapor-liquid critical point, i.e. for water above 374°C and 22 MPa. At supercritical conditions water shows characteristics between steam and liquid water. Supercritical water in particular has the ability to dissolve materials that are normally insoluble in water or steam. It also seems to promote specific chemical reactions that are beneficial for the gasification process (Matsumura, 2002).

Analysis of synthesis gas produced in an atmospheric circulating fluidized bed gasifier from ten residual biomass fuels, e.g. demolition wood, verge grass, cacao shells, revealed around 10% CO, 7% H_2 , 16% CO_2, 2.8% CH_4, and N_2 exceeding 60%, as main constituents (Van der Drift et al., 2001). Within the ten tested samples these concentrations were quite similar, except for the woody fraction of the organic domestic waste, which had a rather low yield in CO and H_2 and consequently contained more N_2 in the synthesis gas (Van der Drift et al., 2001). Faaij et al. (1997a) derived synthesis gas compositions of comparable biomass sources based on lab-scale fuel reactivity tests and subsequent gasifier model calculations, which are slightly higher in dry gas CO content (16-21%) and H_2 content (around 14%), similar in CO_2 content (16%), but considerably lower in N_2 content (around 47%). In another study, the gasification potential of a broad range of biomass wastes and residues in the Netherlands was evaluated (Faaij et al., 1997b), especially waste materials that are available at negative costs, i.e. when landfilling or incineration are the treatment routes, could serve as interesting feed stocks for synthesis gas production. Potential waste streams for gasification include waste paper, demolition wood, verge grass, organic domestic waste, organic rest products from the food industry and sewage sludge (Faaij et al., 1997b), waste products from agricultural crops, e.g. sugarcane waste (Dellepiane et al., 2003) and manure (Young and Pian, 2003). Sewage sludge and manure, however, are difficult materials due to the high nitrogen, sulfur, heavy metal, and high ash content. For use in gasification processes sewage sludge should be

mixed with other waste streams to ensure sufficient calorific value of the resulting synthesis gas (Faaij et al., 1997a). Nevertheless, sewage sludge is an interesting source for synthesis gas production (Midilli et al., 2002) as the overall sludge production is currently rapidly increasing (Werther and Ogada, 1999).

Despite the numerous research activities in the field of biomass gasification still important bottlenecks have to be overcome before biomass gasification can be economical viable for large-scale synthesis gas and energy production (Sutton et al., 2001). Especially the presence of condensable organic compounds (tars) and the resulting gas composition ensures the need for extensive gas cleaning to allow advanced gas utilization. Currently research is focused at catalytic processes for biomass gasification (Sutton et al., 2001).

2.2.2 Chemical catalytic water-gas-shift reaction

The major restriction of synthesis gas application is the presence of CO, which can range from as little as 5% to over 50% in case of coal gasification (Table 2.1). Biological reductive processes utilizing H_2 are generally quite sensitive to CO inhibition, e.g. sulfate reduction with synthesis gas was shown to be hampered by the presence of 5% CO (Van Houten *et al.*, 1996). In PEMFC less than 10 ppm CO is desired in order to ensure economical operation of this type of fuel cells, due to CO poisoning of the platinum catalyst (Ledjeff-Hey et al., 2000). Therefore, it is essential to remove CO, preferentially by conversion to hydrogen. This results in a conversion of energy carrier, but not a significant loss of calorific value as the heat of combustion at standard conditions of H_2 and CO is similar at 286 kJ.mol^{-1} and 283 kJ.mol^{-1}, respectively (Lide, 2001).

In order to convert synthesis gas into a highly purified H_2 gas, the CO present in synthesis gas is reacted with water over a metal catalyst yielding CO_2 and H_2. This reaction is known as the water-gas-shift reaction (reaction 3)

$$CO + H_2O \rightarrow H_2 + CO_2 \qquad\qquad \Delta G° = -20 \text{ kJ.mol}^{-1\,[\#]} \quad (3)$$

[#] $\Delta G°$ value was calculated using the data set of Amend and Shock (2001).

The water gas shift reaction is predominantly carried out in a two-stage catalytic process. The first stage, also known as high temperature shift conversion, proceeds between 315 and 510°C using a reduced iron catalyst. At space velocities of 2000 to 4000 h^{-1} exit CO concentrations ranging from 2-5 % can be obtained. Water to CO ratios of 2 to 4 are used to force the equilibrium towards hydrogen production (Armor, 1999; Bartish and Drissel, 1978). The second stage requires a more active zinc oxide-copper oxide catalyst, which enables operation at lower, thermodynamically more favorable, temperatures. The resulting enriched H$_2$ gas can have a CO-content as low as 0.2%. The residual CO generally is hydrogenated to methane using a reduced nickel catalyst after removal of bulk carbon dioxide (Bartish and Drissel, 1978).

Catalytic processes are generally easily poisoned by hydrogen sulfide (Bredwell et al., 1999). Therefore, a biological alternative, tolerating higher hydrogen sulfide concentrations and operating under far more moderate conditions (pressure, temperature) will become undoubtedly of interest for all industries, which produce or use synthesis gas.

2.3 Occurrence of carbon monoxide

2.3.1 Anthropogenic sources of CO

Besides its occurrence as a major component of synthesis gas, carbon monoxide is an abundant atmospheric pollutant generated to a large extent by incomplete combustion of fossil fuels in domestic and industrial processes, e.g. blast furnace gas contains 25% CO, automobile exhaust gas 0.5 to 12% (Colby et al., 1985). CO occurs in the troposphere at a concentration of 0.1 ppm and in polluted urban areas its concentration has been reported to reach levels of 50-100 ppm (Colby et al., 1985). Riveros et al. (1998) measured average CO concentrations of 4 to 5 ppm in the atmosphere of Mexico City in 1992 and 1993. inside motor vehicles ambient peak CO levels in the late afternoon, as high as 70 ppm have been recorded (Atimtay et al., 2000). Furthermore, CO contributes to ground level ozone and indirect greenhouse warming (King, 1999).

2.3.2 Natural sources of CO

Naturally formed carbon monoxide can be divided in non-biological (abiotic) and biological (biotic) formation processes, which will be discussed separately in the following sections.

Abiotic sources

Photochemical reactions and thermal decomposition of organic substrates are important abiotic CO generating processes. Generation of CO by light may occur by direct photolysis or via radical mechanisms with reactive oxygen species, formed by photosensitizing reactions of molecular oxygen, ozone, or water. Photolysis of dissolved organic matter generates CO in oceans and fresh waters (Zuo and Jones, 1995; Bullister et al., 1982; Conrad et al., 1982; Conrad and Seiler, 1980). CO is formed by oxidation of atmospheric CH_4 by ·OH-radicals. In soil surfaces radical formation is a likely mechanism for CO generation. Abiotic light-independent CO production in soils may occur by auto-oxidation of phenolic compounds and humic acids, which is stimulated by heat, as found by comparing CO formation at 20 and 60ºC (Conrad and Seiler, 1985). One of the major natural sources of CO is its liberation with volcanic gases (Svetlichny et al., 1991a), which may contain 1-2% of CO (Symonds et al., 1994). Numerous other natural sources, most notably the world's oceans, contribute to CO emission to the atmosphere. It has been estimated that, the ambient concentration of CO could be expected to double within 4 to 5 years if it would not be degraded in the atmosphere. The life-time of CO in the atmosphere is about 40 days (Inman et al., 1971). Hence, CO apparently is not accumulating in the earth's atmosphere since ambient concentrations are remaining essentially constant.

Biotic sources

CO can be formed as a metabolite of microbial metabolism, e.g. heme degradation by aerobic microorganisms (Ratliff et al., 2001; O'Brien et al., 1984) and in low amounts by some thermophilic acetogenic bacteria and methanogenic archaea (Svetlichny et al, 1991a). Conrad and Thauer (1983) first reported on the production of trace levels of CO under strictly anaerobic conditions. Lupton et al. (1984) observed the production of small quantities CO by *Desulfovibrio vulgaris* under heterotrophic growth

conditions, which was later also found with *Desulfovibrio desulfuricans* (as reviewed in Davidova et al., 1994). Production of trace levels of CO was observed in the off-gas of a laboratory anaerobic digester fed with waste activated sludge (Hickey et al., 1987). In that reactor CO was produced during methanogenesis and subsequently utilized when the substrate concentration was close to depletion. The production of CO was attributed to the presence of CO dehydrogenase, present in many methanogens, as methanogens that lack CO dehydrogenase activity did not produce measurable quantities of CO in batch culture (Bott et al., 1985). Bae and McCarty (1993) observed CO formation after perturbations of methanogenic reactors fed with acetate and formate. Inconsistencies in CO production after substrate perturbations prevented to use CO as monitoring tool for the control of anaerobic treatment as proposed by Hickey et al. (1987).

2.4 Microbial CO conversion

2.4.1 CO oxidizing microorganisms

CO is metabolized by a wide variety of microorganisms. A sharp division exists between aerobic and anaerobic species, as they contain fundamentally different enzyme systems for CO biotransformation. Aerobic CO oxidizing bacteria may be divided in two groups, i.e. metabolic, in which CO oxidation provides energy for growth, and co-metabolic, in which CO is used as pseudo-substrate for the enzyme system, but does not provide a nutritional value (Colby et al., 1985). The latter is observed during aerobic CO oxidation by methane oxidizing bacteria employing the methane monooxygenase complex, which is rather unspecific with respect to its substrate (Higgins et al., 1980; Daniels et al., 1977). Aerobic metabolic CO oxidizing bacteria, also known as carboxydotrophs, use CO as a source of energy, which is oxidized with O_2 as terminal electron acceptor. These bacteria contain a specific CO tolerant cytochrome b_1 oxidase and O_2 insensitive Mo-Fe-flavin carbon monoxide dehydrogenase. Aerobic CO oxidation is not discussed in detail in this chapter, as it has been extensively reviewed elsewhere (Mörsdorf et al., 1992; Meyer et al., 1990; Meyer and Schlegel, 1983).

Whereas, aerobic CO oxidation merely results in the production of CO_2 and biomass in case of an energy-yielding CO metabolism, anaerobic conversion of CO results in the production of a range of other compounds. Representatives of various trophic groups, e.g. homoacetogens, methanogens and sulfate reducing bacteria, have been identified in CO metabolism. An overview of anaerobic microorganisms known to metabolize CO as sole source of carbon and energy is presented in Table 2.2.

CO conversion by homoacetogens

The reductive acetyl-CoA pathway is essential in acetate formation by homoacetogens (Wood and Ljungdahl, 1991). In this pathway CO_2 functions as electron acceptor. Two CO_2 molecules are reduced to form the acetyl moiety of acetyl-CoA. Further conversion of acetyl-CoA to acetate is needed for the cell to obtain a net energy gain. In the reductive acetyl-CoA pathway one ATP is invested to activate formate. This ATP is regained by substrate level phosphorylation in the conversion of acetyl-CoA to acetate. Additional chemiosmotic processes associated with the reduction of CO_2 to the methyl moiety of acetyl-CoA result in a net energy gain (Diekert, 1990; Hugenholtz and Ljungdahl, 1990). In heterotrophic homoacetogens the oxidative degradation of organic compounds provides reducing equivalents for the reduction of CO_2. In chemolithoautotrophic homoacetogens the reducing equivalents are usual derived from H_2, but in some of these homoacetogens CO can function both as carbon source and energy substrate.

The key enzyme for CO conversions is carbon monoxide dehydrogenase (CODH), which catalyzes the following reaction:

$$CO + H_2O \rightarrow 2\,H^+ + CO_2 + 2\,e^- \tag{4}$$

CODH is present in all CO oxidizing microorganisms (Mörsdorf et al., 1992) and microorganisms that synthesize or cleave acetyl-CoA in a variety of energy yielding pathways (Ferry, 1995). The acetyl-CoA pathway, therefore, is also known as the carbon monoxide pathway (Diekert, 1990). In acetogenic bacteria and methanogens, CODH normally catalyzes the reduction of CO_2 to a bound carbonyl, which then is coupled with a bound methyl group to form acetyl-CoA (Ferry, 1995; Mörsdorf et al., 1992). CODH in homoacetogens is linked with acetyl-CoA synthase (ACS) and forms an ACS/CODH complex.

Table 2.2 Overview of anaerobic microorganisms from different physiological groups that can utilize carbon monoxide as sole energy and carbon source.

CO oxidizing microorganisms[1]	T_{opt} (°C)	pH	t_d (h)	Max P_{CO} (kPa)	Products formed	Orgin	Ref.
Homoacetogens bacteria							
Moorella thermoacetica	55	6.5-6.8	10	72	Acetate, CO_2	Horse faeces	1
Moorella thermoautotrophica	58	6.1	7	214	Acetate, CO_2	Mud and wet soils	2
Clostridium autoethanogenum	37	5.8-6.0	n.r.	100	Acetate, Ethanol, CO_2	Rabbit faeces	3
Oxobacter pfennigii	36-38	7.3	13.9	40	Acetate, n-butyrate	Rumen of cattle	4
Clostridium ljungdahlii	37	6.0	3.8	105	Acetate, CO_2	Chicken yard waste	5
Peptostreptococcus productus	37	7.0	1.5	182	Acetate, CO_2	Sewage sludge digester	6
Acetobacterium woodii	30	6.8	13	n.r.	Acetate, CO_2	Mud	7
Eubacterium limosum	38-39	7.0-7.2	7	152	Acetate, CO_2	Rumen of sheep	7, 8
Butyribacterium methylotrophicum	37	6.0	12-20	120	Acetate, Butyrate, Butanol and ethanol	Sewage sludge digester	9, 10, 11
Methanogens							
Methanosarcina barkeri	37	7.4	65	101	$CH_4 + CO_2$	Anaerobic sewage digester	12
Methanosarcina acetivorans strain C2A	37	7.0	24	100	Acetate, formate, CH_4	Marine mud	13
Methanothermobacter thermoautotrophicus	65	7.4	140	45	$CH_4 + CO_2$	Sewage sludge	14
Sulfate reducing bacteria							
Desulfovibrio vulgaris (strain Madison)	37	n.r.	n.r.	<4.5	Via H_2, CO_2, H_2S	Soil	15
Desulfovibrio desulfuricans	37	n.r.	n.r.	<20	Via H_2, CO_2, H_2S	Mud, soil, rumen sheep	16
Desulfosporosinus orientis	35	7.0	n.r.	<20	H_2S, CO_2	Soil Singapore	17
Desulfotomaculum nigrificans	55	7.0	n.r.	<20	H_2S, CO_2	Soil	17
Desulfotomaculum kuznetsovii	60	7.0	n.r.	<70	Acetate, H_2S, CO_2	Underground thermal water	18
Desulfotomaculum thermobenzoicum subsp. *thermosyntrophicum*	55	7.0	n.r.	<70	Acetate, H_2S, CO_2	Methanogenic sludge	18
Elemental sulfur reducing bacteria							
Thermoproteus tenax[2]	88	5.5	n.r.	n.r.	H_2S, CO_2	Solfatarra mud hole	19

[1]   Species names according to most recent classification. *Methanobacterium thermoautotrophicum* was renamed as *Methanothermobacter thermoautotrophicus* by Wasserfallen et al. (2000) The clostridial species *C. thermoaceticum* and *C. thermoautotrophicum* were reclassified into species of a new genus *Moorella* by Collins et al. (1994). Collins et a. (1994) also proposed the renaming of *C. pfennigii* into *Oxobacter pfennigii*. *Desulfotomaculum orientis* was recently renamed *Desulfosporosinus orientis* (Stackebrandt et al., 1997).

[2]   Only grows in the presence of elemental sulfur as terminal electron acceptor

n.r.   not reported

Ref: 1. Daniel et al. (1990), 2. Savage et al. (1987), 3. Abrini et al. (1994), 4. Krumholz and Bryant (1985), 5. Tanner et al. (1993), 6. Lorowitz and Bryant (1984), 7. Sharak Genthner and Bryant (1987), 8. Sharak Genthner and Bryant (1982), 9. Shen et al. (1999), 10. Lynd et al. (1982), 11. Grehtlein et al. (1991), 12. O'Brien et al. (1984), 13. Rother and Metcalf (2004) 14. Daniels et al. (1977), 15. Lupton et al. (1984), 16. Davidova et al. (1994), 17. Klemps et al. (1985), 18. Parshina et al. (2005), 19. Fischer et al. (1983).

Table 2.2 summarizes the homoacetogens capable to grow on CO as sole substrate. However, Table 2.2 does not summarize all bacteria capable to oxidize CO, as several bacteria found to oxidize CO were either not capable of or not tested to grow on CO as sole substrate. Examples are *Clostridium pasteurianum* capable of CO oxidation at low CO concentrations when growing on glucose (Fuchs et al., 1974) or *Clostridium formicoaceticum* growing on fructose (Diekert and Thauer, 1978). Despite that all homoacetogens employ the ACS/CODH enzyme complex, its presence does not imply the ability to oxidize CO when present as the sole substrate. This is illustrated by the CODH activity, measured as a reduction rate of methylviologen, of 13.3 μmol per minute per mg protein in a strain of *Butyribacterium methylotrophicum* adapted for growth on CO as sole substrate, whereas the CODH activity of the Marburg strain of *Butyribacterium methylotrophicum*, unable to grow on CO alone, amounted to 47.2 μmol per minute per mg protein (Lynd et al., 1982). Several homoacetogens were found capable to oxidize CO with stimulation of cell mass production, when grown on metabolic substrates like H_2/CO_2 or glucose, but were unable to grow at the expense of CO alone (Uffen, 1981). The inability for growth on CO indicates that despite the ability to oxidize CO, the derived electrons could not be coupled to energy conservation as was found for *Thermoanaerobacter kivui* (former *Acetogenium kivui*) during H_2-dependent growth (Yang and Drake, 1990).

Many homoacetogens can grow at high CO concentrations (Table 2.2). The reported values in Table 2.2 do not indicate that higher CO levels in the gas phase

lead to growth inhibition, but merely that higher CO levels were not tested. Nevertheless, homoacetogens are well capable to convert CO not merely to acetate, but to a range of products including ethanol, butyrate and butanol (Table 2.2).

CO conversion by methanogens

The function of the reductive acetyl-CoA pathway in autotrophic methanogens is mainly assimilatory, although part of the CO_2 reduction steps of the pathway are also employed for methanogenesis (Rother and Metcalf, 2004; Fuchs, 1989). In acetoclastic methanogens the reverse reaction of acetate formation occurs, i.e. ACS/CODH catalyzes the cleavage of acetyl-CoA (Ferry, 1995; Mörsdorf et al., 1992). The carboxyl group of acetyl-CoA is oxidized to CO_2 and the released reduction equivalents are used for further reduction of the methyl group to CH_4.

So far, only three representatives of methanogenic archaea have been found capable to grow with CO as the sole source of energy (Table 2.2), i.e. *Methanosarcina barkeri*, *Methanosarcina acetivorans* and *Methanothermobacter thermoautotrophicus* (Rother and Metcalf, 2004; O'Brien et al., 1984; Daniels et al., 1977). CO acts as an electron donor in the reduction of CO_2 to methane by *M. thermoautotrophicus* (Daniels et al., 1977). O'Brien et al. (1984) reported the evolution of H_2 during growth of *M. barkeri* on CO, when the partial pressure of CO in the gas phase exceeded 20 kPa, whereas a net consumption of H_2 occurred below this value. This observation is in line with an early report of Kluyver and Schnellen (1947), in which they suggested that CO conversion by methanogens proceeds via an intermediate production of H_2 and CO_2. The most convincing results concerned the liberation of H_2 during growth on CO with *M. barkeri* in the presence of a strong alkali solution in the headspace for absorption of the produced CO_2 (Kluyver and Schnellen, 1947).

Growth rates of *M. barkeri* and *M. thermoautotrophicus* on CO are considerably lower compared to growth with H_2 as electron donor (O'Brien et al., 1984; Daniels et al., 1977). *M. acetivorans* strain C2A exhibited higher growth rates on CO and may therefore be better capable to convert CO (Table 2.2). *M. acetivorans* strain C2A forms acetate, formate and methane during growth with CO, but with increasing CO pressures it reverts to acetogenesis (Rother and Metcalf, 2004). Methanogens are generally more sensitive to elevated levels of CO and growth as well

as conversion cease rapidly with increasing CO levels (Table 2.2). Nevertheless, *M. barkeri* could be slowly adapted to an atmosphere of 100% CO (101 kPa) by successive transfers (O'Brien et al., 1984).

CO conversion by sulfate reducing bacteria

Despite the large number of sulfate reducing bacteria, reports on their growth with CO or growth in the presence of CO are scarce (Table 2.2). *Desulfovibrio vulgaris* strain Madison (Lupton et al., 1984), the first sulfate reducer reported to be capable to use CO for sulfate reduction, converts CO to H_2 and CO_2 and subsequently uses the H_2 for sulfate reduction. A similar H_2-dependent sulfate reduction was found with *Desulfotomaculum nigrificans*, *Desulfosporosinus orientis* (Klemps et al., 1985) and *Desulfovibrio desulfuricans* (Davidova et al., 1994) with CO as source of energy. Furthermore, *Desulfovibrio baarsii* used low concentrations of CO (1.5% in the gas phase) as carbon source, but was not tested for its capacity to use CO as sole source of carbon and energy (Jansen et al., 1984). All these sulfate reducing bacteria had a rather limited tolerance for CO, i.e. 4.5% for *D. vulgaris* (Lupton et al., 1984) and up to 20% for *D. nigrificans*, *D. orientis* (Klemps et al., 1985) and *D. desulfuricans* (Davidova et al., 1994). The conversion of CO to CO_2 and H_2 probably acts as a CO detoxification pathway, as it did not result in ATP synthesis and did not support growth on CO in the absence of sulfate (Lupton et al., 1984). The CO toxicity is not only the result of an inhibition of hydrogenases by CO, as reported for *D. vulgaris* (Lupton et al., 1984) and *D. desulfuricans* (Davidova et al., 1994), since CO also inhibited sulfate reducers growing on organic substrates (Davidova et al., 1994).

CODH activity is present in many sulfate reducing bacteria, e.g. in *Desulfobacterium autotrophicum* degrading acetate (Schauder et al., 1989). In biochemical tests the presence or absence of CODH activity is used as indicator of the metabolic pathway employed in the complete oxidation of organic compounds to CO_2 or in autotrophic fixation of CO_2. Pathways employed by sulfate reducing bacteria for acetate oxidation or CO_2 fixation are the tricarboxylic acid cycle (TCA) and the acetyl-CoA pathway (Schauder et al., 1986). CODH plays no role in the TCA cycle and representatives of this pathway, in which organic compounds are completely converted to CO_2. Indicative for the presence of the acetyl-CoA pathway is the

absence of the TCA cycle enzyme, 2-oxoglutarate dehydrogenase, and presence of CO dehydrogenase activity (Schauder et al., 1986). However, CO oxidizing activity in cell extracts does not necessarily imply CO utilization (Min and Zinder, 1990).

In general, CO never really was considered a suitable electron donor for sulfate reducing bacteria, likely it was due to the apparent high toxicity of CO. In a recent study it was demonstrated that *Desulfotomaculum kuznetsovii* and *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* are capable of sulfate reduction with hydrogen in the presence of 50% and 70% CO, respectively. Especially *D. thermobenzoicum* subsp. *thermosyntrophicum* showed a high CO tolerance, although sulfate reduction became inhibited above 50% CO in the gas phase (Parshina et al., 2005). Nevertheless, such a high tolerance for CO by any sulfate reducing bacterium has not been reported earlier and it certainly holds a promise for using CO-rich synthesis gas in biotechnological desulfurization.

When grown on a mixture of CO and H_2 the rate of H_2 consumption decreased considerably with increasing CO concentrations (Parshina et al., 2005), despite the fact that these bacteria possess the capacity to grow hydrogenotrophically in the presence of sulfate (Plugge et al., 2002; Nazina et al., 1988). These lower H_2 conversion rates are most probably caused by CO toxicity (Parshina et al., 2005). Inhibition of hydrogenases by CO has been reported previously (Kim et al., 1984; Sharak Genthner and Bryant, 1982; Daniels et al., 1977). Therefore, when grown on CO as sole substrate, the absence of H_2 accumulation indicates that H_2 was not the electron donor for sulfate reduction (Parshina et al., 2005). Since, *D. thermobenzoicum* subsp. *thermosyntrophicum* can not utilize acetate for sulfate reduction a potential role for acetate as intermediate in sulfate reduction with CO can be excluded (Plugge et al., 2002). This provides some evidence that this organism might couple CO oxidation directly to sulfate reduction (Parshina et al., 2005). Possibly, *D. kuznetsovii* can also couple CO oxidation directly to sulfate reduction based on the absence of H_2 accumulation, but this organism might also use CO derived acetate for sulfate reduction. With increasing CO pressures *D. thermobenzoicum* subsp. *thermosyntrophicum* and *D. kuznetsovii* revert to acetogenesis, analogous to *M. acetivorans* C2A (Rother and Metcalf, 2004). Furthermore, these sulfate reducing bacteria were successfully co-cultured with a

hydrogenogenic CO converting bacterium (Parshina et al., 2005). This approach to co-culture a CO converter with a suitable sulfate reducer has not yet received much attention, despite its interesting potential in biotechnological desulfurization. Especially, since many of these CO converting bacteria produce a suitable substrate for sulfate reducing bacteria, e.g. acetate or H_2.

2.4.2 Hydrogenogenic microbial CO conversion

This specific group of microorganisms uses CO as a sole source of carbon and energy source in the absence of an electron acceptor and form equimolar amounts of CO_2 and H_2, analogous to the water-gas-shift reaction described earlier. Growth is usually not supported by H_2/CO_2. The first observation of hydrogenogenic CO conversion dates back to the 1970s (Uffen, 1976). For these microorganisms the terms hydrogenogenic, hydrogenogens and hydrogenogenesis were proposed recently by Svetlichnyi et al. (2001), referring to the type of metabolism, physiological group, and H_2 formation process, respectively. Over the past decade the number of hydrogenogens rapidly increased (Table 2.3). Currently this carboxydotrophic hydrogenogenic metabolism is found in three distinct groups of prokaryotes, i.e. mesophilic gram-negative bacteria, thermophilic gram-positive bacteria, and thermophilic archaea (Table 2.3).

Table 2.3 Hydrogenogenic CO converting microorganisms

Microorganism[1]	T_{Opt} (°C)	pH opt	td (h)	Max P_{CO}[2] (kPa)	Origin	Ref.
Facultative anaerobes						
Rubrivivax gelatinosa	34	6.8-6.9	6.7	101	Lake sediments	1,2
Rhodopseudomonas palustris P4	30	n.r.	2[3]	101	Sludge digester	3
Rhodospirillum rubrum	30	6.8	8.4[4]	101	Brakish ditch	4
Citrobacter sp Y19	30-40	5.5-7.5	8.3	50[5]	Sludge digester	5,6
Obligate (thermophilic) anaerobes						
Carboxydothermus hydrogenoformans	70-72	6.8-7.0	2	101	Hot swamp, Kunashir	7
Carboxydothermus restrictus	70	7.0	8.3	101	Terrestrial hot vent	8
Caldanaerobacter subterraneus subsp. *pacificus*	70	6.8-7.1	7.1	110	Submarine hot vent	9
Carboxydocella thermoautotrophica	58	7.0	1.1	101	Terrestrial hot vent	10
Thermosinus carboxydivorans	60	6.8-7.0	1.2	100	Hot pool, Yellowstone Park	11
Archaea						
Thermococcus strain AM4	82	6.8	n.r.	101	Submarine hot vent	12

[1] Species names according to most recent classification. *Rubrivivax gelatinosa* was formerly classified as *Rhodopseudomonas gelatinosus* as well as *Rhodocyclus gelatinosus* (Willems et al., 1991). *Caldanaerobacter subterraneus* subsp. *pacificus* was originally classified as *Carboxydobrachium pacificum* (Sokolova et al., 2001; Fardeau et al., 2004).

[2] Maximal CO concentration tested, at which CO oxidation proceeds and therefore does not necessarily represent the maximal tolerance of CO for a specfic microorganism

[3] growth was only observed in the light, but no H_2 production was measured in the light; H_2 production could be maintained for prolonged periods when light was eliminated at the late growth phase

[4] The generation time of *Rsp. rubrum* decreased with increasing P_{CO} and the lowest observed generation time was 4.8 hours when initially 25 kPa CO was present

[5] growth and CO conversion to H_2 occurred at 50 kPa, but growth rates were decreasing with increasing P_{CO} (at P_{CO} = 50 kPa, t_d = 8.3 h). H_2 production activity expressed in mmol.(g cells.hour)$^{-1}$ decreased as well with increasing P_{CO}, although at a P_{CO} between 30 and 50 kPa H_2 production activity was similar.

n.r. not reported

Ref: 1. Uffen (1976), 2. Dashekvicz and Uffen (1979), 3. Jung et al. (1999a), 4. Kerby et al. (1995), 5. Jung et al. (1999b), 6. Jung et al. (2002), 7. Svetlichny et al. (1991b), 8. Svetlichny et al. (1994), 9. Sokolova et al. (2001), 10. Sokolova et al. (2002), 11. Sokolova et al. (2004a), 12. Sokolova et al. (2004b).

Facultative anaerobic hydrogenogenic bacteria

Facultative anaerobic bacteria that oxidize CO and evolve H_2 isolated thus far are gram-negative mesophilic bacteria. Hydrogen is only produced under anaerobic conditions upon CO oxidation. Generally growth rates on CO are low and high levels of CO are inhibitory. Non-sulfur purple bacteria form the predominant part of this

group of bacteria. Oxidation of CO coupled to formation of equimolar amounts of H_2 was first discovered with *Rhodopseudomonas gelatinosa* (Dashekvicz and Uffen, 1979; Uffen, 1976), later reclassified as *Rhodocyclus gelatinosa* (Imhoff et al., 1984) and more recently as *Rubrivivax gelatinosus* (Willems et al., 1991). Hydrogenogenic CO conversion was also observed with protein extracts of *Rhodospirillum rubrum* S1 (Uffen, 1981), although the growth rates of *Rhodospirillum rubrum* S1 were considered too low for elucidating the microbiology of CO metabolism. However recently, Kerby et al. (1995) demonstrated that *Rhodospirillum rubrum* in fact was capable of a rapid anaerobic growth on CO in the dark, but only after increasing the nickel concentration in the medium. Two different strains of *R. rubrum* revealed different $NiCl_2$ requirements with concentrations exceeding 75 and 600 μM necessary for growth. Besides different nutrient requirements for growth on CO, the presence of CO_2 may have a stimulatory effect on growth and CO conversion, as recently reported for the homoacetogen *Moorella thermoacetica* (Drake and Daniel, 2004). *R. rubrum* so far is the most studied organism, especially with respect to the properties of its CO dehydrogenase. A major drawback of photosynthetic bacteria in H_2 production from synthesis gas derived CO is the light requirement for optimal cell growth. *Rhodopseudomonas palustris* P4 (Jung et al., 1999a) was found capable of hydrogenogenic CO conversion when incubated anaerobically in the dark, although growth completely ceased in the absence of light. It was postulated that *R. palustris* P4 only obtains maintenance energy from the hydrogenogenic CO conversion (Jung et al., 1999a).

Besides these phototrophic strains only one non-phototrophic gram negative facultative anaerobe was described so far capable to convert CO to H_2, viz. *Citrobacter* strain Y19, isolated from an activated sludge plant (Jung et al., 1999b). However, as the growth of this organism under anaerobic conditions compared to aerobic conditions is low, a two step cultivation of the biomass was proposed, i.e. an aerobic growth phase followed by an anaerobic CO conversion phase (Jung et al., 1999b). Nevertheless, separation of growth and bioconversion complicates the reactor operation considerably.

Obligate anaerobic thermophilic hydrogenogenic bacteria

A rapidly increasing group of carboxydotrophic hydrogenogenic prokaryotes is formed by strict anaerobic thermophiles (Table 2.3). Conversion of CO to H_2 at elevated temperatures has been observed in freshwater as well as marine environments with temperatures ranging from 40 to 85 °C and pH between 5.5 and 8.5 (Bonch Osmolovskaya et al., 1999; Svetlichny et al., 1991a).

All isolated species are capable of chemolithotrophic growth on CO. So far, there exists no evidence of growth inhibition by high levels of CO. Some isolates also grow by fermentation or anaerobic respiration. Because of simultaneous H_2 production and acceptor reduction it is unknown whether CO is a direct electron donor in anaerobic respiration or H_2 acts as an intermediate. *Carboxydothermus hydrogenoformans* was originally described as obligate carboxydotroph (Svetlichny et al., 1991b). Later it was found capable to ferment pyruvate to acetate (Svetlichnyi et al., 1994), as well as more recently discovered that it could respire anaerobically CO as electron donor with different electron acceptors, e.g. iron, nitrate and quinones (Henstra and Stams, 2004). *Caldanaerobacterium subterraneus* subsp. *pacificus* (previously *Carboxydobrachium pacificum*) is the only marine carboxydotrophic bacterial species described so far (Fardeau et al., 2004; Sokolova et al., 2001). It was isolated from a submarine hydrothermal vent. Besides on CO, it also grows organotrophically on several mono- and disaccharides, cellulose and starch. *Thermosinus carboxydivorans* is the only known species with a gram negative cell wall in this group of thermophilic carboxydotrophs (Sokolova et al., 2004a).

Hydrogenogenic CO converting archaea

So far, *Thermococcus* strain AM4 is the only representative of the carboxydotrophic hydrogenogenic archaea, isolated from a hydrothermal vent on the east pacific rise (Sokolova et al., 2004b). Other *Thermococcus* species did not exhibit chemolithotrophic growth with CO (Sokolova et al., 2004b).

2.4.3 Recently discovered anaerobic CO respirations

The possible role of CO as electron donor in anaerobic respiration has received little attention and the number of species known to use CO in anaerobic respiration is still

limited. *Moorella thermoacetica* can grow chemolithotrophically with CO as electron donor and nitrate as electron acceptor (Drake and Daniel, 2004; Frostl et al., 1996). Furthermore, *Thermosinus carboxydivorans* reduces ferric iron and selenite with CO as electron donor (Sokolova et al., 2004a), and *Carboxydothermus hydrogenoformans* reduces fumarate and 9,10-antraquinone-2,6-disulfonate (AQDS) with CO as electron donor (Henstra and Stams, 2004). *C. hydrogenoformans* reduces nitrate, thiosulfate, sulfur, and sulfite with lactate as electron donor, but according to Henstra and Stams (2004) CO might be able to serve as electron donor as well. However, *Thermosinus carboxydivorans* and *Carboxydothermus hydrogenoformans* form H_2 from CO, which might be the actual electron donor for these reductions. In contrast, *Thermoterrabacterium ferrireducens* does not form hydrogen with CO, but is able to reduce AQDS and fumarate with CO. Besides AQDS *T. ferrireducens* may reduce Fe(III), nitrate, sulfite, thiosulfate and sulfur with CO as well, as it does with H_2 or lactate (Henstra and Stams, 2004; Slobodkin et al., 1997).

The exact range of microorganisms capable to use CO is still unclear, as CO utilization is rarely tested in growth studies. Although CO may initially inhibit growth, adaptation to CO can occur after long term incubation or multiple transfers with increasing CO levels (Rother and Metcalf, 2004; O'Brien et al., 1984). Furthermore, growth on CO may require different nutrients or concentrations (Kerby et al., 1995). The ability of CO oxidation to CO_2 seems ubiquitously present in nature and has an ancient origin, as mentioned by Ferry (1995) and Hedderich (2004).

2.4.4   *Key enzymes involved in CO conversion to H_2*

Anaerobic carboxydotrophic hydrogenogenic microorganisms conserve energy by oxidation of CO to CO_2 coupled to reduction of protons to H_2. These reactions are catalyzed by CO dehydrogenase and hydrogenase, respectively. These two enzymes must conserve energy in a yet unknown energy conserving mechanism as they do not fit in classical substrate level phosphorylation (SLP) and electron transfer phosphorylation (ETP) theories (Hedderich, 2004). The Gibbs free energy of the reaction under standard physiological conditions is –20 kJ.mol^{-1} (Table 2.4), insufficient to be directly coupled to ATP formation via SLP (ADP+Pi → ATP -31.8 kJ.mol^{-1}). In ETP an electrochemical proton gradient, i.e. a proton motive force (pmf)

is generated over the cytoplasmic membrane by coupling electron transfer to proton membrane-translocation. Electrons released by oxidation of an electron donor are transferred via a chain of electron carriers to a terminal electron acceptor. E.g. quinones accept electrons and protons at the cytoplasmic side of the membrane and release protons at the periplasmic side of the membrane and transfer electrons to the next component of the chain, thus facilitating proton translocation. However, standard electrode potentials of known quinones fall outside the CO_2/CO ($E°'$ -520 mV) and H^+/H_2 ($E°'$ -414 mV) redox couple (Thauer et al., 1977; Hedderich et al., 2004). It is unlikely that an electron transfer chain composed of quinones and cytochromes facilitates proton translocation between CODH and hydrogenase. Instead it has been proposed that CODH and an unknown type of hydrogenase form one membrane associated enzyme complex that facilitates CO oxidation, proton reduction and proton translocation (Hedderich et al, 2004; Svetlichnyi et al., 2001; Ensign and Ludden, 1991). An overview of CODH and hydrogenase is presented.

Carbon monoxide dehydrogenases (CODH)

CO dehydrogenases (CODHs) of anaerobic prokaryotes are nickel containing, O_2 sensitive enzymes and are either monofunctional or bifunctional (Lindahl, 2002). Monofunctional CODHs catalyze the oxidation of CO, which is part of the energy metabolism and linked to reduction of protons by recently discovered so-called energy converting hydrogenases (ECH; described in the next section) or to the reduction of various other electron acceptors in anaerobic respiration. Recent publications reported about the reduction of sulfate, nitrate, fumarate, Fe(III) and selenate with CO (Parshina et al., 2005; Drake and Daniel, 2004; Sokolova et al., 2004a; Frostl et al., 1996; Henstra and Stams, 2004). The monofunctional CODHs of *R. rubrum* (*CooS*) and *C. hydrogenoformans* involved in H_2 evolution are functionally associated with the iron-sulfur protein *CooF* (Gonzalez and Robb, 2000; Kerby et al., 1992). *CooF* mediates electron transfer from *CooS* to ECH (Soboh et al., 2002; Svetlichnyi et al., 2001).

Bifunctional CODHs catalyze the synthesis of acetyl-CoA or its decarbonylation beside the oxidation of CO. These CODHs form a complex with acetyl-CoA synthase (ACS) and are denominated ACS/CODH. Synthesis of actyl-

CoA by ACS/CODH is a key step in carbon fixation of autotrophic anaerobic prokaryotes, in acetate formation by homoacetogens and in acetate metabolism in sulfate reducing bacteria and methanogenic archaea. The biochemistry of ACS/CODH catalysed acetyl-CoA synthesis has been extensively reviewed recently by Ragsdale (2004) and Lindahl (2002).

Synthesis of actyl-CoA by ACS/CODH is a key step in carbon fixation of autotrophic anaerobic prokaryotes, in acetate formation by homoacetogens and in acetate metabolism in sulfate reducing bacteria and methanogenic archaea. The biochemistry of ACS/CODH catalyzed acetyl-CoA synthesis has been extensively reviewed (Ragsdale, 2004; Lindahl, 2002). ACS/CODH complexes are composed of multiple subunits that together comprise a CODH and a corrinoid-iron-sulfur protein (CoFeSP). While CODH delivers the carboxyl group of acetyl-CoA, CoFeSP carries the methyl group. Decarbonylation of acetyl-CoA allows for complete oxidation of organic substrates to CO_2 by heterotrophic methanogens and sulfate reducers (Ferry, 1995; Wood and Ljungdahl, 1991). A single microbial species may contain multiple CODHs, each with its own physiological function (Lindahl, 2002).

Hydrogenases

Hydrogenases catalyze the reduction of protons to H_2 or the reverse reaction according to:

$$2H^+ + 2e^- \leftrightarrow H_2 \tag{5}$$

The physiological function of hydrogenases is generally restricted to one of these directions and is referred to as hydrogen uptake hydrogenase or hydrogen evolving hydrogenase. Recently the classification and phylogeny of hydrogenases was reviewed by Vignais et al. (2001). Three classes of hydrogenases are recognized based on phylogeny and metal content (also transition-metal content or H_2-activating site content). The first and largest class is formed by the [NiFe]-hydrogenases. The second class, [FeFe]-hydrogenases only contain Fe in their active site, while the third class is formed by hydrogenases that until recently were named 'metal-free' hydrogenases (Berkessel and Thauer, 1995). The latter class was discovered in

methanogens, where they catalyze the reduction of F_{420} with H_2 in complex with methylenetetrahydromenthanopterin dehydrogenase under nickel-deprived conditions (Zirngibl et al., 1992; Afting et al., 1998; Afting et al., 2000). Now they are referred to as iron-sulfur cluster free hydrogenases, since the presence of a novel light sensitive iron-coordinating cluster in this type of hydrogenases was found (Buurman et al., 2000; Lyon et al., 2004; Shima et al., 2004).

Awareness that an unique subclass of [NiFe]-hydrogenases exists has grown in the past decade. These hydrogenases were indicated as energy converting hydrogenases (ECH), after their capacity to couple proton translocation to the reduction of protons or oxidation of molecular H_2, and were recently reviewed by Hedderich et al. (2004). A limited sequence similarity and a deviating enzyme topology mark the main differences between ECH and other [NiFe]-hydrogenases. These ECH are membrane bound enzyme complexes and play a key role in energy generation in the carboxydotrophic hydrogenogenic metabolism (Hedderich et al., 2004).

As mentioned, ECH couple oxidation of H_2 or reduction of protons to translocation of protons over the cytoplasmic membrane. The electrochemical gradient of protons over the membrane is generally referred to as proton motive force (pmf) and is the driving force for ATP synthesis. Translocation of protons by ECH may generate the pmf depending on its direction. Reduction of protons to form H_2 is coupled to the generation of a pmf, which in turn drives ATP synthesis. The number of suitable electron donors for reduction of the protons is limited by the relatively low electrode potential of the H^+/H_2 couple ($E^{o'}$ -414 mV). The CO_2/CO couple ($E^{o'}$ -520 mV) is sufficiently low to drive proton translocation by ECH, but also formate or reduced ferredoxin generated by pyruvate:ferredoxin oxidoreductase in fermentative metabolisms serve as electron donors for ECH (Hedderich et al., 2004; Soboh et al., 2004; Bagramyan and Trchounian, 2003; Sapra et al., 2003). Reduced ferredoxin may donate electrons directly to ECH while CODH and formate dehydrogenase form a complex with ECH. In these complexes a ferredoxin like subunit facilitates electron transfer. In the carboxydotrophic hydrogenogenic metabolism ECH together with CODH play an important role as described for *C. hydrogenoformans* and *R. rubrum* (Hedderich et al., 2004; Fox et al., 1996a,b). These organisms contain similar

enzymatic systems that catalyze the conversion of CO into H_2. Genes that code for the involved enzymes are arranged in two gene clusters that share high sequence similarity between both organisms. One cluster comprises the genes for ECH, the other cluster encodes for a CODH and *CooF*. One functional CO oxidizing H_2-evolving complex is formed with these subunits, as was shown for *C. hydrogenoformans* (Soboh et al., 2002). Coupling of proton translocation to oxidation of CO by *C. hydrogenoformans* and *R. rubrum* enables them to use CO as sole source of energy and to grow with the formation of H_2.

2.5   Biotechnological applications of CO converting microorganisms

Interest in applying suitable biocatalytic processes for the production of useful chemicals from CO or synthesis gas has led to various studies into production of alternative compounds, e.g. methane, acetate, butyrate and other organic compounds (Zeikus, 1983). The conversion of CO to ethanol and butanol was demonstrated for *Butyribacterium methylotrophicum* (Shen et al., 1999) and for a recently isolated *Clostridium* species strain P7 (Rajagopalan et al., 2002). Studies employing methyl viologen and other viologen dyes, as inhibitors of methanogenesis, resulted in the production of formate by *Methanosarcina barkeri* (Mazumder et al., 1985) and methanol by *Moorella thermoacetica* (White et al., 1987). Lapidus et al. (1989) showed that cell free extracts of *Desulfovibrio desulfuricans* can produce methanol, ethanol, acetic acid and C_8-C_{24} paraffins from CO and H_2 at elevated pressures. Incubations of different inocula with mixtures of $H_2/CO/CO_2$, revealed the presence of various acids, ranging from acetic to caproic acid, but only acetic acid and butyric acid were observed in high quantities (Levy et al., 1981). Table 2.4 summarizes thermodynamically possible synthesis gas fermentations, either from CO or H_2/CO.

Table 2.4 Summary of reported reactions with CO and H_2/CO, characteristic for synthesis gas fermentations.

Product	reaction	$\Delta G^{\circ'}$ kJ.mol $CO^{-1\#}$
From CO		
formate	$CO + H_2O \rightarrow HCOO^- + H^+$	-16
acetate	$4\ CO + 2\ H_2O \rightarrow CH_3COO^- + H^+ + 2\ CO_2$	-44
butyrate	$10\ CO + 4\ H_2O \rightarrow CH_3(CH_2)_2COO^- + H^+ + 6\ CO_2$	-44
ethanol	$6\ CO + 3\ H_2O \rightarrow CH_3CH_2OH + 4\ CO_2$	-37
n-butanol	$12\ CO + 5\ H_2O \rightarrow CH_3(CH_2)_3OH + 8\ CO_2$	-40
hydrogen	$CO + H_2O \rightarrow H_2 + CO_2$	-20
methane	$4\ CO + 2\ H_2O \rightarrow CH_4 + 3\ CO_2$	-53
From H_2/CO		
acetate	$2\ CO + 2\ H_2 \rightarrow CH_3COO^- + H^+$	-67
butyrate	$4\ CO + 6\ H_2 \rightarrow CH_3(CH_2)_2COO^- + H^+ + 2\ H_2O$	-80
methanol	$CO + 2\ H_2 \rightarrow CH_3OH$	-39
ethanol	$2\ CO + 4\ H_2 \rightarrow CH_3CH_2OH + H_2O$	-72
n-butanol	$4\ CO + 8\ H_2 \rightarrow CH_3(CH_2)_3OH + 3\ H_2O$	-81
methane	$CO + 3\ H_2 \rightarrow CH_4 + H_2O$	-151

[#] Standard Gibbs free energy changes (273.15 K; 101.325 kPa) were calculated at neutral pH (pH 7 at standard conditions) using the thermodynamic data set compiled by Amend and Shock (2001).

Besides the use of growing cells, also purified enzymes of CO converting organisms, especially CODH, might offer interesting potentials in biotechnology (Ferry, 1995). Purified CODH could be used in biofilters for cleaning the air in underground car parks or in biosensors for CO detectors (Colby et al., 1985). Recently, a CODH from *Moorella thermoacetica* was found to catalyze the reduction of 2,4,6-trinitrotoluene (TNT), an important chemical explosive commonly present in soil of military training sites (Huang et al., 2000). CODH can be applied in dechlorination as well as in the reductive carboxylation of phenols (Ferry, 1995).

With the increasing discovery of fast growing anaerobic isolates capable of hydrogenogenic CO conversion (Table 2.3) together with the current interest in H_2, a biological alternative for the chemical water-gas-shift reaction might represent one of the most interesting applications. In the next sections the use of hydrogenogenic bacteria for the purification of synthesis gas will be discussed, as well as the application of CO converting organisms in biological desulfurization.

2.5.1   *Biotechnological synthesis gas purification*

The actual biotechnological application of hydrogenogenic CO converting bacteria as an alternative for the currently employed chemical water gas shift reaction has received little attention so far. Bredwell et al. (1999) and Vega et al. (1990) studied the design of bioreactors for coal synthesis gas fermentations. They did not study the hydrogenogenic conversion of CO, but conversions of synthesis gas into methane, acetate, ethanol and butanol. Wolfrum and Watt (2002) investigated the application of bioreactors for the production of H_2 from CO, by employing *Rubrivivax gelatinosus* strain CBS (Maness and Weaver, 2002) in a trickle-bed bioreactor. A maximal CO conversion of 74% in continuous experiments was found. This research aimed to estimate design parameters rather than to optimize H_2 production. Recently, an enhanced integrated hydrogen production system was proposed, including biomass gasification, solid oxide and proton exchange fuel cells, biological hydrogenogenic CO conversion by *Rubrivivax gelatinosus* strain CBS and CO_2 sequestering by the same organism when growing in the light (Merida et al., 2004). A limitation of using phototrophic organisms in a bioreactor obviously is that their growth under anaerobic conditions in the dark is usually low compared to growth in the light and light-dark cycles may be required to sustain a sufficiently high population density in the reactor. However, with the discovery of several anaerobic microorganisms lately, showing even higher growth rates on CO alone (Table 2.3), the selection of another microorganism could be considered.

The challenge in designing bioreactors for the commercial conversion of sparingly soluble gaseous substrates is to enhance its mass transfer from the gas phase to the microorganisms that are present in the liquid phase (Bredwell et al., 1999; Vega et al., 1990). This has been the subject of many studies in biological treatment of waste gas and theirresults can be beneficially applied for the purification of synthesis gas. The fundamental difference in biological conversion of waste gas compared with synthesis gas, which is rather purification than pollution mitigation, is the concentration of the compound that requires conversion. Whereas, the concentration of the pollutants in biological waste gas treatment usually is in the lower ppm range, the concentration of CO in synthesis gas may exceed 50% in coal gasification (Table 2.1).

In case of biological waste gas treatment a vast experience in reactor design already is available, from which a future commercial biological hydrogen production from synthesis gas may benefit. In their design, biological waste gas treatment reactors may be divided in 4 basic concepts, i.e. biofilters (Fischer, 2000), biotrickling filters (Cox and Deshusses, 2001; Plaggemeier and Lämmerzahl, 2000), bioscrubbers (Van Groenestijn, 2001; Schippert and Chmiel, 2000), and membrane bioreactors (Ergas, 2001; Reiser, 2000). Bioscrubbers likely are not suited for hydrogenogenic CO conversion in synthesis gas, as these are conventionally used to transfer the pollution to a liquid, which subsequently is converted in a separate reactor and only functions well for water soluble pollutants. Biofilters and biotrickling filters represent more practical options, whereas membrane bioreactors are still in their infancy. The use of biotrickling filters has received attention in waste gas treatment as they are relatively easy to control, with respect to nutrient supply and pH. Biotrickling filters are known to be susceptible for clogging due to excess growth of biomass (Plaggemeier and Lämmerzahl, 2000). Ariga et al. (1986) already pointed out the potential advantages of using honeycomb-monolith bioreactors for bioconversions of gaseous substrates due to their low pressure drop and thin liquid layers. However, this concept has not yet found practical applications in biological gas treatment, although the working principle is quite similar to that of biotrickling filters, i.e. thin liquid layer to enhance mass transfer from the gas phase to the microorganisms. Also the concept of a 'dry' granular activated carbon bio-bed inoculated with strain *Mycobacterium* E3 was investigated for ethene removal from a synthetic waste gas (De Heyder et al., 1994). This study showed that under relative dry conditions the removal rate could be increased with a factor 2.25. This study shows that operational modifications of a conventional biofilter could be beneficial for increasing mass transfer, although supply of nutrients and desired pH value are even more difficult to control than in conventional biofilters.

2.5.2 *Product requirements for synthesis gas purification and product upgrading*

The application potential of biological synthesis gas purification depends first of all on the capabilities of the selected microorganism. When designing a synthesis gas purification system the most important factor is the required product specification,

especially with respect to the permitted remaining CO levels, which will depend on the application of the produced H_2 gas. For application in low temperature PEMFC, the purification requirement is to the level of maximally 100 ppm CO (Ledjeff-Hey et al., 2000), and preferably even less than 10 ppm (Otsuka et al., 2002; Ledjeff-Hey et al., 2000). For other applications, e.g. when H_2 is used in chemical or biological reductive processes, the requirements may be less stringent.

Nevertheless, for most synthesis gas purification processes, the separation of H_2 from the exhaust gas of the bioreactor, which will generally contain compounds such as CO_2, possibly CH_4 and unconverted CO, will be required. In that case the overall selectivity of the H_2 generation plant, including physical-chemical gas separation techniques such as the use of H_2 selective membranes or pressure swing adsorption (Fain, 2000; Koros and Mahajan, 2000) ultimately determines the application potential of the product. The post-treatment method selected will depend on both the achieved exit CO concentrations as well as the product requirements.

Due to the poor energetics of the water gas shift reaction the achievement of sufficiently low CO concentrations in the product gas of the bioreactor may be problematic, especially as at low CO concentrations the driving forces for mass transfer of CO into the liquid phase are low. Therefore, another option is to maintain higher CO concentrations in the hydrogenogenic bioreactor, which are expected to be highly advantageous as it enhances the driving force for mass transfer of the gaseous CO to the hydrogenogenic bacteria. To achieve this, a combined biological and physico/chemical process could be considered in which the CO is biologically converted to H_2 and a high grade H_2 could be derived by physical-chemical gas separation techniques. As separation technique usually the so called pressure swing adsorption (PSA) is preferred when high purity gases are required (Koros and Mahajan, 2000). This PSA comprises a system with fixed beds of different molecular sieves that selectively adsorb gasses at relatively high pressures (500-5000 kPa; Air Liquide technical specifications) and subsequently the adsorbed gases are desorbed at low pressures as an off-gas stream. In this manner the CO content in the gas could be maintained at relatively high levels by recycling the remainder of CO and removing CO_2 and H_2 separately.

The removal of CO_2 from the product gas, e.g. by physical absorption in cold methanol, as in the rectisol wash (Hochgesand, 1970), offers the possibility for selective CO_2 sequestration and geological storage (Gale, 2004), thus preventing its emission to the atmosphere. Liberation of high purity CO_2 could be even useful for reuse in chemical synthesis of e.g. methanol (Pruschek et al., 1997) or in greenhouse horticulture, which nowadays consumes large quantities of natural gas for the production of CO_2. In this respect greenhouse horticulture represents an interesting application for CO_2.

2.5.3   *Utilization of CO containing synthesis gas for biological desulfurization*

Any biological H_2 production process may suffer from the simultaneous occurrence of H_2 consumption processes, i.e. especially when using mixed populations as is the case with anaerobic granular sludge. Successful application of biological H_2 production implies the need to minimize undesired H_2 consumption. The prevention of H_2 consumption by methanogens in practice may be difficult and it may depend on the microbial populations present whether or not a specific inhibition method, e.g. using chemical inhibitors (Chidthaisong and Conrad, 2000; Scholten et al., 2000; Oremland and Capone, 1988) or pretreatment of the sludge prior to inoculation (Oh et al., 2003; Chen et al. 2002), is sufficiently effective. Little is known about the possibility to prevent H_2 consumption by homoacetogens, which may turn out to be even more difficult to suppress once methanogenesis is eliminated. For practical applications, therefore, H_2 production combined with a desired H_2 consumption process, like H_2 utilization for biological sulfate reduction, might result in maximal utilization of H_2 by the desired processes, provided that the sulfate reducers can out-compete the other H_2 consuming microorganisms. Growth kinetics of sulfate reducers generally are superior to that of methanogens with H_2 as electron donor, while also the thermodynamics of sulfate reduction are more favorable (Vallero et al., 2002; Oude Elferink et al., 1994), and consequently sulfate reducers in principle should be capable to out-compete methanogens. However, it should be understood that the overall result of the competition also depends on various other factors, e.g. pH, temperature, medium composition, immobilization characteristics, substrate limitations inside biomass aggregates and the bacterial composition of the seed sludge (Vallero et al.,

2002). Results of various reactor studies revealed that hydrogenotrophic sulfate reducers usually become dominant over hydrogenotrophic methanogens at temperatures around 35ºC (Van Houten et al., 1994; Visser et al., 1993). However, according to Van Houten et al. (1997) at elevated temperatures (55ºC) the outcome of this competition could not be predicted.

Sulfate- or sulfite-rich wastewaters with a low organic matter pollution are generated e.g. in galvanic processes, in the detoxification of metal-contaminated soils, in the mining of heavy metals and coal, and in waste streams generated by flue gas desulfurization (Johnson, 2000). In order to enable the biological desulfurization of these wastewaters a suitable electron donor needs to be supplied in the treatment process. The mining of heavy metals and coal is responsible for the largest quantity of sulfur-rich inorganic wastewater known as acid mine drainage (AMD; Johnson and Hallberg, 2004). This AMD contains high concentrations of dissolved metals and its temperature may vary from 5 to 50ºC (Tsukamoto et al., 2004; Baker and Banfield, 2003; Bond et al., 2000). Biological treatment of AMD is generally performed mesophilically. Treatment of AMD primarily aims at acid consumption in order to neutralize the pH (Johnson, 2000). This can be achieved by biological sulfate reduction, because herewith protons are consumed and a weak acid is produced (H_2S) and subsequently insoluble sulfide precipitates are formed with many heavy metals present in AMD (Johnson and Hallberg, 2004). Besides several so called "passive" biological remediation processes, e.g. aerobic wetlands, permeable reactive barriers and packed bed iron-oxidation bioreactors (Johnson and Hallberg, 2004), there is an increasing interest in "active" high-rate biological sulfate reduction processes for remediation of AMD in off-line sulfidogenic bioreactors. The main advantages of the latter processes are that they are more predictable and allow better control. Additionally, they enable the selective recovery of metals, and reach lower effluent sulfate concentrations (Johnson and Hallberg, 2004). High-rate sulfate reduction processes are successfully applied for remediation of zinc-polluted groundwater at the Budelco zinc refinery in the Netherlands and the selective copper recovery at the Kennecott Bingham Canyon copper mine in Utah, USA (Boonstra et al., 1999). Initially ethanol was used as electron donor at the Budelco zinc refinery, but was later replaced by H_2 and CO_2 as energy and carbon source (Boonstra et al., 1999).

Although high sulfate elimination rates (12 g $S.L^{-1}.day^{-1}$) were achieved with ethanol (De Smul, 1998), the main drawback of its use is the formation of acetate due to incomplete oxidation (Nagpal et al., 2000). Hydrogen is a more economical electron donor for sulfate reduction and it results in the formation of less biomass (Boonstra et al., 1999).

Scrubbing water from flue gas treatment represents another typical example of a sulfur-rich inorganic wastewater. Stringent legislation enacted to prevent or minimize acid deposition from sulfur compounds present in flue gas requires desulfurization of flue gas. SO_2 in flue gas was reported to account for approximately 90% of the anthropogenic SO_2 emissions in 1989 (Brimblecombe et al., 1989). The release of relative high amounts of SO_x leads to the formation of H_2SO_4, which ultimately results in acid deposition and subsequently damages the ecosystem (Vallero et al., 2002; Charlson et al., 1992). The intensive use of fossil fuels will remain an important source of anthropogenic SO_2 emissions. As the natural reserves of coal exceed those of oil or natural gas reserves considerably, the use of coal likely will increase in the near future (Schobert and Song, 2002). One approach to reduce the SO_x contents of flue gas is the gasification of coal to a sulfur-free synthesis gas prior to its combustion. However, end-of-pipe technologies will remain an important measure to prevent SO_x emissions to the atmosphere till the time coal gasification will be implemented on a large scale.

The production of gypsum, a widely applied physical-chemical end-of-pipe technology for SO_x removal from flue gas, results in a voluminous waste product, with little reuse potential due to contamination with heavy metals (Vallero et al., 2002). Biological flue-gas-desulfurization is an attractive alternative method, because of its low operational costs and the fact that relatively pure (>98%) and reusable elemental sulfur is produced, without the need for further wastewater treatment (Janssen et al., 2000). In this biotechnological process the SO_x, mainly present as SO_2, is dissolved in a slightly alkaline solution to form sulfites and minor amounts of sulfate (Janssen et al., 2000). The sulfite and sulfate containing scrubbing water is subsequently subjected to an anaerobic sulfate reduction process, where the oxidized S-compounds are converted into sulfides. In the next step, the sulfide is partially oxidized to elemental sulfur under oxygen limitation (Janssen et al., 1998; Buisman et

al., 1990; Kuenen, 1975) and the insoluble sulfur than can be separated from the water phase (Janssen et al., 2000). Flue-gas-desulfurization scrubbing wastewaters generally are liberated at high temperatures (± 55ºC), because besides SO_2 also heat is transferred to the scrubbing solution. Treatment with (moderate) thermophilic microorganisms is therefore preferred.

Over the past decade a lot of research was undertaken to find cheap and efficient electron donors for the biological treatment of inorganic sulfate-rich wastewaters. Many types of organic waste materials have been considered as potential substrate for the sulfate reducing bacteria, e.g. molasses and whey (Fauville et al., 2004), tannery effluents (Boshoff et al., 2004a), dried algal biomass (Boshoff et al., 2004b), solid waste materials (Chang et al., 2000) and sheep and poultry manures (Gibert et al., 2004). Despite, the fact that these organic substrates have been shown to stimulate sulfate reduction, their use in high-rate bioreactors is rather complicated due to their complexity. Van Houten and Lettinga (1996) already pointed out that organic waste materials are less suitable electron donors, as their use would result in an additional pollution of the wastewater and this would require a supplementary treatment. Since the reported sulfate reduction rates with organic waste materials were rather low, these authors considered the use of cheap bulk chemicals like methanol, ethanol, H_2 gas, or synthesis gas more appropriate. Ethanol (Widdel and Hanssen, 1991; Swezyk and Pfennig, 1990), methanol (Goorissen et al., 2004; Weijma et al., 2003; 2002; 2000) and hydrogen (Colleran et al., 1995; Van Houten et al., 1994) all have been successfully applied in biological sulfate reduction studies. According to a cost estimate made by Van Houten and Lettinga (1996) concerning the use of ethanol and hydrogen as electron donors for sulfate reduction, the use of ethanol would be cheaper for small scale installation (<5 kmol.hour^{-1}), whereas H_2 would be cheaper at larger installations, assuming the use of a high purity H_2. Synthesis gas, a mix of H_2 and CO, is cheaper than pure hydrogen and is widely available as by-product of coal burners. Even low-grade coal (rich in sulfur deposits) can be used safely as the sulfur compounds can be treated within the process as well (Du Preez et al., 1992).

The utilization of synthesis gas for sulfate reduction processes so far received little attention in research, and to our knowledge only three publications appeared (Van Houten et al., 1996; Du Preez and Maree, 1994; Du Preez et al., 1992). Van

Houten et al. (1996) operated a gas lift reactor at 30°C and achieved a sulfate reduction rate in the range of 6-8 g SO_4^{2-}.(L.day)$^{-1}$, with a feed gas containing maximally 20% CO. Du Preez and Maree (1994) reported a sulfate reduction rate of 2.4 g SO_4^{2-}.(L.day)$^{-1}$ with pure CO as the feed gas in a fixed bed bioreactor operated at 35°C. Both groups of researchers speculate on the occurrence of a hydrogenogenic CO conversion, although their results did not support that. Van Houten et al. (1996) observed that the total H_2 consumption was insufficient to account for the total amount of sulfate reduced and acetate produced. The direct conversion of CO to acetate or conversion of CO and H_2 to acetate were not taken into account and could explain the non-stoichiometric utilization of H_2 (Table 2.4). Du Preez and Maree (1994) observed a two-fold higher sulfate reduction rate with pure CO compared to a 90:10 H_2/CO mixture and therefore it is unlikely that H_2 acts as an intermediary electron donor in sulfate reduction when the reactor was fed with CO. In case of hydrogenotrophic sulfate reduction it is expected that sulfate reduction rates are higher since an inhibiting effect of elevated CO concentrations on sulfate reduction is absent (Du Preez and Maree, 1994). The results might be explained by a direct reduction of sulfate with CO or by an intermediate involvement of homoacetogens. Due to the fact that the number of mesophilic homoacetogens capable of CO conversion is rather large and many of these organisms are commonly encountered in anaerobic environments, acetate most probably served as the actual electron donor for the sulfate reduction.

2.6   Conclusions

Biological synthesis gas conversions have a wide range of potential interesting applications, e.g. in the production of a wide-range of valuable chemicals. With the discovery of anaerobic hydrogenogenic microorganisms capable of removing carbon monoxide with concomitant H_2 production, the biotechnological potential of synthesis gas utilization has drastically increased.

The isolation of an increasing number of hydrogenogens (Table 2.3) shows that biotechnological purification of synthesis gas to a pure H_2 gas seems within reach as an attractive alternative to the catalytic processes nowadays employed.

Nevertheless, more research is required to assess the practical applicability of this biological water gas shift reaction. Especially research into the biotechnological utilization of these hydrogenogens has to be performed. The development of a successful biotechnological gas water shift production method, not only depends on the microorganisms and biological reactor, but also on the product requirements and thus its economic value.

Successful application of H_2-rich synthesis gas in biodesulfurization, without the need for prior purification, requires tolerance for the presence of CO. Furthermore, it would be most beneficial to the overall sulfate reduction process when CO within the synthesis gas is used for sulfate reduction as well. Thus, both toxicity and potential metabolic use of CO as electron donor are important factors governing the utilization potential of CO-rich synthesis gas for biotechnological sulfate reduction. In that respect, the recent discovery of more CO tolerant sulfate reducing bacteria indicates that CO-rich synthesis gas may represent an interesting and cheap electron donor for biodesulfurization.

3

Carbon monoxide conversion by anaerobic bioreactor sludges

Abstract

Seven different anaerobic sludges from wastewater treatment reactors were screened for their ability to convert carbon monoxide (CO) at 30 and 55°C. At 30°C, CO was converted to methane and/or acetate by all tested sludges. Inhibition experiments, using 2-bromoethanesulfonate (BES) and vancomycine, showed that CO conversion to methane at 30°C occurred via acetate, but not via H_2. At 55°C, four sludges originally cultivated at 30-35°C and one sludge cultivated at 55°C converted CO rapidly into hydrogen or into methane. In the latter case, inhibition experiments showed that methane was formed via hydrogen as the intermediate.

3.1 Introduction

Anaerobic biological CO conversion can result in various products, e.g. methane, acetate, butyrate, ethanol and butanol (Bredwell et al., 1999). Furthermore, H_2 can be formed from CO via the water-gas-shift reaction (equation 1) performed by phototrophic (Mörsdorf et al., 1992) and anaerobic thermophilic carboxydotrophic microorganisms (Svetlichny et al., 1991b, 1994; Sokolova et al., 2001, 2002, 2004a,b), which all employ a similar CO metabolism with concomitant H_2 production for which recently the term 'hydrogenogenesis' was proposed (Svetlichnyi et al., 2001).

$$CO_{(g)} + H_2O \rightarrow CO_{2(g)} + H_{2(g)} \qquad \Delta G°'= - 20 \text{ kJ.mol}^{-1} \qquad (1)^{\#}$$

Despite the limited number of microorganisms described sofar capable of performing this biological water-gas-shift reaction, enzymes involved in the conversion of CO into CO_2 and H_2 are commonly found in anaerobic microorganisms (Ferry, 1995; Mörsdorf et al., 1992). The key enzyme in the conversion of CO to CO_2, carbon monoxide dehydrogenase, is present in organisms that form or degrade acetate via the acetyl-CoA pathway, also known as the carbon monoxide pathway (Diekert, 1990). Methanogenic microorganisms have also been shown to contain CO dehydrogenases, both involved in hydrogenotrophic, e.g. *Methanothermobacter thermoautotrophicus* (Daniels et al., 1977) and acetotrophic, e.g. *Methanosarcina barkeri* (O'Brien et al., 1984) methanogenesis. As, CO has been overlooked in many physiological studies as a potential metabolic substrate, the occurrence of microbial CO conversion capacity is likely to be more wide-spread than currently known.

In this study, the presence of microorganisms capable to perform the water-gas-shift reaction in full-scale anaerobic wastewater treatment plants was investigated. The potential of seven different anaerobic sludges to degrade CO was determined at

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[#] The standard Gibbs' free energy change at neutral pH and standard conditions ($\Delta G°'$) was calculated using thermodynamic data from Amend and Shock (2001). At 55°C, $\Delta G_{55}' = -22.3$ kJ.mol^{-1}.

30 and 55°C. Specific inhibitors for bacteria and archaea (methanogens) were used to investigate the intermediates involved in CO conversion.

3.2 Materials and Methods

3.2.1 Biomass sources

Granular methanogenic sludge samples were obtained from full-scale anaerobic reactors treating wastewater from paper mills (Eerbeek, Industriewater Eerbeek, The Netherlands), a distillery (Nedalco, Bergen op Zoom, The Netherlands) and a brewery (Heineken, Zoeterwoude, The Netherlands), whereas a granular sulfate-reducing sludge sample was collected from a reactor treating Kraft-pulping wastewater (Norkraft, Montreal, Canada). Furthermore, two suspended biomass samples were obtained from anaerobic gas-lift reactors for biological sulfide production used for the removal of copper, at a pilot plant for acid mine water treatment (AMWT plant operated by Paques B.V., Balk, The Netherlands), and zinc (Budel Zink, Budel, The Netherlands). All these sludges had been cultivated at 30-35°C. Also a sulfate-reducing granular sludge was obtained from a lab-scale methanol fed UASB reactor, operated at 55°C, treating sulfate-rich wastewater (Vallero et al., 2003a).

3.2.2 Experimental set-up

Tests for assessing microbial CO utilization capability under anaerobic conditions were performed in triplicate in temperature controlled non-shaken water-baths in the dark at selected temperatures ranging from 30 to 65°C.

CO was used as sole substrate and the bottles were incubated under an atmosphere of 90 - 100% CO, at a total gas pressure of 1.6 to 1.8 bar, corresponding to an absolute amount of about 4 mmol of CO in the headspace of each bottle. The basal medium contained (in mM): NaCl 5.1, NH_4Cl 5.6, $CaCl_2.2H_2O$ 0.7, $MgCl_2.6H_2O$ 0.5, $Na_2S.9H_2O$ 0.6, yeast extract 500 mg.l^{-1}, and 1 ml.l^{-1} of a trace element solution according to Stams et al. (1993). The medium was buffered using 19.8 mM KH_2PO_4 and the pH was set initially to 7.0 by adding NaOH. Serum bottles of 120 ml, sealed with butyl rubber stoppers, containing 50 ml of basal medium were supplemented with 0.1 to 0.2 g volatile suspended solids (VSS). Sludge samples were washed four times with demineralized-water prior to inoculation, in order to remove

traces of substrate present in the inoculum sludge. The bottles were not shaken. Therefore, the presented rates do not represent the maximal specific conversion rates, since mass transfer from the gas to liquid phase was sub-optimal. During the course of the experiments, the gas pressure in the headspace of the bottles and the biogas composition were monitored. Liquid samples were analyzed for the presence of volatile fatty acids and alcohols at regular time intervals.

Incubations with sterilized sludge (autoclaved for 20 minutes at 121°C) were conducted at 30 and 55°C. Bottles without sludge were incubated as controls for abiotic CO conversion in the presence of the basal medium. To determine the CO conversion route, inhibitor studies were performed using 25 mM 2-bromoethanesulfonate (BES) or 0.07 mM vancomycine (Oremland and Capone, 1988). These selective inhibitors of methanogenic microorganisms and bacteria have been used successfully previously for elucidating the role of bacteria and methanogens in methanol conversion (Weijma et al., 2000; Florencio et al., 1994).

Crushed granular sludge was used to evaluate the effect of increased CO toxicity. By disrupting the granular structure, more microorganisms were expected to be exposed to CO. Crushing was performed by slowly pushing the sludge granules in a syringe through a needle under strictly anaerobic conditions. The used needles, Microlance 3 (BD Medical Systems, Drogheda, Ireland) were 21G2 (0.8 x 50 mm), 23G1 (0.6 x 25 mm) and $25G^5/_8$ (0.5 x 16 mm). By successively using needles with a smaller diameter, a fine suspension of biomass was obtained.

3.2.3 Analysis and chemicals

The pressure in the bottles was determined using a portable membrane pressure unit, WAL 0-4 bar absolute (Wal Mess- und Regelsysteme GmbH, Oldenburg, Germany). The headspace gas composition and methanol and volatile fatty acids (VFA) in the liquid were measured gas chromatographically according to Weijma et al. (2000). VSS was analyzed according to standard methods (APHA, 1995).

Solubilities of CO, CO_2 and CH_4 were calculated using data from Lide (2001), the solubility of H_2 was calculated according to Perry et al. (1997), and the amounts produced or consumed were calculated by taking into account both gas and liquid phases.

All chemicals used were of analytical grade and supplied by Merck (Darmstadt, Germany). CO (99.997 % pure) was supplied by Hoek Loos (Rotterdam, The Netherlands).

3.3 Results

3.3.1 CO conversion at 30°C

All sludge samples, except the thermophilic sludge from the sulfidogenic lab-scale reactor, were tested for anaerobic CO conversion at 30°C and pH 7.0. Although none of the tested sludge samples had been exposed to CO previously, all tested sludges converted CO (Table 3.1) at a partial CO pressure in the headspace of 1.6 to 1.8 bar (95% CO, 5% N_2). Metabolic active microorganisms performed the CO conversion; no conversion was observed in the absence of biomass or with heat-sterilized biomass (data not shown).

Table 3.1   CO conversion rates at 30 and 55°C as well as the amount of sludge supplied.

Inoculum sludge	CO depletion rate at 30°C (mmol.day^{-1})	Biomass concentration (gVSS.bottle^{-1})	CO depletion rate at 55°C (mmol.day^{-1})	Biomass concentration (gVSS.bottle^{-1})
Norkraft	0.48 ± 0.03	0.049 ± 0.008	0	n.d.
AMWT	0.44 ± 0.08	0.019 ± 0.002	0.72 ± 0.33	0.028 ± 0.006
Budelco	0.14 ± 0.06	n.d.	0	n.d.
Eerbeek	0.62 ± 0.12	0.12 ± 0.05	1.04 ± 0.57	0.11 ± 0.04
Heineken	0.37 ± 0.01	0.13 ± 0.01	0.80 ± 0.10	0.084 ± 0.01
Nedalco	0.37 ± 0.05	0.12 ± 0.04	1.32 ± 0.86	0.054 ± 0.001
Lab-scale thermophilic	n.d.	n.d.	0.73 ± 0.06	0.077 ± 0.007

n.d. = not determined

Conversion of CO at 30°C resulted in the production of methane and/or acetate. Norkraft, AMWT and Budelco sludge converted CO to acetate and CO_2, but not to CH_4 (Fig. 3.1). Norkraft sludge gave only minor CO conversion during the first 24 days of incubation (Fig. 3.1A), but from day 24 onwards, the CO conversion rate increased considerably resulting in a complete CO depletion within 30 days (Fig.

3.1A). AMWT sludge gave an almost similar conversion of CO as the Norkraft sludge (data not shown). Budelco sludge converted CO (Fig. 3.1B), but like the Norkraft sludge slowly, i.e. only 50 to 60% of the supplied CO was converted after 38 days, although the CO conversion rate increased somewhat after 30 days.

Based on the amount of CO consumed in each bottle, and the amount of acetate and CO_2 accumulated a carbon recovery in the range of 90 to 100% was found, showing the absence of other major CO conversion products. During the incubation period only trace amounts of H_2 could be detected, although with the AMWT sludge, nevertheless, a small but clear H_2 accumulation manifested (up to 50 µmol per bottle) during the first 22 days. Between day 22 and the termination of this experiment, no H_2 could anymore be detected. In these experiments it is not possible to discriminate between a direct conversion of CO to acetate and an indirect conversion via H_2 and CO_2 as intermediates.

The methanogenic sludges (Eerbeek, Heineken and Nedalco) converted CO significantly faster, and CH_4 was the main product. The lag phase was less than 4 days. CO conversion with Eerbeek is presented in Fig. 3.1C, as the results with Heineken and Nedalco are similar. The carbon recovery in the end products CO_2 and CH_4 exceeded 90% and during the conversion of CO, only traces of H_2 (< 10 µmol) could be detected. Acetate likely is the main intermediate in methane production as indicated by experiments supplemented with 25 mM BES (Table 3.2). Liquid samples taken during the conversion of CO in BES-free incubations revealed the presence of maximal 2 mM acetate, which was completely converted at the termination of the experiments.
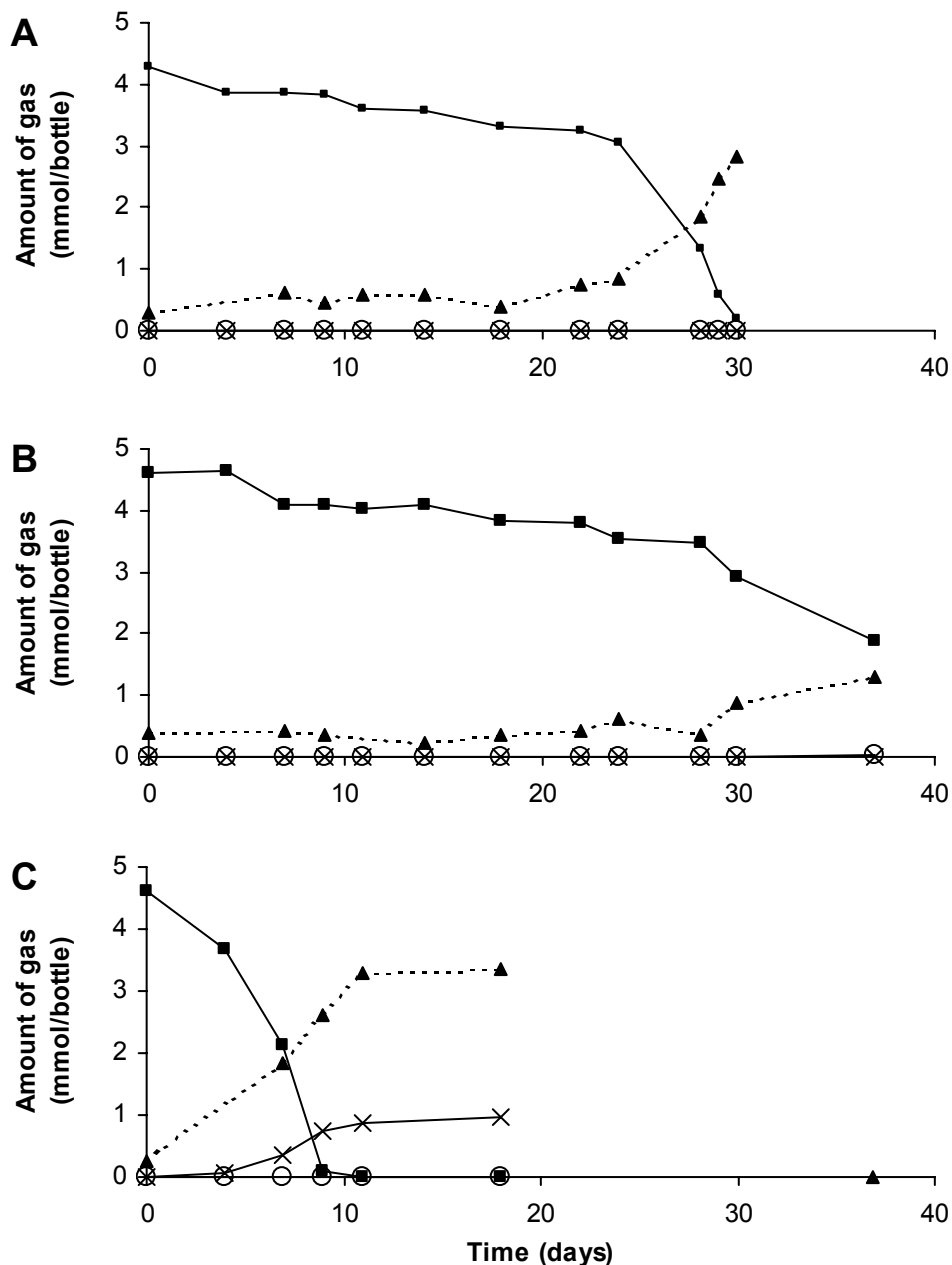
Fig. 3.1 CO conversion in batch incubations performed with Norkraft (A), Budelco (B) and Eerbeek (C) sludge at 30°C with CO as sole substrate ($P_{CO} > 1.6$ bar). Symbols: carbon monoxide (■), carbon dioxide (▲), methane (×) and hydrogen (○).

Direct methane formation from CO was found to be of minor importance. Nedalco sludge did not give any CO conversion in the presence of vancomycine in 55 days of incubation (Table 3.2). Direct conversion of CO into methane was observed only with Eerbeek and Heineken sludge (Table 3.2), although the CO conversion rates were very low (Table 3.2) compared to the CO conversion rates in the absence of

vancomycine (Table 3.1). Moreover, CO conversion rates found with Eerbeek, Heineken and Nedalco sludge in the presence of 25 mM BES (Table 3.2) were almost the same as CO conversion rates in the absence of BES (Table 3.1), confirming that methanogens indeed did not convert CO directly.

The relatively high sensitivity of methanogens to CO manifested clearly in experiments conducted with crushed granular sludge at 30°C. Methane formation was strongly inhibited in crushed sludge samples, but the conversion of CO to acetate proceeded even better (0.89 ± 0.20 mmol.day^{-1}), suggesting that mesophilic methanogens are much more sensitive to CO than homoacetogens.

3.3.2 CO conversion at 55°C

The results summarized in Table 3.1 reveal that five of the tested sludges converted CO ($P_{CO} > 1.6$ bar) at 55°C, whereas this was not the case for two of the sludges samples tested (Budelco and Norkraft sludge) after 30 and 42 days of incubation, respectively. Controls conducted without biomass and with sterilized biomass did not show CO conversion (data not shown).

Eerbeek and Heineken sludge gave a rapid CO conversion with only a short lag phase and a clear H_2 accumulation followed by methane formation (Fig. 3.2A,B). In the presence of 25 mM BES, formation of stoichiometric amounts of H_2 and CO_2, were found with both these sludges (Table 3.2). In the absence of inhibitors, high amounts of H_2 were observed; accounting for 60 to 80% of the CO converted, despite the fact that at the same time H_2 and CO_2 were rapidly converted to methane.

Direct methane formation from CO was absent with both Eerbeek and Heineken sludge, since in incubations supplemented with 0.07 mM vancomycine; CO was not converted within a period of 55 days (Table 3.2). Incubations with Eerbeek and Heineken sludge, supplemented with acetate as sole substrate, showed that acetate was not converted at 55°C after 55 days of incubation (data not shown), indicating that these sludges did not contain thermophilic acetotrophic or carboxydotrophic methanogens.
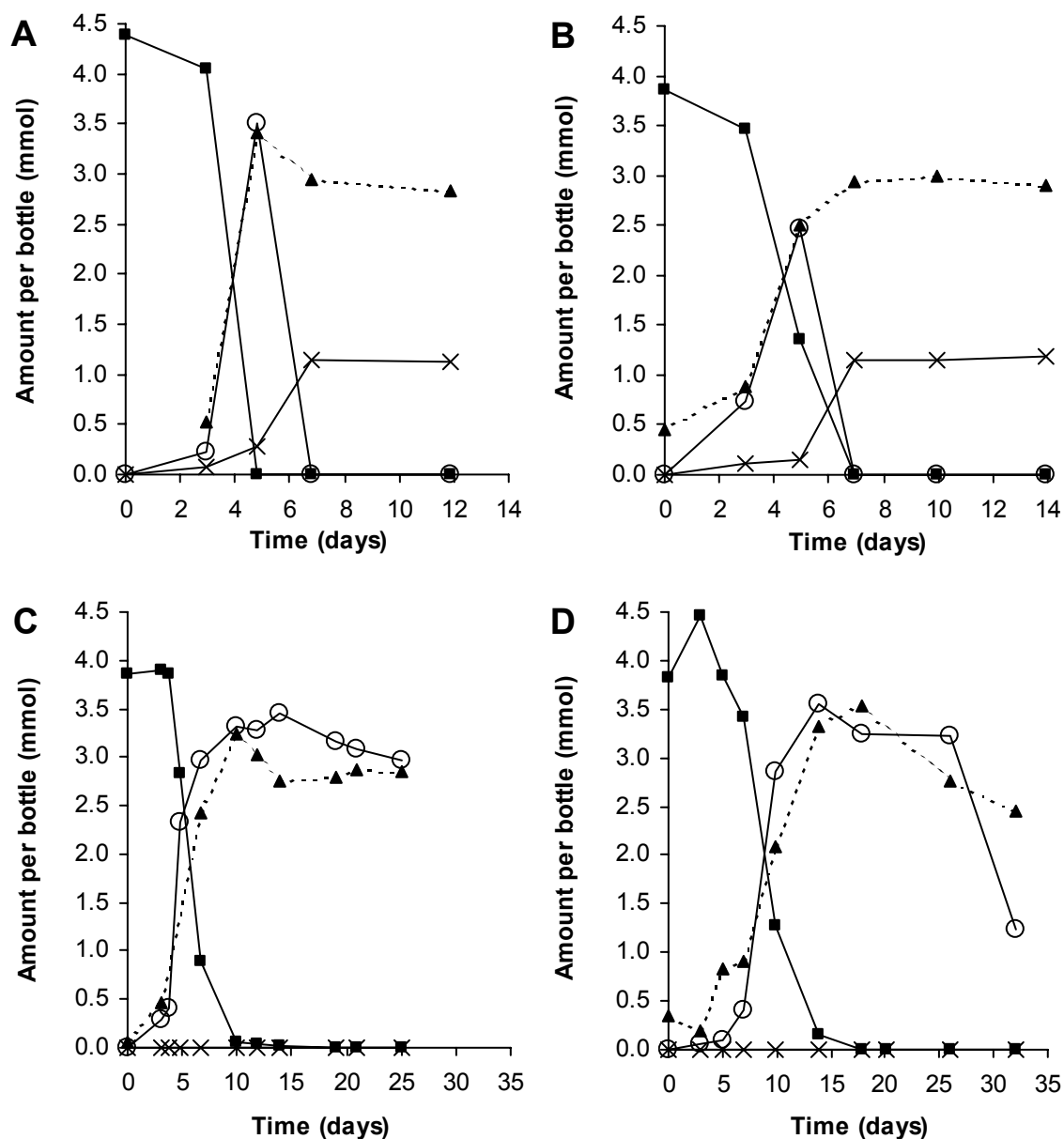
Fig. 3.2 CO conversion in batch incubations performed with Eerbeek (A), Heineken (B), AMWT (C), and Nedalco (D) sludge at 55°C with CO as sole substrate ($P_{CO} > 1.6$ bar). Symbols: carbon monoxide (■), carbon dioxide (▲), methane (×) and hydrogen (○).

Table 3.2 Effect of inhibitors on CO conversion rates and products formed by methane producing sludges at 30 and 55°C. The CO conversion rates are presented as a percentage of the rate in uninhibited assays, i.e. 100% equals the rate in the uninhibited assay.

Inoculum sludge	30°C				55°C			
	BES[a]		Vancomycine[b]		BES[a]		Vancomycine[b]	
	CO conv. rate %	Products formed	CO conv. rate %	Products formed	CO conv. rate %	Products formed	CO conv. rate %	Products formed
Eerbeek	100	Acetate	8	CH_4	100	H_2/CO_2	0	None
Heineken	100	Acetate	3	CH_4	n.d.[*]	H_2/CO_2	0	None
Nedalco	84	Acetate	0	None	n.d.	n.d.	n.d.	n.d.
Lab-scale Thermophilic	n.d.	n.d.	n.d.	n.d.	n.d.[*]	H_2/CO_2	0	None

a = incubation period was 20 days; b = incubation period was 55 days; n.d. = not determined
* = CO conversion observed, but insufficient data points were obtained to determine accurate rates

AMWT and Nedalco sludge rapidly converted CO at P_{CO} of 1.5 − 1.7 bar, resulting in a distinct accumulation of H_2 (Fig. 3.2C,D), but methane formation was not observed. The maximal amount of hydrogen measured indicated that about 90% of the supplied CO was converted into H_2 and CO_2. The amount of produced H_2 and CO_2 after prolonged incubation periods (> 25 days; Fig. 3.2D) declined as a result of formation of acetate. This acetate was not converted in the remaining time of the experiment, indicating the absence of thermophilic acetotrophic methanogens. Incubations with acetate as sole substrate confirmed this observation, as acetate was not converted in 55 days of incubation (data not shown). Thermophilic (55°C) hydrogenotrophic methanogenic activity was present in both AMWT and Nedalco sludge, as methane was rapidly produced in incubations with H_2 and CO_2 (data not shown).

The absence of CH_4 formation in Nedalco and AMWT sludge incubated under a CO containing atmosphere most probably is caused by the particle size distribution. The granules of the Nedalco reactor were relatively small compared to Eerbeek and

Heineken granules, while AMWT sludge was completely dispersed. In order to assess the effect of particle size on the production of methane in CO exposed incubations, a number of tests were conducted with crushed Eerbeek and Heineken sludge. The results of these incubations show that after depletion of CO (within 4 days), methane formation from the produced H_2 and CO_2 did not proceed within 14 days (data not shown). Concentrations of H_2 and CO_2, nevertheless, decreased somewhat during the experiment as a result of acetate formation, i.e. at termination of the experiment about 20% of the produced H_2 and CO_2 was converted into acetate.

Sludge from the thermophilic sulfate reducing lab-scale reactor showed a rapid CO conversion and a short lag phase. The gas composition during the incubation period changed similarly to that found for Eerbeek and Heineken sludges (Fig. 3.2A and B) and is therefore not shown. Both acetate and CH_4 were formed, but acetate was not metabolized further into methane within a period of 35 days. As also further incubations in the presence of 0.07 mM vancomycine did not show methane production in 55 days of incubation, the produced CH_4 merely can originate from H_2 and CO_2. Results of incubations conducted in the presence of 25 mM BES confirmed that CO was converted mainly via H_2 and CO_2 (Table 3.2).

3.3.3 CO conversion routes shift with increasing temperature

The results of an experiment conducted with Eerbeek sludge at different incubation temperatures (30 to 65°C), clearly demonstrate the occurrence of a shift of the CO conversion to H_2 production at increasing temperatures (Fig. 3.3). The highest CO conversion rates of 1.82 ± 0.38 mmol CO.day^{-1} in the experiments with Eerbeek sludge were found around 55°C (Fig. 3.3A), while at 30°C it was 0.62 ± 0.12 mmol CO.day^{-1}. As the supplied biomass amount was nearly equal in all incubations, i.e. 0.14 ± 0.02 gVSS, the calculated specific CO conversion rate (at 55°C) amounted to 13.0 mmol.gVSS^{-1}.day^{-1}, and actually the maximal CO conversion rate very likely even is higher in view of prevailing mass transfer limitations.

At temperatures of 45°C and higher a high H_2 production was observed, in particular around 50 to 55°C (Fig. 3.3B), which coincides with the maximal CO depletion rate. At 50 and 55°C, almost stoichiometric amounts of H_2 were formed. The hydrogen production rates shown in Fig. 3.3B were determined in the presence of 25 mM BES in order to eliminate the effect of H_2 consumption by methanogens.

Unfortunately, it was not possible to eliminate H_2 consumption by acetogens, which leads to an underestimation of H_2 production rates. However, the maximal H_2 production rate measured at 55°C was 1.55 ± 0.38 mmol.day^{-1}, which is only slightly lower than the maximal CO conversion rate found, indicating a nearly stoichiometric production of H_2. The acetate production, whether resulting directly from CO or indirectly via production out of H_2 and CO_2, was small at 50 and 55°C. The methane production rates (Fig. 3.3C) followed the same trend as the CO conversion rates (Fig. 3.3A).

3.4 Discussion

The results of this study reveal that a certain CO conversion capacity is omnipresent in anaerobic sludges from wastewater treatment reactors. CO conversion is performed by metabolically active microorganisms, and that direct conversion of CO by methanogens is negligible. The major difference between CO conversion at mesophilic and thermophilic conditions (55°C) is the shift from the production of acetate and CO_2 to the production of H_2 and CO_2 as major intermediates. This shift in bioconversion route towards H_2 production at elevated temperatures is very similar to that reported for anaerobic methanol mineralization. Methanol is mainly used for direct methanogenesis under mesophilic conditions (Florencio et al., 1994), but it is converted via H_2 and CO_2 as intermediates to methane under thermophilic conditions (Weijma et al., 2000). The difference in CO conversion route is also reflected in the temperature optima found with Eerbeek sludge (Fig. 3.3). The two optima found for methane production most probably are due to the activity of different methanogens, because at 30°C methane is formed by acetotrophic methanogens, whereas at 55°C methane is produced exclusively by hydrogenotrophic methanogens.
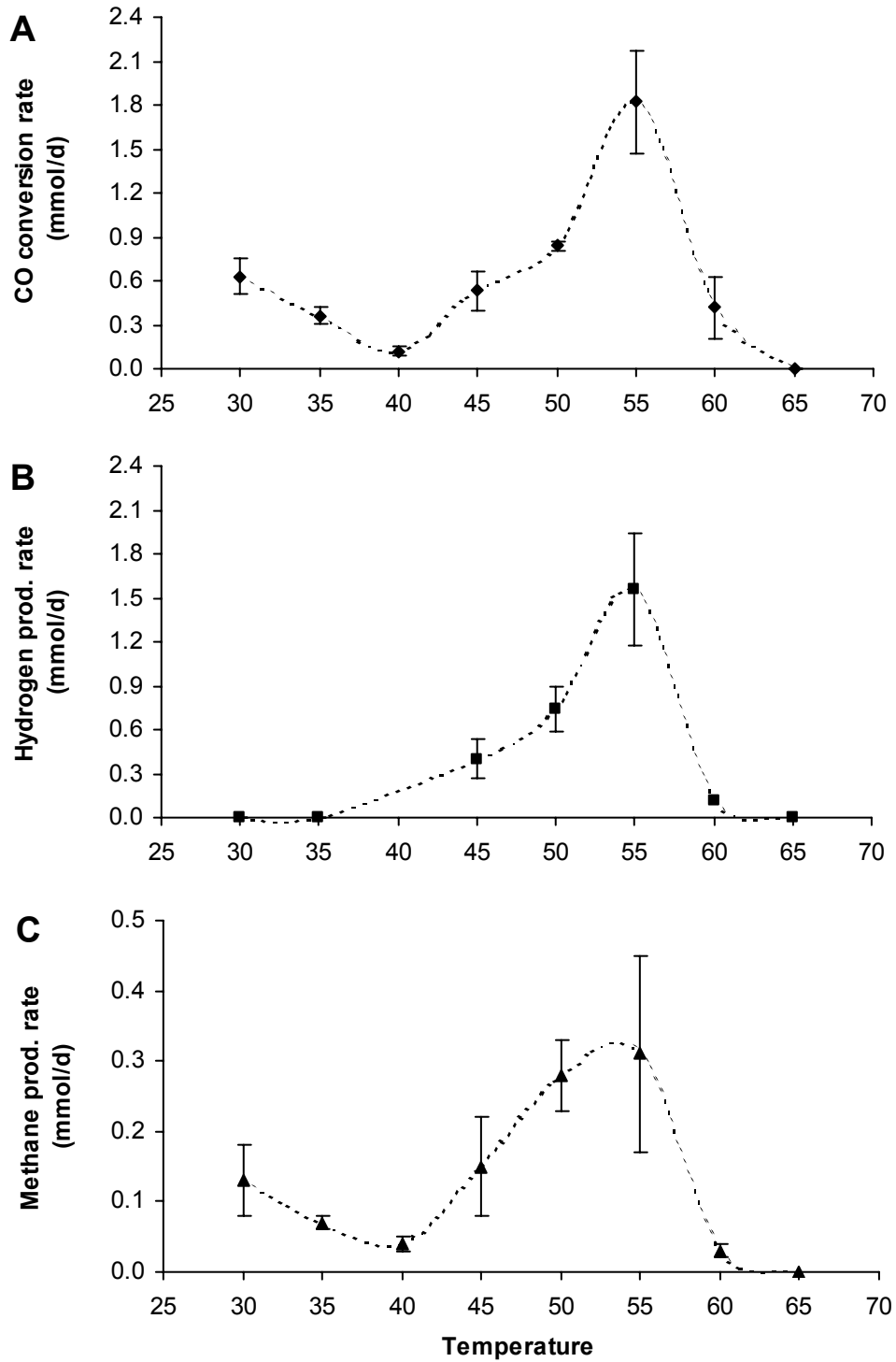
Fig. 3.3 CO conversion and product formation rates with Eerbeek sludge at temperatures ranging from 30 to 65°C, showing the CO conversion rates (A), the hydrogen production rate (B) and methane production rate (C) as function of the temperature.

A slow direct methane formation from CO, at high CO partial pressures (P_{CO} 1.5 – 1.8 bar) merely manifested with Eerbeek and Heineken sludge at 30°C, whereas Nedalco sludge did not produce any methane in the presence of vancomycine at 30°C (Table 3.2). However, even in case direct methane formation from CO would occur, the rates would be so low (Table 3.2), that the quantitative role of methanogens in CO conversion at P_{CO} >1.5 is negligible. The absence of direct methanogenic CO conversion, as found for Nedalco sludge at 30°C, likely can be attributed to a complete inactivation of methanogens due to the high CO concentration in the gas phase (Mörsdorf et al., 1992). Only *Methanosarcina barkeri* sofar has been reported capable to grow at 100% CO in the gas phase after long-term adaptation to slowly increasing CO concentrations (O'Brien et al., 1984). Disrupting the granular structure of the methanogenic sludges resulted in a strongly increased exposed surface area of the active biomass to CO, and therefore will lead to a strong inhibition of methane formation in case acetotrophic methanogens are sensitive to CO concentrations (95%, 1.6 bar), and this apparently is true at 30°C, as was already reported by Mörsdorf et al. (1992).

Comparison of the conversion rates of CO found at 30 and 55°C learns that all sludges show a significantly higher conversion rate under thermophilic conditions (Table 3.1). This despite the fact that a) all the sludges were cultivated at temperatures between 30 to 35°C, and had not been previously adapted to the elevated temperature, and b) the solubility of CO at increased temperatures is lower, i.e. 0.89 mM versus 0.64 mM at 30 and 55°C, respectively (APHA, 1995). Thus, this opens perspectives to use sludges cultivated at 30 to35°C as inocula for thermophilic biotechnological applications.

In the present study, the effect of long-term adaptation to elevated temperature and long-term exposure to CO was not investigated. However, even the mesophilic sludge samples apparently contain high numbers of thermophilic organisms, as the lag-phases of incubations at 55°C are short and CO conversion rates high (Fig. 3.2). Consequently, we may conclude that adaptation presumably will result in a very rapid growth of CO converting populations, leading to higher CO conversion rates, provided mass transfer of CO into the liquid can be maintained at a sufficiently high level.

The microorganisms involved in the conversion of CO to H_2 and CO_2 are not

yet known, and might represent a novel group. The currently known microorganisms capable of converting CO according to the water-gas-shift reaction grow optimally at temperatures differing greatly from the optimum of 55°C found in our experiments. Phototrophic microorganisms capable of converting CO into H_2 and CO_2, *Rhodospirillum rubrum* (Bredwell et al., 1999) and *Rubrivivax gelatinosa,* formerly known as *Rhodopseudomonas gelatinosa* (Uffen, 1983) as well as the facultative anaerobic chemolithotroph *Citrobacter* sp. (Jung et al., 1999b) are characterized by an optimum growth temperature of about 35°C and do not grow at 55°C. Several thermophilic anaerobic microorganisms able to convert CO into H_2 and CO_2, were isolated from hydro thermal vents in the pacific, i.e. *Carboxydothermus hydrogenoformans* (Svetlichny et al., 1991b), *Carboxydothermus restrictus* (Svetlichny et al., 1994), *Caldanaerobacter subterraneus* subsp. *pacificus* (Sokolova et al., 2001) and *Thermococcus* strain AM4 (Sokolova et al., 2004b). These microorganisms grow optimally at 70°C or higher. *Carboxydocella thermoautotrophica* and *Thermosinus carboxydivorans* (Sokolova et al., 2002; 2004a), are more moderately thermophilic hydrogenogens growing up to 68°C, with an optimum between 58-60°C. However, since CO conversion with Eerbeek sludge was apparently absent at a temperature of 65°C and proceeded optimally between 50 and 55°C (Fig. 3.3), suggesting that none of these isolates likely is involved in CO conversion in anaerobic bioreactor sludge. Therefore, the abundance of organisms converting CO to H_2 and CO_2 at 55°C in anaerobic wastewater treatment facilities suggests that CO is converted by some novel group(s) of anaerobic organisms or at least via a non-investigated property of known organisms.

3.5 Conclusions

The results obtained in this research demonstrate that:

1. CO conversion capacity at neutral pH and high P_{CO} levels (> 160 kPa CO), is omnipresent in unadapted anaerobic sludges from bioreactors incubated at both 30°C and 55°C, despite that such high CO levels are not encountered in real environments.

2. CO conversion found with the six tested anaerobic bioreactors in incubations at 30°C all produced acetate as major intermediate of CO conversion, which

with Eerbeek, Heineken and Nedalco sludge was converted by acetotrophic methanogens. Two sludges, i.e. Eerbeek and Heineken, were capable of direct methane production from CO without intermediate formation of acetate at rates of 8 and 3%, respectively, of that for acetate production.

3. Elevation of the incubation temperature from 30 to 55ºC changes the CO conversion routes of unadapted mesophilic sludge from acetate production to H_2/CO_2 production.

4. Disrupting the granular structure revealed high acute toxicity of both mesophilic acetotrophic methanogens and thermophilic hydrogenotrophic methanogens for CO, as with an initial P_{CO} of 160 kPa using crushed sludge no methane production was observed for extended periods, whereas CO converting hydrogenogens and homoacetogens were unaffected. Methane production by granular sludge in incubations with 160 kPa CO demonstrates that growth in granules or biofilms can protect microorganisms from exposure to toxic CO concentrations.

4

Effect of CO, H$_2$ and sulfate on thermophilic (55°C) hydrogenogenic carbon monoxide conversion in two anaerobic bioreactor sludges

Abstract

The conversion routes of CO at 55°C by full-scale grown anaerobic sludges treating paper mill and distillery wastewater were elucidated. Inhibition experiments with 2-bromoethanesulfonate (BES) and vancomycine showed that CO conversion was performed by a hydrogenogenic population producing. H$_2$ and CO$_2$, which subsequently were used by methanogens, homo-acetogens or sulfate reducers dependent on sludge source and inhibitors supplied. Direct methanogenic CO conversion occurred only at low CO concentrations (P$_{CO}$ < 50 kPa) with the paper mill sludge. The presence of H$_2$ decreased the CO conversion rates, but did not prevent the depletion of CO to undetectable levels (< 400 ppm). Both sludges showed interesting potential for hydrogen production from CO, especially since after a 30 minutes exposure to 95°C, the production of CH$_4$ at 55°C was negligible. The paper mill sludge was capable of sulfate reduction with H$_2$, tolerating and using high CO concentrations (P$_{CO}$ > 160 kPa), suggesting that CO-rich synthesis gas could be used as electron donor for biological sulfate reduction.

4.1 Introduction

Carbon monoxide (CO) can support a complex microbial food chain of a variety of trophic groups. CO can be metabolized by methanogens or sulfate reducers, possibly via H_2 and CO_2 as intermediates (Mörsdorf et al., 1992). Hydrogenogens are capable of converting CO into H_2 and CO_2. Furthermore, acetogens are reported to convert CO into acetate (Mörsdorf et al., 1992) or ethanol and butanol (Bredwell *et al*., 1999). The latter compounds can be utilized by other anaerobes. Table 4.1 presents reactions possibly involved in the anaerobic conversion of CO and summarizes their stoichiometry and Gibbs free energy under standard conditions (25°C, 101 kPa) and 55°C.

Table 4.1 Stoichiometry and standard values of Gibbs free energy changes of reactions involved in the anaerobic degradation of carbon monoxide. Reactions were calculated at neutral pH, i.e. pH is 7.00 at 25ºC and 6.58 at 55ºC, using the data set compiled by Amend and Shock (2001).

Reactions	$\Delta G^{0'}$ (25ºC) kJ.mol^{-1}	$\Delta G_{55}'$ (55ºC) kJ.mol^{-1}
Direct		
1. $CO + H_2O \rightarrow CO_2 + H_2$	-20.0	-22.3
2. $4 CO + 2 H_2O \rightarrow 3 CO_2 + CH_4$	-210.8	-207.5
3. $4 CO + 4 H_2O \rightarrow CH_3COO^- + 2 HCO_3^- + 3 H^+$	-165.7	-145.5
Via H_2/CO		
4. $CO + 3 H_2 \rightarrow CH_4 + H_2O$	-150.7	-140.7
Via H_2/CO_2		
5. $2 HCO_3^- + 4 H_2 + H^+ \rightarrow CH_3COO^- + 4 H_2O$	-104.2	-92.7
6. $HCO_3^- + 4 H_2 + H^+ \rightarrow CH_4 + 3 H_2O$	-135.4	-127.5
7. $4 H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4 H_2O$	-152.3	-145.7
Via acetate		
8. $CH_3COO^- + 4 H_2O \rightarrow 2 HCO_3^- + 4 H_2 + H^+$	104.2	92.7
9. $CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31.2	-34.9
10. $CH_3COO^- + SO_4^{2-} \rightarrow 2 HCO_3^- + HS^-$	-48.1	-53.0

A screening of CO conversion in full-scale grown anaerobic sludges showed that CO conversion capacity is ubiquitous in anaerobic bioreactor sludges both at

ambient and elevated temperatures (Chapter 3). Moreover, at elevated temperatures (55°C) H$_2$ was found to be an important intermediate. On the other hand the CO conversion route(s) were not elucidated in detail, and this also applies for the effect of substrate (CO) and product (H$_2$) concentrations. Therefore, two sludges capable of H$_2$ production from CO in the presence of 2-bromoethanesulfonate (BES) were selected to assess the CO conversion routes at 55°C in detail. Potential substrate (CO) and product (H$_2$) inhibition of the biological water-gas-shift reaction was evaluated as well as the fate of sulfate in the presence of CO.

4.2 Materials and Methods

4.2.1 Sludge samples

Granular methanogenic sludge samples were obtained from a full-scale anaerobic reactor treating wastewater from a paper mill (Industriewater Eerbeek, Eerbeek, The Netherlands) and from an alcohol production plant (Nedalco, Bergen op Zoom, The Netherlands). These two sludges were selected from the results of a screening, which revealed that Eerbeek sludge rapidly converted CO to methane via H$_2$/CO$_2$, whereas Nedalco sludge produced H$_2$ and CO$_2$ but not methane (Chapter 3). These sludges were originally cultivated at temperatures between 30 and 35°C, and were not adapted to 55°C prior to the experiments.

4.2.2 Experimental set-up

Tests for assessing the routes of microbial CO utilization at 55°C under anaerobic conditions were performed in non-shaken water baths as previously described (Chapter 3). The basal medium contained (in mM): NH$_4$Cl 5.6, CaCl$_2$.2H$_2$O 0.7, MgCl$_2$.6H$_2$O 0.5, NaCl 5.1, Na$_2$S.9H$_2$O 0.3, yeast extract 500 mg.l^{-1}, and 1 ml.l^{-1} of an acid and alkaline trace element solution according to Stams *et al.* (1993). The medium was buffered at pH 7.0 using 8.2 mM KH$_2$PO$_4$ and 11.4 mM Na$_2$HPO$_4$.2H$_2$O. When H$_2$/CO$_2$ was used, the phosphate concentrations were decreased to 10% of the former and the medium was buffered using 23 mM sodium bicarbonate and about 30 kPa CO$_2$.

To elucidate the routes of CO conversion, a number of inhibition studies were performed using 25 mM 2-bromoethanesulfonate (BES) to inhibit methanogens, or 0.07 mM vancomycine to inhibit the activity of bacteria (Oremland and Capone,

1988). In these experiments CO, acetate (under an atmosphere of N_2), H_2/CO_2 or H_2/CO were used as the substrates. Incubations supplemented with CO as the sole substrate were performed at high ($P_{CO} \geq 160$ kPa), medium ($P_{CO} \pm 40$ kPa) and low ($P_{CO} \leq 6$ kPa) CO partial pressure in order to assess the CO concentration dependency of the biochemical routes. The occurrence of sulfate reduction with CO as the sole substrate was only assessed at high P_{CO} by supplementing 20 mM sodium sulfate to the basal medium. Control experiments were conducted with sterilized biomass (20 minutes at 121°C) and with basal medium without sludge.

Granular sludge was crushed as described previously (Chapter 3) to assess the effect of disruption of the granular structure. Furthermore, the effect of heat treatment (30 minutes exposure time) at different temperatures (75, 95, 100, 105, 110 and 115°C) on CO conversion was investigated with crushed sludge at high P_{CO} (>160 kPa) and 55°C.

4.2.3 Analysis and chemicals

The pressure in the bottles was determined using a portable membrane pressure unit, WAL 0-4 bar absolute (Wal Mess- und Regelsysteme GmbH, Oldenburg, Germany). The headspace gas composition was measured on a gas chromatograph HP 5890 (Hewlett Packard, Palo Alto, USA). The detection limit for CO, with the used settings, was 400 ppm. Volatile fatty acids (VFA) were analyzed on a HP 5890A gas chromatograph (Hewlett Packard, Palo Alto, USA) according to Weijma *et al.* (2000). Sulfide was measured according to Trüper and Schlegel (1964). TSS and VSS were analyzed according to standard methods (APHA, 1995).

Sulfate was measured on a DX-600 IC system (Dionex Corporation, Salt Lake City, USA). The used columns were IonPac AG17 and AS17 4 mm, and were operated at a temperature of 30°C and flow rate of 1.5 ml.min^{-1}. Injection volume was 25 μl. The eluent was made on-line using the EG40 Eluent Generator (Dionex Corporation, Salt Lake City, USA) equipped with a KOH cartridge (Dionex P/N 053921, Salt Lake City, USA) and deionized water as the carrier. The potassium hydroxide concentration of the eluent varied during one run: 1.0 mM from t=0 to t=5min, 20.0 mM from t=5 to 7 min, 40.0 mM from t=7 to 7.1 min and 1.0 mM from 7.1 to 10 min. An ASRS-ULTRA, 4 mm, auto-suppression recycle mode was used.

The detection was based on suppressed conductivity. Prior to analysis, samples were centrifuged and diluted 100 times.

Solubilities of CO, CO$_2$ and CH$_4$ were calculated using data from Lide (2001), solubility of H$_2$ was calculated according to Perry *et al*. (1997), and the amounts produced or consumed were calculated by taking into account both gas and liquid phases.

All chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany). CO (purity 99.997%) was supplied by Hoek Loos (Rotterdam, The Netherlands).

4.3 Results

4.3.1 CO conversion at high partial CO pressure

The results in Fig. 4.1A show that the microbial population present in Eerbeek sludge was capable to convert CO rapidly to methane. H$_2$ was an intermediate, which accumulated temporarily to concentrations exceeding 1.0 mmol per bottle (Fig. 4.1A). In the presence of 25 mM BES, nearly stoichiometric amounts of H$_2$ were formed from CO (Fig. 4.1B), suggesting that the direct conversion of CO into CH$_4$ did not occur at a high P$_{CO}$. Support for this was found in the incubations supplemented with 0.07 mM vancomycine, where no CO conversion could be observed (Table 4.2). The small amounts of methane that accumulated in the gas phase of these bottles most probably originated from endogenic substrates or the supplied yeast extract as in control bottles without CO a similar amount of methane was produced. The carbon recovery at the termination of the incubations in each case slightly exceeded 100% (Table 4.2), most probably due to methane production from endogenic substrates. The results presented in Table 4.2 further show that the conversion of CO to H$_2$ is stoichiometric according to the water-gas-shift reaction (equation 1; Table 4.1), since the maximal amount of H$_2$ nearly equals the initial amount of CO supplied. The slight decrease of H$_2$ in the BES supplemented incubations (Fig. 4.1B) was due to the formation of small amounts of acetate (data not shown).
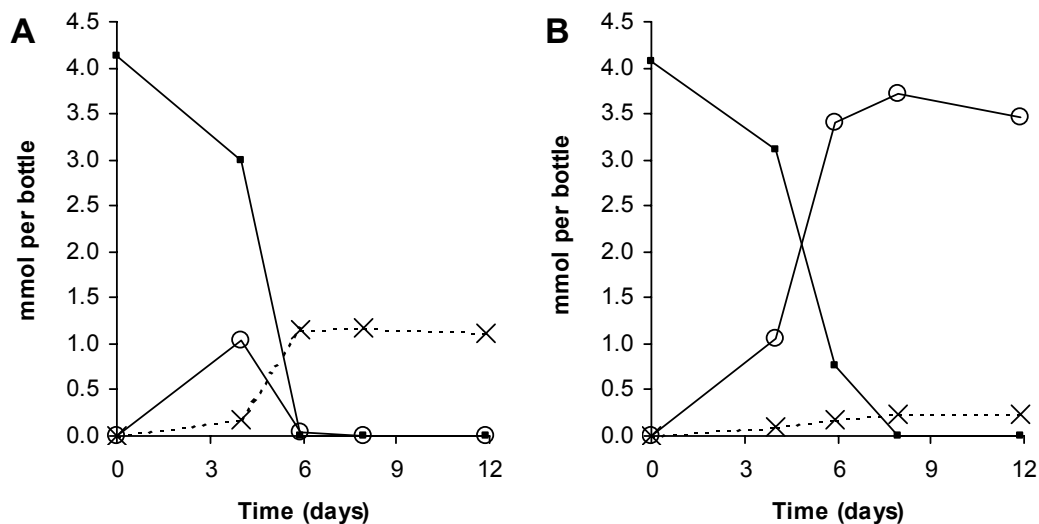
Fig. 4.1 CO conversion at high P_{CO} by granular Eerbeek sludge at 55°C in the absence of any inhibitor (A) and in the presence of 25 mM BES (B). The symbols indicate: carbon monoxide (■), hydrogen (○) and methane (×). Carbon dioxide was only measured at the end of the incubations in order to determine the carbon balance and is therefore not included in the figure.

Nedalco sludge was capable to convert CO rapidly to H_2, whereas methane was not formed, even not in the absence of inhibitors (Table 4.2). Table 4.2 shows that nearly stoichiometric amounts of H_2 accumulated in the gas phase, indicating that CO was solely converted to H_2 and CO_2. At the termination of the experiments, the amount of H_2 decreased slightly as a result of acetate production (Table 4.2). The carbon recovery in each incubation was nearly 100%. As methane formation was negligible in the absence of any inhibitor, methanogens presumably are not involved in CO conversion by Nedalco sludge. This idea was further supported by the absence of CO conversion in the presence of vancomycine (Table 4.2).

Table 4.2   Effect of the inhibitors BES and vancomycine (VM) on CO conversion at 55°C. At the start of the incubations CO was supplied as sole substrate at a P_{CO} of 150 to 160 kPa, resulting in a CO concentration of 85 - 95% in the gas phase (remainder: N_2).

Inhibitor	Start CO	End CO	Max H₂	End H₂	End CH₄	Acetate produced	VSS (g.bottle⁻¹)	Electron recovery (%)
		←		mmol per bottle		→		
Eerbeek Sludge (incubation period 12 days)								
None	4.1	0	1.1 ± 0.1	0	1.2 ± 0.05	n.d.	0.068 ± 0.004	116 ± 2
BES	4.1	0	3.9 ± 0.2	3.6 ± 0.1	0.2 ± 0.04	n.d.	0.074 ± 0.001	109 ± 1
VM	4.1	4.1	0	0	0.03 ± 0.01	n.d.	0.066 ± 0.004	106 ± 1
Nedalco Sludge (incubation period 9 days)								
None	3.8	0	3.8 ± 0.2	3.4 ± 0.2	0.01 ± 0.01	0.1 ± 0.1	0.054 ± 0.001	104 ± 1
BES	4.2	2.4 ± 0.6	n.d.	1.5 ± 0.7	0.01 ± 0.01	0.1 ± 0.03	0.027 ± 0.002	100 ± 3
VM	4.1	4.0 ± 0.05	0	0	0	0	0.026 ± 0.005	97 ± 1

4.3.2 Conversion of acetate and H₂/CO₂ in the absence of CO

Both the sludges were unable to convert acetate when supplied as sole substrate at 55°C within a period of 20 days of incubation (data not shown), indicating the absence of thermophilic acetotrophic methanogens. Furthermore, as no H_2 accumulation was observed in these acetate supplemented incubations, disproportion of acetate into H_2 and CO_2 did not occur.

Eerbeek sludge was capable to convert H_2 and CO_2 supplied as substrate after a 2 day lag phase completely to methane within only 2 days, and acetate concentrations were negligible. However, in the presence of BES, H_2 and CO_2 were converted to acetate after a lag phase of about 7 days and at a considerably lower rate than in case of methane production (data not shown). These results suggest that hydrogenotrophic methanogens in Eerbeek sludge probably out compete homoacetogens for H_2 and CO_2 as substrate. Nedalco sludge was capable to convert

the H_2 and CO_2 supplied as substrate rapidly in methane, indicating the presence of hydrogenotrophic methanogens (data not shown).

4.3.3 CO conversion at medium and low partial CO pressure

Eerbeek sludge converted CO also at P_{CO} of 30 to 50 kPa to methane in the absence of inhibitors. The amount of methane produced was slightly higher than expected based on the supplied CO (according to reaction 1 followed by reaction 6 of Table 4.1), once again likely due to methane production from substrates present in the inoculum or in the medium (Table 4.3). Acetate accumulation was comparable in the presence and absence of CO (Table 4.3), suggesting that acetate formation was not due to CO conversion. In the presence of BES, a considerable production of H_2 manifested, but also small amounts of methane were formed as a result of incomplete inhibition of methanogens by BES (Table 4.3). The difference between acetate production in incubations with basal medium without additional substrate (0.21 mmol) and acetate production in the BES supplemented incubations (Table 4.3) accounts for the loss of H_2. The direct conversion of CO to acetate likely does not occur, as the uninhibited incubations showed an acetate production of 0.21 mmol, equal to the incubations without substrates added (Table 4.3). In the presence of vancomycine, a 25% decrease of the added CO was found and a small amount of methane was formed suggesting that direct methanogenic CO conversion may be involved at $P_{CO} < 0.5$ (Table 4.3). However, not all the CO removed was recovered as methane (Table 4.3). The CO conversion at a low CO pressure (P_{CO} 5 –9 kPa) showed the same trend (Table 4.3), suggesting that the routes at a P_{CO} up to 50 kPa are not affected by the CO concentration.

Table 4.3 CO conversion by Eerbeek and Nedalco sludge at 55°C in the absence and presence of BES or vancomycine (VM) as inhibitors at medium and low P$_{CO}$. The amount of biomass was 0.07 ± 0.003 and 0.03 ± 0.005 gVSS per bottle for Eerbeek and Nedalco sludge, respectively. The incubation time was 9 days.

Inhibitor	P$_{CO}$ start	CO start	CO end	H$_2$ formed	CH$_4$ formed	Acetate amount
	(kPa)	←		mmol per bottle		→
Eerbeek sludge						
Medium P$_{CO}$ (30 - 50 kPa CO; 20 to 25% CO in the gas phase)						
None	0.30 ± 0.01	0.81 ± 0.03	0	0	0.27 ± 0.01	0.20 ± 0.02
BES	0.37 ± 0.05	0.96 ± 0.11	0.07 ± 0.07	0.62 ± 0.04	0.04 ± 0.00	0.25 ± 0.02
VM	0.40 ± 0.05	1.07 ± 0.16	0.76 ± 0.10	0	0.05 ± 0.01	0.02 ± 0.01
Low P$_{CO}$ (5 - 9 kPa CO; 4 to 6% CO in the gas phase)						
None	0.06 ± 0.02	0.17 ± 0.04	0	0	0.07 ± 0.03	0.21 ± 0.01
BES	0.08 ± 0.01	0.22 ± 0.02	0	0.20 ± 0.02	0.04 ± 0.00	0.18 ± 0.01
VM	0.07 ± 0.01	0.20 ± 0.03	0.14 ± 0.03	0	0.03 ± 0.01	0.02 ± 0.00
Nedalco sludge						
Medium P$_{CO}$ (30 - 40 kPa CO; 20 to 25% CO in the gas phase)						
None	0.31 ± 0.03	0.82 ± 0.09	0	0.60 ± 0.12	0.02	0.16 ± 0.01
BES	0.35 ± 0.02	0.89 ± 0.05	0	0.79 ± 0.20	0	0.16 ± 0.02
Vanco.	0.37 ± 0.05	0.97 ± 0.09	0.90 ± 0.10	0	0	0.02 ± 0.01
Low P$_{CO}$ (4 - 6 kPa CO; 3 to 4% CO in the gas phase)						
None	0.05 ± 0.01	0.13 ± 0.03	0	0.13 ± 0.00	0.01 ± 0.00	0.14 ± 0.01
BES	0.05 ± 0.00	0.13 ± 0.00	0	0.17 ± 0.00	0	0.15 ± 0.01
Vanco.	0.05 ± 0.01	0.11 ± 0.01	0.08 ± 0.00	0	0	0.01 ± 0.00

Nedalco sludge produced small amounts of methane both at medium P$_{CO}$ (30 – 40 kPa) and low P$_{CO}$ (4 –6 kPa) (Table 4.3), i.e. in almost equal amounts to those found in the endogenic incubations. The major part of CO was recovered as H$_2$, but not completely, as the amount of H$_2$ recovered could not account for all CO converted (Table 4.3). Part of the produced H$_2$ and CO$_2$ apparently was converted to acetate, since the acetate production was slightly higher than the 0.13 mmol measured in the control incubations (Table 4.3). The direct conversion of CO to acetate at the lowest P$_{CO}$ with Nedalco sludge most likely was absent as the recovery of CO was stoichiometric in H$_2$ (Table 4.3). Any direct conversion of CO into methane was

absent, because the incubations in the presence of vancomycine showed no methane formation (Table 4.3).

4.3.4 Effect of H_2 on CO conversion routes

The presence of H_2 may enable an alternative methanogenic CO conversion route as illustrated by equation 4 in Table 4.1. In order to test the occurrence of alternative metabolic routes of CO and H_2 to CH_4 by methanogens (reaction 4, Table 4.1), Eerbeek sludge was incubated with different amounts of CO and H_2 in the presence of vancomycine. The latter was added to eliminate the effect of CO conversion to H_2 and CO_2 by non-methanogenic microorganisms. The gas phase was initially free of CO_2 in order to exclude hydrogenotrophic methanogenesis. Since Nedalco sludge was unable to produce CH_4 in the presence of CO, tests were exclusively performed with Eerbeek sludge.

In all bottles, the H_2 concentration decreased and CH_4 was produced (Table 4.4). The results in Table 4.4 show, however, that only at low CO concentrations, some CO conversion occurred within 70 days. At $P_{CO} = 8$ kPa, 60 to 70% of the CO was converted. At $P_{CO} = 24$ kPa the conversion was only 20 to 30%, whereas at a $P_{CO} \geq 57$ kPa, no CO conversion was observed. The amount of CH_4 produced was considerably higher than expected from the amount of CO converted (according to both reaction 2 and 4, Table 4.1) and is assumed to be the result of the presence of substrate sources within the sludge or the basal medium. Thus, addition of H_2 apparently did not promote direct methanogenic CO conversion according to reaction 4 (Table 4.4) compared to direct CO conversion to methane in the absence of H_2 (Table 4.3).

Table 4.4 Effect of H$_2$ partial pressure on direct methanogenic CO conversion with Eerbeek sludge in the presence of 0.07 mM vancomycine at 55°C. The composition of the gas phase of each incubation (0, 6, 17, and 34% CO supplied) was analyzed at the start and upon termination of the incubations (after 70 days).

CO start		H$_2$ % Start	CO start	CO end	H$_2$ start	H$_2$ end	CH$_4$ formed
%	P$_{CO}$ (kPa)		←		mmol per bottle		→
0	0	100	0	0	3.79 ± 0.00	2.93 ± 0.00	0.13 ± 0.01
6	0.08	93	0.27 ± 0.02	0.08 ± 0.02	4.00 ± 0.01	2.45 ± 0.04	0.37 ± 0.02
17	0.24	81	0.86 ± 0.03	0.65 ± 0.09	3.93 ± 0.02	2.06 ± 0.04	0.45 ± 0.04
34	0.57	64	2.05 ± 0.01	2.05 ± 0.01	3.84 ± 0.01	1.94 ± 0.06	0.39 ± 0.01

4.3.5 Inhibition of CO conversion by H$_2$

The effect of the presence of H$_2$ on the CO conversion was tested with Eerbeek sludge, i.e. to assess the potential product inhibition by H$_2$. Experiments were performed in duplicate with 12 and 24% CO in the gas phase, the remainder of the gas phase consisting of H$_2$ or N$_2$. In order to maintain a high concentration of H$_2$ during CO conversion, BES was added to some of the bottles with a H$_2$/CO atmosphere. CO was nearly completely removed from all bottles within 7 days (Fig. 4.2A), although it occurred at quite different CO conversion rates. At a CO concentration of 24%, the replacement of nitrogen by hydrogen in the absence of BES resulted in a 50% decreased CO conversion rate (5.52 ± 0.92 mmol.gVSS^{-1}.day^{-1}). In the presence of BES the conversion rate was only 35% of the rate under a CO/N$_2$ atmosphere (3.93 ± 0.46 mmol.gVSS^{-1}.day^{-1}). The CO conversion rate, at 12% CO in the presence of BES (remainder H$_2$), was 1.96 mmol.gVSS^{-1}.day^{-1}, which is only 50% of that with 22-25% CO. The effect of consumption of H$_2$ during CO conversion is rather limited, suggesting that the initial CO/H$_2$ ratio in the different bottles determines the CO conversion rate. Fig. 4.2B shows the Gibbs free energy change (ΔG at 55ºC), estimated for each measured point presented in Fig. 2A.
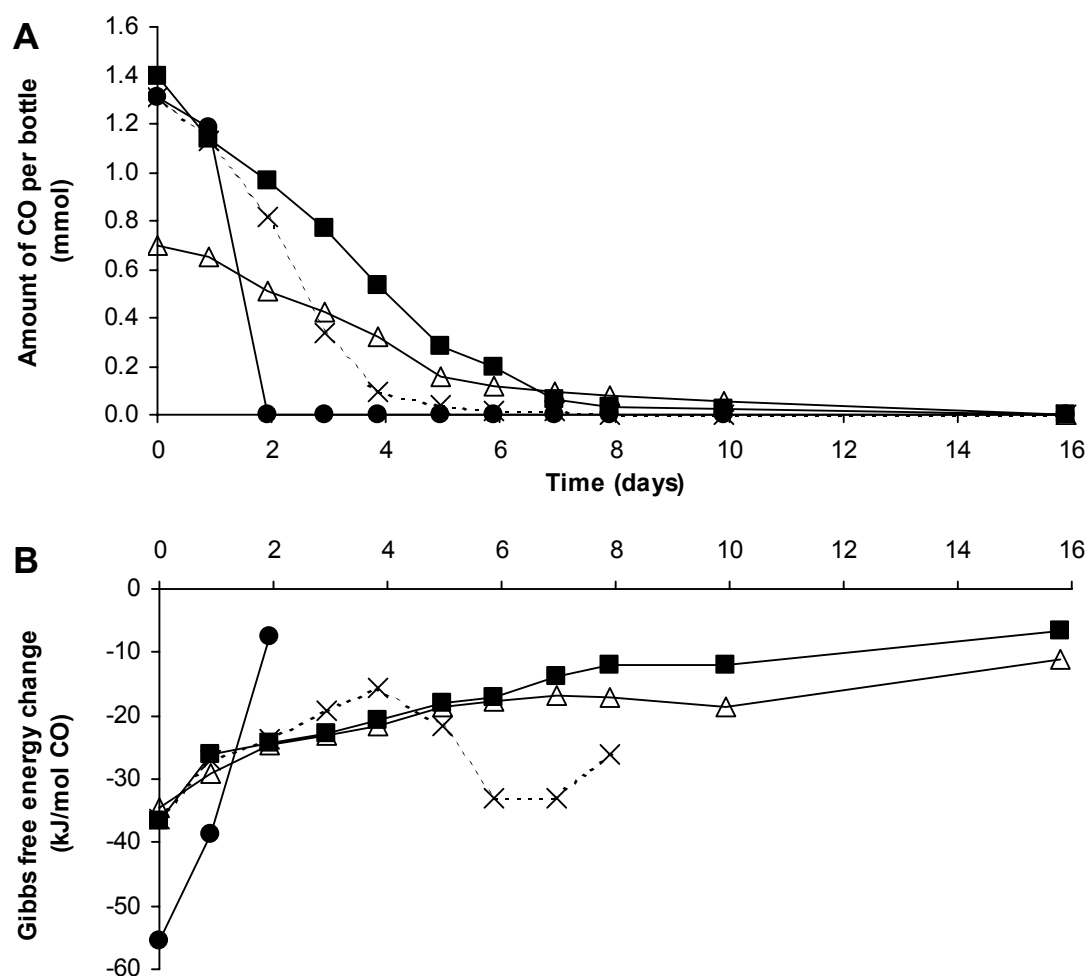
Fig. 4.2 Effect of hydrogen on CO conversion at 55°C with granular Eerbeek sludge. (A) CO depletion in incubations with 24% CO, 71% H_2 and 5% N_2 with BES (■), 24% CO, 73% H_2 and 3% N_2 without BES (×), 12% CO, 78% H_2 and 10% N_2 with BES (Δ), and 24% CO and 76% N_2 without BES (●). (B) Estimated Gibbs free energy values at 55ºC during CO conversion (symbols as in Fig.4.2A). The CO_2 concentration in the gas phase was not measured, but estimated from the carbon balance. The used calculations are presented in the appendix.

At the start, the Gibbs free energy change is highest in the incubations with 24% CO and 76% N_2. This probably results in a higher biomass yield, and consequently higher conversion rate. Consumption of H_2 during CO conversion resulted in a slightly higher CO conversion rate, compared to the BES supplemented incubation. As illustrated in incubations in the absence of BES the consumption of H_2 and CO_2 during CO conversion, resulted in decreased ΔG values (Fig. 4.2B). The Gibbs free energy change for 12 and 24% of CO in the presence of BES showed only slight

differences (Fig. 4.2B), although the rates were lower with decreased CO concentrations (Fig. 4.2A). This is most probably due to a decreased driving force for diffusion, which is largely determined by concentration differences between the gas and liquid phase. The presence of relatively large amounts of H$_2$ did not result in complete inhibition of CO conversion to H$_2$ and CO$_2$, and in each bottle the CO concentration at the end of the experiment was below the CO detection limit of 400 ppm.

4.3.6 Effect of heat treatment of crushed sludge and sulfate reduction capacity

As was shown in Chapter 3 methane formation with crushed Eerbeek sludge, was strongly inhibited by CO, but it was not completely absent as was found in additional experiments (data not shown). Short-term (30 minutes) heat-treatment of granular Eerbeek sludge at 75°C also resulted in a strong but incomplete suppression of methanogenic activity when incubated at 55°C and high P$_{CO}$ (> 160 kPa), whereas no effect on CO conversion was observed (data not shown). A range of temperatures was tested to evaluate the effect of this heat treatment on CO conversion by crushed Eerbeek sludge at 55°C. After exposure to 95°C for 30 minutes, methane formation was absent and all H$_2$ formed from CO was converted to acetate (data not shown). Also exposure of the sludge to treatment temperatures up to 105°C, resulted in a rapid CO conversion without methane formation. Acetate formation started clearly after CO conversion was completed (data not shown). The highest CO conversion rates were found after heat-treatment at 95°C, and they were apparently even higher than those found in the non-treated controls. Treatment at 115°C for 30 minutes resulted in a complete loss of CO conversion.

Following a heat-treatment at 95°C for 30 minutes in the presence of sulfate Eerbeek sludge was capable of reducing sulfate with CO or its conversion products (Fig. 4.3A). This was not observed with Nedalco sludge (Fig. 4.3B), although sulfate reduction with H$_2$/CO$_2$ at 55°C occurred with both Eerbeek and Nedalco sludge (data not shown). Based on sulfate measurements, the sulfate reduction apparently proceeded considerable better than when based on the sulfide measurements. Only 20 to 30% of the sulfate was recovered as sulfide in the medium. Nevertheless, the calculated amount of H$_2$ produced from CO fitted to the sum of H$_2$ remaining at the end of the experiment, the amount used for acetate formation, and the amount

required for the reduction of sulfate. Sulfide analysis of the liquid medium, including the crushed biomass revealed that a large part of the sulfide was present as precipitates or absorbed to the crushed sludge. Taking into account the amount of biomass associated sulfide, corrected for the sulfide amount present in the original inoculum, approximately 85% of the sulfate reduced could be recovered as sulfide.
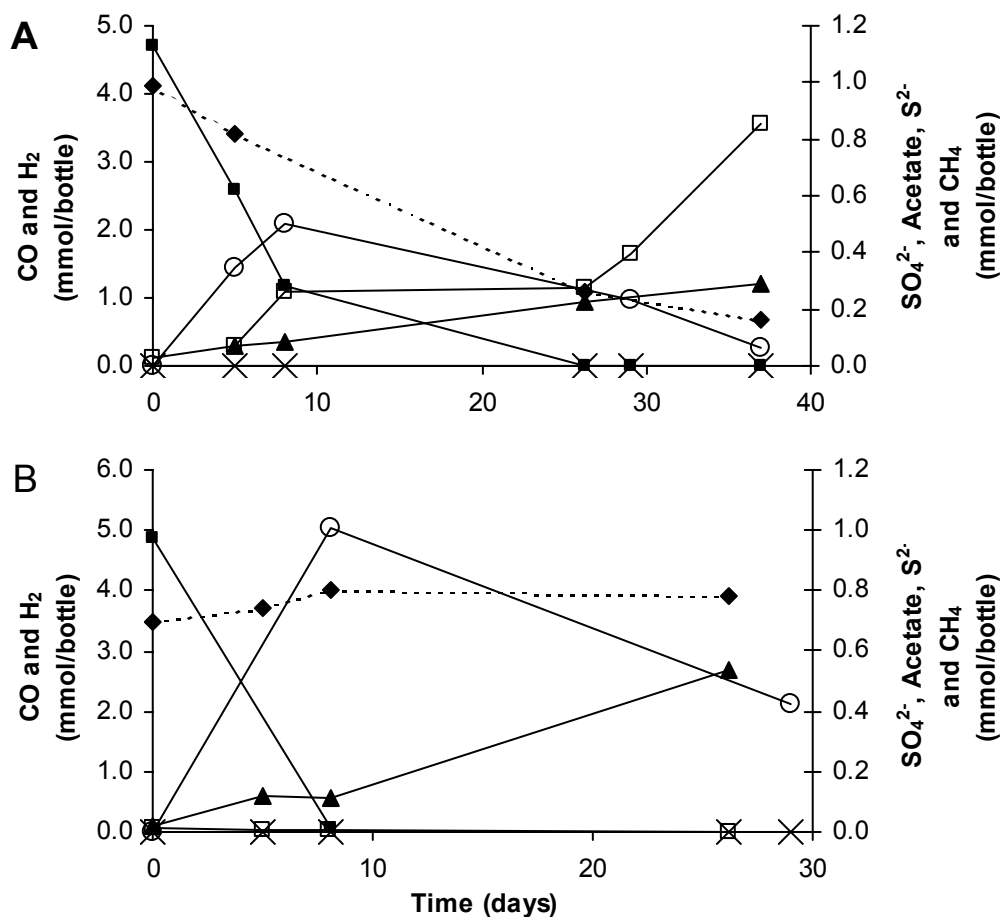

Fig. 4.3 CO conversion at high P_{CO} in sulfate supplemented heat-treated (30 minutes at 95°C) crushed Eerbeek (A) and crushed Nedalco (B) sludge at 55°C. The symbols indicate; carbon monoxide (■), hydrogen (○), methane (×), sulfide (□), sulfate (◆) and acetate (▲).

4.4 Discussion

4.4.1 Effect of CO concentration on CO conversion route

Previous experiments conducted with a number of quite different anaerobic bioreactor sludge samples, showed that at 55°C and P$_{CO}$ > 150 kPa, the CO conversion proceeds via H$_2$ and CO$_2$ (Chapter 3). Independent of the P$_{CO}$ the major part of the CO was converted by hydrogenogens (Svetlichnyi et al., 2001) present within the sludge. At a low P$_{CO}$ a small fraction of the CO was directly converted to methane with Eerbeek sludge (Table 4.3). Complete inhibition of *Methanobacterium thermoautotrophicum*, which is capable to convert CO directly into CO$_2$ and CH$_4$ according to reaction 2 (Table 4.1), occurred at 60 kPa CO (Daniels et al., 1977), whereas *Methanosarcina barkeri* could be slowly adapted to growth at 100% CO (O'Brien et al., 1984). Despite the observed reversibility of CO toxicity as reported by Daniels et al. (1977), methane production with CO exposed Nedalco sludge was not observed in this study. Reversibility of CO toxicity could result in methane production with Eerbeek sludge. However, the short-term CO exposure imposed to crushed Eerbeek sludge resulted in either a complete suppression of methane production or at least dramatic reduction of activity of methanogens. Although the CO was converted with crushed Eerbeek sludge within a few days to values below the detection limit, this relieve of the CO toxicity does not result in a rapid and complete recovery of the methanogenic activity. Apparently CO toxicity is not reversible in crushed Eerbeek sludge. It looks more likely that the higher CO tolerance of the methanogens present in granular Eerbeek sludge can be attributed to a protection mechanism prevailing in sludge granules, for instance the degradation of CO by other microorganisms present in layers at the surface of the granules.

4.4.2 Effect of the presence of H$_2$ on CO conversion

Direct methanogenic CO conversion (reaction 2 or 4, Table 4.1) was not stimulated by the presence of H$_2$, irrespective whether or not CO conversion with H$_2$ is possible at all (reaction 4, Table 4.1). We did not investigate this. Nevertheless, it is clear that the presence of H$_2$ and CO$_2$ as products of the water-shift-reaction (reaction 1, Table 4.1) decreases the Gibbs free energy (ΔG) and therefore will result in decreased CO conversion rates, but not in observable increased final CO concentrations (Fig. 4.2A). CO is removed to concentrations below the detection limit of 400 ppm (e.g. Fig.

4.2A). CO conversion into H_2 and CO_2 yields relatively small amounts of energy for ATP formation (Table 4.1). Initial Gibbs free energy changes are considerably lower when H_2 is present from the start, a situation that would prevail when using synthesis gas (Fig. 4.2B). The differences in ΔG values at the start of these incubations most probably determine the conversion rates. Since the sludge was not adapted to growth on CO, and regarding the absence of hydrogenogenic activity in incubations at 30ºC (Chapter 3), it is clear that in these incubations growth determines the conversion rates. However, since the energy gain of hydrogenogenic CO conversion is rather low, the microorganisms presumably only grow for a short period of time, i.e. as long as the ΔG is sufficiently negative. Thereafter, they may convert CO at a rate uncoupled to growth. It looks that the initial thermodynamical conditions prevailing in the system determine the population size and ultimately the CO conversion rates. At the detection limit of CO (400 ppm), the estimated ΔG is < -7 kJ.(mol CO)$^{-1}$ (Fig. 4.2B). The minimal required ΔG for energy conservation is not known for the microorganisms involved. Hoehler et al. (2001) reported an apparent minimum free energy requirement (threshold ΔG) for hydrogenotrophic methanogens and sulfate-reducing bacteria, of -11 kJ.(mol CH_4)$^{-1}$ and -19 kJ.(mol SO_4^{2-})$^{-1}$, respectively. In chemostat and field studies, catabolic activity was observed when the associated free energy change was at least -10 kJ per mol catabolic reaction (Seitz et al., 1990; Westermann, 1994). This study show that hydrogenogenic CO conversion proceeds to final CO levels below the detection limit of 400 ppm, even in the presence of high levels of H_2, characteristic for synthesis gas. The thermodynamic estimations suggest that the apparent minimum free energy requirement would be even smaller than -7 kJ.mol CO)$^{-1}$. The actual minimal maintenance requirements will ultimately determine the minimal CO concentration that can be reached.

The limiting factor on the conversion rate of gaseous substrates usually is the rate of mass transfer of substrate from the gas phase to the biomass in the aqueous phase (Bredwell et al., 1999). Since, thermodynamics affects the growth of the CO converting population, especially in conversions accompanied with a low energy yield. High CO conversion rates in a bioreactor look only achievable when the CO concentration is maintained at a high level and the biomas retention of the system is such good that dense bacterial populations can develop. Apart from the reactor design, it will be beneficial to apply selective H_2 membranes (Perry et al., 1997) in

combination with CO$_2$ fixation techniques, as these may decrease the ΔG substantially, and by that higher growth and conversion rates can be realized.

4.4.3 Sludge conditioning by heat treatment

Heat treatment of the seed sludge, prior to CO exposure, results in elimination, or at least a dramatic reduction, of one of the undesired side reactions, i.e. methane production. Both heat-treated (30 minutes at 95°C) sludge samples (Nedalco and Eerbeek sludge) seem promising seed sludges for a CO fed hydrogen producing reactor: the heat-treated Eerbeek sludge is also suitable for start-up of thermophilic (55°C) sulfate reducing reactors using synthesis gas as cheap electron donor. Sulfate reducers are reported to be more susceptible to CO toxicity compared to methanogens (Mörsdorf et al., 1992). However, methanogens were severely inhibited in crushed Eerbeek sludge, whereas sulfate reduction proceeded well even after exposing the sludge to a heat-treatment at 95°C (Fig. 4.3A). Some sulfate reducing bacteria, e.g. *Desulfotomaculum* sp., according to Mörsdorf et al. (1992) are capable to metabolize CO at relative low CO concentrations, i.e. up to 20%. This feature of sulfate reduction at high CO concentrations might become extremely attractive for the direct utilization of synthesis gas as a cheap electron donor for sulfate reduction processes.

The cost of a short-term heat treatment likely is rather low, especially when taking into account the prevention of unwanted loss of electron donor to methanogens during reactor operation. The elimination of methanogenesis by a short-term heat treatment is expected to decrease the long-term operational costs considerably. Especially since sulfate reducers are generally characterized by higher specific growth rates, lower affinity constants and higher yields compared to methanogens (Oude Elferink et al., 1993). Nevertheless, at 55ºC a strong competition for hydrogen between sulfate reducing bacteria and methanogens was observed by Van Houten et al. (1997), which was attributed mainly to the high maximal growth rates of thermophilic methanogens. Elimination of methanogenesis is suggested to result in higher electron flow to sulfate reduction and thus in a more cost efficient process for the treatment of sulfate containing wastewaters.

4.5 Conclusions

The results obtained in this chapter demonstrate that:

1. The CO conversion routes by thermophilic populations within the two tested granular sludges were not affected by the partial CO pressure (P_{CO}) in the incubation bottles (4-160 kPa).

2. The initial presence of H_2, which is characteristic for synthesis gas, lowers the ΔG values of hydrogenogenic CO conversions. Thus lower CO conversion rates and ultimately also higher final CO concentrations are expected. Nevertheless, the final concentrations in this study were all below 400 ppm.

3. The presence of sulfate reducing activity in incubations at 55ºC with initially 160 kPa CO and inoculated with crushed Eerbeek sludge and the absence of methane production in the same incubations is promising for a biotechnological application of synthesis gas for sulfate reduction processes at elevated temperatures, e.g. biological flue gas desulfurization.

4. Short-term heat-treatment (95ºC) of the sludge prior to incubation at 55ºC completely eliminated methanogenic activity in batch incubations. It was found to be a more effective method to prevent methanogenesis than disrupting the granular sludge structure.

Appendix: Gibbs free energy change calculations (as used in Fig. 4.2B)

As the CO_2 concentrations in the gas phase were not measured in the course of this experiment they were estimated from the balance of CO_2 production due to CO conversion and CO_2 loss due to consumption by methanogens and/or homoacetogens. The total number of moles CO_2 was recalculated into the partial pressure of CO_2 in the gas phase according to:

$$n_{tot} = n_{gas} + n_{liq}$$

in which n = number of moles CO_2

and:

$$\frac{[CO_2]_{(liq)}}{[CO_2]_{(gas)}} \equiv K_H \cdot RT = 0.4386$$

in which: $[CO_2]$ = concentration CO_2

K_H = Henry constant for CO_2 at 55ºC (= 0.016075 M.bar^{-1})

R = Gas constant = 8.3145 J.mol^{-1}.K^{-1}

T = temperature in Kelvin

$$\frac{n_{liq}}{n_{gas}} = 0.4386 \cdot \frac{V_{liq}}{V_{gas}}$$

in which: n_{liq} = amount of moles CO_2 in the liquid phase

V_{liq} = volume of the liquid phase (in mL)

Rearranging results in:

$$n_{gas} = \frac{n_{tot}}{\left(1 + 0.4386 \cdot \dfrac{V_{liq}}{V_{gas}}\right)}$$

From the amount of moles of CO_2 in the gas phase, the partial pressure of CO_2 (in Pa) in the gas phase was calculated using the ideal gas law:

$$P_{CO_2} = \frac{n_{CO_2,gas} \cdot RT}{V_{gas} \cdot 10^{-6}}$$

Finally, the Gibbs free energy changes were calculated for each measured point according to:

$$\Delta G = \Delta G'_{55} + R \cdot 10^{-3} \cdot T \cdot \ln\left(\frac{P_{H_2} \cdot P_{CO_2}}{P_{CO}}\right)$$

in which the activities of the participating gases were assumed to be equal to its partial pressure in the gas phase (in atm) and the activity of water was set to unity. Furthermore, the amount of gas, when the amount in the headspace was below its detection limit, was set equal to 400 ppm in order to enable calculations. The detection limits of both CO_2 and H_2 were well below this 400 ppm.

5

Desulfotomaculum carboxydivorans; a sulfate reducer capable of hydrogenogenic growth on CO in the presence and absence of sulfate

Abstract

A moderately thermophilic, anaerobic, chemolithoheterotrophic, sulfate-reducing bacterium strain CO-1-SRB was isolated from an anaerobic sludge treating paper mill wastewater. Cells were gram-positive, motile, spore-forming rods. The temperature and pH range for growth were 30-68°C (T_{opt} = 55°C) and pH 6.0-8.0 (pH_{opt} = 7.0), respectively. The unique property of this isolate is that carbon monoxide (CO) could serve as a sole energy and carbon source both in the presence and absence of sulfate. CO was converted to H_2 and CO_2 and in the presence of sulfate the formed H_2 was used for sulfate reduction. Phylogenetic analysis of the 16S rRNA gene sequence placed CO-1-SRB to the genus *Desulfotomaculum* closely resembling *Desulfotomaculum nigrificans* DSM 574[T] and *Desulfotomaculum* sp. RHT-3 (99 and 100% similarity respectively). Nevertheless, the latter strains were completely inhibited at high levels of CO and only metabolized CO in the presence of sulfate. Based on phylogenetic and physiological features it is proposed that strain CO-1-SRB represents a novel species within the genus *Desulfotomaculum*, with the type species *Desulfotomaculum carboxydivorans*. This is the first description of a sulfate reducing microorganism capable of growth under an atmosphere of pure CO with and without sulfate (Type strain CO-1-SRB[T], DSM 14 880[T], VKM B-2319[T]).

5.1 Introduction

Anaerobic conversion of CO has been reported for a large number of microorganisms, including phototrophic, homoacetogenic, methanogenic and sulfate reducing bacteria (Mörsdorf et al., 1992; Davidova et al., 1994). Several anaerobic hydrogenogenic thermophilic bacteria, able to convert CO with H_2O to H_2/CO_2 were isolated from different natural habitats (Svetlichny et al., 1991b, 1994; Sokolova et al., 2001, 2002, 2004a). Recently, the first hyperthermophilic archaeon, capable of hydrogenogenic CO conversion was isolated (Sokolova et al., 2004b). All of the latter bacteria revealed apparent uninhibited growth on CO as they possessed fast generation times when grown at high partial pressures of CO (> 101 kPa) in the gas phase, despite that these conditions are never encountered in real environments.

In contrast to the so-called hydrogenogens (Svetlichny et al., 2001), most anaerobic microorganisms metabolizing CO are sensitive to high levels of CO. In general sulfate reducing bacteria were considered to be highly sensitive for CO toxicity (Mörsdorf et al., 1992; Davidova et al., 1994). Despite that several sulfate-reducing bacteria are capable of CO conversion in concentrations up to 20 kPa, higher concentrations completely inhibited growth (Lupton et al., 1984; Klemps et al., 1985; Karpilova et al., 1983; Mörsdorf et al., 1992; Davidova et al., 1994). Recent experiments with several strains of thermophilic sulfate reducing bacteria demonstrated that *Desulfotomaculum kuznetsovii* and *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* are able to use CO as a sole carbon and energy source up to 50 kPa CO in the gas phase and reduce sulfate with CO (Parshina et al., 2005). No sulfate reducing bacterium, growing on pure CO, was isolated up to date.

Batch experiments at elevated temperatures (55°C) with several mesophilic anaerobic sludges revealed the presence of viable populations of fast growing hydrogenogenic CO-oxidizing bacteria (Chapter 3). Furthermore, the presence of sulfate reduction activity at high CO concentrations, up to 100% CO and 180 kPa (Chapter 4), was not reported previously. For investigation of hydrogenogenic CO conversion mechanisms at moderately thermophilic conditions the isolation of a pure culture from the anaerobic granular sludge was desirable.

5.2 Materials and Methods

5.2.1 Isolation procedure

Anaerobic granular (methanogenic) sludge samples were obtained from a full-scale anaerobic reactor treating wastewater from several paper mills (Industriewater Eerbeek, Eerbeek, The Netherlands). This sludge was originally cultivated at 30-35°C. Laboratory experiments on CO conversion by this sludge were described in Chapter 4.

To obtain an enrichment of hydrogenogenic CO converting bacteria, bottles with crushed Eerbeek sludge were incubated at 55°C. A suspension of crushed granules was obtained as reported previously (Chapter 3) and cultivated in a liquid medium. The medium was prepared as described before (Chapter 4). After a few series of dilution under an atmosphere of 100% CO (at 160-180 kPa) an enrichment culture was obtained that contained at least 3 morphologically different strains of bacteria. Addition of 20 mM sodium sulfate to the dilution series under 160 kPa CO resulted in a suspension of morphologically identical cells. Roll-tubes with the same medium supplemented with 5% agar and pure CO in the gas phase were prepared to obtain separate colonies. Some of the obtained colonies were subsequently inoculated in the liquid medium. The purity of the obtained strain, designated as strain CO-1-SRB, was monitored by phase-contrast microscopy after cultivation on CO with and without sulfate, cultivation on H_2/CO_2 with and without sulfate and product analysis.

5.2.2 Bacterial strains used for comparison

Strain *Desulfotomaculum nigrificans* DSM 574[T] was received from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and *Desulfotomaculum* sp. RHT-3 was kindly provided by Prof. K. Takamizawa (Gifu University, Japan). These strains were cultivated in the medium supplemented with pyruvate or under an atmosphere of H_2/CO_2 in the presence of sulfate.

5.2.3 Physiological characterization of strain CO-1-SRB

Unless otherwise stated, experiments were performed in duplicate. Growth was monitored by measuring the optical density at 600 nm (OD_{600}), using light phase-contrast microscopy, CO consumption, formation of gaseous (H_2 and CO_2) products and H_2S. The temperature range for growth was determined without agitation with

CO as a sole substrate. The NaCl range was obtained at 55°C without agitation. To determine the pH range for growth on CO the concentration of the phosphate buffer was increased to 50 mM.

Potential electron donors for growth were tested (20 mM each if not indicated differently): pyruvate, lactate, glucose, fructose, sucrose, maltose, galactose, serine, alanine, acetate, formate, butyrate, fumarate, benzoate, ethylenglycol, cellobiose, cellulose (filter paper), methanol, ethanol, propanol, butanol, as well as H_2/CO_2 (ratio 80/20%), CO (200 kPa) with (20 mM) and without sulfate. Potential electron acceptors that were tested: sulfate (20 mM), thiosulfate (20 mM), sulfite (2 mM), nitrate (10 mM) and sulfur (2 g/l).

5.2.4 Molecular analysis

Analysis of the 16S rRNA sequence of the isolate was performed by the identification service of DSMZ as described by Parshina et al. (2003). Sequence similarity searches were performed using the BLAST algorithm at http://www.ncbi.nih.gov/blast/ (Altschul et al., 1990). Phylogenetic analysis and tree construction were performed with programs of the ARB software package (Ludwig et al., 2004). The phylogenetic tree was constructed using the neighbor-joining method (Saitou & Nei, 1987), and is based on the results of distance matrix analysis including only those nucleotides between *E. coli* positions 49 to 1387 that are conserved in at least 50% of sequences from relevant members of Gram positive bacteria. The topology of the tree was confirmed using maximum parsimony and maximum likelihood methods as implemented in the ARB software package.

DNA isolation, GC content and DNA-DNA hybridization analyses were done by the identification service of DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and at the Institute of Microbiology (INMI, Russian Academy of Sciences, Moscow, Russia).

In DSMZ DNA was isolated according to the procedure of Cashion et al. (1977). For determination of the GC content the DNA was hydrolyzed with P1 nuclease and the nucleotides were dephosphorylized with bovine alkaline phosphatase (Mesbah et al., 1989). The resulting deoxyribonucleotides were analyzed by HPLC (Shimadzu Corp., Japan) by a method adapted from Tamaoka & Komagata (1984). Calibration was performed with non-methylated Lambda-DNA (Sigma), GC content 49.858 mol% (Mesbah et al., 1989). The GC content was calculated from the ratio of

deoxyguanosine (dG) and thymidine (dT) according to the method of Mesbah et al. (1989). DNA-DNA hybridization was carried out as described by De Ley et al. (1970), with the modification described by Huss et al. (1983) and Escara and Hutton (1980) using a model 2600 spectrophotometer equipped with a model 2527-R thermoprogrammer and plotter (Gilford Instrument Laboratories Inc., Oberlin, Ohio, USA). Renaturation rates were computed with the TRANSFER.BAS program by Jahnke (1992).

In INMI DNA isolation, determination of the GC content and DNA-DNA hybridization of strain CO-1-SRB were performed as described by Parshina et al. (2003).

5.2.5 Chemical analysis

All chemicals used were of analytical grade and supplied by Merck (Darmstadt, Germany). CO (purity 99.997%) was supplied by Hoek Loos (Rotterdam, The Netherlands). H_2 and CO were analyzed by gas chromatography as described by Parshina et al. (2005). CO_2 was analyzed according to Henstra and Stams (2004). Volatile fatty acids were analyzed by HPLC as described by Stams et al. (1993). Sulfide was analyzed according to Trüper and Schlegel (1964). Desulfoviridin was analyzed according to Postgate (1979).

5.3 Results and Discussion

5.3.1 Morphological and physiological characterization of strain CO-1-SRB

Cells of strain CO-1-SRB were rod-shaped with rounded ends (0.5-1.5 μm thick and 5-15 μm long), occurred sometimes paired (Fig. 5.1). Cells of strain CO-1-SRB were motile with "twisting and tumbling" movements. Strain CO-1-SRB formed oval spores, terminal or subterminal. After 6 days of growth in medium supplemented with glucose, colonies were of 0.5 mm in diameter, rhizoid, black.
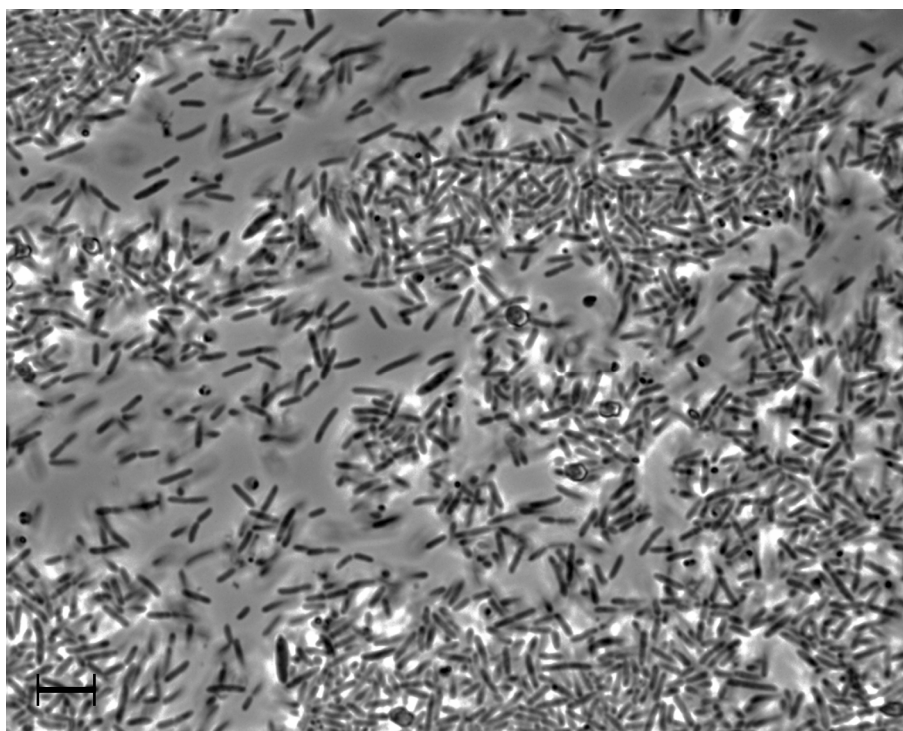
Fig. 5.1 Phase contrast micrograph of a glucose grown culture of strain CO-1-SRB. Bar 10 μm.

Strain CO-1-SRB grew between 30 and 68°C, with an optimum at 55°C. Growth occurred between 0 and 17 g NaCl l^{-1}, with a growth optimum at 0-8 g NaCl l^{-1}. Growth occurred between pH 6.0 and 8.0 with an optimum between 6.8 to 7.2.

Growth in the presence of sulfate was found on CO (200 kPa), H_2/CO_2, pyruvate, glucose, fructose, maltose, lactate, alanine, serine, butyrate (weak), ethanol and glycerol. Very weak growth was observed on yeast extract alone (2 g/l) plus sulfate. Growth in the absence of sulfate was found with CO (200 kPa), pyruvate, lactate, glucose and fructose. No growth in the presence of sulfate was observed on: malate, fumarate, benzoate, cellobiose, galactose, maltose, butyrate and ethylenglycol. No growth was observed in the absence of sulfate on: acetate, malate, fumarate,

glycerol, alanine, ethanol, methanol, formate, butyrate, benzoate, cellobiose, galactose and ethylenglycol. Growth with sulfate, thiosulfate and sulfite as electron acceptor was observed, whereas no growth was found with sulfur and nitrate. Glucose was incompletely oxidized to acetate. Yeast extract (0.5 g l^{-1}) was necessary for growth.

Fig. 5.2 shows the dynamics of growth of strain CO-1-SRB on 160 kPa CO, in the absence and presence of sulfate. The CO conversion rates were similar. Pure CO was converted with stoichiometric production of H_2 and CO_2. The OD at the end of the growth was 0.2, but when CO conversion was coupled to sulfate reduction (Fig. 5.2B), hydrogen and H_2S were formed and a final OD of 0.32 was obtained. No other products were formed during CO conversion. In the presence of CO (160 kPa) and 20 mM of sulfate, sulfide accumulated to concentrations of maximal 6 mM, whereas H_2 and CO_2 were not completely depleted. Despite the cease of sulfide production, most probably as a result of sulfide inhibition, CO was completely converted into H_2 and CO_2 (Fig. 5.2B), indicating that H_2 was used as electron donor for sulfate reduction, rather than CO.

5.3.2 Phylogenetical characterization of strain CO-1-SRB

Phylogenetic analysis revealed that strain CO-1-SRB is highly related to *Desulfotomaculum nigrificans* (99%), based on their 16S rRNA sequences. Gene sequence of 16S rRNA of the closest relatives had the following relatedness with CO-1-SRB (%): *D. acetoxidans* (88.2); *D. ruminis* (93.1); *D. putei* (93.7); *D. aeronauticum* (93.9); *D. nigrificans* (99); *D.* sp. RHT-3 (100). Fig. 5.3 presents the 16S rRNA-based phylogenetic tree constructed using the neighbour-joining method, showing the closest relatives of strain CO-1-SRB.
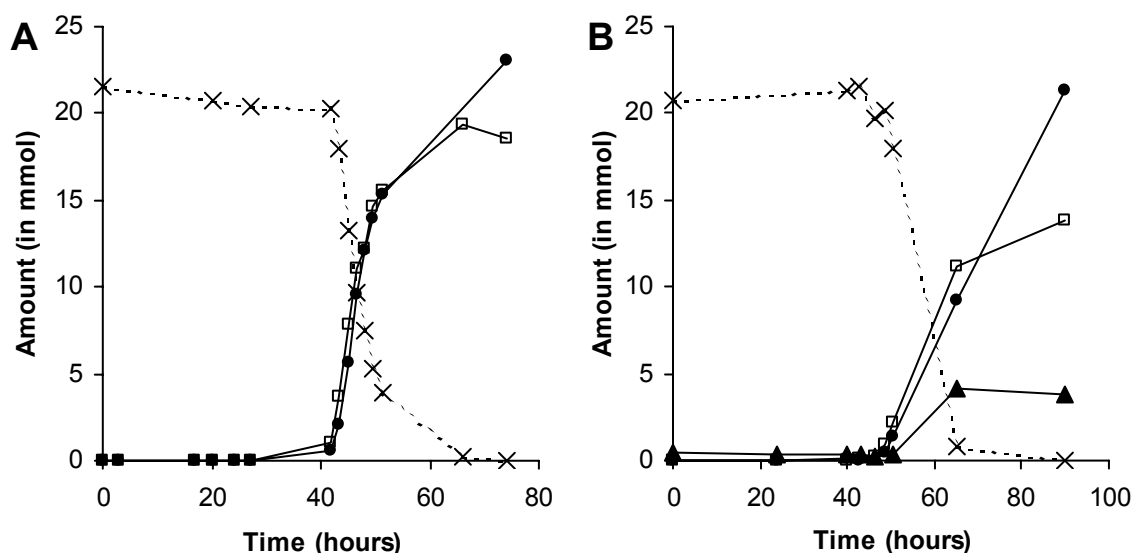
Fig. 5.2   CO conversion by strain CO-1-SRB under an atmosphere of 160 kPa CO in the absence (A) and presence (B) of sulfate. Symbols: carbon monoxide ($\times$), hydrogen ($\square$), carbon dioxide ($\bullet$) and sulfide ($\blacktriangle$).
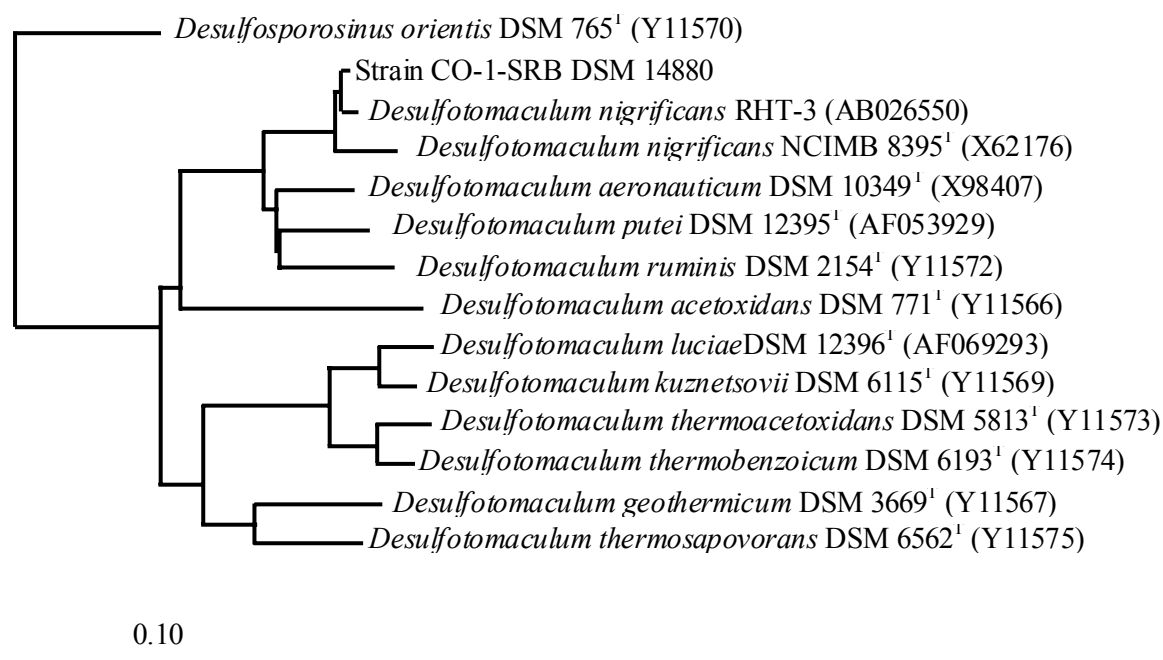

Desulfosporosinus orientis DSM 765[1] (Y11570)
Strain CO-1-SRB DSM 14880
Desulfotomaculum nigrificans RHT-3 (AB026550)
Desulfotomaculum nigrificans NCIMB 8395[1] (X62176)
Desulfotomaculum aeronauticum DSM 10349[1] (X98407)
Desulfotomaculum putei DSM 12395[1] (AF053929)
Desulfotomaculum ruminis DSM 2154[1] (Y11572)
Desulfotomaculum acetoxidans DSM 771[1] (Y11566)
Desulfotomaculum luciae DSM 12396[1] (AF069293)
Desulfotomaculum kuznetsovii DSM 6115[1] (Y11569)
Desulfotomaculum thermoacetoxidans DSM 5813[1] (Y11573)
Desulfotomaculum thermobenzoicum DSM 6193[1] (Y11574)
Desulfotomaculum geothermicum DSM 3669[1] (Y11567)
Desulfotomaculum thermosapovorans DSM 6562[1] (Y11575)

0.10

Fig. 5.3   16S rRNA-based phylogenetic tree constructed by the neighbour-joining method [4], using *E.coli* positions 49 to 1387, and showing the position of strain CO-1-SRB in relation to members of the genus *Desulfotomaculum*. Bar represents 10% divergence.

The GC content of the genome of strain CO-1-SRB after using two different methods was quite similar: 45.6 mol% (DSMZ analysis) and 46.9 mol% (INMI analysis). The GC content of *D. nigrificans* 574[T] was 51.1 mol% and for *D.* RHT-3 46.1 mol% (both INMI analysis). The GC content of *D. nigrificans* 574[T] is significantly higher than of either CO-1-SRB or RHT-3, which suggests to differentiate the latter strains from *D. nigrificans*.

DNA-DNA hybridization of strain CO-1-SRB with *D. nigrificans* DSM 574[T] showed 70.5% relatedness (DSMZ analysis) and 53% (INMI analysis). DNA homology of CO-1-SRB with RHT-3 was 60% (INMI analysis). Moreover, DNA similarity of *D. nigrificans* 574[T] with RHT-3 was 61% (DSMZ) and 52% (INMI). The relatedness of strain CO-1-SRB with *D. nigrificans* determined at DSMZ is just at the threshold for species diversification as determined by the ad hoc committee on reconciliation of approaches to bacterial systematics (Wayne et al., 1987). This indicates that for species diversification additionally considerable physiological differences are required. Nevertheless, when considering the analysis performed at INMI for DNA-DNA hybridization of all three strains among each other, the difference between them was well below this 70%, indicating species diversification.

5.3.3 Comparison of strain CO-1-SRB with its closest phylogenetic relatives

Like *D. nigrificans* 574[T], strain CO-1-SRB did not contain desulfoviridin, i.e. dissimilatory sulfite reductase, which absence is characteristic for the genus *Desulfotomaculum* (Madigan et al., 1997).

The morphological and physiological comparison of the phylogenetically related strains is given in Table 5.1. Strains *D. acetoxidans* (Widdel and Pfennig, 1977)*, D. ruminis* (Campbell and Postgate, 1965) and *D. aeronauticum* (Hagenauer et al., 1997) are mesophilic with a temperature optimum of 36-37°C and are therefore excluded from further comparison. *D. putei* is thermophilic, but does not grow on glucose and fructose in the presence of sulfate and does not ferment lactate, glucose and fructose (Lui et al., 1997). Furthermore, its 16S rRNA sequence revealed a similarity less than 94%, whereas similarities below 97% are generally accepted for diversification of species (Madigan et al., 1997).

Strain CO-1-SRB revealed high 16S rRNA sequence similarity with *D. nigrificans* and *D.* RHT-3 and therefore we focus on a comparison of these three bacteria. *D. nigrificans* does not grow on glycerol with sulfate as electron acceptor

and does not ferment lactate. *D.* RHT-3 (Mori et al., 2000) did not to utilize ethanol in the presence of sulfate (Mori et al., 2000). Sequence similarities of strain RHT-3 to *D. nigrificans*, *D. aeronauticum* and *D. putei* were 96.6, 95.3 and 95% and was therefore assumed to be a new species within the genus *Desulfotomaculum*. However, to our knowledge this bacterium has not been described validly yet. The isolate CO-1-SRB was able to grow on ethanol in the presence of sulfate and weak growth was observed on glycerol with sulfate as electron acceptor. Furthermore, strain CO-1-SRB could actively ferment lactate, glucose and fructose. The most significant difference was the ability to oxidize CO. According to our experiments, both *D. nigrificans* 574^T and RHT-3 were not able to oxidize CO without sulfate. We found that in the presence of sulfate *D. nigrificans* could grow on CO in the concentrations 5-20 kPa, but not higher, which is in agreement with the data of Klemps et al. (1985). Furthermore, *D.* RHT-3, not previously tested for its CO utilizing capacity, was able to grow on CO up to values of 50 kPa. However, strain CO-1-SRB could grow both in the presence and absence of sulfate on pure CO at 200 kPa. Moreover, during growth of *D. nigrificans* and *D.* RHT-3 on CO, hydrogen never was detected in the gas phase. This permits to suggest that these strains may use CO directly for sulfate reduction without intermediate formation of hydrogen, like *D. kuznetsovii* and *D. thermobenzoicum* subsp. *thermosyntrophicum* described recently (Parshina et al., 2005). During growth of strain CO-1-SRB on all studied CO concentrations (5-200 kPa) with sulfate, hydrogen was always an intermediate. During growth on CO without sulfate, hydrogen and carbon dioxide were the only products of CO oxidation.

Table 5.1 Comparison of morphological and physiological characteristics of strain CO-1-SRB and phylogenetically related thermophilic *Desulfotomaculum* species.

Characteristics	CO-1-SRB	*D.* RHT-3	*D. nigrificans*	*D. putei*
Cell diameter (µm)	0.5-1.5	0.8-1.0	0.5-0.7	1.0-1.1
Cell length (µm)	5.0-15.0	3.0-3.3	2.0-4.0	2.0-5.0
Salinity range (g l^{-1})	0-16	n.r.	<20	<20
Salinity opt (g l^{-1})	0-8	n.r.	0	n.r.
T range (°C)	30-68	45-60	30-70	22-65
T opt (°C)	55	55	55	64
pH range	6.0-8.0	6.0-7.5	6-8	6-7.8
pH opt	6.8-7.2	n.r.	7.0-7.9	7.0-7.9
G+C (mol %)	46.9	46.1	49.9 (51.1)	47.1
Fermentative growth:				
CO	+	-	-	n.r.
Pyruvate	+	n.r.	+	+
Lactate	+	n.r.	-	-
Glucose	+	n.r.	-	-
Fructose	+	n.r.	+/-	-
As electron donors:				
CO (%)	+ (200 kPa)	+ (<50 kPa)	+ (<20 kPa +1mM acetate)	n.r.
H$_2$	+	+	+	+
Pyruvate	+	+	+	+
Glucose	+	n.r.	+/-	-
Fructose	+	n.r.	+	-
Lactate	+	+	+	+
Alanine	+	n.r.	+	n.r.
Serine	+	n.r.	n.r.	n.r.
Ethanol	+	-	+	+
Methanol	-	n.r.	-	+/-
Acetate	-	n.r.	-	-
Electron acceptors:				
Na$_2$SO$_4$	+	+	+	+
Na$_2$SO$_3$ (2 mM)	+	+	+	+
Na$_2$S$_2$O$_3$	+	+	+	+
Elemental sulfur	-	n.r.	-	-

+, good growth; +/-, weakly; - no growth; n.r., not reported.

Data obtained from: Mori et al., 2000; Akagi & Jackson, 1967; Liu et al., 1997; Campbell & Postgate, 1965; Campbell & Singleton, 1986; Klemps et al., 1985.

Based on the 16S rRNA sequence strain CO-1-SRB cannot be differentiated from *D. nigrificans* and *D.* sp. RHT-3. DNA-DNA hybridization results suggest that there is sufficient ground to ascribe them as separate species. Furthermore, the GC content of strain CO-1-SRB (46.9%) is significantly lower than that of *D. nigrificans*, which was determined, using the same method, to be 51.1% (INMI). Furthermore, the physiology of CO-1-SRB is clearly different from that of *Desulfotomaculum* sp. RHT-3 and *D. nigrificans*. CO-1-SRB differentiates from *D. nigrificans* by its ability to ferment 100% CO without sulfate in the medium. It is important to emphasize that this is a unique property among sulfate-reducing bacteria. Strains *D. nigrificans* and RHT-3 were not able to grow even on low CO concentrations in the absence of sulfate. Strain CO-1-SRB presents phylogenetic and physiological properties that are clearly different from those of *D. nigrificans* that justifies its description as a new species within the genus *Desulfotomaculum*, for which we propose the name *Desulfotomaculum carboxydivorans* with type strain CO-1-SRB^T.

5.4 Description of *Desulfotomaculum carboxydivorans* sp. nov.

Desulfotomaculum carboxydivorans (N.L. n. carboxydum, carbon monoxyde; L. part. adj. vorans, devouring; N.L. part. adj. carboxydivorans, carbon monoxyde digesting).

Cells are rod-shaped with rounded ends, 0.5-1.5 x 5-15 μm, single or sometimes paired. Cells of strain CO-1-SRB are motile with "twisting and tumbling" movements. Cells form oval spores, terminal or sub-terminal. CO (100% in the gas phase) can serve as a sole electron donor both in the presence and absence of sulfate. Other substrates utilized with sulfate are H_2/CO_2, pyruvate, lactate, glucose, fructose, maltose, ethanol, glycerol, alanine and serine. The bacterium ferments pyruvate, lactate, glucose and fructose. The optimum pH is 6.8-7.2; the optimum temperature is 55°C. The G+C content of the DNA is 45.6 mol%. The type strain is CO-1-SRB (=DSM 14880^T, =VKM B- 2319^T).

6

Physiological study of *Desulfotomaculum carboxydivorans* for biotechnological synthesis gas conversions

Abstract

Recently, *Desulfotomaculum carboxydivorans* was isolated, the first sulfate reducer capable of hydrogenogenic growth on carbon monoxide (CO). In the presence of sulfate the hydrogen formed is used for sulfate reduction. This organism grows rapidly at 200 kPa CO, pH 7.0 and 55ºC, with a generation time of 100 minutes, producing nearly equimolar amounts of H_2 and CO_2 from CO. High specific CO conversion rates, exceeding 0.8 mol CO.(g protein)$^{-1}$.hour^{-1}, make it an interesting candidate for a biological alternative for the currently employed chemical catalytic water-gas-shift reaction to purify synthesis gas (contains mainly H_2, CO and CO_2). Furthermore, as *D. carboxydivorans* is capable of hydrogenotrophic sulfate reduction at partial CO pressures exceeding 100 kPa, it is also a good candidate for biodesulfurization processes at elevated temperatures, e.g. in biological flue gas desulfurization. The determined maximal specific sulfate reduction rate of 32 mmol.(g protein)$^{-1}$.hour^{-1} is promising, although its tolerance for sulfide is rather low and pH dependent, i.e. at pH 7.2 maximally 9 mM sulfide and at pH 6.5 maximally 5 mM. The sulfide toxicity may require special techniques to reduce the sulfide concentration in the reactor system.

6.1 Introduction

Bulk production of hydrogen originates from gasification of fossil fuels, although in principle any organic matter can be gasified to a synthesis gas rich in H_2. Besides H_2, synthesis gas may contain large amounts of carbon monoxide (CO) depending on the type of organic matter (Perry et al., 1997; Armor, 1999). The CO present in synthesis gas represents a substantial amount of energy and simply separation of H_2 from the other synthesis gas components would result in a loss of energy. Therefore, conversion of CO to H_2, according to the water-gas-shift reaction (1) is employed to improve the efficiency of H_2 production processes.

$$CO + H_2O \rightarrow CO_2 + H_2 \qquad \Delta G = -20 \ kJ.mol^{-1\#} \quad (1)$$

Currently, this shift reaction is performed at high temperatures and pressures using chemical catalysts (Czuppon et al., 1995). The discovery of bacteria capable of growth employing a biological analogue of the water-gas-shift reaction holds a promise for the design of a biological alternative for this chemical catalytic reaction (Svetlichny et al., 1991b, 1994; Sokolova et al, 2001, 2002, 2004a,b). These obligate anaerobic hydrogenogenic CO converting organisms sofar were isolated exclusively from thermophilic hot environments related to volcanic activity, which contain small amounts of CO (Symonds et al., 1994). All these isolates showed fast growth on CO with generation times between 1.1 and 8.3 hours at partial pressures of CO in the gas phase exceeding 100 kPa.

Besides the use of H_2 as possible future energy carrier, it has great potential in biotechnological reductive processes, e.g. biodesulfurization of inorganic wastewaters (Van Houten et al., 1994; 1997). Treatment of sulfate rich inorganic wastewater requires addition of an electron donor. Direct use of synthesis gas without a H_2 purification step reduces the operational costs significantly (Van Houten and Lettinga, 1996; Du Preez et al., 1992), provided that the CO present in synthesis gas does not inhibit the biological sulfate reduction process. In general, sulfate reducing bacteria are highly sensitive to CO (Mörsdorf et al., 1992; Davidova et al., 1994). However, *Desulfotomaculum nigrificans*, *Desulfosporosinus orientis* (Klemps et al., 1985) and *Desulfovibrio desulfuricans* (Davidova et al., 1994) are capable to use CO (up to 20 kPa) as electron donor for sulfate reduction.

[#] ΔG value was calculated with the data set compiled by Amend and Shock (2001)

Recently two sulfate reducers, *Desulfotomaculum kuznetsovii* and *Desulfotomaculum thermobenzoicum* subsp. *thermosynthrophicum* were shown capable of CO utilization at CO levels in the gas phase up to 50 kPa (Parshina et al., 2005). The recently isolated *Desulfotomaculum carboxydivorans* is the first sulfate reducer that shows uninhibited growth on 200 kPa CO and 55ºC both in the presence and absence of sulfate (Chapter 5). In the absence of sulfate, it grows hydrogenogenically on CO producing nearly equimolar amounts of H_2 and CO_2 (Chapter 5).

As a result of its unique properties to convert CO to H_2 and sulfate reduction with the hydrogen produced, *D. carboxydivorans* may be applicable in both synthesis gas purification and biodesulfurization at elevated temperatures, e.g. flue gas desulfurization. To evaluate the potential of *D. carboxydivorans* in both processes more detailed information on its physiological features is desired. Here we describe substrate kinetics of the bacterium in the presence and absence of sulfate.

6.2 Materials and Methods

6.2.1 Experimental set-up

Tests for assessing selected physiological features of *D. carboxydivorans* were all performed in a temperature controlled (55°C) shaker-incubator type RFI-125 (Infors AG, Basel, Switzerland) at 200 rpm. The basal medium contained (in mM): NaCl 5.1, NH_4Cl 5.6, $CaCl_2.2H_2O$ 0.7, $MgCl_2.6H_2O$ 0.5, $Na_2S.9H_2O$ 0.6, yeast extract 500 mg.l^{-1}, and 1 ml.l^{-1} of an acid and alkaline trace element solution according to Stams et al (1993). The medium was buffered at pH 7.0 using 8.2 mM KH_2PO_4 and 11.4 mM $Na_2HPO_4.2H_2O$, when CO was the substrate. When H_2/CO_2 was supplied in the gas phase the concentration of the phosphate buffer was decreased to 10% of the former and 23.8 mM sodium bicarbonate was supplemented to buffer the medium at pH 7 with 30 kPa of CO_2 in the gas phase at 55ºC. Physiological tests were performed in serum bottles of 117, 310 or 570 mL containing 50, 100 and 200 mL of basal medium, respectively. The bottles were sealed with butyl rubber stoppers and the gas phase was changed to CO or H_2/CO_2 (80/20). Prior to inoculation with *D. carboxydivorans*, the bottles were autoclaved at 121ºC for 30 minutes.

6.2.2 Experimental design

Experiments to determine the growth curves in the absence or presence of sulfate for *D. carboxydivorans* were performed in 570 mL serum bottles. The gas phase contained about 150 kPa CO and 10 to 20 kPa N_2. Experiments to determine the yield of *D. carboxydivorans* when growing on CO were performed in serum bottles of 117, 310 and 570 mL with different P_{CO} levels. For the assessment of the growth yield of *D. carboxydivorans* with pyruvate, ethanol and H_2/CO_2, experiments (in triplicate) were performed in 117-mL bottles. Experiments with glucose and pyruvate (20 mM) were performed in the absence of sulfate, whereas experiments with ethanol (20 mM) were performed in the presence of 20 mM sulfate. Yield experiments with H_2/CO_2 were performed at 1.6 bar H_2/CO_2 (80:20%) in the presence of 20 mM sulfate. Experiments to determine the threshold CO concentration were performed in 117 mL bottles at P_{CO} varying from about 10 to 50 kPa and 80 to 100 kPa N_2 (total P > 101 kPa to prevent inlet of air upon sampling). The maximal sulfide production was determined in 117 mL bottles, with 200 kPa H_2/CO_2 in the presence of 20 mM sulfate and adjusted bicarbonate buffer to give a starting pH of 7. The effect of the pH on maximal sulfide accumulation was assessed with sulfate and H_2/CO_2 (200 kPa) and varying bicarbonate concentrations to obtain incubations at different initial pH.

6.2.3 Analysis

The pressure in the bottles was determined using a portable membrane pressure unit, WAL 0-4 bar absolute (Wal Mess- und Regelsysteme GmbH, Oldenburg, Germany). The headspace gas composition was measured on a gas chromatograph (GC) HP 5890 (Hewlett Packard, Palo Alto, USA). The detection limit for CO, with the used settings, was 400 ppm. Trace concentrations of CO were determined on a Shimadzu GC 2010, equipped with a methanizer (Shimadzu MTN-1). This GC was further equipped with a Chrompack Molsieve 5Å capillary column of 30 m (0.53 mm; 15μm; CP7544). The temperatures of the oven, injection port and FID detector were 90ºC, 100ºC and 250ºC, respectively. Volatile fatty acids (VFA) and ethanol were analyzed on a HP 5890A gas chromatograph (Hewlett Packard, Palo Alto, USA) according to Weijma *et al.* (2000). Sulfide was measured according to Trüper and Schlegel (1964). Sulfate was measured on a DX-600 IC system (Dionex Corporation, Salt Lake City, USA) as described in Chapter 4. Pyruvate was measured with a Spectrasystem high pressure liquid chromatography system equipped with an autosampler and

refractomonitor. The acids were separated on a Polyspher OAHY column (30 cm by 6.5 mm; Merck, Darmstadt, Germany) in 0.01 N H_2SO_4 at a flow rate of 0.6 ml/min and a column temperature of 60°C. Quantification was performed by differential refractometry. Biomass production was monitored by measuring the optical density at 600 nm (OD_{600}) using a Spectronic 601 spectrophotometer (Milton Roy, Ivyland, USA) and Plastibrand 1.5 mL PMMA cuvettes (Brand GmbH, Wertheim, Germany). The protein concentration was determined according to Bradford (1976).

Solubilities of CO, CO_2 and CH_4 were calculated using data from Lide (2001), solubility of H_2 was calculated according to Perry *et al*. (1997), and the amounts produced or consumed were calculated by taking into account both gas and liquid phases.

All chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany). CO (purity 99.997%) was supplied by Hoek Loos (Rotterdam, The Netherlands).

6.3 Results

6.3.1 Hydrogenogenic CO conversion

Fig. 6.1 presents a typical growth curve of *D. carboxydivorans* at an initial P_{CO} of 160 kPa in the absence of sulfate. Fast growth on CO was observed; the generation time was about 100 minutes. Fig. 6.1 shows that nearly stoichiometric amounts of H_2 and CO_2 are produced. Furthermore, the final concentration of CO is below the detection limit of the standard GC used to measure the gas composition, i.e. below 400 ppm. Though CO conversion proceeds fast, the maximal biomass density is already reached when still 60 kPa CO is present in the gas phase. Due to the limited buffer capacity in these incubations (20 mM phosphate buffer) and the production of CO_2, the pH decreased rapidly (Fig. 6.1B). Growth stopped at pH 6, but CO conversion continued resulting in residual CO levels below 400 ppm.
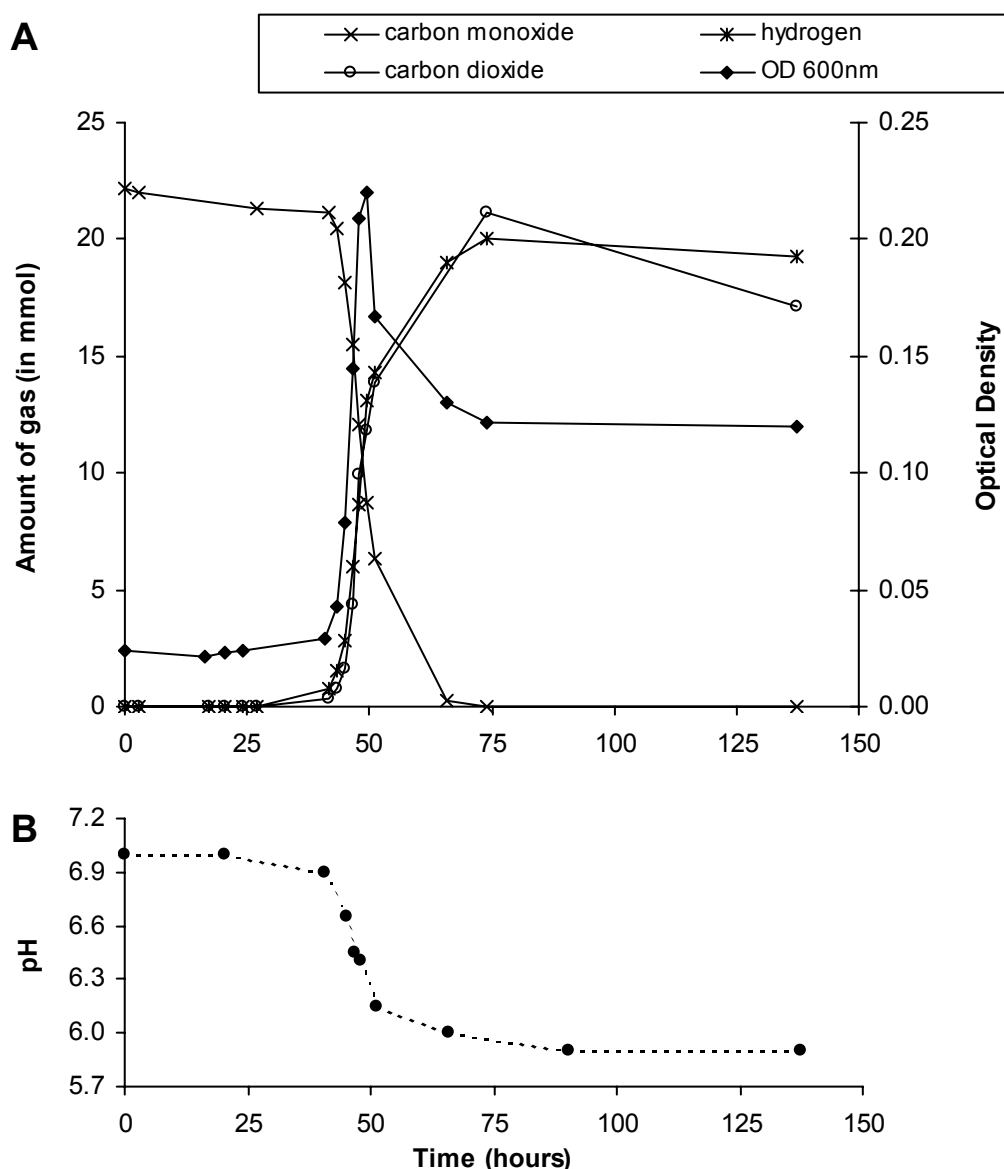
Fig. 6.1   Growth curve of *D. carboxydivorans* grown on 160 kPa CO in the absence of sulfate at 55ºC (A) and the evolution of the pH during CO conversion (B).

From the growth curves an attempt was made to estimate the CO conversion rates. Therefore, CO conversion rates in mmol.hour^{-1} were calculated between each sampling point. The assessed rates for two growth experiments are presented in Fig. 6.2. These rates were related to the average amount of biomass in the corresponding time interval. The amount of biomass production was measured on the basis of the increase in optical density at 600 nm (OD_{600}) and converted into the corresponding amount of protein. The empirical relationship between the amount of protein and the measured OD_{600} for *D. carboxydivorans* is:

$$[protein] = 135 \cdot OD_{600} \tag{1}$$

In which: [protein] = the protein concentration in $mg.L^{-1}$

The obtained CO conversion rates, expressed in $mol\ CO.(g\ protein)^{-1}.hour^{-1}$ during the exponential growth phase are shown in Fig. 6.2 as well as the measured pH values.
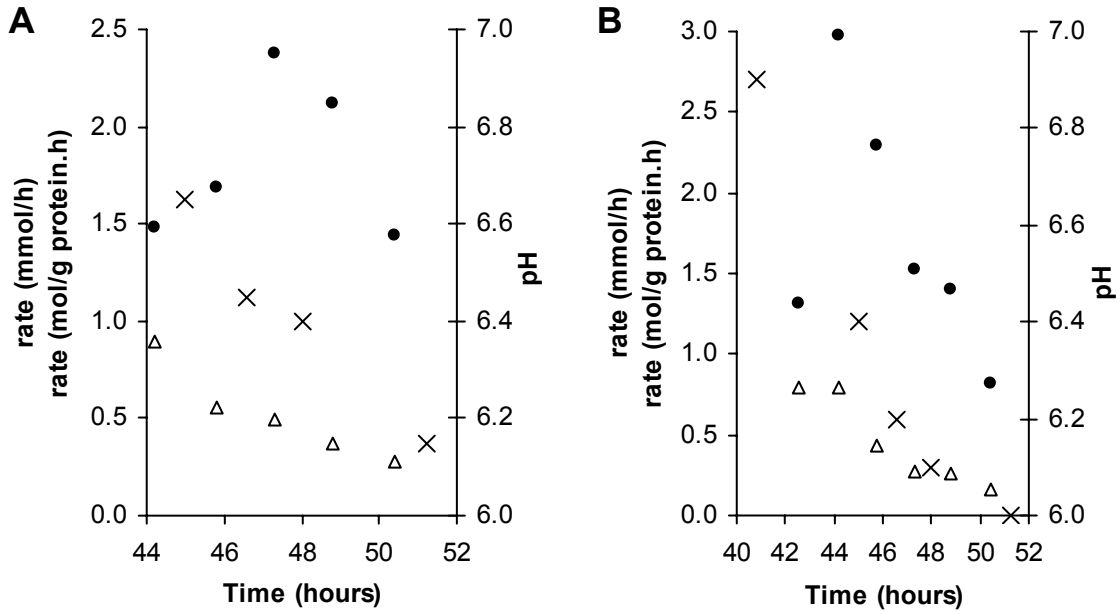
Fig. 6.2   CO conversion rates in the absence of sulfate in $mmol.hour^{-1}$ (•), the corresponding CO conversion rate in $mol.(g\ protein)^{-1}.hour^{-1}$ (Δ) and measured pH values during the course of the incubation (×) for two different growth experiments (A and B), of which A has been shown in Fig. 6.1.

Fig. 6.2 shows that the maximal CO conversion rate, expressed in $mmol.hour^{-1}$ is reached early in the growth phase (as seen from Fig. 6.1). It probably reflects the high CO flux from the gas to the liquid phase and relatively higher CO concentrations in the liquid phase at the start of growth. Due to consumption of CO and subsequent decrease of the pressure, the driving force for mass transfer decreases and the maximal CO flux decreases as well. During the conversion of CO the pH decreases fast, which likely affects the activity of the bacteria. The maximal specific CO conversion rates, expressed per g of biomass protein is extremely high at the start of CO conversion due to the low amount of biomass present, but decreases rapidly due to mass transfer limitations. Thus, it shows that the CO conversion capacity of the bacteria is much higher than the CO flux from the gas to the liquid phase.

6.3.2 Growth yield on CO

From 12 different growth experiments with different amounts of CO in the gas phase, the yield of biomass protein for growth on CO was calculated. For yield determinations, data were used when *D. carboxydivorans* was still growing exponentially as cell lysis occurring after the exponential growth phase would result in an underestimation of the biomass yield. Fig. 6.3 presents the relation between the produced amount of protein and the converted amount of CO.
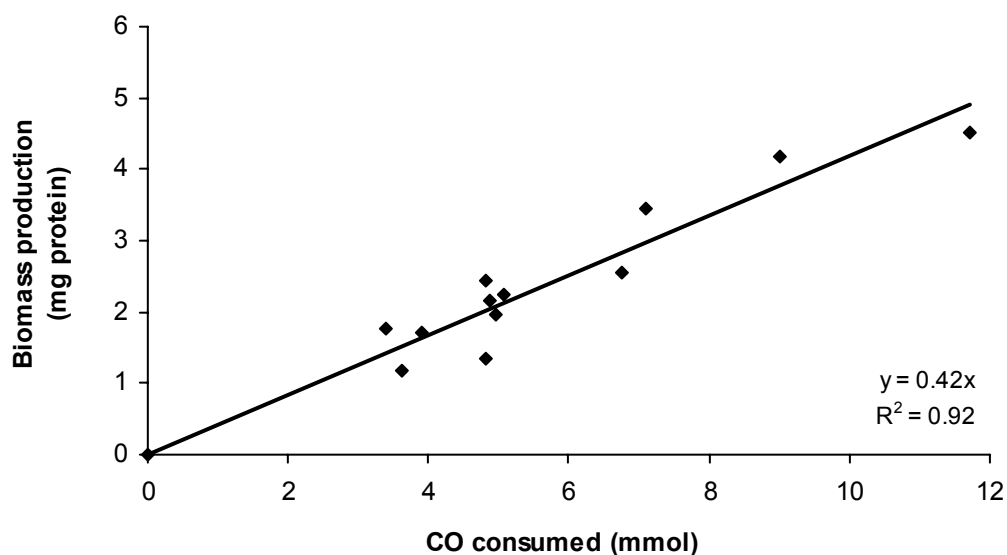
Fig. 6.3   Average protein yield of *D. carboxydivorans* in the absence of sulfate as function of the amount of CO consumed (initial pH = 7.0; T = 55ºC).

A good correlation ($R^2 = 0.92$) between the determined yields from different incubations and the amount of CO consumed was found. The protein yield of *D. carboxydivorans* is 0.42 mg protein produced per mmol CO converted.

6.3.3 Growth of D. carboxydivorans on alternative substrates

D. carboxydivorans is able to grow on a range of substrates both in the presence and absence of sulfate. To evaluate the use of co-substrates for stimulation of growth in a synthesis gas conversion process, growth yields and growth rates for *D. carboxydivorans* with pyruvate, ethanol and H_2 were determined (Table 6.1). The yield with glucose could not be determined due to analytical problems, i.e. interference of phosphate with glucose during analysis. Nevertheless, its high biomass density and fast generation times show that it represents an interesting substrate to stimulate *D. carboxydivorans* biomass production.

Table 6.1 Measured maximal density, yield and generation time of *D. carboxydivorans* on selected substrates

Growth substrate	Coverted substrate (mmol)	OD_{600} increase	Maximal OD_{600}	Yield mg protein·(mmol substrate)$^{-1}$	Generation time (hours)
without sulfate:					
Glucose	n.d.	n.d	0.595	n.d.	6
Pyruvate	1.29 ± 0.05	0.35 ± 0.00	0.433	1.82 ± 0.08	5
with sulfate:					
Ethanol	0.77 ± 0.04	0.24 ± 0.03	0.312	2.13 ± 0.15	19
H_2/CO_2	0.11 ± 0.02	0.02 ± 0.01	0.280	1.34 ± 0.12	8

n.d. = not determined

As both ethanol and H_2 are exclusively used with sulfate as electron acceptor, their potential use is restricted to those processes in which sulfate reduction is the process of interest. The addition of ethanol to stimulate sulfate reduction could be beneficial, although the generation time on ethanol is rather long compared to growth on CO or H_2 with sulfate.

6.3.4 CO threshold concentrations

All incubations depleted CO below the detection limit of the GC that was used in these experiments, which was about 400 ppm. In order to determine the residual CO concentrations at the end of the incubation period more accurately, headspace samples were measured on a GC equipped with a methanizer. Results of analysis of samples from growth curves with an initial P_{CO} of about 150 kPa revealed that the final P_{CO} was about 25 Pa at a final pH ≤ 5.9, which corresponds to a concentration of about 130 ppm at atmospheric pressure. In order to evaluate the effect of thermodynamical limitations on the residual CO concentrations the following equations were used:

$$\Delta G = \Delta G'_{55} + R \cdot 10^{-3} \cdot T \cdot \ln Kp \qquad (2)$$

in which: R is the gas constant (8.3145 J.mol^{-1}.K^{-1}), T is the temperature in Kelvin and the equilibrium constant (Kp) is defined as:

$$Kp = \left(\frac{P_{H_2} \cdot P_{CO_2}}{P_{CO}} \right) \qquad (3)$$

in which P_{CO} is the partial pressure of CO in atm

Table 6.2   Gas analyses at the end of the several incubations, the equilibrium constant (ln Kp) and the ΔG values for reaction 1.

P_{CO} $*10^{-4}$ atm	P_{CO2} atm	P_{H2} atm	ln Kp	ΔG kJ.mol^{-1}
2.92	0.95	1.21	8.27	0.27
2.52	0.91	1.22	8.39	0.59
3.16	0.94	1.23	8.21	0.09
2.48	0.97	0.80	8.05	-0.35

In order to enable that the reaction proceeds at 55ºC, ΔG should be smaller than 0, the value for ln Kp should be < 8.17. The calculated ΔG values are around zero. This indicates that thermodynamic limitations determine the residual CO concentrations. In that case, when starting with lower P_{CO} values, the final CO concentrations are expected to be lower as well. This is in agreement with the results in Table 6.3, which show that the residual CO concentration that can be reached is as low as 4-5 ppm.

   To evaluate whether thermodynamic limitations determine the final CO concentrations, the residual CO concentration calculated at $\Delta G=0$ was compared to the actual measurements of residual CO concentrations after 14 days of incubation in incubations with different initial P_{CO} (Table 6.3). In these calculations the maximal amount of CO_2 and H_2 in the gas phase was calculated, assuming that CO was converted stoichiometrically into these products. The total amount of CO_2 and H_2 were recalculated into the amount of each gas in the gas phase using the following relations:

$$\frac{[gas]_{(liq)}}{[gas]_{(gas)}} = K_H \cdot RT \qquad (4)$$

in which: [gas] = concentration of CO_2 or H_2

K_H = Henry constant at 55ºC

(CO_2: $1.61 \cdot 10^{-4}$ mol.m^{-3}.Pa^{-1}; H_2: $7.65 \cdot 10^{-6}$ mol.m^{-3}.Pa^{-1})

$$R = \text{Gas constant} = 8.3145 \text{ J.mol}^1.\text{K}^{-1}$$

$$T = \text{temperature in Kelvin}$$

$$n_{gas} = \frac{n_{tot}}{\left(1 + K_H \cdot RT \cdot \dfrac{V_{liq}}{V_{gas}}\right)} \tag{5}$$

in which: n_{liq} = amount of moles in the liquid phase

V_{liq} = Volume of the liquid phase (50 mL)

V_{gas} = Volume of the gas phase (67 mL)

The partial pressure of each gas in the gas phase was calculated with the ideal gas law (in atm). When $\Delta G = 0$, the ln Kp is 8.17 and consequently Kp is 3545. Therefore, in order to make the reaction possible, Kp should be smaller than this value.

Table 6.3   Thermodynamic calculations of the minimal CO concentration in the gas phase at different initial P_{CO} compared with trace CO gas measurements of the final CO concentration after 14 days of incubation (initial pH = 7.0; T = 55°C; no sulfate added).

Initial P_{CO} atm	Calculated maximal P_{CO2} atm	Calculated maximal P_{H2} atm	Final pH	Calculated residual CO ppm	Measured residual CO ppm
0.10	0.07	0.09	6.31	2	5
0.17	0.12	0.15	6.20	5	9
0.19	0.13	0.17	6.20	6	4
0.25	0.17	0.22	6.10	11	17
0.28	0.19	0.25	6.11	13	16
0.32	0.22	0.29	5.98	18	17
0.44	0.30	0.39	5.90	33	19
0.50	0.34	0.45	5.88	43	22

The results in Table 6.3 show a good correlation between the calculated and the measured residual CO concentrations, which indicates that the final CO concentration is determined by thermodynamics. Furthermore, as the final concentrations are much lower than the 130 ppm measured with the growth curves, the affinity of *D. carboxydivorans* for CO is not limiting the removal of CO to very low residual CO concentrations. The discrepancies between the calculated and measured values in

Table 6.3 at the two highest initial P_{CO}'s, can be attributed to small losses of gas during sampling.

The initial presence of both H_2 and CO_2, which is typical for synthesis gas, did not prevent conversion of CO to values below the detection limit (400 ppm) of the standard used GC (data not shown). However, the results of calculations show that the initial presence of CO_2 and H_2 will affect the minimal achievable CO concentration. Table 6.4 presents the results of calculations of the minimal CO concentration achievable based on thermodynamics for typical synthesis gas compositions at atmospheric pressure as well as at elevated pressures of 1 MPa. When this CO conversion is performed at elevated pressure, which is beneficial for the rate of mass transfer of CO from the gas to liquid phase, the final CO concentration will increase (Table 6.4).

Table 6.4 Calculated residual CO-content using different typical compositions of synthesis gas. Calculations were performed for batch incubations at P_{atm} (101.3 kPa) and a total pressure of 10 times P_{atm} (1.013 MPa).

Synthesis gas	Typical gas composition (%)			Calculated residual CO in ppm	
	CO	CO_2	H_2	P_{atm}	$10 * P_{atm}$
Coke oven gas	5.6	1.4	55.4	7	747
Water gas	30.0	3.4	31.7	36	3597
Natural gas (steam reforming)	15.5	8.1	75.7	38	3746
Naphta (steam reforming)	6.7	15.8	65.9	29	2840
Partial oxidation of heavy fuel oil	47.0	4.3	46.0	84	8315
Coal gasification	59.4	10.0	29.4	108	10739

6.3.5 Sulfate reduction by D. carboxydivorans

Compared to the hydrogenogenic growth on CO in the absence of sulfate, a considerably higher OD is reached, indicative of a higher biomass yield, because hydrogenotrophic sulfate reduction supports growth of *D. carboxydivorans* (Fig. 6.4). Fig. 6.4 further shows that the S-recovery in sulfide is rather low. When including the calculated amount of H_2S present in the gas phase, about 70% of the sulfate reduced was recovered as sulfide. At pH 6.4, about 66% of the sulfide is present as H_2S. However, as H_2S is relative well soluble in water (i.e. the assessed Henry coefficient of H_2S at 55°C amounts to 0.053 $M.bar^{-1}$ using the data obtained from Lide, 2001), the calculated amount of H_2S in the gas phase is small (<0.1% H_2S at 200 kPa total pressure). Sulfide complexation with metals present in the medium is quantitatively not important and can not increase the recovery by more than 0.5%. For further calculations, sulfate was chosen instead of sulfide as the sulfate analysis looked more accurate, as judged from the good correlation and reproducibility of the sulfate concentrations of three standards analyzed both at the start and end of the analytical run (data not shown).
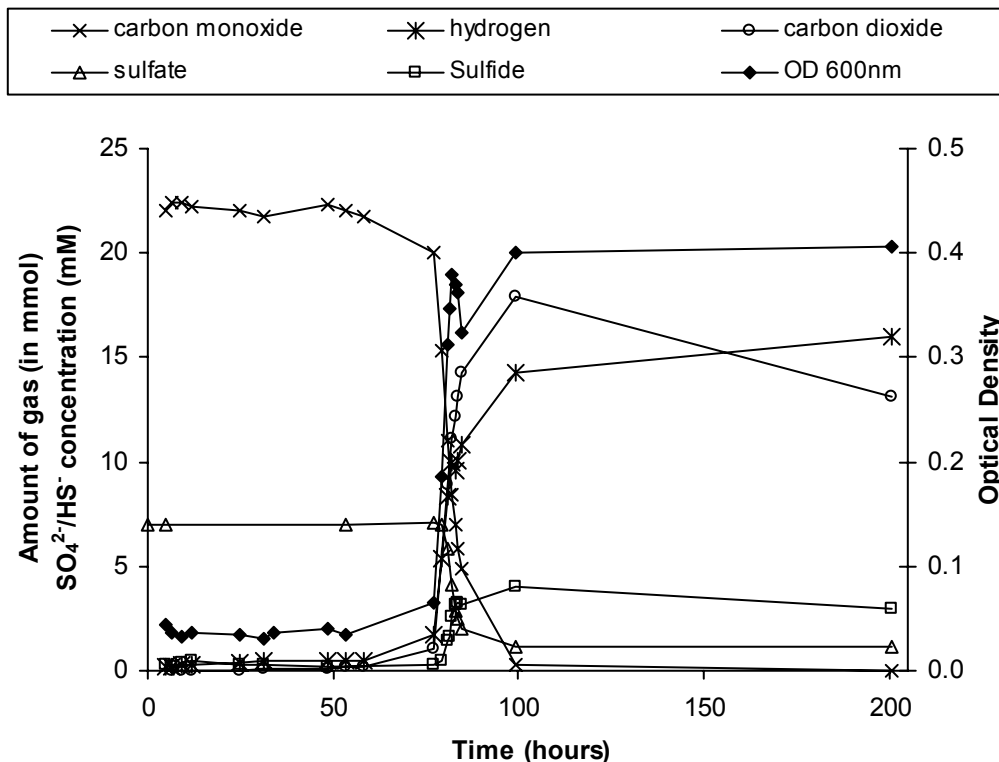
Fig. 6.4   Growth curve of *D. carboxydivorans* grown on 160 kPa CO in the presence of sulfate (initial pH = 7.0; T = 55°C).

Analysis of the growth curves in the presence of sulfate reveals that the sulfide production coincides with the CO conversion. The sulfide concentration already starts to increase when the P_{CO} still exceeds 100 kPa. The maximal obtained sulfide concentration was 4.9 mM at a final pH of 6.5, when CO was supplied in the gas phase. Calculations of the CO conversion rate revealed that the highest rate was 2.6 mmol.hour^{-1}, corresponding to a specific rate of 0.4 mol CO.(g protein)$^{-1}$.hour^{-1}, which is in the same range as presented in Fig. 6.2 for cultures growing in the absence of sulfate. The maximal sulfate reduction rate of 0.3 mmol.hour^{-1} determined from Fig. 6.4 corresponds to 32 mmol SO_4^{2-}.(g protein)$^{-1}$.hour^{-1} based on assessed OD_{600} values.

The biomass yield with CO in the presence of sulfate was calculated using the previously assessed yield on CO. The calculations were performed using the changes measured between the start and the end of the exponential phase, which were 13.57 mmol CO converted, 0.6 mmol sulfate reduced and an OD_{600} change of 0.345 units. The liquid volume of the used serum bottle was about 200 mL. With the assessed yield of 0.42 mg protein.(mmol CO)$^{-1}$ on CO , the amount of CO converted led to the synthesis of 5.7 mg protein. The protein concentration consequently amounts to 28.5 mg.L^{-1}, which corresponds to an OD_{600} change of 0.211. The remainder of the OD increase (0.134 units) likely is the result of hydrogenotrophic sulfate reduction. This amount corresponds to a protein concentration of 18.1 mg.L^{-1} or an absolute amount of 3.6 mg protein. The yield for hydrogenotrophic sulfate-dependent growth then amounts to 6 mg protein.(mmol sulfate reduced)$^{-1}$, or 1.5 mg protein.(mmol H_2 consumed)$^{-1}$, which is in good agreement with the value determined for growth on H_2/CO_2 in the presence of sulfate (Table 6.1). The biomass yield when growing hydrogenotrophically is more than 14 times higher than the value when growing hydrogenogenically, despite that the Gibbs free energy yield for hydrogenotrophic sulfate reduction is only 6.5 times higher than for hydrogenogenic CO conversion as seen from equation (2) and (6).

$$SO_4^{2-} + 4\,H_2 + H^+ \rightarrow \ HS^- + 4\,H_2O \qquad \Delta G'_{55} = -145.7 \text{ kJ.reaction}^{-1} \quad (6)$$

6.3.6 Maximal achievable sulfide concentrations

The results of the incubations fed with CO and sulfate reveal that the maximal sulfide concentration produced is about 5 mM, despite that the CO-derived H_2 was not depleted. This indicates that inhibition due to sulfide prevented the further reduction

of sulfate. Incubations with H_2 and CO_2 as electron donor and carbon source in the presence of sulfate resulted in the accumulation of sulfide up to 10.5 mM. The difference in maximum sulfide concentration in incubations with CO compared to those with H_2/CO_2 likely can be attributed to an effect of the pH. Hydrogenogenic conversion of CO leads to a drop in pH due to CO_2 formation. In incubations with H_2/CO_2 the pH will increase in time, due to the consumption of CO_2 for growth. The effect of the final pH on the maximal sulfide concentration is shown in Fig. 6.5. In these experiments the initial pH was varied between pH 5.7 and 6.5, by adjusting the bicarbonate and partial pressure of CO_2 in the gas phase, which resulted in different final pH values. At the end of the experiments there were still sufficient amounts of H_2 (> 60 kPa) and CO_2 (>100 kPa) present, which confirms that sulfide production did not cease due to a limitation of electron donor or carbon source, but rather due to sulfide inhibition.
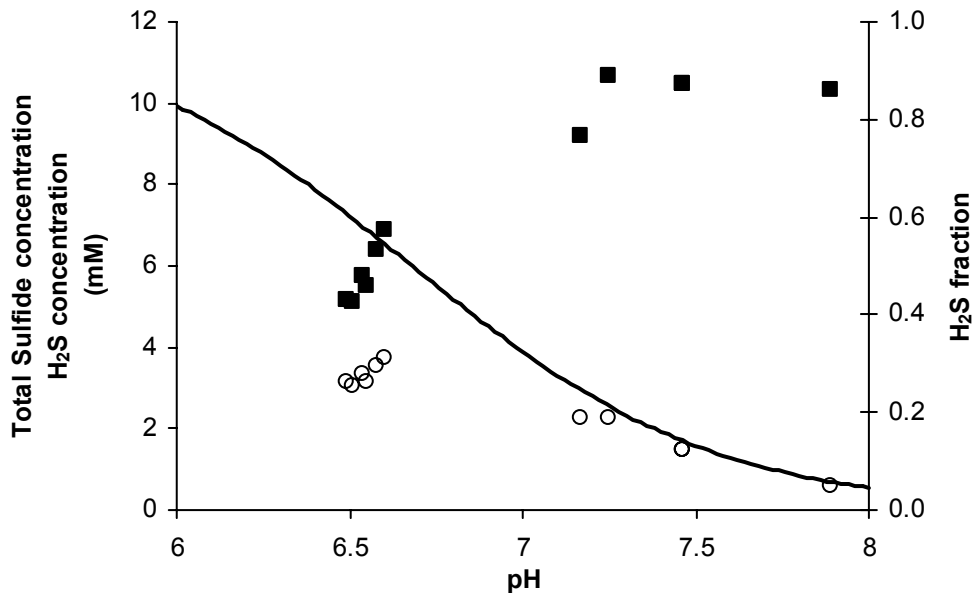
Fig. 6.5 Comparison of the final pH obtained and the produced sulfide concentration (■) in incubations with H_2/CO_2 and 20 mM sulfate. The presented line represents the fraction of total sulfide present in its undissociated form (H_2S) and has been used to determine the amount of H_2S present at the end in each bottle (○).

The results in Fig. 6.5 show a clear increase in sulfide tolerance with increased pH values. With an increase of the pH the fraction H_2S decreases according to:

$$f_{H_2S} = \frac{1}{10^{(pH-pKa)} + 1}$$ (7)

in which the pKa of the reaction: $H_2S \leftrightarrow HS^- + H^+$ is 6.68 at 55ºC (Amend and Shock, 2001).

The sulfide inhibition at pH 6.5 is reached at a total sulfide concentration less than half of the inhibiting concentration at pH 7.8. This most likely can be due to the relatively large contribution of undissociated H_2S at low pH. However, not only H_2S contributes to the inhibition, since the H_2S concentration at pH 7.8 is less than 20% of the H_2S concentration at pH 6.5. This suggests that at high pH values the dissociated form of sulfide (HS^-), contributes to the overall sulfide inhibition as well.

In incubations under an initial gas phase of CO in the presence of sulfate the maximal sulfide concentration measured were in the range of 3.6 – 4.9 mM at pH values of 6.4 – 6.5. When combining these values in Fig. 6.5 it can be seen that these values fit well with those determined in incubations supplemented with H_2/CO_2 at similar pH values.

6.4 Discussion

6.4.1 D. carboxydivorans in synthesis gas purification processes

The fast growth (generation time 100 minutes) and high specific conversion rates (estimated at > 0.8 mol CO.(g protein)$^{-1}$.hour^{-1}) of *D. carboxydivorans* on CO with nearly equimolar production of H_2, makes it an excellent candidate for use in a biological alternative for the chemical water-gas-shift reaction to purify synthesis gas. The CO conversion rates assessed in batch experiments (shaken at 200 rpm) reveal that the highest specific conversion rates are already obtained in the early exponential phase when the biomass protein concentration is still relatively low, which indicated that the conversion was mass transfer limited. In this case the maximal possible flux of CO from the gas phase to the liquid phase, where the bacteria are present, will determine the overall volumetric CO conversion rates.

The flow from the gas to the liquid phase can be described by equation 8:

$$\Phi_{m,CO} = k_L a(C_{CO}^* - C_{CO,L})$$ (8)

In which: $\Phi_{m,CO}$ is the molar flow rate (mol.m^{-3}.s^{-1}); k_La the gas-liquid mass transfer coefficient (s^{-1}); C_{CO}^* the equilibrium concentration for CO in water (mol.m^{-3}) and $C_{CO,L}$ the CO concentration in the liquid phase. When the conversion proceeds with high efficiency and mass transfer limits the biological activity the CO concentration in the liquid phase can be assumed to be equal to zero and then equation 8 reduces to:

$$\Phi_{m,CO} = k_L a \cdot C_{CO}^* \tag{9}$$

C_{CO}^* can be calculated by means of Henry's Law according to:

$$C_{CO}^* = P_{CO} \cdot k_H \tag{10}$$

In which: P_{CO} is the partial CO pressure (Pa) in the gas phase and k_H is Henry's constant for CO, i.e. $6.3*10^{-6}$ mol.m^{-3}.Pa^{-1} at 55ºC (calculated from Lide, 2001). This results in:

$$\Phi_{m,CO} = k_L a \cdot \left(P_{CO} \cdot k_H \right) \tag{11}$$

Thus, the partial pressure of CO (P_{CO}) greatly affects the mass transfer rates of CO to the liquid phase. Bioreactors operated at 1 MPa CO would increase the mass transfer by a factor 10 compared to operation at 100 kPa of CO in the gas phase. Operation of a biological hydrogenogenic CO converting reactor at elevated pressures is not only beneficial for mass transfer rates, but is also desired for the product gas to minimize transportation costs. In batch experiments conducted with Eerbeek sludge, the conversion rates were considerable higher at a higher initial P_{CO} (Chapter 4). In the experiments conducted at the highest tested P_{CO} (160-180 kPa) with both Eerbeek sludge and a pure culture of *D. carboxydivorans* a negative effect on CO conversion by the pressure or CO concentration did not manifest (Fig. 6.1; Chapter 4).

Synthesis gas from steam reforming of light hydrocarbon feeds is produced at pressures between 2.16-2.51 MPa and temperatures in the range of 800-870ºC (Czuppon et al., 1995). In order to minimize the size of the bioreactor it would be beneficial to operate at elevated pressures. Nevertheless, as the synthesis gas needs to be cooled to a temperature around 55ºC, the pressure will decrease from over 2 MPa to values of around 0.7 MPa. Gasification of coal is performed at temperatures exceeding 1300ºC and pressures higher than 2 MPa (Czuppon et al., 1995), respectively, which corresponds to maximal gas pressures of about 0.4 MPa at 55ºC. The affinity of *D. carboxydivorans* for CO is sufficiently high to remove CO to values below 4-5 ppm (Table 6.3), but thermodynamic limitations as a result of high concentrations of H_2 and CO_2 can result in higher exit CO levels (Table 6.4). This for

instance is the case at increased P_{CO} values, as well as when H_2 and CO_2 are present in the feed stock (synthesis gas) (Table 6.4 and equation 6). Post treatment of the product gas is then needed. Gas separation by pressure swing absorption (PSA) or the use of selective gas membranes are the most widely adopted post treatment methods for H_2 purification (Koros and Mahajan, 2000; Perry et al., 1997). These gas separations systems perform best at relatively high pressure, i.e. PSA systems are operated between 0.5-5 MPa and selective gas membranes up to 8 MPa (Air Liquide technical specifications). Especially, the use of gas membranes would enable the constant recovery of H_2 from the system, enabling the recycling of unconverted CO at maintaining a relatively high P_{CO} which facilitates mass transfer of CO. Furthermore, the selective recovery of CO_2 would enable storage in the deep subsurface (Gale, 2004) or reuse in e.g chemical synthesis of methanol (Pruschek et al., 1997) or for stimulating of plant growth in green houses and it also results in more favorable thermodynamics for hydrogenogenic CO conversion. The removal of CO_2 resulted in batch experiments with another hydrogenogenic CO converting bacterium, *Carboxydothermus hydrogenoformans*, in drastically decreased minimal residual CO concentrations (Anne Meint Henstra, personal communication). Although a pressure of 0.7 MPa does not seem very extreme for bacteria, viz. barotolerant microorganisms have been reported to grow at pressures up to 40-50 MPa (Madigan et al., 1997), the effect of high total pressure as well as high P_{CO} still needs to be evaluated. Assuming a synthesis gas pressure of 0.7 MPa at 55ºC for methane steam reforming, which contains about 12% CO (Czuppon et al., 1995), the maximal P_{CO} in the reactor is only 84 kPa, which will not inhibit *D. carboxydivorans*. Similarly, no inhibiting effect of the P_{CO} is expected for coal gas, as its total pressure after decreasing the temperature to 55ºC is about 0.4 MPa with a CO content of around 50% (Czuppon et al., 1995).

In a study that addressed CO conversion in 3 types of reactors it was found that a biotrickling filter gave higher efficiencies than CSTR and bubble column reactors. This was attributed to operational conditions which approach plug flow (Klasson et al., 1992). Furthermore, according to Bredwell et al. (1999) in biotrickling filters the k_La is relatively independent of the gas flow rate for sparingly soluble gasses. In general, increased liquid and gas velocities result in higher mass transfer, resulting in k_La values of up to 0.24 s^{-1} for bubble columns (Bredwell et al., 1999), although such measures are accompanied with increased power demands, which economically/commercially is unattractive. Differences in treatment costs as

evaluated by Zuber et al. (1997), for the scale up of a biotrickling filter and a gas lift reactor, revealed a 50% cheaper treatment when a biotrickling filter is used (Zuber et al., 1997). When selecting a biotrickling filter, the maximal flux of CO could be determined assuming a k_La of 0.015 s^{-1} as determined for this type of reactor in CO fermentations at 37ºC (Klasson et al., 1992). Although modest temperature changes, like the case in the range of 37 to 55ºC, have a negligible effect on the interfacial area (a), they slightly affect k_La due to changes of the medium viscosity (Perry et al., 1997). Nevertheless, taking the value determined by Klasson et al. (1992), the maximal flux of CO to the biomass in a biotrickling filter can be determined as a function of the P_{CO} according to equation 11, i.e. at a P_{CO} of 200 kPa (synthesis gas from coal) the maximal CO flux is 1600 mol.m^{-3}.d^{-1}. This equals to a H_2 production capacity of almost 40 m^3.m^{-3} reactor volume under standard conditions (25ºC and 101.325 kPa). With the conversion of 1600 mol CO, the biomass yield would be almost 690 g protein.day^{-1}. This results in a required CO conversion activity of the biomass of about 0.1 mol CO.(g protein)$^{-1}$.hour^{-1}, which is 8 times lower than the maximal CO conversion rates estimated for *D. carboxydivorans* (Fig. 6.2).

6.4.2 D. carboxydivorans in biological desulfurization processes

D. carboxydivorans enables the direct use of CO gas or CO-rich synthesis gas in biological sulfate reduction processes at elevated temperatures and therefore this bacterium is very interesting for use in flue gas desulfurization. Its extremely high CO conversion rates (> 0.8 mol CO.(g protein)$^{-1}$.hour^{-1}), combined with its high sulfate reduction rates (32 mmol SO_4^{2-}.(g protein)$^{-1}$.hour^{-1}) and its ability to use both CO and H_2, the major constituents of synthesis gas, makes its application highly promising. In flue gas desulfurization, relatively large volumes of water need treatment; the use of gas lift reactors is considered (Van Houten et al., 1994; 1997). Van Houten et al. (1997) determined a k_La value for a gas lift reactor operated at 55ºC of 0.030 s^{-1} for H_2. This k_La (H_2) was converted to a k_La for CO, using the following equation:

$$k_La_{(CO)} = k_La_{(H_2)} \cdot \frac{D_{co}}{D_{H_2}} \qquad (12)$$

The diffusivity (D) of both CO and H_2 was estimated at 55ºC using the Wilke-Chang equation (Perry et al., 1997), estimating a water viscosity at 55ºC (0.5 mPa.s) from Lide (2001) and values for molecular volumes from Treybal (1985). The calculated diffusivity of H_2 at 55ºC amounted to 6.28*10^{-9} m^2.s^{-1} and that of CO to 3.97*10^{-9}

$m^2.s^{-1}$. The resulting value of k_La for CO ($0.019 \ s^{-1}$) used in equation (11) gives a maximal CO flux exceeding 1000 mol $CO.m^{-3}.day^{-1}$. Since four moles of CO are needed to form the required four moles of H_2 for the reduction of one mole of sulfate, the maximal sulfate reduction rate based on the maximal rate of CO supply exceeds 250 mol $sulfate.m^{-3}.day^{-1}$, i.e. corresponding to 25 kg $sulfate.m^{-3}.day^{-1}$. When using synthesis gas, the maximal theoretical sulfate reduction rate is even higher due to the presence of H_2 in the feed gas and the higher k_La for H_2.

However, we found *D. carboxydivorans* suffers from a pH dependent sensitivity for sulfide inhibition, i.e. 9 mM sulfide at pH 7.2 and 5 mM at pH 6.5 causes complete inhibition (Fig. 6.5). Our results demonstrate that most likely not toxicity for CO, but for sulfide determines the sulfate reduction capacity of a CO or synthesis gas fed bioreactor, as sulfide production occurred at CO levels exceeding 100 kPa, which in practice likely will not be reached when using synthesis gas. Therefore, in order to develop a high rate sulfate reducing bioreactor employing *D. carboxydivorans*, additional features to maintain the sulfide concentration below the inhibiting levels undoubtedly are required. In order to maintain sulfide concentrations sufficiently low, operation at elevated pH-values could be considered or application of a stripping process for H_2S from the gas phase. The H_2S stripped from the gas phase could be directly fed to a second micro-aerobic biological reactor where the sulfide is partially oxidized to elemental sulfur (Janssen et al., 2000). Another attractive option that might be considered is the use of H_2S extractive membranes (De Smul and Verstraete, 1999; Chuichulcherm et al., 2001) placed inside the bioreactor mixed liquor, which could result in direct recovery of elemental sulfur when combining with a Fe^{3+}-containing extraction solution.

6.5 Conclusions

The results presented in this chapter demonstrate that:

1. *D. carboxydivorans* grows with a generation time of 100 minutes and a yield of 0.42 mg $protein.(mmol \ CO)^{-1}$ on P_{CO} levels exceeding 160 kPa, producing nearly equimolar amounts of H_2 and CO_2.

2. The maximal CO conversion rate determined for *D. carboxydivorans* of 0.8 mol $CO.(g \ protein)^{-1}.hour^{-1}$ is already reached in the early exponential phase, indicating that its biological activity is limited by mass transfer of CO.

3. The minimal residual CO concentrations that can be achieved are determined by thermodynamics, whereas the affinity of *D. carboxydivorans* is sufficiently high to obtain CO concentrations in the range of a few ppm (< 5 ppm).

4. Hydrogenogenic sulfate reduction by *D. carboxydivorans* tolerates high levels of CO, i.e. sulfate reduction is not inhibited at a P_{CO} of 100 kPa.

5. *D. carboxydivorans* is rather sensitive for elevated sulfide concentrations especially at lower pH-values, i.e. at pH 7.2 sulfate reduction becomes inhibited at 9 mM sulfide and at pH 6.5 already at about 5 mM sulfide.

7

Moderate-thermophilic hydrogenogenic CO conversion in a sulfate fed gas lift reactor: competition between methanogens and sulfate reducers for CO-derived H$_2$

Abstract

Anaerobic methanogenic sludges harbor moderately thermophilic bacteria capable of hydrogen formation from carbon monoxide (CO). Recently, a moderately thermophilic hydrogenogenic CO converting organism was isolated, designated as *Desulfotomaculum carboxydivorans*, which is also capable of sulfate reduction with CO derived H$_2$ at high CO concentrations (>101 kPa). The presence of this bacterium in an unacclimated anaerobic granular sludge from a paper mill wastewater treatment facility holds a promise for the direct use of synthesis gas containing high levels of CO in biological desulfurization at elevated temperatures (55ºC). This study investigated sulfate reduction in a gas lift reactor fed with CO as the sole external electron donor and carbon source inoculated with that anaerobic granular sludge. Despite the high CO conversion capacity of the biomass present, the sulfate reduction capacity was rather limited due to strong competition between methanogens and sulfate reducers for the produced H$_2$. Although, the methanogens appeared to be more sensitive to pH and temperature shocks imposed to the reactor, they could not be completely eliminated. The fast growth rates of the methanogens (generation time of 4.5 hours) enabled a fast recovery from shocks and they rapidly consumed more than 90% of the CO-derived H$_2$.

7.1 Introduction

Coal fueled power plants produce flue gases containing relative high amounts of SO_x, mainly SO_2, representing by far the largest anthropogenic source of SO_2 emissions (Brimblecome et al., 1989). Liberation of SO_x into the earth's atmosphere leads to the production of H_2SO_3 and H_2SO_4, which ultimately results in acid deposition and subsequently, damage to ecosystems (Charlson et al., 1992; Vallero et al., 2002). In order to minimize acid deposition as well as negative effects of SO_x on human health (Komarnisky et al., 2003) from anthropogenic sources, stringent legislations for the amount of sulfurous compounds in flue gas implies the need for SO_x removal.

Biological flue gas desulfurization (Bio-FGD) presents an interesting alternative for physical-chemical removal methods, in view of its low operational costs and the formation of highly purified (>98%) reusable elemental sulfur (Janssen et al., 2000). As organic matter is absent, supply of a suitable electron donor is a prerequisite for Bio-FGD. The economic feasibility depends on the costs of the added electron donor, the electron flow towards sulfate reduction as well as the amount of remaining pollution that has to be removed in a post treatment process. Ethanol (Kalyuzhnyi et al., 1997), methanol (Weijma et al., 2000; Vallero et al., 2003a) and hydrogen (Van Houten et al., 1994, 1997) were shown potential electron donors for biological sulfate reduction processes. Furthermore, synthesis gas consisting of mainly H_2, CO and CO_2 has been suggested as a cheap alternative for H_2 in sulfate reduction processes (Du Preez et al., 1992; Van Houten and Lettinga, 1996). Synthesis gas can be produced by gasification of any type of organic matter. Operational costs of Bio-FGD could be greatly reduced in case of on-site production of synthesis gas from coal or from locally available natural gas, thus minimizing transportation costs. However, depending on the source of feedstock, synthesis gas contains CO, which may exceed 50% in case of coal gasification (Perry et al., 1997).

Despite the ability of some sulfate reducing bacteria to metabolize CO, they are reported to be completely inhibited at partial CO pressures (P_{CO}) exceeding 20 kPa (Mörsdorf et al., 1992, Klemps et al., 1985). Recently, we described a higher CO tolerance for *Desulfotomaculum kuznetsovii* and *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* (Parshina et al., 2005). These bacteria were capable of metabolizing CO up to a P_{CO} of 50 and 70 kPa, respectively. Nevertheless, sulfate

reduction by these bacteria was inhibited above 50 kPa CO as well. The highest tolerance of any sulfate reducer for CO was only discovered recently with the isolation of *Desulfotomaculum carboxydivorans* (Chapter 5). This bacterium seems promising for direct utilization of CO-rich synthesis gas as electron donor for thermophilic sulfate reduction processes without the need for purification prior to its use, especially since it utilizes both CO and H_2 present in synthesis gas (Chapter 6).

In the present study, the use of pure CO as electron donor for sulfate reduction at elevated temperatures (55°C) was investigated, using as inoculum full-scale grown anaerobic granular sludge treating paper mill wastewater from which *Desulfotomaculum carboxydivorans* (Chapter 5) was isolated.

7.2 Materials and Methods

7.2.1 Description of the gas lift loop reactor setup

Continuous flow-through experiments were performed in a conventional gas lift loop reactor (liquid height 0.7 m, inner diameter 0.1 m) with a working liquid volume of 4.0 liter made of glass. The used experimental set-up is shown in Fig. 7.1. The temperature in the bioreactor was controlled between 52 and 55°C, unless otherwise specified, by circulating water from a thermostatic bath (Julabo Labortechnik GmbH, Seelbach, Germany) through the outer mantle of the reactor. The temperature in the reactor was measured just below the effluent using a PT-100 electrode. The reactor contained a plastic internal draft-tube (height 0.45 m, inner diameter 0.062 m, outer diameter 0.070 m). The pH, continuously monitored with a sulfide resistant Flushtrode pH electrode (Hamilton, Hilkomij BV, Rijswijk, the Netherlands), was controlled around pH 6.8, unless specified otherwise, using a Liquisys-P pH controller (Endress and Hauser, Naarden, the Netherlands). 0.1 M NaOH was used to control the pH.
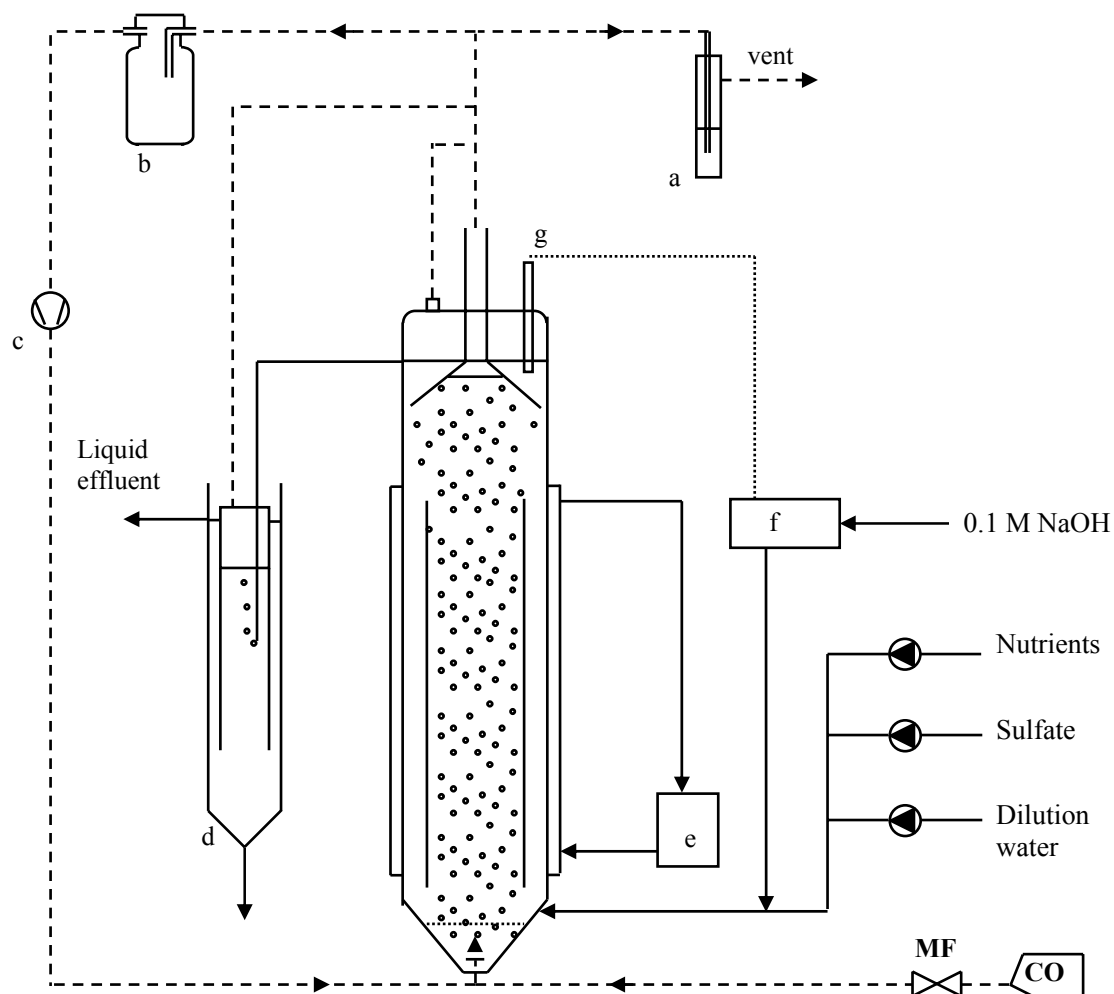
Fig. 7.1 Schematic representation of the used reactor configuration. The set-up consists of an internal draft-tube gas-lift reactor, a water lock in the gas vent (a), condense and foam trap (b), compressor (c), effluent collector with biomass settler and gas return (d), thermostatic bath (e), pH controller (f) and pH electrode (g). The influent CO flow is measured and controlled by a thermal mass flow meter (MF) connected to a control unit.

The gas phase was recycled with a compressor type N726 FT.18 (Neuberger, Freiburg, Germany) at a flow rate of 250 L.h^{-1}. In the gas recycle an empty column was used as condense and foam trap. The influent gas flow of CO was monitored using a mass flow meter type 5850E (Brooks, Veenendaal, the Netherlands), 0-5 L.h^{-1} CO_2. For use with CO the flow was recalculated to 0-6.4 L.h^{-1} CO, using the gas sensor factors for both CO_2 and CO as explained in the installation and operating manual (May 2000, issue 9, Brooks, Veenendaal, the Netherlands). The mass flow

meter was connected to a read-out and control unit (Brooks type 5878, Veenendaal, the Netherlands). All gas tubing was made of 4*6 mm Teflon, except the gas outlet at the top of the reactor, which was made of 6*8 mm Teflon (Rubber BV, Hilversum, the Netherlands) and all gas connections were made of PVDF (Serto AG, Aadorf, Switzerland). Sulfate and nutrients were supplied with a Gilson Minipuls 3 (Gilson International BV, Den Haag, the Netherlands) using PVC tubing and the dilution water was supplied with a Watson Marlow 503 (Watson Marlow, Fallmouth, Cornwall, United Kingdom).

7.2.2 Origin of seed sludge and growth medium

Anaerobic granular sludge was obtained from a full-scale UASB reactor treating wastewater from three paper mills (Industriewater Eerbeek, Eerbeek, the Netherlands). From this sludge *Desulfotomaculum carboxydivorans* was isolated (Chapter 5), a sulfate reducer capable of hydrogenogenic CO conversion and subsequent hydrogenotrophic sulfate and sulfite reduction (Chapter 6).

The reactor was supplied with separate flows of a sulfate stock solution, tap water and a nutrient solution. The nutrient solution contained (in mM): KH_2PO_4, 18.4; $Na_2HPO_4 \cdot 2H_2O$, 25; NH_4Cl, 86; $NaCl$, 79; $CaCl_2 \cdot 2H_2O$, 11.5; $MgCl_2 \cdot 6H_2O$, 7.5; Yeast 1 $g.L^{-1}$ and was further supplemented with 15 mL of an acid trace element solution and 15 mL of an alkaline trace element solution according to Stams et al. (1993). The flows of the nutrient and sulfate stock solution were maintained at about 1.1 $L.day^{-1}$. The flow of tap water varied during the experiments to adjust the hydraulic retention time (HRT).

7.2.3 Experimental design

The gas lift reactor was used in two separate flow-through experiments. In the first experiment, the effect of short-term temperature shocks (at day 18 and 32), operational temperature and pH on sulfate reduction were evaluated. The second experiment was performed to investigate the CO conversion efficiency and electron flow at increased CO loads.

Experiment 1: Effects of heat shocks, operation temperature and pH

In this experiment of 50 days, the sulfate reduction capacity with CO as the sole electron donor was investigated. The reactor was inoculated with 125 g wet sludge, corresponding to a total volatile suspended solids (VSS) amount of 12.1 g VSS (total dry solids 17.1 g). Prior to inoculation, the granular sludge was heat-treated at 95°C for 90 minutes, because it was found in long-term batch incubations (Chapter 4) that the methanogenic activity could be completely eliminated by such a pretreatment. To initiate sulfate reduction and create a low redox potential in the reactor, ethanol (5 – 6 mM) was supplied during the first 7 days. Ethanol was chosen as it was found to support growth of *Desulfotomaculum carboxydivorans* in the presence of sulfate (Chapter 5). From day 6 onwards, pure CO was added to the reactor at an initial flow rate of 40 mmol.h^{-1}. From day 6 until day 32, CO was added in excess to the amount of sulfate, whereas from day 33 onwards sulfate was in excess (Table 7.1). This stoichiometry was determined taking into account that 4 moles of H_2 derived from 4 moles of CO are required to reduce 1 mol of sulfate. An overview of the operating conditions and shocks applied to the gas lift reactor during the first run is presented in Table 7.1. The imposed temperature shocks at day 18 and 33, resulted in enhanced stripping of CO_2 from the reactor liquid, which led to slightly increased pH values (up to pH 7.4).

Table 7.1 Overview of the operating conditions and imposed temperature shocks to the gas lift loop reactor during the first reactor experiment

Day	CO load (mmol.L^{-1}.day^{-1})	Sulfate load (mmol.L^{-1}.day^{-1})	Sulfate (mM)	HRT (h)	T (°C)	Duration (hours)	pH
0-6	0	22	6	9	55	constant	6.8
6-17	125-250	20	6	9	55	constant	6.8
18	0	0	0	-	83	1.5 hour	7.4
18-20	125	21	7	9	55	constant	6.8
21-24	125	21	7	9	55	constant	7.4
25-30	125	21	7 - 12[#]	10-18[#]	60	constant	6.2
30-32	95	21	12	7-10	65	constant	6.2
33	0	0	0	-	78	15 hour	7.4
34-45	27-80	23-30[‡]	6 - 10[‡]	9	55	constant	6.8
46-50	85	30	5	4.7	55	constant	6.8

[#] the sulfate concentration and HRT increased gradually due to malfunctioning of the tap water supply pump; [‡] at day 41 the sulfate concentration in the stock solution was increased, resulting in an increase of the influent sulfate concentration from 6 to 10 mM on day 41.

Experiment 2: CO conversion capacity at increased CO loads

In the second 24 days reactor experiment, the HRT was 14 hours from day 0 to day 12, and then decreased to 6 hours between day 16 and 24. The temperature of the reactor was kept at 52 ± 1ºC. The reactor was inoculated with 50 g wet untreated Eerbeek sludge. The sulfate concentration in the reactor was 15-20 mM between day 0 and 15. Thereafter it was decreased to 10 mM, resulting in a sulfate loading rate between 30 and 37 mmol.L^{-1}.day^{-1}. Until day 17, sulfate was present in excess compared to CO (at the start 80 mmol.L^{-1}.day^{-1} CO was supplied, whereas 120 mmol.L^{-1}.day^{-1} CO would be stoichiometrically required to reduce all sulfate), but after day 17, CO was in excess as a result of the increased CO load. Some CO gas leakage from the reactor system resulted in interruption of feeding during the first 3 days, but from day 3 onwards a continuous flow of CO was supplied to the reactor. Furthermore, in this experiment a constant flow of 8 mmol.h^{-1} N$_2$ gas was added to the reactor to assure a constant small overpressure of the system, thus preventing oxygen inlet from the exhaust, which might occur due to high suction of the gas compressor and loss of gas pressure due to consumption of the introduced CO.

7.2.4 Batch experiments

Batch incubations were performed in 117, 310 or 570 mL serum bottles supplemented with 50, 150 or 200 mL basal medium, respectively. The basal medium contained (in mM): NH_4Cl 5.6, $CaCl_2.2H_2O$ 0.7, $MgCl_2.6H_2O$ 0.5, NaCl 5.1, $Na_2S.9H_2O$ 0.3, yeast extract 500 mg.l^{-1}, and 1 ml.l^{-1} of an acid and alkaline trace element solution according to Stams *et al.* (1993). When using 20 mM acetate or CO (90 – 160 kPa), the medium was buffered at pH 7 using 8.2 mM KH_2PO_4 and 11.4 mM $Na_2HPO_4.2H_2O$. In case H_2/CO_2 was the substrate, 23.8 mM $NaHCO_3$ (together with 30 kPa CO_2) was used to buffer the medium at pH 7 and the phosphate concentrations were lowered to 0.8 mM KH_2PO_4 and 1.1 mM $Na_2HPO_4.2H_2O$. The incubations were performed at 200 rpm in a temperature controlled (55°C) shaker incubator type RFI-125 (Infors AG, Basel, Switzerland). Heat-treatment experiments were performed by heating the sludge for 90 minutes at the desired temperature. Some incubations were supplemented with 20 mM 2-bromoethanesulfonate (BES) to inhibit methanogens or 0.07 mM vancomycine to inhibit bacteria (Oremland and Capone, 1988). Fast gas conversion rates were measured by online pressure determinations, which were recalculated in conversion rates after a gas balance check.

7.2.5 Analysis

The pressure in the bottles was determined using a portable membrane pressure unit, WAL 0-4 bar absolute (Wal Mess- und Regelsysteme GmbH, Oldenburg, Germany). Online gas pressure measurements were performed using a Microcontroller Pressure Data Acquisition System (Electronic Workshop, WUR, Wageningen, the Netherlands) equipped with pressure sensors in the range of 0.9 to 3.0 bar (abs). The pressure sensors were connected with 3 * 0.5 mm PUN tubes (Festo, Delft, the Netherlands) of approximately 1 m length to the shaken 315 ml serum bottles.

The gas composition was measured on a gas chromatograph HP 5890 (Hewlett Packard, Palo Alto, USA). Acetate and other volatile fatty acids (VFA) were analyzed on a HP 5890A gas chromatograph (Hewlett Packard, Palo Alto, USA) according to Weijma *et al.* (2000). Sulfide was measured according to Trüper and Schlegel (1964). VSS was analyzed according to standard methods (APHA, 1995). Sulfate was measured on a DX-600 IC system (Dionex Corporation, Salt Lake City, USA) as described in Chapter 4. The electrical conductivity (EC) was measured in mS/cm

using a microprocessor conductivity meter type LF 196 (WTW GmbH, Weilheim, Germany). Biomass production was monitored by measuring the optical density at 600 nm (OD_{600}) using a Spectronic 601 spectrophotometer (Milton Roy, Ivyland, USA) and Plastibrand 1.5 mL PMMA cuvettes (Brand GmbH, Wertheim, Germany).

Solubilities of CO, CO_2 and CH_4 were calculated using data from Lide (2001), solubility of H_2 was calculated according to Perry *et al.* (1997), and the amounts produced or consumed were calculated by taking into account both gas and liquid phases.

All chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany). The CO (purity 99.9%) used for feeding the gas lift reactor was obtained from Praxair (Indugas BV, Vlaardingen, the Netherlands) and the CO (purity 99.997%) used for batch experiments was supplied by Hoek Loos (Rotterdam, the Netherlands). N_2 (purity 99.999%) and H_2/CO_2 (80%:20%) used in batch experiments were purchased from Indugas (Vlaardingen, the Netherlands).

7.3 Results

7.3.1 *Effects of heat shocks, operation temperature and pH*

Fig. 7.2 presents the operational conditions during the first reactor experiment. The conversion of ethanol, supplied during the first 7 days of reactor operation, exceeded 90% between day 3 and 6 (data not shown) and about 70% of the ethanol was recovered as acetate (Fig. 7.3). The concentration of higher fatty acids was negligible throughout the experiment. The amount of sulfate reduced was somewhat higher than could be explained by the converted amount of ethanol only, suggesting that some acetotrophic sulfate reduction capacity was present. This is in agreement with the consumption of 2 mM acetate (10% of total) in sulfate supplemented batch incubations with heat-treated inoculum sludge for 25 days at 55ºC. In the absence of sulfate, no acetate conversion was observed in these batch incubations.

The ethanol conversion decreased rapidly upon commencing the CO feeding at day 6, with just over 60% ethanol conversion upon termination of the ethanol feeding at day 7 (data not shown). The sulfide concentration in the reactor decreased as well from 2 to 1 mM (Fig 7.3), when starting the CO feeding. This instantaneous decrease in sulfide production is most likely the result of CO inhibition of the

dominant ethanol consuming sulfate reducers. This suggests that the ethanol dosing had not favored the development of *Desulfotomaculum carboxydivorans* or other carboxydotrophic microorganisms present in the starting seed sludge.
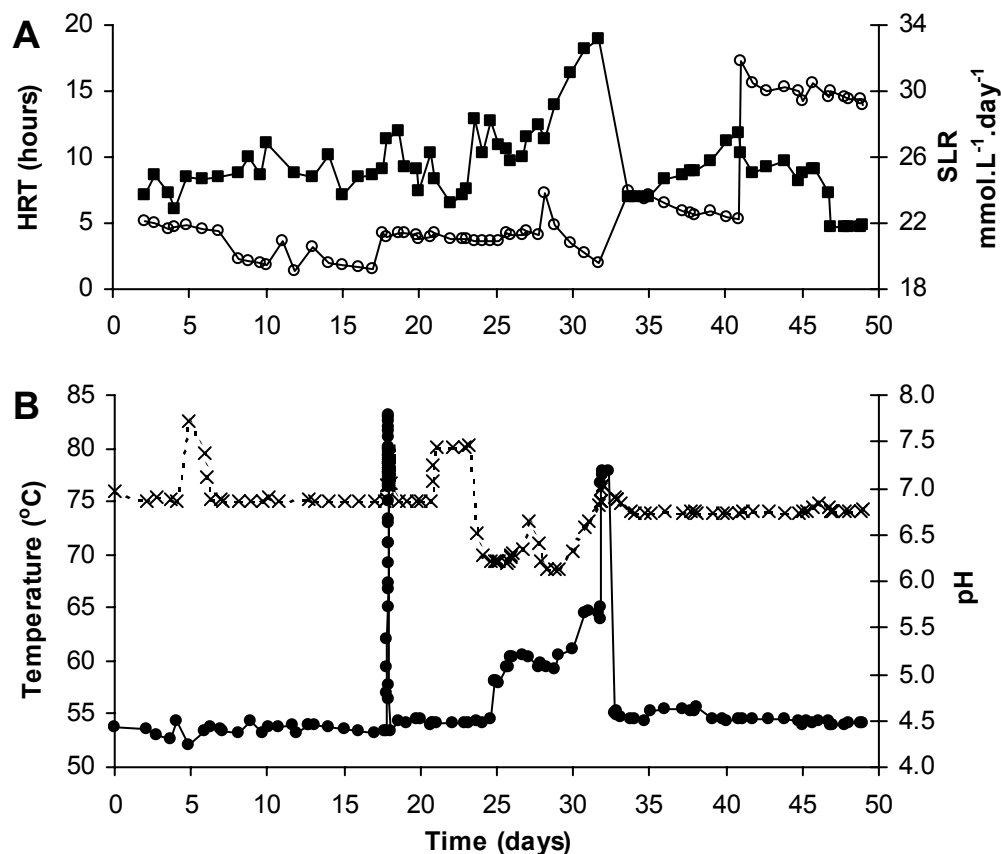
Fig. 7.2 Operational conditions during the first reactor experiment: (A) the applied HRT and sulfate loading rate (SLR) and (B) pH and temperature. Symbols are HRT (■) and sulfate loading rate (○) in A, and pH (×) and temperature (●) in B.
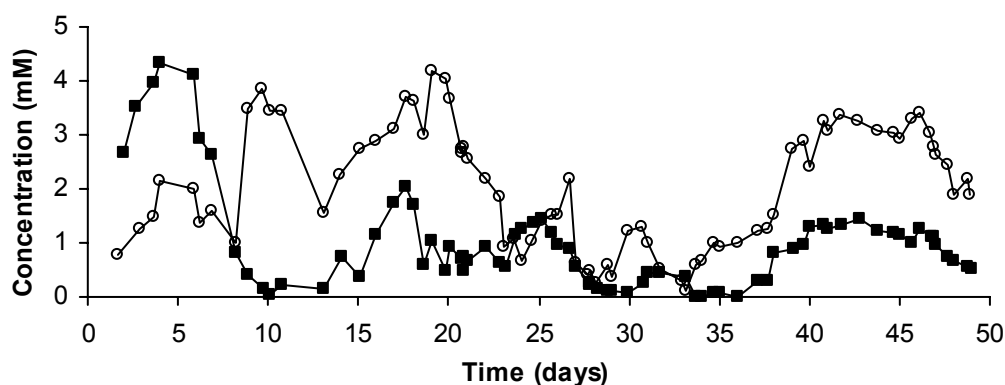
Fig 7.3 The effluent acetate (■) and sulfide (○) concentration during the first reactor experiment.

At day 8, the CO concentration in the effluent gas decreased (Fig. 7.4) and 8% H_2 was detected in the exhaust gas of the reactor, which increased to over 17% within one day (Fig 7.4). This coincided with an increase in sulfide concentration in the effluent from 1 to 3.5 mM (Fig 7.3), and further to 3.8 mM at day 10. The measured sulfate reduction rate at day 10 was about 10 mmol.L^{-1}.day^{-1}. Despite the pre-treatment of the granular sludge at 95°C (90 minutes) methane formation occurred; 5% of methane was measured in the exhaust gas at day 10 (Fig. 7.4). Moreover, after the system was depleted from CO between day 10 and 13 due to gas leakages, resuming the CO flow at 125 mmol.L^{-1}.day^{-1} at day 13 resulted in an almost instantaneous steep increase of the CH_4 content of the exhaust gas, whereas the H_2 levels remained below 2% (Fig. 7.4). The sulfide concentration at day 13 (after resuming CO feeding) amounted to 1.5 mM and then rapidly increased to 3.6 mM at day 18 (Fig. 7.3), corresponding to a sulfate reduction rate of 10 mmol.L^{-1}.day^{-1}. From day 7 onwards, the acetate concentrations in the effluent from the reactor were below 2 mM (Fig. 7.3).
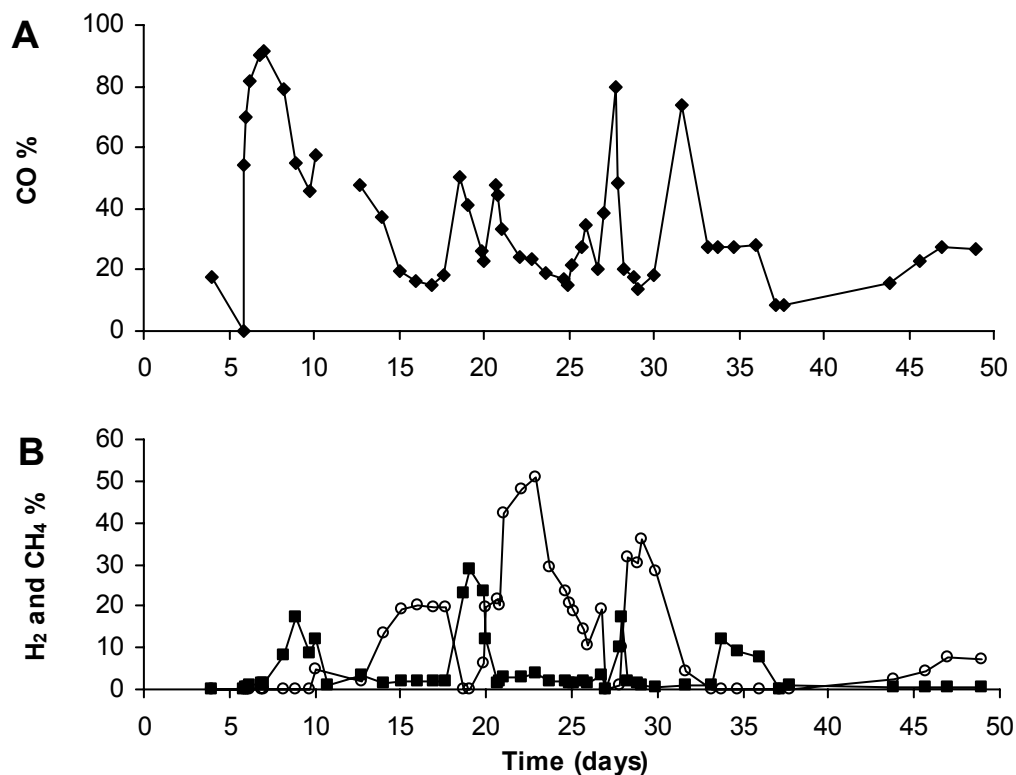
Fig. 7.4   Exhaust gas concentrations of carbon monoxide (A) and hydrogen (■) and methane (○) in (B).

The results in Fig. 7.5 show that at day 17, i.e. at a CO load of 125 mmol.L^{-1}.day^{-1}, 90% of the CO fed to the reactor was converted. Calculations of the electron flow from CO revealed that between day 14 and 18, 50 to 70% of the electron flow was directed to methane and only 30% was used for sulfate reduction. The remainder was recovered in acetate and unconsumed H$_2$ (Fig 7.5). In order to enhance electron donor utilization for sulfate reduction, we investigated the impact of short-term heat shocks imposed to the reactor system.
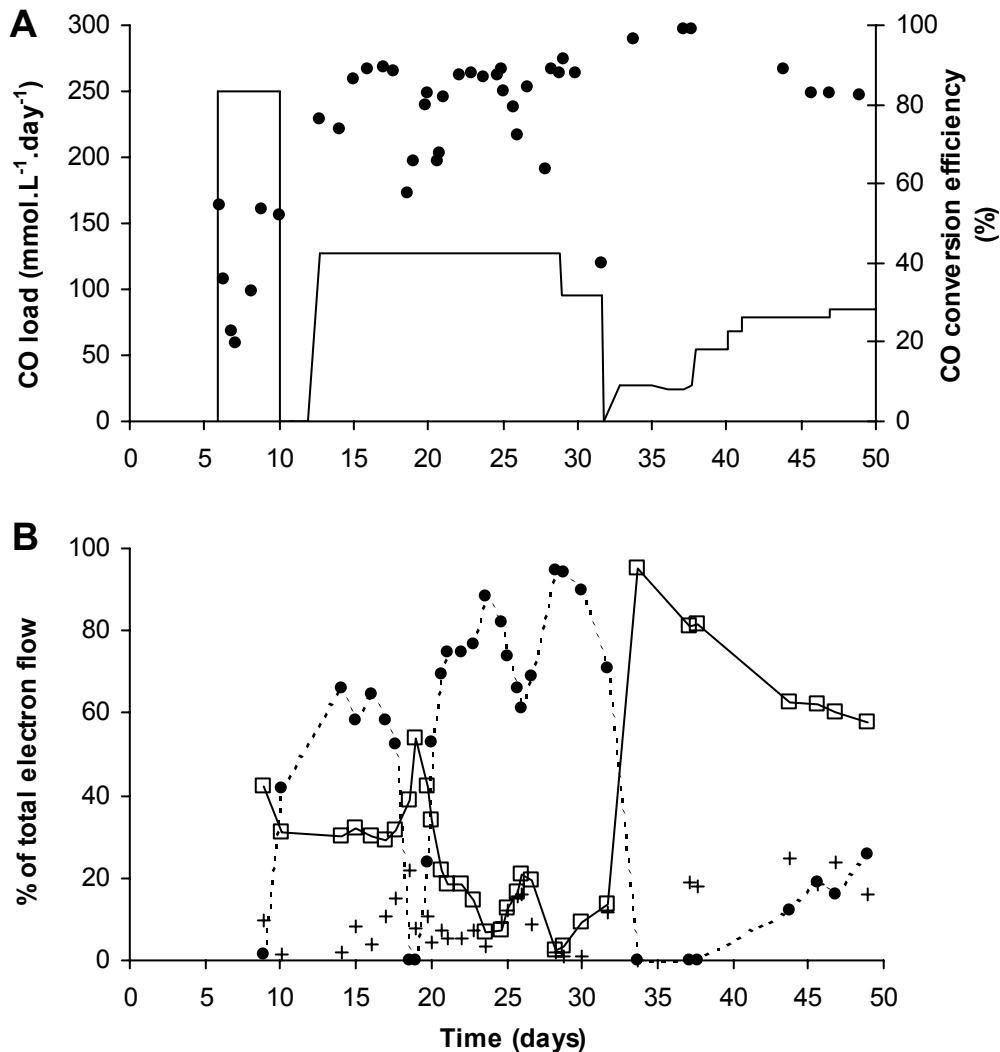
Fig. 7.5   Performance of the gas lift reactor during the first experimental run. (A) the CO loading rate (line) and CO conversion efficiency (●) and (B) the electron flow from CO towards acetate production (+), methane production (●) and sulfate reduction (□).

During the first 1.5 hour heat shock imposed on day 18, the maximum temperature reached inside the reactor was 83°C. Fig. 7.4 shows that directly after resuming the operation at a temperature of 55°C, the H_2 concentration in the exhaust gas increased rapidly, while distinct CH_4 production was absent. This resulted in a maximum of 50% of the electron flow from CO directed towards sulfate reduction (Fig. 7.5) and the remainder to acetate and unconverted H_2. At day 19, the effluent sulfide concentration had increased to 4.2 mM (Fig. 7.3), corresponding to a sulfate reduction rate of 11 $mmol.L^{-1}.day^{-1}$. Nevertheless, already at day 20, methane

production had resumed and from then onwards increased rapidly. The second heat shock imposed at day 33 for a period of 15 hours seemed to have a more lasting effect, although the temperature did not exceed 78°C, because only after 10 days small amounts of methane were detected (Fig. 7.4). This second heat treatment had a positive effect on the electron flow from CO to sulfate reduction (Fig. 7.5), although the amount of CO used for sulfate reduction decreased from 95 to 60%, between day 34 and 44. However, once again, at day 44, a significant recovery of the methanogenic population was observed. The highest sulfate reduction rate (14 mmol. $L^{-1}.day^{-1}$) was found after decreasing the HRT to about 4.7 hours.

In order to reduce the sulfide toxicity, the pH of the reactor mixed liquor was increased from 6.8 to 7.4 at day 21. This seemed to favor methanogenesis as the sulfide concentration in the effluent declined (Fig. 7.3) and the contribution of the electron flow from CO to methane increased (Fig. 7.5). The CO conversion efficiency was not much affected by the increased pH (Fig 7.4). The maximal sulfide concentration measured in the reactor was only 4.2 mM (Fig. 7.3), although sludge samples collected from the reactor at the end of the experiment were capable to produce sulfide concentrations exceeding 11 mM in batch experiments with H_2/CO_2 as the substrate. These higher sulfide concentrations in batch incubations suggests a pH related toxicity effect as in the batch incubations the pH rose during H_2/CO_2 conversion from 7.0 to about 7.8, whereas the highest sulfide concentration in the reactor was measured at pH 6.8. Despite its positive effect on the sulfide concentration, reactor operation at elevated pH values (pH > 7.5) are expected to be unfavorable for CO conversion, as in batch experiments performed at a pH of 7.7, with the reactor seed sludge, no CO conversion was observed within 10 days. Within that period of time, a complete CO conversion at pH 7.0 was observed (data not shown).

At the imposed elevated operation temperatures of 60°C and 65°C between day 25 and 32 (Fig. 7.2), initially, an increased electron flow to sulfate reduction manifested, but still well over 60% was directed to methane formation (Fig. 7.5). Furthermore, as the increased electron flow (Fig. 7.5) coincides with a lower pH (Fig. 7.2), it looks that pH rather than the temperature affects the direction of the electron flow from CO. However, a low pH is not sufficient as even at pH 6.1 (day 29; Fig. 7.2) almost 95% of the electron flow from CO was directed to methane formation

(Fig. 7.5) at a CO conversion efficiency of 90% (Fig. 7.5). A further increase of the temperature to 65°C, at day 30, resulted in a drastically decreased CO conversion efficiency (Fig. 7.5), as well as in a decreased sulfide and methane production, but not in a complete absence of methane formation (Fig. 7.4). This suggests that not the elevated temperature limits methanogenesis, but rather the production of H_2 from CO.

At the end of the reactor experiment about 60% of the electron flow was directed to sulfate reduction and roughly 30% to methane production. H_2 is presumed to be the actual electron donor for both processes. Batch experiments with reactor sludge collected at the end of the reactor experiment revealed that the methanogens consumed H_2 three times faster than sulfate reducers, viz. H_2 consumption rates of 1.4 and 0.4 $mmol.h^{-1}$ were calculated from online pressure measurements in the absence of sulfate and in the presence of sulfate and 20 mM BES, respectively.

The biomass concentration in the reactor was limited due to wash out of granular sludge. The VSS content of the reactor upon termination was 1.6 $g.L^{-1}$. Thus almost 50% of the initial amount of biomass had washed out. Part of the granular structure of the seed sludge was lost after the first reactor run. A clearly visible increase of turbidity of the reactor liquid suggested that a large part of the active biomass was suspended in the reactor medium, rather than immobilized on or in solid particles.

7.3.2 CO conversion capacity at increased CO loads

The operational conditions during the second reactor experiment are presented in Fig. 7.6. The CO flow to the reactor was increased in this experiment from about 80 $mmol.L^{-1}.day^{-1}$ at the start to 420 $mmol.L^{-1}.day^{-1}$ at day 23 (Fig. 7.7). The amount of granular sludge supplied to the system at the start of the experiment was rather small, and most of the active biomass present in the system was finely suspended in the reactor medium and could be monitored by optical density (OD_{600}) measurements. The recorded values for the OD_{600} are presented in Fig. 7.7. The first OD measurement was conducted at day 9 of the experiment, as from that day onwards the reactor liquid did not contain large particles in the effluent anymore that affect the OD measurements.
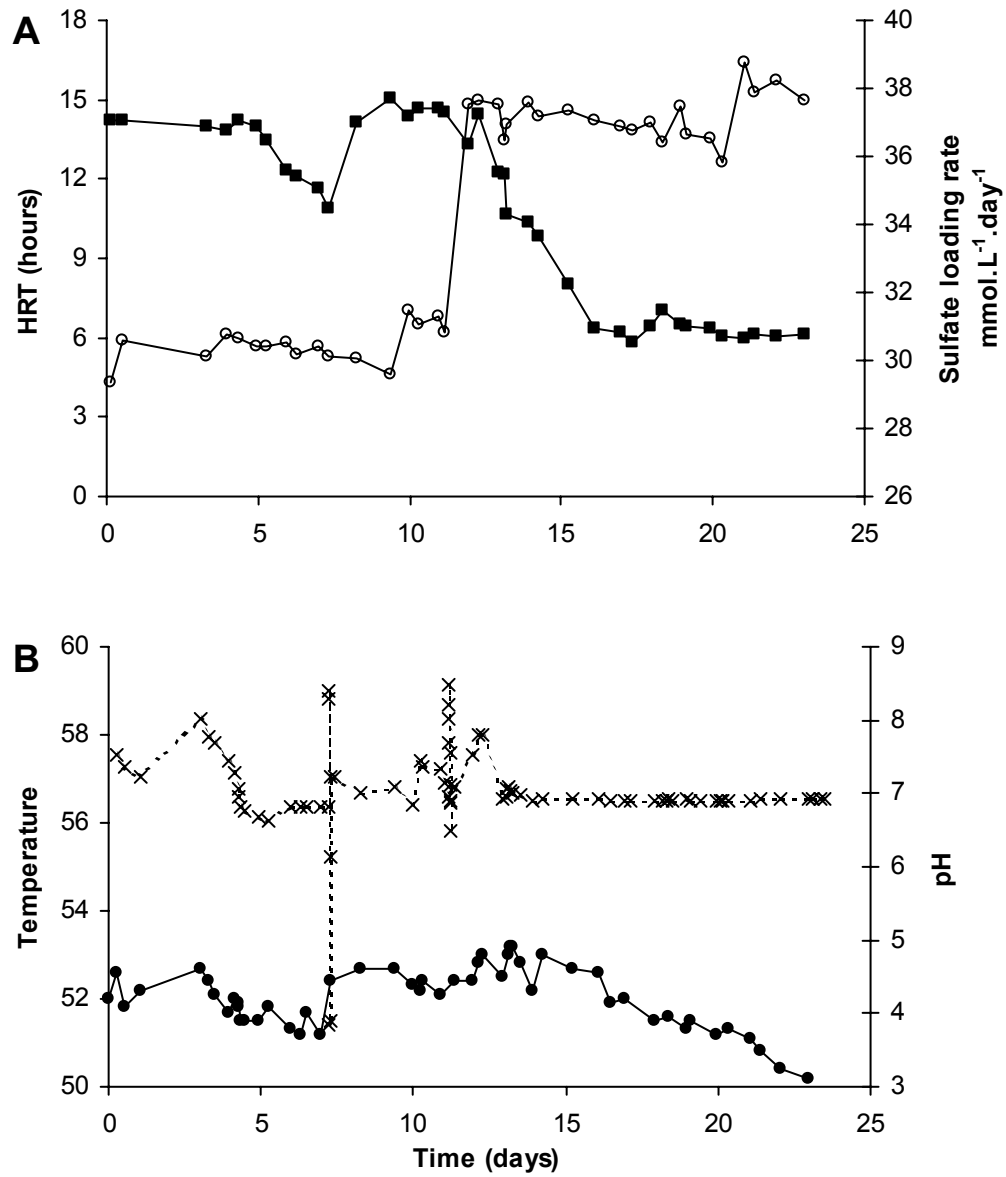
Fig. 7.6 Operational conditions during the second reactor experiment. (A) imposed HRT (■) and sulfate loading rate (○) and (B) pH (×) and temperature (●).
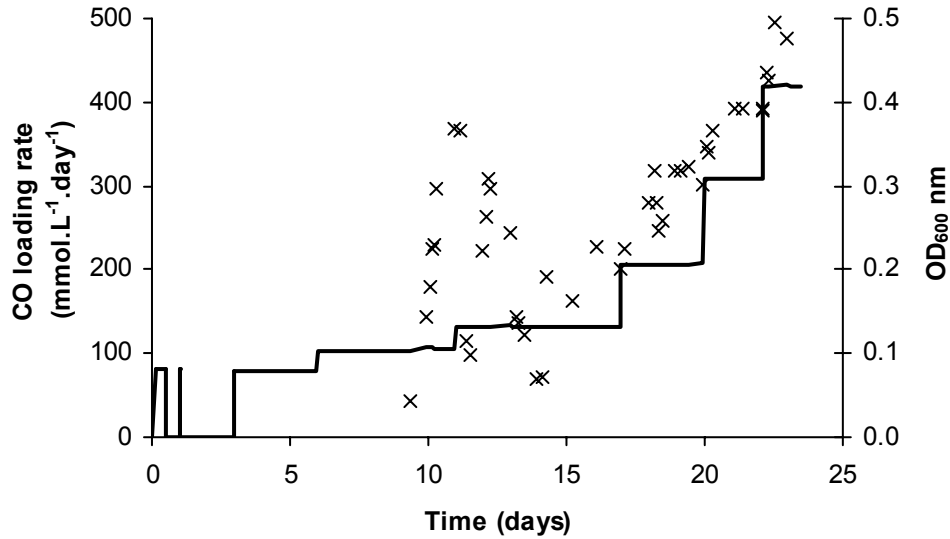
Fig. 7.7 CO loading rate (represented by the line) and measured OD_{600} values ($\times$) during the second reactor experiment.

From the composition of the exhaust gas and the exhaust gas flow, the CO conversion efficiency was calculated as well as the corresponding conversion capacity of CO for the reactor. The results are depicted in Fig. 7.8.
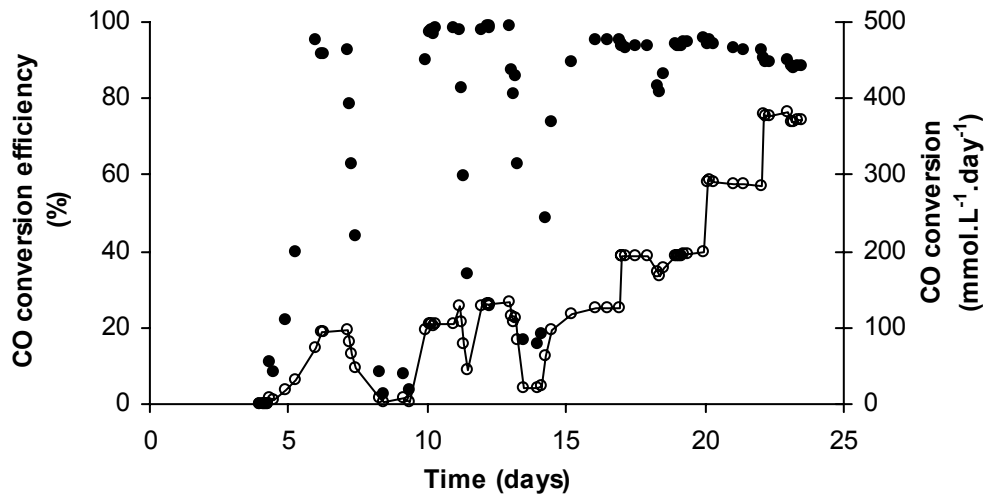

Fig. 7.8 CO conversion efficiency ($\blacksquare$) and CO conversion capacity ($\circ$) during the second reactor experiment.

At day 3, when the continuous feeding with CO was started, the pH was rather high, but it dropped rapidly due to CO_2 production (Fig. 7.6). At day 7 and 11, short-term pH shocks occurred, whereas the pH increased to 7.8 around day 12 (Fig. 7.6). These pH shocks resulted in sharply decreased CO conversion efficiencies (Fig. 7.8).

Generally, the CO conversion recovered rapidly after pH values were recovered at around pH 7.0 (Figs. 7.6B and 7.8). As these pH shocks directly affected the OD_{600} (Fig. 7.7) it presumes that very little biomass retention occurs in the reactor, i.e. the major part of the active biomass is growing suspended in the reactor medium. From day 15 onwards, a stable CO conversion was established and it appears that the rapidly imposed higher CO loading rates did not affect the CO efficiency much. The increased CO conversion rates were directly reflected in the amount of biomass suspended in the reactor (Figs. 7.7 and 7.8). Considerable wash-out of biomass occurs especially at lower HRTs. This is illustrated by the OD_{600} value of 0.37 assessed at day 10 at a HRT of 14 hours and a CO conversion rate of 100 $mmol.L^{-1}.day^{-1}$, whereas at a HRT of 6 hours about 290 $mmol.L^{-1}.day^{-1}$ CO was converted at similar biomass concentrations (Figs. 7.6, 7.7 and 7.8). The latter low value for the biomass content reveals that the CO conversion capacity of the biomass is high.

Fig. 7.9 shows the production of H_2 and CH_4 during the second reactor experiment. The production rates were calculated from the gas composition and the effluent gas flow, which was calculated from the N_2 concentration in the effluent gas and the applied constant in-flow of N_2 to the reactor. Fig. 7.9 suggests that H_2 is the main intermediate of CO conversion as the H_2 concentration peaks in the exhaust gas directly coincide with an increased CO conversion, whereas CH_4 production coincides with the disappearance of H_2 from the exhaust gas. Furthermore, the pH shocks at day 7 and 11 resulted in a longer absence of methane production in the reactor between day 7 and 12. The low methane production between day 13 and 15 most likely is the result of limited availability of H_2 and CO_2 due to a low CO conversion (Fig. 7.8).
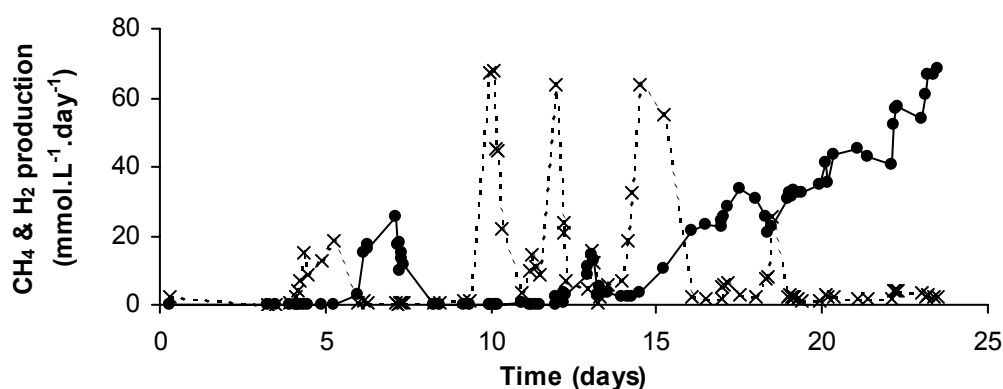
Fig. 7.9 H_2 (×) and CH_4 (●) production during the second reactor experiment calculated from the concentrations in the exhaust gas and the exhaust gas flow.

Fig. 7.10 shows the sulfide and acetate production rates derived from the measured concentrations and the applied HRT.
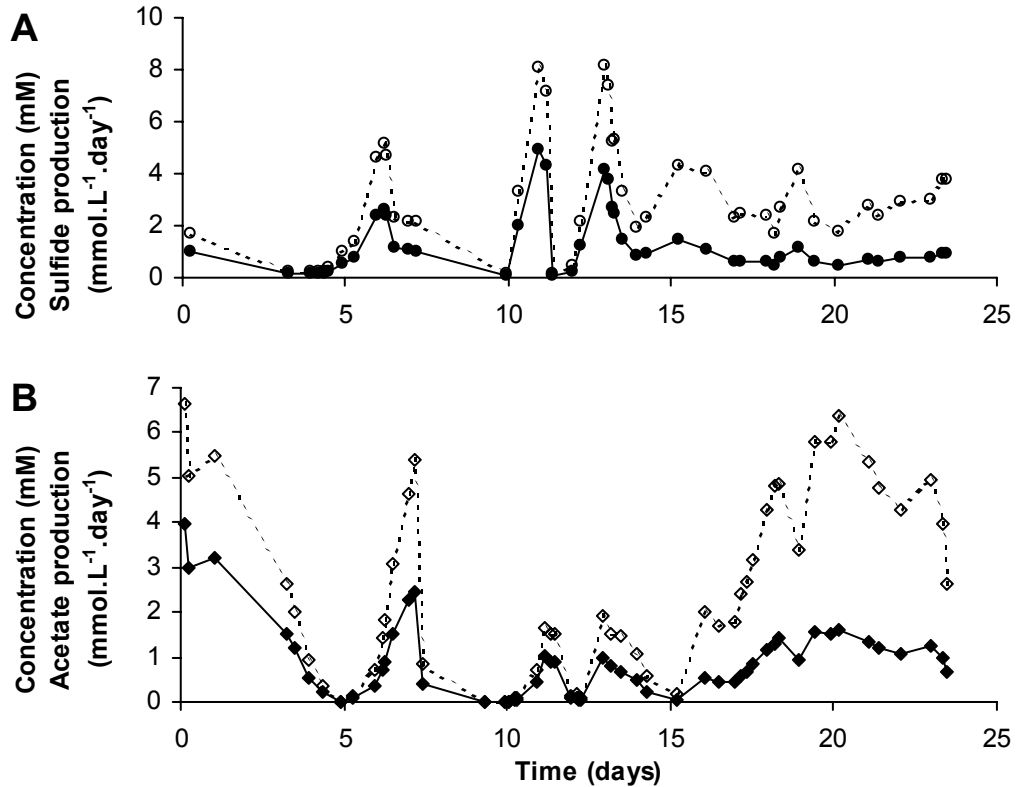

Fig. 7.10 Concentrations and production rates of sulfide (A) and acetate (B) during the second reactor experiment. Closed symbols represent concentrations and open symbols represent the corresponding production rates.

The sulfide concentrations are in general quite low, i.e. the maximal measured sulfide concentration is just below 5 mM at day 11. The sulfur balance in general fits quite well (S-recovery 100 ± 15%), except for a short period around day 11. The amount of sulfide at that day (11), and the calculated corresponding sulfide production rate of 8 $mmol.L^{-1}.day^{-1}$, was much lower than the 12 mM of sulfate reduced, which would correspond to a sulfate reduction rate of 20 $mmol.L^{-1}.day^{-1}$. The acetate concentration is generally low, indicating low acetate production rates. It should be noted that the acetate production during the first 4 days is not due to CO conversion, but rather to decay and wash-out of biomass as CO, the sole external substrate supplied, was not converted in this period. Combining the results in Figs. 7.9 and 7.10 reveals that methane is the major product from CO conversion and that only a minor part of the electron flow is directed to sulfate reduction (Fig. 7.11), except for some small periods when methane production is suppressed by pH shocks.
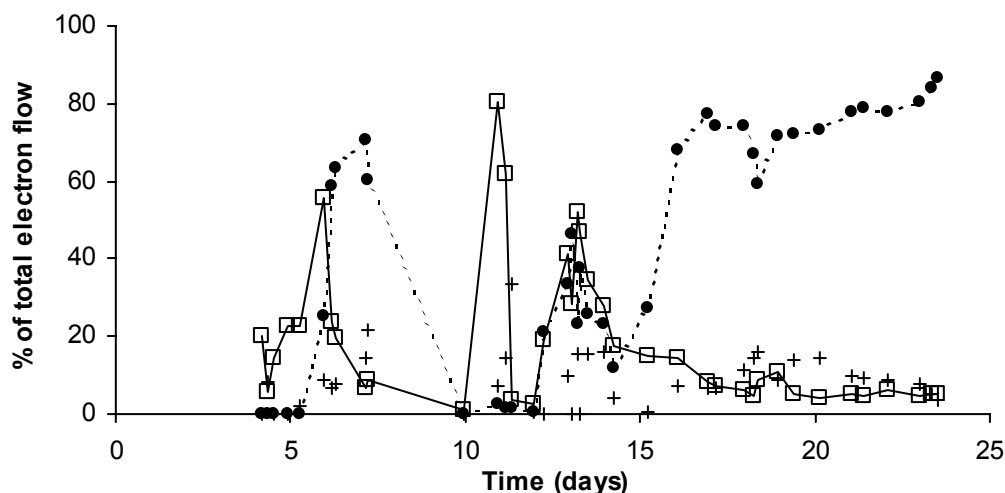
Fig. 7.11   Direction of the electron flow from CO towards acetate production (+), methane production (●) and sulfate reduction (□) during the second reactor experiment.

7.3.3 Effect of short-term heat treatment

The seed sludge, exposed to 95°C for 90 minutes before use, did not produce any methane in batch incubations at 55°C within 60 days using H_2/CO_2 as substrate in the presence of vancomycine. Batch incubations with the same pre-treated sludge produced acetate in the absence of vancomycine. Controls with vancomycine and untreated sludge at 55°C converted H_2/CO_2 completely to methane within 3 days. Incubations with sludge cultivated in the first reactor run and collected at the end of the reactor experiment, which were treated after inoculation at 83°C for 90 minutes and at 78°C for 15 hours, converted H_2/CO_2 within 4 days to methane. This indicates that both heat-treatments applied to the reactor were insufficient to eliminate methanogenic activity.

Incubations with reactor liquid collected from the reactor at the end of experiment 2, revealed that with short term (90 minutes) exposure to a temperature of 80°C, rapid recovery of methane production from H_2/CO_2 occurred when subsequently incubated at 55°C. With short-term exposure of the sludge to temperatures of 85°C and higher, no hydrogenotrophic methane production was observed at 55°C in the presence and absence of vancomycine within a period of 20 days of incubation. In the absence of vancomycine, a 90 minutes heat treatment up to 95°C resulted in acetate production from H_2/CO_2, which was completed within 7 days

of incubation at 55°C. This shows that homoacetogens were not eliminated by such a heat-treatment.

7.3.4 Competition for CO-derived H$_2$

The competition between sulfate reducers and methanogens was investigated in batch experiments with untreated seed sludge. The amount of CO (50 kPa) supplied was low and could produce maximal 3 mM sulfide, as this concentration is presumed not to be inhibitory (Chapter 6). Incubations were performed in the presence of 50 and 120 kPa H$_2$. No CO and H$_2$ could be detected in the gas phase after 6 days. The amount of products formed is presented in Table 7.2.

Table 7.2 shows that only with CO as electron donor in the presence of sulfate the majority of the electron flow is used for sulfide production, whereas with a similar amount of H$_2$ most of it was used for methane production. Most probably, the methanogens are inhibited when CO is supplemented in the gas phase, which is in agreement with the low amount of methane produced in incubations with sulfate compared to the incubations without an external electron donor. Nevertheless, the initial presence of CO did not prevent methane formation in the absence of sulfate, but its presence apparently affects the outcome of the competition between sulfate reducers and methanogens for H$_2$, as CO is converted via an intermediate production of H$_2$. This CO-derived H$_2$ presumably serves as electron donor for sulfate reduction or methane formation.

Table 7.2   Competition between methanogens and sulfate reducing bacteria at 55ºC in untreated inoculum sludge using CO and H_2 as electron donors. The amounts are expressed in mmol per bottle.

Electron donor	Sulfate	E-donor t = 0 (mmol)	Produced methane (mmol)	Produced sulfide (mmol)	E-donor recovery (%)	
					methane	sulfide
None	-	0	0.11 ± 0.01	0	-	-
None	+	0	0.08 ± 0.03	0.02	-	-
CO	-	0.93	0.27 ± 0.06	0	93 ± 6	-
CO	+	0.90	0.06 ± 0.01	0.21 ± 0.01	n.d.	87 ± 6
H_2	-	0.87	0.31 ± 0.03	0	92 ± 8	-
H_2	+	0.88	0.25 ± 0.02	0.06 ± 0.03	78 ± 8	25 ± 10
H_2	+	2.73	0.62 ± 0.04	0.14 ± 0.03	79 ± 5	18 ± 4

n.d. = not determined

At the end of the second reactor experiment, methanogenic and homoacetogenic enrichments were obtained from the biomass present in the reactor by serial dilution under an atmosphere of H_2/CO_2. To obtain a homoacetogenic enrichment the inoculum was first treated at 95ºC for 90 minutes, which eliminated methanogens. Acetate production was not much in the methanogenic enrichments due to the faster growth rates of the methanogens. Growth curves for both enrichments are presented in Fig. 7.12.

The methanogenic enrichment had a generation time of 4.5 hours, whereas the generation time of the homoacetogens was 15 hours. Growth of the methanogenic enrichment at elevated NaCl concentrations revealed that up to 400 mM NaCl (35 mS.cm^{-1}) a full H_2/CO_2 conversion was achieved within 2 days. No measurable inhibitory effects of NaCl were observed. The highest NaCl concentration with complete conversion of H_2/CO_2 to methane was 660 mM (55 mS.cm^{-1}), but conversion was slow (complete conversion > 10 days) with an increased lag phase, requiring 30 days for complete conversion. The homoacetogenic enrichment converted H_2/CO_2 to acetate at maximal 670 mM NaCl (55 mS.cm^{-1}), but due to a limited number of measurements, differences in rates could not be elucidated. Furthermore, batch experiments revealed that methane production proceeded well even at a pH as low as 5.5 (data not shown).
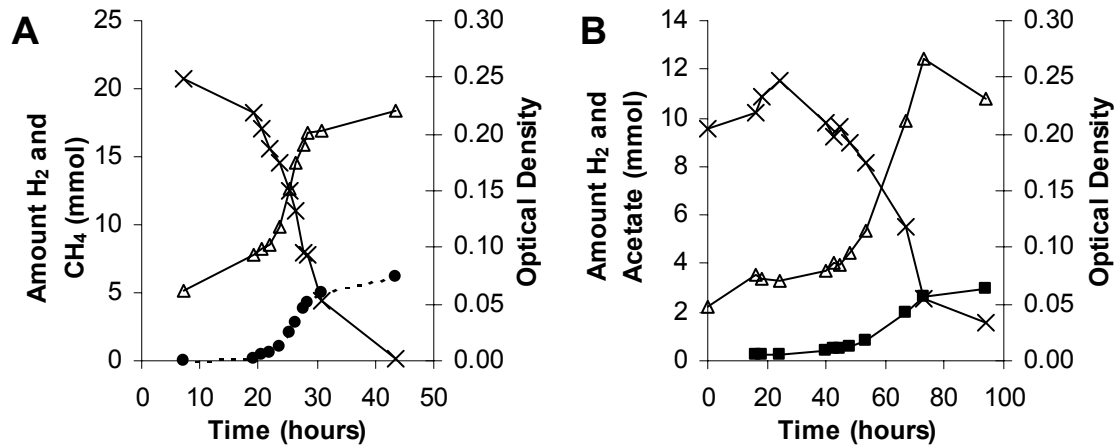
Fig. 7.12 Growth curves of the methanogenic (A) and homoacetogenic (B) enrichment obtained from the reactor at the end of the second experiment. Both enrichments were supplemented with 120 kPa H_2 and 30 kPa CO_2. The difference in H_2 amount at the start is due to the different sizes of the incubation bottles, i.e. 570 and 310 mL. The symbols represent: OD_{600} (Δ); methane (●); hydrogen (×); acetate (■).

7.4 Discussion

7.4.1 CO conversion and biomass retention

In the reactor experiments, high CO conversion efficiencies (up to 99%) were obtained at pH 6.9 (± 0.1) and temperatures between 51 and 55ºC (Figs. 7.5 and 7.8). The short generation time of the hydrogenogenic CO converters present most likely is the reason for the fast recovery of CO conversion upon a pH or temperature shock. *Desulfotomaculum carboxydivorans*, isolated from the same seed sludge (Chapter 5) revealed a generation time of only 1.7 hours (Chapter 6). An initial supply of ethanol for 7 days did not seem to stimulate growth of *D. carboxydivorans* in the reactor, as ethanol conversion as well as sulfide production dropped upon initiating CO feeding. Despite its capability to grow with ethanol (Chapter 5), the rather long generation times (19 hours) in case of growth on ethanol (Chapter 6) did not enable *D. carboxydivorans* to compete with other ethanol utilizing bacteria present in the seed sludge.

CO conversion initially coincided with high amounts of H_2 in the exhaust gas (Fig. 7.4B and 7.9), indicating that CO is converted to H_2 which is the presumed electron donor for sulfate reduction, as well as for methane and acetate formation, as

previously found in batch experiments (Chapter 4). The fast responses after shocks reflect the fast growth and high CO conversion capacity of the biomass. The rapid increased CO load from day 15 onwards in the second reactor experiment (Fig. 7.7) did not result in a significant loss of the CO conversion efficiency (Fig. 7.8). At the highest CO loading rate of 420 mmol.L^{-1}.day^{-1}, the CO conversion efficiency was about 90%, corresponding to a conversion of about 380 mmol.L^{-1}.day^{-1}. As the amount of biomass present in the reactor is rather low (Fig. 7.7), the specific CO conversion capacity of the biomass is obviously extremely high. This is in agreement with the extremely high specific CO conversion capacity estimated for *D. carboxydivorans*, i.e. CO conversion at rates exceeding 0.7 mol CO.(g protein)$^{-1}$.hour^{-1} (Chapter 6). However, as the biomass in the reactor consists of different types of microorganisms, i.e. hydrogenogenic CO converting bacteria, hydrogenotrophic sulfate reducers, methanogens and homoacetogens, the specific conversion rates in the reactor could not be assessed.

7.4.2 Competition for CO-derived H$_2$

The results from the reactor experiments show that despite a significant reduction of the methanogenic population as a result of heat-treatment of the sludge at 95ºC prior to inoculation, methanogens still are capable to out-compete rapidly sulfate reducing bacteria for H$_2$. As this sludge did not produce any methane for at least two months in batch experiments, the methanogenic activity presumably must be attributed to contamination of the reactor. The heat shocks imposed during the first reactor experiment were insufficient to eliminate methanogens. Results of batch experiments demonstrated that at least a temperature exceeding 85ºC is required, a value which was not attained inside the reactor. Also the pH shocks that occurred during the second reactor experiment, although they detrimentally affected the methanogenic activity, did not completely eliminate methanogenesis. However, both heat (Oh et al., 2003) and pH shock treatment (Chen et al., 2002) has been successfully used to eliminate methanogenic activity, and drastically reduced the methanogenic population in our experiments (Figs. 7.2 and 7.4; Figs. 7.6 and 7.9). Nevertheless, their recovery proceeds fast due to incomplete elimination and the fast growth rates of the thermophilic methanogens present. Therefore, methanogens clearly are capable to outcompete sulfate reducing bacteria with respect to CO-derived H$_2$ under moderately

thermophilic conditions. This corroborates with methanol derived H_2, for which Vallero et al. (2003a) reported that about 85% of the methanol derived H_2 was used for methane production in a methanol fed sulfate supplemented reactor operated at 55ºC. Van Houten et al. (1997), also found a strong competition for H_2 between methanogens and sulfate reducers at 55ºC in a H_2/CO_2 fed gas lift reactor.

7.4.3 Alternative means to suppress methanogenesis

For the development of a cost effective sulfate reduction process the loss of electron donor to methanogenesis should be minimized. The methanogenic enrichment obtained from the reactor sludge after the second experiment revealed a short generation time on H_2 (t_d = 4.5 hours), growth at salt concentrations up to 660 mM NaCl, as well as growth and CH_4 production at pH values as low as 5.5. Furthermore, the H_2 consumption rates assessed for the methanogens were three times higher than those found for the sulfate reducing bacteria in reactor sludge samples collected at the end of reactor experiment 1, when sulfate reduction was still dominating the H_2 consumption (Fig. 7.5). Although the occurrence of *D. carboxydivorans* in the reactor sludge was not assessed, its isolation from the seed sludge as well as the capacity of the sludge to reduce sulfate in the presence of CO suggests its presence. When assuming that *D. carboxydivorans* is the sole bacterium responsible for hydrogenogenic CO conversion in the reactor, its characteristics indicate that neither a low pH nor a high salt concentration would be beneficial to stimulate sulfate reduction. In contrast to the methanogenic enrichment, CO conversion by *D. carboxydivorans* was completely inhibited above 300 mM NaCl (Chapter 5). This is in agreement with the observation of Vallero et al. (2003b), who found in thermophilic methanol conversion experiments conducted at NaCl concentrations ranging from 8 to 215 mM a distinctly high sensitivity of sulfate reducers for increased salt concentrations. Similarly, operation of the bioreactor at low pH, as found effective in certain cases at pH-values below 5.5 (Fang and Lui, 2002; Lay, 2000), would not be successful as *D. carboxydivorans* does not grow below pH 5.8 (Chapter 5).

Reactor operation at higher temperatures (60 and 65ºC), which was found to be an effective strategy for maximizing sulfate reduction using methanol (Weijma et al., 2000), did not eliminate methanogenic activity in our experiments (Figs. 7.2 and

7.5). Moreover, at 65ºC the CO conversion efficiency of the reactor rapidly decreased (Figs. 7.2 and 7.5), indicating that operation at higher temperatures is detrimental for CO conversion. This is in agreement with the absence of hydrogenogenic CO conversion at 65ºC found in batch incubations with Eerbeek sludge (Chapter 3). Furthermore, CO toxicity as a means to influence the competition seems only effective at relative high initial P_{CO} of 90 kPa in a batch experiment with unadapted Eerbeek sludge (Table 7.2), as we also found for crushed Eerbeek sludge (Chapter 3). In the reactor experiments, where most of the active biomass grew in finely dispersed form, periods with high CO concentrations in the exhaust gas did not prevent fast recovery of methanogenesis. This is most likely caused by adaptation of the methanogens to CO, as has been reported for other methanogens (O'Brien et al., 1984).

Alternative means to suppress methanogenesis include the use of chemical inhibitors such as 2-bromoethanesulfonate (BES), acetylene (Oremland and Capone, 1988) and chloroform (Chidthaisong and Conrad, 2000; Scholten et al., 2000). However, the use of such chemicals is not desired as these compounds or their degradation products may be released into the environment. Furthermore, as these chemicals are usually applied for pathways studies and not for continuous full scale operations adaptation might result in largely increased operational costs due to an increasing demand. Spontaneous resistance to low concentrations of BES has been reported for several methanogens (Santoro and Konisky, 1987; Haas et al., 1986; Smith, 1983). The use of chemical inhibitors in bioreactor studies has received little attention so far. Sparling et al. (1997) investigated H_2 production in solid substrate anaerobic digesters, in which complete suppression of methane formation, but not H_2 production, was achieved upon addition of 1% v/v acetylene in the headspace. In case of closed treatment systems the use of chemical inhibitors could be considered. Such closed systems look feasible in flue gas desulfurization by reusing the effluent of the biological treatment repeatedly for the scrubbing of SO_x.

For the complete elimination of methanogens a heat treatment at 95ºC for 90 minutes looks to be very effective, at least based on results of batch experiments. Heat treatment is not inhibiting methanogens during reactor operation as would be the case with chemical agents, but rather eliminates methanogens before reactor operation is initiated. Such a heat treatment poses no environmental risk, because no harmful

chemicals will be released. Moreover, a heat treatment in principle needs to be performed only once, whereas chemical inhibitors need to be applied constantly. Despite the positive indication obtained from batch experiments (Chapter 4), results of our continuous reactor experiment did not confirm the effectivity of such a heat treatment, because the desired temperature was not reached. We still consider the heat treatment procedure for suppressing methanogenesis as quite promising for practice.

7.4.4 Sulfate reduction capacity of a CO fed gas lift reactor

Due to the strong competition between sulfate reducing bacteria and methanogens for the produced H_2, the sulfate reduction in the CO fed gas lift reactors remained rather low. In a methanol fed sulfate reducing bioreactor (at pH 7.0 and 55ºC), similar results were found for competition between sulfate reducing bacteria and methanogens, resulting in a methanogenic consumption of 85% of the H_2/CO_2 derived from methanol (Vallero et al., 2003a). Nevertheless, maximal sulfate reduction rates of 20 and 40 $mmol.L^{-1}.day^{-1}$ could be achieved at COD/SO_4^{2-} ratios of 10 and 5, respectively (Vallero et al., 2003a). In the CO fed gas lift reactors, the sulfate reduction/sulfide production rates were generally low, although in one case a maximal sulfate reduction rate of 20 $mmol.L^{-1}.day^{-1}$ was assessed, i.e. one day after an imposed pH shock in the second experiment (day 11). This highest sulfate reduction rate was measured in the absence of methanogenesis. This suggests that the sulfate reduction capacity of a CO fed gas lift reactor operated at 55ºC has potentials once methanogenesis is eliminated. In case of complete elimination of methanogenesis, the maximal assessed amount of CO converted to H_2, i.e. 380 $mmol.L^{-1}.day^{-1}$ (Fig. 7.8), would suffice to reduce over 90 $mmol.L^{-1}.day^{-1}$ of sulfate, a value even slightly higher than reported for thermophilic (55°C) hydrogenotrophic sulfate reduction in a gas lift reactor (Van Houten et al., 1997). Nevertheless, since *D. carboxydivorans*, which presumably is the most important CO converting sulfate reducer in the reactor sludge, was found to be rather sensitive to sulfide (Chapter 6), special measures might be required to maintain sufficient low sulfide concentrations. These measures may include capture of H_2S from the gas recycle or application of a low HRT. A sulfide concentration of 10 mM, which inhibits growth at a pH of about 7.2, would require a HRT as low as 2.7 hours considering a sulfate reduction rate of 90 $mmol.L^{-1}.day^{-1}$. Consequently, despite that CO conversion rates likely can be easily increased (Fig.

7.8) sulfate reduction rates presumably can not be increased much beyond this 90 mmol.L^{-1}.day^{-1}, unless the sulfide concentration can be maintained sufficiently low.

7.5 Conclusions

The results obtained in the present research demonstrate that:

1. Hydrogenogenic CO conversion in a gas lift reactor proceeds with a high efficiency at CO loads as high as 420 mmol.L^{-1}.day^{-1}, despite the presence of little biomass, which reveals a quite high CO conversion potential of moderately thermophilic CO converters present in anaerobic sludge.

2. Methanogens outcompete sulfate reducers for CO-derived H_2 in a gas lift reactor operated at 55ºC, pH 7.0 and HRT > 6 hours.

3. The higher H_2 consumption rates and the higher tolerance for easily controllable environmental conditions (pH, temperature and salinity) of the methanogens present compared to *D. carboxydivorans* combined with their high growth rates requires a total elimination of methanogens from the system (the seed sludge as well as the reactor) in case a sulfate reducing system is pursued.

4. In case methanogens can be completely eliminated the use of CO as electron donor in a sulfate reducing gas lift reactors seeded with Eerbeek sludge has potentials, since already within one day of operation a sulfate reduction capacity of 20 mmol.L^{-1}.day^{-1} can be obtained with only minor amounts of active biomass present.

8

Effect of hydraulic retention time on sulfate reduction in a carbon monoxide fed thermophilic (50-55°C) gas lift reactor

Abstract

Thermophilic sulfate reduction using CO as electron donor with anaerobic granular sludge, from which *D. carboxydivorans* was recently isolated, was investigated in a gas lift reactor at different hydraulic retention times (HRT). Operation at HRT > 9 hours resulted in predominant consumption of the CO-derived H_2 by methanogens (up to 90%) and thus in a poor sulfate reduction efficiency (<15%). At HRTs < 4 hours, the consumption of CO-derived H_2 was dominated by the sulfate reducing bacteria with maximally 95% of the produced H_2 used for sulfate reduction. However, methane production was not eliminated and by changing of the HRT back to > 5.5 hours resulted in a strong increase of the methanogenic activity. CO conversion to H_2/CO_2 with efficiencies up to 98% was achieved at a HRT of 4 hours, whereas at higher HRT the efficiency was nearly 100% most likely due to the increased biomass concentrations as judged from the increased OD_{600} values of the reactor liquid. Sulfate reduction rates of maximally 17 $mmol.L^{-1}.day^{-1}$ were achieved at a HRT of 3 hours, with over 87% of the H_2 produced used for sulfate reduction. The sulfate reduction rates were limited by the amount of CO supplied and the CO conversion efficiency (about 85%) at higher CO loads (106 $mmol.L^{-1}.day^{-1}$), probably as a result of limited biomass retention in the reactor.

8.1 Introduction

The recent finding that some moderately thermophilic sulfate reducing microorganisms show a much higher tolerance for carbon monoxide (CO) than previously thought (Parshina et al., 2005; Chapter 5) is interesting in view of a direct application of synthesis gas as electron donor for sulfate reduction. These organisms can utilize both H_2 and CO, the main components of synthesis gas, as electron donor for sulfate reduction. The presence of thermophilic hydrogenogenic CO converting sulfate reducers in unadapted anaerobic granular sludge (Chapter 4) is promising for the rapid start up of a thermophilic biotechnological flue gas desulfurization process using synthesis gas as electron donor. From an UASB reactor treating paper mill wastewater, *Desulfotomaculum carboxydivorans* was isolated (Chapter 5). This strain showed fast growth on CO at 55ºC with a generation time of 1.7 hours at CO levels exceeding 160 kPa (Chapter 6).

Van Houten et al. (1996) investigated the potential for synthesis gas utilization feeding H_2/CO_2 supplemented with small amounts of CO (up to 20%) to gas lift reactors at 30ºC. With 20% of CO in the feed gas, they achieved sulfate reduction rates as high as 100 $mmol.L^{-1}.day^{-1}$, but the utilization of CO for sulfate reduction in these experiments was not evident. Du Preez and Maree (1994) operated a fixed bed bioreactor at 35ºC fed with CO as sole electron donor and found a sulfate reduction rate of maximally 25 $mmol.L^{-1}.day^{-1}$. Thermophilic sulfate reduction (50-55ºC) with CO as electron donor was shown in a gas lift reactor inoculated with the granular sludge from which *D. carboxydivorans* was isolated (Chapter 7). However, sulfate reduction rates were low and only reasonable rates (up to 20 $mmol.L^{-1}.day^{-1}$) were obtained for a short-term (1-2 days) due to competition for the CO-derived H_2 between thermophilic sulfate reducers and methanogens. This competition resulted in predominant methanogenic H_2 consumption in the gas lift reactor operated at hydraulic retention times (HRTs) between 6 and 14 hours. Similar competition effects at 55ºC leading to methanogenic domination have been reported for methanol derived H_2 (Vallero et al., 2003a) and H_2/CO_2 (Van Houten et al., 1997). In another study performed at 65ºC, sulfate reducers were found to outcompete methanogens for methanol (Weijma et al., 2000). Attempts to operate the CO fed gas lift reactor at

elevated temperatures (up to 65ºC), resulted in decreased CO conversion capacity, whereas methanogens were not eliminated (Chapter 7).

The thermophilic methanogens enriched from the CO fed gas lift reactor operated in the study presented in Chapter 7 exhibited a fast growth rate (generation time 4.5 hours) and a 3 times higher H_2 consumption rate than the sulfate reducers. Comparing physiological characteristics of this methanogenic enrichment with the hydrogenogenic CO converting sulfate reducer, *D. carboxydivorans*, isolated from the inoculum sludge (Chapter 5), showed that although growth of *D. carboxydivorans* was faster (generation time of 1.7 hours; Chapter 6), the methanogens were more robust for environmental changes as pH, temperature and salt concentration. This indicates that these easily controllable environmental conditions can not be used to favor sulfate reduction in CO fed sulfate reducing reactors operated at elevated temperatures.

This chapter describes investigations dealing with the effect of operating a CO fed gas lift reactor inoculated with anaerobic granular paper mill wastewater treating sludge at low (< 4 hours) HRTs. The aim of this work was to assess whether sulfate reduction at low HRT using CO as electron donor is feasible and whether it could promote the development of a predominantly sulfidogenic biomass using granular seed sludge. In a previous study at HRTs between 6 and 14 hours (Chapter 7), sulfate reduction rates were found to be highly instable.

8.2 Materials and Methods

8.2.1 Experimental design

The reactor, set up as described in Chapter 7, was inoculated with a small amount (about 50 g wet weight) of paper mill wastewater treating UASB sludge (Industriewater Eerbeek, Eerbeek, the Netherlands). The nutrients were directly added to the dilution water in order to prevent nutrient limitation due to increased dilution water flow. The composition of the nutrients in the dilution water was (in mM): $NaH_2PO_4 \cdot 2H_2O$, 0.9; K_2HPO_4, 1.3; NH_4Cl, 8.6; NaCl, 7.9; $CaCl_2 \cdot 2H_2O$, 1.2; $MgCl_2 \cdot 6H_2O$, 0.8; Yeast 0.1 g.L^{-1} and was further supplemented with 1.5 mL.L^{-1} of an acid trace element solution and 1.5 mL.L^{-1} of an alkaline trace element solution

according to Stams et al. (1993). From the start until day 73, a small amount of acetate was supplied to the reactor feed as additional carbon source at an influent concentration between 1.5 and 2.4 mM. The sulfate was supplied separately to the reactor at flow rates of about 1.0 L.day^{-1} (day 0-21) and 1.3 L.day^{-1} (day 22-116). The sulfate concentration in this stock solution was 70 mM (day 0-26), 140 mM (day 27-73) and 175 mM (day 91-116).

In order to impose different hydraulic retention times (HRT), the flows of dilution water were varied (Fig. 1). Until day 21, the flow of the dilution water was between 7 and 9 L.day^{-1}, and from day 21 to 106 between 20 and 45 L.day^{-1}. From day 106 onwards, the flow of dilution water was decreased from 14 to 5 L.day^{-1}, whereas the influent flows were stopped, except for NaOH solution for pH control, from day 116 until the end of the experiment. The reactor was temporary stopped twice between day 47 – 55 and day 74 – 90 to evaluate the effect of electron flow upon resuming CO feeding at different HRT.

In order to prevent oxygen inlet via the exhaust gas line, resulting from the high suction of the gas compressor and consumption of the introduced CO, a small amount of N_2 gas was added to the reactor to assure a constant small overpressure of the system. The N_2 flow was 11 mmol.hour^{-1} during the first 35 days, after which it was maintained at 7 mmol.hour^{-1} until day 73. From day 91 until the end of the experiment the N_2 flow was 3 mmol.hour^{-1}.

8.2.2 Batch experiments

During the reactor experiment, batch experiments with 2 to 5 mL of reactor liquid were performed in 117 mL serum bottles sealed with butyl rubber stoppers. In these experiments CO conversion, H_2/CO_2 production and consumption, and utilization of acetate (with and without 8 mM of sulfate) were assessed. The basal medium used in these batch experiments contained (in mM): NH_4Cl 5.6, $CaCl_2.2H_2O$ 0.7, $MgCl_2.6H_2O$ 0.5, NaCl 5.1, $Na_2S.9H_2O$ 0.3, yeast extract 500 mg.l^{-1}, and 1 ml.l^{-1} of an acid and alkaline trace element solution according to Stams *et al.* (1993). When using acetate (6 mM) or 150 kPa CO as feed, the medium was buffered at pH 7.0 using 8.2 mM KH_2PO_4 and 11.4 mM $Na_2HPO_4.2H_2O$. With H_2/CO_2 (80/20 % at a total pressure of 150 kPa) as substrate, 23.8 mM $NaHCO_3$ (together with 30 kPa CO_2) was used to buffer the medium at pH 7.0 and the phosphate concentrations were reduced to 0.8

mM KH_2PO_4 and 1.1 mM $Na_2HPO_4.2H_2O$. To assess the contributions of specific trophic groups of microorganisms, batch experiments were performed with either 25 mM 2-bromoethanesulfonate or 0.07 mM vancomycine to suppress, respectively, the activity of methanogens and bacteria (Oremland and Capone, 1988). The incubations were performed in a temperature controlled (55°C) shaker incubator type RFI-125 (Infors AG, Basel, Switzerland) at 200 rpm. All batch experiments were performed in triplicate.

8.2.3 Analysis

The headspace gas composition was measured on a gas chromatograph HP 5890 (Hewlett Packard, Palo Alto, USA). Acetate and other volatile fatty acids (VFA) were analyzed on a HP 5890A gas chromatograph (Hewlett Packard, Palo Alto, USA) according to Weijma et al. (2000). Sulfide was measured photometrically according to Trüper and Schlegel (1964). Between day 35 and 46 of the reactor experiment, the sulfide was determined using sulfide analysis tubes from Dr. Lange (Hach Lange GmbH, Düsseldorf, Germany). Due to the limited measurement range (0-2 mg.L^{-1}), the samples had to be diluted prior to analysis in 50 mL 0.1 M zinc acetate. When the reagents for the sulfide analysis were prepared in our laboratory (used for all samples except between day 35 and 46), the samples were not diluted separately prior to the analysis. VSS was analyzed according to standard methods (APHA, 1995). Sulfate was measured on a DX-600 IC system (Dionex Corporation, Salt Lake City, USA) as described in Chapter 4. Biomass production was monitored by measuring the optical density at 600 nm (OD_{600}) using a Spectronic 601 spectrophotometer (Milton Roy, Ivyland, USA) and Plastibrand 1.5 mL PMMA cuvettes (Brand GmbH, Wertheim, Germany).

Solubilities of CO, CO_2 and CH_4 were calculated using data from Lide (2001), solubility of H_2 was calculated according to Perry et al. (1997), and the amounts produced or consumed were calculated by taking into account both gas and liquid phases. All chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany). The CO (purity 99.9%) used for feeding the gas lift reactor was obtained from Praxair (Indugas BV, Vlaardingen, the Netherlands), and the CO (purity 99.997%) used for batch experiments was supplied by Hoek Loos (Rotterdam,

the Netherlands). N_2 (purity 99.999%) and H_2/CO_2 (80%:20%) used in batch experiments were purchased from Indugas (Vlaardingen, the Netherlands).

8.3 Results

Fig. 8.1 shows the operation conditions and influent sulfate concentrations applied during the operation of the gas lift reactor as well as the measured OD_{600} of the reactor liquid. During the first 21 days, the temperature was maintained at 54ºC, as measured at the top of the reactor, but afterwards due to increased flow rates the temperature decreased slightly to about 52ºC (Fig. 8.1A). At the start of the reactor experiment the pH was about 7.4, although the set point of the pH controller was set at 6.9; with the production of CO_2 the pH rapidly decreased to the set point value. From day 10 onwards, the pH was kept constant at 6.9.

Fig. 8.1B shows the HRT applied during reactor operation as well as the measured values for the OD_{600}. During the first 20 days, no useful OD measurements could be assessed due to high amounts of suspended solids present in the liquid effluent. At day 21, the HRT was decreased from about 10 hours to 3 hours and this resulted in an enhanced washout of biomass as shown by the OD_{600} values (Fig. 8.1B). Due to loss of pumping capacity, the HRT slowly increased to 4.3 hours, and after replacement of the tubes at day 31 the HRT was about 3.5 hours. At day 57 the reactor was restarted at a HRT of 5.4 hours, which was reduced to less than 4 hours within a day, and at day 67 further to only 2.8 – 3.0 hours. At day 73 the operation of the system was interrupted for a period of 18 days. At day 91 the feeding of the reactor was resumed at a HRT of 2.9 hours, which was further decreased at day 102 to 1.9-2.4 hours. From day 106 onwards, we decided to increase the HRT stepwise to 16 hours at day 114 to assess the effect of HRT on the biomass concentration and CO conversion efficiency. The impact of the increased HRT was positive on the retained amount of biomass as indicated by the OD_{600} values (Fig. 8.1B).

Due to the variations in HRT and sulfate concentration of the stock solution, the sulfate concentration and sulfate loading rates to the reactor varied during the experiment (Fig. 8.1C).
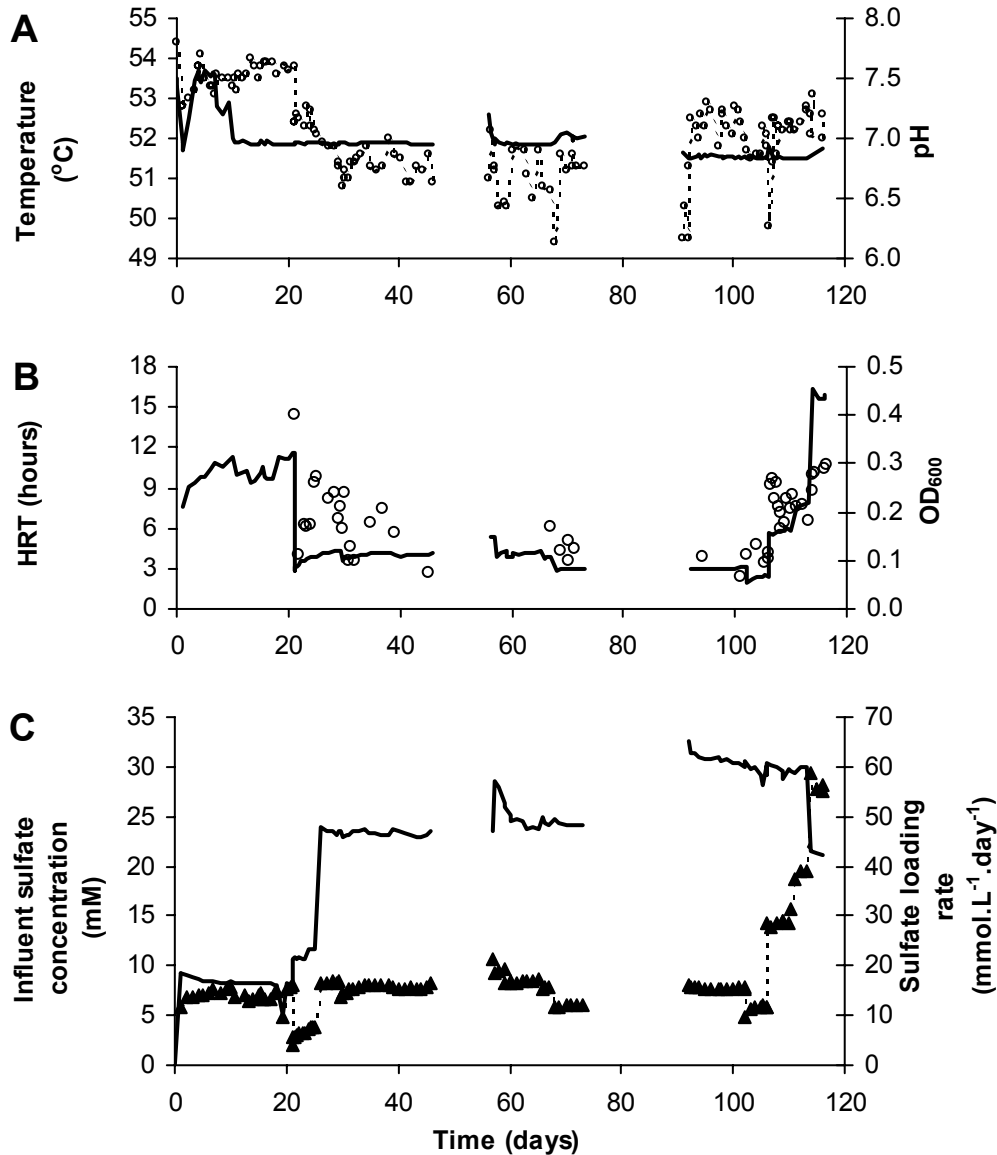
Fig. 8.1   Operational conditions and OD_{600} during the reactor run. (A) The measured pH (line) and temperature (○), (B), the applied HRT (line) and OD_{600} of the reactor liquid (○) and (C) the influent sulfate concentrations (▲) and sulfate loading rates (line).

Fig. 8.2 presents the imposed CO loading rates to the reactor together with the assessed CO conversion efficiency. Already within 4 days CO conversion efficiencies exceeded 87%. The drastic decrease of the HRT at day 21 resulted in short-term accumulation of CO in the exhaust gas, but within three days an efficiency of 87% was again achieved. The efficiency at a low HRT was somewhat lower compared to the period just before day 21, which is presumably due to the limited amount of biomass present at the low HRT (Fig. 8.1B). Nevertheless, between day 102 and 106

at a HRT of 2.0 - 2.4 hours, the CO conversion efficiency exceeded 93% at a CO load of 77 $mmol.L^{-1}.day^{-1}$ (Fig. 8.1B and Fig. 8.2).

Increasing the HRT from day 106 onwards resulted in CO conversion efficiencies exceeding 98%. Thus CO conversion proceeds well at a HRT as low as 2 hours, although the efficiency is slightly lower at such a low HRT. Moreover, the effect of increasing the CO load from 60 to 105 $mmol.L^{-1}.day^{-1}$ at day 68 also resulted in a considerable drop in the CO conversion efficiency from 98 to 86% (Fig. 8.2), but the total amount of CO converted increased, i.e. from 50 to over 90 $mmol.L^{-1}.day^{-1}$. The CO conversion stabilized at about 90 $mmol.L^{-1}.day^{-1}$, which might indicate biomass limitation.
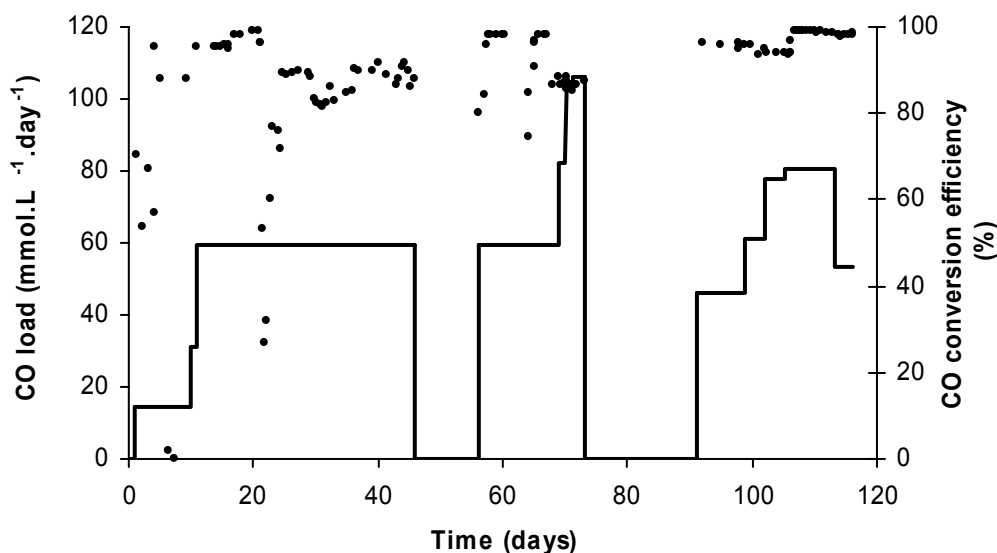
Fig. 8.2   The CO loading rate applied to the reactor (—) and the CO conversion efficiency (•). Note that the reactor was not operated between days 47 – 55 and 74 – 90.

Fig. 8.3 presents the calculated amounts of the gaseous products from CO conversion, i.e. hydrogen and methane. H_2 could only be incidentally detected in high amounts in the exhaust gas, especially at the start and after resuming the CO feed. Methane production rapidly increased after day 11 and between day 11 and 21 the methane production rate amounted to about 15 $mmol.L^{-1}.day^{-1}$. The CO conversion results in production of equimolar amounts of H_2 and CO_2, and methane is presumed to be formed from H_2/CO_2, which requires four moles of H_2 to form one mole of CH_4. Thus, at a CO load of 60 $mmol.L^{-1}.day^{-1}$, maximally 15 $mmol.L^{-1}.day^{-1}$ of methane

could be formed. This indicates that nearly all CO derived reducing equivalents are used for methane production.
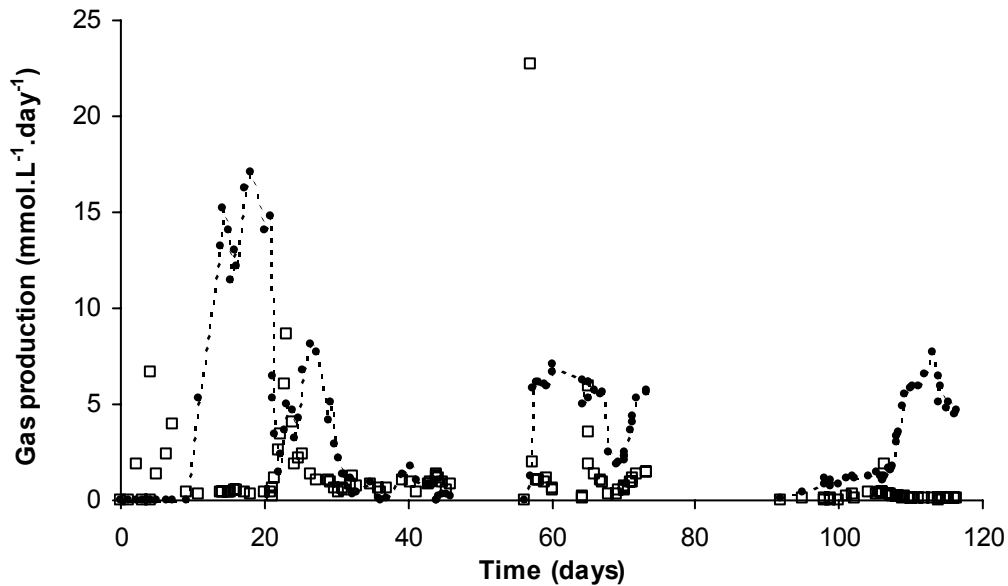
Fig. 8.3   The production of methane (•) and hydrogen (□) as calculated from the exhaust gas composition and the gas flow during flow-through operation of the reactor.

As a result of the drastically decreased HRT at day 21, the CO conversion efficiency decreased for a short period (Fig. 8.2). The rapid recovery of CO conversion coincided with an increased amount of H_2 in the exhaust gas, but methane production already resumed at a maximal production rate of 8 mmol.L^{-1}.day^{-1} at day 26.

Fig. 8.4 presents the sulfide concentration measured in the liquid effluent together with the amount of sulfate removed, i.e. the difference between measured influent and effluent concentrations. The sulfate reduction rates presented in Fig. 8.4B were obtained from the differences in sulfate concentration between the influent and effluent liquid streams. The sulfur balance presented in Fig. 8.4C shows that recovery of HS^- in the liquid effluent generally is high, indicating that just little sulfide is stripped with the gas phase. The H_2S stripping effect apparently does not represent a significant loss of sulfide, which can be attributed to the rather low sulfide concentrations, the low exhaust gas flows, the neutral pH (6.9-7.0) resulting in a limited undissociated H_2S fraction (< 38%), and the relative high solubility of H_2S (Henry's law constant for H_2S at 55ºC is 0.054158 M.atm^{-1}; as calculated from Lide, 2001). Only between day 35 and 46, the sulfide concentrations are significantly lower compared to the calculated amount of sulfate reduced. This discrepancy most

probably can be attributed to the different analytical methods used for sulfide determination; especially the dilution from the methylene blue formation reaction likely resulted in loss of sulfide. The sulfide concentrations were rather low throughout the experiment, with a maximal sulfide concentration of 3 mM measured at day 106.

At day 26, the sulfate loading rate was increased (Fig. 8.1) and methane production rates gradually decreased to values below 1 mmol.L^{-1}.day^{-1} at a HRT of about 4 hours (Fig. 8.3). The sulfide concentration in the effluent increased to about 2 mM (Fig. 8.4A) and the maximal sulfate reduction rate obtained was 17 mmol.L^{-1}.day^{-1}. Apparently at low HRT, sulfate reducers are capable of outcompeting methanogens.

After the first unfed period (day 46-56), methane production occurred at a rate of about 6 mmol.L^{-1}.day^{-1}. The reactor seemed quite sensitive, as can be seen from the sudden drop in CO conversion at day 64, which was followed by an increase of the H$_2$ content in the exhaust gas at day 65 (Fig. 8.3). Initially the methane production seemed to remain unaffected but at day 67 it dropped suddenly (Fig. 8.3), followed by a significant increase in sulfate reduction rate (Fig. 8.4). This might be attributed to the low amount of biomass present and the presumed faster wash out of methanogens (HRT < generation time of the methanogenic population).
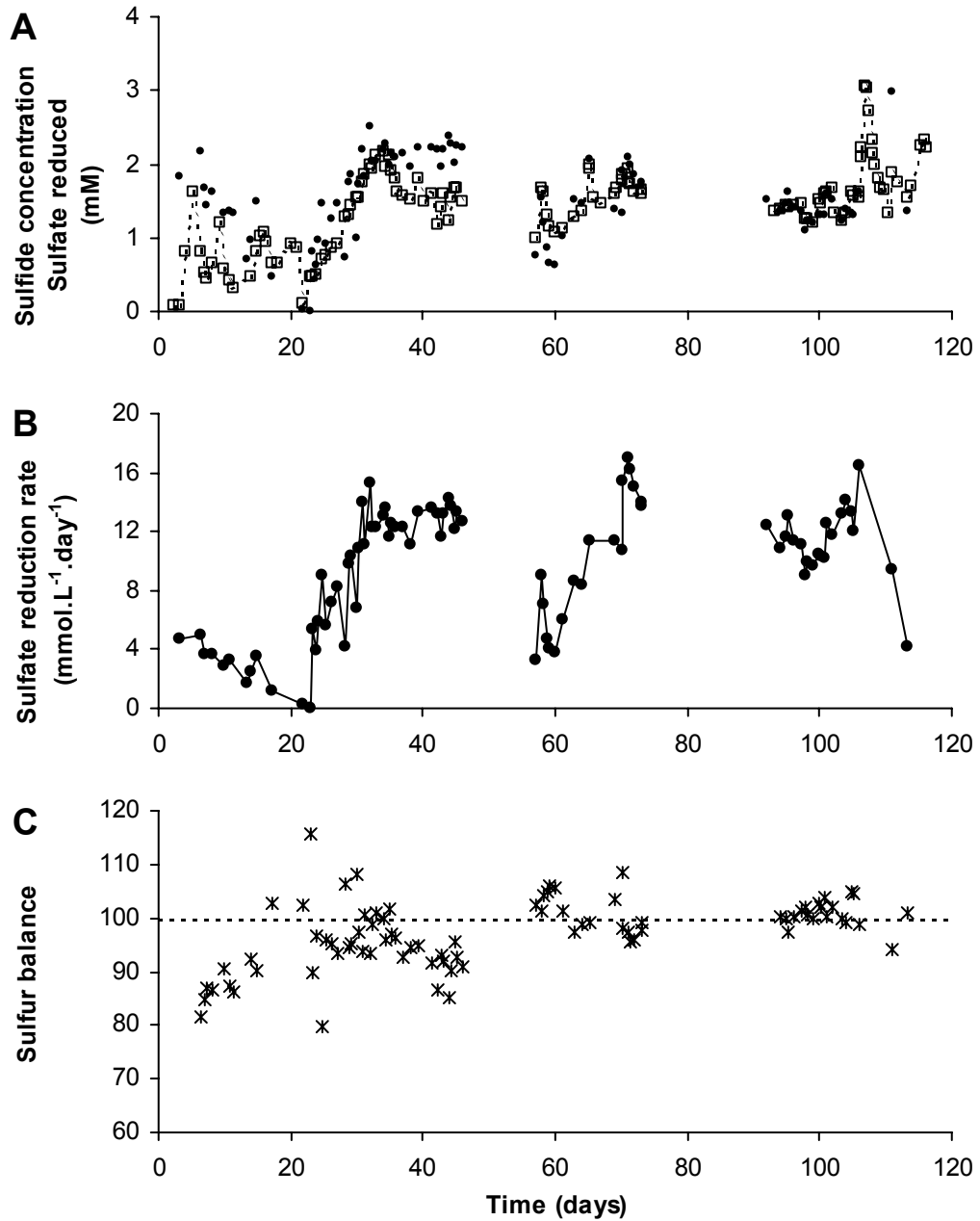
Fig. 8.4 Sulfate reduction during continuous operation of the reactor. (A) effluent sulfide concentration (□) and difference between influent and effluent sulfate concentrations (•), (B) sulfate reduction rate calculated from the sulfate concentrations and (C) the overall sulfur balance between influent and effluent sulfate concentrations and effluent sulfide concentrations.

A short-term slightly elevated HRT of 5.4 hours after resuming the CO feeding to the reactor (at day 56) promoted methane production, whereas resuming the CO feeding at a HRT of 3 hours (at day 91) promoted sulfate reduction (Fig. 8.3). Nevertheless, even at a HRT below 3 hours, methane production increased slowly to about 1.5 mmol.L^{-1}.day^{-1}, which likely is the result of the increased CO load to the reactor between day 98 and 106 (Fig. 8.2). The increases of the HRT from day 106 onwards resulted in rapidly increased methane production rates.

Up to day 73, a small amount of acetate was supplied to the influent with the objective to assess its potential for stimulating biomass growth, by providing a readily available carbon source. Fig. 8.5 shows that at high HRT-values a net production of acetate occurred, but that at low HRT there was a net consumption of acetate, despite the fact that batch experiments conducted with biomass from the reactor revealed that homoacetogens were present. No stimulation of acetate for biomass production was found as the measured OD$_{600}$ at a HRT $<$ 3 hours is similar whether acetate was supplied to the feed or not (Fig. 8.1B and Fig. 8.5). Furthermore, acetate addition did not seem to affect the CO conversion efficiency. Metabolic acetate consumption was tested in batch assays both in the absence and presence of sulfate with biomass from the gas lift reactor, but acetate consumption was not observed.
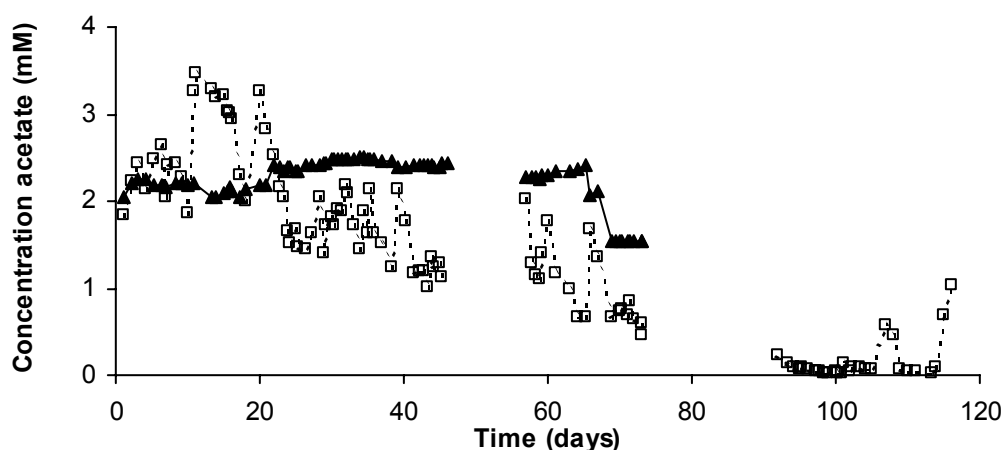

Fig. 8.5   Influent and effluent acetate concentrations during flow-through operation of the gas lift reactor. Symbols present the influent (▲) and effluent (□) concentration.
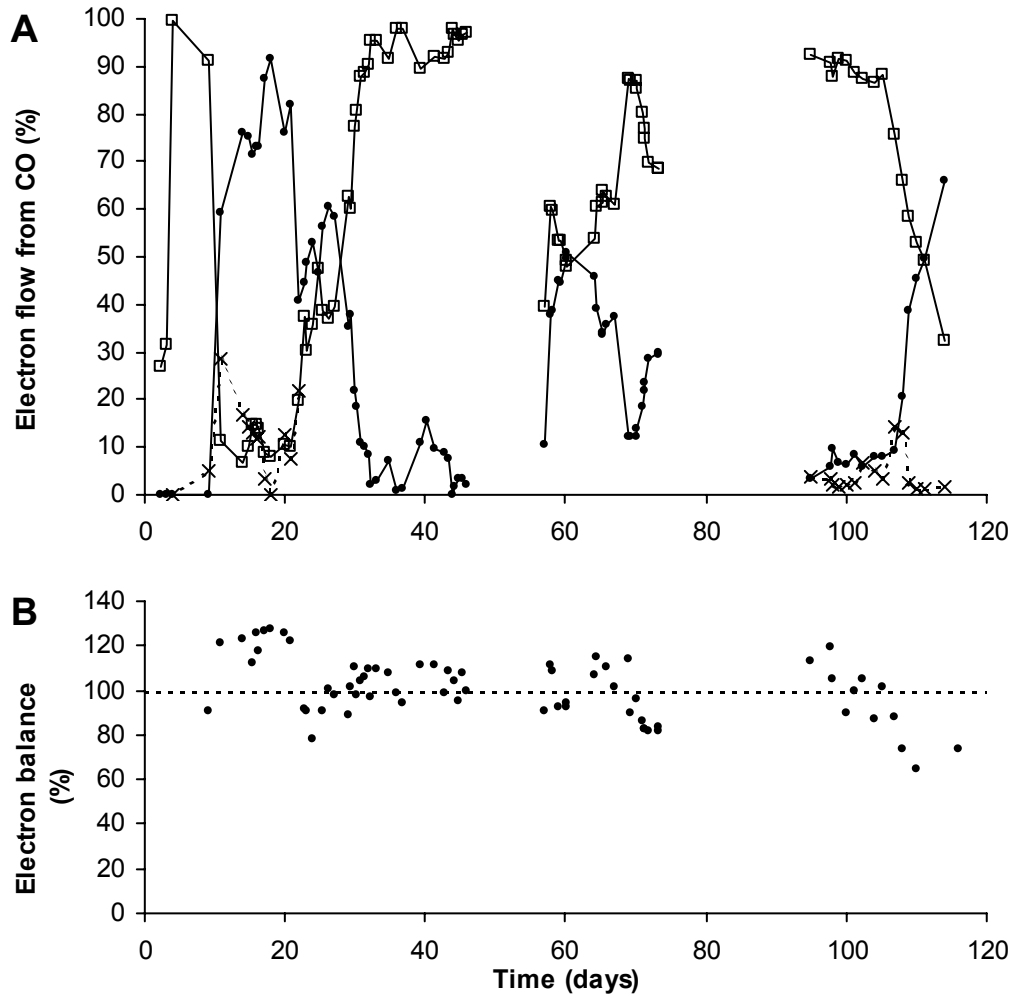
Fig. 8.6 Performance of the CO and sulfate fed reactor during flow through operation. (A) The electron flow from CO to methane production (•), sulfate reduction (□) and acetate production (×) and (B) the electron balance.

Fig. 8.6 shows the electron flow from CO towards production of methane, sulfate reduction and acetate production. A net acetate production was only found during the first 21 days and between days 91 and 116 (Fig. 8.5). Therefore, an electron flow towards acetate production could only be calculated for these periods (Fig. 8.6). The electron flow towards H_2 production is not shown in Fig. 8.6, as only incidentally significant amounts of H_2 were observed in the exhaust gas, i.e. during the initiation of CO conversion (see for example day 57 in Fig. 8.3). In general, the electron flow of CO towards unconsumed H_2 was below 2% of the total electron flow. Fig. 8.6 further presents the electron balance, viz. the recovery in products relative to the amount of CO converted. The electron balance shows that the recovery of CO in its products fits quite well.

Furthermore, the results in Fig. 8.6 show that after resuming CO feeding the imposed HRT affects the electron flow. Resuming CO feeding at a short-term slightly elevated HRT (1 day at a HRT of 5.4 hours at day 56; Fig. 8.1B) results in an increased electron flow towards methanogenesis (up to 50%), despite the fact that upon stopping the CO feeding at day 46, the electron flow was directed for 95% towards sulfate reduction. In contrast, resuming the CO feeding at a HRT of 3 hours (day 91; Fig. 8.1B) results in an electron flow towards sulfate reduction, which even exceeded 90%, but it did not result in decreased CO conversion rates (Fig. 8.2). Therefore, in order to promote the development of a sulfate reducing biomass, the start up at a HRT of 3 hours is preferred over short-term elevated HRT to increase the biomass concentration.

The low amount of H_2 found in the exhaust gas shows that the produced H_2 is effectively consumed. Results of batch experiments with biomass from the gas lift reactor, sampled at day 33, 42 and 45, showed that CO was converted to H_2 and that direct CO consumption by methanogens did not occur in vancomycine inhibited incubations (within 20 days; data not shown). This suggests that methanogens were inable to grow on CO. Nevertheless, in the presence of H_2/CO_2 (ratio 80/20 at 150 kPa) and small amounts of CO (5-20 kPa) in vancomycine inhibited assays, the CO was slowly (20 kPa in 20 days) consumed after depletion of H_2/CO_2. Perhaps these methanogens are capable to grow very slowly on CO or they can only use it as energy source. Nevertheless, some CO consumption by the methanogens may explain the slightly higher CO conversion efficiency when methanogenesis prevailed in the reactor.

From day 116 to 123, the reactor liquid feed to the reactor was interrupted, except for the NaOH solution for pH control. The drastically increased HRT enabled us to assess the maximal sulfide accumulation and its possible effect on CO conversion. The amount of NaOH consumed at a CO loading rate of 53 $mmol.L^{-1}.day^{-1}$ was about 1 $L.day^{-1}$, resulting in a HRT of about 4 days. The OD_{600} in the reactor increased rapidly to values up to 1.5 and the CO conversion efficiency exceeded 98%. The amount of sulfate present in the reactor when the feeding was stopped amounted to about 22 mM. Fig. 8.7 presents the sulfide and acetate concentrations in the reactor liquid as well as the methane production rates from day 116 until the end of the reactor experiment.
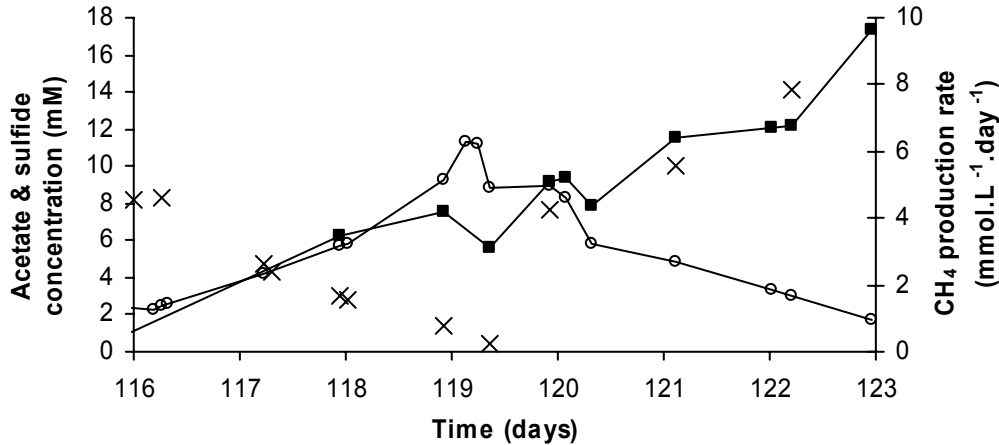
Fig. 8.7 Sulfide (○) and acetate (■) concentrations and methane production rates (×) in the gas lift reactor when no liquid influent was supplied to the reactor, except for NaOH for neutralization of the pH.

Despite the fact that the electron flow towards methane production increased in the last days of the operation (Fig. 8.6A), operation of the reactor at a drastically increased HRT (from 15 hours to 4 days) resulted in declining methane production. These reduced methane production rates coincided with an accumulation of sulfide up to 11 mM in the reactor liquid. Such high sulfide concentrations did not seem to affect the CO conversion efficiency as this remained unchanged at values exceeding 98%. After day 119, the sulfide concentration started to decrease slowly as a result of sulfate depletion in the reactor liquid and gradually led to increased methane production rates. The sulfide concentration gradually decreased due to dilution with NaOH solution for pH control as well as enhanced H_2S stripping by the produced methane.

8.4 Discussion

8.4.1 Operation at low HRT to promote sulfate reduction in a CO-fed gas lift reactor

Previous research demonstrated that operational conditions such as pH, temperature and salt concentration were unsuccessful as tools to promote sulfate reduction in a CO-fed gas lift reactor (Chapter 7). The maximal sulfate reduction rate of 20 mmol.L^{-1}.day^{-1} could not be maintained for prolonged periods due to strong competition for

the CO-derived H_2 between sulfate reducers and methanogens (Chapter 7). This study demonstrates that at a relatively high HRT ($\geq$ 9 hours), the produced H_2 is predominantly used by methanogens (up to 90%) and only up to 15% for sulfate reduction, resulting in sulfate reduction rates of less than 4 mmol.L^{-1}.d^{-1}, which even decreased in time (Fig. 8.4B). Operation of the CO-fed sulfate reducing gas lift reactor at a HRT below 4 hours resulted in increased H_2 consumption by the sulfate reducing bacteria, i.e. maximally 95% of the produced H_2 was used for sulfate reduction (Fig. 8.6). Compared to the incidentally reasonable sulfate reduction rates achieved in Chapter 7, the sulfate reduction at low HRT looks more stable for prolonged periods (Fig. 8.4B). However, operation of the reactor at low HRT drastically decreased the biomass concentration, because in these systems of gas lift reactors, biomass retention on the basis of biofilm growth on the reactor wall presumably plays only a marginal role; upon decreasing the HRT on day 21, the OD_{600} values dropped and also CO conversion, sulfate reduction and methane production were nearly absent (Figs. 8.2, 8.3 and 8.4). The CO conversion efficiencies found at a HRT of 4 hours (maximally 98%) are similar to those found at a HRT of about 10 hours (maximally 99%), although the effect of a limited amount of biomass resulted in decreased CO conversion efficiencies at increased CO loads, i.e. maximally 88% conversion at a CO load of 105 mmol.L^{-1}.day^{-1} (day 73; Fig. 8.2).

8.4.2 Biomass retention

The performance capacity of a CO-fed sulfate reducing gas lift reactor at HRT < 4 hours, obviously is limited by the low biomass concentration in the mixed liquor and the fact that hardly any sludge immobilization occurs in conventional types of reactor systems. The biomass retention could be enhanced by adding a proper carrier material to the reactor, e.g. pumice particles, because these were successfully used in experiments with a H_2/CO_2 fed sulfate reducing gas lift reactor at 30ºC (Van Houten et al., 1995). However, in another study they found that addition of 5 to 20 kPa CO detrimentally affected the aggregates, because they became smooth and large in the presence of CO (Van Houten et al., 1996). Upon addition of 5 kPa CO, the overall sulfate reduction rate dropped from maximally 145 mmol.L^{-1}.day^{-1} to less than 80 mmol.L^{-1}.day^{-1}. Increased rates with application of external biomass recycling

demonstrated that biomass retention was the rate limiting step (Van Houten et al., 1996). Moreover, it should be understood that improved biomass retention likely will result in a predominance of methanogens in the bioreactor, similar as was the case in the operation of the bioreactor at HRTs > 6 hours (Fig. 8.1 and 8.6; Chapter 7). Clearly, the operation at low HRT did not eliminate methanogenesis. A system based on improved biomass retention by using carrier material may work when methanogens can be completely eliminated from the seed sludge prior to the start of the reactor operation, e.g. by heat treatment of the sludge (Oh et al., 2003; Chapter 4). Nevertheless, reactor experiments showed that incomplete heat treatment results in rapid recovery of thermophilic methanogens due to their high growth rates (Chapter 7).

In view of the need for biomass retention, the use of a submerged anaerobic membrane bioreactor could represent an interesting option for the use of specific microorganisms (Vallero et al., 2005). Such a reactor configuration was successfully used in a study of biological sulfate reduction at 33 ($\pm$ 1) ºC and high salinity, i.e. 0.9 M NaCl (electrical conductivity of 60-70 mS.cm^{-1}). Vallero et al. (2005) used a pure culture of *Desulfobacter halotolerans* (Brandt and Ingvorsen, 1997), which reduces sulfate with acetate as electron donor at high salinity. Attempts to bioaugment this bacterium in anaerobic granular sludge were unsuccessful (Vallero et al., 2004), but operation of a submerged anaerobic membrane bioreactor inoculated with this bacterium were quite promising, because sulfate reduction rates of 68 mmol.L^{-1}.day^{-1} were achieved. Assuming a protein content of roughly 50%, this corresponds to a specific sulfate reduction rate of 0.11 mol.(g protein)$^{-1}$.day^{-1}. In their reactor *D. halotolerans* had formed small flocs, which is likely to have decreased the overall specific sulfate reduction rates due to mass transfer limitations. Their specific sulfate reduction rate was considerably higher than the rates reported in bioreactors inoculated with mixed biomass sources (Vallero et al., 2005). Comparison of specific rates with suspended pure cultures and biomass in thick biofilms or granules is nevertheless somewhat disputable. In the latter case, only a small fraction of the biomass can be maximally active, whereas most of the biomass is substrate limited or inactive.

When we assume that *D. carboxydivorans* represented most of the biomass in our reactor, as the electron flow from CO is for 80% directed to sulfate reduction (Fig.

8.6), the estimated specific sulfate reduction rates were between 0.7 and 1.0 mol.(g protein)$^{-1}$.day^{-1} (using the equation for biomass given in Chapter 6). In these calculations, any biomass retention by attachment on the reactor walls was not taken into account, which may significantly reduce these specific sulfate reduction rates. Nevertheless, the sulfate reduction rate for *D. carboxydivorans* as determined in Chapter 6 amounted to almost 0.8 mol.(g protein)$^{-1}$.day^{-1}, suggesting that such high specific rates with this organism seem possible.

The generation time of *Desulfobacter halotolerans* at 0.9 M NaCl is about 40 hours at 30ºC (Brandt and Ingvorsen, 1997). In comparison, the generation time of *D. carboxydivorans* on CO at 55ºC is 1.7 hours (Chapter 6), suggesting that the start up of a thermophilic CO fed sulfate reducing submerged membrane bioreactor will likely be considerably faster. Furthermore, the successful application of N$_2$ sparging to minimize membrane fouling (Vallero et al., 2005) would not be required when operating a gas lift reactor fed with gaseous substrates. Therefore, the application of biomass retention membranes in a CO or synthesis gas fed sulfate reducing bioreactor has a high potential, as it enables the use of a pure culture of *D. carboxydivorans*.

8.4.3 Prospects for synthesis gas utilization in thermophilic desulfurization processes

For a successful utilization of synthesis gas for biological sulfate reduction, the utilization of both H$_2$ and CO is important. In general, the toxicity of CO for sulfate reducing bacteria is reported to be high (Mörsdorf et al., 1992; Davidova et al., 1994), although recently more CO tolerant organisms were found (Parshina et al., 2005). Nevertheless, the CO concentrations in the bioreactor will be rather low, as a result of CO conversion and synthesis gas composition (Perry et al., 1997), which will reduce its toxicity. However, successful application of synthesis gas implies the utilization of CO derived electrons for sulfate reduction and thus CO needs to be used as substrate by the sulfate reducers present, e.g. *Desulfotomaculum kuznetsovii* or *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* (Parshina et al., 2005) or CO needs to be converted to a useful substrate for the sulfate reducers present. In the latter case, the conversion of CO to H$_2$ would be the most feasible, and several bacteria have been isolated capable of such hydrogenogenic CO conversion

(Svetlichnyi et al, 1991b; 1994; Sokolova et al., 2001; 2002; 2004a,b; Chapter 5). Especially, *D. carboxydivorans* would be an interesting candidate, as this organism can convert both the CO to H_2 and subsequently use the H_2 for sulfate reduction (Chapter 5). However, *D. carboxydivorans* is rather sensitive for sulfide (Chapter 6), which has been reported for several other *Desulfotomaculum* species (Widdel and Hansen, 1991). The sulfide tolerance of *D. carboxydivorans* depends strongly on the pH as indicated by a maximal sulfide accumulation in batch experiments of about 5 mM at pH 6.5 and about 10 mM at pH >7.2 (Chapter 6). Apparently for the utilization of synthesis gas not the CO concentrations, but rather the sulfide concentrations determines the toxicity. Thus, additional measures to maintain the sulfide concentrations sufficient low might be required. Operation of a sulfate fed gas lift reactor utilizing CO as electron donor at a HRT smaller than 4 hours could promote sulfate reduction and would further minimize sulfide accumulation. In this respect the near complete CO conversion in the reactor attained at sulfide concentrations as high as 11.3 mM is promising for sulfate reduction using synthesis gas (Fig. 8.7).

8.5 Conclusions

The results presented in this chapter demonstrate that:

1. In a CO fed sulfate reducing gas lift reactor operated at 55ºC, stable sulfate reduction rates (up to 17 mmol.L^{-1}.day^{-1}) could be attained at HRT < 4 hours, with over 87% of the CO-derived H_2 used by the sulfidogens. In contrast, at an HRT of 9 hours more than 90% of the electrons from CO lead to methane formation.

2. Acetate addition did not promote biomass production, CO conversion or electron flow towards sulfate reduction.

3. The very high specific CO conversion rates (> 5.6 mol.(g protein)$^{-1}$.day^{-1}) and sulfate reduction rates (> 0.75 mol.(g protein)$^{-1}$.day^{-1}) suggest that the bacteria present have a high potential to be used in a high rate sulfate reduction process at 55ºC , when biomass retention of the desired sulfate reducing bacteria is applied.

4. The observed maximal sulfide accumulation of 11 mM at pH 6.9 at a CO conversion efficiency exceeding 98% seems very promising for practical

application of a high rate sulfate reduction process at elevated temperatures (50-55ºC). Especially, as the maximal tolerable sulfide concentration rather than the CO concentration is presumed to determine the sulfate reduction capacity of a CO fed bioreactor.

9

General discussion and summary

9.1 Introduction

The main objective of the research presented in this thesis was to assess the potentials of biological hydrogenogenic CO conversion, i.e. the biological conversion of CO with H_2O to H_2 and CO_2. A biological CO conversion process is interesting for the purification and utilization of synthesis gas, which contains mainly H_2, CO and CO_2. Bulk production of H_2 relies on the gasification of organic matter which results in the production of synthesis gas. In order to produce a high purity H_2 gas from synthesis gas a further purification is required. Biological hydrogenogenic CO conversion represents a potential alternative for the currently employed chemical catalytic water gas shift reaction which is conducted at high temperatures using chemical catalysts. A large scale cost effective H_2 production is desired not merely for its use as a future energy carrier, but also because it is an important chemical reactant in reductive processes in both chemical industry as well as in specific wastewater treatment processes. For the latter purpose, especially the use of H_2 as electron donor in biological sulfate reduction of sulfate/sulfite rich wastewaters which are low in organic matter content has been shown successful both at moderate and elevated temperatures (Van Houten et al., 1994; 1997). In contrast to the production of valuable chemicals, wastewater treatment generally does not result in the production of valuable compounds, which implies that the used electron donor should be cheap. Synthesis gas as electron donor has been proposed as a cheap alternative for H_2 in biotechnological sulfate reduction processes (Van Houten and Lettinga, 1996;

DuPreez and Maree, 1994; DuPreez et al., 1992). The combination of hydrogenogenic CO conversion and hydrogenotrophic sulfate reduction enables the use of synthesis gas as electron donor without the need for a prior H_2 purification step and maximizes the utilization of synthesis gas components as electron donor. Therefore hydrogenogenic CO conversions may find two major applications in biotechnology, i.e. a biological H_2 purification process and a direct utilization of synthesis gas in biotechnological desulfurization. This thesis focuses on both these biotechnological processes in order to evaluate their suitability.

This chapter summarizes and discusses the results on the studies concerning the occurrence of hydrogenogenic CO conversion, its microbiological aspects and practical aspects of biotechnological H_2 purification, as well as synthesis gas utilization in biodesulfurization.

9.2 Occurrence of hydrogenogenic CO-converting bacteria in sludges from anaerobic wastewater treatment plants

Chapter 3 reports on the results of a screening study of hydrogenogenic CO converting activity, i.e. hydrogenogenesis (Svetlichnyi et al., 2001), in six readily available anaerobic bioreactor sludges, originating from full-scale bioreactors operated at 30-35°C and treating different types of wastewater. One sludge was obtained from a laboratory scale methanol fed thermophilic sulfate reducing UASB reactor. All full-scale sludge samples incubated at 30°C converted CO to methane and/or acetate. Inhibition experiments, using 2-bromoethanesulfonate (BES) and vancomycine, showed that CO conversion to methane at 30°C occurred via acetate, not via H_2. Direct methane formation from CO was only observed with two sludge samples. Results of incubations supplemented with vancomycine in order to inhibit homoacetogens (Oremland and Capone, 1988) showed that the direct conversion of CO to methane proceeded extremely slow (Table 3.2; Chapter 3) and thus we concluded that this route was of minor importance for the overall CO conversion. The occurrence of acetogenic CO conversion at 30°C is not surprising regarding the reported number of mesophilic homoacetogens capable of CO metabolism (Table 2.2; Chapter 2). Hydrogen production, on the other hand, has only been described sofar for a few mesophilic bacteria, i.e. some phototrophic organisms (Uffen, 1976;

Dashekvicz and Uffen, 1979; Jung et al., 1999a ; Kerby et al., 1995) and a facultative anaerobic bacterium as well (Jung et al., 1999b). However, all these organisms have in common that their growth under anaerobic conditions in the dark is rather slow.

Surprisingly the incubations at 55°C revealed a rather smooth evolution of H_2 upon CO conversion with five of the seven sludge samples tested. In case methane was generated in CO supplemented incubations, results of inhibition experiments revealed that CO was converted to H_2, but that the direct methane formation from CO did not occur in any incubation conducted at an initial partial CO pressure (P_{CO}) exceeding 160 kPa (Chapter 3). Despite four of the H_2 producing sludges had been cultivated at 30 to 35ºC, the lag phase for hydrogenogenic CO conversion at 55ºC was very short. This suggests short generation times of the responsible microorganisms. The results of Chapter 3 reveal that hydrogenogenic CO conversion at high P_{CO} is a rather common phenomenon, despite all anaerobic thermophilic hydrogenogenic microorganisms isolated so far originated from locations with volcanic activity (Table 2.3; Chapter 2). According to Symonds et al. (1994) such locations contain up to 1.5%, of CO. On the other hand the key enzymes required for hydrogenogenic CO conversion, i.e. carbon monoxide dehydrogenase (CODH) and hydrogenases, are widespread in anaerobic microorganisms (Chapter 2). CODH not merely functions in the CO oxidation, but it is also one of the key enzymes in the synthesis or cleavage of acetyl-CoA (Ferry, 1995).

9.3 Effect of P_{CO} and H_2 on CO conversion routes at 55ºC

All experiments presented in Chapter 3 were performed at P_{CO} levels exceeding 160 kPa. This high partial pressure may affect the conversion of CO, because it is known that CO acts as an inhibitor of some types of hydrogenases (Davidova et al., 1994; Kim et al. 1984; Lupton et al., 1984; Daniels et al., 1977) and some sulfate reducing bacteria growing on organic substrates as well (Davidova et al., 1994). Chapter 4 reports on the results of investigations of CO conversion routes at 55ºC as a function of initial P_{CO} and the presence of H_2. From the hydrogenogenic CO converting sludge samples, two were selected for a more detailed study, viz. Eerbeek (paper mill) and Nedalco (distillery) sludge. The main difference between these sludge samples was the absence of methane formation with Nedalco sludge under an atmosphere of 150 kPa CO. The investigation showed that the CO conversion routes were not dependent

on the supplied amount of CO, although at P_{CO} values up to 50 kPa some direct conversion of CO to methane occurred in the presence of vancomycine with Eerbeek sludge (Table 4.3; Chapter 4). Nevertheless, the rate of this methanogenesis from CO was so low that its contribution to the overall CO conversion pathway at 55°C (Fig. 1) could be considered insignificant. The absence of direct CO conversion at high P_{CO} (>150 kPa) is attributed to a toxic effect of CO for these methanogens, which most likely also prevented the production of methane in Nedalco sludge. Nedalco sludge rapidly converted H_2/CO_2 into methane, but traces of CO, left after CO conversion was completed, were apparently sufficient to inhibit methanogenesis completely. The possible route of direct methane production from the reduction of CO with H_2 (question mark in Fig. 9.1), could not be distinguished from a direct methanogenic CO conversion route from CO alone, but regarding the very similar rates the presence of H_2 does not stimulate methane production. The main reactions involved in CO conversion are shown in Fig. 9.1 and reactions are tabulated in Table 9.1.
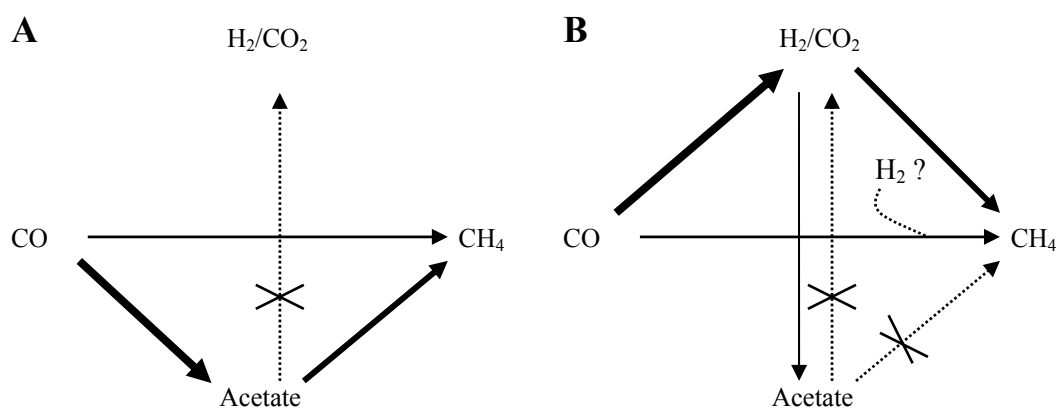
Fig. 9.1   Schematic presentation of the main CO conversion routes at 30 (A) and 55°C (B) as observed with several anaerobic full-scale wastewater treating sludge samples. The dotted lines with cross represent conversion routes that were shown to be absent.

Table 9.1 Stoichiometry and standard values of Gibbs free energy changes of reactions involved in the anaerobic conversion of carbon monoxide. Gibbs free energy changes were calculated using the data set compiled by Amend and Shock (2001).

Reactions	$\Delta G^{0'}$ (25ºC) kJ.mol^{-1}	$\Delta G_{55}'$ (55ºC) kJ.mol^{-1}
1) $CO + H_2O \rightarrow CO_2 + H_2$	-20.0	-22.3
2) $4\ CO + 2\ H_2O \rightarrow 3\ CO_2 + CH_4$	-210.8	-207.5
3) $4\ CO + 4\ H_2O \rightarrow CH_3COO^- + 2\ HCO_3^- + 3\ H^+$	-165.7	-145.5
4) $2\ HCO_3^- + 4\ H_2 + H^+ \rightarrow CH_3COO^- + 4\ H_2O$	-104.2	-92.7
5) $HCO_3^- + 4\ H_2 + H^+ \rightarrow CH_4 + 3\ H_2O$	-135.4	-127.5
6) $CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31.2	-34.9

The energy yield of hydrogenogenic CO conversion is relatively low and as a result of H_2 and CO_2 production, the Gibbs free energy change declines along with the conversion of CO. Furthermore, the initial presence of large amounts of H_2 as is typical for synthesis gas, decreases the energy yield of CO conversion further. This is illustrated in Fig. 9.2, which presents theoretical Gibbs free energy changes of hydrogenogenic CO conversion (reaction 1; Table 9.1) and the effect of the initial presence of H_2 and/or CO_2.

The presence of either H_2 or CO_2 decreases the overall Gibbs free energy change (ΔG). The slightly higher decrease of ΔG in the presence of CO_2 is due to the higher solubility of CO_2. Results of experiments with Eerbeek sludge revealed that the initial presence of H_2 decreased the CO conversion rates (Chapter 4), but it did not prevent the depletion of CO to undetectable levels (about 400 ppm for the gas chromatograph used). The observed differences in CO conversion rates can be attributed to the initial differences in ΔG values, which likely resulted in higher biomass yields and consequently higher conversion rates. The thermodynamic estimations suggest that the apparent minimum free energy requirement would be even smaller than –7 kJ.mol CO^{-1}, as this value was estimated assuming a final CO concentration of 400 ppm (Fig. 4.2B; Chapter 4).

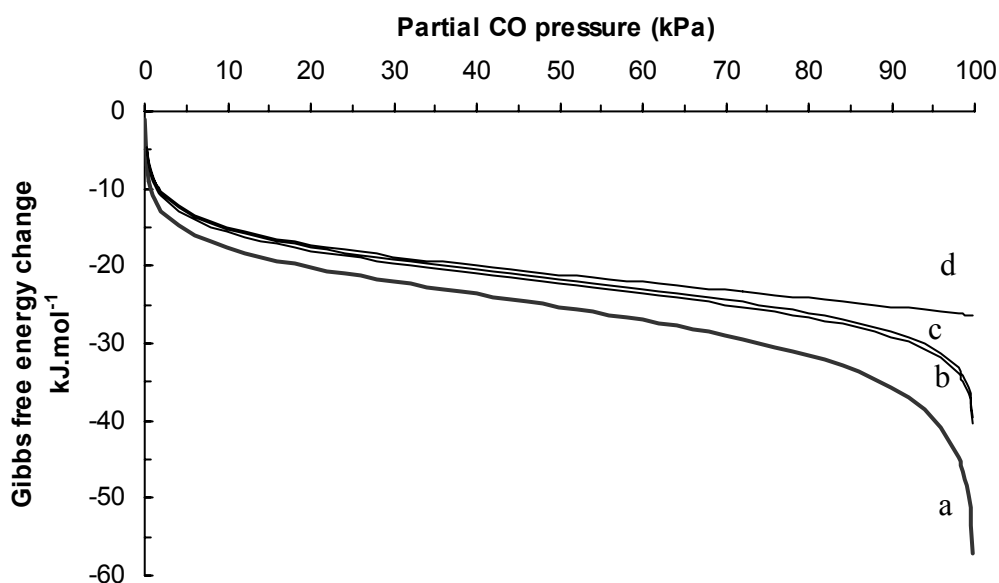Fig. 9.2   Theoretical evolution of the Gibbs free energy change at 55ºC during the conversion of 100 kPa CO to H_2 and CO_2 and the effect of the initial presence of H_2 and/or CO_2. The different hypothetical gas compositions are: only CO (a), CO + 100 kPa H_2 (b), CO + 100 kPa CO_2 (c) and CO + 50 kPa H_2 and 50 kPa CO_2 (d). Calculations performed for batch incubations (gas volume of 70 mL; liquid volume of 50 mL).

9.4 Sulfate reduction in the presence of CO

The results presented in Chapter 4 show that Eerbeek sludge was capable of sulfate reduction when incubated at $P_{CO} > 160$ kPa, suggesting that CO-rich synthesis gas could be used as electron donor for biological sulfate reduction. In general, sulfate reducing bacteria were found to be highly sensitive for CO toxicity (Mörsdorf et al., 1992; Davidova et al., 1994). Despite the capability of various sulfate-reducing bacteria to convert CO at concentrations up to 20 kPa, higher concentrations were found to inhibit completely growth (Lupton et al., 1984; Klemps et al., 1985; Karpilova et al., 1983; Mörsdorf et al., 1992; Davidova et al., 1994). However recently we found in our laboratory (Parshina et al., 2005) that the organisms *Desulfotomaculum kuznetsovii* and *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* can use CO as sole carbon and energy source for sulfate reduction up to 50 kPa CO in the gas phase.

Chapter 5 describes the isolation and characterization of a moderately thermophilic sulfate reducer, strain CO-1-SRB, isolated from Eerbeek sludge. The unique property of this isolate is that carbon monoxide (CO) serves as a sole energy and carbon source, both in the presence and absence of sulfate. CO was converted to H_2 and CO_2 and in the presence of sulfate the formed H_2 was used for sulfate reduction. Cells were gram-positive, motile, spore-forming rods. The temperature and pH ranges for growth were 30-68°C (T_{opt} = 55°C) and pH 6.0-8.0 (pH_{opt} = 7.0), respectively. Phylogenetic analysis of the 16S rRNA gene sequence placed CO-1-SRB to the genus *Desulfotomaculum*, closely resembling *Desulfotomaculum nigrificans* DSM 574[T] and *Desulfotomaculum* sp. RHT-3 (Mori et al., 2000), i.e. 99 and 100% similarity respectively. Nevertheless, the latter strains were completely inhibited at high levels of CO and only metabolized CO in the presence of sulfate, whereas strain CO-1-SRB grew well at 200 kPa CO in the headspace. Based on phylogenetic and physiological features strain CO-1-SRB was described as a new species, i.e. *Desulfotomaculum carboxydivorans* (Chapter 5).

As a result of its unique properties to convert CO to H_2 and in the presence of sulfate to reduce sulfate hydrogenotrophically, *D. carboxydivorans* may be applicable in both synthesis gas purification and biodesulfurization at elevated temperatures, e.g. flue gas desulfurization. In Chapter 6 a detailed physiological study of *D. carboxydivorans* is presented, focusing at its use in biotechnological synthesis gas conversions. *D. carboxydivorans* grows rapidly at 200 kPa CO, pH 7.0 and 55ºC, with a generation time of 100 minutes, producing nearly equimolar amounts of H_2 and CO_2 from CO and H_2O. At the start of batch incubations extremely high specific CO conversion rates, exceeding 0.8 mol CO.(g protein)$^{-1}$.hour^{-1}, were observed, which rapidly decreased as a result of increased biomass concentrations. This indicates that CO mass transfer from the gas to the liquid phase limits the CO conversion rate and not the activity of the biomass. The biomass yield measured as protein yield of *D. carboxydivorans* amounted to 0.42 mg protein produced per mmol CO converted (Fig. 6.3; Chapter 6). As *D. carboxydivorans* was capable to grow on various substrates, the biomass yield on selected substrates, which potentially are interesting for use as co-substrate to stimulate biomass production, was determined. When H_2 production is desired, the relatively fast generation times on glucose and pyruvate with a relatively high biomass yield (Table 6.1; Chapter 6), suggests their use as co-substrate could increase the H_2 producing biomass concentration considerably. In the presence of

sulfate, a higher biomass yield is obtained with both ethanol and H_2 as electron donors compared to CO (Table 6.1; Chapter 6), although the generation time on ethanol is rather long (19 hours) and H_2 will be present as a CO conversion product or as synthesis gas constituent. Perhaps for both H_2 production and biodesulfurization processes employing CO or synthesis gas, the most appropriate co-substrate for biomass formation would be glucose.

Analysis of the batch incubations in the presence of sulfate revealed that sulfide production occurs at a P_{CO} exceeding 100 kPa (Fig. 6.4; Chapter 6). The highest CO tolerance of a sulfate reducer was reported to be 50 kPa (Parshina et al., 2005). The determined maximal specific sulfate reduction rate of 32 mmol.(g protein)$^{-1}$.hour^{-1} is promising for biodesulfurization processes at elevated temperatures, although its tolerance for sulfide is rather low and pH dependent, i.e. maximally 9 mM and 5 mM sulfide at pH 7.2 and pH 6.5, respectively. The growth of *D. carboxydivorans* with sulfate, thiosulfate and sulfite as electron acceptor (Chapter 5) is interesting in view of the treatment of flue gas scrubbing water as the major part of SO_x forms sulfite upon dissolving in the scrubber solution (Janssen et al., 2000).

9.5 Application of synthesis gas in thermophilic biotechnological desulfurization

For large scale installations (> 5 kmol sulfate.h^{-1}), on-site produced high purity H_2 gas from methane steam reforming was suggested to be a more economical electron donor than ethanol (Van Houten and Lettinga, 1996). Although methane steam reforming results in a typical synthesis gas containing relatively low amounts of CO, i.e. about 15% (Table 2.1; Chapter 2), additional purification in order to obtain a highly purified H_2 is a prerequisite. Furthermore, depending on the source of feedstock, synthesis gas may contain CO levels exceeding 50%, as e.g. in case of coal gasification (Table 2.1; Chapter 2). In case further purification of the synthesis gas is not required prior to its use in biodesulfurization the production costs will reduce greatly, without decreasing the sulfate reduction capacity of the synthesis gas as both H_2 and CO are being utilized. The recent finding that some thermophilic sulfate reducing microorganisms have a much higher tolerance for carbon monoxide than previously thought (Parshina et al., 2005; Chapter 5), is interesting in view of a direct application of synthesis gas as electron donor for thermophilic sulfate reduction. The presence of a hydrogenogenic CO converting sulfate reducer, i.e. *D. carboxydivorans*, in granular

sludges (Chapter 5) seems promising for the rapid start up of a thermophilic biotechnological flue gas desulfurization using synthesis gas as electron donor.

Thermophilic sulfate reduction with CO as electron donor was observed in a gas lift reactor inoculated with Eerbeek granular sludge (Chapter 7). Despite the high CO conversion capacity of the biomass present, the sulfate reduction capacity was rather limited due to strong competition between methanogens and sulfate reducers for the CO-derived H_2. Although the methanogens appeared to be more sensitive to pH and temperature shocks imposed to the reactor, they could not be completely eliminated. As a result of the high growth rates of the methanogens (generation time about 4.5 hours), their recovery from shocks proceeded fast and they readily consumed over 90% of the CO-derived H_2. The results presented in Chapter 7 indicate that suppressing methanogens is of utmost importance when a sulfate reducing system is desired.

The results presented in Chapter 3 suggest that methanogens are quite sensitive to CO, since methane production was absent when granular sludge was crushed. However, this suppression of methanogenesis is presumably only a short-term effect of CO toxicity as reactor sludges produced methane from CO despite that the sludge was finely dispersed (Table 7.2; Chapter 7). Adaptation of methanogens to increasing levels of CO has been reported (O´Brien et al., 1984). Furthermore, investigations in the utilization of CO-derived H_2 revealed that the methanogens consume H_2 at higher rates than the sulfate reducers and are further capable of growth at more extreme conditions of pH and salinity than *D. carboxydivorans*, the presumed sulfate reducer present (Chapter 7). The complete elimination of methanogenesis from the sludge could be obtained by treating the sludge for a short-term at high temperatures (90 minutes at 95ºC; Chapter 4) as found in batch experiments. With this procedure living vegetative cells are killed, but spores remain almost unaffected. Elimination of methanogenesis has been achieved successfully by heat-treatment (Oh et al., 2003) or acid-base treatment (Chen et al., 2002). However, pretreatment of the sludge alone was not sufficient to prevent methanogenesis in the reactor experiment presented in Chapter 7, due to the presence of a small number of methanogens in the reactor and their fast generation times. Nevertheless, we consider the application of such a heat-treatment as quite promising for practice, especially, since such a heat treatment does not pose any environmental risk, i.e. potentially harmful chemicals are not released. Moreover a heat treatment in principle needs to be performed only once.

In contrast, the application of chemical inhibitors, such as 2-bromoethanesulfonate (BES), acetylene (Oremland and Capone, 1988) and chloroform (Chidthaisong and Conrad, 2000; Scholten et al., 2000), represents a potential risk when they are released into the environment. Furthermore, adaptation might result in increased operational costs due to an increasing demand of chemical inhibitors to maintain the same level of inhibition. Application of continuous dosing of 2 g.L^{-1} BES to a bioreactor showed to be ineffective for suppressing of methanogenesis as complete inhibition of methanogenesis was obtained for two days only (Weijma et al, 2002). In contrast, Sparling et al. (1997) successfully inhibited methane formation in a H$_2$ producing solid substrate anaerobic digester, by the addition of 1% v/v acetylene in the headspace.

In the CO fed gas lift reactors the sulfate reduction rates were generally low, although a maximal (short-term) sulfate reduction rate of 20 mmol.L^{-1}.day^{-1} was assessed in the absence of methanogenesis (Chapter 7). This suggests that the sulfate reduction capacity of a CO fed gas lift reactor operated at 55ºC has potentials once methanogenesis is eliminated. In case of complete elimination of methanogenesis, the maximal assessed amount of CO converted to H$_2$, i.e. 380 mmol.L^{-1}.day^{-1} (Fig. 7.8; Chapter 7) could hypothetically reduce more than 90 mmol.L^{-1}.day^{-1} of sulfate, which is slightly higher than the sulfate reduction rate reported in a thermophilic (55°C) H$_2$/CO$_2$ fed gas lift reactor (Van Houten et al., 1997). Assuming that *D. Carboxydivorans* is responsible for both hydrogenogenic CO conversion and sulfate reduction in the reactor, its sensitivity towards sulfide (Chapter 6) might require special measures to maintain sufficient low sulfide concentrations. These measures may include capture of H$_2$S from the gas recycle or application of a low HRT. A sulfide concentration of 10 mM, which inhibits growth at a pH of about 7.2 (Fig. 6.5; Chapter 6), would require a HRT as low as 2.7 hours considering a sulfate reduction rate of 90 mmol.L^{-1}.day^{-1}.

The results in Chapter 8 show that sulfate reduction at low HRT (< 3 hours) is feasible and promotes stable sulfate reduction (Fig. 8.4B; Chapter 8). Despite the high CO conversion efficiencies (> 95%) obtained at this HRT and most of the CO-derived H$_2$ used for sulfate reduction (> 87%), methanogens remained present and upon increasing the HRT methanogenic activity rapidly increased at the expense of sulfate reduction. The maximal sulfate reduction rate achieved was about 17 mmol.L^{-1}.day^{-1}, and the rate was limited by the amount of CO supplied and the CO conversion

efficiency (about 85%) at higher CO loads (106 mmol.L^{-1}.day^{-1}), probably as a result of limited biomass retention in the reactor. Nevertheless, at low HRT a more stable sulfate reduction was observed (Chapter 8) compared to the results presented in Chapter 7. The results in Chapter 8 clearly indicate the potential of CO as electron donor for stable sulfate reduction at elevated temperatures (55ºC), although special attention has to be paid to biomass retention as well as elimination of methanogens. Biomass retention using carrier material as e.g. employed by Van Houten et al. (1997) ensures the need for a complete elimination or suppression of methanogens. In view of the need for biomass retention, the use of a submerged anaerobic membrane bioreactor (SAMBaR) represents an interesting option as shown by Vallero et al. (2005) for sulfate reduction at high salinity using a pure culture of *Desulfobacter halotolerans*. The short generation time of *D. carboxydivorans* on CO at 55ºC (1.7 hours; Chapter 6) compared to that of *Desulfobacter halotolerans* (t_d = 40 hours) suggests that the start up of a thermophilic CO fed sulfate reducing submerged membrane bioreactor may be fast. In order to increase the biomass concentration of *D. carboxydivorans*, the supply of some glucose during the start-up phase could be considered (Chapter 6). When we assume that *D. carboxydivorans* is the most dominant sulfate reducer in the biomass of the reactor presented in Chapter 8, the estimated specific sulfate reduction rates would be between 0.7 and 1.0 mol.(g protein)$^{-1}$.day^{-1} (Chapter 8). The sulfate reduction rate for *D. carboxydivorans* determined in Chapter 6 (Fig. 6.4) amounted to almost 0.8 mol.(g protein)$^{-1}$.day^{-1}, suggesting that such high specific rates with this organism seem possible. Therefore, the application of biomass retention membranes in a CO or synthesis gas fed sulfate reducing bioreactor has potential using either a pure culture of *D. carboxydivorans* or a heat treated sample of Eerbeek sludge.

9.6 Design of a biological synthesis gas purification process

One of the major points in the design of a biological synthesis gas purification process is the required product specification, i.e. especially the permitted levels of CO. In Chapter 6, the biological achievable minimal CO concentrations with a pure culture of *D. carboxydivorans* were found to be determined by the thermodynamics rather than by effects of pH or CO affinity of the bacterium. Thermodynamic calculations performed with the results obtained in Chapter 4 already indicate that the minimal ΔG

for CO conversion is < -7 kJ.(mol CO)$^{-1}$ (Fig. 4.2B) as at this value the CO concentration is below the detection limit of 400 ppm for the GC used in that experiment. This is lower than the values obtained from chemostat and field studies, in which catabolic activity was observed when the associated free energy change was at least -10 kJ per mol catabolic reaction (Seitz et al., 1990; Westermann, 1994). Nevertheless, CO conversion by *D. carboxydivorans* proceeds as long as $\Delta G < 0$, which suggests a very efficient coupling of energy conservation to CO conversion. The results presented in Chapter 6 show that the assessed minimal CO concentration could be even as low as 4-5 ppm (Table 6.3; Chapter 6), depending on the initial gas composition and P_{CO}. Thermodynamic calculations, assuming that CO conversion stops at $\Delta G = 0$, were compared to actual measurements of CO concentration, showing a good correlation between them (Table 6.3; Chapter 6). The initial composition, i.e. the presence of H_2 and CO_2 as is typical of synthesis gas, affects the minimal achievable CO concentrations (Table 6.4; Chapter 6). This is illustrated by the minimal CO concentration for synthesis gas derived from coal, i.e. 120 ppm at a total gas pressure of 101 kPa, whereas at 1 MPa the minimal CO concentration cannot be decreased to less than 1% (Chapter 6).

Low exit CO concentrations imply that the CO concentrations in the gas phase of the reactor are low as well, which negatively affects the mass transfer of CO from the gas to the liquid phase. This would result in the need to use voluminous reactors. Operation of a synthesis gas purification reactor at high P_{CO} would greatly enhance the mass transfer, but results in higher exit CO concentrations. From the growth curve of *D. carboxydivorans* (Fig. 6.1; Chapter 6) it is evident that the CO conversion activity of the biomass is much higher than the CO flux to the liquid phase as the maximal CO conversion rates of 0.8 mol CO.(g protein)$^{-1}$.hour^{-1} (Fig. 6.2; Chapter 6) were obtained at the start of the incubations when the biomass concentration was low. Synthesis gas purification at high P_{CO} requires the need for a gas separation technology to provide a high purity H_2 gas or selective removal of one of the products to maintain $\Delta G < 0$. The latter could be achieved by physical absorption of CO_2, e.g. by hot potassium carbonate or other processes using amine, selexol, sulfinol or rectisol solutions (Czuppon et al., 1995). This offers the possibility for selective CO_2 sequestration and geological storage (Gale, 2004), thus preventing its emission to the

atmosphere. Liberation of high purity CO_2 could be even useful for reuse in chemical synthesis of e.g. methanol (Pruschek et al., 1997) or in greenhouse horticulture.

The combination of separation processes with a biological CO conversion process would enable high rate CO conversions due to high mass transfer of CO from the gas to the liquid phase at high P_{CO}. Complete CO conversions could be obtained by recycling the unconverted CO back to the reactor after removal of both CO_2 and H_2. The hydrogen present in the product gas coming from the bioreactor can be separated from the other gasses by e.g. a pressure swing adsorption (PSA) process or by selective gas separation membranes (Koros and Mahajan, 2000; Perry et al., 1997). Although, PSA is usually preferred when high purity gases are required (Koros and Mahajan, 2000), the use of gas separating membranes seem particularly attractive to be used in a synthesis gas purification process. The reason is its simplicity and continuous operation in-line with the bioreactor, whereas PSA requires the use of multiple units to assure a continuous operation. The H_2 purity of the product gas in PSA can be up to 99.9999% at a H_2 recovery between 50-95%, whereas with the use of membranes the purity is slightly lower at maximally 99.9% H_2 at recoveries up to 98% (Air Liquide technical specifications). PSA systems operate between 500-5000 kPa and for selective gas membranes pressures up to 8 MPa can be handled (Air Liquide technical specifications). This indicates a further interest for operation of the bioreactor at elevated pressures as synthesis gas is also produced at high pressures (> 2 MPa; Czuppon et al., 1995), and alternating depressurizing-pressurizing represents additional operation costs. However, the upper limit of P_{CO} needs to be determined to demonstrate the feasibility of operating a bioreactor for synthesis gas at relatively high pressures. A proposed synthesis gas purification process employing both H_2 separation and selective CO_2 recovery is schematically depicted in Fig. 9.3.
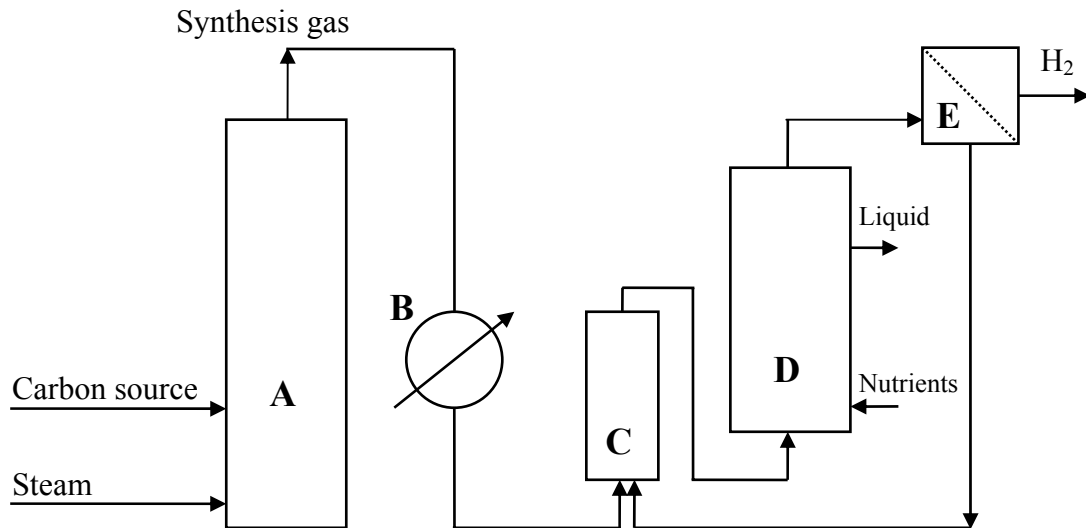
Fig. 9.3   Proposed synthesis gas purification process including steam reforming reactor (A), Heat recovery (B), CO_2 absorption (C), biological CO conversion reactor (D) and selective recovery of a high purity H_2 gas using a gas selective membrane (E).

For the hydrogenogenic CO converting bioreactor two concepts are particularly interesting, i.e. biotrickling filters (Plaggemeier and Lämmerzahl, 2000) and monolith biofilm reactors (Ebrahimi et al., 2005; Ariga et al., 1986). Synthesis gas purification in principle requires only a small liquid stream, which supplies the biomass with nutrients and prevents too high salinity. Furthermore, thin liquid films are beneficial for the mass transfer of gaseous compounds towards the biomass. In both reactor types the biomass may be growing as a biofilm or the use of trickling cell suspensions over the surface of the carrier material could be considered. Bioreactors using cell suspensions, consisting of individual cells or small flocs have lower mass transfer resistances compared to biofilm reactors, as the only mass transfer barrier in cell suspension bioreactors is transfer of the compounds from the gas to the liquid phase (Nicolella et al., 2000). In Chapter 6 the theoretical volumetric CO conversion rate in a biotrickling filter assuming a k_La of 0.015 s^{-1} (Klasson et al., 1992) was estimated at 40 m^3.m^{-3} reactor volume under standard conditions (25ºC and 101.325 kPa) with synthesis gas derived from coal gasification at P_{CO} of 200 kPa. Since the CO content of coal derived synthesis gas is about 50%, it represents a treatment of 80 m^3.m^{-3} reactor volume of synthesis gas.

9.7 Recommendations for further research

The occurrence of thermophilic microorganisms capable of high-rate hydrogenogenic CO conversion as well as sulfate reduction in the presence of high levels of CO enables the use of CO containing synthesis gas in biotechnological applications, such as synthesis gas purification or the use of CO rich synthesis gas as electron donor in the treatment of sulfate rich wastewaters.

In view of the development of a biological synthesis gas purification process laboratory scale continuous experiments should be conducted to determine the long-term operation, methods to minimize or rather eliminate H_2 consumption, preferred type of seed material (sludge versus pure cultures) as well as the need or possibility to operate under sterile conditions when using a pure culture of e.g. *D. carboxydivorans*. Furthermore, the results from such experiments should be compared to the currently employed chemical catalytic reactors to estimate the economic benefits of any biological system.

With regards to the use of *D. carboxydivorans* or Eerbeek sludge in synthesis gas fed thermophilic sulfate reducing bioreactors, attention should be paid especially to the sulfide toxicity and competition for H_2 between the various microorganisms present. Assuming that special measures are required to maintain the sulfide concentration low this will result in additional costs. The same would hold when using more complex and expensive bioreactor types such as the SAMBaR (Vallero et al., 2005), to be able to work with cell suspensions as well as to prevent the introduction of H_2 consuming microorganisms provided that competition cannot be directed to sulfate reduction. Thereafter it should be evaluated whether the direct use of CO containing synthesis gas has economical benefits compared to the use of highly purified H_2 or methanol.

9´

Samenvatting en discussie

9.1´ Inleiding

Het doel van het in dit proefschrift gepresenteerde onderzoek was om de mogelijkheden van biologische hydrogenogene omzetting van koolmonoxide (CO), d.w.z. de biologische omzetting van CO in waterstof (H_2) en kooldioxide (CO_2), te evalueren. Een biologisch proces voor de omzetting van CO is interessant voor de zuivering van synthesegas, hetgeen voornamelijk H_2, CO en CO_2 bevat. Grootschalige productie van H_2 komt voornamelijk tot stand door gasificatie van organisch materiaal tot een waterstofrijk synthesegas. Teneinde een zeer puur H_2 gas te produceren uit synthesegas is een verdere zuivering noodzakelijk. Biologische hydrogenogene CO omzetting biedt een mogelijk alternatief voor de huidig toegepaste chemisch-katalytische water-gas-shift reactie, die bij hoge temperaturen wordt uitgevoerd gebruikmakend van chemische katalysatoren. Een grootschalige kosteneffectieve H_2 productie is niet alleen belangrijk met het oog op een eventuele rol van waterstof als toekomstige energiedrager, maar ook omdat H_2 een belangrijke chemische reagens is in reductieve processen, zowel in de chemische industrie als in specifieke afvalwaterbehandelingsprocessen. Vooral het gebruik van H_2 als elektronendonor in de biologische sulfaatreductie van sulfaat-/sulfietrijk afvalwater met weinig organisch materiaal is succesvol gebleken (Van Houten et al., 1994; 1997). Afvalwaterbehandeling resulteert, in tegenstelling tot de productie van chemicaliën, in het algemeen niet in de productie van waardevolle componenten, zodat de gebruikte elektronendonor goedkoop en effectief moet zijn. Synthesegas zou een goedkoop

alternatief kunnen zijn voor zuiver H_2 als elektronendonor in biotechnologische sulfaatreductieprocessen (Van Houten en Lettinga, 1996; DuPreez en Maree, 1994; DuPreez et al., 1992). De combinatie van hydrogenogene CO omzetting met hydrogenotrofe sulfaatreductie maakt het gebruik mogelijk van synthesegas als elektronendonor zonder de noodzaak om het synthesegas vooraf te zuiveren, waarbij zowel H_2 als CO gebruikt kunnen worden voor sulfaatreductie. Dientengevolge zouden hydrogenogene CO omzettingen twee belangrijke toepassingen in de biotechnologie kunnen vinden, te weten biologische synthesegas zuivering en het direct gebruik van synthesegas als elektronendonor in biotechnologische ontzwavelingsprocessen.

9.2´ Aanwezigheid van hydrogenogene CO-omzettende bacteriën in slibben van anaërobe afvalwaterbehandelingsinstallaties

In Hoofdstuk 3 zijn de resultaten gepresenteerd van een studie naar de aanwezigheid van CO omzetting in zes anaërobe bioreactorslibben, gecultiveerd op verschillende typen industrieel afvalwater bij temperaturen van 30-35°C. Daarnaast is de CO omzettingcapaciteit getest van een slib afkomstig uit een laboratoriumschaal methanolgevoede thermofiele sulfaatreducerende UASB. Alle geteste slibmonsters uit de praktijkinstallaties, wanneer geïncubeerd bij 30°C, zetten CO om in methaan en/of acetaat. Experimenten met specifieke remmers voor methanogene archaea of bacteriën, waarbij 2-broomethaansulfonaat (BES) en vancomycine gebruikt zijn, lieten zien dat de CO omzetting in methaan via acetaat verloopt. Directe methaanvorming vanuit CO is uitsluitend geobserveerd met twee slibmonsters. Resultaten van incubaties, waaraan vancomycine is toegevoegd om homoacetogenen te remmen (Oremland en Capone, 1988), lieten een erg langzame directe omzetting van CO in methaan zien en daarom hebben we geconcludeerd dat deze route van ondergeschikt belang is voor de algehele CO omzetting. De aanwezigheid van acetogene CO omzetting bij 30°C is niet verrassend als men kijkt naar het aantal mesofiele homoacetogenen beschreven, die kunnen groeien met CO (Tabel 2.2; Hoofdstuk 2). Waterstofproductie is tot dusver slechts beschreven voor een paar mesofiele bacteriën, te weten een aantal fototrofe organismen (Uffen, 1976; Dashekvicz en Uffen, 1979; Jung et al., 1999a ; Kerby et al., 1995) en een facultatief

anaërobe bacterie (Jung et al., 1999b). Echter, al deze bacteriën hebben gemeen dat hun groei onder anaërobe condities in het donker behoorlijk traag is. Tot onze verrassing lieten de incubaties bij 55°C een duidelijke productie van H_2 zien als gevolg van CO omzetting met vijf van de zeven geteste slibmonsters. Wanneer methaan gevormd werd in incubaties met CO, werd de CO via H_2 omgezet in methaan zoals experimenten met remmers aangetoond hebben. De directe omzetting van CO in methaan trad in geen enkele incubatie op, uitgevoerd op een initiële partiële CO druk (P_{CO}) groter dan 160 kPa (Hoofdstuk 3). Ondanks dat vier van de succesvol H_2 producerende slibben oorspronkelijk gecultiveerd waren bij temperaturen van 30 tot 35ºC, was hun lagfase voor hydrogenogene CO omzetting bij 55ºC erg kort. Dit veronderstelt korte generatietijden van de verantwoordelijke micro-organismen. De resultaten in Hoofdstuk 3 tonen aan dat hydrogenogene CO omzetting bij hoge P_{CO} een veelvoorkomend fenomeen is, ondanks dat alle anaërobe thermofiele hydrogenogene micro-organismen tot dusver geïsoleerd afkomstig waren van locaties met vulkanische activiteit (Tabel 2.3; Hoofdstuk 2). Volgens Symonds et al. (1994) kunnen zulke locaties tot 1.5% CO bevatten. Echter de enzymen benodigd voor hydrogenogene CO omzetting, te weten koolmonoxide-dehydrogenase (carbon monoxide dehydrogenase; CODH) en hydrogenasen, komen veel voor in anaërobe micro-organismen (Hoofdstuk 2). CODH heeft niet uitsluitend een functie in CO oxidatie, maar vormt tevens één van de belangrijkste enzymen in de synthese of splitsing van acetyl-CoA (Ferry, 1995).

9.3´ Effect van P_{CO} en H_2 op de CO omzettingsroutes bij 55ºC

Alle experimenten gepresenteerd in Hoofdstuk 3 zijn uitgevoerd bij een P_{CO} groter dan 160 kPa. Zo´n hoge partiële druk zou de omzetting van CO kunnen beïnvloeden, omdat bekend is dat CO een remmer is voor verschillende hydrogenasen (Davidova et al., 1994; Kim et al., 1984; Lupton et al., 1984; Daniels et al., 1977) en bovendien voor een aantal sulfaatreducerende bacteriën die op organische substraten groeien (Davidova et al., 1994). Hoofdstuk 4 beschrijft de resultaten van een studie naar CO omzettingsroutes bij 55ºC als een functie van de initiële P_{CO} alsmede de aanwezigheid van H_2. Uit de hydrogenogene CO omzettende slibmonsters zijn twee geselecteerd voor nadere studie, Eerbeek (papierindustrie) en Nedalco (distilleerderij) slib. Het grote verschil tussen deze beide slibmonsters was de afwezigheid van

methaanproductie wanneer geïncubeerd onder 150 kPa CO. Deze studie toonde aan dat de CO omzettingsroutes niet bepaald worden door de hoeveelheid CO aanwezig, alhoewel bij P_{CO} waarden tot 50 kPa een langzame directe omzetting van CO in methaan optrad met Eerbeek slib. Aangezien de snelheid van deze methanogene route zo laag was, is aangenomen dat deze bijdrage aan de algehele CO omzetting bij 55ºC nihil is (Fig. 9.1´). De afwezigheid van directe CO omzetting bij een hoge P_{CO} (>150 kPa) is toegeschreven aan een toxisch effect van CO voor deze methanogenen, hetgeen waarschijnlijk ook de productie van methaan met Nedalco slib heeft voorkomen, aangezien dit slib wanneer geïncubeerd met H_2/CO_2 wel degelijk methaan vormt. Een mogelijk directe methaanproductie als gevolg van de reductie van CO met H_2 (vraagteken in Fig. 9.1´), kon niet onderscheiden worden van directe methanogene CO omzetting vanuit CO alleen, maar gelijkaardige snelheden voor methaanproductie suggereren dat H_2 de directe methanogene CO omzetting niet stimuleert. De belangrijkste reacties zijn weergegeven in Fig. 9.1´ en de reactievergelijkingen in Tabel 9.1´.
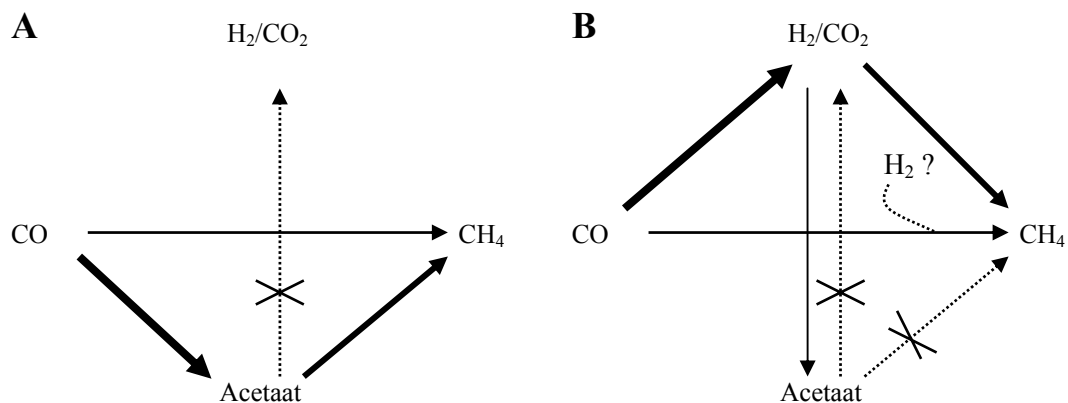

Fig. 9.1´   Schematische weergave van de belangrijkste CO omzettingsroutes bij 30ºC (A) en 55ºC (B) zoals gevonden voor verschillende anaërobe afvalwaterbehandelingsslibben. Stippellijnen geven de omzettingroutes aan die niet gevonden zijn.

Tabel 9.1´ Stoichiometry en standaard waarden voor de Gibbs vrije energie veranderingen van de reacties betrokken bij de anaërobe omzetting van koolmonoxide. De Gibbs vrije energie veranderingen zijn berekend met de gegevens gecompileerd door Amend en Shock (2001).

Reacties	$\Delta G^{0'}$ (25ºC) kJ.mol^{-1}	$\Delta G_{55}^{'}$ (55ºC) kJ.mol^{-1}
1) $CO + H_2O \rightarrow CO_2 + H_2$	-20.0	-22.3
2) $4\ CO + 2\ H_2O \rightarrow 3\ CO_2 + CH_4$	-210.8	-207.5
3) $4\ CO + 4\ H_2O \rightarrow CH_3COO^- + 2\ HCO_3^- + 3\ H^+$	-165.7	-145.5
4) $2\ HCO_3^- + 4\ H_2 + H^+ \rightarrow CH_3COO^- + 4\ H_2O$	-104.2	-92.7
5) $HCO_3^- + 4\ H_2 + H^+ \rightarrow CH_4 + 3\ H_2O$	-135.4	-127.5
6) $CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31.2	-34.9

De energie opbrengst van de hydrogenogene CO omzetting is relatief laag en als gevolg van H_2 en CO_2 productie, daalt de Gibbs vrije energie verandering met de voortgaande omzetting van CO. Bovendien zal de aanwezigheid van grote hoeveelheden H_2, zoals typerend is voor synthesegas, de energie opbrengst van de CO omzetting verder verlagen. Dit is geïllustreerd in Fig. 9.2´, waarin de theoretische Gibbs vrije energie verandering weergegeven wordt voor de omzetting van CO (reactie 1; Tabel 9.1´) in een batch systeem en tevens het effect van de aanwezigheid van H_2 en/of CO_2 in het gevoede gasmengsel.

De aanwezigheid van ofwel H_2 of CO_2 verlaag**t** de Gibbs vrije energie verandering (ΔG). De iets grotere verlaging van de ΔG in aanwezigheid van CO_2 is het gevolg van de betere oplosbaarheid van CO_2 in water. Resultaten van experimenten met Eerbeek slib tonen aan dat de initiële aanwezigheid van H_2 de omzettingssnelheden van CO verlagen (Hoofdstuk 4), maar niet dat de uiteindelijk bereikte CO concentraties boven de detectielimiet lagen (ongeveer 400 ppm voor de gebruikte gaschromatograaf). De geobserveerde verschillen in CO omzettingssnelheden kunnen toegeschreven worden aan de verschillen in ΔG aan het begin van de incubaties, hetgeen waarschijnlijk geresulteerd heeft in hogere biomassa opbrengsten en daardoor hogere omzettingssnelheden. Thermodynamische berekeningen suggereren dat de minimale ΔG waarbij nog CO omzetting plaatsvindt kleiner is dan -7 kJ.mol CO^{-1}, de waarde geschat bij een uiteindelijke CO concentratie van 400 ppm (Fig. 4.2B; Hoofdstuk 4).
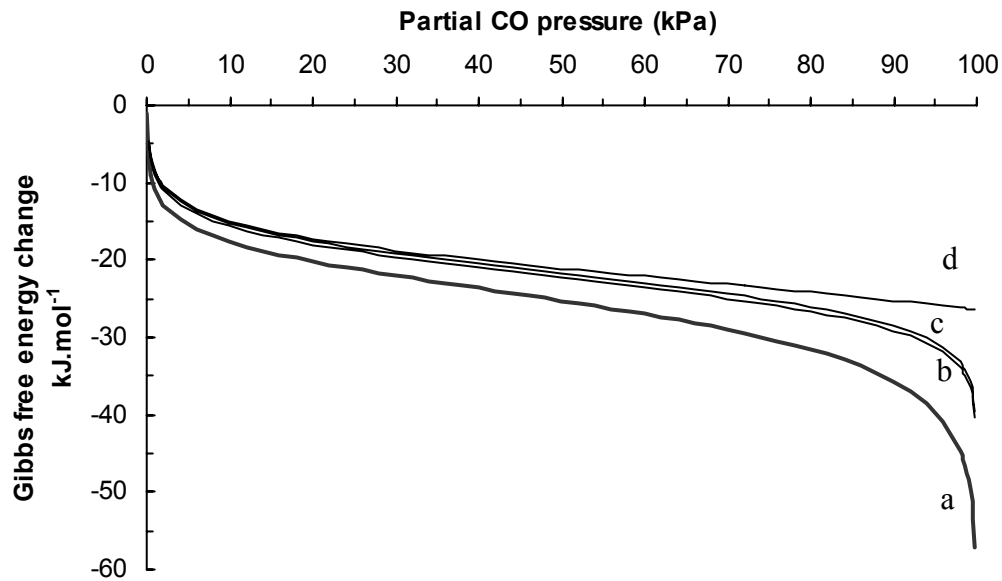
Fig. 9.2′   Theoretisch verloop van de Gibbs vrije energie verandering op 55ºC tijdens de omzetting van 100 kPa CO in H_2 en CO_2 en het effect van de aanwezigheid van H_2 en/of CO_2 vanaf het begin van de incubatie. De verschillende hypothetische gas composities zijn: alleen CO (a), CO + 100 kPa H_2 (b), CO + 100 kPa CO_2 (c) en CO + 50 kPa H_2 en 50 kPa CO_2 (d). Berekeningen uitgevoerd voor batch incubaties (gas volume van 70 mL; vloeistof volume van 50 mL).

9.4′ Sulfaatreductie in aanwezigheid van CO

De resultaten gepresenteerd in Hoofdstuk 4 laten zien dat Eerbeek slib in staat is tot sulfaatreductie wanneer geïncubeerd bij een $P_{CO} > 160$ kPa, hetgeen suggereert dat CO-rijk synthesegas gebruikt zou kunnen worden als elektronendonor voor biologische sulfaatreductie. In het algemeen is gevonden dat sulfaatreducerende bacteriën erg gevoelig zijn voor toxiciteit van CO (Mörsdorf et al., 1992; Davidova et al., 1994). Ondanks dat verschillende sulfaatreducerende bacteriën CO kunnen gebruiken in concentraties tot 20 kPa, is voor hogere concentraties CO gerapporteerd dat de groei volledig geremd wordt (Lupton et al., 1984; Klemps et al., 1985; Karpilova et al., 1983; Mörsdorf et al., 1992; Davidova et al., 1994). Echter, (komma toevoegen) recentelijk hebben we in ons laboratorium gevonden dat de organismen *Desulfotomaculum kuznetsovii* en *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* CO kunnen gebruiken als koolstof en energiebron voor sulfaatreductie tot 50 kPa CO in de gasfase (Parshina et al., 2005).

Hoofdstuk 5 beschrijft de isolatie en karakterisatie van een gematigd thermofiele sulfaatreduceerder, stam CO-1-SRB, geïsoleerd uit Eerbeek slib. De unieke eigenschap van dit isolaat is dat CO kan dienen als koolstof en energiebron bij hoge P_{CO}, zowel in aanwezigheid als afwezigheid van sulfaat. CO is omgezet in H_2 en CO_2 en in aanwezigheid van sulfaat wordt het gevormde H_2 gebruikt voor sulfaatreductie. De cellen zijn grampositieve, beweeglijke, sporenvormende staafjes. De temperatuur en pH voor groei liggen tussen 30-68°C (T_{opt} = 55°C) en pH 6.0-8.0 (pH_{opt} = 7.0), respectievelijk. Fylogenetische analyse van het 16S rRNA plaatste CO-1-SRB in het geslacht *Desulfotomaculum*, veel gelijkenis vertonend met *Desulfotomaculum nigrificans* DSM 574[T] en *Desulfotomaculum* sp. RHT-3 (Mori et al., 2000), te weten 99 en 100% respectievelijk. Echter, laatstgenoemde twee worden volledig geremd bij hoge concentraties CO en gebruiken CO alleen in de aanwezigheid van sulfaat, terwijl stam CO-1-SRB goed groeit bij 200 kPa CO in de gasfase. Gebaseerd op fylogenetische en fysiologische eigenschappen is stam CO-1-SRB beschreven als een nieuwe bacteriesoort, *Desulfotomaculum carboxydivorans* (Hoofdstuk 5).

Als gevolg van zijn unieke eigenschap om CO met water om te kunnen zetten in H_2 en in de aanwezigheid van sulfaat dit sulfaat hydrogenotrofisch te reduceren, zou *D. carboxydivorans* toepasbaar kunnen zijn in zowel synthesegas zuivering als in biologische ontzwaveling van afvalwaters bij hogere temperaturen, zoals bijvoorbeeld rookgasontzwaveling. In Hoofdstuk 6 wordt een gedetailleerde fysiologische studie van *D. carboxydivorans* gepresenteerd. *D. carboxydivorans* groeit snel bij 200 kPa CO, pH 7.0 en 55ºC, met een generatietijd (verdubbelingstijd) van 100 minuten, waarbij equimolaire hoeveelheden H_2 en CO_2 van CO worden geproduceerd. Aan het begin van batch incubaties werden extreem hoge specifieke CO omzettingsnelheden geobserveerd (> 0.8 mol CO.(g eiwit)$^{-1}$.uur^{-1}), die snel daalden als gevolg van de toename van de biomassaconcentratie. Dit toont aan dat massaoverdracht van CO van de gas naar de vloeistoffase en niet de activiteit van de biomassa de CO omzettingssnelheid beperkt. De biomassaopbrengst gemeten als de eiwitopbrengst van *D. carboxydivorans* was 0.42 mg eiwit geproduceerd per mmol omgezet CO (Fig. 6.3; Hoofdstuk 6). Omdat *D. carboxydivorans* op verschillende substraten kan groeien, is de biomassaopbrengst op een aantal geselecteerde substraten, die potentieel interessant zijn om te gebruiken als co-substraat om de biomassaproductie te stimuleren, bepaald. Indien H_2 productie gewenst is, dan zou het gebruik van glucose

en/of pyruvaat de biomassaproductie kunnen stimuleren gezien de relatief hoge biomassaopbrengsten alsmede de korte generatietijden (Tabel 6.1; Hoofdstuk 6). In aanwezigheid van sulfaat resulteert de toevoeging van zowel ethanol als H_2 als elektronendonor in hogere biomassaopbrengsten dan de CO omzetting alleen (Tabel 6.1; Hoofdstuk 6), alhoewel de verdubbelingstijd met ethanol nogal lang is (19 uur). Waarschijnlijk zal glucose voor zowel H_2 productie uit synthesegas en biologische ontzwavelingsprocessen met CO of synthesegas, het meest gunstige co-substraat voor biomassavorming zijn.

Analyse van de batch incubaties in aanwezigheid van sulfaat laat zien dat sulfide productie aanvangt bij een $P_{CO} > 100$ kPa (Fig. 6.4; Hoofdstuk 6). De hoogst gerapporteerde CO tolerantie voor een sulfaatreduceerder was 50 kPa (Parshina et al., 2005). De maximale specifieke sulfaatreductiesnelheid van 32 mmol.(g eiwit)$^{-1}$.uur^{-1} gemeten voor *D. carboxydivorans* is veelbelovend voor biologische ontzwavelingsprocessen bij hogere temperaturen, hoewel de tolerantie voor sulfide nogal laag en pH afhankelijk is, namelijk maximaal 9 mM en 5 mM sulfide bij pH 7.2 en pH 6.5, respectievelijk. De groei van *D. carboxydivorans* met sulfaat, thiosulfaat en sulfiet als elektronenacceptor (Hoofdstuk 5), is interessant met het oog op rookgasontzwaveling, omdat een groot deel van de SO_x sulfiet vormt wanneer het opgelost wordt in een alkalische oplossing (Janssen et al., 2000).

9.5´ Toepassing van synthesegas in thermofiele biotechnologische ontzwaveling

Voor grootschalige installaties (> 5 kmol sulfaat.h^{-1}) is ter plaatse geproduceerd zeer zuiver H_2 gas voorgesteld als een economisch gunstiger elektronendonor dan ethanol (Van Houten en Lettinga, 1996). Hoewel de omzetting van methaan in synthesegas resulteert in een gas mengsel met relatief lage CO concentraties, namelijk ongeveer 15% (Tabel 2.1; Hoofdstuk 2), is een aanvullende zuivering om een zeer zuiver H_2 gas te verkrijgen noodzakelijk. Wanneer een ander organisch materiaal gebruikt wordt voor de productie van synthesegas kunnen de CO concentraties aanzienlijk hoger zijn tot concentraties groter dan 50%, zoals bijvoorbeeld in het geval van kolenvergassing (Tabel 2.1; Hoofdstuk 2). Wanneer een dergelijke zuivering van het synthesegas niet noodzakelijk is voor het gebruikt kan worden in de biologische ontzwaveling zullen de kosten aanzienlijk lager zijn, zonder dat de sulfaatreductiecapaciteit van het synthesegas vermindert, aangezien zowel H_2 als CO benut worden. De recente

ontdekking dat er thermofiele sulfaatreducerende micro-organismen zijn die een veel hogere CO tolerantie hebben dan vooralsnog aangenomen was (Parshina et al., 2005; Hoofdstuk 5), is interessant met het oog op een directe toepassing van synthesegas als elektronendonor voor thermofiele sulfaatreductie. De aanwezigheid van een hydrogenogene CO omzettende sulfaatreduceerder, namelijk *D. carboxydivorans*, in korrelslib (Hoofdstuk 5) lijkt veelbelovend voor een snelle opstart van een thermofiele biotechnologische rookgasontzwaveling met synthesegas als elektronendonor.

Thermofiele sulfaateductie met CO als elektronendonor was geobserveerd in een gas lift reactor geïnoculeerd met Eerbeek korrelslib (Hoofdstuk 7). Ondanks de hoge CO omzettingscapaciteit van de aanwezige biomassa, was de sulfaatreductiecapaciteit nogal beperkt vanwege de sterke competitie tussen methanogenen en sulfaatreduceerders voor de H_2 geproduceerd uit CO. Hoewel de methanogenen gevoeliger leken voor pH en temperatuurschokken toegepast op de reactor, konden ze op deze wijze niet geëlimineerd worden uit de reactor. Als gevolg van de hoge groeisnelheden van de methanogenen (verdubbelingstijd ongeveer 4.5 uur) was het herstel van de methanogene populatie snel en daarmee ook de methanogene consumptie van de H_2. De resultaten gepresenteerd in Hoofdstuk 7 tonen aan dat de onderdrukking van de methanogenen van het grootste belang is wanneer een sulfaatreducerend systeem gewenst is.

De resultaten in Hoofdstuk 3 suggereren dat methanogenen vrij gevoelig zijn voor CO, omdat met een gesuspendeerd slib methaanproductie afwezig was. Echter, deze onderdrukking van methanogenese is waarschijnlijk een CO toxiciteitseffect dat slechts van korte duur is aangezien reactorslib ondanks zijn gesuspendeerde aard methaan produceerde (Tabeel 7.2; Hoofdstuk 7). Adaptatie van methanogenen aan toenemende CO concentraties is reeds eerder beschreven (O´Brien et al., 1984). Daarnaast is gevonden dat de methanogenen aanwezig in de reactor H_2 aanzienlijk sneller consumeren dan de sulfaatreduceerders en dat ze bovendien kunnen groeien bij meer extreme condities wat betreft pH en zoutconcentratie dan *D. carboxydivorans*, waarvan aangenomen wordt dat het de sulfaatreduceerder is in de reactor (Hoofdstuk 7). De volledige eliminatie van methanogenese kon verkregen worden door het slib voor te behandelen op hoge temperatuur (90 minuten bij 95ºC; Hoofdstuk 4). Met deze procedure worden levende vegetatieve cellen gedood, maar blijven sporen vrijwel onaangetast. Echter, voorbehandeling van het slib alleen was onvoldoende om methanogenese in de reactor experimenten gepresenteerd in Hoofdstuk 7 te

voorkomen, vanwege de aanwezigheid van een klein aantal methanogenen in de reactor en de korte verdubbelingstijden. Desondanks beschouwen we de toepassing van zo'n hittebehandeling interessant, temeer omdat een dergelijke behandeling in principe slechts eenmalig hoeft worden uitgevoerd en bovendien geen risico vormt voor het milieu, d.w.z. dat er geen potentieel toxische chemicaliën gebruikt worden die in het milieu terecht kunnen komen. De toepassing van chemische remmers, zoals bijvoorbeeld 2-broomethaansulfonaat (BES), acetyleen (Oremland en Capone, 1988) en chloroform (Chidthaisong en Conrad, 2000; Scholten et al., 2000), vormen een potentieel risico wanneer ze in het milieu terechtkomen Bovendien kan adaptatie resulteren in toenemende operationele kosten door een toenemend verbruik om dezelfde mate van remming te bewerkstelligen. De toepassing van continue dosering van 2 g.L^{-1} BES aan een bioreactor was ineffectief voor de onderdrukking van methanogenese, omdat een volledige remming slechts twee dagen kon worden bereikt (Weijma et al, 2002). Daarentegen hebben Sparling et al. (1997) methaanvorming succesvol kunnen remmen in een H$_2$ producerende anaërobe vergister van vast afval met de toevoeging van 1% v/v acetyleen in de gasfase.

In de CO gevoede gas lift reactoren waren de sulfaatreductiesnelheden in het algemeen laag, hoewel een maximale (kortstondige) sulfaatreductiesnelheid van 20 mmol.L^{-1}.dag^{-1} gemeten is in de afwezigheid van methanogenese (Hoofdstuk 7). Dit suggereert dat de sulfaatreductiecapaciteit van een CO gevoede gas lift reactor bedreven bij 55ºC potentieel heeft als eenmaal methanogenese geëlimineerd is. In het geval dat een complete eliminatie van de methanogenese verkregen kan worden dan zou de maximale omgezette hoeveelheid CO, te weten 380 mmol.L^{-1}.dag^{-1} (Fig. 7.8; Hoofdstuk 7) hypothetisch voldoende zijn om meer dan 90 mmol.L^{-1}.dag^{-1} sulfaat te reduceren, hetgeen iets hoger is dan de gerapporteerde sulfaatreductiesnelheid in een thermofiele (55°C) H$_2$/CO$_2$ gevoede gas lift reactor (Van Houten et al., 1997). Aannemende dat *D. carboxydivorans* verantwoordelijk is voor zowel de hydrogenogene CO omzetting alsmede de sulfaatreductie in de reactor, dan zou de sulfide toxiciteit (Hoofdstuk 6) speciale voorzieningen noodzakelijk kunnen maken om deze sulfide concentraties voldoende laag te houden. Maatregelen kunnen de afvangst van H$_2$S uit de gasrecirculatie zijn of de toepassing van een lage hydraulische verblijfstijd (HRT). Een sulfide concentratie van 10 mM, welke de omzetting volledig remt bij een pH van ongeveer 7.2 (Fig. 6.5; Hoofdstuk 6), zou een HRT vereisen van 2.7 uur bij een sulfaatreductiesnelheid van 90 mmol.L^{-1}.dag^{-1}.

Hoofdstuk 8 toont dat sulfaatreductie bij lage HRT (< 3 uur) mogelijk is en dat het bovendien een stabiele sulfaatreductie bevordert (Fig. 8.4B; Hoofdstuk 8). Ondanks de hoge CO omzettingsefficiëntie (> 95%) bij deze HRT en het gegeven dat het merendeel van de geproduceerde H_2 gebruikt wordt voor sulfaatreductie (> 87%), blijven de methanogenen aanwezig en nadat de HRT verhoogd is neemt hun activiteit snel toe ten koste van de sulfaatreductie. De maximale sulfaatreductiesnelheid behaald was ongeveer 17 mmol.L^{-1}.dag^{-1}, welke gelimiteerd was door de hoeveelheid CO gevoed en de CO omzettingsefficiëntie (ongeveer 85%) bij hogere CO belastingen (106 mmol.L^{-1}.dag^{-1}), waarschijnlijk als een gevolg van beperkte biomassaretentie in de reactor. Niettemin is bij lage HRT een stabielere sulfaatreductie gevonden (Hoofdstuk 8) dan in de resultaten gepresenteerd in Hoofdstuk 7. Hoofdstuk 8 laat duidelijk zien dat CO een potentieel interessante elektronendonor kan zijn voor stabiele sulfaatreductie bij hogere temperaturen (55ºC), alhoewel speciale aandacht moet worden besteed aan biomassa retentie en eliminatie van methanogenen. Biomassa retentie door middel van het gebruik van een drager materiaal zoals bijvoorbeeld toegepast door Van Houten et al. (1997) maakt de noodzaak van een volledige eliminatie of onderdrukking van methanogenen groter. Met het oog op de benodigde biomassa retentie zou het gebruik van een anaërobe ondergedompelde membraan bioreactor (SAMBaR; Vallero et al., 2005) een interessante optie kunnen zijn. Dit principe is succesvol toegepast voor sulfaatreductie bij hoge zoutconcentraties gebruik makend van een reincultuur van *Desulfobacter halotolerans* (Vallero et al., 2005) De korte verdubbelingtijden van *D. carboxydivorans* op CO bij 55ºC (1.7 uur; Hoofdstuk 6) in vergelijking met die van *Desulfobacter halotolerans* (t_d = 40 uur) toont aan dat de opstart van een thermofiele CO gevoede sulfaatreducerende SAMBaR waarschijnlijk een korte periode zal behoeven. Om de biomassa concentratie van *D. carboxydivorans* te vergroten kan de toevoeging van glucose tijdens de opstart overwogen worden (Hoofdstuk 6). Wanneer we ervan uitgaan dat *D. carboxydivorans* de dominante sulfaatreduceerder is in de biomassa aanwezig in de reactor in Hoofdstuk 8, dan zou de geschatte specifieke sulfaatreductiesnelheid tussen 0.7 and 1.0 mol.(g eiwit)$^{-1}$.dag^{-1} liggen (Hoofdstuk 8). De sulfaatreductiesnelheid bepaald voor *D. carboxydivorans* in Hoofdstuk 6 (Fig. 6.4) was bijna 0.8 mol.(g eiwit)$^{-1}$.dag^{-1}, zodat zo'n hoge specifieke snelheid met dit organisme mogelijk lijkt. Daarom lijkt de toepassing van biomassaretentie met membranen in een CO of synthesegas gevoede sulfaatreducerende bioreactor

potentieel te hebben met gebruik van ofwel een reincultuur van *D. carboxydivorans* of een hittebehandeld Eerbeek slibmonster.

9.6´ Ontwerp van een biologisch synthesegas zuiveringsproces

Eén van de belangrijkste onderdelen in het ontwerp van een biologische synthesegas zuivering wordt gevormd door de vereiste product specificaties, met name de toegestane CO concentraties. In Hoofdstuk 6 is gevonden dat de biologisch bereikbare minimale CO concentraties met een reincultuur van *D. carboxydivorans* bepaald worden door de thermodynamica en niet zozeer door de affiniteit van de bacterie. Thermodynamische berekeningen toegepast op de resultaten verkregen in Hoofdstuk 4 lieten al zien dat de minimum ΔG voor CO omzetting kleiner is dan -7 kJ.(mol CO)$^{-1}$ (Fig. 4.2B); bij deze waarde voor ΔG is de minimale CO concentratie gelijkgesteld aan de detectielimiet van 400 ppm CO voor de GC gebruikt in dit experiment. Dit is lager dan de waarden verkregen uit chemostaat en veldstudies, waarin catabolische activiteit werd waargenomen indien de geassocieerde vrije energie verandering tenminste -10 kJ per mol catabolische reactie bedroeg (Seitz et al., 1990; Westermann, 1994). Niettemin verloopt CO omzetting door *D. carboxydivorans* zolang $\Delta G <0$, hetgeen een zeer effectieve koppeling tussen energieconservatie en CO omzetting suggereert. De resultaten gepresenteerd in Hoofdstuk 6 laten zien dat de minimale residuele CO concentratie tenminste zo laag als 4-5 ppm kan zijn (Tabel 6.3; Hoofdstuk 6), afhankelijk van de initiële gassamenstelling en P_{CO}. Thermodynamische berekeningen, ervan uitgaande dat CO omzetting stopt bij $\Delta G = 0$, zijn vergeleken met actuele metingen van de CO concentratie, waarbij een goede correlatie gevonden werd (Tabel 6.3; Hoofdstuk 6). De initiële samenstelling, bijvoorbeeld de aanwezigheid van H_2 en CO_2 zoals typerend is voor synthesegas, beïnvloedt de uiteindelijke minimale CO concentratie (Tabel 6.4; Hoofdstuk 6). Dit is geïllustreerd door de minimale CO concentratie die behaald kan worden met synthesegas gemaakt met kolen, 108 ppm bij een totale gasdruk van 101 kPa, terwijl bij 1 MPa de residuele CO concentratie niet tot beneden 1% verwijderd kan worden (Tabel 6.4; Hoofdstuk 6).

Lage uitgaande CO concentraties impliceren dat de CO concentraties in de gasfase van de reactor eveneens laag zullen zijn, hetgeen de massaoverdracht van CO

van de gas naar de vloeistoffase beperkt. Dit leidt tot grote reactorvolumes. De bedrijfsvoering van een synthesegas zuiveringsreactor bij hoge P_{CO} zou de massaoverdracht positief beïnvloeden, maar leidt tot hogere uitgaande CO concentraties. Aan de hand van de groeicurven van *D. carboxydivorans* (Fig. 6.1; Hoofdstuk 6) is het vanzelfsprekend dat de CO omzettingsactiviteit van de biomassa veel groter is dan de CO overdracht naar de vloeistoffase, omdat de maximale CO omzettingsnelheid van 0.8 mol CO.(g eiwit)$^{-1}$.uur^{-1} (Fig. 6.2; Hoofdstuk 6) al vroeg in de exponentiële groeifase bereikt is wanneer de biomassaconcentratie nog heel laag is. Synthesegaszuivering bij hoge P_{CO} vereist het gebruik van gasscheidingstechnologie teneinde een zeer zuiver H_2 gas te verkrijgen of tenminste de selectieve verwijdering van één van de producten om $\Delta G < 0$ te houden. Het laatste zou tot stand kunnen komen door de fysische absorptie van CO_2, bijvoorbeeld met heet kaliumcarbonaat of andere processen gebruikmakend van amine, selexol, sulfinol of rectisol oplossingen (Czuppon et al., 1995). Dit biedt tevens de mogelijkheid tot CO_2 fixatie en geologische opslag (Gale, 2004), en kan daarmee CO_2 emissie naar de atmosfeer voorkomen. Het vrijkomen van zuiver CO_2 zou zelfs bruikbaar kunnen zijn in chemische synthese van bijvoorbeeld methanol (Pruschek et al., 1997) of in de glastuinbouw.

De combinatie van scheidingsprocessen met een biologische CO omzetting zou hoge CO omzettingen mogelijk kunnen maken vanwege de relatief grote massaoverdracht van CO van de gas naar de vloeistoffase bij hoge P_{CO}. Volledige CO omzetting zou verkregen kunnen worden door het niet omgezette CO te recirculeren naar de reactor nadat zowel CO_2 als H_2 uit het productgas zijn afgescheiden. De waterstof aanwezig in het productgas zou afgescheiden kunnen worden door middel van bijvoorbeeld een pressure swing adsorptie (PSA) proces of door een selectief gasscheidingsmembraan (Koros and Mahajan, 2000; Perry et al., 1997). Hoewel gewoonlijk het PSA proces geprefereerd wordt wanneer zeer zuiver H_2 gewenst is (Koros and Mahajan, 2000), lijkt het gebruik van gasscheidingsmembranen met name interessant voor een toepassing in synthesegas zuivering. De reden hiervoor is dat het eenvoudig toegepast kan worden in de recirculatiegasstroom van de reactor, terwijl de toepassing van een PSA proces het gebruik van meerdere units nodig maakt om een continue bedrijfsvoering te kunnen garanderen. De zuiverheid van het geproduceerde H_2 met een PSA proces kan 99.9999% bereiken bij een H_2 terugwinning tussen 50-95%, terwijl bij gebruik van membranen de zuiverheid iets lager is (maximaal 99.9%)

bij een terugwinning tot 98% (Air Liquide technische specificaties). PSA systemen kunnen worden toegepast tussen 500-5000 kPa en selectieve gasscheidingsmembranen kunnen een druk tot 8 MPa aan (Air Liquide technische specificaties). Dit vormt een extra reden om de bioreactor bij hogere druk te bedrijven. Bovendien wordt synthesegas geproduceerd bij hoge druk (> 2 MPa; Czuppon et al., 1995), en alternerend druk verlagen en verhogen zal resulteren in toenemende operationele kosten. De bovenlimiet voor P_{CO} zal echter nog bepaald moeten worden om te kunnen bepalen tot welke druk een CO conversie bioreactor kan worden bedreven. Een mogelijk synthesegas zuiveringsproces, waarin zowel de H_2 afscheiding en selectieve CO_2 terugwinning is opgenomen, is schematisch weergegeven in Fig. 9.3'.
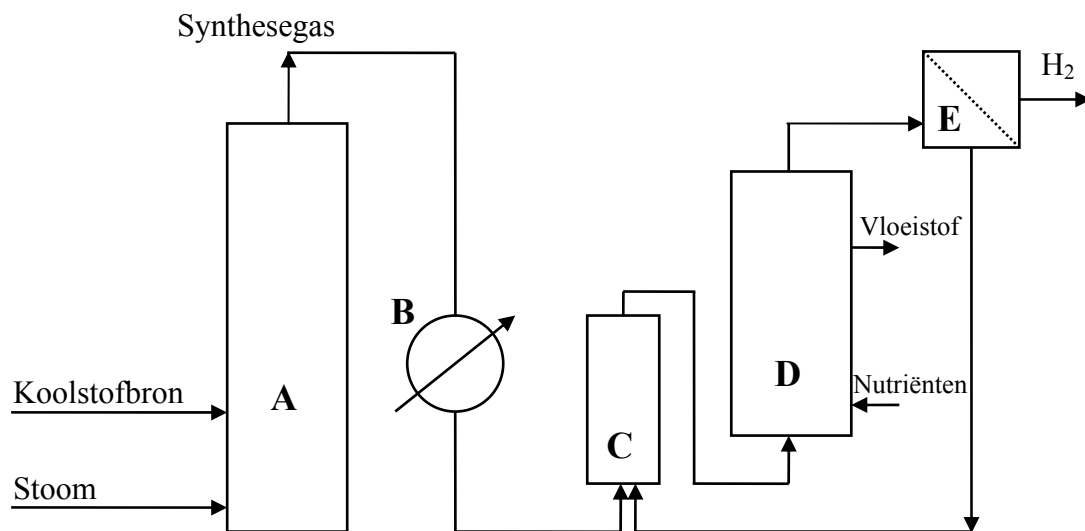
Fig. 9.3' Voorgestelde synthesegas zuiveringsproces inclusief synthesegas productie reactor (A), warmte terugwinning (B), CO_2 absorptie (C), biologische CO omzettingsreactor (D) en selectieve afscheiding van zeer zuiver H_2 gas met behulp van een membraan (E).

Voor de hydrogenogene CO omzetting lijken twee bestaande bioreactor concepten bijzonder interessant, te weten biotrickling filters (Plaggemeier en Lämmerzahl, 2000) en monolith biofilm reactoren (Ebrahimi et al., 2005; Ariga et al., 1986). Synthesegas zuivering zou in principe slechts een klein vloeistof influent behoeven om de biomassa van nutriënten te voorzien en om accumulatie van zouten te voorkomen. Dunne vloeistoffilms zijn bovendien voordelig voor de massaoverdracht van het gas naar de biomassa. In beide reactor typen kan de biomassa als biofilm groeien of kan

de biomassa in suspensie over het oppervlak gesproeid worden. Bioreactoren gebruik makend van cel suspensies, bestaande uit individuele cellen of kleine vlokken, hebben een lagere massaoverdrachtsweerstand dan biofilm reactoren, omdat de enige massaoverdrachtsweerstand in cel suspensie reactoren de overdracht van de gas naar de vloeistoffase is (Nicolella et al., 2000). In Hoofdstuk 6 is de theoretische volumetrische CO omzettingssnelheid in een biotrickling filter bepaald, uitgaande van een k_La van 0.015 s^{-1} (Klasson et al., 1992), die geschat is op 40 m^3.m^{-3} reactor volume onder standaardcondities (25ºC en 101.325 kPa) met synthesegas afkomstig van kolenvergassing bij een P$_{CO}$ van 200 kPa. Omdat de CO concentratie van kolen geproduceerde synthesegas ongeveer 50% is, correspondeert dit met een synthesegas behandeling van 80 m^3.m^{-3} reactor volume.

9.7´ Aanbevelingen voor nader onderzoek

De aanwezigheid van thermofiele micro-organismen, die in staat zijn tot snelle hydrogenogene CO omzettingen en tevens sulfaat reduceren in de aanwezigheid van hoge concentraties CO, maakt het gebruik van synthesegas in biotechnologische toepassingen mogelijk. Voorbeelden hiervan zijn de zuivering van synthesegas en het gebruik van CO-rijk synthesegas als elektronendonor voor de behandeling van sulfaatrijk afvalwater.

Om een biologische synthesegas zuivering verder te ontwikkelen zouden op laboratorium schaal continue experimenten verricht moeten worden voor een nadere bepaling van de lange termijn resultaten. Tevens verdient aanbeveling om mogelijke methoden om H$_2$ consumptie te minimaliseren of liever te elimineren te bestuderen, alsmede het beste type entmateriaal (slib of reincultuur) en de noodzaak om al dan niet de reactor onder steriele condities te bedrijven. Bovendien zouden de resultaten van deze experimenten moeten leiden tot een vergelijking van een dergelijk biologisch systeem met de momenteel toegepaste chemische katalytische reactoren om de eventuele economische voordelen in te schatten.

In het geval van het gebruik van *D. carboxydivorans* of Eerbeek slib in synthesegas gevoede thermofiele sulfaatreducerende bioreactoren, zou speciaal aandacht besteed moeten worden aan de sulfide toxiciteit alsmede de competitie voor H$_2$ door de verschillende aanwezige micro-organismen. Wanneer wordt aangenomen dat speciale voorzieningen om de sulfide concentraties laag te houden noodzakelijk

zijn, dan zal dit resulteren in hogere behandelingskosten. Hetzelfde geldt wanneer complexere en duurdere bioreactoren gebruikt moeten worden, zoals bijvoorbeeld het SAMBaR type (Vallero et al., 2005). Dit om te kunnen werken met celsuspensies onder steriele condities indien de competitie voor H_2 met slib niet in de richting van sulfaatreductie gestuurd kan worden. Daarna zou bepaald moeten worden of het directe gebruik van CO bevattend synthesegas economische voordelen oplevert in vergelijking met het gebruik van bijvoorbeeld puur H_2 gas of methanol.

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Curriculum Vitae

Jan Sipma was born on February the 5th 1972 in Leek, the Netherlands. He obtained his BSc degree in environmental sciences in 1994 at the Prof. H.C. van Hall Instituut in Groningen. In 1994, he continued his education in environmental technology at Wageningen University (former Wageningen Agricultural University). In 1998 he received his MSc degree, with the specialization treatment and reuse of wastewater. During his MSc he performed two research studies anaerobic wastewater treatment at the sub-Department of Environmental Technology ('The effect of staging on the anaerobic degradation of acetate in sulfidogenic reactors' and 'Thermophilic acidification and sulfate reduction at pH 6').

After finishing his MSc study, he worked for three months on anaerobic wastewater treatment at Kirin brewery, in Hiroshima, Japan (employed by the engineering and consultancy company Grontmij Water en Reststoffen BV, in De Bilt, the Netherlands). From August 1998 until April 2000 he was employed as scientific researcher at the sub-Department of Environmental Technology (Wageningen University) on a project financed by Paques BV (Balk, the Netherlands) and UOP LLC (Des Plaines, Illinois, USA). In this project, aerobic and anaerobic biodegradation of mercaptans was investigated, aiming at the development of a biological alternative for treatment of volatile organic sulfur containing waste streams from the petrochemical industry. In September 2000 he started his PhD research at the sub-Department of Environmental Technology (Wageningen University). At this moment the author works as a postdoc at Escola Superior de Biotecnologia (Universidade Católica Portuguesa) in Porto, Portugal where he conducts research on the treatment of halogenated compounds in wastewater.

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Picture on the back cover: Glucose grown culture of *Desulfotomaculum carboxydivorans* (photo by Sonja Parshina)

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