Renze Tjeert van Houten

Biological sulphate reduction with synthesis gas

Proefschrift

ter verkrijging van de graad van doctor,
op gezag van de rector magnificus,
dr. C.M. Karssen
in het openbaar te verdedigen
op woensdag 11 september 1996
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen

Promotor: dr.ir. G. Lettinga
bijzonder hoogleraar in de anaërobe waterzuivering

Stellingen

1. Sulfaat-reducerende bacteriën zijn zeer goed in staat aggregaten te vormen met een bezinksnelheid die groot genoeg is om biomassa retentie in gas-lift reactoren mogelijk te maken.
   Dit proefschrift

2. Het effect van de storende toxiciteit van CO voor sulfaat-reducerende bacteriën kan significant worden tegengegaan door deze bacteriën samen met *Acetobacterium* sp. te laten groeien.
   Dit proefschrift

3. De meeste biofilm modellen staan ver van de experimentele werkelijkheid en hebben derhalve weinig wetenschappelijke waarde.
   Dit proefschrift

4. De door Elferink *et al.* gebruikte term 'substrate affinity constant' voor de Monod parameter K_m is onjuist, daar de 'substrate affinity constant' een theoretisch af te leiden grootheid is met een fysische betekenis en de werkelijke Monod parameter K_m een fit-parameter voor celgroei is zonder werkelijke fysische betekenis.
   Oude Elferink, S.J.W.H. et al. 1993. FEMS Microbiol. Rev. 15:119-136
   Monod, J. 1949. Ann. Rev. Microbiol. 3:371-394

5. De door du Preez *et al.* getrokken conclusies omtrent de reactiesnelheden van sulfaat reductie op waterstof zijn uiterst twijfelachtig daar in hun publikatie tevens melding wordt gemaakt van continue lekkage van waterstof uit de experimentele opstelling.
   du Preez, L.A. and J.P. Maree. 1994. Wat. Sci. Technol. 30(12):275-285

6. De conclusies van Kargi en Toprak, dat de keuze van dragerdeeltjes voor fluïde bed bioreactoren zich zou moeten richten op deeltjes met een zo groot mogelijk oppervlak en een zo groot mogelijke biomassa retentie, zijn weinig vernieuwend.
   Kargi, F. and H. Toprak. 1994. J. Chem. Tech. Biotechnol. 59:201-204.
   Heijnen, J.J. 1984. Biological industrial wastewater treatment - minimizing biomass production and maximizing biomass retention. Ph.D. thesis, Delft Technical University, The Netherlands

7. Een goede inleiding is voor herhaling vatbaar.
   Plumb, P. et al. 1990. Appl. Biochem. Biotechnol. 24/25:785-797
   Deshmane, V. et al. 1993. Appl. Biochem. Biotechnol. 39/40:739-752
   Dasu, B. et al. 1993. Fuel 72:1705-1714
   Lee, C.-M. and K.L. Sublette. 1994. Appl. Biochem. Biotechnol. 45/46:417-428

8. Het is voor de lezer van het proefschrift van C.J. van Ede niet duidelijk op welke wijze zijn stelling "(Numerieke) modellen voor de beschrijving van biofilms zijn vaak nodeloos ingewikkeld", met verwijzing naar zijn eigen proefschrift, moet worden uitgelegd.
   van Ede, C.J. 1994. Bioconversions catalyzed by growing immobilized bacteria. Ph.D. thesis, Groningen State University, The Netherlands

9. Het grote aantal naamswijzigingen van micro-organismen in de afgelopen jaren toont aan dat het soortbegrip, en de soortdefinitie, in de microbiologie nodig moeten worden herzien.

10. Aangezien het in het midden- en kleinbedrijf (MKB) ontbreekt aan de nodige 'kennisdragers' zijn voor deze categorie bedrijven de grote technologische instituten van groot belang voor de noodzakelijke kennistransfer van universiteit naar bedrijf.

11. Het is een zorgwekkende ontwikkeling dat het huidige economische bestel in Nederland geen plaats (meer) biedt aan bedrijven die produkten zonder aanzienlijke toegevoegde waarde - in de vorm van kennis - produceren.

12. De steeds frequenter gehouden 'vrolijkheidssessies' van diverse populaire NLP-goeroe's, ter overwinning van een negatief zelfbeeld, gaan volledig voorbij aan de ernst en duur van sommige, eerder in het leven opgedane, traumatische ervaringen.

13. Derhalve zijn deze sessies in ernstige vorm van oplichterij.

14. Het is op z'n minst merkwaardig te noemen dat de Nederlandse overheid enerzijds het autogebruik wil reduceren doch anderzijds de arbeidsmarkt flexibel maakt door het opstellen van kortdurende, projectgebonden, arbeidscontracten te stimuleren.

15. Aan Nederlandse universiteiten wordt men eenvoudiger hoogleraar dan universitair docent.

16. De conclusie dat waterverontreiniging negatief zou werken op de vruchtbaarheid van de mens lijkt in tegenspraak met waarnemingen bij de vakgroep Milieutechnologie.

17. Corpulente mensen zijn niet dik, ze bezitten een grotere ruimtelijke kwaliteit.

Stellingen behorende bij het proefschrift "Biological sulphate reduction with synthesis gas" van Renze T. van Houten.

Wageningen, 11 september 1996

Abstract

Van Houten, R.T. (1996). Biological sulphate reduction with synthesis gas. Ph.D. thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

The objectives of this thesis are (1) to study the feasibility of using synthesis gas as electron donor and carbon source for biological sulphate reduction and (2) to develop criteria for design and operation of gas-lift bioreactors for sulphate reduction using immobilized biomass.
At appeared that sulphate-reducing bacteria, grown on H_2/CO_2, formed stable biofilms on pumice particles. Biofilm formation was not observed when basalt particles were used. However, use of basalt particles led to the formation of aggregates of sulphate-reducing biomass. The sulphate-reducing bacteria grown on pumice particles easily adapted to free H_2S concentrations up to 450 mg/L. These high free H_2S concentrations caused reversible inhibition rather than acute toxicity. When free H_2S concentrations were kept below 450 mg/L, a maximum sulphate conversion rate of 30 g SO_4^{2-}/L.d could be achieved after only 10 days of operation. Gas to liquid mass transfer capacity of the reactor determined this maximum sulphate conversion rate.
Furthermore, biological sulphate reduction appeared to be applicable within a pH range of 5.5 to 8.0, with an optimum near pH 7.5. The pH affected aggregate configuration and diameter. At pH 7.0, the average Sauter mean diameter of the aggregates was 1.5 mm. Moreover, phase-contrast and SEM microscopy showed highly branched aggregate surfaces. A pH increase led to increased surface irregularity without affecting the particle diameter. A pH decrease caused a decreased surface irregularity and changed the aggregate Sauter mean diameter from 1.50 mm at pH 7.0 to 2.26 at pH 5.5. However, the pH did not have a significant effect on the biomass composition. Examination of the bacterial composition of the aggregates by phase-contrast microscopy, SEM microscopy as well as enrichments showed that at all pHs *Desulfovibrio* sp. and *Acetobacterium* sp. were the most abundant micro-organisms.
When sulphate reduction was carried out with synthesis gas as electron donor and carbon source, the reactor performance was strongly affected. Addition of 5% CO negatively affected the overall sulphate conversion rate, i.e. it dropped from 12 - 14 g SO_4^{2-}/L per day to 6 - 8 g SO_4^{2-}/L per day. However, a further increase of CO to 10 and 20% did not further deteriorate the process. With external biomass recycling the sulphate conversion rate could be improved to 10 g SO_4^{2-}/L per day. Therefore biomass retention clearly could be regarded as the rate limiting step. Furthermore, CO affected the aggregate shape and diameter. SEM photographs showed that rough aggregates, pre-grown on H_2/CO_2, changed into smooth aggregates upon addition of CO. Addition of CO also changed the aggregate Sauter mean diameter (d_{32}) from 1.7 mm at 5% CO to 2.1 mm at 20% CO. After addition of CO, a layered biomass structure developed. *Acetobacterium* sp. were mainly located at the outside of the aggregates, whereas *Desulfovibrio* sp. were located inside the aggregates.
Additionally, thermophilic (55 °C) sulphate and sulphite reduction was studied. The results of the experiments clearly demonstrated that sulphate conversion rates up to 7.5 g SO_4^{2-}/L per day can be achieved. With sulphite a reduction rate of 3.7 g S/L per day was obtained, which equals a sulphate conversion rate of 11.1 g SO_4^{2-}/L per day. Under the applied conditions, a strong competition for hydrogen between hydrogenotrophic sulphate-reducers, designated as *Desulfotomaculum* sp., and hydrogenotrophic methanogens was observed. Growth of the mixed culture was totally inhibited at a H_2S concentration of 250 mg/L. Poor attachment of sulphate-reducing bacteria was observed in all experiments. The biomass concentration did not exceed 1.2 g/L, despite the presence of 50 g/L of pumice.
Based in the abovementioned results it is concluded that both aims of the thesis are attained. First, biological sulphate reduction appears to be feasible. Additionally, a number of criteria for design and operation of gas-lift bioreactors for sulphate reduction were developed and discussed. Finally, several recommendations for future research are given.

Keywords: sulphate reduction • biofilm • aggregation • immobilization • gas-lift reactor • mass transfer

Preface

The title of the present publication is, like most titles, inaccurate. I have not attempted to present all features of biological sulphate reduction using synthesis gas, but concentrated my efforts on applied aspects of biofilm formation and aggregation, kinetics, mass transfer and rate optimization.

Chapter 1 presents the historical background to current research on applications of biological sulphate reduction. Also its relevance for society is discussed.

Chapter 2 describes reactor engineering aspects of biological sulphate reduction using hydrogen as energy source and carbon dioxide as carbon source.

Microbial aspects of sulphate reduction with hydrogen and carbon dioxide are investigated in *Chapter 3*. Special attention is paid to the microbial composition of the obtained aggregates.

Additionally, *Chapter 4* presents results concerning the optimization of biological sulphate reduction using real synthesis gas as combined electron donor and carbon source.

In *Chapter 5* thermophilic sulphate and sulphite reduction with hydrogen and carbon dioxide is investigated.

Finally, *Chapter 6* concludes this thesis with a general discussion of the results obtained. In addition, an outlook is given to future research.

The chapters in this thesis are presented as independent contributions (published or to be published), each of which can be read separately. Necessarily this means some repetition of theories and methods. Each chapter has been closed with literature references and, if necessary, a list of symbols used.

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Several people have directly or indirectly contributed to this thesis. I would like to thank them here, because this thesis would not have appeared without their sustained help.

First of all my 'boss', Gatze Lettinga. His stimulating enthusiasm and optimism supported my research a great deal.

Secondly, my students who joined the smelly world of sulphate reduction. In order of appearance, Marriët, Ingeborg, Geert, Lars, Sandra, Hielke and Shang. All of them worked with great enthusiasm and devotion to their specific part of research. As a result, most of their work has been published.

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It was a pleasure to work together with Stefanie Oude Elferink and Fons Stams of the Department of Microbiology. Our discussions were a rich source of ideas, some of which have been published.

Adriaan van Aelst, who created most beautiful SEM-pictures, which contributed significantly to the understanding of population dynamics in sulphate-reducing aggregates.

My roommates and colleagues, Adrie Veeken and Sjon Kortekaas, thanks to them Friday afternoon started sometimes in the middle of the week.

Also very fruitful for my research were the several discussions with Bert Hamelers, who showed me some real basic principles of scientific research, resulting in always animating and interesting discussions.

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A dedicated analytical team at the Department of Environmental Technology has supported me a great deal: Ilse Bennehey and Johannes van der Laan for the chromatographic determinations; and Jo Ackerman-Jacobs, Sjoerd Hobma for their willingness to help otherwise.

Additionally, I want to thank all other colleagues and students of the Department of Environmental Technology for creating a very pleasant (non)working atmosphere. VIPs among them were Miriam, Robbert and Jules.

Furthermore, I want to express my gratitude to the people of Mediaservice for their rapid and accurate delivery of slides and photographs. Special thanks belong to Boudewijn van Veen. His immense knowledge of, and enthusiasm for computer assisted image analyses and desk-top publishing software often resulted in a complete over-kill of information on my part.

Acknowledgements should likewise be extended to all the staff of the technical services and glass works, who made any required experimental set-up.

Finally, I am greatly indebted to my wife for her unerring support, even in periods when I was under stress. Agatha, without your never exhausted love and care, life would not have been as complete as it is now. You gave a special meaning to the term 'productivity of a Ph.D. student', resulting in three magnificent children. It has been a tough period for you, suffering the brutishness to which I sank in order to create something that I hope is worthwhile. Therefore, this thesis should actually have two author's names. Unfortunately, universities have their own rules. That's a great pity !

Contents

1

General introduction

Scope of this thesis

A number of industries, in particular chemical, metallurgical and mining industries, produce wastewater containing sulphate or sulphite. Biological sulphate reduction, combined with sulphide removal, may be a feasible method for removal of these sulphur compounds. Since the aforesaid wastewaters do not contain organic compounds, sulphate reduction can only proceed when an electron donor and a carbon source are added. The objectives of this thesis are (1) to study the feasibility of using synthesis gas as a combined electron donor and carbon source for biological sulphate reduction and (2) to develop criteria for design and operation of gas-lift bioreactors for sulphate reduction using immobilized biomass.

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1.1 Background

Since the beginning of man's existence, his activities have influenced the natural biogeochemical cycles of elements. With increasing industial and urban development, the impact of man's actions on the natural cycles has become substantial. To maintain these cycles, there is increasingly more need for rational management of wastes generated by human activity.

Because human life is dependent on water, wastewater treatment is one of the oldest forms of waste management. Biological processes have found wide application in wastewater treatment, because they are substantially cheaper than physical or chemical methods. Especially aerobic processes are widely employed for the removal or conversion of oxygen demanding organic pollutants, sulphur and nitrogen compounds. Until the 1970s, anaerobic wastewater treatment was not considered as a feasible and attractive alternative to aerobic treatment.

During the last decades, however, interest in anaerobic wastewater treatment has increased strongly. A break-through in the application of anaerobic wastewater treatment came with the development of reactors with high biomass retention[70,27,26,38]. The most widely applicated system is undoubtedly the Upflow Anaerobic Sludge Blanket reactor, developed by Lettinga and co-workers[39] in the 1970s, in cooperation with the potato starch and beet sugar refining industries.

Anaerobic wastewater treatment was initially developed for the treatment of organic wastewater of the food industry. However, as the technology matured, applications to other types of wastewater have been explored. The effect of different constituents of industrial wastewater - like aldehydes[85], heavy metals[57], halogenated hydrocarbons[54], aromatic hydrocarbons[18], long-chain fatty acids[85], sulphur compounds[59] and wood constituents[68] - have received considerable research attention. It appears that many of the toxic compounds that are frequently present in industrial wastewaters do not necessarily rule out the possibility of anaerobic treatment. In many cases, subtoxic concentrations of such compounds can be tolerated or anaerobic bacteria can adapt to the toxic concentrations. Moreover, the toxic compounds may undergo transformations by anaerobic bacteria that lead to compounds of decreased toxicity and, for certain compounds, adjusting the environmental conditions (i.e. pH) can minimize the inhibition effect. Major problems arise in anaerobic treatment, when industrial wastewaters with high sulphate or sulphite content are treated. Under appropriate conditions, sulphate can be converted into hydrogen sulphide by sulphate-reducing bacteria. This biological sulphate reduction has several disadvantages:

- soluble hydrogen sulphide concentrations, if sufficiently high, reduce the amount of methane produced from a given amount of organic waste;

- hydrogen sulphide is inhibitory to several types of anaerobic bacteria, effecting the efficiency of the treatment process;
- part of the hydrogen sulphide is transferred to the biogas, causing corrosion problems in engines and boilers;
- the remainder of the hydrogen sulphide will be present in the effluent causing lower purification efficiency and malodor problems.

In the past, sulphate reduction was considered unfavourable because of the above mentioned problems. Therefore, several studies aimed at inhibition of sulphate reduction during anaerobic wastewater treatment[65].

1.2 Sulphate containing wastewaters

At present, there is an increasing attention for sulphate reduction processes which can be attributed to some interesting potentials, i.e.:

- when combined with sulphide removal, sulphate reduction can be used as a biological method for sulphate removal;
- formation of metallic sulphide precipitates can remove or alleviate toxicity of heavy metal ions;
- sulphite toxicity during anaerobic treatment can be elevated by reduction of sulphite to hydrogen sulphide.

To what extend sulphate reduction will predominate over methanogenesis, will depend on the organic substrate (Chemical Oxygen Demand) to sulphate ratio of the wastewater and the type of organic substrate as well. Wastewaters from the fermentation industry, starch industry, edible oil industry and pulp and paper factories contain high concentrations of sulphate, sulphite or other sulphur compounds. The C.O.D. to sulphate ratio in these wastewaters may be low enough to degrade most of the C.O.D. via sulphate reduction. Thus organic compounds and sulphate are removed with hardly any concomitant methanogenesis. This subject has been discussed extensively by Oude Elferink *et al.*[59] and Visser[79].

Sulphate reduction processes may become particularly important when removal of sulphate from wastewater is necessary, and they do not contain any organic compound. Examples of this category of wastewater are waste sulphuric acid from chemical, metallurgical, farmaceutical and mining industries, and sulphite liquor from flue-gas scrubbing. Table 1.1 provides general information about the wastewater composition in some of the aforementioned industries.

Currently, most of these industrial wastewaters are either discharged to local sewage works or neutralized with lime, which results in the production of large quantities of qypsum. In view of environmental considerations both options now have to be reconsidered. Biological sulphate reduction could represent an attractive alternative for these methods, provided that a proper electron donor and carbon source can be made available.

Table 1.1. Some examples of the composition of industrial wastewater containing sulphate or sulphite.

branch of industry	sulphate [g/L]	sulphite [g/L]	pH	ref.
chemical industry	0.2 - 50	0 - 5	1 - 7	
metallurgical industry	0.2 - 50	0 - 25	1 - 7	
mining industry	0.2 - 20	-	2 - 7	42,35,22,4
sulphite liquor from flue-gas scrubbing	1 - 2	1 - 2	7.5	2

An interesting and elegant example of a process in which sulphate reduction could play a key-role, is bioleaching of heavy metal contaminated solid wastes. In this process, schematically represented in Figure 1.1, conversions of the biological sulphur cycle are employed [17,74].

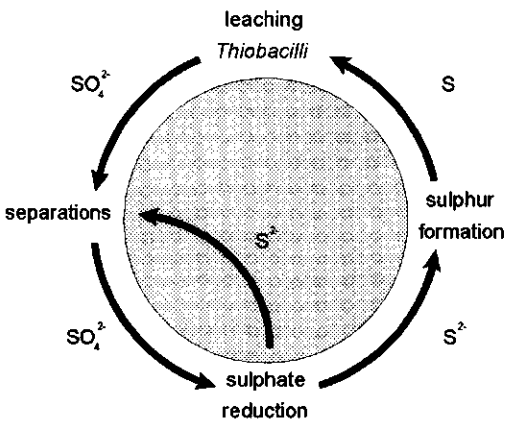

Figure 1.1. Sulphur cycle in the bioleaching process.

In this process, waste materials are leached with sulphuric acid, produced from sulphur by acidophilic *Thiobacilli* spp. Then, the heavy metals, extracted from the waste, are present in an inorganic sulphate rich wastestream. After removal of the heavy metals by sulphide precipitation, this waste sulphuric acid is recycled in two stages to solid sulphur. In the first stage, sulphate is biologically converted to hydrogen sulphide. The second stage converts hydrogen sulphide to solid sulphur by colourless sulphur bacteria, which is a proven technology[9,10]. The produced sulphur sludge can be reused for the bioleaching of waste materials. In this way, the biological sulphur cycle is closed and no sulphate is discharged to the environment.

1.3 Selection of electron donor for biological sulphate reduction

As mentioned before biological sulphate reduction is only possible if a proper electron donor is present in the sulphate containing wastewater. The economic feasibility of this process depends on two factors:

- cost of the added electron donor, per unit sulphate converted to sulphide.
- the added electron donor should cause little, if any, remaining pollution, which always should be easy to remove.

Low cost electron donors to be considered are organic waste materials like molasses, vinasses and whey, as well as high quality bulk chemicals like hydrogen gas, methanol and ethanol. Because the selection of electron donors partly will depend on the physiology of the bacteria involved, some relevant information on the microbiology of sulphate-reducing bacteria is presented.

Microbiology of dissimilatory sulphate reduction

Physiology

The characteristic feature of sulphate-reducing bacteria is the capacity to use sulphate as the terminal electron acceptor during the anaerobic oxidation of substrates. During electron transport, energy is gained by electron transport phosphorylation. A proposed pathway for the dissimilatory reduction of sulphate to sulphide by sulphate-reducing bacteria is shown in figure 1.2.

Sulphate-reducing bacteria usually produce sulphide as the sole final product. Only in two cases, excretion of minor amounts of sulphite or thiosulphate as end products was reported[20,76]. Since the free sulphate anion is not a suitable electron acceptor, it is first activated by ATP

sulphurylase; the product is adenosine-5'-phosphosulphate or APS. APS is the actual electron acceptor which is converted to bisulphite and AMP. The bisulphite formed is then reduced to sulphide.

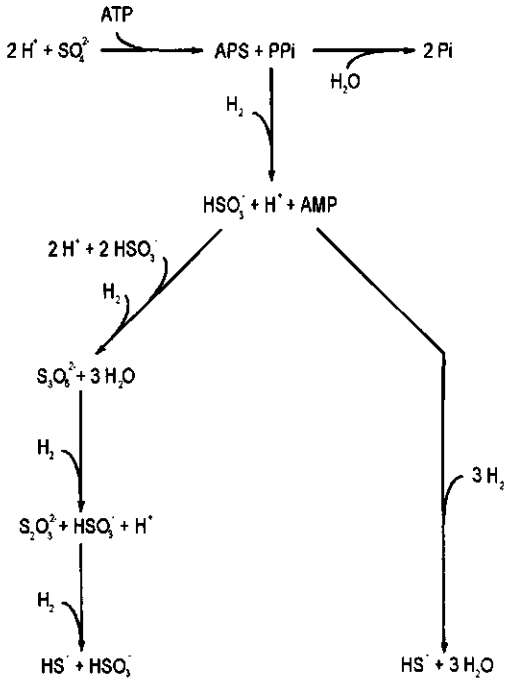

Figure 1.2. *Possible pathways for the dissimilatory reduction of sulphate to sulphide by sulphate-reducing bacteria (adapted from Faugue et al.[19]).*

Sulphate-reducing bacteria are capable of using hydrogen and several organic compounds as electron donors. For detailed information, the reader is referred to Widdel[80,81], Widdel and Bak[82], Widdel and Hansen[83]. Some of the reactions carried out by sulphate-reducing bacteria are listed in Table 1.2.

***Table 1.2.** Some sulphate-reducing reactions carried out by sulphate-reducing bacteria.*

			$\Delta G^{o'}$ * [kJ/mol]
$4\ H_2 + SO_4^{2-} + H^+$	→	$HS^- + 4\ H_2O$	-38.1
$Acetate^- + SO_4^{2-}$	→	$2\ HCO_3^- + HS^-$	-47.6
$Propionate^- + ¾\ SO_4^{2-}$	→	$Acetate^- + HCO_3^- + ¾\ HS^- + ¼\ H^+$	-37.7
$Butyrate^- + ½\ SO_4^{2-}$	→	$2\ Acetate^- + ½\ HS^- + ½\ H^+$	-27.8
$Lactate^- + ½\ SO_4^{2-}$	→	$Acetate^- + HCO_3^- + ½\ HS^- + ½\ H^+$	-80.0
$Ethanol + ½\ SO_4^{2-}$	→	$Acetate^- + ½\ HS^- + ½\ H^+ + H_2O$	-66.4

* $G^{o'}$-values are taken from Thauer *et al.*[73]

Both autotrophic and heterotrophic growth on hydrogen is possible. The classical *Desulfovibrio* sp. require acetate as carbon source, whereas e.g. *Desulfobacterium* sp. can use CO_2 as the only carbon source[82,83]. Many sulphate-reducing bacteria can degrade short-chain fatty acids; some oxidize these fatty acids completely to carbon dioxide, while others oxidize these compounds to acetate. Other substrates, which can be completely or incompletely degraded by sulphate-reducing bacteria, are branched-chain fatty acids, long-chain fatty acids, several alcohols, organic acids - e.g. lactate and malate - and aromatic compounds like benzoate, catechol, phenol, indole and aniline[80,81,82,83]. Therefore, it appears that several types of electron donors potentially can be selected. However, one should consider that also other micro-organisms - like methanogens and acetogens - are able to use these substrates, or at least intermediates (like acetate) formed during their degradation. Then, it is necessary to consider the thermodynamics of the conversion reactions and, more importantly, the kinetic properties of the sulphate-reducing bacteria as well as the competing micro-organisms. Figure 1.3 showes a scheme for this competition.

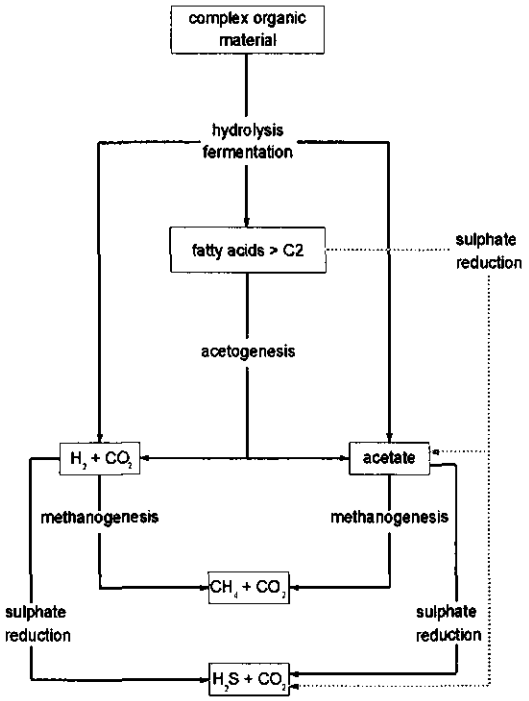

Figure 1.3. Substrate competition between sulphate-reducing and methanogenic or acetogenic bacteria.

Competition

Competition between sulphate-reducing bacteria, methanogens and acetogens has been reviewed extensively[59,65] and we will suffice with a brief description here. An examination of literature data clearly shows that methanogens will be outcompeted for hydrogen, because sulphate-reducing bacteria generally combine a higher maximum specific growth rate with a lower affinity constant and a higher yield[59]. Information available with regard to acetate utilization is less clear. The kinetic data from ecological studies[43,84,3,69] seem to indicate an advantage for sulphate-reducing bacteria in marine and freshwater sediments. However, for anaerobic digesters it is less clear how acetate is degraded. Experiments conducted with granular sludge from UASB reactors gave conflicting results. Therefore, a complete conversion of acetate to methane, even in presence of an excess of sulphate, has been reported[28,29,30,88,78], while the results of other studies revealed that acetoclastic sulphate-reducing bacteria will become

important[1,77]. Until now it is still unknown whether these conflicting results are due to the kinetic properties of the bacteria involved, to a combination of kinetics and transport phenomena in the biofilm or to a difference in adherence capacity of the various competing bacteria to carrier materials or bacterial aggregates.

Possible electron donors for biological sulphate reduction

Electron donors available in large quantities can be divided roughly into two groups:
- organic waste materials
- bulk chemicals

Waste materials

Organic waste materials are less suitable as electron donors for two reasons. Firstly, the use of these materials is generally accompanied by an additional pollution of the wastewater and therefore a supplementary treatment process is needed to remove the remaining pollutants in order to create a clean effluent. Although use of organic waste materials has been proposed in the literature before the 1980s[11,12,75], most of these studies with this category of electron donors were carried out during the last decade. Maree and Strydon[45] used molasses, sewage sludge and pulp mill wastewater as electron donors. They found relatively low sulphate conversion rates - less than 2.5 g $SO_4^{2-}/L_{reactor}.d$ - while it appeared also difficult to remove all the C.O.D. from the wastewater. Particularly pulp mill wastewater contained poorly biodegradable compounds and, as a result, the effluent contained 18 g C.O.D/L. Further research of Maree *et al.*[46-51] was mainly based on the use of molasses. All the proposed processes consisted of two or three stages. Sulphate is reduced to hydrogen sulphide in the primary anaerobic stage in the presence of excess molasses. Remaining, poorly degradable, soluble organic matter is removed in the second stage, which is either an aerobic or an anaerobic stage. Part of the pollution even may consist of non-biodegradable organic matter and then a non-biological post-treatment step may be required. Maximum achieved sulphate conversion rates were up to 6.4 g SO_4^{2-}/l.d. Similar sulphate conversion rates - 4.1 g SO_4^{2-}/l.d - were obtained with cheese-whey as electron donor[56] but also in that case an additional effluent-polishing is needed.

Besides these aforesaid pollution problems, other problems might occur as well. Intermediates and/or end products of the degradation of organic matter, for example hydrogen, alcohols and lower fatty acids are used by either sulphate reducing-bacteria or acetogenic and methanogenic bacteria. Thus competition will arise between these three groups of bacteria, as mentioned before.

Bulk chemicals

The use of relatively pure, fully degradable bulk chemicals offers a number of substantial advantages. First of all, no remaining pollution will exist and therefore any post-treatment is not required. Secondly, pure chemicals have a well defined composition, hence it is less difficult to understand, to describe and to predict the bioprocess of sulphate reduction. Additionally, results are easier to compare with literature data. For large scale use, the chemicals obviously should be as cheap as possible. Possible candidates for use are: ethanol, methanol and synthesis gas (i.e. gas mixture of H_2, CO and CO_2).

Ethanol represents a substrate that supports fast growth of sulphate reducing bacteria[61,71,83]. Ethanol is rapidly oxidized to acetate, by sulphate reducing bacteria according to:

$$2\ CH_3CH_2OH + SO_4^{2-} \rightarrow 2\ CH_3COO^- + H^+ + HS^- + 2\ H_2O$$

Acetate is used as carbon source or is oxidized further to carbon dioxide:

$$CH_3COOH + SO_4^{2-} \rightarrow 2\ HCO_3^- + HS^- + H^+$$

Methanogenic bacteria however, can also use acetate and hydrogen as substrate and therefore sulphate-reducing bacteria and the methanogenic bacteria will compete for the use of acetate.

A cheaper possible chemical to be used is methanol. Methanol is already widely used for denitrification in wastewater treatment processes[64]. Although its use as electron donor for sulphate reduction is described in literature, all of the isolated organisms were found to have very low growth rates. Moreover, according to some authors[6,55], methanol only can be used as an electron donor, thus an additional carbon source has to be added. An important additional complicating factor is that probably sulphate-reducing bacteria, methanogenic bacteria and acetogenic bacteria will compete for the use of methanol. Oremland and Polcin[58] even showed that methanogenic bacteria can out-compete sulphate-reducing bacteria for methanol and therefore it is doubtful whether methanol is a suitable electron donor for sulphate reduction in an engineered environment. It is clear that more information is needed on substrate utilization and competition in order to determine the feasibility of methanol as electron donor.

The third option is the use of synthesis gas (gas mixture of H_2, CO and CO_2), already described in the literature by Du Preez and co-workers[62]. The advantage of using this electron donor is the

presence of hydrogen, representing a direct electron donor for sulphate-reducers. Sulphate-reducing bacteria will certainly outcompete methanogenic bacteria for hydrogen[59,65]. Carbon dioxide can be used as a carbon source[83]. However, the presence of carbon monoxide might introduce some problems. Little is known about the capability of sulphate-reducing bacteria to grow (autotrophically) on carbon monoxide. Jansen *et al*[31] report growth of *Desulfovibrio baarsii* on carbon monoxide (1.5% v/v) with formate as electron donor, but unfortunately this strain is unable to use H_2 as an electron donor. According to Karpilova *et al.*[33] and Mukhitova *et al.*[52] carbon monoxide inhibits growth of the sulphate-reducing bacteria *Desulfovibrio desulphuricans*, *Desulfovibrio baculatus* and *Desulfovibrio africanus*. Inhibition of growth was more or less proportional to the carbon monoxide concentration. Lupton *et al.*[44] showed that *Desulfovibrio vulgaris* strain Madison was able to utilize CO as electron donor when present at a concentration up to 4% (v/v) in the gas phase. CO consumption was accompanied by the production of H_2, which in turn was consumed. However, concentrations exceeding 4.5% (v/v) CO were inhibitory to growth, probably due to inhibition of hydrogenase or electron carriers involved in the reduction of sulphate. *Desulfotomaculum* species were reported to grow slowly in presence of CO as the only energy source at concentrations of 5-20% (v/v) in the culture head-space. CO concentrations exceeding 20% (v/v) inhibited growth completely[36].
Karpilova *et al.*[33] note that cell suspensions and cell extracts of *Desulfovibrio desulphuricans* and *Desulfovibrio baculatus* were capable of oxidising CO to CO_2. This water-shift reaction:

$$CO + H_2O \quad \rightarrow \quad CO_2 + H_2$$

was described earlier by Yagi[86] and Yagi and Tamyia[87] for *Desulfovibrio desulphuricans*. The same shift reaction can also be carried out by non-sulphate-reducing micro-organisms growing on carbon monoxide. For example Kluyver and Schnellen[37] used *Methanosarcina barkerii* for this shift conversion. Levy *et al.*[41] described the production of CO_2 from CO by mixed-culture anaerobes. Du Preez *et al.*[62] also reported about the oxidation of CO to CO_2 by micro-organisms living symbiotically with sulphate reducing bacteria. Klasson *et al.*[34] report results of mixed-culture studies employing the photosynthetic bacterium *Rhodospirrilum rubrum* for converting CO to CO_2 and H_2 by the said water shift reaction. The main value of this water-shift reaction is clearly that CO is "converted into" H_2, which is the perfect electron donor for sulphate reduction. Thus the H_2 to $(CO+CO_2)$ ratio of synthesis gas is increased during the biological process.

Another possibility to reduce CO toxicity, is the use of homo-acetogenic bacteria, like *Acetobacterium* spp.[67] and Clostridium spp.[16]. These micro-organisms reduce two molecules of CO or CO_2 to acetate. The acetate produced by these organisms can be used as carbon source for the sulphate-reducing bacteria, while obviously the toxicity of CO for sulphate-reducing bacteria is reduced. A disadvantage of using this type of co-culture might be that the produced acetate is consumed by non-sulphate reducing micro-organisms. The outcome of such competition is highly complex. Factors such as substrate transport inside aggregates; the site of sulphate reduction and its proximity to the site of homo-acetogenesis and pH gradients etc. can have significant but so far greatly unknown effects on the populations involved in the sulphate reduction process.

From the above discussion, it is obvious that for practice it would be very attractive if a process for sulphate reduction could be developed which uses synthesis gas, and where (autotrophic) sulphate-reducing bacteria grow symbiotically with anaerobic bacteria which convert CO either into H_2 and CO_2 or into acetate, in this way preventing CO toxicity for the sulphate-reducing bacteria.

Sources of synthesis gas

The term synthesis gas is applied to a gas mixture containing mainly H_2, CO and CO_2, with minor levels of other components, such as methane. Synthesis gas is used as a major building block in the synthesis of chemicals and fuels. Natural gas, petroleum liquids, coal and biomass may all be readily reformed or partially oxidized to produce synthesis gas suitable for further use. Steam reforming of natural gas generates synthesis gas rich in hydrogen. The primary reactions carried out in methane steam reforming are:

$$CH_4 + H_2O \rightarrow CO + 3\,H_2$$

$$CO + H_2O \rightarrow CO_2 + H_2$$

Heavier feedstocks can be reformed in the same manner, yielding different quantities of CO and H_2 based on their composition. Gasification is the term commonly applied to the partial oxidation of liquid and solid fuels. Generally the following set of reactions proceed:

$$\text{Fuel} + O_2 \rightarrow CO + H_2 + \text{Heat}$$
$$\text{Heat} + \text{Fuel} + \text{Steam} \rightarrow CO + H_2$$
$$CO + H_2O \rightarrow CO_2 + H_2$$

Depending on the fuel being processed, the actual full set of reactions occurring is quite complicated, the more because a complex process like thermal pyrolyses is an important reaction step.

An important factor governing the possibility of using synthesis gas is the $H_2/(CO+CO_2)$ ratio of the gas. By selecting the proper feedstock and applying the proper technology, it is possible to prepare synthesis gas of the desired composition. Usually the synthesis gas composition is altered by addition of CO_2 from an external source to increase the CO concentration. If synthesis gas is produced from heavy oil or coal, the gas is rich in CO; here water gas shifting is used to convert externally added steam to hydrogen at the expense of CO.

Typical compositions of synthesis gas are given in Table 1.3. With natural gas feedstocks, the synthesis gas is rich in hydrogen. Steam reforming of natural gas to produce hydrogen and synthesis gas has been practised commercially since 1930. It is the most widely used method for hydrogen manufacturing in the United States. As for biological sulphate reduction, synthesis gas should contain as much hydrogen as possible, natural gas certainly is an attractive feedstock for synthesis gas production.

***Table 1.3.** Typical composition of synthesis gas.*

component	± 95% conversion of methane [vol. %][23]	heavy oil oxidation [vol. %][23]	coal gasification [vol. %][23,60]	biomass gasification [vol. %][5]
H_2	75.7	46.0	33.0	33.7
CO	15.5	47.0	55.0	30.0
CO_2	8.1	4.3	11.0	26.6
N_2+A	0.2	1.4	0.6	3.2
CH_4	0.5	0.3	0.1	4.9
H_2S	-	1.0	0.3	-
Others	-	-	-	1.6
$H_2/(CO+CO_2)$ volume ratio	3.2	0.90	0.50	0.60

Economy of electron donors

Based on the previous discussion about possible electron donors for sulphate reduction, two electron donors - ethanol and synthesis gas - will be subjected to a simplified economical analysis. Hereto, two molar sulphate reduction rates were selected, representing a small scale and a large scale application. In an existing relatively small biotreatment system - at Budelco B.V., Budel, The Netherlands - the applied reduction rate amounts to 2 kmol sulphate/h[66]. For biological flue-gas desulphurization the required sulphate reduction rate might be up to 85 kmol/h [2]. Therefore, sulphate reduction rates of 2 kmol/h and 20 kmol/h have been used in the calculations below.
The reduction reaction of sulphate with ethanol as the electron donor is as follows:

$$2\,CH_3CH_2OH + SO_4^{2-} \rightarrow 2\,CH_3COO^- + H^+ + HS^- + 2\,H_2O$$

$$2\,CH_3COO^- + 2\,SO_4^{2-} \rightarrow 4\,HCO_3^- + 2\,HS^- \quad +$$

$$2\,CH_3CH_2OH + 3\,SO_4^{2-} \rightarrow 4\,HCO_3^- + 3\,HS^- + H^+ + 2\,H_2O$$

Considering two boundary situations:
- ethanol is completely converted into bicarbonate
- ethanol is only oxidized to acetate

molar ratios of ethanol to sulphate between 2/3 and 2 are obtained, respectively. Taking an average value of 1 for the ethanol to sulphate ratio, 2 kmol ethanol/h is needed for a small treatment system and 20 kmol ethanol/h for a large treatment system, or 2.2 tons ethanol per day and 22 tons ethanol per day respectively.
Ethanol can be produced by destillation of crude oil or by fermentation of raw sugars. In general ethanol produced by destillation is slightly cheaper. The average price of ethanol is estimated at Dfl 1000,- per ton (96% pure)[7].
For synthesis gas as carbon and energy source the situation is somewhat different. The reaction stoichiometry is:

$$4\,H_2 + SO_4^{2-} \rightarrow HS^- + 3\,H_2O + OH^-$$

The molar ratio of hydrogen to sulphate is 4. For the two situations chosen we then need 8 kmol H_2/h and 80 kmol H_2/h respectively. Based on literature data[15], the cost of hydrogen

has been calculated for sulphate reduction rates between 1 and 30 kmol sulphate/h, see Figure 1.4. The price of synthesis gas is based on literature data for on-site high purity hydrogen production from methane steam-reforming[15]. Therefore it is a worst case calculation because production of synthesis gas - which represents a less pure form of hydrogen gas - will be cheaper.

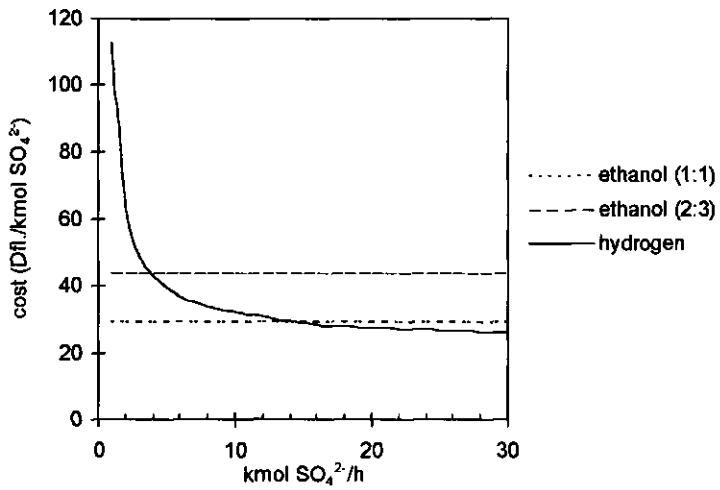

***Figure 1.4.** Cost of electron donor as a function of the capacity of the biotreatment system.*

The estimated cost of the two most promising electron donors is graphically represented in Figure 1.4, which is of course a simplified representation of the actual cost. Ethanol is cheaper for small scale applications (i.e. < 5-10 kmol sulphate/h). In large biotreatment systems (i.e. > 5-10 kmol sulphate/h) use of synthesis gas becomes distinctly cheaper. Since there is a trend towards large scale applications of sulphate reduction - e.g. flue-gas desulphurization - we selected synthesis as the electron donor and carbon source to be investigated.

1.4 Preliminary reactor selection

For selection of the proper reactor type for sulphate reduction with synthesis gas, the main factors determining the choice of the reactor type comprise:
- required mixing regime
- biomass activity or concentration
- number of physical phases in the system
- mass transfer of hydrogen

Required mixing regime
Results of kinetic studies on sulphate-reducing bacteria showed that the toxicity resulting from H_2S can be described by a relatively simple model with linear inhibition kinetics[63]. Generally speaking, in case product inhibition will occur, a system with plug flow characteristics is preferred[40]. However, in case the system should treat an acid wastewater with pH-values down to 2.0, or a sulphite containing wastewater, a well-mixed reactor liquid is needed.
Sulphate-reducing bacteria generally do not grow below pH 6.0 [80] and therefore low pHs in the reactor should be prevented. As far as sulphite is concerned, it is known that concentrations above 500 mg SO_3^{2-}/L become inhibitory to sulphate-reducing bacteria[8] and it is clear that using a well-mixed reactor, the sulphite concentrations remain at the average effluent level, which will be significantly lower than the influent concentration. In this way sulphite inhibition is prevented.

Biomass activity or concentration
To obtain the maximum sulphate conversion rate, a reactor system should be selected which offers a very high retention of active biomass. Consequently we need to immobilize viable biomass. A very cheap and attractive option for retaining biomass is growing the organisms as well settling aggregates, i.e. like is practice in UASB systems. Evidence has been obtained that also sulphate-reducing bacteria are well able to attach to solid particles and to grow in sludge aggregates[1,77].
In order to improve the settability of aggregates, it might be useful to employ carrier material. In literature, the use of a number of carrier types is described[26]. Almost all the carriers used are sandlike materials. Sometimes Al_2O_3, activated carbon or synthetic resins are successfully used. Until now hardly any systematic research has been carried out to define optimal characteristics of the carrier surface in anaerobic gas-lift reactors, but for anaerobic filter reactors and to some extent also for fluidized bed reactors some information has been published[53,72]. From this

information it can be concluded that biomass immobilization is strongly influenced by the surface composition and roughness, and by the wastewater composition (especially Ca^{2+} concentration) as well. However, clear practical guidelines are not yet available. Therefore, we decided to use pumice and basalt because these materials are cheap and seem to be effective carriers.

There are also indications that the carrier diameter has a strong influence on biofilm formation. For example, the start-up using sand of 0.35 mm in diameter was found to proceed much faster than that with sand of 0.75 mm in diameter[24,25,32]. The reason is thought to be the lower liquid shear and collision intensity with smaller particles. For convenience (i.e. availability) we chose carrier particles in the range of 0.2 -0.5 mm.

Number of physical phases

Because synthesis gas is a gaseous electron donor, we obviously are dealing with a three phase system. As mentioned above, the liquid phase should be well-mixed. Also, the solid phase should be well-suspended to prevent any settling of aggregates at the bottom of the reactor. However, as intensive mixing by stirring will give high shear forces and therefore high aggregate attrition, the reactor should be well-mixed by gentle means to minimize attrition of the biomass aggregates.

Mass transfer of hydrogen

Since hydrogen is poorly soluble in aqueous solutions, the mass transfer of hydrogen might be the rate limiting step. Hence, reactor selection should aim at maximizing the hydrogen mass transfer rate, and therefore we need a high gas hold-up and a small bubble diameter.

Based on the above considerations we decided to investigate the potentials of a gas-lift loop reactor, because it is a well-mixed non-stirred reactor. The options of a three phase bubble column or three phase fluidized bed were rejected because the rate of liquid circulation in gas-lift reactors depends on, and is determined by, the gas flow rate, whereas in the bubble column and fluidized bed reactors the liquid flow rate is independent of gas flow. Thus in the latter types, large liquid throughputs are not possible without large liquid recycle rates. In gas-lift reactors, however, quite high linear liquid velocities can be generated without the need for any external recirculation mechanism. Also, the gas velocities for liquid blow-out conditions are lower in bubble columns than in gas-lift reactors. As a result of these effects the operating range of the gas-lift reactors in terms of admissible gas and liquid superficial velocities is broader than for the bubble column reactors[13].

Preliminary reactor design

Two types of gas-lift reactors can be distinguished: the internal loop gas-lift reactor and the external loop gas-lift reactor. Based on general considerations of the effect of reactor geometry and fluid properties on reactor performance, a comparison between the internal-loop and external-loop configurations was made by Chisti[13]. He presented general guidelines for making a preliminary selection with respect to the best suitable type of gas-lift reactor for a given application. The main difference between the two types is the lower overall gas hold-up and higher superficial liquid velocity in the external loop gas-lift reactors. Due to the lower gas hold-up the volumetric mass transfer rate of external types will be lower compared to the internal ones. Since gas-to-liquid mass transfer of hydrogen is assumed to be one of the major rate limiting steps in sulphate reduction, an external gas-lift reactor was chosen as the model reactor in the investigations.

The geometry of the laboratory reactor is furthermore determined by the ratio of the riser to downcomer cross-sectional areas and the ratio of height to diameter of the reactor. Minimum mixing times are generally obtained at riser-to-dowcomer area ratios between 0.6 and 4.0[13]. In our case riser and column diameter are chosen such that the riser-to-downcomer cross-sectional areas is 0.75. To minimize the wall-effects a reactor height to diameter ratio of 5 is used[13].

1.5 Conclusions

Organic waste materials are unsuitable electron donors for sulphate reduction in practice, because considerable pollution will remain in the wastewater, partially consisting of non-biodegradable organic matter. Moreover, in the degradation of these raw materials a competition will occur between sulphate-reducing, acetogenic and methanogenic bacteria, thus reducing the sulphate removal efficiency.

Of the fully degradable bulk chemicals, ethanol and synthesis gas may both represent feasible electron donors. However, synthesis gas avoids the problem of competition between sulphate reducing bacteria and methanogenic bacteria, and therefore prevents 'loss' of electron donor.

From an economical point of view ethanol seems a cheaper electron donor for small scale sulphate reduction (i.e. < 5-10 kmol sulphate/hr) and synthesis gas for large scale applications (i.e. > 5-10 kmol sulphate/hr). Since there is a trend towards large scale application of sulphate reduction - e.g. flue-gas desulphurization - synthesis gas was selected as electron donor in our studies

1.6 References

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2

Engineering aspects of biological sulphate reduction using hydrogen and carbon dioxide

Abstract

Feasibility and engineering aspects of biological sulphate reduction in gas-lift reactors were studied. Hydrogen and carbon dioxide were used as energy and carbon source. Attention was paid to biofilm formation, sulphide toxicity, sulphate conversion rate optimization and gas-liquid mass transfer limitations. Sulphate-reducing bacteria formed stable biofilms on pumice particles. Biofilm formation was not observed when basalt particles were used. However, use of basalt particles led to the formation of aggregates of sulphate-reducing biomass. The sulphate-reducing bacteria, grown on pumice, easily adapted to free H_2S concentrations up to 450 mg/L. These high free H_2S concentrations caused reversible inhibition rather than acute toxicity. When free H_2S concentrations were kept below 450 mg/L, a maximum sulphate conversion rate of 30 g SO_4^{2-}/L.d could be achieved after only 10 days of operation. Gas to liquid hydrogen mass transfer capacity of the reactor determined the maximum sulphate conversion rate.

Published as: van Houten, R.T., Hulshoff Pol, L.W., Lettinga, G. 1994. Biological sulphate reduction using gas-lift reactors fed with hydrogen and carbon dioxide as energy and carbon source. Biotechnol. Bioeng. **44:** 586-594.

2.1 Introduction

Due to more stringent environmental legislation, there is an increasing demand for cheap technologies for remediation of heavy metal containing solid wastes. Heavy metal containing solid wastes are ubiquitous: examples are mineral wastes on mine sites all over the world; flue ashes and bottom ashes from waste incineration plants; and biological sludges from both communal and industrial sewage works. Most of these wastes are disposed of on controlled sites at high cost, awaiting the development of new cleaning technologies. These depositions may be seen as chemical time bombs, eventually threatening to contaminate ground and surface water supplies.

One promising detoxification technology is bioleaching of the contaminated solid wastes, with use of conversions of the biological sulphur cycle. The waste materials are leached by acidophilic *Thiobacilli* spp., which convert sulphur to sulphuric acid. After removal of the solid wastes and solubilized heavy metals, the inorganic wastestream containing sulphate is recycled. This sulphate recycling process will be carried out in two stages. In the first stage, sulphate is biologically converted to hydrogen sulphide. The second stage converts hydrogen sulphide to solid sulphur by colorless sulphur bacteria, which is a proven technology[3,4]. The produced sulphur sludge can be reused for the bioleaching of contaminated solid wastes. In this way, the biological sulphur cycle is closed and no sulphate is discharged to the environment. Research on this integrated biological treatment system was carried out by five research groups within the European Community (Doddema, H.J. *et al.* 1991. Remediation of contaminated soils and waste materials. EC STEP-project CT 90-0073).

Research on this sulphate recycling may be applicable to other sulphuric acid containing wastestreams. A number of industries, in particular chemical and mining industries, also produce wastewaters containing sulphuric acid. Most of these wastewaters are either discharged to local sewage works or neutralized with lime yielding (contaminated) gypsum. From an environmental point of view both options have to be reconsidered.

Since the previously mentioned waste sulphuric acids contain no organic compounds, biological sulphate reduction can only take place when electron donor and carbon source are added to the sulphate containing wastestream. On the basis of available cost information, availability and remaining pollution of the added energy and carbon source, we selected synthesis gas for the electron donor (van Houten, R.T. 1991. Selection of electron donors for sulphate reduction. Desk-study EC STEP-project CT 90-0073). In practice, composition of synthesis gas is rather undefined, as outlined in chapter 1. Hydrogen concentration is generally above 30% (v/v).

Carbon monoxide and carbon dioxide concentrations can vary from 0 to 60% and 0 to 30% respectively[14]. To determine possible toxic effects of carbon monoxide in the future, we used, in this study, a gas mixture of only hydrogen and carbon dioxide (80% and 20%, respectively).

There is a vast amount of literature available about the growth and metabolism of sulphate-reducing bacteria with hydrogen as energy source. A summary of the most relevant kinetic data is given by Rinzema and Lettinga[16] and Widdel[25]. More recent literature about microbial aspects of sulphate reduction with hydrogen and carbon dioxide is presented in three comprehensive reviews of Widdel and Hansen[24], Widdel[23], Widdel and Bak[22].
No literature data are available about the ability of sulphate-reducing bacteria to form biofilms or biomass granules in turbulent three-phase systems, in particular gas-lift reactors. In addition, little is known about engineering aspects of biological sulphate reduction in such three-phase systems. Du Preez *et al.*[6] carried out a study using a packed bed reactor with synthesis gas as energy and carbon source. However no attention was paid to gas-liquid mass transfer, sulphide toxicity and process optimization. Stucki and co-workers[19] performed a detailed study on reactor design and process optimization using two-phase fixed-bed reactors, but they used acetate as energy and carbon source.

The purpose of the present study was to investigate feasibility and reactor engineering aspects of sulphate reduction in gas-lift reactors using natural immobilized biomass grown on hydrogen and carbon dioxide as energy and carbon source. Particular attention was paid to biofilm formation, sulphide toxicity, sulphate conversion rate optimization and gas-liquid mass transfer limitations. A model medium with defined mineral nutrients was used. Theoretical aspects of the reactor system are presented in the Appendix.

2.2 Materials and methods

Experimental set-up.
The experimental set-up is shown in Figure 2.1. The laboratory gas-lift reactor (liquid height 0.700 m, inner diameter 0.100 m) was made of glass and was temperature controlled at 30 °C by running water through the outer mantle of the reactor. Reactor contained a plastic internal draft-tube (length 0.450 m, inner diameter 0.062 m, outer diameter 0.070 m). The biologically active reactor volume was 4.5 liters. Pumice particles (Aqua-volcano d: 0.2 - 0.5 mm, density ca. 2440 kg/m^3, Aquatechniek bv, Papendrecht, Holland) or basalt particles (d_{50}: 0.29 mm,

density ca. 2900 kg/m^3, Gist-Brocades nv, Delft, Holland) were used as carrier material. pH was controlled with a sulfide-resistant Flushtrode® pH-electrode (Hamilton, Hilkomij bv, Rijswijk, Holland) connected with a pH controller. The pH-electrode performance was checked every week, although no recalibration during the experiments was necessary.

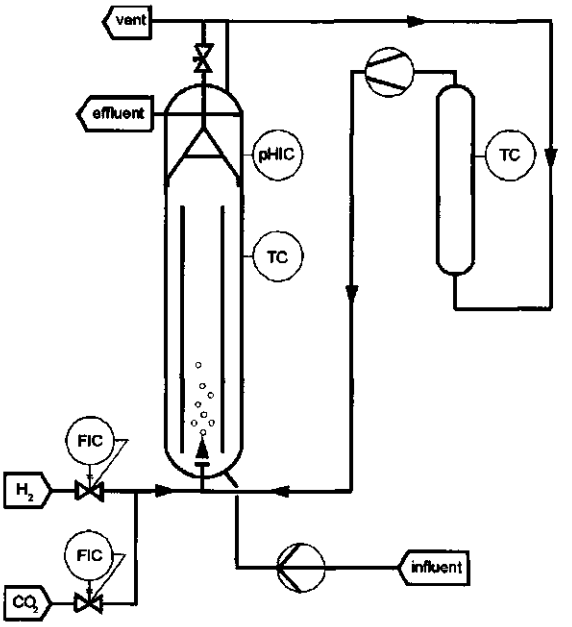

Figure 2.1. Flow sheet of the experimental set-up. This set-up was used throughout all three experiments. The set-up consists of an internal draft-tube gas-lift reactor and a buffer column, connected by a gas recycle. FIC: flow indication and control; pHIC: pH indication and control; TC: temperature control.

The gas phase was recycled with a compressor (Verder type N 726 FT 18, Verder bv, Vleuten, Holland) at a flow rate of 350 L/h. Gas recycle contained a glass column which was used as foam trap in case heavy foaming occurred. The column was temperature controlled at 30 °C.
Influent gas flows of H_2 and CO_2 were monitored with two mass flow controllers (Brooks thermal mass flowmeter, type 5850 E, 30.0 Ln/h (Ln refers to liters at normal conditions, i.e. 101325 Pa and 273.15 K) and type 5850 TR, 3.00 Ln/h respectively; Brooks Instruments, Veenendaal, Holland) connected to a read-out/control unit (Brooks type 5878, Brooks

Instruments, Veenendaal, Holland). Effluent gas flow was regularly determined with a glass-tube (calibrated inner diameter 0.07570 m) equipped with a Teflon® piston (calibrated outer diameter 0.07567 m) with a mercury seal.
Medium was pumped into the reactor with a Watson-Marlow peristalic pump (type 503 S, Smith & Nephew Watson Marlow, England). All tubings of the gas inlet, gas recycle and gas outlet were made of stainless steel 316 L. All tubings of the liquid influent and liquid effluent were made of Marprene® tubing (Smith & Nephew Pharmaceuticals Ltd, England).
In all cases, pH and temperature were kept constant at 7.0 and 30 °C, respectively. Since sulphate reduction at pH 7 is accomplished by hydroxyde production according to:

$$8\ H_2 + 2\ SO_4^{2-} \rightarrow H_2S + HS^- + 5\ H_2O + 3\ OH^-$$

the pH was controlled with a 1 M HCl solution.

Start-up.
Before the start of each experiment the reactor system was made anaerobic by flushing overnight with a gas mixture containing 99% H_2 and 1% CO_2 at a total flow rate of 2.43 Ln/h. Low flow of CO_2 was needed to prevent acidification of the medium. Then 2 crystals of $Na_2S \times xH_2O$ (x=7-9) were added to the reactor to remove the last traces of oxygen. Decolorization of the redox indicator resazurin indicated complete anaerobic conditions. When sulphate reduction started, accomplished by hydroxyde production, the incoming CO_2 concentration was increased step-wise to obtain a gas mixture of 80% H_2 and 20% CO_2.

Biomass.
The reactor was inoculated with 100 mL crushed anaerobic granular sludge that had been grown on a mixture of volatile fatty acids and sulphate as described previously[1]. Before inoculation the anaerobic sludge was pre-cultivated for one month on a gas mixture of 80% (v/v) H_2 and 20% (v/v) CO_2. Biofilm growth was examined regularly by light microscopy. Particle size was measured according to the image analysis technique described by Visser *et al.*[21].

Medium.
The growth medium contained (in g per 1 liter of demineralized water): Na_2SO_4, 4.97; $Na_2HPO_4 \times 2H_2O$, 0.53; KH_2PO_4, 0.41; NH_4Cl, 0.30; KCl, 0.37; $MgCl_2 \times 2H_2O$, 0.10; $CaCl_2 \times 2H_2O$, 0.11; $NaHCO_3$, 1.2; resazurine, 0.00025; vitamins 0.2 mL/L; acidic trace elements 1 mL/L and alkaline trace elements 1 mL/L. Vitamins solution and trace elements

solutions were prepared as described previously[18]. All chemicals used were of extra pure quality and supplied by Merck (Darmstadt, Germany), except for the gases, which were supplied by Hoekloos (Schiedam, Holland).

Analytical methods.

Acetic acid was determined with a gas chromatograph (Hewlett Packard 5890A) equipped with a glass column (2 m x 4 mm) packed with Supelcoport (100-200 mesh), coated with 10 % Fluorad FC 431. Temperature of the column, injection port and flame ionization detector were 130, 200 and 280 °C, respectively. Nitrogen gas saturated with formic acid was used as carrier gas at a flow rate of 50 mL/min. All samples were centrifuged (3 min, 9500 *g*) and fixed by adding a formic acid solution (6% v/v, dilution 1:1).

Sulphate and thiosulphate were determined with an HPLC equipped with a VYDAC Ion Chromatography column (cat# 302 IC, 250 x 4.6 mm). The temperature of the column and detector (Waters 431 conductivity detector) were 20 °C and 35 °C respectively. As eluent 0.018 M potassium biphtalate with 2.5% (v/v) acetonitrile was used. The eluent flow rate was 1.2 mL/min. All samples were fixed by adding a 0.1 M zinc-acetate solution (dilution 1:1), centrifuged (3 min, 9500 *g*) and diluted down to a concentration below 500 mg/L with demineralized water.

Sulphide was determined colorimetrically using a slightly modified method adapted from Trüper and Schlegel[20].

Gas phase composition (CO_2, H_2S, CH_4 and N_2) was determined with a gas chromatograph (Fisons Instruments GC 8000) equipped with two columns: 1.5 m x 1/8" teflon packed with Chromosorb 108 (60-80 mesh) and 1.2 m x 1/8" stainless steel packed with mol. sieve 5A (60-80 mesh). The columns were connected parallel with a split of 1:1. Helium was used as carrier gas. The total carrier gas flow rate was 45 mL/min. The temperature of the columns, injection port and thermal conductivity detector were 40 °C, 140 °C and 100 °C respectively. Hydrogen was determined a gas chromatograph (Hewlett Packard 5890), using a 1.5 m x 1/8" stainless steel column packed with mol. sieve 5A (60-80 mesh). The temperature of this column, injection port and thermal conductivity detector were 40 °C, 125 °C and 110 °C respectively. Argon was used as carrier gas at a flow rate of 20 mL/min.

2.3 Results

The results of the first experiment are shown in Figure 2.2. In this experiment 250 g of pumice was used as carrier material. The influent sulphate concentration was 0.035 M. After inoculation the reactor was started in batch mode. Only a gas feed of 2.43 Ln/h, containing 99% H_2 and 1% CO_2, was supplied. After one day the reactor was operated continuously at a hydraulic retention time of 27 h. At day 5 the hydraulic retention time was decreased to 13.5 h. At the same time the gas feed composition was changed to 94% H_2 and 6% CO_2 at a total flow rate of 2.55 Ln/h. The hydraulic retention time was further decreased to 4.5 h at day 7. Also the gas feed composition was changed to 80% H_2 and 20% CO_2 at a total flow rate of 3.30 Ln/h. After 11 days of operation a steady-state sulphate conversion rate of 14 g SO_4^{2-}/L reactor.d was reached. No methane or fatty acids could be detected.

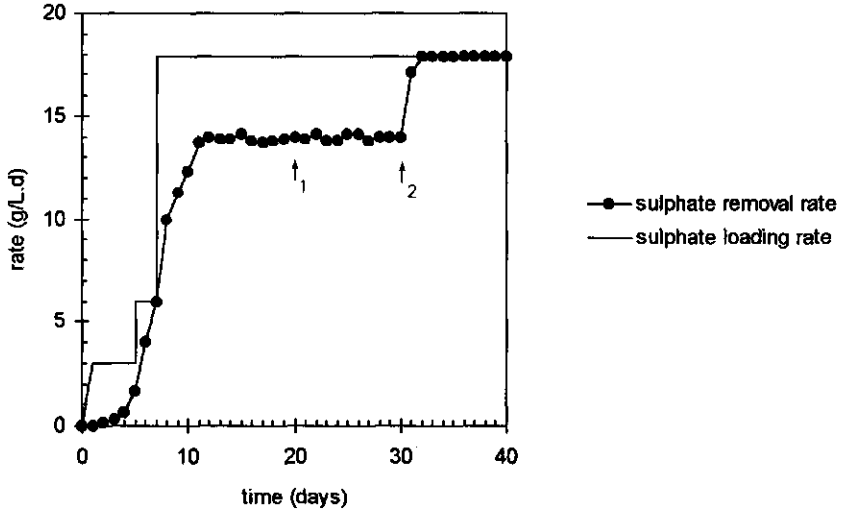

Figure 2.2. Sulphate loading rate and sulphate conversion rate during the first experiment; 250 g pumice was used as carrier material. Hydrogen concentration in the reactor was increased between arrow 1 and arrow 2. Arrow 2 indicates doubling of the total gas flow rate.

During the first 12 days of the experiment biofilm growth was examined by light microscopy (Fig. 2.3). The pictures indicate that growth started in the pores of the particles and then

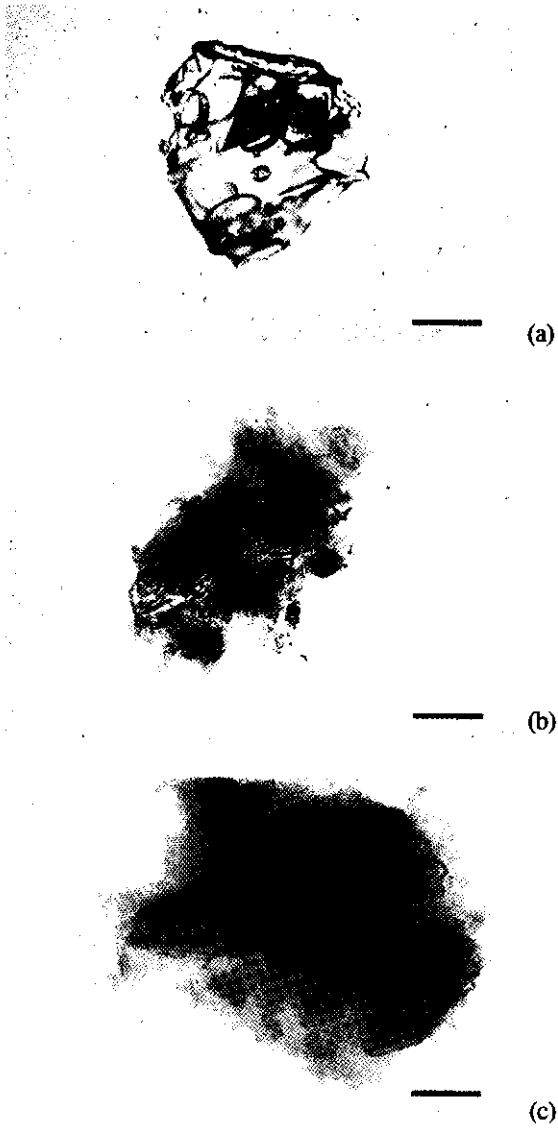

Figure 2.3. Biofilm formation on pumice. Pictures taken during the first experiment. (a) Start of experiment; (b) 8 days of operation; 12 days of operation. Bar length is 100 μm .

gradually more surface was covered as time passed. After 15 days of operation, no bare carrier particles were observed by light microscopy. Gram staining of resuspended biofilms indicated the presence of Gram (+) rods and Gram (-) vibrios.

During the first steady-state the total liquid sulphide concentration remained constant at a level of 894 mg total S/L (± 21 mg/L). By measuring the effluent gas flow regularly, as described in the Materials and Methods section, we were able to check the mass balances (see Appendix) of sulphur and hydrogen quantitatively. The results are given in Table 2.1.

Table 2.1. Conversion rates obtained during the steady-states of the three experiments. The values are calculated according to the steady-state mass balances (A.5), (A.6) and (A.7) given in the Appendix.

experiment	time (days)	$-R'^{a}_{S}$ (mol/L.d)	$-R'^{a}_{H2}$ (mol/L.d)	R'^{a}_{P} (mol/L.d)
1	13	0.145	0.607	0.155
	15	0.147	0.608	0.151
	17	0.148	0.609	0.148
2	25	0.144	0.617	0.156
	28	0.141	0.587	0.157
	35	0.186	0.730	0.190
3	13	0.222	0.864	0.206
	17	0.221	0.882	0.196
	20	0.222	0.864	0.203

[a] R' denotes R (given in the Appendix) expressed in mol/L.d.

The established steady-state of the sulphate conversion rate could be due to either mass transfer limitation of hydrogen or product inhibition by H_2S. A possible hydrogen limitation was tested by changing, at day 20, composition and flow rate of the incoming gas in such a way that the hydrogen concentration in the gas phase of the reactor increased, at a constant total effluent gas flow rate. Thus no extra H_2S was removed. Increase of sulphate conversion rate then could mean that the steady-state was caused by hydrogen limitation. Figure 2.2 shows that no increase in sulphate conversion rate occurred.

Since the concentration of undissociated H_2S seemed to determine the steady-state, the incoming gas flow was increased at day 30 to 6.60 Ln/h. At the same time the incoming gas composition was reset at 80% H_2 and 20% CO_2. Figure 2.2 clearly shows the effect of H_2S stripping. The

sulphate conversion rate increased within two days to 17.9 g SO_4^{2-}/L.d, which equals the loading rate. The total sulphide concentration in this second steady-state was 760 mg S/L (± 13 mg/L). No sulphate could be detected in the effluent. However, during this period of sulphate limitation, acetate was produced up to a steady level of 467 mg/L (± 10 mg/L).

Similar results were obtained in a second experiment with basalt as carrier material. In this experiment 500 g basalt was used to obtain a similar particle surface area compared to pumice. The process conditions (i.e. loading rates, pH and temperature) and start-up procedure were the same as in the first experiment. Only the gas recycle flow rate was increased to 550 L/h in order to suspend all the basalt. The results are shown in Figure 2.4.

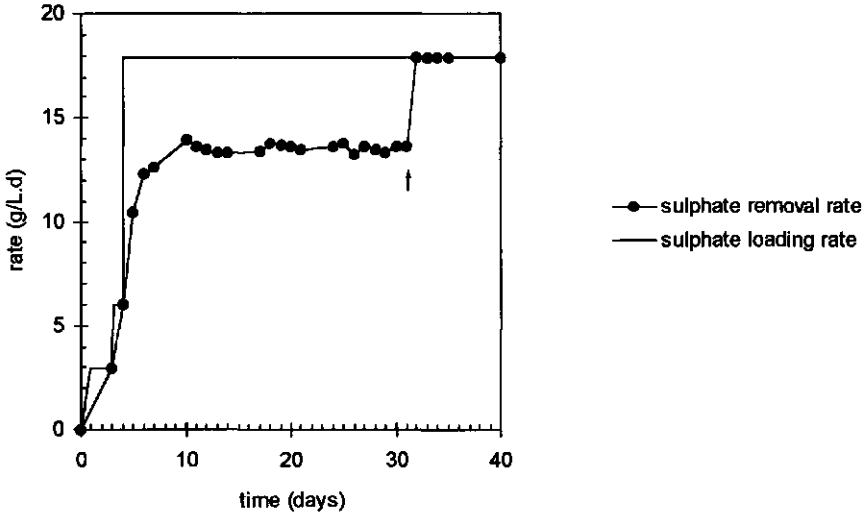

Figure 2.4. Sulphate loading rate and sulphate conversion rate during the second experiment; 500 g basalt was used as carrier material. The arrow indicates doubling of the total gas flow rate.

At day 4 a hydraulic retention time of 4.5 h was applied. At the same time the incoming gas flow consisted of 80% H_2 and 20% CO_2 at a flow rate of 3.30 Ln/h. After 10 days of operation a steady sulphate conversion rate of 13.5 g SO_4^{2-}/L.d was obtained. The total liquid sulphide concentration was 910 mg S/L (± 46 mg/L). At day 30 the incoming gas flow was increased to 6.60 Ln/h. The system immediately responded by an increase of the sulphate conversion rate to

17.9 g SO_4^{2-}/L.d. The total liquid sulphide concentration during this second steady-state was 781 mg S/L (± 18 mg/L). Again acetate was detected up to a concentration of 189 mg/L.
Another interesting phenomenon was observed during this experiment. No biofilm growth occurred although the sulphate conversion rate was the same as in the previous experiment. However formation of small, d < 1.0 mm, aggregates was observed (Fig 2.5.). Although these aggregates did not contain any carrier particles, only particles with a diameter less than 0.2 mm were washed-out. However, attempts to grow the aggregates in a gas-lift reactor without carrier material failed.

Figure 2.5. Formation of aggregates of sulphate reducing bacteria. Picture taken at day 20 of experiment 2. Bar lenght is 100 μm.

High H_2S removal rates, without the expense of relative high effluent gas flow rates, can be achieved by applying shorter hydraulic retention times. Since in the previous experiments the final total liquid sulphide concentration was below the measured inhibitory level of 900 mg S/L, we decided to perform an experiment with a hydraulic retention time of 2.25 h. The results are shown in Figure 2.6.
In this third experiment we used 250 g of pumice as carrier material. The influent sulphate concentration was 0.050 M. The start-up procedure was similar to the procedure in the previous experiments. At day 4 the hydraulic retention time was decreased to 4.5 h. At the same time the gas flow, containing 80% H_2 and 20% CO_2, was increased to 6.60 Ln/h. After 10 days, a steady-state sulphate conversion rate of 21.2 g SO_4^{2-}/L.d was achieved. This resulted in a total liquid sulphide concentration of 907 mg S/L (± 20 mg/L). After 20 days of operation the

hydraulic retention time was decreased to 2.25 h. This increased sulphate conversion to 31.3 g SO_4^{2-}/L.d at day 24. The total liquid sulphide concentration decreased to 761 mg S/L (± 13 mg/L). However this conversion rate readily decreased to 24.9 g SO_4^{2-}/L.d at day 32. In the mean time, biomass wash-out occurred. Increase of the hydrogen gas phase concentration in the reactor restored the sulphate conversion rate to 29.5 g SO_4^{2-}/L.d after 36 days of operation, indicating that the biomass suffered mass transfer limitation of hydrogen. Since hydrogen transfer from gas to liquid phase could not be increased further, the experiment was stopped.

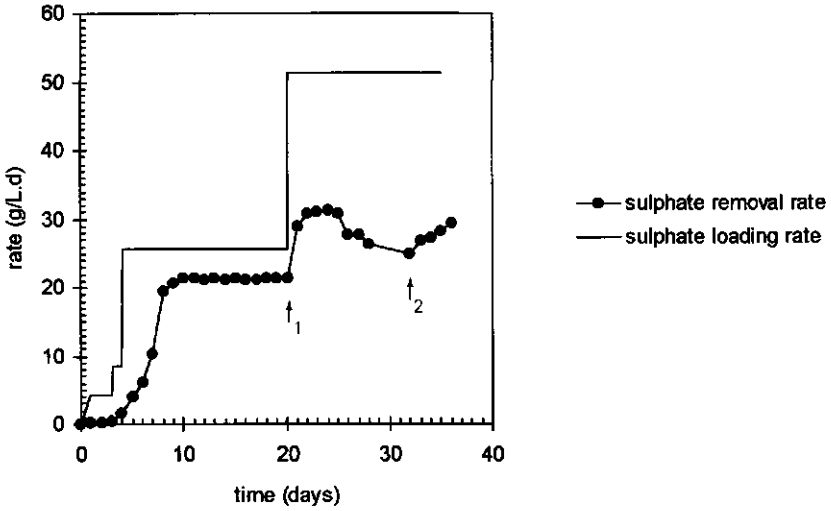

Figure 2.6. Sulphate loading rate and sulphate conversion rate during the third experiment; 250 g pumice was used as carrier material. Hydraulic retention time was decreased from 4.5 to 2.25 h at arrow 1. Arrow 2 indicates that the hydrogen concentration in the reactor was increased from 70% (v/v) to 85% (v/v).

2.4 Discussion

Our experimental results reveal that sulphate-reducing bacteria are able to form stable biofilms. Figure 2.3 clearly shows biofilm formation on pumice under the prevailing turbulent flow conditions. The picture made during the second experiment (Fig. 2.5) shows that sulphate-reducers are even able to form small aggregates, with a diameter less than 1.0 mm. From the liquid upward velocity in the gas-liquid-solid separator on top of the reactor we can calculate the

minimum settling velocity of the sludge granules. After day 4, liquid flow through the reactor was 1 l/h. Reactor diameter was 0.100 m and diameter of the gas collector was 0.098 m. From this values we can calculate a minimum settling velocity of the granules of 3.2 m/h.

Although pure and mixed cultures of sulphate-reducing bacteria cultivated in the laboratory often aggregate or stick to surfaces[12,25], the ability of sulphate-reducers to form biofilms or sludge granules in turbulent anaerobic reactors has not been described before. The results of very recent experiments[21] conducted in Upflow Anaerobic Sludge Blanket reactors support our results.

Most published experimental results on sulphate reduction were obtained with mixed populations of methanogenic bacteria and sulphate-reducing bacteria. In these mixed systems methanogens are thought to be responsible for the ability to form stable biofilms and granules. The filamentous nature of the predominant acetotrophic methanogen *Methanosaeta* sp.[26] or the specific hydrophobic properties of some methanogens[9] are thought to be key factors in biofilm and granule formation.

Most experiments dealing with the technical aspects of sulphate reduction without concomitant methanogenesis were carried out in fixed bed reactors[6,19]. In these fixed bed reactors, biomass is retained by entrapment rather than attachment. This holds in particular for the experiments of Stucki *et al*[19]. During their experiments the biomatrix was mixed once a day to assure a more homogeneous distribution of the active biomass. Also, scanning electron microscopy showed preferentially entrapment of biomass in the pores of the glass beads. However, all their attempts to operate a fluidized bed reactor with sand particles as carrier material failed.

No methane production occurred in any of our experiments. Even incubation of biomass samples in 120 ml batch vials, containing 50 ml of growth medium without sulphate and a headspace of 80% H_2 and 20% CO_2 showed no methanogenic activity after 4 weeks. Therefore we can assume that no methanogens were present in the active biomass. Thus the sulphate-reducing bacteria had to adhere by themselves.

The question remains why small agregates are formed when basalt is used as carrier material. Differences in surface properties between pumice and basalt particles may be a possible explanation. Because pumice particles have a very 'open' structure, like sponges, bacteria easily enter the pores of the pumice particles. In these pores liquid turbulence is significantly less so the bacteria have time to adhere to the surface of the particles. Once adhered, the bacteria can form micro-colonies that can grow out of the pores, thus forming a complete biofilm. The pictures in Figure 2.3 support the above-mentioned mechanism, generally proposed for biofilm formation.

Basalt particles however, have a rough surface without deep pores. When bacteria attach to the surface, they still have to cope with high liquid turbulence. Thus before the bacteria are able to attach to the surface, they are already abraded from the particle. This may act as a driving force for the bacteria to stick together instead of sticking to a surface. In this way, aggregates can be formed so the bacteria still retain in the reactor.

The results of our experiments show that the biomass adapts easily to total sulphide levels of 900 mg total S/L. The relative distribution of H_2S and HS^- at pH 7.0 is about 1:1 (pK_a of H_2S is 7.0 at 25 °C), thus all the steady-states in the experiments were obtained at free H_2S levels of about 450 mg/L. Although the pH within the biofilm may be different, at least the outer region of the biofilm has to cope with these high H_2S concentrations. This outer region also suffers from attrition, so apparantly biofilm growth is still possible at these high free H_2S concentrations. The results also show an immediate response of the biomass to removal of free H_2S, so no reactivation of the biomass was needed as was suggested by Stucki and co-workers[19].

The admissible free H_2S concentrations we found are much higher than generally reported in literature. Except for the results of Isa *et al*[7], who found only 50% inhibition at concentrations exceeding 1000 mg free H_2S/L, total inhibition of growth is generally obtained at concentrations below 550 mg free H_2S/L[10,13,15,19]. Our experiments demonstrate that effluent concentrations of 450 mg free H_2S/L can be obtained at neutral pH, which facilitate the biological sulphide oxidation stage of the sulphate recycling process.

Sulphate conversion rates up to 30 g SO_4^{2-}/L.d so far only have been reported by Stucki *et al.*[19]. They used three types of reactors (i.e. 1 L fixed-bed reactor, 10 L fixed-bed reactor and a 3.6 L statically mixed reactor). Only in the experiments in the 1 liter fixed-bed reactor a high sulphate conversion rate of 60 g SO_4^{2-}/L.d was reached after 100 days of operation. Their other experiments were carried out in the other two reactor types. In these reactors sulphate conversion rates of 15 g SO_4^{2-}/L.d were obtained after 73 and 60 days of operation respectively. These results demonstrate the advantage as well as the disadvantage of using acetate as energy and carbon source. Using acetate mass transfer limitation is insignificant thus in principle high sulphate conversion rates can be achieved. However, the growth rate of sulphate-reducing bacteria on acetate is rather poor[16,25] so start-up of the reactors may be time-consuming.

Our experiments show that a sulphate conversion rate of 30 g SO_4^{2-}/L.d can easily be obtained after 10 days of operation and without the necessity to remove high amounts of sulphide, as was done by Stucki *et al*[19]. Therefore, our results show the advantage of sulphate reduction with hydrogen, as the growth rate of hydrogenotrophic sulphate-reducing bacteria is in general relatively high[16,25].

In all the experiments we observed acetate production during sulphate limitation. The acetate can be produced by either acetogenic sulphate-reducing bacteria, i.e. *Desulfotomaculum* sp., or homo-acetogenic bacteria. Further experiments on the microbial nature of the biofilms and aggregates, should reveal which microbial population is responsible for acetate production.

In the third experiment a maximum sulphate conversion rate of 31.3 g SO_4^{2-}/L.d was obtained. Because incoming and outgoing flows of hydrogen were measured, the mass transfer rate of hydrogen can be calculated according to equations A5, A6 and A7 given in the Appendix. Using these equations the hydrogen transfer rate was 1.23 mole H_2/L.d. Since 0.03 mole H_2/L.d. still left the reactor unconverted, the results obtained during the period of increased hydrogen concentration indicate that gas to liquid mass transport limitation was governing sulphate conversion rates exceeding 30 g SO_4^{2-}/L.d. Assuming that the liquid bulk concentration of hydrogen was zero, the volumetric mass transfer coefficient, $k_L.a$, can be calculated as described in the Appendix. The volumetric mass transfer coefficient was calculated at 0.026 s^{-1}. This value corresponds well with the obtained experimental values ranging from 0.018 to 0.035 s^{-1} (see Appendix, Table A.I). Also $k_L.a$ values, measured in two- and three-phase systems, reported in literature[2,5,17] vary between 0.01 and 0.04 s^{-1}, depending on particle type and particle loading.
Summarizing the results of the experiments it can be concluded that gas mixtures of hydrogen and carbon dioxide are very useful substrates for biological removal of sulphate from sulphuric acid containing waste waters. High sulphate reduction rates can be obtained within a short period of time.

2.5 Conclusions

The results in this chapter clearly demonstrate that:

1) Sulphate-reducing bacteria are able to form stable biofilms under turbulent flow conditions, as observed from growth of sulphate-reducing bacteria on pumice particles. Use of basalt particles led to the formation of granules of sulphate-reducing biomass.
2) Growth of sulphate-reducing bacteria is still possible at free H_2S concentrations up to 450 mg/L. These high free H_2S concentrations cause reversible inhibition rather than acute toxicity.
3) When free H_2S concentrations are kept below 450 mg/L, a maximum sulphate conversion rate of 30 g SO_4^{2-}/L.d can be achieved after only 10 days of operation.

4) Gas to liquid hydrogen mass transfer capacity of the reactor determines the maximum sulphate conversion rate.

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2.6 Appendix

In this appendix theoretical aspects of the reactor system are presented. In addition to the used kinetics and mass balances, the calculation of volumetric mass transfer coefficients and experimental determination of the physical volumetric mass transfer coefficient are also given. In the following paragraphs 'reactor' refers to the gas-lift reactor including gas recycle and buffer column.

Kinetics

The kinetics of sulphate reduction are based on the following assumptions:

- Growth of biomass proceeds according to Monod-kinetics with simultaneous inhibition by the product, H_2S. Instead of using true Monod-kinetics it is assumed that reaction kinetics for each substrate are of zeroth order in substrate concentration. Since the Monod-constants for hydrogen and sulphate are very small[25], at least 100 times smaller than actual reactor concentrations, this assumption is justified.
- Product inhibition proceeds according to first order inhibition kinetics[8]:

$$\mu = \mu_{max} \cdot (1 - \frac{c_P}{c_{P,max}}) \qquad (A.1)$$

with $c_{P,max}$ = 16 mol/m^3 [13 15]. Since not much actual information about inhibition kinetics is available, this is a reasonable first guess.

- Product formation is directly coupled to biomass production. This assumption is trivial in case of dissimilatory sulphate reduction.
- Substrate consumption for maintenance is incorporated in the overall biomass yield.

With the assumptions formulated above, the reaction equations become:

$$\begin{aligned} R_X &= \mu \cdot c_X \\ R_S &= -Y_{S/X} \cdot \mu \cdot c_X \\ R_{H_2} &= -Y_{H/X} \cdot \mu \cdot c_X \\ R_P &= Y_{P/X} \cdot \mu \cdot c_X \end{aligned} \qquad \text{(A.2)}$$

Mass balances

For the reactor the following assumptions are made:

- Gas and liquid phase of the reactor are ideally mixed.
- The volume of the reactor is constant.
- Liquid flow passing through the reactor is constant.
- Under steady-state conditions, neither biomass nor product accumulation is assumed.
- Gas-side mass transfer resistance is negligible. This is generally true for low-soluble gases like hydrogen.
- External (i.e. around biofilms or granules) mass transfer limitations are neglected. With an external mass transfer film thickness typically in the order of ten microns for aqueous systems, this assumption usually will be valid.

The mass balance, for component j, over the liquid phase of the reactor then becomes:

$$V\frac{d\,c_{j,L\,out}}{dt} = Q_L \cdot c_{j,L\,in} - Q_L \cdot c_{j,L\,out} + k_L.a\left(\frac{c_{j,G\,out}}{m} - c_{j,L\,out}\right).V + R_j.V \qquad \text{(A.3)}$$

The mass balance, for component j, over the gas phase becomes:

$$V\frac{d\,c_{j,G\,out}}{dt} = Q_G \cdot c_{j,G\,in} - Q_G \cdot c_{j,G\,out} - k_L.a\left(\frac{c_{j,G\,out}}{m} - c_{j,L\,out}\right).V + R_j.V \qquad \text{(A.4)}$$

During steady-state, and with the assumption that the bulk liquid hydrogen concentration in the reactor is negligible, we can write down the simplified mass balances for hydrogen:

$$Q_{G\,in} \cdot c_{H_2,G\,in} - Q_{G\,out} \cdot c_{H_2,G\,out} = -R_{H_2}.V \qquad \text{(A.5)}$$

sulphate:

$$Q_L \cdot c_{S,L\,in} - Q_L \cdot c_{S,L\,out} = -R_S.V \qquad \text{(A.6)}$$

and hydrogen sulphide:

$$- Q_{L} \cdot c_{P,L\,out} - Q_{G\,out} \cdot c_{P,G\,out} = R_P \cdot V \qquad (A.7)$$

According to the reaction stoichiometry of the reaction equation for sulphate reduction:

$$8\ H_2 + 2\ SO_4^{2-} \rightarrow H_2S + HS^- + 5\ H_2O + 3\ OH^- \qquad (A.8)$$

$$-R_S = -\frac{1}{4} R_{H_2} = R_P \qquad (A.9)$$

The steady-state reaction rates of all the three experiments are calculated according to equations (A.5),(A.6) and (A.7). The results are given in Table 2.1.

Mass transfer coefficients

The difference in hydrogen mass flow rate between incoming and outgoing gas flow, given in (A.5), equals the amount of hydrogen transferred to the liquid phase. Therefore we can write:

$$Q_{G\,in} \cdot c_{H_2,G\,in} - Q_{G\,out} \cdot c_{H_2,G\,out} = k_L.a.\frac{c_{H_2,G\,out}}{m}.V \qquad (A.10)$$

Since, during steady-state, all parameters are known we can calculate the volumetric mass transfer coefficient k_L.a for hydrogen.

The pure physical volumetric mass transfer coefficient has been measured with the CO_2 absorption method according to Mehta and Sharma[11]. During these experiments the gas-lift reactor contained two phases (gas-liquid) or three phases. No biomass was present. The liquid phase had the same ionic strength as the biologically active reactor. The results of the k_L.a measurements are shown in Table A.I.

***Table A.I.** Mass transfer coefficients as a function of gas recycle flow rate.*

gas recycle flow rate (L/h)	k_L.a (two-phase sytem) (s^{-1})	k_L.a (three-phase system) (s^{-1})
53	0.024	0.008
299	0.032	0.016
462	0.035	0.018
547	0.037	0.021

[a] In the three-phase system a particle loading of 250 g pumice was applied.

There is a clear drop in $k_L.a$ values for the three-phase system, compared to the two-phase system. This is caused be increased coalescence of the gas bubbles by addition of small carrier particles[5].

2.7 Nomenclature

a	specific surface area of reactor	(m^2/m^3)
c_{H2}	hydrogen concentration	(mol/m^3)
c_P	product, i.e. sulphide concentration	(mol/m^3)
c_{Pmax}	maximum sulphide concentration at which growth occurs	(mol/m^3)
c_S	sulphate concentration	(mol/m^3)
c_X	biomass concentration	(mol/m^3)
k_L	liquid-side mass transfer coefficient	(m/s)
m	gas/liquid solubility coefficient	(-)
Q_G	gas flow rate	(m^3/s)
Q_L	liquid flow rate	(m^3/s)
R_{H2}	hydrogen consumption rate	($mol/m^3.s$)
R_P	sulphide production rate	($mol/m^3.s$)
R_S	sulphate consumption rate	($mol/m^3.s$)
R_X	biomass production rate	($mol/m^3.s$)
V	reactor volume	(m^3)
$Y_{H/X}$	yield coefficient (hydrogen consumed per biomass produced)	(mol/mol)
$Y_{P/X}$	yield coefficient (sulphide formed per biomass produced)	(mol/mol)
$Y_{S/X}$	yield coefficient (sulphate consumed per biomass produced)	(mol/mol)
m_{max}	maximum specific growth rate	(1/s)

subscript

G	gas phase
in	incoming
j	component j
L	liquid phase
out	outgoing

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3

Microbiological aspects of biological sulphate reduction using hydrogen and carbon dioxide

Abstract

Microbiological aspects of biological sulphate reduction in gas-lift reactors were studied. Hydrogen and carbon dioxide were used as energy and carbon source. Biomass retention was obtained by aggregation and natural immobilization on pumice particles. Biological sulphate reduction on H_2/CO_2 appeared to be applicable within a pH range of 5.5 to 8.0 with an optimum near pH 7.5. The pH affected aggregate configuration and diameter. At pH 7.0, the average Sauter mean diameter of the aggregates was 1.5 mm. Moreover, phase-contrast and SEM microscopy showed highly branched aggregate surfaces. A pH increase led to increased surface irregularity without affecting the particle diameter. A pH decrease caused a decreased surface irregularity and changed the aggregate Sauter mean diameter from 1.50 mm at pH 7.0 to 2.26 at pH 5.5. However, the pH did not have a significant effect on the biomass composition. Examination of the bacterial composition of the aggregates by phase-contrast microscopy, SEM microscopy as well as enrichments showed that at all pHs Desulfovibrio species and Acetobacterium species were the most abundant micro-organisms.

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3.1 Introduction

As shown in chapter 2, sulphate reduction on hydrogen and carbon dioxide at pH 7.0 resulted in high sulphate conversion rates up to 30 g SO_4^{2-}/L per day. Biomass retention was obtained by aggregation and natural immobilization of the sulphate-reducing biomass on pumice particles. Complete sulphate removal could be achieved. However, we also found that sulphate limitation of the biomass will result in acetate production.

Since homo-acetogenic growth of sulphate-reducing bacteria has sofar been observed by only a few authors[9,11], the presence of homo-acetogenic bacteria producing acetate should be considered. Homo-acetogenic bacteria are quite common in anaerobic ecosystems and are able to produce substantial amounts of acetate[5]. Moreover, the presence of sulphate-reducing bacteria which use hydrogen as electron donor and acetate/CO_2 as carbon source is likely. These sulphate reducers are described in detail by Widdel and Bak[19]. Brysch *et al.*[3] have already reported the existence of such co-cultures, obtained during enrichment of autotrophic sulphate-reducing bacteria. However, their co-cultures apparently only grew in suspension and were not studied in more detail.

The purpose of the present study was to investigate microbial aspects of sulphate-reducing aggregates growing on H_2/CO_2 gas-mixtures. Special attention was paid to the effect of pH on the activity, as well as the morphology, of the aggregates. In addition, the microbial composition of the aggregates was studied in order to assess whether, in the cultivated aggregates, homo-acetogenic bacteria produce acetate as the substrate for growth of sulphate-reducing bacteria. A model medium with defined mineral nutrients was used throughout all experiments.

3.2 Materials and methods

Experimental set-up.

The experimental set-up was described in detail previously[8]. The biologically active reactor volume was 4.5 L. All experiments were carried out at 30 °C. The gas recycle flow rate was 350 L/h. Pumice particles (Aqua-volcano 0.2-0.5 mm, Sauter mean diameter d_{32} = 0.38 mm, density ca. 2440 kg/m^3, Aquatechniek bv, Papendrecht, Holland) were used as carrier material.

Start-up.

Start-up of the reactor experiments was performed as described before[8].

Biomass.

The reactor was inoculated with 100 mL aggregate suspension cultivated on a gas mixture of H_2 and CO_2 (80:20, vol/vol) as described previously[8]. Aggregate formation was examined by phase-contrast microscopy (Olympus BH-2, Paes Nederland bv, Zoeterwoude, The Netherlands).

Scanning electron microscopy (SEM).

Aggregates for scanning electron microscopy were stored at 4°C before fixation. The particles were fixed for 3 hours in 2.5% glutaraldehyde. After rinsing three times with demi-water the aggregates were post-fixed for 1.5 hours in 1% osmium tetroxide. After rinsing again with demi-water, the particles were stored overnight in demi-water and dehydrated next day in an ethanol series (10-30-50-70-90-100%, 20 min. per step) and subsequently critical-point dried with CO_2. After gold/palladium sputtercoating the aggregates were examined on a SEM (JSM 6300F, Jeol).

Particle size distribution.

Particle size distribution was measured according to an image analysis technique. Ca. 0.25 mL well-mixed aggregates were brought into a Petri dish (d = 6 cm). Pictures of the Petri dishes (minimum 4 per sample of 1000 particles) were digitalized and analyzed on a MAGISCAN 2 image analyzer (Applied Imaging, Tyne & Wear, UK) using the image-analysing software package GENIAS (v4.6, 1993, Applied Imaging, Tyne & Wear, UK). Assuming ideal spherical particles, the radius was calculated from the two-dimensional projection of the particles. Particles smaller than 0.05 mm diameter were neglected in the calculations. A volume surface mean diameter or Sauter diameter was used to estimate mean particle size. In this case, Sauter mean diameter is given according to:

$$d_{32} = \frac{\sum_{i=1}^{n} d_i^3}{\sum_{i=1}^{n} d_i^2}$$

where:

d_{32} = Sauter mean diameter

n = number of measurements, normalized with respect to sample volume.

The mean biofilm thickness (d_m) was calculated according to:

$$\delta_m = \frac{d_{32,a} - d_{32,p}}{2}$$

where:

$d_{32,a}$ = Sauter mean diameter of the aggregate

$d_{32,p}$ = Sauter mean diameter of the carrier particles.

Medium.

The growth medium contained (in g per 1 litre of demineralized water): Na_2SO_4, 4.97; $Na_2HPO_4.2H_2O$, 0.53; KH_2PO_4, 0.41; NH_4Cl, 0.30; KCl, 0.37; $MgCl_2.2H_2O$, 0.10; $CaCl_2.2H_2O$, 0.11; $NaHCO_3$, 4.0; resazurine, 0.00025; vitamins 0.2 mL/L; acidic trace elements 1 mL/L and alkaline trace elements 1 mL/L. Vitamins solution and trace elements solutions were prepared as described previously[17]. All chemicals used were of extra pure quality and supplied by Merck (Darmstadt, Germany), except for the gases, which were supplied by Hoekloos (Schiedam, The Netherlands).

Enrichment of trophic groups.

Samples for enrichment of bacteria were taken from the reactors once they were operated at a steady sulphate conversion[8]. For enrichment of different types of organisms (e.g. homo-acetogens or sulphate reducers) 4 serial dilutions were made in triplicate. The dilutions ranged from 10 up to 10^{10th}. As inoculum 5 mL (10% v/v) crushed aggregates suspension was used. All incubations were performed at 30 °C in 120-mL serum bottles, containing 50 mL of growth medium supplemented with $Na_2S \times 9H_2O$ (0.48 g/L). Vitamins were filter sterilized; other compounds were sterilized by heat.

Several conditions were applied. In series 1,2 and 3 a gas-phase of 180 kPa H_2-CO_2 (80:20, vol/vol) was used, while in series 4 a gas-phase of 180 kPa N_2-CO_2 (80:20, vol/vol) was used. In series 2 sulphate was omitted. In series 3 and 4 sodium acetate was added up to concentrations of 2 mM and 20 mM respectively. After inoculation, bottles were incubated while being shaken at 100 rpm. The cultures were examined by phase-contrast microscopy for microbial growth. After growth, substrate conversion and product formation were analyzed.

Analytical methods.

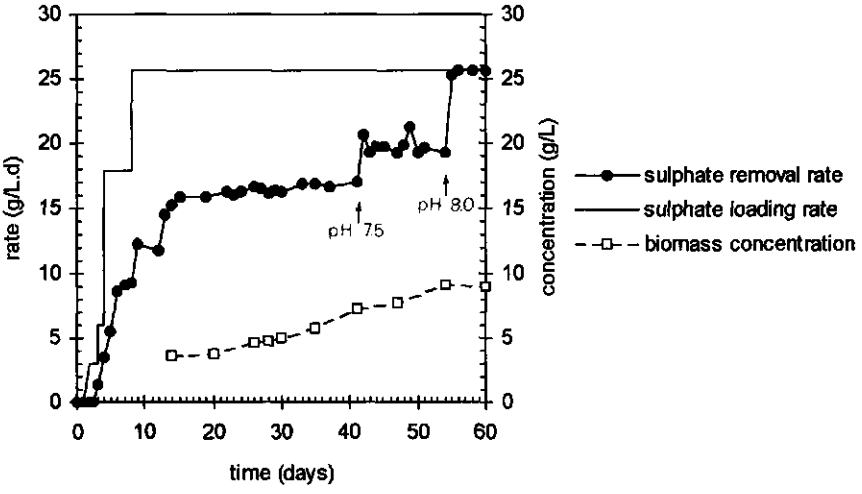
Kjeldahl-nitrogen and Volatile Suspended Solids (VSS) were determined according to *Standard Methods* [1]. Biomass concentration, in g/L, was calculated from Kjeldahl-nitrogen according to a general formula $CH_{1.8}O_{0.5}N_{0.2}$ for biomass composition[15]. Tests for desulfoviridin were carried out according to Postgate[13]. Gas phase composition, acetic acid, sulphate and sulphide were all determined as described previously[8].

3.3 Results

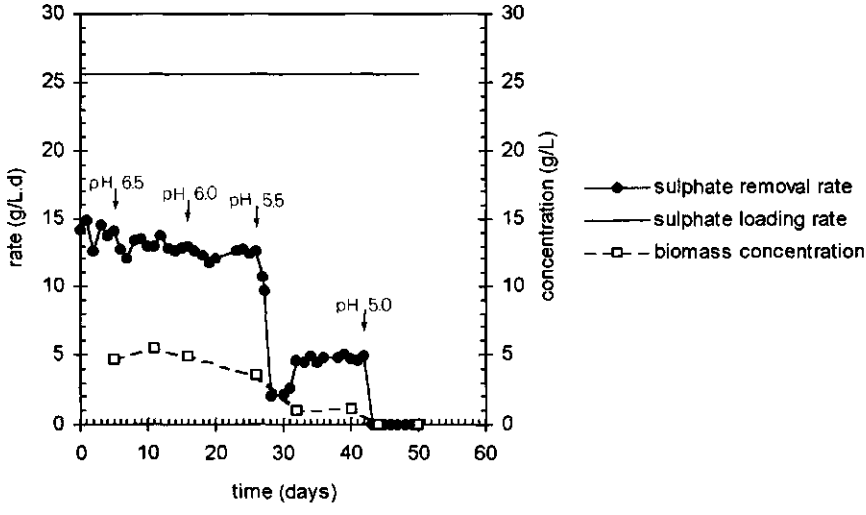
Effect of pH on the overall sulphate conversion rate and specific activity of the biomass.
Two experiments were carried out to study the effect of pH on the overall sulphate conversion rate and the specific activity of the biomass. In the first experiment the pH of the reactor was increased stepwise from 7.0 to 8.0, and in the second experiment decreased stepwise from 7.0 to 5.0. In both experiments 250 g of pumice was used as carrier material. Throughout these experiments samples were taken regularly for determination of total reactor biomass, effluent biomass, particle size distribution and SEM.

The results of the first experiment are shown in Figure 3.1 (a). The initial influent sulphate concentration was 3.36 g/L at an initial reactor pH of 7.0. After inoculation, the reactor was started in batch mode. Only a gas feed of 2.94 Ln/h, containing 90% H_2 and 10% CO_2, was supplied. After 3 days, the continuous operation of the reactor was started at a hydraulic retention time of 13.5 h and the gas feed composition then was changed to 80% H_2 and 20% CO_2 at a total flow rate of 3.30 Ln/h. The hydraulic retention time was decreased further to 4.5 h at day 5. The influent sulphate concentration was changed to 4.8 g/L at day 8. At day 21 the influent gas flow rate was increased to 6.60 Ln/h. After about 25 days of operation a steady sulphate conversion rate of 16 g SO_4^{2-}/L reactor per day was reached. No methane or fatty acids could be detected. The effluent biomass concentration increased from 0.10 g/L at day 20 to 0.15 g/L at day 41.
At day 41, the reactor pH was raised to 7.5. After this pH increase, the effluent biomass concentration increased from 0.15 g/L to 0.18 g/L.
After 54 days, the reactor pH was further increased from 7.5 to 8.0. The sulphate conversion rate now approached the sulphate loading rate. However, during this period more voluminous sludge aggregates were formed resulting in a reactor failure at day 60, due to clogging of the transition of the downcomer to riser area.

Results of the second experiment are shown in Figure 3.1 (b). The experiment was carried out with the restarted reactor from the previous experiment, therefore the restart period is not shown. Until day 5, the effluent biomass concentration was 0.1 mg/L. No methane or fatty acids could be detected.
The reactor pH was decreased to pH 6.5 at day 5. During this second period the reactor biomass and effluent biomass concentrations remained constant at an average of 5.0 g/L and 0.1 mg/L, respectively.



(a)



(b)

Figure 3.1. (a) Effect of pH increase on overall sulphate conversion rate and biomass concentration in the reactor. (b) Effect of pH decrease on overall sulphate conversion rate and biomass concentration.

The reactor pH was further decreased to pH 6.0 at day 16. The overall sulphate conversion rate decreased only slightly to 12.5 g SO_4^{2-}/L per day. Also the effluent biomass concentration became slightly lower, i.e. it dropped to 0.07 mg/L.
A further decrease of the pH to 5.5 at day 26, caused a dramatic deterioration in the overall sulphate conversion rate. Also the reactor biomass concentration decreased significantly to 1.0 g/L at day 32 and the effluent biomass concentration amounted only 0.01 mg/L. Small amounts (up to 0.1%) of methane were found in the gas phase of the reactor.
Lowering the reactor pH to 5.0 at day 42, resulted in a complete deterioration of the biological activity in the reactor.

General characterization of the aggregates.
Most of the aggregates from the reactor operated at pH 7.0 were spherical with an irregular surface, often divided in sub-regions. Sometimes large branches were formed. The diameter of the aggregates was in the range of about 0.2 - 2.0 mm with a mean Sauter diameter of 1.46 mm. A typical size distribution of the aggregates is given in Figure 3.2.

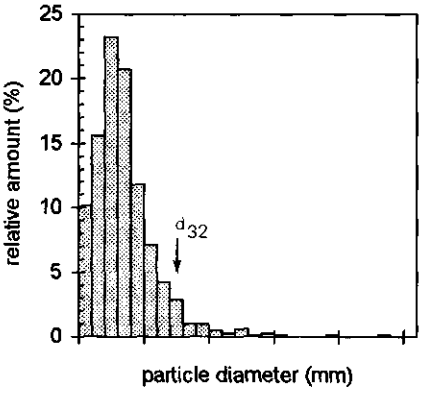


Figure 3.2. Typical size distribution of aggregates grown at pH 7.0. The Sauter mean diameter was 1.46 mm.

From this size distribution a mean biofilm thickness of 0.54 mm was calculated. During steady sulphate conversion, no bare carrier particles were observed. However, several particles did not contain any pumice grains in the centre of the particles. The colour of the aggregates was dark

grey to brownish. SEM also showed an irregular aggregate surface with several fissures. The biomass consisted of vibrios and small rods. The ratio of vibrios to rods was typically in the order of 20:1. More detailed micrographs clearly showed the morphology of the predominant micro-organisms (Figure 3.3). Generally a complete biofilm was observed but quite often parts had sloughed off of the particle surface.

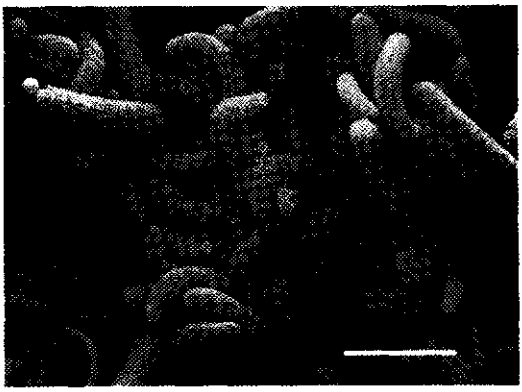
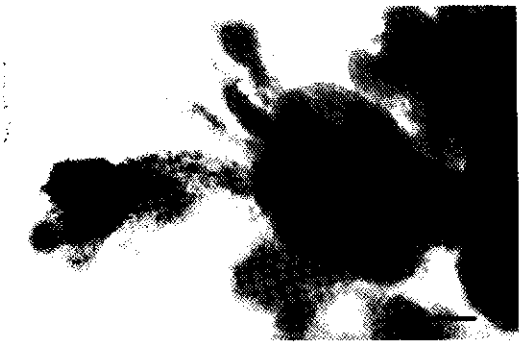
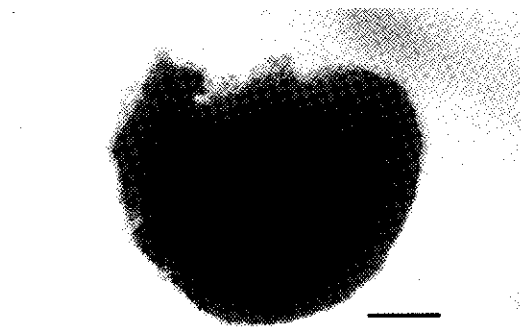


Figure 3.3. *SEM micrograph showing clearly the morphology of the microorganisms. Bar lenght is 1.5 μm.*

During the first reactor experiment the biofilm morphology changed when the pH was increased. At pH 7.0 the biofilm surface was irregular with large protrusions. The biofilm surface became even more irregular when the pH was increased, resulting in more voluminous sludge at pH 8.0. The biomass composition was not affected by pH levels beyond 7.0. The Sauter mean diameter remained rather constant at 1.50 mm, throughout the whole experiment.
During the second reactor experiment, the biofilm morphology was strongly affected by the imposed lower pH values. The decrease of pH caused a decrease of surface irregularity, compared to the aggregates cultivated at pH 7.0, resulting in smooth aggregates at pH 5.5. This change in surface structure is shown in Figure 3.4 (a), (b). Throughout the second experiment no significant change in the biomass composition was observed either. Only at pH 5.5, some filamentous and spirillum-shaped bacteria appeared in the biomass. On the other hand, particle size was affected by lower pH-values. The Sauter mean diameter increased from 1.50 mm at pH 7.0 to 2.26 mm at pH 5.5.



(a)



(b)

Figure 3.4. Change in the aggregate surface structure upon pH decrease. (a) Picture taken during growth at pH 7.0. (b) Picture taken during growth at pH 5.5. Bar lenght is 0.75 mm.

Microbial composition analysis.

Observation by phase-contrast microscopy and electron microscopy of aggregates and enrichment cultures revealed that two types of, morphologically distinct, bacteria predominated. The enrichments showed the following results.

In series 1 (H_2/CO_2 + sulphate) no acetate production was observed. The biomass consisted of mobile Gram (+) rods (0.7 x 3-5 mm) and mobile Gram (-) vibrios (0.5 x 2-3 mm). The desulfoviridin (DV) test was positive.

In series 2 (H_2/CO_2 - sulphate) acetate was produced up to 20 mM. No other volatile fatty acids were produced. The biomass consisted of mobile Gram (+) rods (0.7 x 3-5 mm); the DV-test was negative. Incubation of the bacteria from the highest dilution in sulphate containing medium did not result in sulphide formation. These highest dilution was susceptible to pasteurization (5 or 10 min. at 80 °C).

In series 3 (H_2/CO_2 + sulphate + 2 mM acetate) acetate was consumed slowly, while sulphate reduction took place. The biomass was similar to that of series 1. The DV-test was positive.
In series 4 (N_2/CO_2 + sulphate + 20 mM acetate) no growth was observed, even after 4 weeks of incubation.
The biomass of the aggregates in the reactor consisted of Gram (+) rods (0.7 x 3-5 mm) and Gram (-) vibrios (0.5 x 2-3 mm). The DV-test was also positive.

3.4 Discussion

From the data of the experiments at pH 7 and higher, it is apparent that biological sulphate reduction on H_2/CO_2 proceeds very well under these conditions. As expected from literature data[12,14], an increase of pH beyond 7 causes a higher overall sulphate removal rate mainly due to the reduced toxicity of hydrogen sulphide. Conversely, from the data on the retained biomass concentrations during the steady sulphate conversion rates at pH 7.0, 7.5 and 8.0, a decrease of the overall activity, from 4.2 g SO_4^{2-}/g biomass per day to 2.1 g SO_4^{2-}/g biomass per day, can be calculated. Since neither substrate nor product concentrations altered significantly during the separate pH conditions, this drop in activity was not expected.
A possible explanation could be that during the steady operation conditions, the number of *Acetobacterium* sp. decreases in time, due to the higher susceptibility of the *Acetobacterium* sp. to high hydrogen sulphide concentrations, relative to the sulphate-reducing bacteria. As a result, the growth rate of the homo-acetogenic bacteria might become lower than its 'viable biomass' retention time. As a consequence, these bacteria will vanish slowly from the biomass. Since the *Desulfovibrio* sp. are always limited by the amount of acetate produced[3], the overall specific activity of the biomass will decrease with time. This hypothesis is supported by results obtained in batch experiments carried out with the enriched homo-acetogens (data not shown). Incubation of these bacteria in sulphate depleted medium, supplemented with different amounts of sulphide resulted in complete inhibition at pH 7.0 at an H_2S concentration of approximately 500 mg/L.
Furthermore, a stepwise increase of pH furthermore affected the aggregate morphology. A higher pH caused an increase of surface irregularity of the aggregates, via the formation of highly branched surfaces. The latter might have been caused by a low substrate concentration. Similar results have been obtained by other authors[4,6]. At this low substrate concentration a kind of chemotaxis may occur by which the specific surface area increases. In such a way, real substrate limitation is postponed. According to previous results[8], substrate limitation occurred at sulphate removal rates exceeding 30 g SO_4^{2-}/L reactor per day.

Lowering of pH to pH 6.5 and 6.0 only affected the overall sulphate removal rate slightly. This small effect can be explained by assuming that the decrease in specific activity of the outer region of the aggregates was compensated by the increased activity of the inner region of the aggregates. Due to the reduced consumption rate of the growth limiting substrate in the outer region, more substrate then became available to the inner region of the aggregates. Similar effects have been observed by van Lier[10] in their measurements on thermostability of thermophilic anaerobic granules. They found that the high stability of the reactors to temperature changes was caused by the above-mentioned effect.
The same hypothesis might also explain the rather dramatic drop in overall sulphate conversion rate observed upon a pH shift from 6.0 to 5.5 and 5.0. At pH 5.5 all biomass in the aggregate takes part in the process, although at a low specific activity, and at pH 5.0 no activity is left.
In view of the above observations it is interesting to note that the average aggregate diameter increased while the surface irregularity decreased, when the reactor was operated at pH 5.5. Most likely, a decrease of the growth rate (i.e. low substrate utilization) results in a less branched outer region of the aggregates.

Phase-contrast micrographs, see Figure 3.4 (a), showed highly branched aggregate-surfaces at pH 7.0 and higher. The typical sub-unit structure of these aggregates may be caused by *in situ* clumping of small aggregates to larger units. This hypothesis is supported by the fact that sometimes large aggregates were found, containing several pumice particles in the centre of the aggregate. SEM micrographs also indicated an irregular biofilm surface, although not as clear as from the light micrographs. This is probably due to the fixation procedure, which generally caused some sticking of branches to the aggregates. The occurrence of the branched biofilm surface probably is the result of the low dissolved hydrogen concentrations prevailing in the system. Highly branched structures would facilitate substrate (i.e. hydrogen) transport into the inner regions of the biofilm and therefore such a biofilm structure would be favoured by substrate limited growth conditions, as suggested by Christensen *et al.*[4].
Beeftink and Staugaard[2] concluded that biofilm-covered sand grains only represent an intermediate type of aggregate, and that matured aggregates would be essentially free of sand grains. We did not find, however, any loss of pumice particles other than loss by sampling, which was only a very small fraction. Since - during steady operation - size distribution did not change significantly, the biomass increase in the experiments can be attributed mainly to an increase of the number of particles resulting from the formation of aggregates not containing carrier particles. Severe sloughing of biofilms occurred, which presumably limited the maximum diameter of the aggregates. As a consequence of this, sloughing generated biofilm

fragments with sufficiently high settling velocities to enable biomass retention. Therefore, pumice-containing aggregates are likely to be retained completely in a gas-lift reactor, as long as the excess sludge discharge can be omitted.

The enrichments clearly showed that sulphate reduction is performed by Gram (-) vibrios growing on hydrogen as the electron donor and CO_2/acetate as the carbon source. Besides, the bacteria contain desulfoviridin. Therefore the bacteria can be designated as members of the genus *Desulfovibrio* [19]. Homo-acetogenic activity was caused by non-sporeforming, mobile Gram (+) rods, which are not able to reduce sulphate. Besides acetate, no other volatile fatty acids were produced. Therefore these bacteria can be designated as members of the genus *Acetobacterium* [5,16].

Isolation of stable cultures of *Desulfovibrio* and *Acetobacterium* has been reported before by Brysch *et al.*[3]. Their enrichments in liquid mineral media with H_2/CO_2 and sulphate consistently yielded mixed cultures of non-autotrophic, acetate-requiring *Desulfovibrio* sp. and autotrophic, acetate-producing *Acetobacterium* sp. The cell ratio was approximately 20:1 respectively. Their cultures grew in suspension with a doubling time of 8.5 h.

Our experimental results reveal that this type of culture is able to form stable aggregates. Figure 3.3 shows a SEM micrograph of the biomass. Bacterial counting showed that even the cell ratio closely resembled the cell ratio found by Brysch *et al.*[3]. From experiments carried out with suspended biomass (data not shown) we calculated a doubling time of 14 h, which is slightly higher than the value of 8.5 h found by Brysch.

Apparently, the aggregate formation occurring in our reactors was related to the short liquid retention time imposed on the system. A short liquid retention time results in a high selection pressure on the system and will favour bacterial adhesion, while non-adhering bacteria will be largely removed by wash-out. Similar principles for aggregate formation were applied by Heijnen[7], who advocated to decrease the liquid retention time below the minimal doubling time of the considered bacterial population. In the experiments of Brysch *et al.*[3], microorganisms were grown in batch-cultures, for which obviously no selective pressure for adhesion exists.

3.5 Conclusions

In summary, it can be concluded that biological sulphate reduction is possible in the pH range of 5.5 to 8.0, with an optimum near pH 7.5. Moreover, it can be concluded that biological sulphate reduction in gas-lift reactors fed with hydrogen and carbon dioxide will result in the

development of mixed cultures of *Desulfovibrio* sp. and *Acetobacterium* sp. These mixed cultures are well able to form stable aggregates consisting of either aggregated biomass, or biofilm on carrier particles.

Acknowledgements

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4

Biological sulphate reduction using synthesis gas

Abstract

Biological sulphate reduction was studied in lab-scale gas-lift reactors. Synthesis gas (gas mixtures of $H_2/CO/CO_2$) was used as energy and carbon source. The required biomass retention was obtained by aggregation and immobilization on pumice particles. Special attention was paid to the effect of CO addition on the sulphate conversion rate, aggregation and aggregate composition. Addition of 5% CO negatively affected the overall sulphate conversion rate, i.e. it dropped from 12 - 14 g SO_4^{2-}/L per day to 6 - 8 g SO_4^{2-}/L per day. However, a further increase of CO to 10 and 20% did not further deteriorate the process. With external biomass recycling the sulphate conversion rate could be improved to 10 g SO_4^{2-}/L per day. Therefore biomass retention clearly could be regarded as the rate limiting step. Furthermore, CO affected the aggregate shape and diameter. SEM photographs showed that rough aggregates, pre-grown on H_2/CO_2, changed into smooth aggregates upon addition of CO. Addition of CO also changed the aggregate Sauter mean diameter (d_{32}) from 1.7 mm at 5% CO to 2.1 mm at 20% CO. After addition of CO, a layered biomass structure developed. Acetobacterium sp. were mainly located at the outside of the aggregates, whereas Desulfovibrio sp. were located inside the aggregates.

Published as: van Houten, R.T., van der Spoel, H., van Aelst, A.C., Hulshoff Pol, L.W., Lettinga, G. 1996. Biological sulphate reduction using synthesis gas as energy and carbon source. Biotechnol. Bioeng. **50**:136-144.

4.1 Introduction

In the previous two chapters, we already investigated gas mixtures of H_2/CO_2, enabling us now to assess the effect of CO.

Although use of carbon monoxide as substrate for sulphate reduction has been described in literature before[2,7,9], most of the reported studies dealt with elucidation of biochemical pathways of CO-utilization. Only Du Preez *et al.*[11] described more practical applications of CO as substrate for sulphate reduction. However, in their study little attention was paid to the identification of the dominant micro-organisms and the effect of CO on the biomass composition. Furthermore, only a low sulphate conversion rate, up to 1.6 g SO_4^{2-}/L reactor per day was obtained.

The study presented in this chapter deals with investigations concerning the optimization of biological sulphate reduction with use of real synthesis gas as electron donor. Special attention was paid to the influence of CO on the sulphate conversion rate and aggregate formation. In addition, size distribution and microbial composition of the aggregates were studied as a function of the CO concentration. A model medium with defined mineral nutrients was used.

4.2 Materials and methods

Experimental setup.

The experimental setup is shown in Figure 4.1, and was previously described in detail[3]. The biologically active volume of the reactor was 4.5 L. All experiments were carried out at 30 °C and pH 7.0. The gas recycle flow rate was 350 L/h. Pumice particles (Aqua-volcano 0.2-0.5 mm, density ca. 2440 kg/m^3, Aquatechniek bv, Papendrecht, The Netherlands) were used as carrier material.

Start-up.

Start-up of the reactor experiments was performed as described previously[3].

Biomass.

The reactor was inoculated with 100 mL aggregate suspension. These aggregates had been cultivated on a gas mixture of H_2-CO_2 (80:20, vol/vol) as described previously[3]. Aggregate formation was examined by phase-contrast microscopy, as described before[3,4].

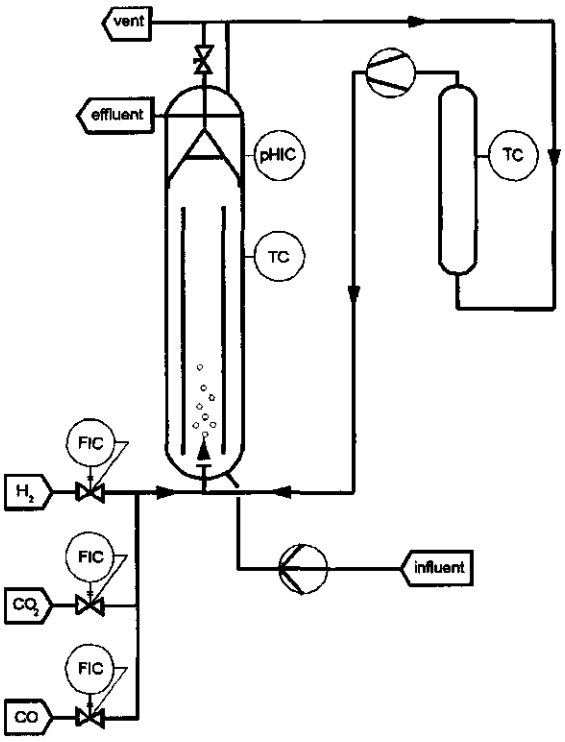

Figure 4.1. Flow sheet of the experimental set-up. This set-up was used throughout the experiments. The set-up consists of an internal draft-tube gas-lift reactor and a buffer column, connected by a gas recycle. FIC: flow indication and control; pHIC: pH indication and control; TC: temperature control.

Scanning electron microscopy (SEM).

Aggregates were prepared for and examined with scanning electron microscopy as described before[4].

Particle size distribution.

Particle size distribution was measured according to an image analysis technique as described previously[4].

Medium.

The growth medium contained (in g per 1 liter of demineralized water): Na_2SO_4, 4.97; $Na_2HPO_4.2H_2O$, 0.53; KH_2PO_4, 0.41; NH_4Cl, 0.30; KCl, 0.37; $MgCl_2.2H_2O$, 0.10; $CaCl_2.2H_2O$, 0.11; $NaHCO_3$, 4.0; resazurin, 0.00025; vitamins 0.2 mL/L; acidic trace elements 1 mL/L and alkaline trace elements 1 mL/L. Vitamins solution and trace elements solutions were prepared as described previously[14]. All chemicals used were of extra pure quality and supplied by Merck (Darmstadt, Germany), except the gases, which were supplied by Hoekloos (Schiedam, Holland).

Analytical methods.

The biomass concentration, in g/L, was calculated from Kjeldahl-nitrogen as described previously[4]. Tests for desulfoviridin were carried out according to Postgate[10]. Gas phase composition, acetic acid, sulphate and sulphide were all determined as described previously [3].

4.3 Results

The results of the first experiment are shown in Figure 4.2. In this experiment 250 g of pumice was used as carrier material. The influent sulphate concentration was 3.36 g/L. After inoculation the reactor was operated in batch mode. A gas feed of only 2.43 Ln/h, containing 99% H_2 and 1% CO_2, was supplied. After one day the operation of the reactor was changed to a continuous mode at a hydraulic retention time of 27 h. At day two, the hydraulic retention time was decreased to 13.5 h. Simultaneously, the gas feed composition was changed to 94% H_2 and 6% CO_2 at a flow rate of 2.55 Ln/h. The hydraulic retention time was further decreased to 4.5 h at day 3. Also, the gas feed composition was changed to 80% H_2 and 20% CO_2 at a flow rate of 3.30 Ln/h. After 9 days of operation a steady sulphate conversion rate was reached. No methane or fatty acids could be detected. During this first period, the total liquid sulphide concentration remained constant at a level of 849 mg total S/L ($\pm$ 41 mg/L). The biomass concentration in the reactor was 3.0 g/L.

In the next period we started to study the effect of CO on the sulphate conversion rate. For this purpose, the gas phase composition was changed at day 17, to 80% H_2, 15% CO_2 and 5% CO. Figure 4.2 clearly shows the detrimental effect of CO. The sulphate conversion rate gradually declined to 9.4 g SO_4^{2-}/L per day at day 35. In addition, also the biomass concentration dropped to 1.4 g/L.

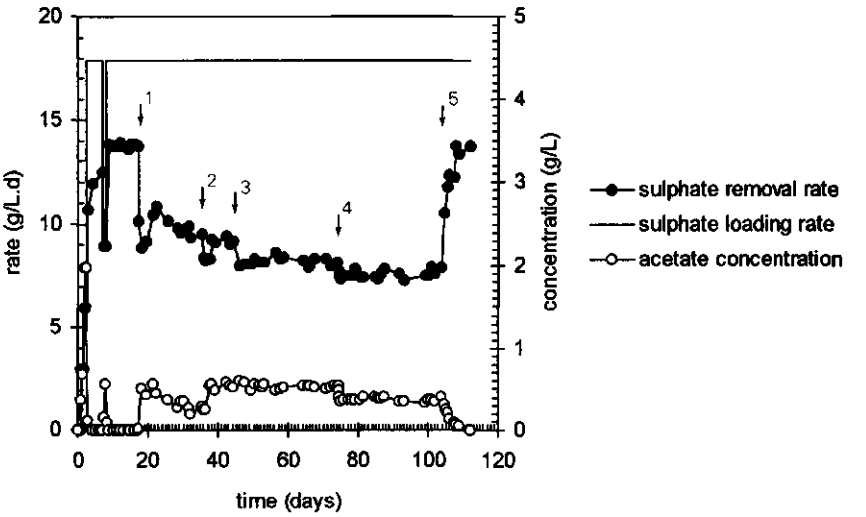

Figure 4.2. Sulphate loading rate, sulphate conversion rate, acetate concentration and biomass concentration during the first experiment; 250 g pumice was used as carrier material. Start H_2/CO_2; Arrow 1, $H_2/CO_2/CO$ 80:15:5; Arrow 2, $H_2/CO_2/CO$ 80:10:10; Arrow 3, H_2/CO 80:20; Arrow 4, feed of medium without bicarbonate; Arrow 5, H_2/CO_2 80:20 again.

At day 35, the CO concentration was increased further to 10%. This apparently hardly affected the system because the sulphate conversion rate declined only slightly, while the biomass concentration remained constant at 1.4 g/L.

From day 45 onwards the gas phase composition was changed to 80% H_2 and 20% CO. This change also hardly affected the process. Only a small decrease of the sulphate conversion rate was found. The biomass concentration also remained constant at 1.5 g/L.

At day 74, bicarbonate was left out of the medium to study, if there was, any conversion of CO into CO_2. Depletion of bicarbonate caused a slight decrease of the sulphate conversion rate as well as the acetate concentration. CO_2 was detected in the gas phase up to a steady level of 0.8%. Any clear effect on the biomass concentration could not be observed. By measuring and analyzing the effluent gas flow regularly during the steady periods from day 50 to 70 and day 80 to 100, we could check the mass balances (see Appendix) for H_2, CO and sulphur compounds quantitatively. The results are given in Table 4.1.

To test the (ir)reversibility of the CO toxicity, CO was replaced completely by CO_2 at day 104. The results clearly reveal that the sulphate conversion rate recovered within five days. In the

same period acetate disappeared completely from the effluent. Moreover, the reactor biomass concentration increased from 1.5 g/L at day 104 to 2.0 g/L at day 112.
In presence of CO, biofilm growth on the pumice particles proceeded poorly but aggregates were formed instead. Even though these aggregates generally did not contain carrier particles, only aggregates smaller than 0.2 mm were washed-out.

Table 4.1. Conversion rates obtained during the steady periods of the first experiment. The values are calculated according to the steady-state mass balances (A.3),(A.4),(A5)and (A.6) given in the Appendix.

time (days)	$-R'^{a}_{S}$ (mol/L.d)	$-R'^{a}_{H2}$ (mol/L.d)	$-R'^{a}_{CO}$ (mol/L.d)	R'^{a}_{P} (mol/L.d)	$R'^{a}_{Ac^-}$ (mol/L.d)
13	0.141	0.572	n.d.[b]	0.138	0.000
17	0.139	0.565	n.d.	0.136	0.000
52	0.085	0.410	0.103	0.080	0.053
65	0.083	0.425	0.110	0.075	0.045
72	0.083	0.434	0.097	0.079	0.047
77	0.078	0.390	0.092	0.070	0.033
81	0.077	0.381	0.100	0.067	0.037
91	0.078	0.407	0.105	0.072	0.031

[a] R' denotes R (given in the Appendix) expressed in mol/L.d.
[b] n.d. = not determined.

In view of the observed poor biofilm formation we decided to carry out a second experiment in which only 50 g of pumice was used. The imposed process conditions (i.e., loading rates, pH and temperature) and start-up procedure were the same as in the first experiment. The results are shown in Figure 4.3.
During the first 6 days the hydraulic retention time was stepwise decreased to 13 h. Starting from day 6, a hydraulic retention time of 4.5 h was applied. Simultaneously the incoming gas flow was set to 80% H_2 and 20% CO_2 at a flow rate of 3.30 Ln/h. During the first period of the experiment, the biomass concentration in the reactor amounted to 2.4 g/L. Immediately after the change in gas phase composition at day 16, the system responded by a drop in the sulphate conversion rate. Moreover, the biomass concentration dropped to 1.4 g/L, while acetate was produced.
From day 34 onwards, the CO concentration was increased further to 10%. Again any clear effect could not be observed. Both the sulphate conversion rate as well as the acetate

concentration remained almost constant. Also, the biomass concentra-tion remained unchanged at 1.3 g/L.

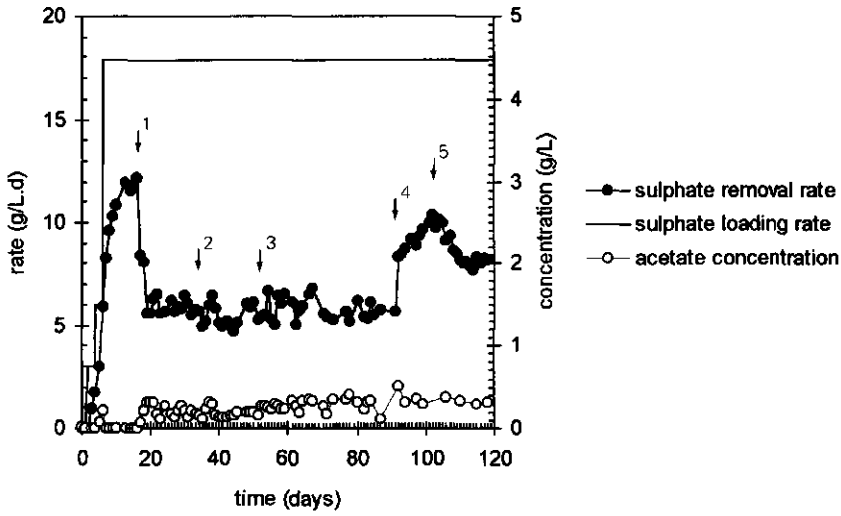

Figure 4.3. *Sulphate loading rate, sulphate conversion rate,acetate concentration and biomass concentration during the second experiment; 50 g pumice was used as carrier material. Start H_2/CO_2; Arrow 1, $H_2/CO_2/CO$ 80:15:5; Arrow 2, $H_2/CO_2/CO$ 80:10:10; Arrow 3, H_2/CO 80:20; Arrow 4, start of the recycling of externally settled biomass; Arrow 5, end of the recycling of external settled biomass.*

Except for the acetate concentration, which increased slightly, any noticeable change also was absent after the CO concentration was increased to 20% at day 52.

Since the retainable amount of biomass presumably represented one of the main factors governing the conversion potential of the process, we decided to investigate the effect of recycling of washed-out biomass. For this purpose the reactor was combined with an external settler from which settled small biomass aggregates were recycled to the bioreactor. These recycled small aggregates could possibly serve as germs for aggregation. The biomass recycling was started at day 91. Indeed, immediately following the start of the recycling, the sulphate conversion rate increased. Concomitantly, the biomass concentration increased from 1.3 g/L to 2.3 g/l at day 93 and 2.6 g/L at day 104. After termination of the biomass recycling, its concentration in the reactor slowly declined to 2.1 g/L. Apparently because of that, the sulphate

conversion rate dropped too. The results of the calculated mass balances of this experiment are given in Table 4.2.

Table 4.2. Conversion rates obtained during the steady periods of the second experiment. The values are calculated according to the steady-state mass balances (A.3),(A.4),(A5)and (A.6) given in the Appendix.

time (days)	$-R'^{a}_{S}$ (mol/L.d)	$-R'^{a}_{H2}$ (mol/L.d)	$-R'^{a}_{CO}$ (mol/L.d)	R'^{a}_{P} (mol/L.d)	R'^{a}_{Ac-} (mol/L.d)
10	0.122	n.d.[b]	n.d.	n.d.	0.000
22	0.068	0.291	0.022	0.058	0.016
31	0.063	0.231	0.019	0.055	0.014
42	0.054	0.209	0.022	0.040	0.014
49	0.062	n.d.	n.d.	n.d.	0.019
62	0.053	n.d.	n.d.	n.d.	0.027
83	0.062	0.290	0.091	0.049	0.035

[a] R' denotes R (given in the Appendix) expressed in mol/L.d.
[b] n.d. = not determined.

During the experiment the aggregate size distribution was measured regularly. The results, shown in Figure 4.4, reveal that due to the CO addition apparently the size of the aggregates increased. The mean Sauter diameter (d_{32}) increased from 1.7 mm at 20% CO_2 and 1.8 mm at 5% CO to 2.1 mm at 20% CO.

SEM micrographs showed, upon addition of CO, a clear change in the biomass composition of the aggregates (see Fig. 4.5). In the presence of CO, the surface of the aggregates became smooth and the predominant microorganisms started to grow in a layered structure. The rod-shaped *Acetobacterium* sp. were predominant at the outside of the aggregate, whereas the vibrio-shaped *Desulfovibrio* sp. were at the inside of the aggregates.

4.4 Discussion

The results of both experiments show that after about 10 days a steady sulphate conversion rate of 12 - 14 g SO_4^{2-}/L per day was reached. We described earlier[3], that on a gas mixture of H_2/CO_2, this steady conversion rate is mainly determined by the sulphide inhibition. After changing the gas phase composition to 80% H_2, 15% CO_2 and 5% CO, in both experiments the

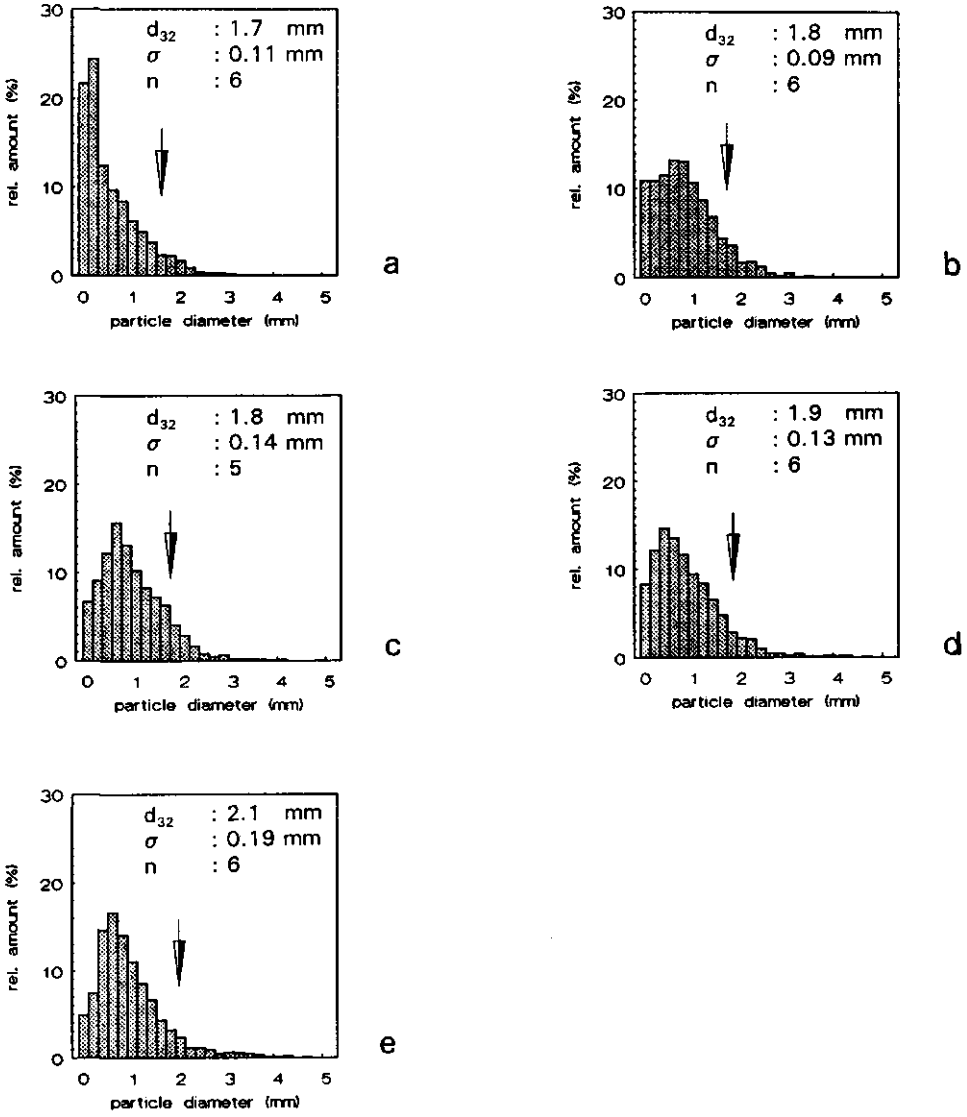

Figure 4.4. Size distributions of aggregates grown at different CO concentrations. (a) Size distribution of aggregates grown on H_2/CO_2. (b) Size distribution of aggregates grown on H_2/CO (5%, 12 days). (c) Size distribution of aggregates grown on H_2/CO (5%, 18 days). (d) Size distribution of aggregates grown on H_2/CO (10%, 18 days). (e) Size distribution of aggregates grown on H_2/CO (20%, 31 days). d_{32}: Sauter mean diameter, indicated by the arrow; σ : standard deviation; n: number of samples.

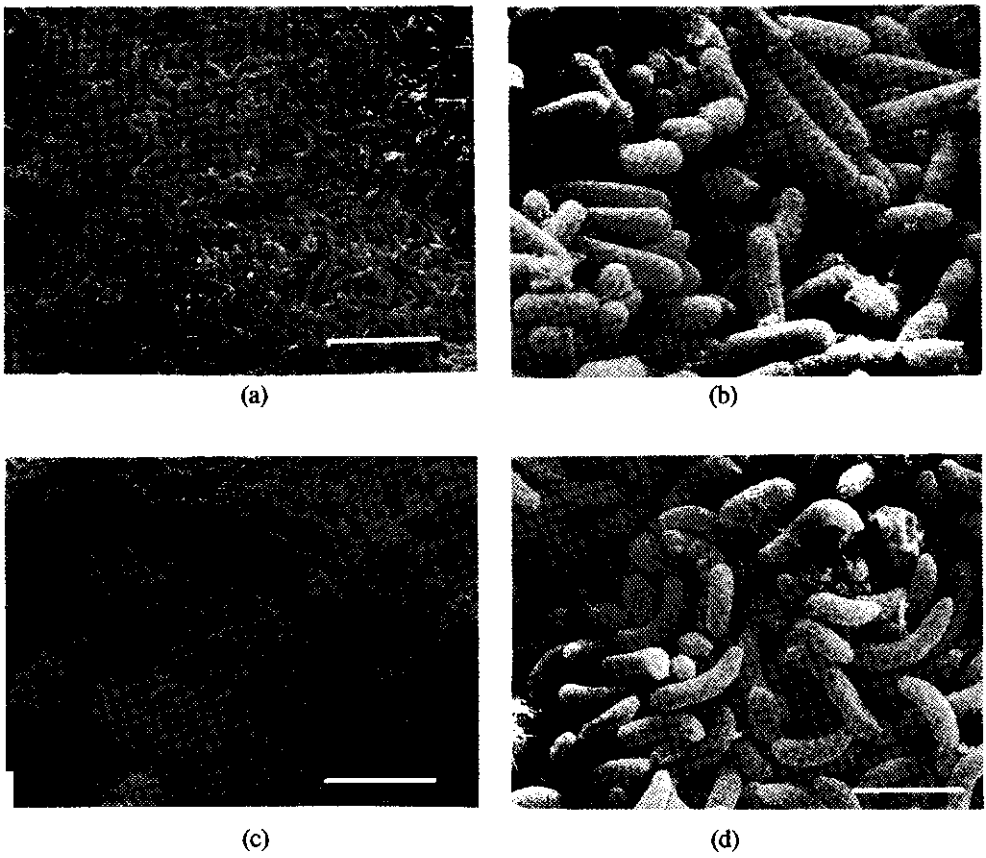

Figure 4.5. *SEM micrographs showing the layered population structure of the aggregates grown on H2/CO (20%, 31 days). (a) Surface of an aggregate. (b) Detail of the surface, showing the predominance of* Acetobacterium *sp. (c) Inside of the aggregate. (d) Detail of the inside, showing the mixed population of* Desulfovibrio *sp. and* Acetobacterium *sp. Bar lengths are 8.5 μm (a,c) and 1.5 μm (b,d) respectively.*

sulphate conversion rate dropped to 6 - 8 g SO_4^{2-}/L per day. However, a further increase of the CO concentration to 10 and 20% clearly did not affect the sulphate conversion rate. The drop in the sulphate conversion rate due to the addition of 5% CO mainly can be attributed to the fact that small aggregates are washed out, resulting in a lower biomass hold-up in the system. When the CO concentration is increased up to 10% and 20%, plausibly hydrogen pene-trates deeper into the aggregates due to the increased toxicity resulting from CO. In this way the increased CO toxicity can be compensated by the increased participation of the biomass inside the aggregate to the conversion process. Similar phenomena were observed for pH[4] and temperature[6] changes in comparable aggregates.

The results of the second experiment show that recycling of washed-out biomass to the bioreactor improves the sulphate conversion rate substantially. From this observation, a high biomass retention is evidently a prerequisite for accomplishing higher sulphate conversion rates. Therefore, priority should be set to the development of bioreactor systems that meet this condition.

The sulphate conversion rates with H_2/CO as energy and carbon source presented in this study have not been reported so far. In fact, only Du Preez *et al.*[11,12] reported about sulphate reduction experiments using H_2/CO gas mixtures. The maximum sulphate conversion rate found by them amounted to 2.4 g SO_4^{2-}/L per day, using pure CO. With H_2/CO gas mixtures they obtained a maximum sulphate conversion rate of 1.6 g SO_4^{2-}/L per day.

The results of Du Preez seem contradictionary. Actually, they also found[12] that decreasing the H_2/CO ratio from 90:10 to 50:50 caused a reduction of the sulphate conversion rate, while it increased again after resuming the feed with 90:10 H_2/CO. This, to our opinion, also points to a toxicity at higher CO concentrations. Therefore, their results also support the general idea that CO is a quite toxic compound. In this context their reported higher sulphate conversion rate with pure CO certainly is peculiar.

The results of the mass balances in Table 4.1 and 4.2 reveal that more H_2 is used then stoichiometricallly is needed for sulphate reduction ($R_{H2}/R_{SO4} > 4$). This can be explained by the formation of acetate according to the reaction[15]:

$$2HCO_3^- + 4H_2 + H^+ \rightarrow CH_3COO^- + 4H_2O$$

However, the additional amount of H_2 consumed does not suffice for the produced amount of acetate. It is therefore likely that some of the added CO was converted to CO_2, according to the equation[2]:

$$CO + H_2O \rightarrow CO_2 + H_2$$

The hypothesis is supported by the experimental results of the first experiment in which, during the period where only CO (without CO_2 or bicarbonate) was added, up to 0.8% CO_2 was found in the gas phase. Moreover, from Table 4.1 it also appears that CO was not used as electron donor for sulphate reduction, because the amount of CO converted is stoichiometrically nearly equal to the amount of acetate produced plus the additional amount of H_2 generated.

A satisfactory biofilm formation on pumice particles and subsequently also aggregation was observed during the start-up of both experiments on a gas mixture of H_2/CO_2. As described previously[3,4], the external surface of these biofilms and aggregates had a 'hairy' appearance. After addition of CO to the gas mixture, the hairy structures on the external surface disappeared and the aggregates became smooth.
The observed significant change in surface structure looks very similar to the surface change of an H_2/CO_2 fed culture after the pH was decreased below 7.0[4]. Lowering of the pH resulted also in a disappearance of the hairy structures and some loss of biomass attachment capacity to carrier particles was observed that time.
Conceivably, the loss of protrusions is due to the lowered growth rate of the microorganisms after addition of CO. Due to this lowered growth rate more hydrogen (the growth limiting substrate on H_2/CO_2) becomes available to the bacteria and consequently there is less need for a high surface area of the aggregates.

The observed increase in the Sauter mean diameter after CO-addition (see Fig. 4a to 4e) should be interpreted with great care because it might result from the wash-out of all the fine aggregates. The relative amount of large aggregates obviously increased but the absolute aggregate size in fact increased only slightly. In fact the biomass concentration dropped significantly when 5% CO was added to the gas feed. Remarkably, no further decrease of the biomass concentration occurred upon a further increase of the CO-fraction in the gas mixture. Apparently, the structure of the aggregates did not further deteriorate, neither decreased the number of the remaining aggregates.

Upon the change in the influent gas-flow from 20% CO_2 to 20% CO, a clear change in the biomass population was observed. As we described before[4], the biomass grown on H_2/CO_2, mainly consisted of *Desulfovibrio* sp. and *Acetobacterium* sp. which were homogeneously distributed throughout the aggregate. However, after addition of CO, a layered biomass structure developes (see Fig.4.5). The *Acetobacterium* sp. predominate at the outside of the aggregates, whereas the *Desulfovibrio* sp. are located at the inside of the aggregates.

This change in bacterial composition can be explained by a difference in sensitivity toward CO. *Desulfovibrio* sp. seem to be more susceptible to carbon monoxide inhibition than *Acetobacterium* sp. According to Lupton[8] *Desulfovibrio vulgaris* can only grow at CO concentrations less than 4.5% while Karpilova[5] reported for *Desulfovibrio desulfuricans* that growth stopped when 20% CO is present in the gas mixture. In contrast, *Acetobacterium woodii* was found to be able to grow with a doubling time of 13 h, at a gas-phase concentration up to 30% CO[13]. Additionally, this organism, when maintained on H_2/CO_2, did not require any adaptation period to grow with CO[13]. From these literature data it can be concluded that stronger inhibition of the *Desulfovibrio* sp. is the main reason for the observed change in the spatial distribution of the microorganisms. At higher CO levels, the *Desulfovibrio* sp. cannot survive in the outer region of the aggregates and will be replaced by *Acetobacterium* sp. This results in a relative increase of the amount of *Acetobacterium* sp. As these bacteria convert CO mainly into acetate, CO is removed when it diffused into the inner region of the aggregate. In this way growth of *Desulfovibrio* sp. in the inner part of the granule becomes possible. More detailed studies are required for the verification of this hypothesis.

The above mentioned difference in sensitivity for CO also may explain the acetate accumulation in the reactor liquid in the presence of CO. On H_2/CO_2, the growth of the *Desulfovibrio* sp. is limited by the acetate production rate of the *Acetobacterium* sp.[1,4]. However, in presence of CO the sulphate-reducing bacteria are strongly inhibited, therefore leaving part of the produced acetate unconverted.

Although it is well known that methanogens also can use CO as a substrate[9], no methane production was observed in any of our experiments. Even incubation of biomass samples in 120 mL batch vials, containing 50 mL of growth medium without sulphate and a headspace of 80% H_2 and 20% CO, showed no methanogenic activity after 4 weeks. Therefore, we can assume that no methanogens were present in the active biomass.

4.5 Conclusions

The results of the research presented in this paper clearly demonstrate that:

1) Growth of sulphate-reducing bacteria is still possible at a gas phase CO concentration up to 20%, although such a CO concentration causes a reversible type of inhibition.
2) Sulphate conversion rates up to 10 g SO_4^{2-}/L per day can be achieved at a biomass concentration of only 2.6 g/L.
3) Carbon monoxide is not used as electron donor for microbial sulphate reduction.

4) The consortium of sulphate-reducing bacteria and homo-acetogenic bacteria forms smooth aggregates upon addition of CO. A layered structure is formed within the aggregates. The homo-acetogens are predominant at the outside of the aggregate and the sulphate-reducers are located at the inside of the aggregate.

Acknowledgements

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4.6 Appendix

In this appendix the used mass balances of the reactor are given. The reactor is defined here as the gas-lift reactor including gas recycle and buffer column.
For the reactor the following assumptions are made:
- Gas and liquid phase of the reactor are ideally mixed.
- The volume of the reactor is constant.
- Liquid flow passing through the reactor is constant.
- Under steady-state conditions, neither biomass nor product accumulation is assumed.
- Gas-side mass transfer resistance is negligible. This is generally true for low-soluble gases like hydrogen and carbon monoxide.
- External (i.e. around biofilms or aggregates) mass transfer limitations are neglected. With an external mass transfer film thickness typically in the order of ten microns for aqueous systems, this assumption usually will be valid.

The mass balance, for component j, over the liquid phase of the reactor then becomes:

$$V\frac{d\,c_{j,L\,out}}{dt} = Q_L \cdot c_{j,L\,in} - Q_L \cdot c_{j,L\,out} + k_L.a\left(\frac{c_{j,G\,out}}{m} - c_{j,L\,out}\right).V + R_j.V \qquad (A.1)$$

The mass balance, for component j, over the gas phase becomes:

$$V\frac{d\,c_{j,G\,out}}{dt} = Q_G \cdot c_{j,G\,in} - Q_G \cdot c_{j,G\,out} - k_L.a\left(\frac{c_{j,G\,out}}{m} - c_{j,L\,out}\right).V + R_j.V \qquad (A.2)$$

During steady-state, and with the assumption that the bulk liquid hydrogen and carbon monoxide concentrations in the reactor are negligible, we can write down the simplified mass balances for hydrogen:

$$Q_{G\,in} \cdot c_{H_2,G\,in} - Q_{G\,out} \cdot c_{H_2,G\,out} = -R_{H_2} \cdot V \qquad (A.3)$$

carbon monoxide:

$$Q_{G\,in} \cdot c_{CO,G\,in} - Q_{G\,out} \cdot c_{CO,G\,out} = -R_{CO} \cdot V \qquad (A.4)$$

sulphate:

$$Q_L \cdot c_{S,L\,in} - Q_L \cdot c_{S,L\,out} = -R_S \cdot V \qquad (A.5)$$

and hydrogen sulphide:

$$-Q_L \cdot c_{P,L\,out} - Q_{G\,out} \cdot c_{P,G\,out} = R_P \cdot V \qquad (A.6)$$

According to the reaction stoichiometry of the reaction equation for sulphate reduction:

$$8\ H_2 + 2\ SO_4^{2-} \rightarrow H_2S + HS^- + 5\ H_2O + 3\ OH^- \qquad (A.7)$$

$$-R_S = -\frac{1}{4} R_{H_2} = R_P \qquad (A.8)$$

The steady-state reaction rates of all the three experiments are calculated according to equations (A.3), (A.4), (A.5) and (A.6). The results are given in Table 4.1 and 4.2.

4.7 Nomenclature

a	specific surface area of reactor	(m^2/m^3)
c_{H2}	hydrogen concentration	(mol/m^3)
c_{CO}	carbon monoxide concentration	(mol/m^3)
c_P	product, i.e. sulphide concentration	(mol/m^3)
c_S	sulphate concentration	(mol/m^3)
k_L	liquid-side mass transfer coefficient	(m/s)
m	gas/liquid solubility coefficient	(-)
Q_G	gas flow rate	(m^3/s)
Q_L	liquid flow rate	(m^3/s)
R_{Ac-}	acetate production rate	($mol/m^3.s$)

R_{H2}	hydrogen consumption rate	$(mol/m^3.s)$
R_{CO}	carbon monoxide consumption rate	$(mol/m^3.s)$
R_P	sulphide production rate	$(mol/m^3.s)$
R_S	ulphate consumption rate	$(mol/m^3.s)$
V	reactor volume	(m^3)

subscript

G	gas phase
in	incoming
j	component j
L	liquid phase
out	outgoing

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5

Thermophilic sulphate and sulphite reduction using hydrogen and carbon dioxide

Abstract

Feasibility of thermophilic (55 °C) sulphate and sulphite reduction with H_2 and CO_2 gas-mixtures was studied in gas-lift reactors, which contained pumice particles as carrier material. Particular attention was paid to biomass retention and the competition between hydrogenotrophic sulphate-reducers and other hydrogeno-trophic thermophiles. A model medium with defined mineral nutrients was used.

The results of the experiments clearly demonstrate that sulphate conversion rates up to 7.5 g SO_4^{2-}/L per day can be achieved. With sulphite a reduction rate of 3.7 g S/L per day was obtained, which equals a sulphate conversion rate of 11.1 g SO_4^{2-}/L per day. Under the applied conditions, a strong competition for hydrogen between hydrogenotrophic sulphate-reducers, designated as Desulfotomaculum sp., and hydrogeno-trophic methanogens was observed. The outcome of the competition could not be predicted. Growth of the mixed culture was totally inhibited at a H_2S concentration of 250 mg/L. Poor attachment of sulphate-reducing bacteria was observed in all experiments. The biomass concentration did not exceed 1.2 g/L, despite the presence of 50 g/L of pumice. The reason for this phenomenon remains to be understood.

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5.1 Introduction

The release of sulphur dioxide (SO_2) into the atmosphere, as a result of the increasing use of sulphur-containing fossil fuels, has become a major concern. Emission of SO_2 primarily leads to deposition of acid substances. This acid rain or dry acid deposition disturbs several sensitive ecosystems. Additionally, excessive levels of SO_2 in the atmosphere are associated with a sharp increase in acute and chronic respiratory diseases[16].

There are several chemical engineering solutions to the problem of SO_2 emissions from the combustion of sulphur-bearing fuels. The most common flue gas desulphurization technology at present is based on the wet-scrubbing process with use of solid throwaway adsorbents, such as limestone and dolomites[19,28]. During this type of process however, large amounts of gypsum, i.e. calcium sulphate ($CaSO_4$), are produced resulting in disposal problems since only part of the gypsum is used as construction material.

Several alternative, more promising technologies for flue gas desulphurization are currently developed[1]. These technologies are all based on recovery processes. They include wet regenerable scrubbing processes like the Amine process and catalytic oxidation processes e.g., the copper oxide process. However, these processes are more expensive than existing processes and remove only up to 95% of the SO_2.

Recently, a new biotechnological process has been proposed for flue gas desulphurization[5], which likely is cheaper than existing processes and can remove up to 98% of the SO_2. Moreover, instead of gypsum or waste sorbent, this process produces sulphur that potentially can be reused in industry. The process consists of one alkaline SO_2 absorption step and two biological steps. In the first biological step, sulphate and sulphite are biologically converted to hydrogen sulphide. In the second stage hydrogen sulphide is converted to solid sulphur by colorless sulphur bacteria[3,4,13].

It has been demonstrated in chapters 2, 3 and 4, that mesophilic sulphate-reducing bacteria are well able to aggregate in gas-lift reactors fed with H_2/CO_2 or H_2/CO gas mixtures. A maximum sulphate conversion rate of 30 g SO_4^{2-}/L per day could be achieved after only 10 days of operation. The hydrogen mass transfer capacity of the reactor determined the maximum sulphate conversion rate[9]. Sublette *et al.* have shown that mesophilic reduction of sulphite, with pure cultures of hydrogenotrophic sulphate reducers and hydrogen as the electron donor, is a feasible process, although the obtained sulphite reduction rate is quite low, i.e. 0.12 g S/L per day[6,18,23]. Little is known, however, about the potential applications of thermophilic reduction of sulphate and sulphite with hydrogen as the electron donor. Only a few reports have appeared, describing thermophilic sulphate reduction with VFA mixtures as electron donor and carbon source[22,29]. No

literature is available on thermophilic sulphate reduction in gas-lift reactors. Since the presence of sulphite might also lead to either a chemical reaction with sulphide resulting in thiosulphate formation[8], or biological disproportionation with sulphate and sulphide as products[2,17], a thermophilic system with this electron donor is an interesting object of research.

The aim of the present research was to determine the feasibility of thermophilic (55 °C) sulphate and sulphite reduction in gas-lift reactors fed with H_2 and CO_2 gas-mixtures. Particular attention was paid to biomass retention and the competition between hydrogenotrophic sulphate-reducers and other hydrogenotrophic thermophiles. Furthermore, the possible dismutation of sulphite in this system was studied. A model medium with defined mineral nutrients was used.

5.2 Materials and methods

Experimental set-up.
The experimental set-up was described in detail in chapter 2. The biologically active volume of the reactor was 4.3 L. All experiments were carried out at 55 °C and pH 7.0. The gas recycle flow rate was 350 L/h. Pumice particles (Aqua-volcano 0.2-0.5 mm, density ca. 2440 kg/m^3, Aquatechniek bv, Papendrecht, Holland) were used as carrier material.

Start-up.
Before the start of each experiment the reactor system was made anaerobic by flushing overnight with a gas mixture containing 99% H_2 and 1% CO_2 at a flow rate of 2.43 Ln/h (Ln refers to liters at normal conditions, i.e., 101325 Pa and 273.15 K). Low flow of CO_2 was needed to maintain the pH between 6.5 and 7.0. Then about 0.1 g Na_2S . xH_2O (x=7-9) was added to the reactor to remove the last traces of oxygen, as indicated by decolorization of the redox indicator resazurin. When sulphate reduction started, the incoming CO_2 concentration was increased step-wise to obtain a gas mixture of 80% H_2 and 20% CO_2.

Biomass.
The reactor was inoculated with biomass aggregates from a 7.5 m^3 pilot plant for biological flue-gas desulphurization at the 600 MW coal-fired power plant Amer-8 at Geertruidenberg, The Netherlands. The seed aggregates had been cultivated on ethanol at a loading rate of about 1-2 kg S/m^3 per day, containing 1/3 of sulphate and 2/3 of sulphite. Aggregate formation was examined by phase-contrast microscopy (Olympus BH-2, Paes Nederland bv, Zoeterwoude,

The Netherlands). Enrichments were carried out as described in chapter 3, except for the incubation temperature, which was 55 °C. The determination of the disproportionation activity was carried out according to Bak and Pfennig[2].

Medium.

The basal growth medium contained (in g per 1 liter of demineralized water): Na_2SO_4, 4.97; $Na_2HPO_4.2H_2O$, 0.53; KH_2PO_4, 0.41; NH_4Cl, 0.30; KCl, 0.37; $MgCl_2.2H_2O$, 0.10; $CaCl_2.2H_2O$, 0.11; $NaHCO_3$, 4.0; resazurin, 0.00025; vitamins 0.2 mL/L; acidic trace elements 1 mL/L and alkaline trace elements 1 mL/L. Vitamins solution and trace elements solutions were prepared as described previously[27]. If used, sulphite was dosed additionally from a separate 2.0 M stock solution kept under a nitrogen atmosphere. All chemicals used were of extra pure quality and supplied by Merck (Darmstadt, Germany), except the gases, which were supplied by Hoekloos (Schiedam, Holland).

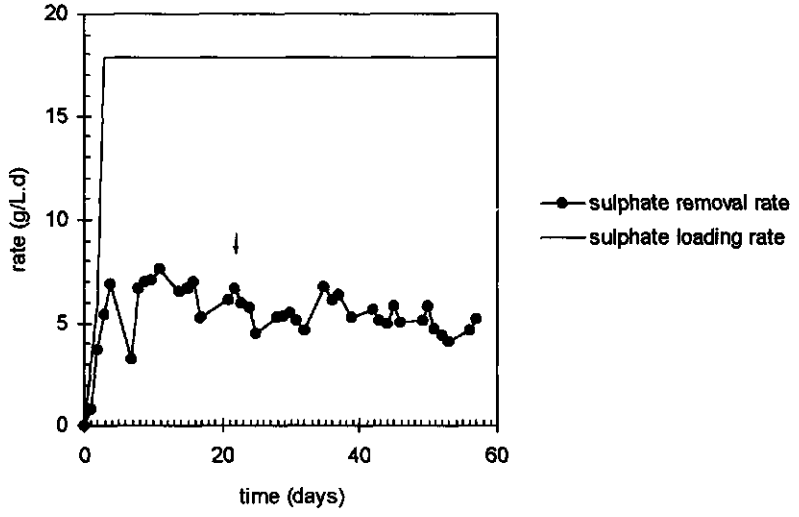
Analytical methods.

Biomass concentration, in $g/L_{reactor}$, was calculated from Kjeldahl-nitrogen as described in chapter 3. Tests for desulfoviridin were carried out according to Postgate[24]. Gas phase composition, acetic acid, sulphate and sulphide were all determined as described in chapter 2. The sulphite concentration was determined semi-quantitative using test strips (Merckoquant® cat. nr. 1.100-13).

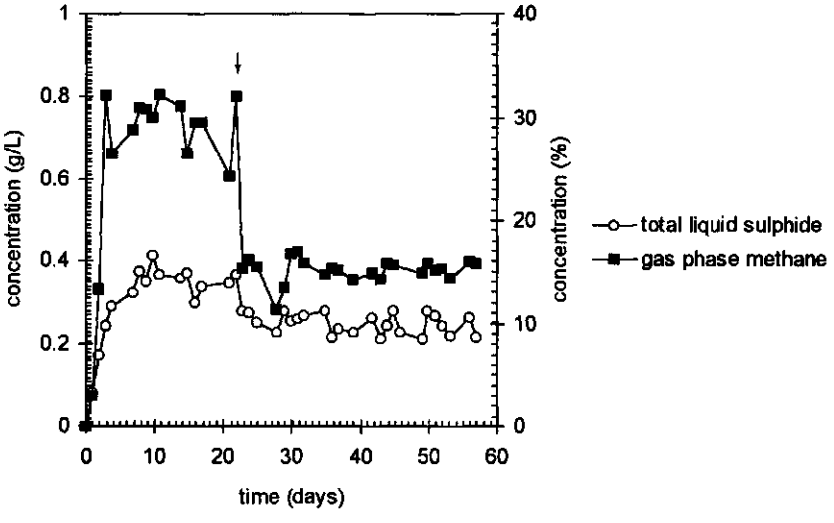
5.3 Results

The first investigation concerned the assessment of the feasibility of thermophilic sulphate reduction in gas-lift reactors merely fed with H_2 and CO_2 and sulphate. In this experiment 250 g of pumice was used as carrier material. The total influent gas flow rate was 3.30 Ln/h. From day 3 onwards, a hydraulic retention time of 4.5 h was applied. The results of the experiment are shown in Figure 5.1.

After 10 days of operation, the sulphate conversion rate reached 7.0 g SO_4^{2-}/L per day, despite the poor biofilm formation attained at that time. Even after 20 days, the majority of the carrier particles were still bare. The attached biomass concentration then amounted to only 0.95 g/L. The suspended biomass concentration was 0.08 g/L. Enrichments showed that autotrophic, Gram (+), endospore-forming bacteria were the dominant sulphate-reducing micro-organisms. Therefore, these micro-organisms can be tentatively designated as *Desulfotomaculum* sp.[31].



(a)



(b)

Figure 5.1. *(a) Sulphate loading rate, sulphate conversion rate; (b) liquid sulphide concentration and relative methane concentration during the first experiment. The arrow indicates doubling of the incoming gas flow rate.*

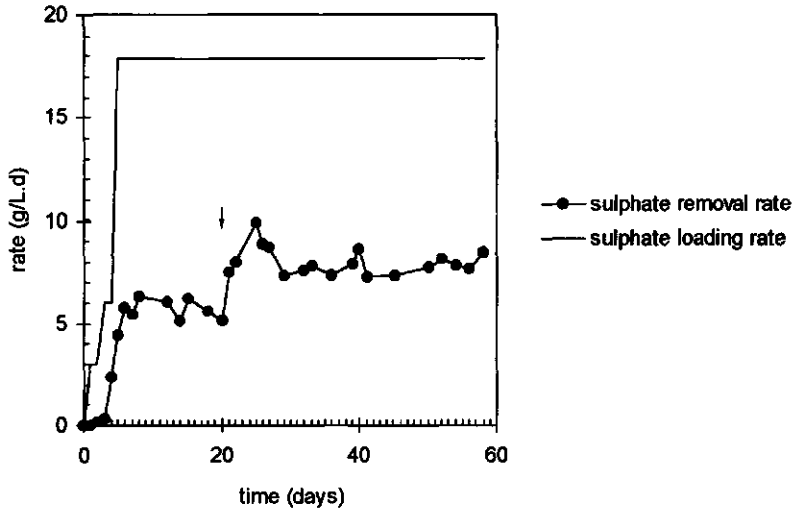
In order to check for a possible hydrogen mass transfer limitation, the influent gas flow rate was doubled at day 22. This didn't result in any significant effect.
The results also show that large amounts of methane were produced. From day 10 until day 22, the gas phase of the reactor contained approximately 30% methane. This percentage dropped to 15% upon the doubling of the influent gas flow rate at day 22. Apparently, the overall methane production rate remained constant throughout the whole experiment. By analysing the effluent gas flow on regular basis the overall mass balances were checked. The conversion rates, calculated from the steady-state mass balances, are given in Table 5.1.

Table 5.1. *Conversion rates obtained during the steady periods of the first experiment. The values are calculated according to the steady-state mass balances (A.5), (A.6), (A.8) and (A.9) given in the Appendix.*

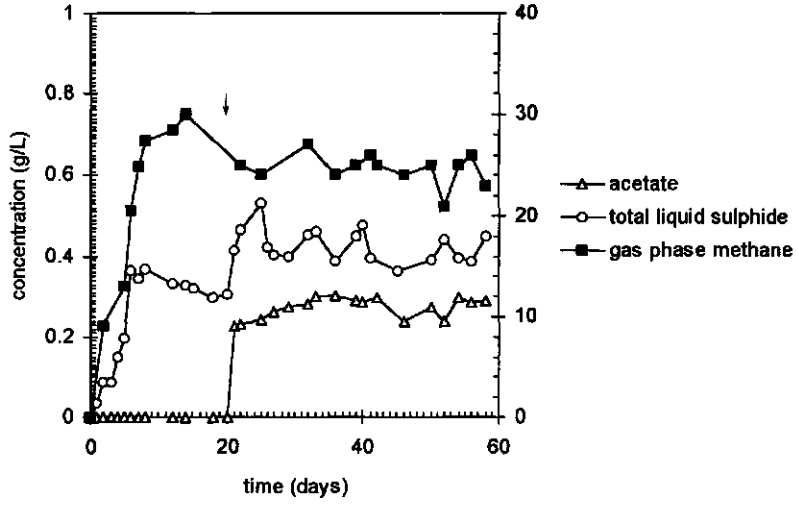
time (days)	$-R'^{a}_{SO4}$ (mol/L.d)	$-R'^{a}_{H2}$ (mol/L.d)	R'^{a}_{P} (mol/L.d)	R'^{a}_{CH4} (mol/L.d)
9	0.073	0.540	0.071	0.061
16	0.073	0.565	0.065	0.064
30	0.058	0.538	0.060	0.060
45	0.060	0.534	0.057	0.065
57	0.054	0.525	0.050	0.059

[a] R' denotes R (given in the Appendix) expressed in mol/L.d.

As Widdel reported in his review article a stimulatory effect of the addition of (reduced) short-chain fatty acids on the sulphate reduction rate of hydrogen-utilizing *Desulfotomaculum* sp.[31], it was decided to assess the effect of adding lactate, pyruvate and acetate.
The effect of lactate addition is shown in Figure 5.2. In that experiment 250 g of pumice was used as the carrier material. Start-up procedure, pH and influent sulphate concentration of this experiment were similar to the previous experiment. The influent gas flow consisted of 80% H_2 and 20% CO_2 at a total flow rate of 3.30 Ln/h. From day 5 onwards, a hydraulic retention time of 4.5 h was applied.
Starting from day 20, sodium lactate was dosed to the influent solution at a rate of 4.5 mmol/h. The effect was very clear. The overall sulphate conversion rate increased from 6 g SO_4^{2-}/L per day to 7.5 g SO_4^{2-}/L per day. Also acetate was produced at a rate of 4.5 mmol/h. The biomass concentration in the reactor did not increase significantly. The attached and suspended biomass



(a)



(b)

Figure 5.2. (a) Sulphate loading rate, sulphate removal rate; (b) liquid acetate, liquid sulphide concentration and relative methane concentration during the second experiment. The arrow indicates the start of addition of sodium lactate.

concentration were 1.0 g/L and 0.09 g/L respectively, throughout the whole experiment.
The addition of sodium lactate influenced the methane production rate only slightly. The methane concentration in the gas phase dropped from 30% to about 25%. This latter concentration remained constant throughout the rest of the experiment. Results of the calculations of the conversion rates from the overall mass balances are given in Table 5.2.

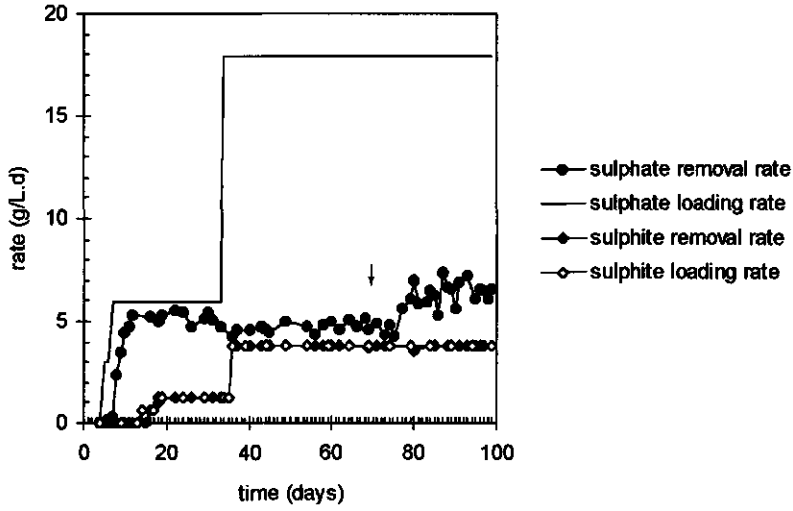
Table 5.2. Conversion rates obtained during the steady periods of the second experiment. The values are calculated according to the steady-state mass balances (A.5), (A.6), (A.8) and (A.9) given in the Appendix.

time (days)	$-R'^{a}_{SO4}$ (mol/L.d)	$-R'^{a}_{H2}$ (mol/L.d)	R'^{a}_{P} (mol/L.d)	R'^{a}_{CH4} (mol/L.d)
8	0.066	0.135	0.070	0.065
15	0.065	0.125	0.062	0.067
32	0.079	0.131	0.079	0.065
45	0.076	0.145	0.072	0.060
56	0.080	0.139	0.070	0.062

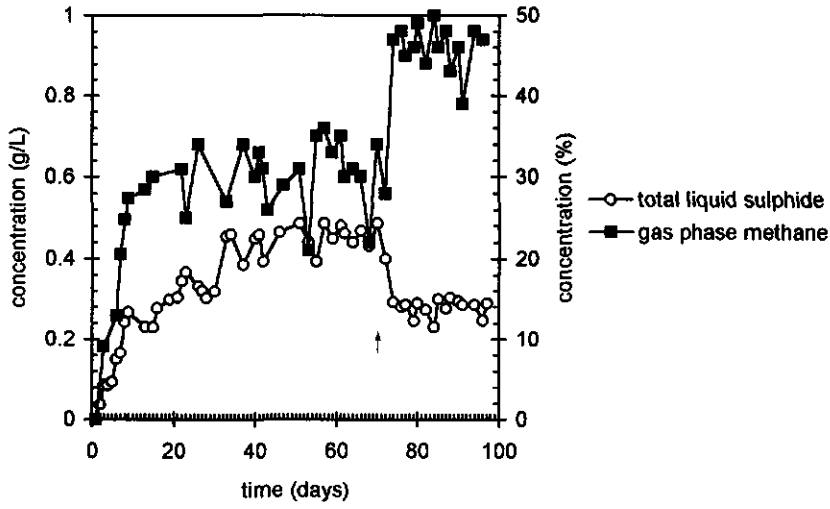
[a] R' denotes R (given in the Appendix) expressed in mol/L.d.

In two separate experiments, of which data are not shown, we investigated the addition of pyruvate and acetate. Neither pyruvate nor acetate showed any clear effect on the overall sulphate conversion rate, while also the methane production did not change.

Since in practice, a mixture of sulphate and sulphite will be obtained from the flue-gas absorption step, we also conducted an experiment using such a feed solution. The results are depicted in Figure 5.3. Start-up procedure, pH and influent sulphate concentration were similar to the previous experiments.
Sulphite was added from day 9 onwards up to an influent concentration of 8.5 mM. The hydraulic retention time applied was of 13.5 h during the period of day 3 to day 29. It was reduced to 4.5 h after 29 days. After 10 days of operation, a steady sulphate conversion rate of 4.8 g SO_4^{2-}/L per day was obtained. This sulphate conversion rate did not change upon the reduction in hydraulic retention time after 29 days.



(a)



(b)

Figure 5.3. *(a) Sulphate loading rate, sulphate removal rate, sulphite loading rate, sulphite removal rate; (b) liquid sulphide concentration and relative methane concentration during the third experiment. The arrow indicates doubling of the influent liquid flow rate.*

The results clearly demonstrate that all sulphite was reduced throughout the whole experiment, because sulphite could not be detected in the effluent. The thiosulphate concentration remained constant at a level of about 15 mg/L. In order to determine the prevalence of possible disproportionation activity in the reactor, biomass samples were subjected to batch incubations according to the method described in the Materials and Methods section. In none of the experiments was any disproportionation activity found.
The results also reveal that, large amounts of methane were produced. From day 10 until day 70, the gas phase contained approximately 32% methane. The values of the conversion rates calculated from the overall mass balances are given in Table 5.3.

Table 5.3. Conversion rates obtained during the steady periods of the third experiment. The values are calculated according to the steady-state mass balances (A.5), (A.6), (A.8) and (A.9) given in the Appendix.

time (days)	$-R'^{a}_{SO4}$ (mol/L.d)	$-R'^{a}_{SO3}$ (mol/L.d)	$-R'^{a}_{H2}$ (mol/L.d)	R'^{a}_{P} (mol/L.d)	R'^{a}_{CH4} (mol/L.d)
22	0.052	0.016	0.492	0.065	0.062
40	0.048	0.047	0.540	0.091	0.050
50	0.050	0.047	0.531	0.095	0.047
69	0.046	0.047	0.535	0.096	0.051
81	0.066	0.047	0.614	0.110	0.051
93	0.068	0.047	0.611	0.112	0.049

[a] R' denotes R (given in the Appendix) expressed in mol/L.d.

At day 70, the liquid flow rate was doubled, at an unchanged sulphate and sulphite loading rate. The sulphate conversion rate then instantaneously from 4.8 g SO_4^{2-}/L per day to 6.4 g SO_4^{2-}/L per day. The overall methane production rate remained unaffected (see Table 5.3).

5.4 Discussion

The results clearly demonstrate that sulphate conversion rates upto 7.5 g SO_4^{2-} /L per day (= 2.5 g S/L per day) can be achieved, and even a reduction rate up to 3.7 g S/L per day or 11.1 g SO_4^{2-}/L per day when the feed contained sulphite. Based on the established biomass

concentration, which was in the range of 0.9 to 1.2 g/L, the calculated overall activity of the biomass amounted to 2.8 to 3.1 g S/g biomass per day.

These values are much higher than previously found for thermophilic sulphate reduction. Sublette *et al.* reported sulphite reduction rates on hydrogen up to 0.12 g S/L per day[6,18,23] or 0.36 g SO_4^{2-}/L per day. From their data, assuming that one gram of protein equals two grams of biomass, an overall activity of 0.2 g S/g biomass per day can be calculated. It is clear that the mixed culture present in our reactors exerted a higher activity than the pure *Desulfotomaculum orientis* culture used by Sublette *et al.*

The values found are also significantly higher than those under mesophilic conditions on H_2/CO_2, which ranged from 0.7 to 1.4 g S/g biomass per day[9,10]. A possible explanation could be that in the mesophilic experiments only part of the biomass participated in the process. The mesophilic biofilm thickness was typically in the order of 0.5 mm[10], conceivably causing substrate limitation in the interior of the aggregate. During thermophilic conditions only poor aggregation was observed, resulting in thinner biofilms and therefore an overall more active biomass.

A strong competition for hydrogen between hydrogenotrophic sulphate-reducers and hydrogenotrophic methanogens was observed in all experiments. Based on reported literature values for μ_{max} and K_m of some sulphate-reducers and methanogens, given in Table 5.4, it is

Table 5.4. *Selected growth kinetics of thermophilic hydrogenotrophic sulphate-reducing bacteria and methanogenic bacteria. All reported data were measured at 55 °C.*

bacterial strain	μ_{max} (1/day)	K_S (μM)	Yield[a] (g/mol H_2)	reference
Sulphate reducers				
Desulfotomaculum	1.4 - 2.3	2	n.r.	25
Methanogens				
Methanobacterium	4.2 - 8.4	80 - 120	2.05	7,15,26,32
Methanococcus	7.2 - 12.8	n.r.	n.r.	12,14,33
Enrichment	7.92	n.r.	n.r.	30

[a] The yield is given in gram cell dry weight per mol H_2.

[b] n.r. = not reported.

likely that a strong competition between sulphate-reducers and methanogens at higher temperatures occurs. Mainly because of the high μ_{max}-values of the latter micro-organisms. Since thermophilic sulphate reducers possibly have a lower K_s-value and a lower threshold value as well, based on the results of Schmidt and Ahring[25], a long-term exposure to hydrogen-limiting conditions might result in a pure sulphate-reducing system.
Significant homo-acetogenesis could not be detected. These observations are very similar to those of Nozhevnikova and Kotsuyrbenko[20]. According to their insights on the temperature dependency of methanogenesis and acetogenesis, methanogenesis from hydrogen would predominate at high temperatures (>45°C), while at low temperature conditions (<20°C) homo-acetogenesis would become increasingly important at decreasing temperature.

In our experiments we could observe hardly any effect of the addition of fatty acids on the ratio of sulphate reduction to methanogenesis. Only the addition of lactate increased the sulphate reduction rate, although any stimulatory effect on sulphate reduction was not observed since lactate was just used as an electron donor in addition to hydrogen. Therefore addition of lactate is only useful for increasing the development of sulphate-reducing sludge, such as in the start-up period to control the initial competition between sulphate-reducers and methanogens.
Although there possibly exists a strong competition for hydrogen, still a conversion rate of 3.7 g S/L per day could be obtained. However, it should be clear that this conversion rate can only be improved by firstly decreasing the methanogenesis.
The results of the mass balance calculations, see Table 5.3, show that at the abovementioned conversion rate, 0.61 mol H_2/L per day is consumed and consequently has been transferred from the gas into the liquid phase. Assuming that the bulk liquid concentration of hydrogen was zero at that time, the volumetric mass transfer coefficient, k_L.a, can be calculated according to the method described previously[9]. The volumetric mass transfer coefficient calculated in this way, amounted to 0.030 s^{-1} assuming a value of 60 for the gas-liquid solubility coefficient for hydrogen. This value corresponds well with the k_L.a-values obtained at 30 °C indicating that hydrogen mass transfer was the limiting factor at this stage[9]. Therefore, the sulphate reduction rate can only be improved by decreasing the methane production rate.

In the third experiment, we found that all sulphite present in the feed was converted to sulphide. No sulphite could be detected in the effluent. According to Krämer and Cypionka[17] several sulphate-reducing bacteria are capable of fermenting sulphite to sulphate and hydrogen sulphide. Since this disproportionation activity cannot be deduced from the results of the continuous reactor experiments, batch tests had to be conducted to find out whether or not

disproportionation activity was present. Within the applied experimental period of two weeks of the batch experiments, no disporportionation activity could be detected. Therefore it can be concluded that all the sulphite was reduced to hydrogen sulphide directly. Additionally, the sulphite reduction rate was higher than the rate of chemical reaction of sulphite with hydrogen sulphide because the thiosulphate concentration remained always below 15 mg/L.
Since all the disproportionating sulphate-reducers so far, grow in the mesophilic temperature range, an interesting remaining question is whether or not thermophilic sulphate-reducing bacteria are in general capable of fermenting sulphur compounds. Additional research is necessary to provide an answer on this question.
The results of the third experiment show that growth of the predominant *Desulfotomaculum* sp. is completely inhibited at a H_2S concentration of 250 mg/L. This inhibiting H_2S concentration is half of that found under mesophilic conditions[9,10]. According to results of other investigations, this could be due to the presence of different sulphate-reducing bacteria. For example, Widdel[31] reported that growth of *Desulfotomaculum* sp. generally becomes inhibited at 7 to 10 mM H_2S (225-320 mg/L), whereas under mesophilic conditions *Desulfovibrio* sp. are the dominant hydrogenotrophic sulphate-reducing bacteria, which are less susceptible to H_2S inhibition[9,10]. The observed inhibition of the *Desulfotomaculum* sp. is apparently reversible because an increase in the H_2S removal rate, by shortening the hydraulic retention time, caused an immediate increase in the sulphate reduction rate, see Figure 5.3.

In all our experiments we noticed that bacterial attachment was poor. Although 50 g/L of pumice was present as carrier material, the biomass concentration did not exceed 1.2 g/L. The reason for this phenomenon remains to be investigated. Future research should therefore focus on improved biomass retention and selection of alternative thermophilic sulphate-reducing bacteria, tolerant to higher levels of hydrogen sulphide.

5.5 Conclusions

1) The results of the presented experiments clearly demonstrate that volumetric sulphate conversion rates upto 7.5 g SO_4^{2-}/L per day can be achieved. In case also sulphite is present, a sulphate/sulphite reduction rate of 3.7 g S/L per day can be obtained, which equals a theoretical sulphate conversion rate of 11.1 g SO_4^{2-}/L per day.
2) When both sulphite and sulphate are present, sulphite is preferentially used as electron acceptor.

3) A strong competition for hydrogen between hydrogenotrophic sulphate-reducers and hydrogenotrophic methanogens occurs in thermophilic sulphate and sulphite removal removal systems with hydrogen as the electron donor.
4) The maximum achievable sulphate conversion rate is determined by the gas-to-liquid hydrogen mass transfer rate.
5) The predominant sulphate-reducing Desulfotomaculum sp., present in the sludge, are inhibited at a H2S concentration of 250 mg/L.
6) Aggregation of sulphate-reducing bacteria in thermophilic gas-lift reactors is worse than in mesophilic ones.

Acknowledgements

We would like to thank Dr. L.W. Hulshoff Pol for valuable discussions regarding the manuscript. This work was supported by the IOP-Milieutechnologie programme, grant no. IOP 90-209 and Paques bv, Balk, The Netherlands.

5.6 Appendix

In this appendix some theoretical aspects of the reactor system are presented. In the following, 'reactor' refers to the gas-lift reactor including gas recycle and buffer column. In the considerations given below, the following assumptions are made:
- the gas and liquid phase of the reactor are ideally mixed.
- the volume of the reactor is constant.
- the liquid flow passing through the reactor is constant.
- the reactor is essentially in a steady-state.

The mass balance over the reactor, for component j, then becomes:

$$Q_L \cdot c_{j,L\,in} + Q_{G\,in} \cdot c_{j,G\,in} - Q_L \cdot c_{j,L\,out} - Q_{G\,out} \cdot c_{j,G\,out} = R_j \cdot V \qquad \text{(A.1)}$$

According to the reaction equations for sulphate reduction:

$$8\ H_2 + 2\ SO_4^{2-} \rightarrow H_2S + HS^- + 5\ H_2O + 3\ OH^- \qquad \text{(A.2)}$$

sulphite reduction:

$$6\ H_2 + 2\ SO_3^{2-} \rightarrow H_2S + HS^- + 3\ H_2O + 3\ OH^- \qquad \text{(A.3)}$$

and methanogenesis:

$$4\ H_2 + H^+ + HCO_3^- \rightarrow CH_4 + 3\ H_2O \qquad \text{(A.4)}$$

and the assumption that the bulk liquid hydrogen concentration in the reactor is negligible, we can write down the mass balance for hydrogen:

$$Q_{G\,in} \cdot c_{H_2,G\,in} - Q_{G\,out} \cdot c_{H_2,G\,out} = -R_{H_2} \cdot V \qquad \text{(A.5)}$$

sulphate:

$$Q_L \cdot c_{SO_4,L\,in} - Q_L \cdot c_{SO_4,L\,out} = -R_{SO_4} \cdot V \qquad \text{(A.6)}$$

sulphite:

$$Q_L \cdot c_{SO_3,L\,in} - Q_L \cdot c_{SO_3,L\,out} = -R_{SO_3} \cdot V \qquad \text{(A.7)}$$

hydrogen sulphide:

$$-Q_L \cdot c_{P,L\,out} - Q_{G\,out} \cdot c_{P,G\,out} = R_P \cdot V \qquad \text{(A.8)}$$

and methane:

$$-Q_{G\,out} \cdot c_{CH_4,G\,out} = R_{CH_4} \cdot V \qquad \text{(A.9)}$$

The steady-state reaction rates of all the three experiments are calculated according to equations (A.5), (A.6), (A.7), (A.8) and (A.9). The results are given in Table 5.1 to Table 5.4.

The difference in hydrogen mass flow rate between incoming and outgoing gas flow, given in (A.5), equals the amount of hydrogen transferred to the liquid phase. Assuming that the gas-side mass transfer resistance is negligible, we can write:

$$Q_{G\,in} \cdot c_{H_2,G\,in} - Q_{G\,out} \cdot c_{H_2,G\,out} = k_L.a.\frac{c_{H_2,G\,out}}{m} \cdot V \qquad \text{(A.10)}$$

Since, during steady-state, all parameters are known we can calculate the volumetric mass transfer coefficient $k_L.a$ for hydrogen.

5.7 Nomenclature

a	specific surface area of reactor	(m^2/m^3)
c	concentration	(mol/m^3)
k_L	liquid-side mass transfer coefficient	(m/s)
m	gas/liquid solubility coefficient	(-)
Q	flow rate	(m^3/s)
R	reaction rate	($mol/m^3.s$)
V	reactor volume	(m^3)

subscript

G	gas phase
H_2	hydrogen
in	incoming
j	component j
L	liquid phase
out	outgoing
P	product, i.e. sulphide
S	sulphate

5.8 References

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6

General discussion

Abstract

This chapter discusses the results obtained and presented in the previous chapters in view of the aims of thesis: 1) to obtain more (fundamental) knowledge about the feasibility of biological sulphate reduction with synthesis gas. 2) to develop criteria for design and operation of gas-lift bioreactors for sulphate reduction using immobilized biomass.
It appears that both aims are attained. First, biological sulphate reduction with synthesis gas is feasible. Additionally, a number of criteria for design and operation of gas-lift bioreactors for sulphate reduction are briefly discussed. Finally, several recommendations for future research are given.

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6.1 Introduction

In the past, research on anaerobic wastewater treatment has been mainly focused on the treatment of concentrated wastewaters containing readily degradable organic compounds. Considerable progress has been made and at this time hundreds of 'high-rate' anaerobic reactors are operating successfully.

Current research focuses more and more on the treatment of specific industrial wastewaters, particularly of those not considered previously, i.e. bearing compounds which were known as 'harmful' or 'non-degradable'.

Treatment of inorganic, sulphate containing wastewater is such an example. Fundamental and applied research on biological sulphate reduction is required to demonstrate the potential of this process in industrial wastewater treatment. As outlined in chapter 1, biological sulphate reduction with synthesis gas was investigated for two reasons:

- to obtain more (fundamental) knowledge about the feasibility of biological sulphate reduction using synthesis gas.
- to develop criteria for design and operation of gas-lift bioreactors for sulphate reduction based on immobilized biomass.

The results presented in the previous chapters will be discussed here in view of both aims.

6.2 Engineering aspects

Conversion rates

As outlined in chapter 1, sulphate conversion rates needed in practice, should be in the range of 2 to 85 kmol SO_4^{2-}/h. When comparing the experimental results of chapter 2 with these numbers, the required reactor volumes would range from 150 m^3 to 6000 m^3. However, when the negative effect of CO (chapter 4) and the thermophilic data of chapter 5 are taken into account, the volumes would increase significantly, up to 900 m^3 to 36000 m^3. These volumes are likely out of economic feasibility.

Consequently, the sulphate reduction rates need further improvement in order to make the process attractive for application. Regarding the discussion about the observed conversion rates in the previous chapters it can be concluded that the major bottlenecks for achieving substantial rate optimization are the gas-to-liquid hydrogen mass transfer, the kinetics (e.g. growth rate) of the micro-organisms and the biomass retention. These bottlenecks will be discussed separately in the next paragraphs.

Mass transfer

As hydrogen is a poorly soluble gas at atmospheric pressures, the hydrogen mass transfer capacity and performance is of great importance for the application of gas-lift reactors in wastewater treatment. Since the hydrogen mass transfer resistance can be located at two positions: the gas-liquid interface and the liquid-solid interface, both volumetric mass transfer coefficients will be discussed separately below.

Volumetric gas-liquid mass transfer coefficient ($k_L.a$)

Several investigations have been carried out on determining the mass transfer capacity of gas-lift reactors. Some relevant reviews are given by Blenke[6], Heijnen and van't Riet[20], Chisti[11] and Joshi *et al.*[24]. Most of these results, if not all, are obtained with air/oxygen-water systems. Quite often pure water was used in the experimental studies, and then good correlations or models, with a relative high predictability for future experiments and scale-up, were obtained. However, accuracy vanishes when the correlations are applied to 'real-life' systems, i.e. gas-lift reactors with biomass and wastewater. Clearly, an experimental base for the description of hydrogen transfer and the estimation of hydrogen mass transfer coefficients in such gas-lift reactors is lacking in the literature.

Nevertheless, it is interesting to discuss the experimentally determined volumetric mass transfer coefficients, presented in chapter 2, in view of the available literature data and correlations. The assessed $k_L.a$ values in the laboratory gas-lift reactor are presented in Figure 6.1.

From figure 6.1 it appears that the gas hold-up in the two-phase system is slightly higher than the gas hold-up in the three-phase system. Since the gas hold-up in gas-lift reactors is closely related to the bubble rise velocity and superficial gas velocity, the bubble rise velocity U_b can be calculated with (Heijnen and van't Riet[20]):

$$\varepsilon = U_{Gs}/U_b$$

provided that the flow is homogeneous, which is a reasonable assumption for our system. The calculated U_b amounts to 0.16 m/s and 0.18 m/s for the two-phase and three-phase systems respectively. The bubble rise velocity in the three-phase system is higher because the average bubble diameter is larger, which can be attributed to the presence of small carrier particles in this system. Small suspended carrier particles ($d_p \approx 1$ mm) generally promote coalescence of gas bubbles[14]. This also explains the drop in $k_L.a$ values for the three-phase system, compared to the two-phase system.

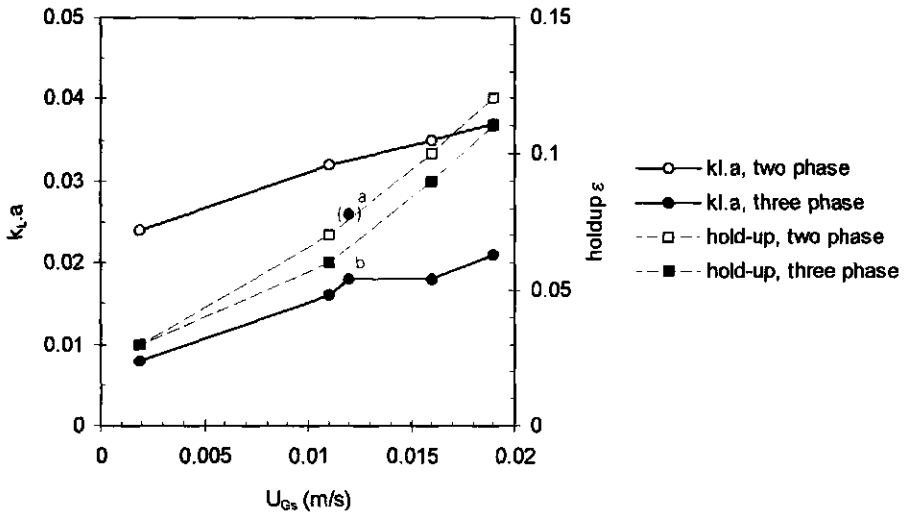


Figure 6.1. *Experimentally determined values for $k_L.a$ and overall gas hold-up ε as a function of the superficial gas velocity U_{Gs} in the reactor. [a]: determined in the biological active system, value shown between brackets; [b]: value normalized to CO_2.*

The following relation holds for the volumetric mass transfer coefficient $k_L.a$:

$$k_L.a = 6\varepsilon.k_L/d_{32b}$$

in which d_{32b} is the Sauter mean bubble diameter and ε is the gas hold-up. This theoretical relation implies that, for any particular set of data, the log-log plots of $k_L.a$ against ε should have a slope near 1, with intercept $(6.k_L/d_{32b})$. Therefore, this relation can be used to check the validity of the experimental data on gas hold-up and mass transfer coefficients. However, in our case the slope deviates strongly from unity, it increases from 1.3 to 1.7 with increasing hold-up (figure not shown). This indicates that the average bubble diameter is not constant throughout one set of experiments. Very likely, wall effects in the small laboratory reactor have influenced bubble formation and concomitantly also the $k_L.a$ measurements. Any comparison with literature data is therefore not realistic although the overall $k_L.a$ values are well in between the reported literature values, as outlined in the appendix of chapter 2.

Volumetric liquid-solid mass transfer coefficient ($k_{L,S}.a$)

In the chapters 2,3 and 4 it was assumed that the external mass transfer resistance of hydrogen to an aggregate is negligible compared to the conversion of hydrogen inside the aggregate. In this paragraph we will elucidate this assumption. First the mass transfer coefficient from the bulk liquid to the aggregate is estimated and thereafter the hydrogen conversion rate in the aggregate.

The flux of a compound, in our case hydrogen, from the liquid phase to the surface of an aggregate is generally described as the product of a mass transfer coefficient and a concentration difference, which is the driving force of the flux:

$$J_{H2} = k_{L,S}.(C_{L,H2} - C^{i}_{L,H2})$$

According to the film theory[28], mass transfer results from diffusion through a stagnant liquid boundary with film thickness δ. When the concentration profile of the substrate is linear, the mass transfer coefficient is equal to:

$$k_{L,S} = D_{H2}/\delta$$

The thickness of the liquid boundary layer depends on the reactor and aggregate geometry and the hydrodynamic conditions. In general the thickness is derived from (semi-)empirical relations for the dimensionless Sherwood number. The Sherwood number, *Sh*, represents the ratio of external mass transport by convection and diffusion:

$$Sh = k_{L,S}.d_P/D_{H2}$$

The Sherwood number for suspended solid particles can be calculated from the relation of Ranz and Marshall[32] provided that the Reynolds number exceeds a value of 30.

$$Sh = 2 + 0.57\ Re_p^{1/2}\ Sc^{1/3}$$

The Reynolds number can be calculated from the Galileo number, defined as:

$$Ga = g.d_P.\rho_L(\rho_P-\rho_L)/\eta^2$$

Using the physical constants valid for our system, at 30 °C [22]:

D_{H2}	$= 4.0*10^{-9}$	m^2/s
ν	$= 8.0*10^{-7}$	m^2/s
ρ_L	$= 995$	kg/m^3
ρ_P	$= 1500$	kg/m^3
d_P	$= 1.5*10^{-3}$	m
η	$= 0.797*10^{-3}$	kg/m.s
$C_{L,H2}$	$= 0.52$	mol/m^3 (m = 54, $P_{G,H2}$ = 70% sat.)

we get:

$Ga = 2.6*10^4$ and therefore (Ranz and Marshall[32]):

$$Re = 0.153*Ga^{0.71} = 200$$

Assuming furthermore that the liquid-solid interfacial hydrogen concentration is 0, the surface flux becomes:

$$J_{H2} = 6.6*10^{-5} \quad mol/m^2.s.$$

According to the theoretical considerations of Tijhuis[37] concerning substrate penetration depth in aggregates, and assuming zeroth order kinetics, the corresponding maximal surface flux can be calculated using:

$$J_{H2} = Y'_{S/X}.\mu.C_X.[r_p^3 - (r_p - \delta)^3]/3\ r_p^2$$

with:	r_p	$= 0.75*10^{-3}$	m.
	μ	$= 3*10^{-5}$	1/s.
	$Y'_{S/X}$	$= 0.5$	mol H_2/kg biomass.
	C_X	$= 100$	kg/m^3
	δ	$= 3*10^{-5}$	m.

The maximum surface flux thus calculated is:

$$J_{H2} = 4.4*10^{-8} \quad mol/m^2.s.$$

Since this value is distinctly smaller than the physical flux calculated from the film theory, it is clear that the external mass transfer resistance can be neglected.

In case of an operational temperature of 55 °C, as was applied in the experiments presented in chapter 5, the same procedure is used. Using the following physical constants[22]:

D_{H2}	$= 4.4*10^{-9}$	m^2/s	
ν	$= 5.1*10^{-7}$	m^2/s	
ρ_L	$= 986$	kg/m^3	
η	$= 0.50*10^{-3}$	kg/m.s	
$C_{L,H2}$	$= 0.48$	mol/m^3	(m = 54, $P_{G,H2}$ = 70% sat.)

the surface flux then becomes:

J_{H2}	$= 8.2*10^{-5}$	$mol/m^2.s$

This value is of the same order of magnitude as the value calculated for 30 °C. Since the true kinetics of the thermophilic micro-organisms only increase with a factor of 50, $\mu \approx 1.5*10^{-3}$ 1/s see chapter 5 Table 5.4, we can still assume that the external mass transfer resistance is negligible compared to the maximum biological reaction rate..

It might be argued that these conventional chemical engineering considerations about liquid-solid mass transfer are a little out of line with reality since it was shown in the previous chapters that the surface of the aggregates is irregular with moving protrusions on top of it. Indeed they are. Very recently, Yang and Lewandowski[43] reported about experimentally determined mass transfer coefficients *inside* heterogeneous biofilms. Their results show that the liquid-solid mass transfer coefficient increases just above an irregular biofilm surface. This suggests local flow instability in this region. Therefore, this raises strong doubts about the assumption used in conventional theories that a stagnant liquid boundary layer would exist around the aggregates. If local flow instabilities exist, the liquid-solid mass transfer rate will be higher than the rate calculated according to 'conventional' chemical engineering theories.

Kinetics

From an engineering point of view, inhibition of sulphate-reducing bacteria by H_2S seems to be insignificant under mesophilic conditions. High liquid H_2S concentrations up to 15 mM can be

tolerated without any clear irreversible negative effects on the microbial population. Therefore loading rates up to 30 mol total sulphide/$m^3_{reactor}$ per h can be applied to the aerobic sulphur production stage, which is near to the maximum capacity of that system[23]. Therefore, the mesophilic system is a promising technology, ready for application.
Under thermophilic conditions inhibitory levels of H_2S are much lower. According to results obtained in chapter 5, a complete inhibition of the sulphate reducers already occurred at 8 mM H_2S. From a practical point of view, such a hydrogen sulphide concentration is too low because the subsequent aerobic sulphur production stage would be severely hydraulic limited[23]. Consequently, optimization of the kinetics of the sulphate reduction process is needed to improve its perspectives for near future application. The hydrogen sulphide inhibition might be reduced by working at a higher pH (lower H_2S and higher HS^-), provided that the bacteria tolerate this higher pH.

Due to analytical difficulties, no reliable kinetic data are available that permit a correlation of the growth rate of sulphate reducers or homo-acetogens with the actual *dissolved* CO concentration. Therefore, only qualitative considerations can be made. Literature data suggest[13] that total inhibition of sulphate reducing bacteria occurs at gas phase CO concentrations between 4% and 20%. In our case, we did not observe such a strong inhibition, rather, we found the same overall biomass activity at 20% CO as at 0% CO. This indicates the advantage of using balanced immobilized 'ecosystems', consisting of symbiotic microbial populations in which a substrate - possible toxic to one trophic group - is converted by another trophic group. Further research is needed in order to obtain useful information about the growth kinetics of sulphate reducing bacteria in presence of (higher levels of) CO.

It is clear that competition between mesophilic hydrogenotrophic sulphate reducers and other mesophilic hydrogenotrophs, e.g. methanogens or acetogens, is insignificant, because any methanogenic activity could be not detected during our mesophilic experiments.
However, the situation is completely different under thermophilic conditions. The results of these experiments - presented in chapter 5 - reveal that methanogens strongly compete with sulphate-reducing bacteria for hydrogen, resulting in a 50% electron flow to methanogenesis. This strong competition might due to the relative slow growth of the *Desulfotomaculum* sp. present, however, as only a very limited amount of data is available on the growth kinetics of thermophilic *Desulfotomaculum* sp., additional research on the kinetics is necessary to verify the hypothesis given.

For the application of sulphate reduction processes in the mining and galvanic industries, toxicity of heavy metals is of great importance. However, toxicity of heavy metals to sulphate reducing bacteria has not been studied extensively. So far, only the studies of Capone *et al.*[10] and Ueki *et al.*[40] are available, dealing with heavy metal toxicity towards sulphate reducing bacteria. Hardly any comprehensive kinetic studies on heavy metal inhibition have been carried out. This can be partly due to the complexity of the phenomena involved, because the actual speciation of the individual metals in anaerobic cultures is often difficult to measure.
The fact that heavy metals are toxic to sulphate reducing bacteria may be expected, in view of the inhibitory effects of heavy metals towards many enzymes and micro-organisms[18]. On the other hand, most of the heavy metals will precipitate in the presence of hydrogen sulphide since these sulphides are insoluble, e.g. the solubility constants of CuS and HgS are 10^{-36} and 10^{-53} respectively[33]. In view of that, low toxicity towards sulphate reducing bacteria might be expected, as indeed was observed by e.g. Lawrence and McCarty[25].

Scale-up

The general problem during scale-up of bioreactors is to maintain an appropriate micro-environment for the micro-organisms, since this affects the overall performance of the full-scale reactor. This change in micro-environment is often due to changes in hydrodynamic conditions, which affect the rate of mass transfer to the biomass and influence the shear on the aggregates. It therefore also changes the substrate or product concentrations, consequently affecting the kinetics of the bacteria. In the following paragraphs this item will be discussed in view of the experimental results presented in the previous chapters.

For airlift loop reactors already a substantial amount of information about scale-up is available, at least as far as oxygen mass transfer and hydrodynamics of the reactor are concerned[6,11,21]. However, with hydrogen gas-lift reactors still some basic questions on gas-liquid and liquid-solid mass transfer have to be answered, as discussed in the previous paragraphs.
Particularly, information is lacking on the influence of biomass(constituents) on the gas-liquid mass transfer rate. It is known that most of the bacterial cell wall components act as surfactants, causing coalescence of gas bubbles. On the other hand, this phenomenon might be opposed by the presence of salts. Salts are known to turn water into a non-coalescing liquid, and therefore it would enhance the dispersion of hydrogen in the liquid phase. This would result in a considerable higher gas-to-liquid hydrogen mass transfer rate. Since such effects will be encountered in a number of applications, proper design rules for scale-up of hydrogen gas-lift reactors for various wastewater treatment applications are still lacking.

In our laboratory scale reactor, a superficial gas velocity of 1-2 m/s was applied. Using the equations given by Chisti[11] and Heijnen and van der Lans[21] the calculated liquid upward velocity is 0.30 m/s. This value is in qualitative agreement with the visual observations that only gas bubbles smaller than 1 mm, which have a bubble rise velocity of about 0.25 m/s [20], were carried along the downcomer. Bubbles with a diameter of 1-2 mm are kept in the riser to downcomer transition area as a stagnant bubble swarm, whereas larger bubbles left the reactor on top. The calculated liquid upward velocity is also in accordance with those calculated for reactor larger than the 4.5 L laboratory reactor[11,20]. Therefore, the shear forces opposed to the biomass likely were of the same order of magnitude as the shear forces occurring in large scale reactors, indicating that the laboratory biofilms might have been identical to those that would prevail in larger reactors. However, also on this topic additional scale-up research is necessary to verify this hypothesis.

In nearly all of the experiments in the previous chapters it appeared to be difficult to retain biomass aggregates without carrier particles. Since such aggregates always are produced, and since they constitute a substantial fraction of the total biomass, the settler should be designed such that these aggregates will be retained. Because the liquid velocity in the settler area of the laboratory reactor was 3.2 m/h (see chapter 2), this means that the full-scale upward velocity in the settler should not exceed 3.0 m/h.

A phenomenon that can obscure proper scale-up is wall-growth. Often, wall growth is observed in laboratory reactors. Since the surface to volume ratio of these reactors is much smaller than at full scale, wall growth can contribute significantly to the overall performance of the laboratory reactor.
Visible wall growth was absent in our reactor. However, to be sure about the insignificance, we have calculated the possible contribution of the effect of a 50 μm layer of bacteria, which is assumed to be 'not visible', using the kinetic parameters of page 102 and a reactor surface of 0.4 m^2. A contribution to the overall sulphate reduction rate of 0.015 g SO_4^{2-}/L per day was found, which is less than 0.5 % of the total sulphate reduction rate. It is clear that in our case wall growth can be neglected.

Future perspectives
A very interesting field of research is to improve the understanding of liquid-solid mass transfer rates just around and inside microbial aggregates and biofilms. More insight is required about the hydrodynamics around and inside irregular biofilms and aggregates. Since these aggregates

also prevail in sulphate reduction, on both small and large scale[9], this improved understanding is important for rate optimization and therefore for full-scale application of sulphate reduction processes.
The development of new processes for biological sulphate reduction can be greatly facilitated when a comprehensive set of reliable kinetic data, on parameters like m_{max}, K_s, yield and maintenance factors, is made available, because only then adequate predictions concerning the kinetics of sulphate reducing micro-organisms at relevant practical conditions can be performed.

Finally, obviously also further scale-up research is of major importance. Particularly, the application to flue-gas desulphurization requires a 10^6-fold scale-up, putting high demands upon available design rules for scale-up. So, additional research on hydrogen mass transfer in gas-lift reactors is needed. Especially the influence of growing biomass and high salt concentrations on the hydrogen mass transfer rate needs to be investigated since hardly any relevant literature data can be found at this moment.

6.3 Microbial aspects

Micro-organisms
The studies of the enrichment cultures in chapter 3 clearly show that mesophilic sulphate reduction is performed by *Desulfovibrio* sp. Homoacetogenic activity was caused by *Acetobacterium* sp. According to Brysch *et al.*[8] this co-culture will always be obtained during mesophilic enrichment on H_2/CO_2 gas mixtures. If so, some problems will be encountered when other process conditions are imposed to the system. So a shift in the microbial population might occur, resulting in other biomass characteristics.
Problems might arise in treating hot wastewaters, wastewaters containing high sulphate concentrations or wastewaters with a low pH. It was shown in chapter 5 that strong competition between *Desulfotomaculum* sp. and methanogens will occur at thermophilic conditions. The reason for this phenomenon remains to be investigated. Additionally, treatment of wastewater containing more than 10 g/L of sulphate might result in H_2S concentrations that exceed 15 mM (i.e. > 0.5 g/L). Most known sulphate reducing micro-organisms do not grow at such high hydrogen sulphide concentrations[42]. Furthermore, treatment of wastewater with low pH (< 2) might be difficult. Proton elimination capacity of the system, resulting from sulphate reduction, might not suffice, leading to acidification of the system and consequently a poor reactor performance.

Future perspectives

Future research should be focused at sulphate reduction with CO as electron donor, because this would open the possibility to use (cheap) synthesis gas from coal gasifications units, which has a H_2/CO ratio smaller than 0.5.

Additionally, studies of bacterial aggregates have raised several questions about the species composition and structure, and the activity of the individual trophic groups present in the aggregates. Traditional microbiological techniques and conventional microscopy are often insufficient means to answer these questions. However, new identification techniques, like *in situ* 16S rRNA probing and denaturing gradient gel electrophoresis (DGGE) of PCR-amplified DNA and mRNA, might elucidate the organization of microbial aggregates and give the opportunity to monitor population dynamics during perturbations under process conditions[1,31,41].

A whole new area of research is the application of new genera of sulphate reducing bacteria, isolated from extreme ecological niches. Examples are recently isolated thermophilic sulphate reducing archea, like *Archaeoglobus* sp.[7,34,35]. Since these micro-organisms are related to early forms of life, they may posses rather peculiar, useful metabolic routes, while they also may tolerate 'extreme' environmental conditions like low pH or high salt concentrations.

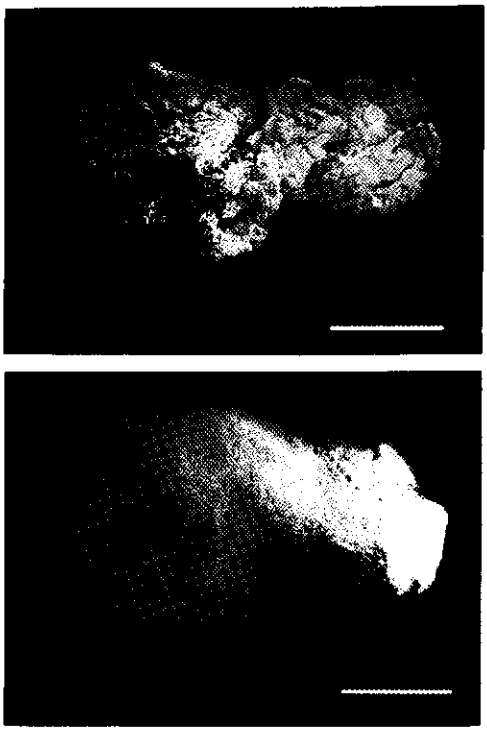
6.4 Aggregate structure and dynamics

Obviously, biomass retention is essential for the operation of gas-lift reactors. Although in recent years in this respect large improvements have been made[3,19,37], several aspects of biomass retention are still poorly understood. This is particularly true for the ability of sulphate-reducing bacteria to form biofilms or aggregates in turbulent three phase systems. This paragraph discusses some specific features of aggregate structure and population dynamics, on the basis of the experimental results presented in chapter 2, 3 and 4.

Structures

The results obtained with H_2/CO_2 at pH 7.0 (chapters 2 and 3), reveal that sulphate-reducing bacteria can form stable biofilms on pumice particles. In addition, carrier-free aggregates were formed. Phase-contrast microscopy and SEM observations showed highly branched surfaces with sometimes large protrusions (see Fig. 6.2a).

Results of experiments at different pH-values (chapter 3), reveal that the pH affects aggregate configuration and diameter, i.e. higher values lead to increased surface irregularity (likely due to reduced hydrogen sulphide toxicity), without affecting the particle diameter. A lower pH reduces the surface irregularity and increased the aggregate Sauter mean diameter.



(a)

(b)

Figure 6.2. SEM micrographs of sulphate-reducing aggregates. (a) Aggregate grown on H_2/CO_2. (b) Aggregate grown on H_2/CO. Clearly the surface if the aggregate changed upon addition of CO. Bar length 500 mm.

The results in chapter 4 show that CO affected the aggregate shape and diameter. SEM micrographs showed that rough aggregates, pre-grown on H_2/CO_2, changed into smooth aggregates upon addition of CO. An example is given in Figure 6.2b. Addition of CO also increased the aggregate Sauter mean diameter. Additionally, although the hydrodynamics in the reactor remained unchanged, the detachment rate was half of the rate observed before CO addition.

The observations made in all our experiments show the same clear trend. Under conditions of optimal growth, i.e. pH 7.0 and CO absent, the biofilm or aggregate surface is rough with sometimes large protrusions. When the growth rate decreases due to the presence of some inhibitory compound, i.e. CO, H_2S, or excess of H^+ (low pH), the biofilm or aggregate surface becomes smooth. The detachment rate of the aggregates is also reduced at the lowered growth rate, e.g. by 50% upon changing from CO_2 to CO. The observed phenomena are most apparent in case of inhibition with carbon monoxide.

In recent years, similar observations have been made by Beeftink and Staugaard[2]. They found that aggregates producing butyric acid from glucose had a loose and hairy appearance, while propionate and acetate producing aggregates had a much smoother aggregate surface. The observed hairy structures were attributed to the high substrate consumption rate, indicated by the high production rate of gaseous metabolites during butyrate production. Such an open structure would facilitate substrate transport into the interior of the aggregates.
According to Tijhuis *et al.*[38,39] there exists a relationship between surface substrate loading rate and aggregate morphology. At a high surface substrate loading rate and under conditions of low detachment forces, hairy and rough aggregates would develop. These hairy aggregates also were found to be weaker than smooth aggregates. They postulated that the growth rate of the bacteria in the protrusions is high, due to the high surface to volume ratio which probably reduces diffusion limitation of the substrates. Higher surface substrate loading rates could therefore result in higher growth rates, weaker aggregates and higher detachment rates.

Hypothesis
In our experiments the growth rate of the micro-organisms was clearly influenced by either the pH or the fraction of CO present. Particularly, the addition of CO causes a decrease in the growth rate of the bacteria[13,29]. Simultaneously, the protrusions disappear from the aggregate surface, as shown in Figure 6.2a,b. Clearly, there is a relationship between the growth rate of the bacteria and the aggregate morphology, since the hydrodynamic conditions in the reactor always remained constant.
Based on the observed differences in the aggregate surface structure between fast-growing and slow-growing aggregates under the same hydrodynamic conditions, the following hypothesis might be proposed.
The protrusions formed will be sensitive to shear forces. Consequently, they are easily abraded from the aggregate, resulting in relative high detachment rates. However, due to the high growth rate of the micro-organisms present, the protrusions are replaced quickly by new ones.

Thus, a rough aggregate surface prevails. When the growth rate of the involved bacteria decreases, as is the case with CO addition, the abraded branches will not be replaced by new protrusions. This results in a smooth aggregate surface and a lower detachment rate.

Similar hypotheses were proposed by Tijhuis *et al.*[39]. Based on their experimental results, they proposed two hypotheses.
In the first hypothesis (A) they assume that there exists a gradient in strength within the aggregate, due to a gradient in growth rate over the depth of the biofilm. The fast growing outer shell of the biofilm is assumed to produce less exopolymer than the inner part of the biofilm, and therefore the outer biofilm would be weaker than the inner part, since exopolymers are supposed to be responsible for the strength of the aggregates. Consequently, the outer part of the aggregate would be much more susceptible to shear forces than the slowly growing inner part of the aggregate. As a result, the fast growing outer shell of the aggregate will be sheared off during the substrate depleted period. After that, the stronger, inner part of the aggregate is left and hence the detachment rate will decline.
Their second hypothesis (B) is based on the observation that the surface of most of the aggregates was knobbly or rough with sometimes large protrusions. As these protrusions particularly will be sensitive to shear forces, they are easily removed from the aggregate. Under conditions of substrate depletion the number of protrusions will decrease and, therefore, also the rate of detachment. Then the aggregate surface will change from rough to smooth. This is in contrast to hypothesis A, where the roughness of the aggregate surface does not necessarily change upon substrate depletion.
It might be argued that, since we did not measure the amount of exopolymer in the aggregate, hypothesis A might also be possible. However, according to hypothesis A the diameter of the aggregates should decrease after substrate depletion or growth inhibition. This is not observed. In contrast, upon addition of CO or growth at a pH below 7.0, the Sauter mean aggregate diameter increased from 1.6 mm to 2.0 mm (chapters 3 and 4). Thus, hypothesis A is not valid.

The question why the non-inhibited micro-organisms tend to grow as aggregates with a rough or hairy surface still remains. An explanation could be (chapter 3) that the micro-organisms show a kind of chemotaxis, by which they tend to increase the specific surface area in order to postpone possible substrate limitation. This suggestion is in agreement with the observation made by other authors[12,15], that this type of aggregates only prevails during substrate limitation. More detailed studies, however, are required for the verification of this hypothesis.

Population dynamics

A related phenomenon is the observed change in the spatial distribution of the micro-organisms upon addition of CO. As discussed in chapter 4, the *Desulfovibrio* sp. cannot survive in the outer regions of the aggregates at higher CO levels. Therefore, they will be replaced by *Acetobacterium* sp. This results in a relative increase of the amount of *Acetobacterium* sp. As these bacteria can convert CO into either acetate or CO_2, they will remove CO on its way into the inner region of the aggregate. Thus, growth of *Desulfovibrio* sp. in the inner part of the aggregate still can proceed. For the aggregates grown on H_2/CO this change in population distribution might also be the reason for the observed change in surface structure. However, in the experiments in which the pH was changed to values below 7.0, see chapter 3, we also observed the development of a smooth aggregate surface, but without any measurable change in the heterogeneous population distribution. Therefore, it is not plausible that the change in surface structure during growth on H_2/CO is determined by the change in population distribution.

Summarizing it can be concluded that the surface structure of the sulphate-reducing aggregates depends on the growth rate of the micro-organisms present. A high growth rate will yield a rough and hairy aggregate surface, while at a low growth rate the aggregate surface will become smooth, because then detachment of the large protrusions from the aggregate surface will proceed.

The surface structure does not depend on the type of bacteria present at the surface, i.e. sulphate-reducing bacteria or homo-acetogenic bacteria.

6.5 Modelling aspects

State of the art

There is a vast amount of literature available on modelling mass transfer into and inside biofilms and aggregates. Nearly all of these biofilm models assume that mass transfer inside the biofilm or aggregate is diffusion controlled and that the external, liquid-solid mass, transfer is dominated by convective flow. Consequently, it is believed that mass transfer inside biofilms can be described by molecular diffusivity as the only parameter. External mass transfer from bulk liquid to the aggregate surface is commonly approximated by assuming that the liquid boundary layer above the aggregate surface is uniform and stagnant.

Mass transport of substrates into biofilm or aggregate is traditionally modelled by Monod kinetics to describe the rate substrate utilization, and Fick's first law for mass transfer by diffusion:

$$J_i = -D_i \frac{dC_i}{dx}$$

An implicit assumption in these models is that the pH remains constant throughout the aggregate. However, using Fick's law to describe ionic fluxes creates the problem of assigning effective ionic-diffusion coefficients to incorporate ionic interactions. Furthermore, the pH-profile in the biofilm or aggregate depends on the diffusion of all ions and on the neutral species of the dissociating electrolytes (e.g. H_2S). Thus, ionic fluxes must be described using the Nernst-Planck equation, which equation accounts for the flux of ions due to a concentration gradient *and* an electrostatic potential gradient:

$$J_i = -D_i\left(\frac{dC_i}{dx} + z_i C_i \frac{F\nabla_x \psi}{RT}\right)$$

In the absence of an externally applied potential, the electrostatic potential gradient accounts for the interactions between ions as they diffuse through a medium. Because of the requirement of electroneutrality of the medium, ions moving with different diffusivities induce temporary charge separation which is restored by the electrostatic forces between these ions.
Therefore, recently, the pool of existing models has been extended by Flora *et al.*[16,17] They developed a detailed biofilm model accounting for the diffusion of neutral and ionic species. Since several biological systems deal with ion transport, e.g. nitrification/denitrification and sulphate reduction processes, these models may give an improvement in our understanding of the basic diffusion processes occurring in biofilms.

Bottlenecks

The abovementioned 'simple' models for mass transport in biofilms have been challenged by recent literature reports that reveal that structures of biofilms or aggregates are not uniform but consist of microcolonies separated by, sometimes large, pore channels[4,5,27,36,43]. According to this concept, mass transport in the interstitial voids is enhanced by convective flow, while mass transfer inside the microcolonies would be entirely due to molecular diffusion. Therefore, the mechanism of local mass transport is determined by the structural heterogeneity of the aggregates.

Consequently, mass transfer rates can differ significantly at different locations in the aggregate due to variability in pore structure and thus spatial variability in reactivity of the aggregate. Rather, mass transfer rates depend on complex interactions between local microbial activity, aggregate structure and hydrodynamics. The situation is further complicated because biofilms are viscoelastic, i.e. they behave partly viscous (as liquids) and partly elastic (as solids), and microscale hydrodynamics near such surfaces have not been characterized analytically. Thus, there is no adequate mathematical model describing mass transfer in a growing three-dimensional irregular structure.
Apart from difficulties in describing mass transfer into (and growth of) microbial aggregates properly, experimental evidence shows that detachment from a growing biofilm system is a stochastic process[39], rather then a continuous process. So, detachment cannot be described by the linear models for detachment, summarized by Tijhuis *et al.*[39], which are currently available.

For the process of sulphate reduction in microbial aggregates nearly all described bottlenecks are encountered. First, diffusive mass transport in sulphate reducing aggregates cannot be described with simple Fick-diffusion since several ions and pH gradients are involved. By using the Nernst-Planck equation that problem could be eliminated, However, hardly any data are available on the influence of pH and liquid CO concentration on the kinetics of sulphate reducing bacteria. Moreover, as was shown in the previous chapters, sulphate-reducing aggregates often have an irregular surface with large protrusions and interstitial voids, resulting in two- or three-dimensional differences in substrate concentrations in the aggregate. Any, conventional modelling would therefore yield concentration profiles that are averaged over various locations, possibly resulting in e.g. a false prediction of the spatial distribution of microbial species. Additionally, it was shown in this chapter that detachment of biomass is growth rate dependent and much more complex than the previously published linear models.

Future perspectives
Modelling sulphate reduction in microbial aggregates will be greatly facilitated when a comprehensive reliable set of kinetic data becomes available. Hardly any literature data can be found at present, that describe adequately the influence of pH, H_2S and liquid CO concentration on the kinetic parameters of hydrogenotrophic sulphate reducing micro-organisms.

Furthermore, there is a clear need for new concepts in order to improve our notion of mass transfer into biofilms. Irrespective of the question whether or not it is possible to describe the complexity in reliable models, it would be worthwhile to consider stochastic models for growth

and mass transport, which are much more common in the field of polymer sciences, surface chemistry and crystallisation.

On the other hand however we might conclude from an engineering point of view that, although short-comings exist, current models are well capable of describing a variety of practical processes. Thus, the significance of engineering results from any new sophisticated model with a better physical background should be viewed in that light.

6.6 Conclusions

This thesis provides experimental results that show the feasibility of biological sulphate reduction with synthesis gas as electron donor and carbon source. Additionally, valuable tools have been obtained for the design of large-scale reactors for biological sulphate reduction.
Concerning the practical needs, outlined in chapter 1, the next logical step would be the application of the process under practical conditions. Fortunately, various attempts are presently made. So two 5 m^3 pilot-plants are currently operated, one treating sulphite-liquor from an alkaline flue gas scrubber at a 600 MW power plant in Geertruidenberg, The Netherlands, the other treating mine leachate from an huge open copper pit in Kennecott, Utah, USA. This pilot plant research will be the best test case for the design rules given in this thesis.

6.7 Nomenclature

C_i	substrate concentration i in the bulk liquid	(mol/m^3)
$C_{L,H2}$	substrate concentration in the bulk liquid	(mol/m^3)
$C^i_{L,H2}$	substrate concentration at the surface of the aggregate	(mol/m^3)
C_X	biomass concentration	(kg/m^3)
d_P	characteristic length of the system, in our case the average aggregate diameter	(m)
d_{32b}	Sauter mean bubble diameter	(m)
D_{H2}	diffusion coefficient of hydrogen	(m^2/s)
D_i	diffusion coefficient of component i	(m^2/s)
F	Faraday's constant	(C/m)
J_{H2}	hydrogen flux to the surface	($mol/m^2.s$)

J_i	flux to the surface of component *i*	(mol/m^2.s)
k_L	liquid-side mass transfer coefficient	(m/s)
$k_L.a$	volumetric liquid-side mass transfer coefficient	(1/s)
$k_{L,S}$	liquid-solid mass transfer coefficient	(m/s)
$k_{L,S}.a$	volumetric liquid-solid mass transfer coefficient	(1/s)
$P_{G,H2}$	partial pressure of hydrogen	(kg.m/s)
r_p	radius of the aggregate	(m)
R	universal gas constant	(J/mol.K)
T	absolute temperature	(K)
U_b	bubble rise velocity	(m/s)
U_{Gs}	superficial gas velocity	(m/s)
$Y'_{S/X}$	yield coefficient (hydrogen consumed per biomass produced)	(mol/kg)

dimensionless numbers

Re_p	Reynolds number of the aggregates	($\rho.v.d_P/\eta$)
Sc	Schmidt number	(ν/D_{H2})

greek

δ	thickness of the stagnant boundary layer	(m)
ε	gas hold-up	(-)
η	dynamic viscosity	(kg/m.s)
μ	specific growth rate	(1/s)
ν	kinematic viscosity	(m^2/s)
ρ_P	density of the liquid	(kg/m^3)
ρ_L	density of the aggregates	(kg/m^3)
ψ	electrostatic potential	(V)

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Samenvatting

In een aantal sectoren van de industrie, in het bijzonder de chemische en metallurgische industrie en de mijnbouw, ontstaan afvalwaterstromen die veel sulfaat en/of sulfiet bevatten. Biologische sulfaat-reductie, gecombineerd met sulfide-verwijdering, lijkt een aantrekkelijke techniek om deze zwavelcomponenten te verwijderen. Omdat de genoemde afvalwaterstromen geen 'complexe' organische stoffen bevatten, kan biologische sulfaat-reductie echter alleen plaatsvinden wanneer een elektronen donor en koolstofbron worden toegevoegd. De belangrijkste doelen van dit proefschrift zijn derhalve (1) het onderzoeken van de haalbaarheid van het gebruik van synthese gas (een mengsel van H_2, CO en CO_2) als elektronen donor en koolstofbron en (2) het ontwikkelen van regels voor ontwerp en bedrijfsvoering van gas-lift bioreactoren voor sulfaat-reductie met geïmmobiliseerde biomassa.

Om de giftigheid van CO vast te kunnen stellen is het mesofiele onderzoek gesplitst in twee delen. Allereerst is, als een soort basisgeval, onderzoek uitgevoerd met H_2/CO_2 als elektronen donor en koolstofbron. Daarna is onderzoek gedaan met echt synthese gas.

Het in dit proefschrift beschreven onderzoek met H_2/CO_2 onder mesofiele condities heeft aangetoond dat met dit substraat een mesofiele mengcultuur wordt verkregen waarin *Desulfovibrio* sp. en *Acetobacterium* sp. de meest voorkomende bacteriën zijn. Deze bacteriepopulatie vormt stabiele biofilms op puimsteen deeltjes. Biofilm vorming op basalt deeltjes is nauwelijks waargenomen. Gebruik van basalt leidt voornamelijk tot de vorming van aggregaten zonder drager deeltjes, hetgeen op zich ook erg aantrekkelijk kan zijn.
De gevormde biofilms en aggregaten hebben een zeer ruw oppervlak met poliepvormige uitstulpingen. De Sauter gemiddelde diameter van de (dragervrije) aggregaten is 1.5 mm. De bacteriën in de aggregaten zijn bestand tegen vrije H_2S concentraties tot 450 mg/L. Deze hoge H_2S concentraties veroorzaken geen acute vergiftiging maar reversibele remming. Wanneer de vrije H_2S concentratie beneden 450 mg/L wordt gehouden, wordt binnen 10 dagen na opstart een maximale sulfaat-omzettingssnelheid van 30 g SO_4^{2-}/L per dag bereikt. Deze maximale omzettingssnelheid wordt bepaald door de gas-vloeistof stofoverdrachtscapaciteit van de laboratorium reactor.

Verder onderzoek met H_2/CO_2 toonde aan dat sulfaat-reductie toepasbaar is in een pH-bereik van 5.5 tot 8.0, met een optimum rond pH 7.5. De pH beïnvloedt de verschijningsvorm en

diameter van de aggregaten. Een pH-verhoging leidt tot een toename van de oppervlakteruwheid, maar niet tot een verandering in de diameter van de aggregaten. Een pH-verlaging veroorzaakt een afname van de oppervlakteruwheid, met een gelijktijdige toename van de Sauter gemiddeld diameter van 1.5 mm bij pH 7.0 tot 2.2 mm bij pH 5.5. De pH veranderingen hebben geen meetbare invloed op de microbiële samenstelling van de aggregaten. Fase-contrast microscopie, elektronenmicroscopie en ophopingsculutres tonen aan dat bij alle pHs *Desulfovibrio* soorten en *Acetobacterium* soorten de meest voorkomende bacteriën zijn.

Wanneer sulfaat-reductie wordt uitgevoerd met synthese gas als elektronen donor en koolstofbron, worden de prestaties van de reactor sterk beïnvloed. Toevoeging van 5% CO leidt tot een daling van de sulfaat-reductiesnelheid van 12 - 14 g SO_4^{2-}/L per dag tot 6 - 8 g SO_4^{2-}/L per dag. Verder toename van het percentage CO van 10% naar 20% geeft geen verder verslechtering van het proces. Met externe biomassa bezinking en recirculatie kan de sulfaat-reductiesnelheid worden verbeterd tot 10 g SO_4^{2-}/L per dag. Hieruit blijkt dat onder deze omstandigheden de biomassa retentie de snelheidsbepalende stap is. Koolmonoxyde beïnvloedt ook de verschijningsvorm en diameter van de aggregaten. Scanning elektronenmicroscopie toonde aan dat ruwe aggregaten, gegroeid op H_2/CO_2, na toevoeging van CO veranderen in gladde aggregaten. Gelijktijdig neemt ook de Sauter gemiddeld diameter toe van 1.7 mm bij 5% CO tot 2.1 mm bij 20% CO. Daarnaast ontstaat na toevoeging van CO een gelaagde biomassa structuur waarbij de *Acetobacterium* sp. voornamelijk in de buitenste laag van de biofilm groeien, en de *Desulfovibrio* sp. voornamelijk binnenin de aggregaten groeien.

Gebaseerd op de waargenomen verschillen in de oppervlakte-structuur van snelgroeiende en langzaamgroeiende aggregaten (onder gelijke hydrodynamische condities) is een hypothese opgesteld, die is gestoeld op de veronderstelling dat de oppervlakte structuur van de aggregaten afhangt van de groeisnelheid van de bacteriën in de aggregaten.

Als laatste onderwerp is in dit proefschrift de thermofiele sulfaat- en sulfiet-reductie op H_2/CO_2 onderzocht. De resultaten van de experimenten laten duidelijk zien sulfaat-reductiesnelheden tot 7,5 g SO_4^{2-}/L per dag kunnen worden behaald. Met sulfiet wordt een reductiesnelheid van 3,7 S/L per dag bereikt, wat overeenkomt met een sulfaat-reductiesnelheid van 11,1 g SO_4^{2-}/L per dag. Deze maximale sulfaat-reductiesnelheid wordt bepaald door de remming door H_2S. De groei van de verkregen mengcultuur wordt volledig geremd bij een vrije H_2S concentratie van 250 mg/L.

Onder de toegepaste condities treedt ook, in tegenstelling tot wat onder mesofiele condities is waargenomen, een sterke competitie om waterstof op tussen waterstofminnende sulfaat-reduceerders, *Desulfotomaculum* sp., en waterstofminnende methanogene bacteriën. De uiteindelijke uitkomst van deze competitie kan op grond van de behaalde resultaten niet worden voorspeld. Verder is tijdens alle experimenten een slechte biomassa retentie waargenomen. De hoogst gemeten biomassa concentratie is 1,2 g/L, ondanks de aanwezigheid van puimsteen als dragermateriaal. De reden voor dit verschijnsel is vooralsnog onbekend.

Gebaseerd op de behaalde resultaten is geconcludeerd dat beide doelen van het proefschrift zijn bereikt. Ten eerste blijkt biologische sulfaat-reductie met synthese gas als elektronen donor en koolstofbron goed mogelijk te zijn. Daarnaast zijn verschillende regels voor ontwerp en bedrijfsvoering van gas-lift reactoren voor sulfaat-reductie verkregen en besproken.
Wat betreft de praktische toepassing zou de volgende, meest logische stap de toepassing van het proces onder praktische condities zijn. Daartoe worden op dit moment een aantal pogingen ondernomen. Er zijn twee 5 m^3 'pilot-plants' in bedrijf. De ene behandelt waswater uit een alkalische rookgas scrubber van een 600 MW kolengestookte elektriciteitscentrale in Geertruidenberg, Nederland. De andere behandelt percolatie-water van mijnafval van een grote open kopermijn in Kennecott, Utah, USA. Dit 'pilot-plant' onderzoek zal de beste test zijn voor de in dit proefschrift gegeven regels voor ontwerp en bedrijfsvoering van gas-lift reactoren voor sulfaat-reductie.

Curriculum vitae

The author of this thesis was born on 23 August 1967 in Warffum. After finishing grammar school in 1985, he started studying Chemical Engineering at the Groningen State University. He obtained his M.Sc. degree in Chemical Engineering in 1990. The topic of his master thesis was on the enhancement of gas to water mass transfer rates by a dispersed organic phase. In 1991 he also obtained his M.Sc. degree in Chemistry at the Groningen State University. The topic of this masters thesis was on the high cell density cultivation of poly(3-hydroxyalkanoate)-producing *Pseudomonas olevorans*.

In August 1991, he moved to the Wageningen Agricultural University, Department of Environmental Technology, to start the Ph.D. research described in this thesis. From 1994 until the end of his Ph.D. in October 1995, he worked also part-time with Paques Environmental Technology BV as a process specialist. During this job, he was responsible for the process development of biological processes for sulphate removal from wastewater.

After October 1995, the author works with the TNO Institute of Environmental Sciences, Energy Research and Process Innovation. He is responsible for the project management and scientific coordination of industrial wastewater research within the Department of Environmental Biotechnology.