The Fate of Methanol in Thermophilic-Anaerobic Environments

Paula Loureiro Paulo

Promotor

Prof. dr. ir. G. Lettinga

hoogleraar in de anaerobe zuiveringstechnologie en hergebruik van afvalstoffen

Co-promotor

Dr. ir. J. B. van Lier

onderzoeker bij de sectie milieutechnologie en directeur Lettinga Associates Foundation

Samenstelling promotie commissie

Prof. dr. M. L. Florencio, Federal University of Pernambuco, Brazil

Dr. ir. A. J. H. Janssen, Paques b. v., Balk

Prof. dr. ir. I. M. C. M. Rietjens, Wageningen University

Prof. dr. ir. A. J. M. Stams, Wageningen University

The Fate of Methanol in Thermophilic-Anaerobic Environments

Paula Loureiro Paulo

Proefschrift

Ter verkrijging van de graad van doctor

Op gezag van de rector magnificus

van Wageningen Universiteit,

Prof. dr. ir. L. Speelman,

in het openbaar te verdedigen

op woensdag 18 december 2002

des namiddags te half twee in de Aula

CIP-DATA KONINKLIKE BIBLIOTHEEK, DEN HAAG

Author: Paulo, P. L.

Title: The fate of methanol in thermophilic-anaerobic environments

ISBN: 90-5808-756-5

Publication year: 2002

Subject headings: anaerobic, methanogenesis, methanol, pathway, thermophilic, UASB.

Thesis Wageningen University, Wageningen, The Netherlands – with references - with summary in English and Dutch.

Propositions

1. The concept "simple" may express well the structure of methanol but certainly not its anaerobic conversion pathway.
This thesis

2. The influence of bicarbonate and phosphate in the anaerobic biodegradation of methanol under thermophilic conditions exceeds by far the pH-buffering effect.
This thesis

3. Bicarbonate is crucial for achieving both a stable and efficient anaerobic conversion of methanol under thermophilic conditions at a temperature of 55 °C.
This thesis

4. Contrary to the ideas of Nishio et al. (1993) methanogenic-sludge granulation can proceed quite well on methanol.
This thesis
Nishio N., R. G. Silveira, K. Hamato and S. Nagai (1993) High rate methane production in a UASB reactor fed with methanol and acetate. J. Ferment. Bioeng. 75(4):309-313
Bhatti Z. I., K. Furukawa and M. Fujita (1993) Treatment performance and microbial structure of a granular consortium handling methanolic waste. J. Ferment. Bioeng. 76(3):218-223
Bhatti Z. I., K. Furukawa and M. Fujita (1995) Methanogenic granular sludge formation in an upflow anaerobic sludge blanket reactor treating synthetic methanolic waste. World J. Microbiol. Biotechn. 11(6):631-637

5. Remember that not getting what you want is sometimes a wonderful stroke of luck.
His Holiness The Dalai Lama

8. You have one mouth and two ears, use them at that proportion.
Chinese saying

7. The only way to find the limits of the possible is by going beyond them to the impossible.
Arthur C. Clarke (Clarke's Second law)

8. High expectations are usually followed by disappointment.

9. To fight against Friesian stubbornness is a lost battle.

Propositions belonging to the thesis entitled "The fate of methanol in thermophilic-anaerobic environments".

Paula Loureiro Paulo
Wageningen, 20 November 2002.

Aos meus pais

À memória de meu irmão Edson (1961-1993)

Abstract

Paulo, P.L. (2002) The fate of methanol in thermophilic anaerobic environments. **Doctoral Thesis, Wageningen University, The Netherlands, 126 Pages.**

Methanol is a simple C1-compound, which sustains a complex web of possible degradation routes under anaerobic conditions. Methanol can be the main pollutant in some specific wastewaters, but it is also a compound that may be formed under natural conditions, as intermediate in the decomposition of organic matter. The information available in literature enables one to design a satisfactory application of a stable high-rate mesophilic-methanogenic reactor system, but the same does not apply for thermophilic conditions. The main objective of this thesis was to assess the feasibility of treating methanol-containing wastewater under thermophilic (55 °C) conditions in a single-step UASB-reactor. The research was focused on the stability of the reactor performance and on the environmental factors that may play a role on the anaerobic conversion of methanol including pH, inorganic carbon and trace metals. Good reactor performance was achieved at organic loading rates (OLR) up to 47.3 $gCOD.L^{-1}.d^{-1}$ in a bicarbonate buffered medium, with 93% of methanol removal where the major end product was methane. Moreover, the accumulation of volatile fatty acids (VFA), often reported as a drawback, was not detected. The assessed physical characteristics of the cultivated sludge showed that a good quality, well settleable granular sludge, was cultivated and retained in the reactor. Further, the stability of the system was studied. When the bicarbonate buffered-reactor was exposed to specific environmental stress situations (temperature drop, overloading and no feeding), the performance was temporarily affected during the shocks but the system promptly recovered, after the normal conditions were restored. On the other hand, when the methanol conversion was studied in a bicarbonate-deprived medium (either phosphate buffered or with automatic addition of NaOH, neutral pH range), the reactor performance was poor, and the system was quite sensitive to disturbances, even at low OLR. When phosphate was present in the medium, acetate accumulation manifested, indicating that phosphate inhibited the acetotrophic microorganisms present in the consortium. The cultivated thermophilic consortium showed to be sensitive to pH shocks, both acidic (pH 4) and alkaline (pH 9.5). A recovery of methanogenesis was not possible by simply restoring the reactor pH, besides, the addition of bicarbonate at this stage, stimulated the formation of acetate. A proposed reactor-recovery strategy, based on the stepwise addition of bicarbonate, however, was found to be very effective to recover methanogenesis from methanol from complete failure or reactor upset caused by pH shock, even in case where (homo)acetogens were outcompeting methanogens. To obtain an insight in the degradation pathway of methanol and better understanding of the influence of the parameters mentioned above, a detailed study using specific inhibitors, and nuclear magnetic resonance (NMR) technique was conducted. Results showed that about 50% of methanol was directly converted to methane by the methylotrophic methanogens and 50% via the intermediates H_2/CO_2 and acetate. The results also indicated that inorganic carbon ($\Sigma([HCO_3^-] + [CO_2])$) is required as "electron" (H_2) sink as well as cosubstrate for efficient and complete chemical oxygen demand (COD) removal. Furthermore, we studied the importance of cobalt to the thermophilic cultivated consortium in continuous experiments and in a cobalt-deprived enrichment culture. The cobalt requirement of our cultivated consortium was lower as compared to that of a mesophilic-methylotrophic consortium. For the cobalt-deprived enriched culture, 0.1 µM of cobalt was found to be the most appropriate concentration, leading to the highest methanol conversion rate with methane as sole end product from methanol.

The information contained in this thesis enables a successful application of the UASB reactor for methanol-containing wastewaters under thermophilic conditions. For that purpose, it is also recommended the use of bicarbonate for the treatment of methanol-containing wastewater where the syntrophic conversion via H_2/CO_2 is involved. Another important recommendation is that, to develop a balanced consortium with methane as the target end product, cobalt and bicarbonate should always be stepwise introduced to the system.

Table of contents

1

General introduction

Methanol sources

Methanol is widely produced in nature by anaerobic microorganisms responsible for complex aromatic hydrocarbon biodegradation [46]. Methanol is a major product of microbial growth on pectin [103] which together with hemicellulose make up the abundant methoxylated polysaccharides in plant tissues. Furthermore, due to the solubility of methanol in water, methanol molecules are bioavailable to microorganisms, which can utilise them as a source of carbon and energy. The importance of methanol and methylated amines as methane precursors in estuarine, intertidal sediments is variable, due to the abundance of decomposing plant materials in the sediment system [33, 59].

World-wide, the over 90 existing methanol plants produce over 11 billion US gallons of methanol annually. The typical feedstock used in the production of methanol is natural gas. Methanol can also be made from renewable resources such as wood, municipal solid wastes and sewage. In a typical plant, methanol production is carried out first by the conversion of natural gas in the synthesis gas consisting of CO, CO_2, H_2O and hydrogen. This is usually accomplished by the catalytic reforming of feed gas and steam. Methanol is then synthesised under pressure in a catalytic process and the crude methanol is purified to chemical grade by distillation. Each of these steps can be carried out in a number of ways and various technologies offer a spectrum of possibilities, which may be most suitable for any desired application. Methanol is a chemical building block used to produce formaldehyde, acetic acid and a variety of other chemical intermediates. A significant amount of methanol is also used to make MTBE (methyl tertiary butyl ether), an additive used in cleaner-burning gasoline. Methanol is also considered a potential candidate for an alternate supply of hydrogen for vehicular fuel cell applications [51, 71]. Studies on the production of synthetic natural gas (SNG) via chemical conversion of methanol to methane have been carried out [53, 86]. The increase in methanol production may reduce its cost and consequently stimulates either chemical or biological methanation from methanol [87] representing another possibility for energy generation.

Methanol sources and use in biotechnology

Methanol has been detected in low concentration as a constituent of landfill leachates [131], or as a product of thermophilic acidification of dairy wastewater [145]. In higher concentration it may be found in formaldehyde production plants, which represents the largest single end use for methanol. Formaldehyde concentration can be found up to 10000 $mg.L^{-1}$ in the wastewater streams, and is often accompanied by methanol [55]. In addition, methanol was found to be an intermediate product in the anaerobic degradation of formaldehyde [41]. Kraft pulping mill condensate is highly polluted, containing remainders of terpenes, aldehydes, ethanol, reduced sulphur compound and methanol as the main organic pollutant [25] with its concentration ranging from 1.5 to 24.5 $g.L^{-1}$ [78].

A recent review of Weijma and Stams [136] reported the potential of methylotrophic organisms or anaerobic sludge to (co)metabolically degrade a large number of toxic or waste chemicals in laboratory studies.

Methanol can also be used as electron donor for sulphate-reducing processes such as flue-gas desulphurisation and for the treatment of acid mine drainage. Under mesophilic conditions, such an application does not seem attractive, since sulphate does not sufficiently affect the conversion of methanol to methane [136]. On the other hand, under thermophilic conditions (65 °C), Weijma et al. [135] found that methanol mainly was used for sulphate reduction in an expanded granular sludge bed (EGSB)-reactor.

In the United States, over 100 wastewater treatment plants currently use methanol for anaerobic denitrification. Methanol is more expensive for nitrogen removal than other carbon sources such as brewery wastes, molasses and whey, but in many cases methanol is preferred, as it is totally utilised without accumulation of undesirable intermediates [94].

High rate anaerobic wastewater treatment

The anaerobic wastewater treatment process is a successful and well-established technology applied for the degradation of organic matter [67]. Due to the long retention times of the active biomass, the upflow anaerobic sludge blanket (UASB)-reactor is also suitable for the development of bacterial consortia capable of degrading xenobiotics [132]. Recently, anaerobic microorganisms have been discovered capable to degrade compounds previously considered recalcitrant [27]. A wide variety of reactor designs have been developed throughout the years making possible the application of the anaerobic system for a broader range of wastewater types and temperature.

Thermophilic anaerobic treatment

Process water and wastewater temperatures in many manufacturing processes range from 50 to 70 °C and in certain processes it even may exceed 90 °C [66]. Anaerobic treatment at high

temperatures has been investigated for more than one century, particularly for the treatment of slurries and solid waste, and over the last decade, many researches investigated thermophilic high rate reactors [124]. Nevertheless, biological treatment for industrial wastewaters is applied almost exclusively under mesophilic conditions, and so far very few full-scale thermophilic anaerobic systems have been installed. According to Ahring et al. [2] the rate of thermophilic digestion exceeds that of mesophilic systems and applicable residence times may approach one-third of those of mesophilic digesters. At thermophilic temperatures, substrates are more accessible for biodegradation and consequently the gas yields are enhanced compared to mesophilic process. Good results have been obtained in laboratory scale experiments [3, 4, 66, 92, 96] showing that the thermophilic anaerobic treatment comprises an attractive alternative for treating high strength wastewater, especially when they are discharged at high temperatures. Thus, capital and operating costs associated with pre-cooling for a mesophilic treatment can be avoided.

However, several drawbacks of thermophilic reactors have been reported, such as a high susceptibility to temperature fluctuations [150, 151], feed interruption [138] and shock loading [106, 110]. Another drawback often reported is a relatively high effluent volatile fatty acids (VFA) concentration [150]. According to Van Lier et al. [128], the occurrence of poor quality effluents can be attributed to the applied process technology rather than to the thermophilic digestion process itself. The high VFA-effluent concentration can be overcome by applying staged anaerobic reactor systems [125]. Nevertheless, also the biomass retention is a critical point when applying thermophilic treatment, due to the lower liquid viscosity and the occasional occurrence of less stable thermophilic aggregates [128]. These drawbacks might be the reason for the still low popularity for the implementation of full-scale thermophilic reactors.

The anaerobic conversion of methanol: microbiological aspects

Methanol is a simple C1- compound that under anaerobic conditions can potentially support a complex food chain. However, we will particularly focus on methanogenic and

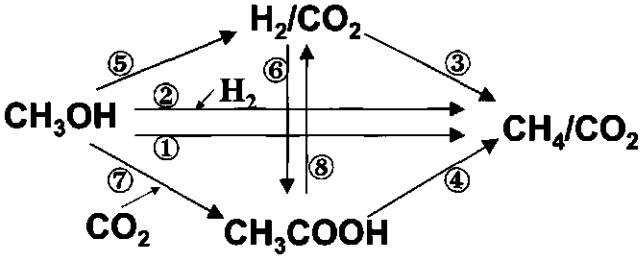


Figure 1 Potential individual pathways of the anaerobic conversion of methanol.

(homo)acetogenic pathways that represent the main topic of this thesis.

Figure 1 illustrates the possible pathways of methanol conversion, and Table 1 the stoichiometries and Gibbs free energy changes (at 55 °C) of the reactions involved. Methanol can be directly converted to methane by methylotrophic methanogens (Reaction 1) [122] and it may be reduced to methane with H_2 (Reaction 2) [121].

Table 1 Reactions possibly involved in the anaerobic conversion of methanol and the Gibbs free-energy changes[a].

Reaction			$\Delta G°'_{55\,°C}$ kJ/reaction
Methanogenesis			
1. - $4\,CH_3OH$	$\rightarrow$	$3\,CH_4 + CO_2 + 2\,H_2O$	-326
2. - $CH_3OH + H_2$	$\rightarrow$	$CH_4 + H_2O$	-113
3. - $CO_2 + 4\,H_2$	$\rightarrow$	$CH_4 + 2\,H_2O$	-125
4. - $CH_3COO^- + H_2O$	$\rightarrow$	$CH_4 + HCO_3^-$	-35
Homoacetogenesis			
5. - $CH_3OH + H_2O$	$\rightarrow$	$3\,H_2 + CO_2$	13
6. - $2\,CO_2 + 4\,H_2$	$\rightarrow$	$CH_3COOH + 2\,H_2O$	-90
7. - $4\,CH_3OH + 2\,CO_2$	$\rightarrow$	$3\,CH_3COOH + 2\,H_2O$	-221
8. - $CH_3COO^- + 4\,H_2O$	$\rightarrow$	$2\,HCO_3^- + 4\,H_2 + H^+$	90
Sulphate reducers			
9. - $4\,CH_3OH + 3\,SO_4^{2-}$	$\rightarrow$	$4\,HCO_3^- + 3\,HS^- + 4\,H_2O + H^+$	-385
10. - $CH_3COOH + SO_4^{2-}$	$\rightarrow$	$2\,HCO_3^- + HS^-$	-55
11. - $4\,H_2 + SO_4^{2-} + H^+$	$\rightarrow$	$HS^- + 4\,H_2O$	-145
12. - $4\,HCOOH + SO_4^{2-} + H^+$	$\rightarrow$	$HS^- + 4\,HCO_3^-$	-141

[a] Energy changes at 55 °C were calculated by using the van't Hoff equation, standard free energy of formation and standard enthalpy of compounds [18, 121].

Another possible transformation represents the conversion to acetate by (homo)acetogens (Reaction 7), provided that sufficient CO_2 is available [72] followed by acetate cleavage to methane by aceticlastic methanogens [88]. Acetic acid can also be oxidised to H_2/CO_2 [45, 65, 152]. When the H_2 concentration is kept low by syntrophic partnership, methanol can be oxidised to H_2 and CO_2 [46] followed by either methanogenesis performed by the hydrogenotrophic methanogens [23] or (homo)acetogenesis [102]. When sulphate or nitrate is present as an electron acceptor, methanol can be used by sulphate- [23, 84] and nitrate reducing bacteria [97].

Substrate competition

In natural systems, including anaerobic bioreactors, the activity of anaerobic microorganisms which are always present as mixed cultures, depend to a great extent on the co-operation of several metabolic types of bacteria in feeding chains. Substrate competition among the microorganisms for available substrate may be intense. In the simplest case, the outcome of a competitive interaction depends on thermodynamics, rates of nutrient uptake, inherent metabolic rates, and growth rates [15]. These factors are of crucial importance in the prediction of which bacterial population will become predominant. The degradation route of methanol and its final fate in an anaerobic environment may become entirely different when environmental conditions change, but it also depends weighty on the history of the sludge. In the absence of nitrate, sulphate or oxidised metal ions like Fe^{3+} and Mn^{4+}, methanogens and acetogens are the expected predominant group of microorganisms in the anaerobic conversion of methanol [28]. Acetic acid or H_2/CO_2 do not act as an important intermediate in the methanol degradation in mesophilic conditions [29, 40]. According to Weijma and Stams [136], direct methanogenesis from methanol seems to be the predominant mineralisation route under mesophilic conditions both in the absence and the presence of sulphate. However, by contrast, at higher temperature syntrophic conversion seems to be important [23, 135]. Moreover, specific syntrophic interactions proceed, i.e. instead of competing for the same nutrient, some microorganisms co-operate in performing a particular transformation that each separate organism can not carry out alone as, for instance the oxidation of methanol to H_2/CO_2. In most anoxic ecosystems, the rate-limiting step in methanogenesis from organic compounds is not the terminal step of methane formation but instead, the steps involved in the production of acetate and H_2 by the syntrophs. Growth rates of syntrophic fatty acid oxidisers are very slow. As soon as H_2 is formed during their fermentations, it is quickly consumed by a methanogen, a homoacetogen, or a sulphate reducer [15]. These types of microbial interactions are crucial to the competitive success of certain anaerobic bacteria.

Competition for methanol

For the growth of homoacetogens on methanol, sufficient free CO_2 needs to be available as electron acceptor. Some CO_2 is produced in the direct conversion of methanol to methane enabling homoacetogens to grow even when any external source of CO_2 such as bicarbonate is not added to the system. However, the amount of CO_2 produced only suffices the formation of a maximum amount of acetic acid equal to 33% of the consumed methanol (Table 1).

Similar growth rates have been reported for acetogens and methanogens grown on methanol [39, 74, 98, 122, 152, 153]. For a mixed culture cultivated on methanol at 30 °C, growth rate in a bicarbonate sufficient medium was about the same for both groups but the affinity for

methanol of the methylotrophic methanogens was approximately 60-fold higher compared to the acetogens [29].

Competition for acetate

The syntrophic conversion of acetate to methane via H_2/CO_2 is reported to represent the major metabolic pathway under thermophilic and extreme thermophilic conditions, despite the unfavourable thermodynamically situation, at least under standard conditions ($\Delta G'_{55\,°C}$ 90 kJ/reaction), [148, 151, 152]. Possibly, as in high temperature habitats the aceticlastic methanogenesis might become less significant, the electron flow goes from acetate through the C_1 pool and hydrogen towards methane [102].

The first isolated syntrophic organism was the thermophilic (58 °C) strain AOR [65]. An interesting feature of this strain is that it both can produce and degrade acetic acid with using probably the same biochemical reaction apparatus, just depending on the prevailing concentration of substrates and products [102]. Recently, two new thermophilic homoacetogenic bacteria able to convert acetate syntrophically were isolated. *Thermoacetogenium phaeum* gen. nov. sp. nov. was isolated from a thermophilic (55 °C) anaerobic methanogenic reactor treating kraft-pulp mill wastewater and was capable to convert methanol (among others) into acetate, and acetate into methane, when living in coculture with hydrogenotrophic methanogens [45]. *Thermotoga lettingae* sp. nov., was isolated from a thermophilic sulphate-reducing bioreactor operated at 65 °C with methanol as sole substrate and was able to degrade methanol to CO_2 and H_2 in syntrophy with *Methanothermobacter thermoautotrophicus* ΔH or *Thermodesulfovibrio yellowstonii*. Growth on acetate in coculture with *Methanothermobacter thermoautotrophicus* ΔH was also observed, though it proceeds slowly [6].

Competition for H_2

The importance of hydrogen increases with temperature. The hydrogen partial pressure according to Zinder [148] would be 5 to 10 times higher under thermophilic than mesophilic conditions. In anaerobic environments, H_2 is consumed by methanogenic or also by sulphate-reducing and homoacetogenic bacteria [38]. H_2 concentrations are usually extremely low in such environments [114] and microorganisms using hydrogen are outcompeted by others that more effectively utilise traces of hydrogen. Sulphate reducers are capable to outcompete the hydrogenotrophic methanogens for hydrogen in the presence of sulphate, because of their higher affinity and higher growth yield [1, 140]. When sulphate is not present, methanogens likely predominate over homoacetogens due to their lower threshold value [66] and substrate affinity [101]. Under standard conditions the energy yield from the methanogenic hydrogen oxidation exceeds that of the homoacetogenic hydrogen oxidation, which might mean that homoacetogens have little chance to compete successfully against methanogens for hydrogen

at limiting concentrations [102]. However, at low temperatures, homoacetogens producing acetic acid from H_2/CO_2 seem to play a major role. At temperatures below 20 °C, the known species of hydrogen oxidising methanogens are not significantly active [147], and homoacetogens appear to be less restricted in this respect, improving the chance of homoacetogens to compete successfully against methanogens for hydrogen. Under such temperatures, homoacetogens appear to take over significant parts of hydrogen oxidation in paddy soil and lake sediments [19, 21].

Environmental conditions

Besides thermodynamics and growth rate, environmental conditions have to be taken into consideration with respect to the competition between the various bacterial species in a mixed culture, since they play a very important role in the competition for substrate among the microorganisms. Under mesophilic conditions, the factors found to be important for the anaerobic conversion of methanol are, the presence of cobalt in the media, the methanol concentration in the reactor, the pH inside the reactor, the level of bicarbonate and concentration of undissociated VFA [28]. Under thermophilic conditions, hydrogen is believed to play a major role in the pathway concerning the conversion of methanol. Complex metabolic interactions then may prevail between the microorganisms in mixed cultures. Small changes in hydrogen partial pressure may alter the spectrum of products provided that the microorganisms have alternative pathways [16, 63, 83]. Moreover, according to Kleerebezem and Stams [60] in anaerobic fermentations where 2 or 3 hydrogen molecules have to be released per mole of substrate, small changes in the hydrogen partial pressure, may have a significant impact on substrate conversion rates. Changes in environmental conditions may cause shift in the microbial composition of mixed cultures and may wipe completely out microorganisms if the abnormal condition persists. The main impacting environmental factors are briefly discussed below.

pH and inorganic carbon species ($\Sigma([HCO_3^-] + [CO_2])$)

Sodium bicarbonate is commonly supplied in order to increase the bicarbonate alkalinity. It is a compound safe to handle, it dissolves easily in water and dosage errors (especially in excess) do not affect digester operation [69]. The pH and bicarbonate concentrations are considered as key system variables affecting the production of hydrogen and formate during shock loads [133]. The use of sodium bicarbonate is applied, for instance, for promoting solid-state refuse fermentation. By supplying 2.5% $NaHCO_3$ (w/w) a balanced acetogenesis and methanogenesis could be achieved, particularly because methanogenesis then was accelerated by a factor of 6 [54].

Bicarbonate plays an important role on the anaerobic conversion of methanol, not only as a pH buffer, but also as source for CO_2, which is required as co-substrate in the

(homo)acetogenesis of methanol [30, 72]. Carbon dioxide is produced when methylotrophic methanogenesis of methanol occurs, and as a result (homo)acetogenesis can proceed, although just to a limited extent for the amount of CO_2 produced does not support high acetogenesis. In case the buffer capacity of the system is insufficient, the production of acetic acid obviously will cause sharp drop in the pH. The concentration of bicarbonate present in the system has been shown to influence the fate of methanol under mesophilic conditions [30]. The pH is probably the main operational control parameter in the anaerobic conversion of methanol. Under mesophilic conditions using a one-stage UASB reactor, the elementary pathways of the conversion of methanol into methane were shown to be governed by the pH. At neutral pH-values *Methanosarcina* species predominates and the accumulation of VFA is therefore, insignificant. However, when the pH is maintained between 5.0 - 6.0, accumulation of acetate takes place and then *Methanothermobacter/ methanobrevibacter* genera predominate [12]. In the mesophilic range a successful methanogenesis was achieved even in an UASB reactor operated at pH 4.2 [32]. When treating a methanolic wastewater in an UASB-reactor at 40 °C, Bhatti et al. [14] found that during the first 40 days of operation the pH could be maintained around 6.0 – 6.3 without any addition of external buffer, but thereafter the pH dropped to 5.5 within three days. They found that the pH could be restored by the addition of 2.52 $g.L^{-1}$ $NaHCO_3$ without build up of VFA in the effluent.

Trace elements

Trace elements are required as micronutrients in the anaerobic digestion processes as they are essential constituents of cell components. All methanogens tested so far were found to require iron, nickel and cobalt for growth [47, 95, 99, 108, 119, 120]. Moreover, iron was reported to enhance sludge granulation [89, 107]. Bacteria compete for trace elements when these are limiting, and it may be expected that species with a low (or not) requirement or a high affinity for limiting trace elements will eventually dominate [134]. The effect of cobalt on the anaerobic mesophilic degradation of methanol has been studied in detail by Florencio et al. [29, 31]. Methylotrophic methanogens predominated in their consortium, and they found that cobalt greatly enhanced both methanogenesis and acetogenesis from methanol. The optimal concentration of cobalt for growth and activity of the methanol utilising methanogens and acetogens was 0.85 μM. From literature reports, it is known that 98% of the total cobalt content in the cells is present in corrinoids [115]. *Methanosarcina barkeri*, a methylotrophic organism, is the methanogen which contains the highest corrinoid concentration, and it is even higher when cells are grown on methanol [62]. This explains the high requirement of cobalt for the systems governed by a methylotrophic-methanogenic pathway. A maximum methanol removal of 51% was reached over a 100 days experimental period, when the feed of a mesophilic UASB reactor was cobalt deprived [31]. For the growth of *Methanobacterium thermoautotrophicum* on H_2/CO_2 as sole carbon source Schönheit et al. [105], found that

nickel requirement exceeds that of cobalt. However, growth in the medium was observed without supplying any cobalt but the addition of 0.01 μM of cobalt distinctly enhanced growth.

Acetate

The accumulation of acetic acid (consequently of acetate) in bicarbonate buffered medium may occur when methanol is converted by (homo)acetogens. It is well known from literature that the free acetic acid (the undissociated fraction) is quite inhibitory for methanogenesis of VFA-substrates [36, 37, 127]. The reported values of the toxic concentration of free acetic acid, which is a function of both the total acetate concentration and the pH, varies greatly for pure cultures and sludges, as well as for the different temperature ranges. Moreover, the extent of inhibition is also specie specific. It depends - as far as different types of sludge are concerned, on the dominant population present in the sludge and on the actual pH [66]. It therefore comprises a quite complex matter, and the figures mentioned below are quite case specific. Van Lier et al [126] in an experiment with a thermophilic sludge, reported that already about 1 mM of free acetic acid inhibited the methane formation by 50%. Yamaguchi et al [144] reported for thermophilic methylotrophic methanogens a complete inhibition at 8.9 mM undissociated acetic acid whereas 4 mM caused partial inhibition. Under mesophilic conditions, a distinct inhibition of methylotrophic methanogens at free acetic acid concentrations of 5.4 mM at a pH around 5 was found [30].

The State of the art concerning the anaerobic treatment of methanol-containing wastewaters

The first comprehensive investigations dealing with the feasibility of anaerobic treatment of methanol containing wastewater using the UASB reactor technology, were conducted by Lettinga et al. [68, 70] and dealt with mesophilic temperature range. Attention was focused on the effect of environmental factors like pH, bicarbonate alkalinity and the presence of one or more trace elements. These factors were found to be of crucial importance in the anaerobic fermentation of methanol. Years later, a detailed research focused on these factors was conducted by Florencio [28]. Cobalt was found to be the only trace element which greatly enhanced methanogenesis from methanol [29, 31]. The investigations led to the conclusion that all three factors mentioned above determine the fate of methanol in the anaerobic conversion occurring in UASB reactors. They can be used as a tool to steer the system to the final product desired. Methanogens generally will win the competition over acetogens if either the reactor methanol concentration, the inorganic carbon content and/or the cobalt concentration are maintained low. A significant acetogenesis only will predominate under conditions of high methanol concentration in the reactor, when inorganic carbon in the form

of CO_2 is available, e.g. supplied, cobalt is available and when methanogens are inhibited [30].

Table 2 shows some of the relevant results reported in literature. In most of these investigations a high methanol removal efficiency was achieved and in most cases it was mainly converted into methane. From the dominant microorganisms found in the cultures, it appears that the main pathway of methanol conversion under mesophilic conditions is performed by the methylotrophic methanogens. This was supported by studies performed by Florencio et al. [29] and Gonzalez-Gil et al. [40] who assessed the metabolic route of methanol degradation under mesophilic conditions by using specific inhibitors.

To date, no studies using high rate technology under thermophilic conditions with merely methanol as substrate were conducted, but a few investigations have been conducted (Table 2) for kraft evaporator condensate under thermophilic conditions (53 °C). Methanol comprises the main organic pollutant in this type of wastewater. A fairly satisfactory COD removal was achieved when using a fixed-film bed packed with pumice stone. However, these investigations did not provide a good insight in the conversion of methanol itself, due the complexity of the kraft evaporator condensate.

Although a high conversion to methane is often obtained in properly designed and well operated reactors [12, 30, 35, 68, 77], the accumulation of VFA was found to represent a problem of concern [14, 29, 70, 142-144]. This was the case for both mesophilic and thermophilic conditions. For mesophilic conditions, the available information in literature suffices to enable the implementation of a stable methanogenic process using the anaerobic high-rate reactor technology.

Reasons why accumulation of VFA takes place are discussed in literature. One possibility is that, methanol-grown mesophilic organisms are not able to metabolise acetate in the presence of methanol [109]. Under thermophilic conditions, Yamagushi et al. [144] observed that, when methanol grown cultures incubated for more than a week after exhaustion of methanol, were inoculated to fresh methanol or acetate medium, the methanogen was able to grow with methanol but not with acetate. When methanogens, which had lost their acetate utilising ability, were inoculated to methanol-containing medium, the acetate-utilising ability was recovered.

The production of acetate by methanogenic bacteria was also taken into consideration by Yamagushi and Minami [143] and by Westermann et al. [137]. Enzyme activity was proposed to monitor and control the stability in the thermophilic anaerobic digestion of methanol-containing wastewater [142].

A well known important feature of sludge bed reactors, concerns their ability to form granular sludge, consisting of very well settleable, stable granules composed of well balanced

micro-ecosystems [48]. Due to the high temperatures, the process stability of thermophilic processes seems to be poorer than that of mesophilic, while the immobilisation of bacteria also seems to be more difficult under thermophilic conditions [123]. The formation of a dispersed type of sludge might partly be attributed to the higher degree of sludge mineralisation under thermophilic conditions which results in the formation of a lower amount of extracellular polymers [104] that are believed to play an important role in bacterial adhesion. Under mesophilic conditions, sludge granulation can be easily accomplished when methanol is a major pollutant [11, 35, 70], and it seems to be independent on the type of seed sludge, or the dominant micro-organism present in the sludge. It also has been reported that the addition of methanol in the start-up of a lab-scale UASB treating dairy waste, aids rapid biomass granulation [17].

Table 2 Anaerobic wastewater treatment of methanol-containing wastewaters in high-rate reactors.

Reactor type	Carrier material	T [a] (°C)	Substrate	OLR gCOD.L^{-1}.d^{-1}	Substrate removal (% COD)	Dominant organism	Reference
UASB[b]		30	methanol	2-24	30-93	nr[c]	[70]
UASB		37	methanol, acetate (7.5:1.0)[d]	127	91	*Methanosarcina* –like and *Methanosaeta*	[87]
UASB		37	methanol	21.4	80	*Methanosarcina*	[12]
UASB		37	propionate, methanol (6.7:1)	12.7	Propionate – 93 methanol - 99	*Methanosarcina*	[35]
UASB		37	propionate, methanol (1:2.6)	39.5	prop.- 25 methanol- 85	*Methanosarcina*	
UASB		40	methanol	2-52	50-79	*Methanobacterium* or *Methanobrevibacter*	[14]
UASB		30	methanol[e]	24	54	*Methanosarcina*-like	[32]
UASB		30	methanol	8-9	87	*Methanosarcina*-like	[31]
Fixed bed	Pumice stone	53	kraft evaporator condensate[f]	13 - 38	87	nr	[78]
Fixed bed	Pumice stone	53	methanol, sulphate	9 - 12	78 - 100	nr	[79]
IC[g]		35	kraft evaporator condensate	10-18	80 - 85	nr	[25]

[a]T, Temperature; [b]UASB, upflow anaerobic sludge blanket reactor; [c]nr, not reported; [d]COD-substrate proportion; [e]experiment conducted at pH 4.2; [f]60 – 90% of the COD-load of kraft evaporator condensate consists of methanol [136]; [g]IC, internal recirculation reactor, full scale.

Scope and outline of this thesis

The main objective of this thesis is to assess the feasibility of treating methanol-containing wastewater under thermophilic conditions in a single-step UASB-reactor. The studies are based on the information available in literature, although this mainly concerns anaerobic treatment under mesophilic conditions. We attempted to take into consideration all the drawbacks related to anaerobic conversion of methanol in general and thermophilic anaerobic treatment particularly. We also focused the investigations on relevant microbiological and biotechnological aspects.

Chapters 2 and 3 deal with the feasibility of thermophilic anaerobic treatment of methanol-containing wastewater at a temperature of 55 °C, focusing the research on start up, process stability, maximal permissible load(s), biomass washout and resistance to environmental shocks. The quality of the cultivated sludge was assessed by a physical-chemical characterisation and assessment of the specific methanogenic activity on methanol, acetate and H_2/CO_2.

The investigations presented in **Chapter 4** are directed on the thermophilic anaerobic conversion of methanol under acidic conditions as well as the effects of the supply/deprivation of bicarbonate on the performance of the system, i.e. the stability and the pathway of the conversion of methanol.

In **Chapter 5** we propose a strategy to recover reactor performance after serious upset or total failure based on the findings of Chapter 4. The strategy was tested with both acid and alkaline stressed sludges.

Chapter 6 deals with a detailed study to elucidate the pathway of the degradation of methanol by our mixed cultivated consortium. Nuclear magnetic resonance (NMR) spectroscopy technique was used to analyse the incorporation routes of methanol into acetate. These results contribute to a better understanding of the degradation routes of methanol and they serve for optimising the (thermophilic) treatment process of methanolic containing wastewater, as they also provide the possibility to predict the effect of various environmental conditions to which the system can be exposed.

Chapter 7 describes the effect of cobalt and trace metals deprivation in continuous experiments and particularly on the specific methanogenic activity of the cultivated sludge. By using a cobalt-deprived enrichment, we assessed the influence of cobalt deprivation and its reintroduction to the medium on the competition of (homo)acetogens and methanogens on methanol degrading thermophilic consortia.

2

Thermophilic anaerobic digestion of methanol in a UASB reactor

Abstract

A 5.1 L laboratory scale upflow anaerobic sludge blanket (UASB) reactor, was operated at 55 °C over 130 days in order to investigate the feasibility of treating methanol-containing wastewater under thermophilic conditions, focussing on start-up and process stability. Batch assays were conducted to elucidate the most probable pathway for methanol conversion. The results demonstrated a good performance, with a chemical oxygen demand (COD) removal averaging 82% throughout the experiment. No significant volatile fatty acids (VFA) accumulation was detected in the effluent, even with bicarbonate concentration exceeding 20 mM. Acetate was the main component of the VFA at relatively low organic loading rates (OLR). At high OLR, the main components were propionate and butyrate. Reactor performance was hardly affected when the system was exposed to non-optimal conditions, i.e., temperature drop, overloading and no feeding. Good thermophilic granular sludge was retained in the reactor. Washout of biomass was not severe during the experiment. From the pathway analysis it could be concluded that indirect pathway plays an important role in the methanol degradation by the cultivated consortia.

A modified version of this chapter has been published as: **Paulo, P.L., B. Jiang, S. Rebac, L. Hulshoff-Pol and Lettinga, G.** (2001) Thermophilic anaerobic digestion of methanol in UASB reactor, Water Sci. Technol., **44**(4), 129-136.

Introduction

Methanol can be the main pollutant in some specific wastewaters and may be formed under natural conditions as intermediate in the decomposition of organic matter. Coal-gasification plants, evaporator condensate of pulp and paper industries, potato-starch producing factories and landfill leachates are examples of wastewater where methanol can be present. Moreover, it represents a cheap, easy to handle electron donor for biological processes, such as sulphate reduction [136]. High-rate anaerobic digestion of evaporate condensate with methanol concentrations ranging from 1.5 to 24.5 $g.L^{-1}$ had been studied [77-79, 142]. This evaporate is discharged at high temperatures. In such case thermophilic anaerobic treatment would be an attractive option, avoiding the required pre-cooling for mesophilic treatment. Thermophilic treatment is also an alternative for mesophilic digestion due to the higher metabolic rates of the bacteria involved and, consequently, the theoretical higher maximum specific methanogenic activities [124]. The results obtained with thermophilic treatment of various types of wastewaters are very promising [124]. Nevertheless, many authors have reported several drawbacks of thermophilic reactors, such as: high susceptibility to temperature increases, feed interruptions and shock loads.

The mesophilic anaerobic treatment of methanolic wastewaters has been investigated by many researchers [12, 29, 35, 70, 78, 87] but so far, very little is known about the thermophilic methanol conversion.

The feasibility of the anaerobic treatment of methanolic wastewaters seems to remain questionable and doubts about the operation of a stable treatment process still persists, due to unpredictable accumulation of VFA in the effluent. Such accumulation may cause failure of the treatment process due to inhibition of the methanogens, especially at low pH-values. Technically, methylotrophic methanogens are not inhibited at low pH. The degree of inhibition is strongly dependent on the concentration of the undissociated form of the fatty acids.

The main objective of the research described in this Chapter was to investigate the feasibility of treating methanol-containing wastewater under thermophilic condition (55 °C), focusing on start up, process stability, and the assessment of the probable pathway of methanol conversion to methane.

Material and methods

Continuous experiment

The thermophilic anaerobic degradation of methanol was studied in continuous and batch reactor systems. A Glass UASB reactor equipped with a water jacket, with a working volume of 5.1 L was used (Figure 1). The reactor was equipped with a double wall connected to a 55

°C waterbath recirculator (Julabo, MB-Basis, Germany). Biogas was collected in a gas-solid-liquid separator and led through a waterlock filled with a 16% NaOH solution to remove CO_2 from the gas. Thereafter the gas passed through a column filled with soda lime pellets with indicator.

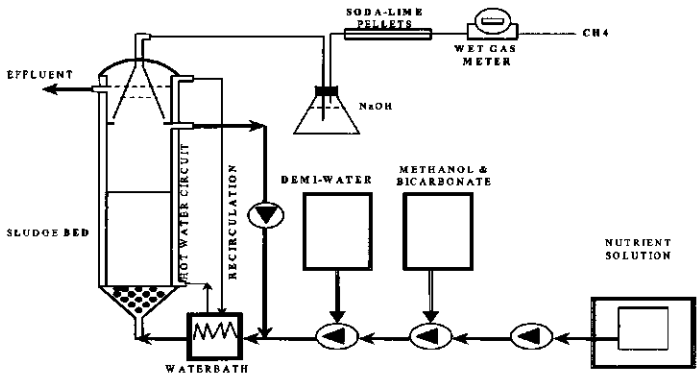


Figure 1 Schematic view of the experimental set-up.

Subsequently, the gas flow was measured with a wet-type precision gas meter (Meterfabriek Dordrecht, The Netherlands). Methane (CH_4) production was continuously measured. The hydraulic retention time (HRT) was calculated based on the flow rate of effluent. Influent and effluent samples were taken twice per week to analyse the methanol and VFA concentration. The sludge bed height was measured every day. The biogas composition and suspended solid COD were measured when the OLR was increased. The reactor was inoculated with 1170 g granular wet sludge from a pilot plant UASB reactor treating paper mill wastewater at 55 °C (Paques Biosystems BV, Balk, The Netherlands). Table 1 presents the operating conditions of the UASB-reactor. The OLR was always increased when about 90% of methanol conversion was achieved. Effluent recirculation was imposed to the system. Methanol was used as sole organic carbon source. The concentration in the stock solution was 2.7 $gCOD.L^{-1}$ (day 0-60) and 5.4 $gCOD.L^{-1}$ (day 61-130). 0.33 g bicarbonate was added per 1g $methanol.L^{-1}$, to ensure pH stability.

The reactor was supplemented with macro and micro-nutrients. 2.22 ml of a nutrient stock solution was supplied for each gram influent $COD.L^{-1}$, stock solution contained ($g.L^{-1}$): From day 0-27: NH_4Cl (0.28), $K_2HPO_4.3H_2O$ (0.27), Na_2S (0.032), $CaCl_2.2H_2O$ (0.01), yeast (0.1) and 1 millilitre of trace elements solution. From day 28-130: NH_4Cl (7.5), $K_2HPO_4.3H_2O$ (2.12), $MgSO_4.H_2O$ (1.5), $CaCl_2.2H_2O$ (0.3), yeast (0.5) and 4.5 millilitre of trace elements solution.

The trace elements solution contained ($mg.L^{-1}$): $FeCl_2.4H_2O$ (2000), H_3BO_3 (50), $ZnCl_2$ (50), $CuCl_2.2H_2O$ (38), $MnCl_2.4H_2O$ (500), $(NH_4)6MoO_{24}.4H_2O$ (50), $AlCl_3.6\ H_2O$ (90),

$CoCl_2.6H_2O$ (2000), $NiCl_2.6H_2O$ (92), $Na_2SeO_3.5H_2O$ (194), EDTA (1000), Resazurine (200), HCl 36% (1%). All chemicals were of analytical grade.

Table 1 Operating conditions and performance data of the 5.1 L UASB reactor treating methanol at 55 °C (values given as average of each phase).

		PHASE I	PHASE II	PHASE III
Period (days)		0 - 41	42 - 60	61 – 130
Methanol concentration	($gCOD.L^{-1}$)	2.7	2.7	6
HRT	(h)	33	10	3
OLR	($gCOD.L^{-1}.d^{-1}$)	2	8.1	28.4
Sodium Bicarb. (influent)	($mEq.L^{-1}$)	11	10.7	17.4
pH (effluent)		6.49	6.60	6.69
CH_4 production rate	($gCOD.L^{-1}.d^{-1}$)	1.1	7.4	24.6
VFA (effluent)	($gCOD.L^{-1}$)	0.030	0.026	0.040
Methanol Removal	(% Inf.)	76	87	83

Batch experiments

Activity Assays

120-ml glass serum vials were filled with 50 ml basal medium containing ($g.L^{-1}$): NH_4Cl (0.28), $K_2HPO_4.3H_2O$ (0.33), $MgSO_4.7H_2O$ (0.1), $CaCl_2.2H_2O$ (0.01), yeast (0.1) and one millilitre of trace elements solution. Before adding the sludge and substrate, all bottles containing basal medium were incubated in a waterbath with shaker (TUV, GLF 1083, Germany) at 55 °C and 50 rpm. Sludge was added to the vials at a volatile suspended solids (VSS) concentration of about 1 $g.L^{-1}$. Methanol or acetate was added as the substrate at a concentration of 2 $gCOD.L^{-1}$. When methanol was used as a substrate, 2.52 g $NaHCO_3$ per litre of basal medium was added, to ensure pH stability. The vials were sealed with butyl rubber stoppers and the gas headspace was flushed for 5 minutes with N_2/CO_2 (70:30). After various periods of time, gas samples were taken and analysed for CH_4. The pH, as well as the amount of VSS in each bottle was measured after the test was completed. The specific methanogenic activity (SMA) was calculated from the linear increase of the CH_4 concentration in the beginning of the experiment, when no lag phase was observed, divided by the amount of VSS. The assay was performed in triplicate, using the bottles without substrate as blank.

Pathway analysis

The presence of specific bacterial subpopulations in the sludge was studied by using batch activity test to which specific inhibitors, 30 mM bromoethane sulphonic acid (BESA, Sigma, USA) and 0.25 $g.L^{-1}$ Vancomycin (Acros, Belgium) were added for blocking a metabolic

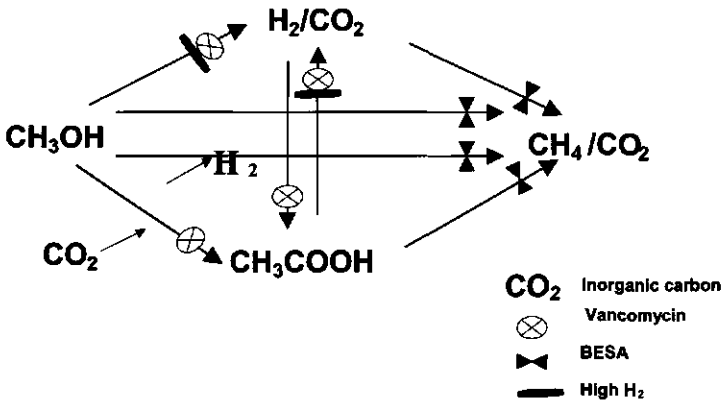


Figure 2 Diagram of blockage of potential individual pathways of methanogenic metabolism of methanol by inhibitors (After Florencio [29]).

pathway. Figure 2 represents the general strategy used for blocking the competitive reactions.

The 120-ml serum vials were filled with 25 ml basal medium when H_2/CO_2 was the substrate. Pure H_2 was injected to give a pressure of 1.74 atm, equivalent to 2.0 g $COD.L^{-1}$. When methanol and acetate were used as the substrate, the experimental set-up and conditions of the assays were the same of the activity test, except the shaking speed (100 rpm).

The bottles were placed horizontally in a water-bath to optimise mass transfer of hydrogen from gas to liquid phase. Liquid and gas samples were taken periodically to analyse substrate consumption and product formation. The apparent substrate affinity K_m and maximum substrate degradation activity V_{max} on methanol and acetate were estimated using the same conditions of the specific methanogenic activity assay. Apparent K_m and V_{max} were estimated from the substrate depletion curve, by using a Michaelis-Menten derived equation and a non-linear regression routine for parameter estimation.

Analyses

Liquid samples for methanol and VFA analysis were centrifuged at 17,000 x *g* for 5 minutes, diluted with a 3% formic acid solution, and stored at 4 °C. VFA was determined by chromatography. The GC (HP 5890A, Palo Alto, USA) was equipped with 2 m × 4 mm glass column, packed with Supelcoport (100-200mesh) coated with 10% Fluorad FC 431.

Operating temperatures were: column, 130 °C; injection port, 200 °C; flame ionisation detector, 280 °C. N_2 saturated with formic acid at 20 °C is used as carrier gas (30 ml). Methanol was analysed in the same way as VFA except for the oven temperature, which was 70 °C.

Biogas composition (CH_4, CO_2, N_2) was determined with a Packard Becker GC model 433 (Delft, The Netherlands) equipped with two columns connected in parallel (split 1:1): 1.5m × 1/8" Teflon packed with Chromosorb 108 (60-80mesh) and 1.2 m × 1.8" stainless steel packed with molecular sieve 5A (60-80 mesh). Helium was used as a carrier gases (45 $ml.min^{-1}$). Temperatures were column, 40 °C; injection port, 100 °C; and hot wire detector, 100 °C. Injection volume was 100 µL.

Methane was determined in a Packard-Becker 438/S gas chromatograph (Delft, The Netherlands). Injection volume was 100 µL. A 2 m × 2 mm stainless steel column was used packed with Poropak Q (80-100 mesh) The temperatures of the column, injection port and flame ionisation detector were 60, 200 and 220 °C, respectively. N_2 was used as carrier gases (20 $ml.min^{-1}$).

Hydrogen was determined by GC with a Hewlett Packard 5890 gas chromatograph equipped with a thermal conductivity detector (TCD) and molecular sieve 25H (60-80 mesh). Column size: 1.5 m × 6.4 mm. Argon was used as carrier gas at a flow rate of 25 $ml.min^{-1}$. Temperature were: column, 40 °C; injection port 110 °C; and detector 125 °C. Injection volume was 100 µL or 1000 µL depending on the concentration.

The gas samples were taken by a pressure-lock syringe (Alltech, USA). The gas standards were incubated at 55 °C in order to prevent unexpected errors [58].

VSS, total suspended solids (TSS) and ash were analysed according to the Standard Methods [5].

COD was analysed using the micro-method as described by Jirka and Carter [52]. The soluble COD was measured by filtrated sample through 0.2 µm membrane filter (N16, D-37528, Dassel, Germany).

pH was determined daily with Knick type 510 pH-meter (van Oortmerssen, The Hague, The Netherlands).

Results and discussion

The performance of the reactor

Table 1 describes the operating conditions and performance data of the reactor. During the first 28 days of operation, the reactor presented a poor performance, with total VFA accumulation up to 563 $mgCOD.L^{-1}$. From day 28 onwards, the nutrient stock solution was

replaced and the performance of the reactor improved immediately. No significant accumulation of VFA was detected in the overall continuous experiment, even when relatively high OLRs were applied (Table 1, Figure 3). Less than 2% of the influent COD was detected as VFA after day 40, when total VFA concentration was kept below 100 $mgCOD.L^{-1}$.

In Phase II, the reactor accommodated immediately to the OLR increase. Decrease in the methane production rate was detected in some periods, however it could be attributed to the gas outlet clogging by floc biomass. The sludge bed increased constantly and gradually, even though floc and spongy biomass was washed out due to the high gas loading rates. Significant disintegration of granular sludge occurred at this phase. In phase III, the reactor took more time to recover from the increasing OLR due to some undesirable temperature and load shocks. The performance recovered however, without any retardation for the temperature shock (day 77, 35 °C, during about 17 h) and overloading shock (day 87, 67 $gCOD.L^{-1}.d^{-1}$, about 21 hours).

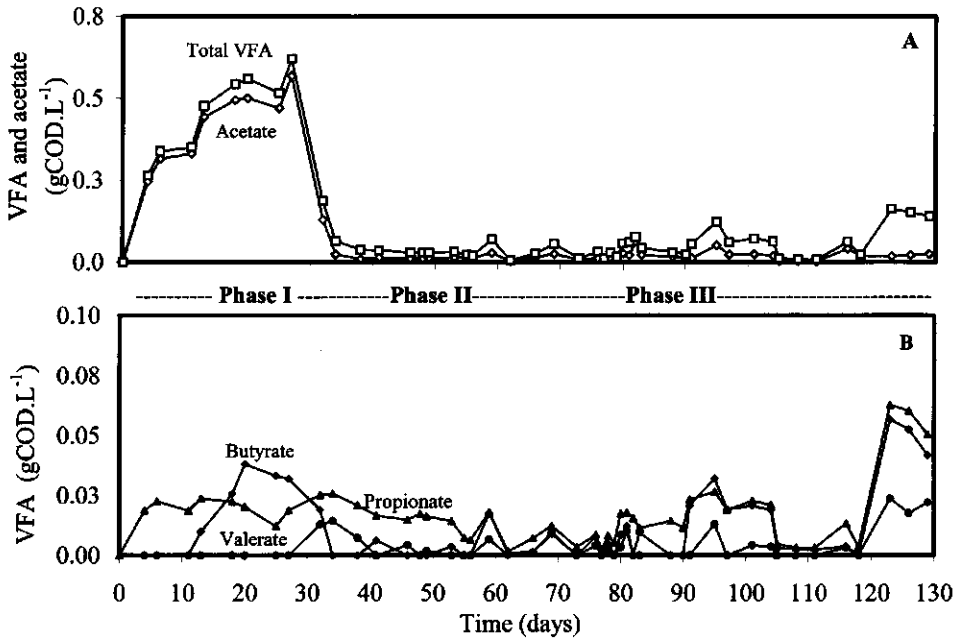


Figure 3 The concentration of total VFA and acetate (**A**), propionate, butyrate and valerate (**B**) in the effluent: Butyrate= $iC_4 + nC_4$, Valerate= $nC_5 + bC_5$, respectively.

The recovery from a period without feed supply (day 96, about 7 h) took 3 days, suggesting high maintenance energy requirement. These results show that, the reactor performance is quite stable when exposed to non-optimal conditions.

Theoretical calculation was applied to compare the biomass washout and bacterial growth, based on the biomass COD conversion factor of 1.45 and biomass yield of 0.05 gVSS. $gCOD^{-1}$ [91]. The bacterial growth was higher than the biomass washout (Figure 4), explaining the continuous increase in the sludge bed in Phases II and III (suspended solid COD was not measured during Phase I).

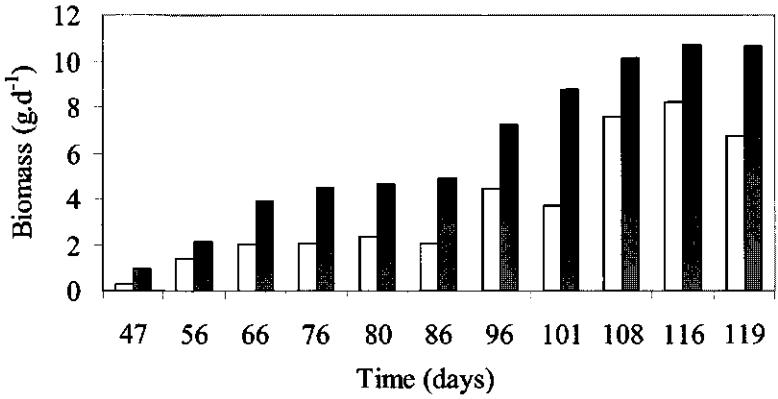


Figure 4 Comparison of measured biomass washout (left bar) and theoretical calculated bacterial growth (right bar).

Methanogenic activity

After adaptation on methanol, the SMA on this substrate increased by 167%; conversely, the SMA on acetate decreased by 28% (Table 2), indicating the growth of a new population. The apparent substrate affinity K_m and maximum substrate degradation rate $V_{max,}$ of the cultivated sludge were estimated (Table 3). The high affinity for acetate indicates that acetate could be converted rapidly, if produced from methanol. It also explains why acetate concentration was always low in the effluent. The non adapted population to methanol coupled with the lack of nutrients, might explain the poor performance of the reactor during the first 28 days of the experiment.

Table 2 The specific methanogenic activity for the different sludges.

Sludge type	pH	T (°C)	Substrate	SMA (g COD.gVSS^{-1}.d^{-1})
Seed sludge	7.0	55	Methanol	0.42 (0.01)
	7.0	55	Acetate	1.17 (0.05)
Cultivated sludge	7.0	55	Methanol	1.13 (0.05)
	7.0	55	Acetate	0.84 (0.03)

Standard deviation is given between brackets as mean value of triplicate culture.

Pathway analysis

Methanol was converted to methane within about one day when no inhibitor was supplied. Addition of vancomycin to the medium resulted in a decrease in the SMA by 55% (Table 4). By the addition of BESA to the medium, methanogenesis from methanol completely ceased. Notably, only after a lag phase of 2 days, methanol was stochiometrically converted to acetate. Nonetheless, a significant contribution of the methanol-acetate or methanol-H_2/CO_2 pathway in the methanol degradation yet cannot be excluded. Results show that the SMA on acetate was 1.4 times higher than the (homo)acetogenic activity, indicating that a methanol conversion via acetate might occur without the build up of acetate in the medium.

Table 3 The apparent substrate affinity K_m and maximal substrate degradation rate V_{max} of cultivated sludge.

Substrate	K_m (g COD.L^{-1})	V_{max} (g COD.g VSS^{-1}.d^{-1})
Methanol	0.043 (0.01)	2.28 (0.15)
Acetate	0.123 (0.02)	1.41 (0.03)

Standard deviation is given between brackets as mean value of duplicate culture.

The obtained results give a general indication about the methanol conversion via the different pathways. Syntrophic conversion seems to play an important role in the methanol degradation by the cultivated consortia.

Table 4 The effects of specific inhibitors on methanogenic or acetogenic activity of the cultivated sludge.

Substrate	Methanol			Acetate		H_2/CO_2		
Inhibitor	-	BESA	Vancomycin	-	BESA	-	BESA	Vancomycin
Methanogenesis	1.13	0.0	0.51	0.84	0.0	2.20	0.0	2.25
($gCOD.gVSS^{-1}.d^{-1}$)	(0.01)		(0.01)	(0.05)		(0.2)		(0.01)
Acetogenesis		0.60						
($gCOD.gVSS^{-1}.d^{-1}$)		(0.04)						

Standard deviation is given between brackets as mean value of triplicate culture.

The hydrogenotrophic methanogenic activity found in our cultivated sludge, was relatively high. Hydrogenotrophic methanogens, play a key role in the overall process by maintaining the very low partial pressure of H_2 (< 10 Pa), necessary for the metabolism of the syntrophic bacteria. This high activity coupled to the high acetotrophic activity, and high apparent affinity for acetate is essential to keep the concentrations of acetate and higher VFA in the effluent low, when methanol is converted by non-methylotrophic bacteria.

Conclusions

- The satisfactory reactor performance at an OLR up to 47.3 $gCOD.L^{-1}.d^{-1}$ and a 3.2 h HRT demonstrates the feasibility of the thermophilic treatment of methanol-containing wastewater by using a one stage UASB reactor.
- No significant VFA accumulation was detected in the effluent, even with bicarbonate concentration exceeding 20 mM. Acetate was the main component of the VFA at relatively low OLR (below 20 $gCOD.L^{-1}.d^{-1}$), and at high OLR (above 30 $gCOD.L^{-1}.d^{-1}$), propionate and butyrate were the main VFAs accumulating.
- The reactor was characterised by a stable performance even when exposed to non-optimal conditions, such as, a temperature drop (to 35 °C), overloading (67 $gCOD.L^{-1}.d^{-1}$) and no feeding (during 7 hours). The recovery from interruption in feed supply required more time than from the other two shocks.
- The thermophilic granular sludge was appropriately retained in the reactor. Biomass washout was low throughout the experimental period.

Acknowledgements

We would like to thank Jim Field and Jules van Lier for the valuable comments and critical remarks in the manuscript. This work was supported by "Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq" (Project n° 201055/97-0), an entity from the Brazilian Government for the development of Science and Technology.

3

Start-up of a thermophilic methanol-fed UASB reactor: change in sludge characteristics

Abstract

Experiments were performed to study the change in sludge characteristics and sludge granulation during the start-up of a thermophilic methanol-fed upflow anaerobic sludge blanket (UASB) reactor. The laboratory scale reactor, was inoculated with thermophilic granular sludge and operated at 55 °C over 130 days, at organic loading rates (OLR) varying from 2.7 to 47 $gCOD.L^{-1}.d^{-1}$. Physical characterisation was performed for both the seed and the cultivated sludge. Results demonstrated that a good quality, well settleable granular sludge was cultivated and retained in the reactor, allowing an OLR of 47 $gCOD.\ L^{-1}.d^{-1}$ with 93% of methanol removal, where 79% was converted into methane (CH_4). By a community analysis of the cultivated consortium, high numbers of rod-shaped hydrogenotrophic methanogens were enumerated. Biomass washout coincided with a high specific gas load, but was not detrimental to the system in the conditions tested.

A modified version of this chapter has been published as: **Paulo, P.L., B. Jiang, K. Roest, , J.B. van Lier and G. Lettinga** (2002) Start-up of a thermophilic methanol-fed UASB reactor: change in sludge characteristics, Water Sci. Technol., **45**(10) 145-150.

Introduction

Immobilisation of methanogenic consortium is of crucial importance in the so-called anaerobic 'high-rate' reactors, where the solids retention time (SRT) is uncoupled from the hydraulic retention time (HRT). An important feature of the generally applied sludge bed reactors, is the formation of granular sludge that culminates in the form of settleable, stable and balanced bio-granules [48]. Regarding high temperature conditions, it is often reported that process stability and immobilisation of bacteria are more difficult to achieve under thermophilic conditions than under mesophilic conditions, as briefly reviewed by van Lier [123]. The formation of dispersed sludge might partly be attributed to the higher degree of sludge mineralisation under thermophilic conditions which, consequently, results in a lower amount of extracellular polymers [104] that are believed to play an important role in bacterial adhesion.

There has been a limited number of studies conducted on the thermophilic granulation mechanism, compared with the extensive quantity of studies on mesophilic granulation [116, 138]. Successful sludge granulation on methanol-fed UASB reactors operating under mesophilic conditions has been reported by several researchers [13, 35, 70]. However, to our knowledge, any reference with respect to granulation in thermophilic reactors when using methanol as substrate, could not be found in the literature.

The objectives of this study were to investigate the change in sludge characteristics and the quality of the sludge formed in a UASB- reactor treating methanol-containing wastewater under thermophilic conditions (55 °C), starting with thermophilic granular sludge.

Material and methods

Continuous experiments

Table 1 Operating conditions of the 5.1 L UASB reactor treating methanol at 55 °C (values given as average of each phase).

		Phase I	**Phase II**	**Phase III**
Period (days)		0 - 41	42 - 60	61 – 130
Methanol concentration	($gCOD.L^{-1}$)	2.7	2.7	6
HRT	(h)	33	10	3
OLR	($gCOD.L^{-1}.d^{-1}$)	2	8.1	28.4
$NaHCO_3$ (influent)	($mEq.L^{-1}$)	11	10.7	17.4
pH (effluent)		6.5	6.6	6.7

The sludge was cultivated in a 5.1 L glass UASB reactor. The reactor was inoculated with 1170 g granular wet sludge from a pilot plant UASB reactor treating paper mill wastewater at

55 °C (Paques Biosystems BV, Balk, The Netherlands) that was originally inoculated with mesophilic granular sludge from a UASB reactor, treating paper mill wastewater at 40 °C. Effluent recirculation was imposed to the system. Table 1 presents the operating conditions of the UASB reactor. A complete description of the continuous experiment is presented in Chapter 2.

Physical characterisation

Physical characterisation was determined for both the seed and the cultivated sludge. For the cultivated sludge, samples were taken from the reactor at the end of the experiment (day 131), when the reactor was operated at an OLR of 47.3 gCOD. $L^{-1}.d^{-1}$ and a HRT of 3.2 h. Prior to analyse, seed sludge samples were stored at 4 °C. Size distribution, density and settling properties of the sludge were determined by using a modified sedimentation balance as described by Hulshoff Pol [48]. The granule strength was measured with a 'tension and compression' test apparatus (Overload Dynamics S900, Schiedam, The Netherlands).

Analyses

A detailed description of the analytical procedures has been presented in Chapter 2.

Co^{2+} and Ni^{2+} were determined by flame atomiser in an Atomic Absorption Spectrometer (Varian model SpectraA 300, Springvale, Australia). The burning gas for the flame was a mixture of air: acetylene (2:1). The extraction of trace elements from the sludge was done according to Lustenhouwer and Hin [73].

Most probable number (MPN) technique is described elsewhere [9]. MPN series were made in basal bicarbonate buffered medium according to Stams et al. [112].

Results and discussion

Sludge characteristics

Overall reactor performance and kinetic data on the acetate and methanol consuming methanogenic activity were shown in Chapter 2. Results showed a decrease in the acetate consuming methanogenic activity by 28%; while the specific methanol conversion rate increased by 167%. The sludge properties changed significantly during the 130 days of continuous reactor operation (Table 2), indicating that new biomass was retained in the reactor and attached to the granular inoculum. The colour of the sludge changed gradually during reactor operation from black to light brown-yellowish.

There was a considerable increase in the average size of the granules but a significant reduction in their density. This reduction was probably due to the fact that the seed sludge was obtained from a pilot plant treating paper mill wastewater with a water hardness of 700 mg $Ca.L^{-1}$. TSS and ash content of the seed sludge were also higher, i.e., 37.1% and 72.4%

for the seed sludge and 21.4% and 48.6% for the cultivated sludge, respectively. It should be noted that the seed sludge consisted of a large inorganic fraction, likely sand and/or $CaCO_3$ precipitates that accumulated in the anaerobic reactor. Growth of new biomass without a concomitant increase in the inorganic fraction may have resulted in a lower strength and settling velocity of the cultivated sludge compared to the seed sludge.

Table 2 Physical and chemical characteristics of the seed and cultivated sludge.

	Seed Sludge Day 0		**Cultivated Sludge** Day 131	
Average diameter (mm)	1.71	(0.05)	2.14	(0.06)
Density (kg/m^3)	1246.3	(11.7)	1088.6	(4.7)
Average settling velocity (m/min)	4.35	(0.14)	2.75	(0.11)
Strength (kN/m^2)	491.1	(5.1)	202.4	(6.4)
TSS (% of wet sludge)	37.1	(0.7)	21.4	(0.7)
VSS (% of TSS)	27.6	(0.4)	51.5	(0.3)
Ash (% of TSS)	72.3	(0.4)	48.6	(0.3)
Cobalt (μg/gTSS)	11.8	(0.1)	28.2	(0.2)
Nickel (μg/gTSS)	23.8	(0.7)	16.4	(0.4)

Standard deviation is given between brackets, as mean value of triplicate measurements.

The importance of calcium on methanogenic granules formation has been demonstrated in several studies [49, 76]. Other study [43], has shown that the removal of calcium from the granules, reduced their strength or caused complete disintegration.

The nickel and cobalt concentrations changed significantly. Nickel concentration decreased from 23.8 to 16.4 μg/gTSS, and cobalt increased from 11.9 to 28.2 μg/gTSS. It is remarkable that cobalt concentration was 22.4 times lower than the sludge treating methanol under mesophilic conditions, where methanol was directly converted to methane by methylotrophic methanogens [31]. Our previous study (Chapter 2) indicates that in the current cultivated consortium, the syntrophic conversion of methanol to methane via H_2/CO_2 very likely play a role. This is confirmed further by a community analysis of the sludge. Using the most probable number technique, high numbers (10^9 per ml sludge) of rod-shaped hydrogenotrophic methanogens were enumerated. With methanol, grows up to the 10^{-8} dilution of sarcina-shaped microorganisms was observed. Research is in progress to enrich and identify by molecular biological techniques the microorganisms, which degrade methanol in syntrophic association with methanogens. Interestingly, a lower requirement for cobalt has been reported for the thermophilic hydrogenotrophic methanogen *Methanothermobacter thermoautotrophicus*; for growth on H_2 and CO_2 as sole energy and carbon source, the requirement for nickel was found to be higher than for cobalt and molybdenum [105].

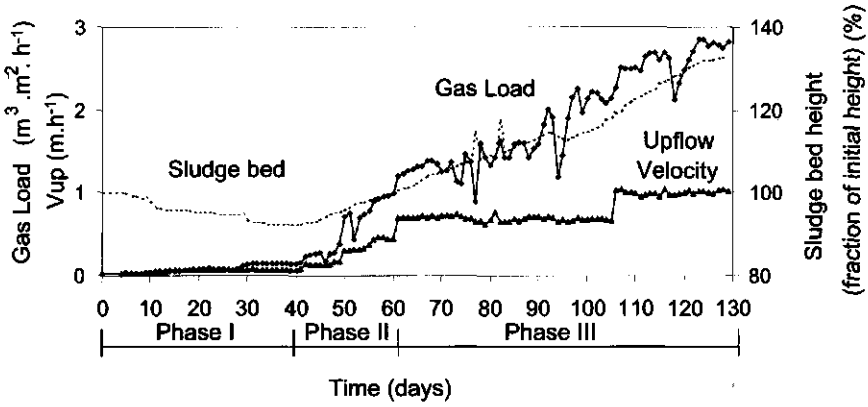


Figure 1 The profiles of upflow velocity, gas loading rate and sludge bed height during the experimental period.

The suspended solids COD concentration was analysed during phases II and III. No suspended solids were introduced into the reactor through the influent; thus the suspended

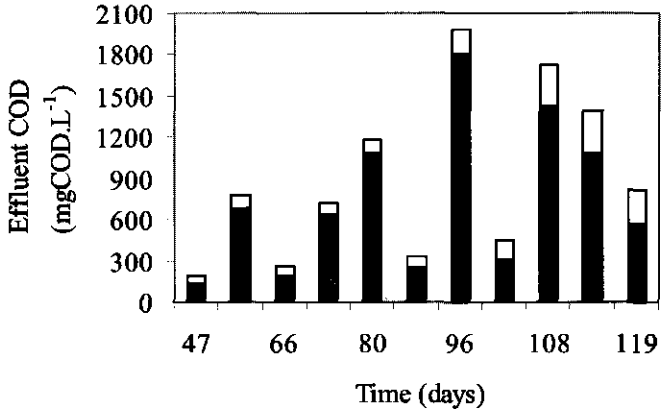


Figure 2 Effluent COD concentration. The total COD is expressed by the whole bar, in which the upper blank part is ssCOD and lower black part is soluble COD.

solids measured are merely washed-out biomass. However, it remains unclear whether the washed-out biomass comes from the original inoculum or from the newly grown biomass.

The washout of biomass increased after day 106, when the recirculation ratio and OLR were increased to 3.5 and 47.3 $gCOD.L^{-1}.d^{-1}$, respectively, resulting in a relative high upflow velocity and high gas load (Figure 1).

Average SS-COD concentration was 286.2 mg $COD.L^{-1}$ in this period (last twenty days of phase III), i.e., 3.7 times higher than during the other periods (Figure 2). The rinsed fraction could be characterised as fluffy and spongy biomass, while granular sludge was effectively retained by the system. The bacterial growth, determined by theoretical calculation (Chapter 2) was in average 30% higher than the biomass washout, explaining the continuous increase of the sludge bed (Figure 1). The washout of sludge, particularly in phase III, may be explained by the increased turbulence while it was practically absent in phase II as shown in Figure 1.

Formation of good quality granular sludge depends, amongst others, on the application of appropriate selection pressure for granular growth [48]. Quite contrasting results are found in literature concerning sludge granulation. Bhatti et al. [14], suggests that bacterial aggregation/biogranulation may vary considerably with the type of substrate, the metabolic pathway and the cultivation conditions. Grotenhuis [42] found that attached growth preferentially occurred in a mixed bacterium consortium of hydrogen producing bacteria and hydrogenotrophic methanogens under mesophilic conditions. The same was observed by Syutsubo et al. [117] in recent study, where the presence of a symbiotic microbial community between acetogens and hydrogen utilising methanogens showed to be of great importance for enhancement of thermophilic granulation.

From Table 3 it can be concluded that sludge granulation was successfully achieved under mesophilic conditions when methanol was present in the substrate composition, independently of the type of seed sludge or the dominant micro-organism. Sludge characteristics are comparable to those found in the present study at 55 °C, indicating that methanogenic-sludge granulation in thermophilic UASB reactors is very well possible with methanol as the sole substrate.

Table 3 Characteristics of granular sludge cultivated in UASB reactors under mesophilic conditions.

Seed sludge	Substrate	T (°C)	Mean diameter (mm)	Settling velocity ($cm.s^{-1}$)	Ash cont. (%)	Dominant organism	Ref
Type: Fine-suspended floc biomass. Treated methanolic ww.	Synthetic methanolic wastewater (180 days)	37 ± 2	1– 2	1.6	16.5	*Methanosarcina*-type	[13]
Type: Granular Brewery ww.	Synthetic methanolic wastewater (120 days)	40 ± 1	2.31	2.54	13.2	*Methanobacterium* or *Methanobrevibacter*	[14]
Type: Granular Butyrate-propionate mix.	Synthetic methanol 73.3% - propionate 27.7%. mixture (120 days)	37	0.80	2.22	42.6	*Methanosarcina spp.*	[35]

Conclusions

- Good quality, well settleable granular sludge was cultivated and retained in the reactor, allowing an OLR of 47 $gCOD.L^{-1}.d^{-1}$ with 93% of methanol removal, where 79% was converted into CH_4.
- Biomass washout was closely related to a high specific gas load. Washout was not detrimental to the system at the conditions tested.

Acknowledgements

We would like to thank Salih Rebac for his technical assistance and advises with the physical sludge characterisation and Look Hulshoff Pol for valuable discussion. This work was supported by "Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq" (Project n° 201055/97-0), an entity from the Brazilian Government for the development of Science and Technology.

4

The anaerobic conversion of methanol under thermophilic conditions: pH and bicarbonate dependence

Abstract

The thermophilic (55 °C) anaerobic conversion of methanol was studied in an unbuffered medium (pH 4 ± 0.2) and in a phosphate buffered medium (pH 6.4 ± 0.1), in both cases without bicarbonate addition. Our cultivated sludge consortium was unable to degrade methanol under acidic conditions. During the 160 days of continuous operation of an upflow anaerobic sludge blanket (UASB) reactor (R1), at an organic loading rate (OLR) of 6 $gCOD.L^{-1}.d^{-1}$ and pH around 4, only 5% of the applied methanol load was consumed and no methane (CH_4) was detected. However, hydrogenotrophic methanogens were found to be resistant to exposure to such conditions. In the end of the trial, the hydrogenotrophic methanogenic activity of the sludge was 1.23 ± 0.16 $gCOD.gVSS^{-1}.d^{-1}$ at neutral pH. With methanol as the test substrate, the addition of bicarbonate led to acetate accumulation. A second reactor (R2) was operated during 303 days at OLRs ranging from 5.5 to 25.4 $gCOD.L^{-1}.d^{-1}$, to assess the conversion of methanol at neutral pH (phosphate buffered) in a bicarbonate deprived medium. The reactor performance was poor with a methanol-COD removal capacity limited to about 9.5 $gCOD.L^{-1}.d^{-1}$. The system appeared to be quite susceptible to any type of disturbance, even at low OLR. The fraction of methanol-COD converted to CH_4 and acetate was found to be unaffected by the OLR applied. At the end of the trial, the outcome of the competition was about 50% methanogenesis and 50% (homo)acetogenesis.

Paulo, P.L., G. C. Villa, J.B van Lier and G. Lettinga (2002) *Submitted*

Introduction

The degradation route of methanol and its final fate in an anaerobic environment may be entirely different when different environmental conditions are applied. Direct methanogenesis from methanol seems to be the predominant mineralisation route under mesophilic conditions both in the absence and the presence of sulphate [136]. The results presented in chapters 2 and 3 indicate that the syntrophic conversion via H_2/CO_2 also plays a role in the methanol conversion to methane by our cultivated consortium. Growth and activity of the H_2 consumers and the H_2 producers, are assumed to be limited by the partial pressure of H_2 in their natural habitat, for thermodynamical reasons [21]. According to Kleerebezem and Stams [60] for anaerobic fermentation where 2 or 3 hydrogen molecules have to be released per mole of substrate, small changes in the hydrogen partial pressure may have a strong impact on substrate conversion rates.

Bicarbonate plays an important role in the anaerobic conversion of methanol, since it is required for the acetogenesis of methanol [72]. Florencio et al. [30, 32] studied the effects of bicarbonate on the competition between methanogens and (homo)acetogens for methanol under mesophilic conditions. According to their findings, (homo)acetogenesis merely proceeds in the presence of bicarbonate, high methanol concentrations and undissociated volatile fatty acids (VFA). They also found that, the mesophilic conversion of methanol to CH_4 without the addition of bicarbonate can proceed both under acidic conditions (pH 4.2), and at neutral pH using phosphate buffer. Accumulation of VFA under these conditions did not occur [30, 32]. According to Bhatti et al. [14] a methanolic wastewater could be treated in an UASB-reactor at 40 °C, without any addition of external buffer. The consortium could hold a pH around 6.0 – 6.3 for a period of 40 days. Thereafter the pH dropped to 5.5 during three consecutive days and could be restored by the addition of 2.52 $g.L^{-1}$ $NaHCO_3$, without build up of VFA in the effluent. Our results described in Chapter 2 revealed a good conversion of methanol to CH_4 under thermophilic (55 °C) conditions in a bicarbonate-supplied system. Even when exposing the system to temperature drop, overloading and unfed conditions, the performance remained almost unaffected and recovery always proceeded rapidly once normal operational conditions were restored.

In the present Chapter we investigated the feasibility of thermophilic anaerobic conversion of methanol under acidic conditions. The effects of the bicarbonate addition or deprivation on the performance, stability and the pathway of the conversion of methanol were also assessed.

Material and methods

Experimental set-up

The experiments were conducted in two UASB reactors, R1 (operated for 190 days) and R2 (operated for 303 days), with a total volume of 0.92 L and 0.3 L, respectively.

The reactors were immersed in a glass waterbath (Julabo- MB-Basis, Germany) maintaining the reactors temperature at 55 °C. Biogas was collected and led through a waterlock filled with a 20% NaOH solution and a column filled with soda lime pellets with indicator in order to remove CO_2 from the gas. Subsequently, the gas passed through a Mariotte flask system containing water for quantification of the CH_4 production. The displaced water was collected in plastic containers. The influent was pumped through the reactors with a peristaltic pump (Watson-Marlow 505S, Falmouth Cornwall, UK). Basal medium was introduced in the influent line using a vertical axis peristaltic pump (Gilson Minipuls 3, France). The bottom of the reactors was filled with glass marbles to ensure uniform influent distribution in the reactors.

Inoculum

R1 was inoculated with 14 g volatile suspended solids (VSS) and R2 with 9.3 gVSS anaerobic thermophilic (55 °C) granular sludge from a lab scale UASB reactor, described in detail in Chapters 2 and 3. The assessed specific methanogenic activities (SMA) for the inoculum sludge on methanol, acetate and H_2/CO_2 were, respectively: 1.13 , 0.84 and 2.24 $gCOD.gVSS^{-1}.d^{-1}$.

Medium

The concentration of methanol in the stock solution varied according to the desired OLR. The reactor was supplemented with macro and micronutrients. 2.22 ml of a nutrient stock solution was supplied for each gram influent $COD.L^{-1}$, the stock solution contained ($g.L^{-1}$): NH_4Cl (7.5), K_2HPO_4 (2.12), $MgSO_4.7H_2O$ (1.5), $CaCl_2.2H_2O$ (0.3), yeast (0.5) and 4.5 millilitre of trace elements solution. The composition of the trace elements solution is presented in Chapter 2. All chemicals were of analytical grade and all solutions were prepared with demi-water.

Continuous experiment

Reactor R1 was operated during the entire experiment (160 days) at an OLR of 6 $gCOD.L^{-1}.d^{-1}$, a hydraulic retention time (HRT) of 7 h and methanol concentration of 1.8 $gCOD.L^{-1}$. No alkalinity was added in the basal medium during this period and the pH in the reactor was kept around 4. NaCl (1 $g.L^{-1}$) was added to the basal medium, since the alkalinity free medium does not contain sodium, an essential ion for all methanogens [93].

Reactor R2 was started up at an OLR of $5gCOD.L^{-1}.d^{-1}$, at a HRT of 12 h and at a methanol concentration of 1.8 $gCOD.L^{-1}$. The OLR was stepwise increased to 20 $gCOD.L^{-1}.d^{-1}$, by decreasing HRT (first 20 days) and increasing the methanol concentration. The feed was supplied with 0.33g of $NaHCO_3$ per each gram of methanol. L^{-1}, to ensure pH stability (day 1-77). From day 113-303, when bicarbonate was not supplied to the reactor, the pH was

maintained around 6.3-6.5 by using a phosphate buffered solution using 4.75 g $Na_2HPO_4.2H_2O$ and 5.45 g KH_2PO_4 per litre (70 mM).

For both reactors the CH_4 production was continuously measured. The HRT was calculated based on the flow rate of the effluent. Influent and effluent samples were taken twice per week to analyse the methanol and VFA concentration. The biogas composition was measured every 15 days or when the OLR was increased.

Batch experiments

Activity assays

Activity tests were performed with the sludge sampled at day 160 from R1 and days 76 and 300 from R2. The SMA was assessed using methanol, acetate and H_2/CO_2 as substrates. Such tests were also used to assess the influence of bicarbonate addition/deprivation on the methanol conversion pathway, either by adding or depriving $NaHCO_3$ from the medium and CO_2 from the headspace.

The sludge bed was gently mixed before sampling in order to get a representative sludge sample. The samples were rinsed with anaerobic pre-heated (55 °C) medium to remove remaining carbon source.

Glass serum vials of 120-ml were used when the substrate was methanol or acetate and 250-ml bottles when the substrate was H_2/CO_2. Serum vials and bottles were filled with 50 ml basal medium containing ($g.L^{-1}$): NH_4Cl (0.28), $K_2HPO_4.3H_2O$ (0.33), $MgSO_4.7H_2O$ (0.1), $CaCl_2.2H_2O$ (0.01), yeast (0.1) and one millilitre of trace elements solution. Before adding the sludge and substrate, all vials and bottles containing basal medium were incubated in a waterbath with shaker (TUV, GLF 1083, Germany) at 55 °C and 50 rpm. When H_2/CO_2 was used as the substrate, shaking speed was 100 rpm and the bottles were placed horizontally in the waterbath, in order to optimise mass transfer of hydrogen from the gas to the liquid phase.

Washed sludge was added into the vials and bottles at a VSS concentration of about 2 $g.L^{-1}$ beneath the liquid surface by means of a 5 ml automatic pipette (Gilson, Villiers, France), with a plastic tip of which the narrow opening was cut off. Methanol or acetate was added as the substrate at a concentration of 2 $gCOD.L^{-1}$. 6.72 g $NaHCO_3$ per litre of basal medium was added, to ensure pH stability. When an assay required bicarbonate deprived medium, phosphate buffer 8.31 g $Na_2HPO_4.2H_2O$ and 2.72 g KH_2PO_4 per litre (72 mM) was used instead and headspace was flushed with pure N_2.

Final pH was neutralised to 7.0 by adding a concentrated HCl solution. The vials were sealed with butyl rubber stoppers and the gas headspace was flushed with N_2/CO_2 (70:30), or pure N_2 depending on the assay. When H_2/CO_2 was the substrate, the headspace was flushed with 1.05 atm of H_2/CO_2 (80:20), equivalent to 2 g $COD.L^{-1}$. Liquid and gas samples were taken periodically to analyse substrate consumption and product formation. The pH, as well as the

amount of VSS in each bottle was measured after the test was completed. The SMA was calculated from the linear increase of the CH_4 concentration in the beginning of the experiment, when no lag phase was observed, divided by the amount of VSS. All assays were performed in triplicate, using the bottles without substrate as blank.

Analysis

A detailed description of the analytical procedures has been presented in Chapter 2.

Results

Reactor R1- acidic conditions

Continuous experiment

Due to the absence of any buffer in the feed, the pH of the reactor was around 4 during the whole experiment. During its 160 days of continuous operation, less than 5% of methanol was consumed and any CH_4 could not be detected (data not shown). During the first 25 days of the operation, the acetate concentration was about 5.5 mM, which dropped to 0.6 mM during the last 85 days of operation.

Batch experiment

Figure 1 shows the results of the batch experiment performed with sludge sampled at day 160. In the presence of bicarbonate, only a small fraction of methanol was converted into methane. The main product was acetate when bicarbonate was present. In the absence of bicarbonate, acetate plus methane were the products representing only 40% of the total methanol consumed. When bicarbonate was absent methanol conversion started after 3 days. It should be noticed that despite the same conditions were applied, the sludge behaved differentially (Figure 1A and 1B). In the experiment shown in Figure 1A a faster conversion took place from day 7 onwards. That might be attributed to some slight differences in the sludge concentration in the bottles.

When CO_2 was provided to the headspace (30% of the headspace volume) in the bicarbonate free experiment bottles, the response was immediate and acetate was formed, representing 85% of the consumed methanol. No CH_4 was detected. Feeding the bicarbonate supplied system with a mixture of H_2/CO_2 once methanol had been converted to acetate (which was not further consumed), led to a very rapid and complete conversion of H_2/CO_2 into CH_4. Such a rapid conversion of H_2/CO_2 was also observed when the sludge supplied with bicarbonate was immediately fed with this substrate mixture.

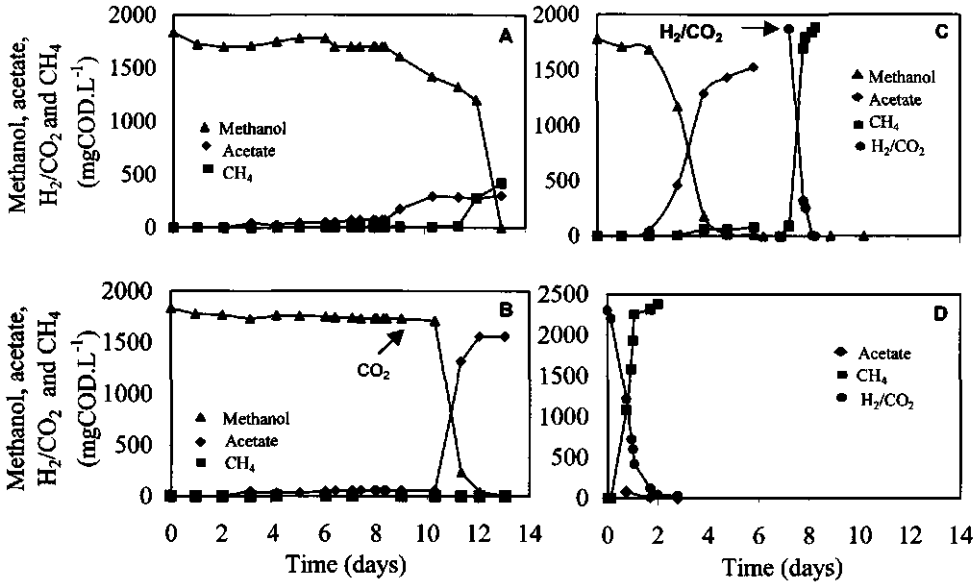


Figure 1 The course of methanol and H_2/CO_2 conversion and product formation, under different conditions during batch experiment performed with sludge sampled from reactor R1 at day 160. **A**: Bicarbonate deprived, no posterior addition of CO_2; **B**: Bicarbonate deprived, 30% of CO_2 added in the headspace at day 10; **C**: Bicarbonate supplied, headspace flushed with H_2/CO_2 (80/20) at day 7.4; **D**: Bicarbonate supplied, H_2/CO_2 used as substrate.

Reactor R2

Continuous experiment

Period 1

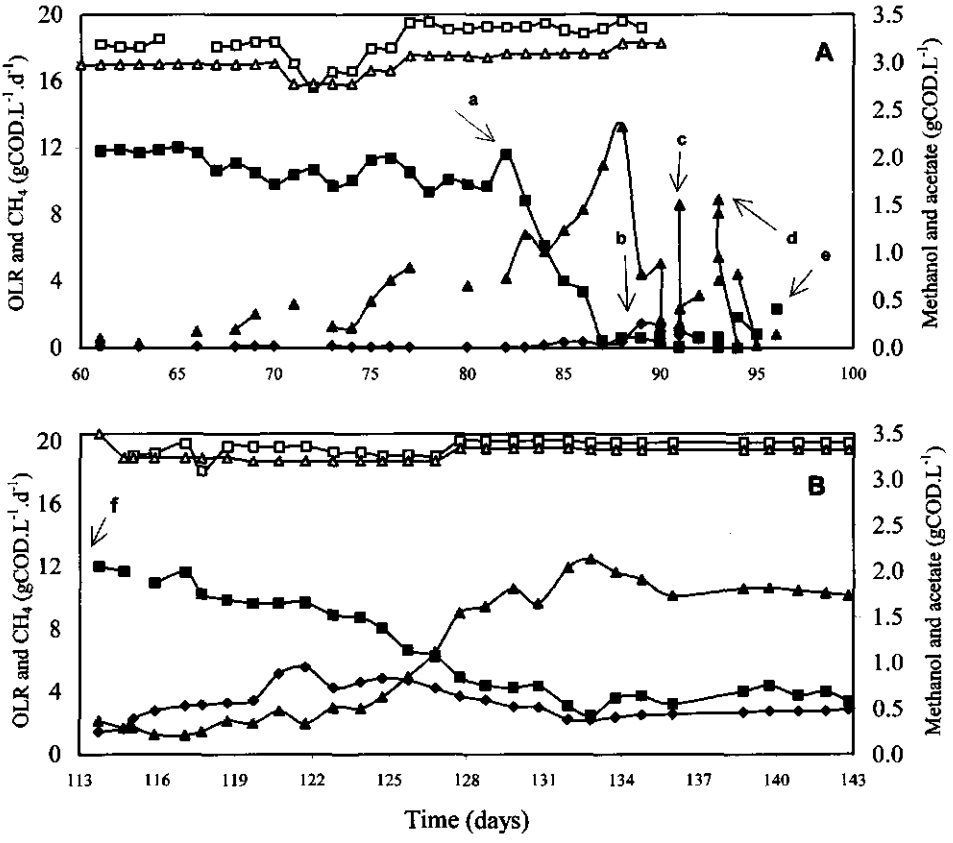


Figure 2 Performance response of reactor R2 on bicarbonate deprivation under 2 different situations. **(A)** Bicarbonate addition is stopped and no buffer is used. **a**: bicarbonate is deprived; **b**: bicarbonate is added; **c**: bicarbonate is deprived and phosphate buffer is used; **d**: bicarbonate is added, reactor operated in a batch mode and reinoculated; **e**: running at normal conditions. **(B)** Bicarbonate addition is stopped and phosphate buffer is used (pH 6.5). **f**: bicarbonate is deprived. Symbols: OLR (❑), methanol-COD_{in} (△), methanol-COD_{ef} (▲), acetate- COD_{ef} (♦) and methane (■).

Table 1 The outcome of competition between methanogens and acetogens during the different phases of the continuous operation of reactor R2.

Period	Days	OLR	MeOH removal	COD removal	Methane[a]	Acetate[b]	Sum[c]	Proportion methane	Proportion acetate
		($gCOD.L^{-1}.d^{-1}$)	(%)	(%)	(%)	(%)			
Regular[d]	105 -112	20.2	92.1	84.0	61.8	8.1	69.9	0.9	0.1
		(0.4)	(5.8)	(7.5)	(5.9)	(5.1)			
I.1	113-133	19.7	74.7	56.6	41.3	18.1	59.4	0.7	0.3
		(0.5)	(20.0)	(19.8)	(16.0)	(6.3)			
I.2	134-143	19.9	45.3	31.6	18.9	13.7	32.6	0.6	0.4
		(0.0)	(2.7)	(2.1)	(1.9)	(0.9)			
II	165-201	14.4	66.5	52.2	22.0	14.4	36.4	0.6	0.4
		(2.0)	(8.4)	(5.0)	(7.5)	(4.2)			
III	202-230	25.4	37.5	27.9	9.6	9.7	19.3	0.5	0.5
		(1.6)	(5.5)	(5.3)	(3.2)	(0.9)			
IV	231-302	5.5	86.7	62.8	27.4	23.9	51.4	0.5	0.5
		(1.1)	(15.5)	(13.9)	(11.2)	(5.5)			

[a] – methane (%) = 100 *[methane produced ($gCOD.d^{-1}$)/ $MeOH_{in}$ ($gCOD.d^{-1}$)]
[b] – acetate (%) = 100 *[acetate produced ($gCOD.d^{-1}$)/ $MeOH_{in}$ ($gCOD.d^{-1}$)]
[c] – sum = a + b
[d] – Period related to reactor running under normal conditions (bicarbonate addition)
Standard deviation is given between brackets

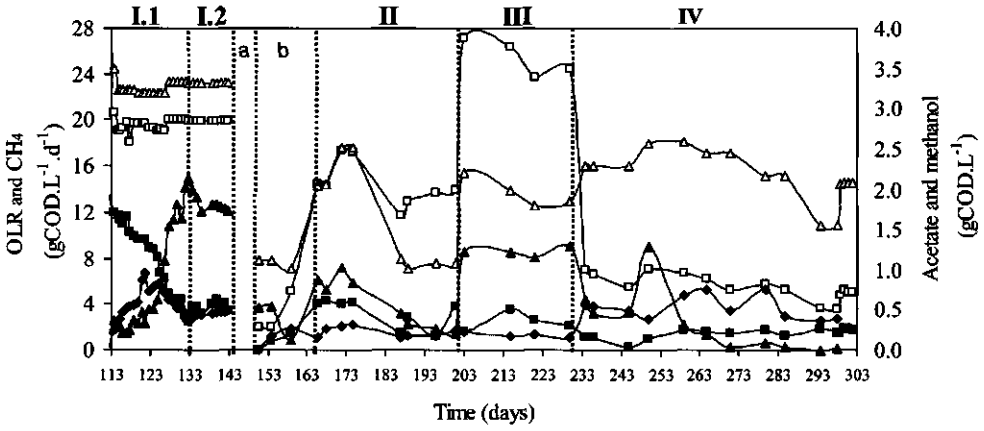


Figure 3 Operational parameters and efficiency of reactor R2 operating during 303 days under bicarbonate deprivation. Symbols: OLR (❑), methanol-COD_{in} (△), methanol-COD_{ef} (▲), acetate- COD_{ef} (♦) and methane (■). **a**: operation discontinued; **b**: new start up. Other phases are detailed in Table 1.

After 81 days of continuous operation, bicarbonate was deprived from the reactor feed. In the period prior day 81, 91% of the methanol COD was removed, of which 70% was converted to CH_4 and 1.2% to acetate.

After depriving the bicarbonate, the reactor moved into a complete failure (Figure 2A). The pH dropped from 6.7 to 4.0 and CH_4 production ceased completely within 5 days. Moreover, a washout of biomass was observed. In an attempt to recover the reactor pH we reintroduced bicarbonate in the feed, but acetate accumulation took place (Figure 2A, arrow b). The system was deprived of bicarbonate again and phosphate buffer was used instead and the reactor was operated in batch mode (Figure 2A, arrow c). As no significant recovery was established, the reactor was reinoculated at day 93 with 3.86 gVSS (representing 41% of original inoculum) of the seed sludge, and was operated in batch mode until CH_4 production restored.

Period 2

From day 96 to 113, the reactor was operated in a continuous mode under the same conditions as in period 1 (before omission of bicarbonate), until an OLR of 20 $gCOD.L^{-1}.d^{-1}$ was reached. Compared with the previous phase under the same conditions, the reactor efficiency was about the same, except for acetate accumulation, which increased by about 4.7%. At day 114, once again the system was deprived of bicarbonate (Figure 2B) and the pH was maintained around 6.3 - 6.4, by using phosphate buffer.

Under these conditions methanol conversion remained but, CH_4 production dropped and the acetate accumulation increased. It took 19 days until the system reached a stable performance. The acetate production increased, representing 13.7% of the methanol COD influent. Methanol-COD removal dropped from 84% to 32%.

To check whether the imposed OLR would affect the competition between methanogenesis and (homo)acetogenesis when the reactor was bicarbonate deprived, reactor R2 was operated during 190 days while the OLR was imposed randomly and varied from 5.5 to 25.4 $gCOD.L^{-1}.d^{-1}$ (Figure 3). Table 1 shows the outcome of the competition for the applied conditions.

Batch experiments

The SMA tests were performed with the sludge sampled at the end of the trial. Figure 4 shows the course of methanol conversion and the products formed both in the absence and in the presence of bicarbonate. In the absence of bicarbonate, the SMA on methanol was 0.60 ± 0.09 $gCOD.gVSS^{-1}.d^{-1}$ while in the presence of bicarbonate it was only 0.13 ± 0.02 $gCOD.gVSS^{-1}.d^{-1}$. No methanogenic activity was observed with acetate. The SMA on H_2/CO_2 was also measured, being 2.13 ± 0.03 $gCOD.gVSS^{-1}.d^{-1}$.

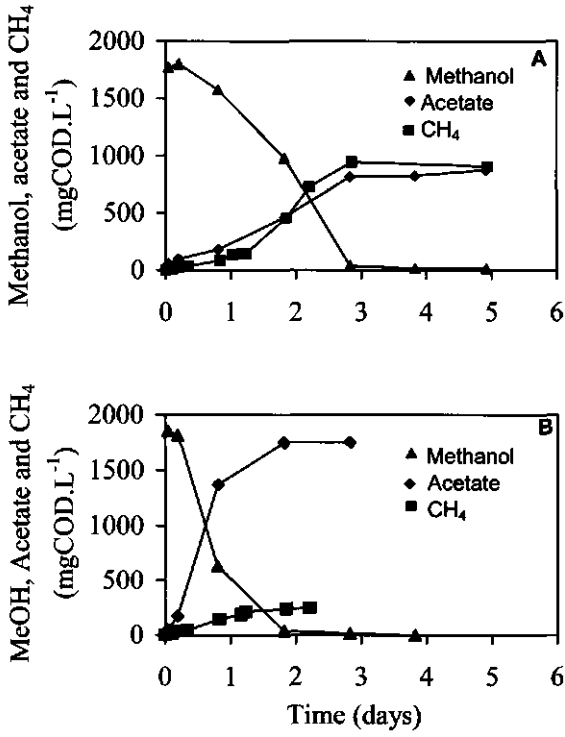


Figure 4 The course of methanol conversion and products formation during activity assay performed with sludge sampled from reactor R2 at the end of continuous run (day 303). **A**: No bicarbonate supplied; **B**: Bicarbonate supplied.

Discussion

The effect of pH

The results obtained in the present investigations reveal that our sludge consortium is unable to degrade methanol under acidic conditions. This may be due to the low pH, the presence of undissociated acids, the lack of bicarbonate or the complete washout of the microorganisms responsible for methanol degradation. Based on the assessed acetate concentration in the beginning of the reactor operation, the estimated amount of undissociated acid is 4.6 mM. It is difficult to clarify whether this concentration was toxic. The values reported in literature for the toxicity of weak acids vary greatly both for pure cultures and sludges, as well as for the different operational temperatures. Moreover, the extent of inhibition also is highly specie

specific, and consequently it depends on the dominant population present in the sludge and on the actual pH. For thermophilic methylotrophic methanogens, a complete inhibition was evident at 8.9 mM undissociated acetic acid [144]. Under mesophilic conditions, methylotrophic methanogens were inhibited at 5.4 mM at a pH value around 5 [30]. In the present investigation we found prompt recovery of the activity when the pH medium was raised to neutral values. This indicates that viable microorganisms were still present in the sludge.

pH resistance

The hydrogenotrophic methanogenic bacteria present in the sludge were, apparently, the least affected by the long term exposure to the acidic conditions. The methanogenic activity on H_2/CO_2 was 1.23 ± 0.16 gCOD.gVSS^{-1}.d^{-1}, representing more than 50% of the initial activity. Somehow, the concerning organisms could withstand a period of 140 days in an acid environment. The sludge was cultivated in a pH range of 6.4 - 6.7 before it was exposed to pH 4. The so-called 'acid habituation' or the 'adaptive acid tolerance response' phenomenon, described by Hall et al. [44] might explain the resistance of our sludge. According to their description, the bacterial cells grown at a moderately acid pH or temporary exposed to low pH, resist killing at low pH much better than cells grown at pH 7. For our sludge, we do not know to what extent the pH inside the granules was affected but, most probably, it was considerably lower than the optimum pH range for growth of thermophilic methanogens (6.5 – 8.0) and (homo)acetogens (5.8 – 7.0) [134]. Several mechanisms for maintaining the intracellular pH to minimise the stress from non-optimal extracellular pH are quoted in literature [24]. One option involves energy-requiring systems by which ions are actively 'pumped' across the cell membrane. The required energy to sustain such condition might be derived from substrate conversion. The estimated 5% of methanol consumed in our experiment possibly was used for the survival and maintenance of the microorganisms. The observed acetate accumulation in the batch experiments indicates that the acetotrophic microorganisms could not resist such conditions and were killed/washed out.

The role of bicarbonate

The results of the experiments dealing with pathway studies indicate that part of the methane produced by the cultivated thermophilic (55 °C) consortium proceeds via H_2/CO_2. However how much proceeds from the oxidation of methanol (Reaction 1) and from acetate (Reaction 4 followed by Reaction 7) could not be quantified.

				$\Delta G_{55°C}$ kjoule/reaction
Reaction 1	$CH_3OH + 2\,H_2O$	$\rightarrow$	$3\,H_2 + HCO_3^- + H^+$	13
Reaction 2	$4\,H_2 + 2\,HCO_3^- + H^+$	$\rightarrow$	$CH_3COO^- + 4\,H_2O$	- 90

Reaction 3	$4\,H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3\,H_2O$	- 125
Reaction 4	$4\,CH_3OH + 2\,HCO_3^- \rightarrow 3\,CH_3COO^- + 4\,H_2O + H^+$	- 220
Reaction 5	$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	- 113
Reaction 6	$4\,CH_3OH \rightarrow 3\,CH_4 + HCO_3^- + H_2O + H^+$	- 325
Reaction 7	$CH_3COO^- + 4\,H_2O \rightarrow 4\,H_2 + 2\,HCO_3^- + H^+$	90
Reaction 8	$CH_3COO^- + H_2O \rightarrow CH_4 + H_2O$	- 35

Theoretically, inorganic carbon is not needed for the partial syntrophic conversion of methanol into CH_4 via H_2/CO_2 (Reactions 1 and 3). The formation of acetate from methanol is only possible when an oxidised cosubstrate such as CO_2, CO, formate or acetate is present [146]. Consequently, the deprivation of bicarbonate affects in a first moment the rate of Reaction 4, at least at the initial stage because CO_2 has to be formed via Reaction 1 and/or 6. On the other hand, we observed that the lack of bicarbonate also delays the conversion of methanol to H_2/CO_2. During batch experiments, when no bicarbonate is supplied, the build up of H_2 in the headspace occurs (data not shown). The lack of bicarbonate also reflects in the overall reactor performance. The methanol-COD removal capacity of the present system was found to be limited to about 9.5 $gCOD.L^{-1}.d^{-1}$, which is quite low if compared with our bicarbonate-supplied system. In the latter, a satisfactory removal was accomplished up to considerably high OLRs (Chapter 2). As the contribution of the direct conversion of methanol in our system represent about 50% of the total methane formed, 50% of the formation of methane will depend on the occurrence of the (homo)acetogenic step which, in this case, is restricted by the available amount of bicarbonate.

The effect of bicarbonate on the competition between methanogens and (homo)acetogens

Our results indicate that when the system is bicarbonate-deprived, (homo)acetogens are able to compete with hydrogenotrophic methanogens for CO_2. In case all H_2/CO_2 produced from methanol (or methanol directly) is stoichiometrically converted to CH_4, the remaining CO_2 would enable the formation of acetate to a maximum of 33% of the converted methanol (via Reaction 4). However, the results show that, after 200 days of continuous operation, 50% of the consumed methanol (found as product) was converted to acetate and 50% to CH_4. During the whole period of continuous operation, the CO_2 contained in the gas phase never exceeded 1% (data not shown), indicating that all CO_2 produced was nearly consumed. Higher substrate affinity [101], lower threshold value [66] and thermodynamic equilibrium favour methanogens over homoacetogens in the competition for H_2/CO_2. On the other hand, according to Schink [100], homoacetogens are superior to other fermenting bacteria with respect to their ability to use CO_2 as an external electron acceptor, and they also have the ability to change between various substrates and can use them simultaneously. Our results indicate that, when the consortium is exposed to stress condition (i.e., the absence of

bicarbonate), the (homo)acetogenic bacteria seem to be capable to compete with hydrogenotrophic methanogens for CO_2.

It has to be taken into consideration that, when (homo)acetogens can compete with hydrogenotrophic methanogens for CO_2 (from Reaction 1), H_2 will accumulate in the system (considering that acetate is formed via reaction 4) and a H_2 sink will be required. Reaction 5 would be the simplest way to dispose of the built up H_2. If part of the acetate formed is converted to methane via H_2/CO_2 (Reaction 7), extra CO_2 would be available to hydrogenotrophic methanogens. Considering that Reaction 7 would proceed, which is possible since, the syntrophic conversion of acetate to methane via H_2/CO_2, seems to represent an important metabolic pathway under thermophilic and extreme thermophilic conditions [148, 151, 152], the contribution of Reaction 4 in the system might be higher than we can quantify, simply because part of the formed acetate will be further converted to methane. The ability of (homo)acetogens to compete with methanogens in the UASB-experiment under bicarbonate deprivation was confirmed when batch experiments were conducted in the end of the continuous trial in a bicarbonate supplied medium. The results reveal that the methanogens only get the opportunity to compete with (homo)acetogens under reactor conditions due to bicarbonate limitation.

Acetate accumulation

The consortium lost its ability to degrade acetate. At time 0, the SMA on acetate was 0.84 ± 0.05 $gCOD.gVSS^{-1}.d^{-1}$ while at the end of the trial, methanogenic activity on acetate was not detected. In addition, in the batch experiment any CH_4 formation from acetate could not be detected. Evidence was obtained that the use of 70 mM of phosphate buffer in the long term-continuous experiment negatively affected the acetotrophic microorganisms present in the consortium. For batch experiments where a bicarbonate-deprived medium was required, we usually used phosphate buffer (72 mM). In the experiments conducted with this procedure, we observed a distinct accumulation of acetate. In an additional continuous UASB-experiment in a bicarbonate-deprived medium, we kept the pH close to neutral by using an automatic pH controller and a NaOH solution instead of phosphate buffer. Also here, the poor performance and instability of the system were observed, although without any acetate accumulation while operating the system under optimal conditions (data not shown). Conrad et al. [20] reported the inhibition of acetotrophic methanogenesis by phosphate ($\geq$ 20 mM) in experiments conducted with washed excised rice roots incubated in phosphate buffer under anaerobic conditions.

Overall performance

Under mesophilic conditions a high methanol concentration in combination with addition of bicarbonate lead to acetate accumulation [30]. Cord-Ruwisch and Ollivier [22] found that in a

mesophilic coculture of *S. acidovorans* with hydrogenotrophic methanogens, the percentage of CH_4 produced from methanol depend on the type of the hydrogenotrophic methanogen specie. The results of our thermophilic continuous experiment show that, in the case of a bicarbonate deprived system, competition between methanogens and (homo)acetogens does not depend on the methanol concentration in the reactor. The fraction of Methanol-COD_{in} converted into CH_4 and acetate changed throughout time, and the outcome of the competition was the same under high (25 $gCOD.L^{-1}.d^{-1}$) or low (5 $gCOD.L^{-1}.d^{-1}$) loading conditions. Moreover, when bicarbonate was not supplied to the system, the reactor performance remained poor, and the system also showed to be quite sensitive to any disturbance, even under low OLR conditions. The batch experiment performed with the sludge sampled in the end of the trial presented the same pattern of methanol conversion. In spite of the low CH_4 production found in the reactor R2 by the end of the experiment, the SMA on H_2/CO_2 was the same as assessed at time 0, indicating that under prevailing conditions in the reactor they were outcompeted by (homo)acetogens. On the other hand, the SMA on methanol (without addition of bicarbonate) dropped to half of its initial value, while the (homo)acetogenic activity, which was not detected before, was 0.55 ± 0.02 $gCOD.gVSS^{-1}.d^{-1}$ in the end of the experiment.

Our results strongly indicate that the addition/deprivation of bicarbonate governs the pathway of methanol conversion in our consortium. Indirectly, it acts as a H_2 sink, helping to keep the pH_2 value low, so that the reaction becomes thermodynamically favourable and methanol can be converted to H_2/CO_2. Moreover, the results indicate that just the partnership with hydrogenotrophic methanogens does not suffice for the establishment of a high rate of methanol conversion. Bicarbonate is also used together with methanol for the acetate production, which contributes to the amount of methane formed when operating the system under optimal conditions. The fact that the hydrogen partial pressure may change the spectrum of products in case the microorganisms also have alternative pathways [16, 63, 83], might explain the higher contribution of acetate as an intermediate compound in the methanol conversion in the reactor when operating the system without bicarbonate addition.

It is clear that CH_4 is the final fate of methanol for our thermophilic cultivated consortium, and that acetate just accumulates under specific conditions, e.g. when methanogens are inhibited by BESA, phosphate, free acetic acid or when pH_2 is higher than usual.

Summarising, our studies reveal that the treatment of methanolic-containing wastewater by the cultivated consortium is not worthwhile without the addition of bicarbonate, since a buffer is anyway required to maintain the pH in the neutral pH range. Besides, the reactor performance is indubitable better and more stable when bicarbonate is supplied.

Acknowledgements

We would like to thank A.J.M. Stams for reviewing the manuscript. This work was supported by "Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq" (Project n° 201055/97-0), an entity from the Brazilian Government for the development of Science and Technology.

5

Bicarbonate dosing: a tool to performance recovery of a thermophilic methanol-fed UASB reactor

Abstract

The thermophilic-anaerobic treatment of methanol-containing wastewater in an upflow anaerobic sludge blanket (UASB) reactor, was found to be quite sensitive to pH shocks, both acid and alkaline. The results of the recovery experiments of sludge exposed to an alkaline shock, indicated that the addition or deprivation of sodium bicarbonate ($NaHCO_3$) in the medium, plays an important role on the competition of methanogens and (homo)acetogens for methanol. In addition, caution has to be taken when using $NaHCO_3$ for buffering methanol-containing wastewaters, since its introduction in the system will favour (homo)acetogenesis when proper conditions are not established. Based on these results, a recovery strategy for methanogenesis was proposed where bicarbonate is supplied stepwise, and the reactor is operated in a batch mode. This strategy was found to be appropriate, i. e. the results revealed that the recovery of methanogenesis on methanol from a reactor upset or complete failure caused by pH shock is possible, even in systems where (homo)acetogens are outcompeting methanogens. The time and the number of feedings required will depend on the degree of deterioration of the sludge.

Paulo, P.L., J.B van Lier and G. Lettinga Presented at the VII Latin-American workshop and Seminar on Anaerobic digestion, Merida, Mexico, October, 2002.

Introduction

In some of our previous studies (Chapter 2), a satisfactory conversion of methanol to CH_4 under thermophilic (55 °C) conditions was achieved, without any considerable accumulation of volatile fatty acids (VFA). Not even with the supply of $NaHCO_3$ concentrations exceeding 20 mM. When the system was exposed to specific environmental stress situations (temperature drop, overloading and no feeding), the performance remained almost unaffected and the system promptly recovered as soon as normal conditions were restored. Recently, however, we studied the methanol conversion in a bicarbonate-deprived medium (phosphate buffered, pH 6.4 ± 0.1). The reactor performance was poor, and the system was quite sensitive to disturbances, even under low organic loading rates (OLR). From the methanol converted into products, 50% was converted to CH_4 and 50% to acetate. The same consortium was not able to degrade methanol under acidic conditions (Chapter 4).

For our cultivated thermophilic sludge (Chapter 2), about 50% of the methanol is converted to methane directly by methylotrophic methanogen, and the remaining is converted into methane either via acetate or H_2/CO_2. Therefore, the consortium would be basically composed of the (homo)acetogens, methylotrophic, hydrogenotrophic and acetoclastic methanogens, where competition for methanol, acetate and H_2/CO_2 may take place.

Under mesophilic conditions, where the direct conversion of methanol to CH_4 seems to be the main degradation route [136], methanogens are the predominant trophic group when bicarbonate is not supplied to the medium, because the meagre endogenous resources of bicarbonate generated by methanogens cannot support significant (homo)acetogens [30]. The conversion of methanol to H_2/CO_2 is limited due to the thermodynamics of the metabolic reactions, which are very sensitive to the H_2 partial pressure [21].

Sodium bicarbonate is commonly used to provide bicarbonate alkalinity. It can be recommended for that purpose since it is safe to handle, it dissolves easily in water and dosage errors (especially in excess) do not affect digester operation [69].

Considering the facts that, the main bottlenecks of methanol and thermophilic treatment are the accumulation of VFA and process instability, we attempted in the present study, to develop a proper strategy for recovery of methanogenesis from a serious pH-upset or even total failure, both to acid and alkaline exposures. The strategy was focused on supply of bicarbonate, since in the particular case of the thermophilic anaerobic treatment of methanol, the addition/deprivation of bicarbonate plays an important role on the competition between methanogens and (homo)acetogens.

Material and methods

Continuous experiments

Reactor R1

The experiment was conducted with a 0.9 L UASB-reactor, which was operated during 154 days. Reactor was immersed in a waterbath (Julabo- MB-Basis, Germany) which maintained the reactor temperature at 55 °C. Biogas was collected and led through a waterlock filled with a 20% NaOH solution and a column filled with soda lime pellets with indicator in order to remove CO_2 from the gas. Subsequently, the gas passed through a Mariotte flask system containing water for quantification of the methane production. Reactor was inoculated with 14 g volatile suspended solids (VSS) anaerobic-thermophilic (55 °C) granular sludge from a lab scale UASB reactor (5.1 L), fed with methanol as sole organic carbon source and bicarbonate buffered (Chapters 2 and 3). Specific methanogenic activities (SMA) for the inoculum sludge on methanol, acetate and H_2/CO_2 were, 1.13, 0.84, and 2.24 $gCOD.gVSS^{-1}.d^{-1}$, respectively. Methanol was used as sole organic carbon source. The concentration in the stock solution varied according to the desired OLR. The reactor was supplemented with macro and micro-nutrients (Chapter 2).

Tentative recovery strategy

Two different sludges were used to assess the adequate recovery strategy: sludge originating from the reactor described above, which was exposed to an alkaline pH shock and an acidic sludge.

The acidic sludge was cultivated at an OLR of 6 $gCOD.L^{-1}.d^{-1}$ in a bicarbonate deprived medium (reactor pH 4 ± 0.2). During the 160 days of continuous operation, less than 5% of methanol removal was observed and no methane was detected (data not shown). SMA at the end of the trial (pH 7 and bicarbonate supplied) on H_2/CO_2 was 1.23 $gCOD.gVSS^{-1}.d^{-1}$. At the same conditions, but using methanol as substrate, no methanogenic activity was detected and methanol was completely converted to acetate (Chapter 4).

The recovery strategy was applied in the end of both continuous reactors run. The main point of the strategy was that reactor was operated in batch mode, until complete depletion of the methanol applied and in the end of each feeding, liquid phase was completely replaced. The number of feedings, methanol and bicarbonate concentration applied are summarised in Table 1.

Batch experiments

The batch experiments were performed as described in Chapter 4.

Analysis

A detailed description of the analytical procedures for determination of methanol, acetate, biogas composition, hydrogen, CH_4 and VSS has been presented in Chapter 2.

Results and Discussion

Prior to imposing the alkaline pH shock to reactor R1, the system seemed to be overloaded. Only 60% of methanol COD was removed and 43% was converted to CH_4. No accumulation of VFA was observed (data not shown). At day 32, the pH in the system raised to 9.5 and then methanogenesis ceased. Figure 1 shows the reactor behaviour during the first attempt to the performance recovery.

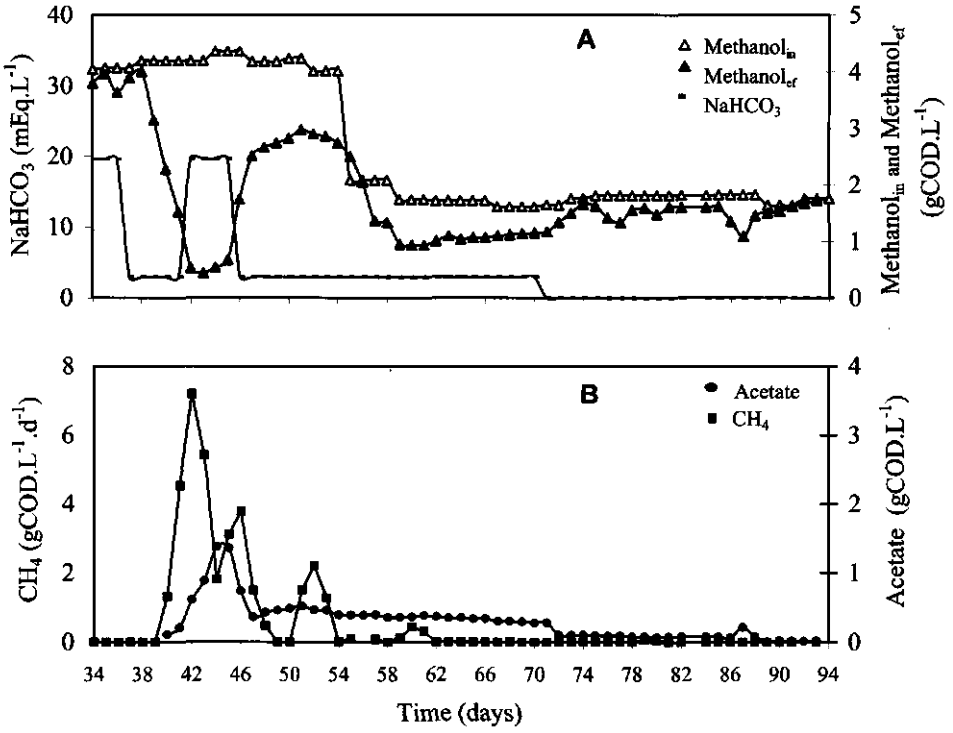


Figure 1 Operational parameters and performance response of the reactor under continuous operation, during the first tentative of performance recovery after being exposed to an alkaline pH shock.

After decreasing the OLR and the bicarbonate concentration and setting the pH to neutral (day 37), CH_4 production restored, but also acetate started to accumulate and consequently, the pH dropped to 5.7. When bicarbonate concentration was returned to 20 mEq.L^{-1} the pH restored but, with the addition of bicarbonate, competition for methanol manifested between

methanogens and (homo)acetogens and methane production dropped again (Figure 1B). It was quite clear that (homo)acetogenesis was restricted by the bicarbonate concentration. The profile of acetate accumulation followed that of bicarbonate addition (Figure 1). Methanogenesis could not be recovered under such conditions, not even after decreasing the OLR to 6 $gCOD.L^{-1}.d^{-1}$ and bicarbonate concentration to 3 $mEq.L^{-1}$. Nevertheless, 2 peaks of CH_4 production (day 52 and 60) were observed.

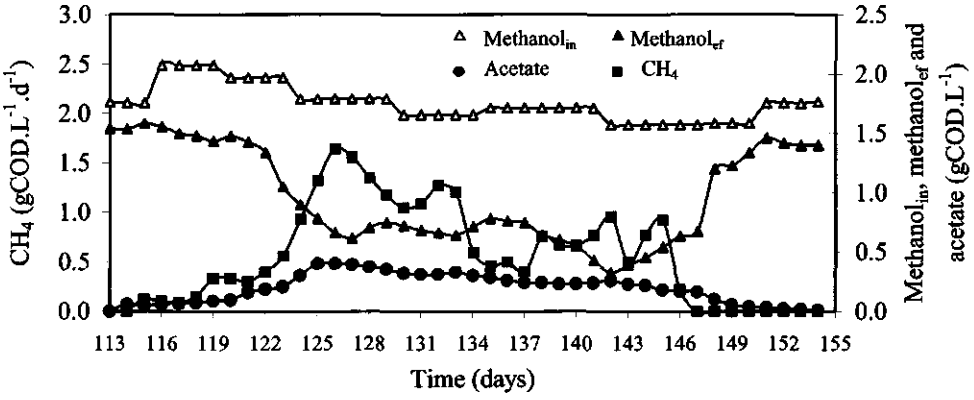


Figure 2 Operational parameters and performance data of the reactor operating during 40 days, under bicarbonate deprivation at neutral pH.

In an attempt to avoid the competition between methanogens and (homo)acetogens due to the presence of bicarbonate, from day 71 onwards, we exposed the system to bicarbonate-deprived conditions and the pH was automatically controlled by supplying NaOH. It took about 45 days until any response could be observed. The reactor was operated during 40 days. Performance was poor, with an average methanol removal of 60%, where 16% was converted to acetate. The CH_4 production was unstable during the whole trial, the maximum reached was 1.64 $gCOD.L^{-1}.d^{-1}$ (Figure 2). The reactor was quite sensitive to disturbances, even small variations in the methanol influent concentration or flow disturbed the system.

The results obtained are comparable with those we have found in batch experiments with the seed sludge (cultivated on methanol with addition of bicarbonate) and the sludge which was cultivated under acidic conditions without addition of bicarbonate (Figure 3). In the experiment with the seed sludge, we found accumulation of acetate merely when the experiments were performed in a phosphate buffered medium (neutral pH) without addition of bicarbonate. For that case the complete methanol conversion required 7 days. 2/3 of the recovered product was methane and 1/3 was acetate (Figure 3A). For the acidic sludge (Figure 3B), following a 2 days lag phase, methanol was completely converted to acetate

when batch experiments were performed under neutral pH (phosphate buffered) and bicarbonate supplied medium.

The results described in Chapter 4 indicate that the cultivated consortium requires bicarbonate for full methanogenesis, but at the same time the addition of bicarbonate leads to the accumulation of acetate, in case the environmental conditions are not appropriate. Therefore, we based the strategy for recovering the reactor performance on a stepwise reintroduction of bicarbonate to the system. According to the reaction stoichiometry (equations presented in Chapter 1), 0.63 g HCO_3^- per each 1 g methanol-COD is required for the complete conversion of methanol into acetate.

We observed that the sludge still exerted methanogenic activity, and the methanogens were outcompeted by (homo)acetogens under reactor conditions. Based on this, we hypothesised that by limiting (homo)acetogenesis by the amount of supplied bicarbonate, the normal conversion process would prevail, and the methanogens would have a chance to develop provided that optimal conditions developed in the batch-mode system. If methanogenesis takes place, part of the produced CO_2 will be left over, but to a limited extent. Once methanogenesis is recovered and proper environmental conditions can be maintained in the system, (homo)acetogens would not outcompete methanogens for methanol and the formed acetate would be quickly consumed. (see Chapter 2). The results of the 2 sludge samples tested are presented in Table 1.

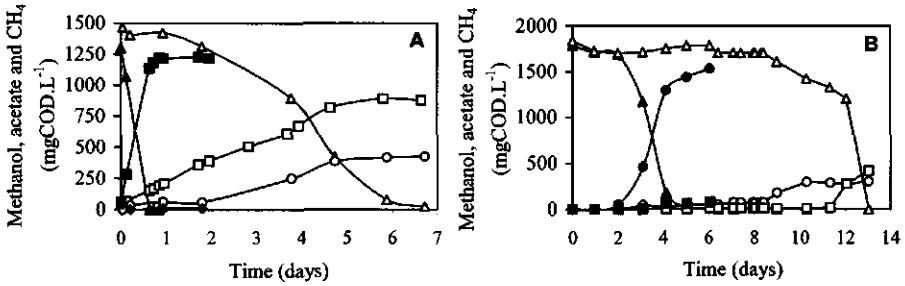


Figure 3 The course of methanol conversion with bicarbonate supplied and deprived medium. Two different sludges were tested: **A**: sludge cultivated with supplied bicarbonate and neutral pH, **B**: sludge cultivated at acid pH without supplementation of bicarbonate. Symbols: Bicarbonate supplied: methanol (▲), acetate (●) and methane (■); Bicarbonate deprived: methanol (△), acetate (○) and methane (□).

Table 1 The recovery strategy applied to a sludge cultivated under acidic conditions and to the sludge submitted to an alkaline pH shock.

Sludge tested	Feeding (number)	Methanol applied ($gCOD.L^{-1}$)	HCO_3^- ($g.L^{-1}$)	Duration (days)	Acetate accumulated ($gCOD.L^{-1}$)
Acidic sludge	1	2.0	0.44	4	0.52
	2	1.6	0.64	3	0.58
	3	1.6	0.64	2	0.71
	4	1.6	0.93	3	0.92
	5	2.0	0.93	3	0.93
	6	2.0	1.28	4	0.75
	7	2.0	1.28	6	0.02
	8	2.0	1.28	6	0.05
Alkaline sludge	1	1.8	0.44	1	0.45
	2	1.7	0.44	1	0.59
	3	2.3	0.44	6	0.02

For the acidified sludge, 8 feedings were required for the complete recovery of the methanogenic activity, but in the 6^{th} feeding CH_4 already was detected in the reactor headspace. The initial pH of the medium for each batch was around 7.5 but pH dropped to around 5.5 in the end of each assay due to the acetate accumulation, except for the last 3 feedings, where pH was constant. For the alkaline sludge, phosphate was added to the medium in the first feeding, in order to avoid the pH drop. The bicarbonate concentration was the same for all feedings. At the end of the third feeding accumulation of acetate did not manifest, and 73% of the methanol consumed was converted to CH_4 (Figure 4). Results clearly show that when operating in batch mode under proper conditions, i.e., neutral pH and low pH_2 values, growth of methanogens proceeded and they could compete with (homo)acetogens, despite the apparent growth rate in our mixed culture was slightly higher for acetogens than for methanogens under sufficient bicarbonate conditions (data not shown). The conversion pattern was similar as found by Cord-Ruwisch and Ollivier [22] in experiments with the coculture of *S. acidovorans* and *Methanospirillum hungatei*.

The sludge exposed to acidic conditions was more difficult to recover. When acetogens were outcompeting methanogens (feedings 1-6), the degradation process in each batch lasted much longer than found for the sludge exposed to the alkaline sludge under the same conditions. However, when the sludge recovered its methanogenic activity (feeding 7-8 acidic sludge and feed 3 for alkaline sludge), the degradation lasted about the same period of time for both sludges (6 days).

The low reaction rate (6 times longer) and the lower methanol recovery as products compared with the seed sludge (about 20% lower), indicate that the degree of inhibition/inactivation of the microorganisms was high. As a consequence, the period of time and energy required

either to recover activity or to grow also were high. Nevertheless, recovery still is possible, but the time and the number of feedings required will depend on the degree of deterioration of the sludge. For the acidic sludge, for instance, after applying the recovery strategy, the methanogenic activity on methanol was 1.37 ± 0.11 $gCOD.gVSS^{-1}.d^{-1}$, which is somewhat higher than the one found for the seed sludge.

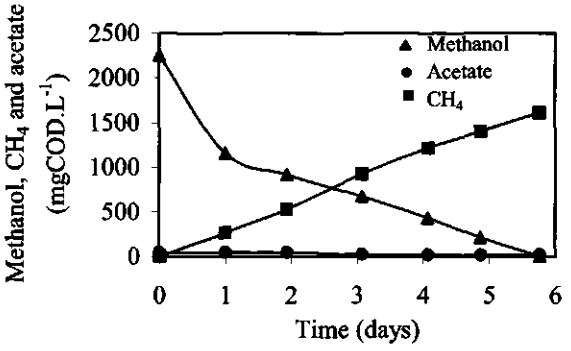


Figure 4 The course of methanol conversion and product formation during the last feeding when operating in batch mode, for tentative reactor recovery using a sludge exposed to an alkaline shock.

Conclusions

- The anaerobic thermophilic conversion of methanol in an automatic pH-controlled UASB-reactor, operated under bicarbonate deprivation is poor and unstable when the system has been exposed to high pH.
- Caution has to be taken when using $NaHCO_3$ for buffering methanol-containing wastewaters. Its introduction in the system will favour acetogenesis when proper conditions had not been established.
- The recovery of methanogenesis on methanol from a reactor upset or total failure is possible, even in systems where (homo)acetogens are outcompeting methanogens, provided that the proper strategy is applied. The time and the number of feedings required will depend on the degree of deterioration of the sludge.

Acknowledgements

This work was supported by "Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq" (Project n° 201055/97-0), an entity from the Brazilian Government for the development of Science and Technology.

6

Pathways of methanol conversion in a thermophilic anaerobic- (55 °C) sludge consortium

Abstract

The pathway of methanol conversion by a thermophilic anaerobic consortium was elucidated by measuring the fate of carbon in the presence and absence of bicarbonate and specific inhibitors. Results indicated that about 50% of methanol is directly converted to methane by the methylotrophic methanogens and 50% via the intermediates H_2/CO_2 and acetate. The deprivation of inorganic carbon species ($\Sigma([HCO_3^-] + [CO_2])$) in a phosphate buffered system, reduced the rate of methanol conversion. This suggests that bicarbonate is required as an "electron" (H_2) sink as well as a cosubstrate for efficient and complete chemical oxygen demand (COD) removal. Nuclear magnetic resonance (NMR) spectroscopy technique was used to investigate the route of methanol conversion to acetate in bicarbonate sufficient and bicarbonate depleted environments. The proportions of [1,2-^{13}C] acetate, [1-^{13}C]acetate, and [2-^{13}C]acetate were determined. Methanol was preferentially incorporated into the methyl group of acetate, whereas HCO_3^- was the preferred source of the carboxyl group. A small amount of the added $H^{13}CO_3^-$ was reduced to form the methyl group of acetate and a small amount of the added $^{13}CH_3OH$ was oxidised and found in the carboxyl group of acetate when $^{13}CH_3OH$ was converted. The recovery of [^{13}C]carboxyl groups in acetate from $^{13}CH_3OH$ was enhanced in bicarbonate deprived medium. The small amount of label incorporated in the carboxyl group of acetate when $^{13}CH_3OH$ was converted in the presence of BESA, indicates that methanol can be oxidised to CO_2 prior to acetate formation. These results indicate that methanol is converted through a common pathway (Acetyl-CoA), on the one hand being reduced to the methyl group of acetate and on the other hand oxidised to CO_2, with CO_2 being incorporated as the carboxyl group of acetate.

Paulo, P.L., A. J. M. Stams, J. A. Field, C. Dijkema, J.B van Lier and G. Lettinga (2002) *Submitted*

Introduction

Insight into the flow of carbon is essential for a good understanding and optimisation of the anaerobic wastewater treatment processes. The insights can be utilised to predict the effect of environmental conditions on system stability and product formation.

Several industrial waste streams contain high levels of methanol such as evaporative condensates from the pulp- and paper-industry [78]. Methanol may also be formed by natural conditions as an intermediate in the decomposition of organic matter [46, 103]. Methanol also represents a cheap electron donor for biological processes such as denitrification, sulfate reduction or reductive dechlorination [136]. The first report of methanol degradation under anaerobic conditions was in 1979 in a study by Lettinga et al [70].

Although methanol is a simple C1- compound, it supports a complex web of possible degradation routes under anaerobic conditions as indicated in Table 1 and Figure 1.

Table 1 The reported reactions and estimated free energy changes possibly involved in the anaerobic degradation of methanol at 55°C.

Reaction	$\Delta G'_{55°C}$ kJ/reaction
1 - $4\,CH_3OH \rightarrow 3\,CH_4 + HCO_3^- + H^+ + H_2O$	-326
2 - $CH_3OH + H_2 \rightarrow CH_4 + H_2O$	-113
3 - $4\,CH_3OH + 2\,HCO_3^- \rightarrow 3\,CH_3COO^- + H^+ + 4\,H_2O$	-221
4 - $CH_3OH + 2\,H_2O \rightarrow 3\,H_2 + HCO_3^- + H^+$	13
5 - $2\,HCO_3^- + 4\,H_2 + H^+ \rightarrow CH_3COO^- + 4\,H_2O$	-90
6 - $HCO_3^- + 4\,H_2 + H^+ \rightarrow CH_4 + 3\,H_2O$	-125
7 - $CH_3COO^- + 4\,H_2O \rightarrow 2\,HCO_3^- + 4\,H_2 + H^+$	90
8 - $CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-35

Methanol can be directly metabolised by methanogens and (homo)acetogens. However, it can also be oxidised to H_2 and CO_2 provided a low pH_2 is sustained by hydrogenotrophic methanogens (Table 1). The degradation route of methanol and its final fate in an anaerobic environment depend on specific environmental conditions as well as the history of the anaerobic consortium. Florencio et al. [29] and Gonzalez-Gil et al. [40] assessed the metabolic route of methanol degradation in anaerobic sludge under mesophilic conditions by using specific inhibitors. Their results indicated that methanol is converted directly to methane by the methylotrophic methanogens. A syntrophic route via intermediates H_2/CO_2 followed by hydrogenotrophic methanogenesis does not appear to be an important route during methanol degradation under mesophilic conditions [29, 40]. According to a review of Weijma and Stams [136], direct methanogenesis from methanol seems to be the predominant mineralisation route under mesophilic conditions both in the absence and the presence of

sulphate. By contrast, at higher temperature syntrophic conversion likely predominates [23, 135]. Our preliminary studies (Chapters 2 and 3) indicate that the conversion via the intermediaries H_2/CO_2 and acetate play a role in the conversion of methanol to methane.

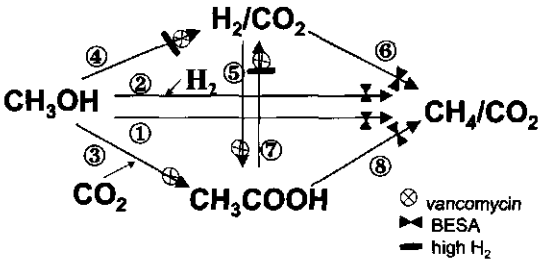


Figure 1 The diagram of blockage of potential individual pathway of methanogenic metabolism of methanol by inhibitors. Reactions representing each of the pathways are presented in Table 1.

High-rate anaerobic digestion of evaporate condensate with methanol concentrations ranging from 1.5 to 24.5 $g.L^{-1}$ has been studied [77, 78, 80, 142]. Process instability and/or volatile fatty acids (VFA) accumulation are often reported when treating methanolic wastewaters. In previous studies (Chapter 2) we were capable to achieve a good conversion of methanol to CH_4 under thermophilic (55 °C) conditions in a bicarbonate-supplied medium.

The purpose of this study was to elucidate the pathways of methanol conversion under thermophilic (55 °C) conditions in a granular sludge mixed consortium from a thermophilic methanol degrading bioreactor. Nuclear magnetic resonance (NMR) spectroscopy technique was used to analyse the incorporation routes of methanol into acetate.

Materials and methods

Biomass

All experiments were performed with freshly collected methanogenic granular sludge cultivated in a lab scale upflow anaerobic sludge blanket (UASB) reactor operated at 55 °C using methanol as sole organic substrate in a mineral bicarbonate buffered medium (Chapters 2 and 3). The sludge bed was gently mixed before sampling to obtain a representative and homogenous sample. Sampled sludge was rinsed with anaerobic pre-heated (55 °C) medium to remove residual carbon source.

Activity assays

The presence of specific bacterial subpopulations in the sludge was studied by using batch activity tests to which specific inhibitors (30 mM of bromoethanesulfonic acid - BESA and/or 0.25 $g.L^{-1}$ vancomycin) or specific conditions were applied. Figure 1 describes the general strategy applied for blocking certain specific reactions.

Experimental set-up

120-ml glass serum vials were used when the substrate was methanol or acetate and 250-ml bottles with H_2/CO_2 as substrate. Serum vials and bottles were filled with 50 ml basal medium as described in Chapter 2. Before adding the sludge and substrate, all vials and bottles containing basal medium were incubated in a water bath with shaker (TUV, GLF 1083, Germany) at 55 °C and 50 rpm. When H_2/CO_2 was used as the substrate, shaking speed was 100 rpm and the bottles were placed horizontally in the waterbath, to optimise mass transfer of hydrogen from the gas to the liquid phase. To ensure pH stability, 80 mM $NaHCO_3$ was added to the basal medium. When an assay required bicarbonate deprived medium, phosphate buffer (72 mM, pH 7.2) was used instead and headspace was flushed with pure N_2 or pure H_2, depending on the assay. Washed sludge was added into the bottles at a volatile suspended solids (VSS) concentration of about 2 $g.L^{-1}$ beneath the liquid surface by means of a 5 ml automatic pipette (Gilson, Villiers, France), with a plastic tip of which the narrow opening was cut off. Methanol (37 mM) or acetate (33 mM) was supplied, to provide a concentration of around 1.8 – 2.0 g $COD.L^{-1}$. The vials were sealed with butyl rubber stoppers and the gas headspace was flushed for 5 minutes with N_2/CO_2 (70:30), pure N_2 or pure H_2 depending on the assay. When H_2/CO_2 was the substrate, the headspace was flushed with 1.05 atm of H_2/CO_2 (80:20), equivalent to 2 g $COD.L^{-1}$. Liquid and gas samples were taken periodically to analyse substrate consumption and product formation. The pH, as well as the amount of VSS in each bottle was measured after completion of the test. The methanol depletion rate, specific acetogenic activity (SAA) and specific methanogenic activity (SMA) were calculated from the linear decrease or increase of the different compounds in the vials, divided by the amount of VSS present in each bottle, measured by the end of the experiment. All assays were performed in triplicate, using the bottles without substrate as blank.

NMR experiments

To assess the route of acetate formation, 47 mM $^{13}CH_3OH$ and 80 mM $NaH^{13}CO_3$ (final concentrations, 99% ^{13}C) were used in several combinations with unlabelled substrates (details are presented in Table 3). Basal medium (12 ml) was distributed into 30-ml vials. Excess phosphate (80 mM) was used in all experiments (except in the one where reactor conditions were simulated). The vials headspace was flushed with N_2/CO_2 (70:30) except for the bicarbonate deprived treatment where pure N_2 was used instead. At the end of the experiment the pH was raised to 12 by injecting a concentrated NaOH solution into the assay bottle before withdrawing the sample, in order to absorb all CO_2 into the liquid phase. To verify methanol consumption and product formation over time, a complete set of experiment with the same conditions was run with unlabelled substrates. All experiments were performed in duplicate. Conditions and procedures were the same as those described previously for the pathway conversion assay, unless otherwise stated. At time 0, after 5 days of experiment and

after complete depletion of methanol (7 days), 500 µL samples were withdrawn for analysis. Samples were centrifuged at 17 000 x *g* for 10 minutes and 50 µL of D_2O was added to 450 µL of centrifuged sample. Samples were stored at -20 °C until NMR analysis. Prior to analysis, samples were defrosted and transferred to 5 mm o.d. NMR-tubes containing 80 µL of 500 mM dioxane solution. The proton-decoupled ^{13}C-NMR-spectra of the samples were recorded at 125.76 MHz on a AMX-500 NMR spectrometer (Bruker, Germany). For each spectrum 10,000 transients (4 h) were accumulated and stored on disc using 32 K data points, a 45° pulse angle (pulse duration 5 µs) and a delay time of 1.5 seconds between the pulses. The measuring temperature was maintained at 20 °C and the chemical shift belonging to the dioxane carbon nuclei (67.4 ppm) was used as an internal standard. The deuterium in the samples (8.6% v/v) was used for field lock. A balance of ^{13}C-labelled compounds was calculated by relating the areas of the observed resonances to the areas in the spectrum of a sample containing 10 mM $^{13}HCO_3^-$ and 300 mM of acetate (1.11% natural abundance) measured under identical conditions with dioxane (500 mM) as an internal standard.

Analysis

A detailed description of the analytical procedures has been presented in Chapter 2.

Chemicals

All chemicals were of analytical grade. Most of them were obtained from Merck (Darmstadt, Germany). Vancomycin was obtained from Acros Organics (Geel, Belgium) and BESA from Sigma chemical Co. (St. Louis, Missouri, U.S.). The ^{13}C labelled compounds were obtained from Isotec (Miamisburg, Ohio, U.S).

Results

Methanol conversion by thermophilic sludge

Figure 2 shows the effect of the different conditions imposed on the rate of methanol consumption and the product formed. Without addition of inhibitors and in the presence of bicarbonate, methanol was completely converted to CH_4 within one day, without accumulation of acetate. With vancomycin present in the medium, no lag phase was observed but total conversion of methanol took double time than the latter. When the inhibitor was BESA, about 2 days of lag phase was observed before acetate production started.

The effect of bicarbonate deprivation and phosphate addition on the rate of methanol consumption and product formation is shown in Figure 3.

When the medium was bicarbonate deprived and phosphate buffered, and the bottle headspace was flushed with pure N_2, methanol conversion took 7 days, where 2/3 of the recovered products were methane and one third was acetate. The addition of pure H_2 in the

headspace caused serious inhibition in the methanol conversion and almost no reaction was observed.

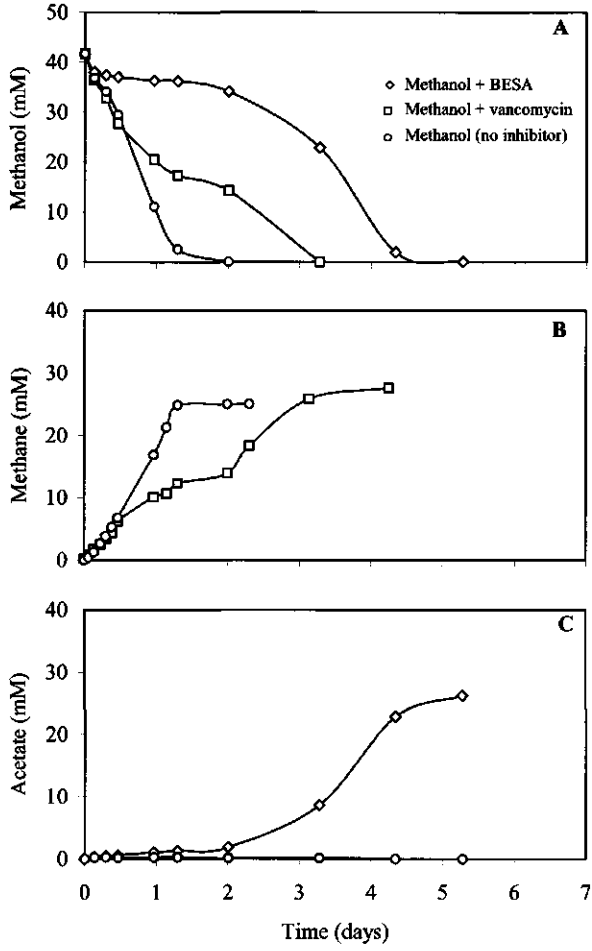


Figure 2 The evolution of the methanol depletion and product formation by the mixed cultivated consortium, under different experimental conditions in a bicarbonate buffered system (80 mM $NaHCO_3$ and N_2/CO_2 in the headspace). (**A**) Methanol (**B**) Methane and, (**C**) Acetate (methane was not detected when BESA was present in the medium and acetate was not detected in the presence of vancomycin).

Table 2 presents the methanol depletion rate, specific activity of methanogens and acetogens in the mixed culture for methanol, acetate and H_2/CO_2 for all conditions tested as well as a COD balance. When no inhibitors were applied, methanogenic activity was detected for all substrates tested. Compared with the methanogenic activity on methanol, the activity with H_2/CO_2 was 2 times higher and the activity with acetate was somewhat lower. The addition of

vancomycin to the medium did not affect the methanogenic activity on H_2/CO_2. However, when methanol was the substrate, methanogenic activity dropped to half when vancomycin was present. The methanogenic activity remained unchanged when the latter test was performed in the presence of surplus H_2.

When H_2/CO_2 was the substrate and BESA was present, half of the substrate was consumed but only 1.7 mM of acetate was detected in the end of the experiment.

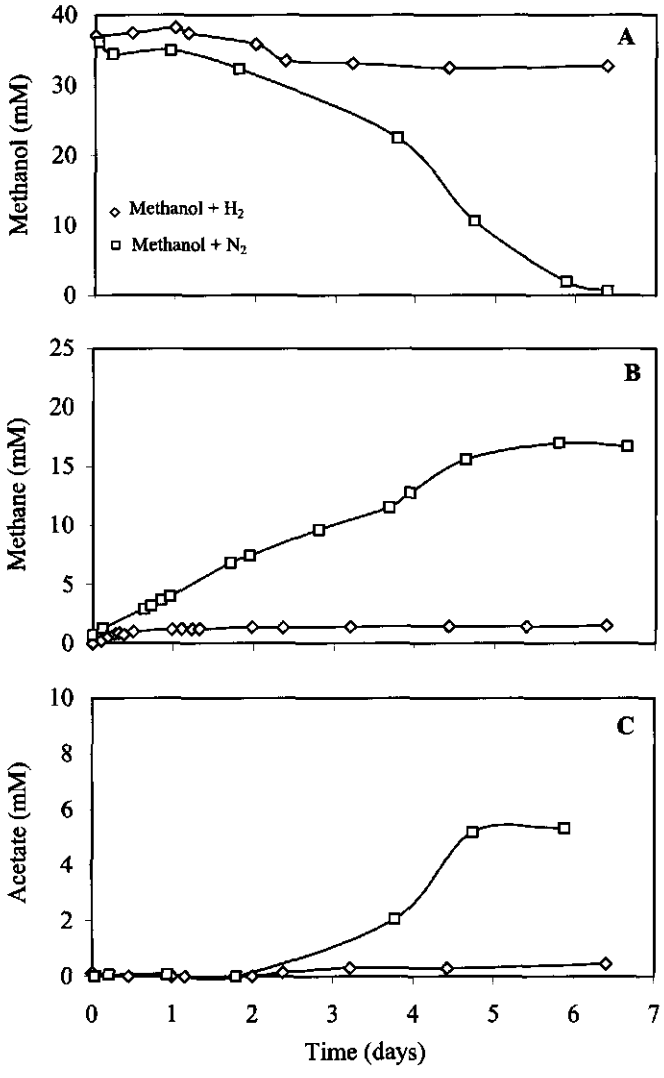


Figure 3 The evolution of the methanol depletion and product formation by the mixed cultivated consortium, under different experimental conditions in a phosphate buffered system (72 mM) deprived of inorganic carbon specie. **(A)** Methanol **(B)** Methane and, **(C)** Acetate.

Acetate build-up, in considerable amounts, was only observed when methanol was used in bicarbonate-deprived medium buffered with phosphate or when BESA was supplied. In the latter case, the net acetogenic activity was about 50% of the methanogenic activity (based on COD) measured when no inhibitor was applied, and the shape of the curve resembled a growth curve. Accumulation of traces of H_2 was observed for all experiments using BESA and when medium was bicarbonate deprived.

NMR Experiment

The amounts of ^{13}C label incorporated into the methyl and carboxyl positions of acetate were determined (Table 3). With $^{13}CH_3OH$ and HCO_3^-, the ^{13}C of methanol was mainly incorporated into the methyl of acetate (84% of the total label in acetate) but also some label was found in the carboxyl group (16% of the total label in acetate). When $H^{13}CO_3^-$ was used together with CH_3OH the ^{13}C of bicarbonate was predominantly incorporated into the carboxyl group of acetate (82% of label in acetate) but also 18% of ^{13}C in acetate was found in its methyl group. The percentage label in the carboxyl group of acetate increased dramatically to 51% when the experiment was performed with $^{13}CH_3OH$ in a bicarbonate-deprived medium, while 67% of the acetate was labelled in both carbons. In the control experiment, where both substrates were labelled, 60% of acetate was labelled in the methyl group and 40% of the acetate was labelled in the carboxyl group. Only 53% of the acetate produced was labelled in both carbons. The substrates used were at minimum 99% ^{13}C enriched, meaning that the possibility of getting double-labelled acetate was 98%. For all treatments, except for the case where bicarbonate was deprived, bottles headspace contained 30% of $^{12}CO_2$ gas, which might have contributed to the formation of acetate with unlabelled group. A certain endogenous contribution of carbon from the sludge, e.g. $CaCO_3$ or sludge substrate mineralised to CO_2 can also be considered. The later addition of BESA plus a pool of bicarbonate (either labelled or unlabelled) did not significantly alter the total amount of acetate formed or the fraction distribution of the labels (Table 3). No acetate was detected in the experiment performed without the addition of phosphate (simulating the operational reactor conditions at pH 7). Table 4 shows product formation, substrate remaining and COD recovery at day 5 of the experiment.

Table 2 Methanol depletion rate, specific (homo)acetogenic and methanogenic activity of the cultivated consortium for the specific bacterial subpopulations, and COD balance.

Substrate/ cosubstrate	Inhibitor	Gas in the head-space	Methanol depletion rate mgCOD.gVSS^{-1}.d^{-1}	(Homo)acetogenic activity mgCOD.gVSS^{-1}.d^{-1}	Methanogenic activity mgCOD.gVSS^{-1}.d^{-1}	Substrate remaining	Products formed mgCOD.L^{-1}			Recovery (%)
							CH_4	Acetate	H_2	
Bicarbonate buffered treatments										
1- Methanol + HCO_3^-	-	N_2/CO_2	-1001.0 (81.9)	[a]nd	1113.0 (79.6)	nd	1628.0	nd	0.08	82
2- Methanol + HCO_3^-	Besa	N_2/CO_2	[b]- 439.7 (39.6)	[b]596.8 (41.7)	nd[a]	nd	nd	1797.0	12	92
3- Methanol + HCO_3^-	Vancomycin	N_2/CO_2	- 506.5 (58.7)	nd	512.2 (8.3)	nd	1765.0	nd	0.04	90
4- Acetate + HCO_3^-	-	N_2/CO_2	-	nd	840.6 (46.3)	9.7	1463.0	nd	0.04	74
5 -Acetate + HCO_3^-	Besa	N_2/CO_2	-	nd	nd	1991.0	nd	nd	12	101
6- H_2/CO_2 + HCO_3^-	-	H_2/CO_2	-	nd	2199.9 (209.4)	nd	1995.0	105.0		100
7- H_2/CO_2 + HCO_3^-	Besa	H_2/CO_2	-	nd	nd	1027.0	nd	141.0		59
8- H_2/CO_2 + HCO_3^-	Vancomycin	H_2/CO_2	-	nd	2247.6 (135.9)	nd	1991.0	nd		100
Phosphate buffered treatments										
9- Methanol	-	N_2	-227.1 (25.2)	86.25 (14.7)	157.8 (22.4)	nd	1141.0	336.0	[c]ND	98
10- Methanol + H_2	-	H_2	-72.8 (11.0)	nd	99.9 (4.3)	MeOH-1571 H_2-633	119.0	14.0		86

[a] nd- Not detected

[b] Measurement taken after growth occurred

[c] ND- Not determined.

Table 3 The fractional distribution of ^{13}C atoms found in acetate for the mixed consortium, cultivated on methanol in bicarbonate buffered medium at 55 °C, when exposed to 80mM of phosphate.

	Fractional distribution of ^{13}C atoms in acetate							
Substrate label position	$^{13}CH_3$-COOH	CH_3-$^{13}COOH$	$^{13}CH_3$-$^{13}COOH$	$\Sigma^{13}CH_3$	$\Sigma^{13}COOH$	$\Sigma^{13}CH_3/\Sigma^{13}COOH$	Total recovery[c] as acetate (%)	Total recovery[d] as HCO_3^- (%)
$^{13}CH_3OH + HCO_3^-$	0.74	0.06	0.20	0.84	0.16	5.25	40	35
$CH_3OH + H^{13}CO_3^-$	0.11	0.74	0.14	0.18	0.82	0.20	12	49
$^{13}CH_3OH + H^{13}CO_3^-$	0.34	0.14	0.53	0.60	0.40	1.51	19	37
$^{13}CH_3OH$[a]	0.16	0.17	0.67	0.49	0.51	0.96	6	23
$^{13}CH_3OH$[b] + later addition of HCO_3^- + BESA	0.73	0.08	0.19	0.82	0.18	4.60	37	26
CH_3OH[b] + later addition of $H^{13}CO_3^-$ + BESA	0.07	0.73	0.20	0.17	0.83	0.20	12	52

[a] Medium was bicarbonate deprived.

[b] BESA and bicarbonate were added after 24 h of running experiment.

[c] Total recovery of [^{13}C]carbon in acetate = carbon recovered as [^{13}C]acetate/[^{13}C]carbon added

[d] Total recovery of [^{13}C]carbon in bicarbonate = carbon recovered as [^{13}C]bicarbonate/[^{13}C]carbon added

Label recovered as CH_4 gas was not quantified

Results are based on the last sampling after 7 days of experiment, when methanol was depleted

Data represent mean values from duplicate incubations for the different conditions applied and standard deviation represents less 10% of the mean value.

Table 4 Product formation, substrate remaining and COD recovery after 5 days for the NMR experiment when the cultivated consortium was exposed to 80 mM of phosphate buffer.

Substrate/ [a]cosubstrate	Inhibitor	Gas in the headspace	Methanol applied ($mgCOD.L^{-1}$)	Products formed ($mgCOD.L^{-1}$)		Methanol remaining ($mgCOD.L^{-1}$)	[b]Recovery (%)
				CH_4	Acetate		
Methanol + HCO_3^-	-	N_2/CO_2	2263	193	1497	340	90
Methanol + HCO_3^-	Besa	N_2/CO_2	2250	nd	1207	204	63
MeOH	-	N_2	2269	931	257	306	66

[a] Cosubstrate was added as 80 mM of $NaHCO_3$

[b] Recovery = 100 (Acetate + CH_4 + methanol remaining)/methanol applied

For all tests reaction started after 1 day of lag phase.

Discussion

The pathway of methane formation

Preliminary studies had already indicated that both indirect and direct route play a role on the pathway conversion of methanol to methane by our thermophilic cultivated consortium (Chapter 2). However, the results were not sufficient to elucidate the importance of each, and whether the indirect conversion was via H_2/CO_2 or acetate. The drop of 50% of the SMA on methanol (and methanol depletion rate) when vancomycin was present in the medium confirms that about half of methanol is converted directly to methane by the methylotrophic methanogens. The formation of acetate from methanol is only possible when inorganic carbon species are present as cosubstrate [72]. The fact that acetate could be formed without inorganic carbon addition clearly indicates endogenous sources of bicarbonate either from the production of CO_2 (Reactions 1 or 4) or from carbonates in the sludge or mineralisation of sludge organic matter. Acetate is certainly an intermediate in the conversion of methanol to methane by the cultivated consortium. The relatively high SMA on acetate as substrate, and the delay and severe inhibition on the methanol conversion when medium was phosphate buffered and bicarbonate deprived confirm that. The accumulation of acetate when acetotrophic microorganisms are inhibited by phosphate is also a strong indication that acetate is always formed but quickly consumed when comparing the results with the bicarbonate supplied (no phosphate added) medium. However, the growth-like curve observed after about 2 days of lag phase when the medium was supplied with BESA, suggests growth other than activity, since methanol was not straightway converted into acetate. Indicating then, that the initial population of methylotrophic acetogens is rather small and therefore, the indirect conversion is not represented only by Reaction 3.

Addition of BESA may also have hindered the conversion of methanol to H_2/CO_2 since it blocks the hydrogenotrophic methanogens, that are of utmost importance to keep the low pH_2 during the conversion of methanol to H_2/CO_2. The importance of Reaction 1 on the indirect conversion of methanol was evident when excess H_2 was supplied to a bicarbonate-deprived medium in the presence of phosphate. In this case, the effect caused on methanol conversion was much stronger than the effect on the bicarbonate deprived medium alone. This fact clearly reveals that from the indirect pathway, a greater part of methane formed in the system depends on the oxidation of methanol to H_2/CO_2. This fact can also be supported by the 2.6 times higher SMA on H_2/CO_2 than the SMA on acetate.

Minor pathways

Methanol conversion to hydrogen may lead to complex metabolic interactions between microorganisms in mixed cultures [136]. At a high hydrogen partial pressure (homo)acetogens reduce CO_2 with H_2 to form acetate [139] while the bacteria which are able to oxidise acetate to CO_2 and H_2 were shown to be (homo)acetogens as well [149]. Thus, the (homo)acetogenic pathway is largely reversible. In a mixed culture and balanced consortium, pH_2 is expected to be low to enable the methanol oxidation to H_2 and CO_2. As a high hydrogen partial pressure is required for the reduction of CO_2 with H_2 to acetate by (homo)acetogens, the oxidation of methanol to H_2 and CO_2 followed by (homo)acetogenesis from H_2 and CO_2 is not likely to occur [134]. This could be confirmed by the absence of (homo)acetogenic activity when the substrate was H_2/CO_2, even when BESA was present in the medium. Nevertheless, some acetate was detected in the end of the experiment. H_2/CO_2 and methanol were utilised to estimate the apparent specific growth rate of the (homo)acetogens present in our mixed consortium. The (homo)acetogens were not able to convert H_2/CO_2 after 14 days of incubation, whereas methanol was completely depleted within 6 days of experiment (data not shown). The growth with H_2/CO_2 has been reported for nearly all (homo)acetogens, except for *Clostridium formicoaceticum*, *Syntrophococcus sucromutans*, strain TMBS4, and *Clostridium magnum* [100]. Rapid conversion of H_2/CO_2 to acetate was noted in mesophilic methanol-degrading anaerobic sludge consortia [29]. It seems that for our cultivated consortia Reaction 5 does not play an important role.

The reversal of the acetate formation reaction to form hydrogen and CO_2 has been documented for the thermophilic strain AOR [65]. Recently, two new thermophilic (homo)acetogenic bacteria able to convert acetate syntrophically were isolated. *Thermoacetogenium phaeum* gen. nov. sp. nov. [45] and *Thermotoga Lettingae* sp. nov., [6]. The syntrophic conversion of acetate to methane via H_2/CO_2 is reported as the major metabolic pathway under thermophilic and extreme thermophilic conditions [148, 151, 152]. The acetate consumption during the experiment with BESA seemed to be inhibited by the built up H_2 in the headspace, indicating that a syntrophic association between acetate

oxidisers and H_2 – utilising methanogens could be responsible for some portion of the overall acetate elimination by the cultivated consortium. However, if the contribution of Reaction 7 was significant, acetate accumulation would not be detected (or would be very low) in the presence of phosphate, since phosphate seems to inhibit the acetoclastic methanogens but not the hydrogenotrophic methanogens and (homo)acetogens, which has been also observed by Conrad et al. [20]. That would indicate that, the relatively high SMA on acetate (840.6 ± 46.3 $mgCOD.gVSS^{-1}.d^{-1}$), would represent mostly the acetoclastic methanogens and that most part of the formed acetate would be converted directly to methane.

The acetate formation

NMR techniques were applied to investigate the route of methanol conversion to acetate in bicarbonate sufficient and bicarbonate depleted media. Kerby et al. [56], when studying the transformations of $^{13}CH_3OH$ by *Butyribacterium methylotrophicum* with in vivo ^{13}C-NMR, found that methanol in the presence of CO_2, CO or formate, predominantly labels the methyl group of acetate. Their results also showed that a small fraction of methanol is oxidised to CO_2 and subsequently incorporated into the carboxyl group. The results obtained in our investigations are in good agreement with their findings. In a bicarbonate sufficient medium, methanol was preferentially incorporated into the methyl group of acetate, whereas CO_2 was the preferred source of the carboxyl group. In a bicarbonate-deprived medium, the label recovery in the carboxyl group of acetate was enhanced. In addition, a small fraction of the added $H^{13}CO_3^-$ was reduced to the methyl group of acetate and a small fraction of the added $^{13}CH_3OH$ was oxidised to the carboxyl group of acetate. The fact that some methanol label appears in the carboxyl group and some CO_2 label appears in the methyl group, indicates the reversibility of the acetyl-CoA cleavage pathway. Wood [141] also found a randomerisation of the ^{13}C label by *Clostridium thermoaceticum*, indicating that there may be total synthesis of acetate from CO_2. It seems reasonable to assume that our mixed cultivated consortium synthesises acetate through a pathway similar to the one proposed by Kerby et al. [56]. According to their model (Figure 4), single carbon substrates enter a common metabolic route at distinct points and are differentially transformed to acetyl-CoA. Methanol and CO represent the immediate methyl and carbonyl precursors for acetyl-CoA synthesis and are precursors for the synthesis of CO_2. Acetyl-CoA is the direct precursor for acetic acid. The 26-fold higher ratio $\Sigma^{13}CH_3/\Sigma^{13}COOH$ when using $^{13}CH_3OH + HCO_3^-$ compared with $CH_3OH + H^{13}CO_3^-$, reveals that the main flow of carbon is through the shortest part of the proposed model and a small fraction follows a longer way, involving more enzymatic steps. However, when medium is bicarbonate deprived the ratio $\Sigma^{13}CH_3/\Sigma^{13}COOH$ is only 5.5 folds higher comparing $^{13}CH_3OH + HCO_3^-$ with $^{13}CH_3OH$.

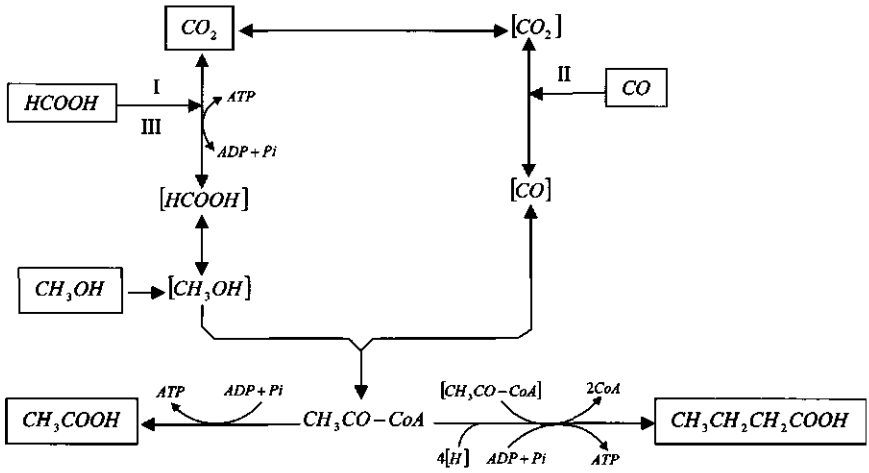


Figure 4 Single carbon catabolism flow model proposed by Kerby et al. [56] for acetogenic bacteria that synthesise acetate or butyrate from single-carbon compounds. This scheme predicts that two distinct formyl-level intermediates, [HCOOH] and [CO] are linked by formate, CO_2, and a carboxyl intermediate [CO_2]. The roman numerals indicate the following enzymatic activities: I, formate dehydrogenase; II, CO dehydrogenase; and III, formyl-THF synthetase.

This is due to the fact that acetate formation from methanol requires bicarbonate. In the absence of externally supplied bicarbonate, acetate formation from methanol is dependent on the bicarbonate formed by methylotrophic methanogens or methanol oxidation to H_2/CO_2. The small amount of label incorporated in the carboxyl group of acetate together with the pool of labelled bicarbonate recovered when $^{13}CH_3OH$ was converted in the presence of BESA, indicate that methanol can be oxidised to CO_2 prior to acetate formation.

General implications

It is clear that, independently of the main route, all routes (with a much lesser extent to Reactions 5 and 7) play an important role on the conversion of methanol for our cultivated consortium. The pathway of methane formation in methanogens has many similarities with the pathway of acetate synthesis in (homo)acetogens. According to Ljungdahl [72] in methanogens, the acetyl-CoA is used for the synthesis of cell carbon in reactions similar to those used by the (homo)acetogens. In a study on methanol conversion by *Eubacterium limosum* Van der Meijden et al [129], proposed that methylotrophic acetogens and methylotrophic methanogens use similar enzymes in the first step of methanol conversion. In the case of the present sludge, where half of methanol is converted to methane by the methylotrophic methanogens and half depends on the methylotrophic acetogens for a first

step before conversion to methane, interactions are quite complex. Pacaud et al. [90] reported that the growth of *E. limosum* B2 on methanol-CO_2 was regulated by the HCO_3^- concentration in the medium. As the syntrophic conversion of H_2/CO_2 is also involved in our process, small changes in the hydrogen partial pressure may have a strong impact on substrate conversion rates [60]. The balance among the microorganisms present in the sludge is of paramount importance since it determines the reaction that governs the pathway.

Acknowledgements

This work was supported by "Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq" (Project n° 201055/97-0), an entity from the Brazilian Government for the development of Science and Technology.

7

The effect of cobalt on the anaerobic thermophilic conversion of methanol

Abstract

The importance of cobalt on the anaerobic conversion of methanol under thermophilic conditions was studied in three parallel lab scale UASB-reactors. Reactors R1, R2 and R3, were fed with methanol in a bicarbonate-buffered medium, supplied with iron and macronutrients: R1 all metals supplied (control); R2: cobalt deprived; R3: all metals deprived. In the 136 days of continuous experiment, a drop in the performance was observed during the last 30 days, particularly to reactor R3, where both methanol removal and methane formation dropped by 6.4% and 14%, respectively, as compared to the control-reactor R1. When the medium was cobalt deprived, acetate was not produced and the enriched consortium lost its capacity to degrade acetate, indicating that the acetotrophic microorganisms were washed out. The addition of 0.5 μM of cobalt to a cobalt-deprived enrichment culture, lead to acetate accumulation. The results obtained in this study indicate that the mixed consortium demands a proper amount of cobalt, and its addition to a concentration of 0.1 μM leads to the highest methanol conversion rate with methane as sole end product from methanol.

Paulo, P.L., B. Jiang, D. Cysneiros, A. J. M. Stams and G. Lettinga (2002) *Submitted*

Introduction

Trace metals are crucial for the performance of biological waste and wastewater treatment. All methanogens that have been tested so far, appear to require iron, nickel and cobalt for growth [47, 95, 99, 108, 119, 120]. Iron is also reported to enhance sludge granulation [89, 107]. The requirement of trace elements has been studied for a wide variety of substrates using a diversity of cultures such as granular sludge, biofilms, cocultures and pure cultures. The optimal concentrations found, vary enormously [7, 40, 50, 57, 61, 81, 105, 118]. Such variations are explained by the variety of methanogens, each having unique trace metals requirement, which likely also depends on the type of substrate utilised. Although the specific activity of biomass depends upon many factors, the lack of a single trace element can severely limit the overall process [111]. On the other hand, a higher concentration may lead to toxicity, due to the formation of unspecific complex compounds in the cell [85], resulting in inhibition of methanogens [10, 26]. The effect of cobalt on the anaerobic degradation of methanol under mesophilic conditions has been studied by Florencio et al. [29, 31]. Methylotrophic methanogens predominated in their consortium and they found that cobalt greatly enhanced both methanogenesis and acetogenesis from methanol. The optimal concentration of cobalt found for growth and activity of methanol utilising methanogens and acetogens was 0.85 μM.

In our studies dealing with the conversion of methanol under thermophilic conditions (55 °C) using sodium bicarbonate as a buffer and with sufficient micro and macronutrients, we have achieved a high reactor performance, with good sludge granulation and low volatile fatty acids (VFA) accumulation (Chapters 2 and 3). The metals were added in excess to ensure availability to microorganisms, even when eventual metals precipitation or chelation could take place. In the operation of a continuous reactor, a much lower metal concentration may be required than the amount required in a batch system, because more biological ligands may be produced [8, 64]. According to Gonzalez et al. [40] the continuous addition of nutrients ensures free metal availability for the biomass. The concentration of cobalt used in our continuous experiment mentioned above was 8.4 μM. That is 10 times higher than the optimum found by Florencio et al. for methylotrophic methanogens that, due to the high amount of corrinoids content [62] are known to demand much higher amount of cobalt than the other methanogenic species.

In the present study, we assessed the effect of cobalt deprivation and the influence of its reintroduction to the medium on the competition between (homo)acetogens and methanogens on methanol degrading thermophilic mixed consortium.

Material and methods

Continuous experiment

The experiments were conducted in three 0.3 L-UASB reactors operated for 136 days. The reactors were immersed in a glass waterbath (Julabo- MB-Basis, Germany) maintaining the reactors temperature at 55 °C. Biogas was collected and led through a waterlock filled with a 20% NaOH solution and a column filled with soda lime pellets with indicator to remove CO_2 from the gas. Subsequently, the gas was passed through a Mariotte flask system containing water for quantification of the methane (CH_4) production. The displaced water was collected in plastic containers. The reactors were inoculated with a methanogenic thermophilic (55 °C) granular sludge cultivated in a lab scale UASB reactor, which was fed with methanol as the sole organic carbon source and buffered with bicarbonate (Chapters 2 and 3). The reactors were inoculated with 7 g volatile suspended solids (VSS) of sludge each.

The influent was pumped into the reactors with a peristaltic pump (Watson-Marlow 505S, Falmouth Cornwall, UK). Basal medium was introduced in the influent line using a vertical axis peristaltic pump (Gilson Minipuls 3, France). The bottom of the reactors was filled with glass marbles to ensure uniform influent distribution in the reactors. The 3 reactors were operated under identical conditions except for the trace elements composition. Reactor R1 was supplied with all metals. Reactor R2 was deprived of cobalt addition and reactor R3 was deprived of all metals except for iron, which was present in all media. Methanol was used as sole organic carbon source. The concentration in the stock solution varied according to the desired organic loading rate (OLR). To ensure pH stability, $NaHCO_3$ (0.33 g) was added per 1-g methanol L^{-1}. The reactors were supplemented with macro and micronutrients. 2.22 ml of a nutrient stock solution was supplied for each gram influent $COD.L^{-1}$, the stock solution contained (mM): NH_4Cl (140), K_2HPO_4 (12), $MgSO_4.7H_2O$ (6), $CaCl_2.2H_2O$ (2), vitamin solution (10 ml), iron solution (6.5 ml) and trace elements solution (4.5 ml). Iron solution contained (mM): $FeCl_2.4H_2O$ (761). The trace elements solution contained (mM): H_3BO_3 (100), $ZnCl_2$ (54), $CuCl_2.2H_2O$ (9), $MnCl_2.4H_2O$ (21), $Na_2MoO_4.2H_2O$ (37), $CoCl_2.6H_2O$ (75), $NiCl_2.6H_2O$ (100), Na_2SeO_3 (8). The solutions also contained ($g.L^{-1}$): EDTA (10), Resazurine (2) and HCl 36% (1%). The vitamin solution contained ($mg.L^{-1}$): biotin (20), niacin (200), pyridoxine (500), riboflavin (100), thiamine (200), p-aminobenzoic acid (100) and pantothenic acid (100). The solution used for reactor R2 had the same composition except for cobalt, which was deprived. The 3 reactors were started up at an OLR of about 3.5 $gCOD.L^{-1}.d^{-1}$, hydraulic retention time (HRT) of 8 h and a methanol concentration of 1.4 $gCOD.L^{-1}$. The OLR was stepwise increased until 9.8 $gCOD.L^{-1}$, by increasing the methanol concentration and decreasing the HRT. For all reactors, CH_4 production was continuously measured. The HRT was calculated based on the flow rate of the effluent. Influent and effluent samples were taken twice per week to analyse the methanol

and VFA concentration. The biogas composition was measured every 15 days or when the OLR was increased.

Batch experiments

Specific methanogenic activity (SMA) tests were conducted to assess the effect of cobalt and metals deprivation and the addition of cobalt after long term deprivation on the cultivated culture. The effect on the competition between methanogens and acetogens was also assessed. Activity tests were performed with the seed sludge and sludge sampled from the 3 reactors at the last day of the continuous experiment (day 136).

30-ml glass vials were used when the substrate was methanol or acetate and 250-ml bottles when the substrate was H_2/CO_2. Vials and bottles were filled with 12 ml and 50 ml, respectively of basal medium containing (mM): $NaHCO_3$ (80), NH_4Cl (5.2), $K_2HPO_4.3H_2O$ (1.1), $MgSO_4.7H_2O$ (0.4), $CaCl_2.2H_2O$ (0.1), trace elements solution (100 µL), iron solution (250 µL) and vitamin solution (1000 µL). Final pH was neutralised to 7.0 by adding a concentrated HCl solution. Before adding the sludge and substrate, all vials and bottles containing basal medium were incubated in a waterbath with shaker (TUV, GLF 1083, Germany) at 55 °C and 50 rpm. When H_2/CO_2 was used as the substrate, shaking speed was 100 rpm and the bottles were placed horizontally in the waterbath, in order to optimise mass transfer of hydrogen from the gas to the liquid phase. Washed sludge was added to the vials and bottles to a VSS concentration of about 2 $g.L^{-1}$ beneath the liquid surface by means of a 5 ml automatic pipette (Gilson, Villiers, France), with a plastic tip of which the narrow opening was cut off. Methanol (37 mM) or acetate (33 mM) was added as the substrate, to provide a concentration of about 2.0 $gCOD.L^{-1}$. The vials were sealed with butyl rubber stoppers and the gas headspace was replaced with N_2/CO_2 (70:30), using a Manifold Gas Exchanger System. When H_2/CO_2 was the substrate, the headspace was replaced with 1.05 atm of H_2/CO_2 (80:20), equivalent to 2 g $COD.L^{-1}$. Liquid and gas samples were taken periodically to analyse substrate consumption and product formation. The pH, as well as the amount of VSS in each bottle was measured after the test was completed. The SMA was calculated from the linear increase of the CH_4 concentration in the beginning of the experiment, when no lag phase was observed, divided by the amount of VSS. All assays were performed in triplicate, using bottles without added substrate as blank.

Enrichment culture

A cobalt-limited culture was enriched starting from freshly grown methanogenic thermophilic sludge (55 °C) which was the same used as seed sludge for the reactors. The sludge was crushed under anaerobic conditions and transferred to 120-ml serum vials containing 50 ml of a sterile anaerobic medium as described by Stams et al. [113], which was cobalt deprived. Medium also contained (L^{-1}): acid trace elements solution (1000 µL), alkaline trace elements

solution (1000 µL), acid nickel solution or acid cobalt solution (1000 µL) (or both, depending on the experiment), and vitamin solution (1000 µL). The acid trace elements solution contained (mM): $FeCl_2.4H_2O$ (7.5), H_3BO_3 (1.0), $ZnCl_2$ (0.5), $CuCl_2.2H_2O$ (0.1), $MnCl_2.4H_2O$ (0.5) and HCl (50). The alkaline trace elements solution contained (mM): $Na_2MoO_4.2H_2O$ (0.1), $Na_2WO_4.2H_2O$ (0.1), Na_2SeO_3 (0.1) and NaOH (10). The acid nickel solution contained (mM): HCl (50) and $NiCl_2.6H_2O$ (0.1). The acid cobalt solution contained (mM): HCl (50) and $CoCl_2.6H_2O$ (0.5). The vitamin solution was the same as used for the continuous experiment. Methanol was added at a concentration of 28 mM and the gas headspace was replaced with 1.7 atm of N_2/CO_2 (70:30). Bottles were incubated at 55 °C and transfers were consecutively made after total methanol depletion in the bottles, with a total of 7 transfers. Methane formation was still observed, but time between the transfers increased progressively from 7 days (first transfer) until 90 days (last transfer).

To assess the culture behaviour on the reintroduction of cobalt to the medium, the cobalt-deprived enriched culture was transferred (10% v/v) to 250-ml bottles containing 45 ml of fresh medium. Cobalt concentrations varied: 0, 0.5 and 40 µM. Methanol (117 mM final concentration) was added as the substrate. This experiment was performed in quadruplicate. A second experiment was performed to find the optimal cobalt concentration for the cobalt-deprived enrichment culture. Procedures were the same except for the cobalt concentrations used, which were 0.01, 0.05, 0.1 and 0.2 µM. This experiment was performed in triplicate. For both experiments, samples, 300 µL of liquid and 200 µL of gas were taken periodically to analyse substrate consumption and product formation.

To minimise cobalt contamination, all glassware used was soaked for 3 days in a 4 M HNO_3 solution and rinsed abundantly with nanopure water before use and, all solutions were prepared with nanopure water. all chemicals used were of analytical grade and most of them were purchased from Merck (Darmstadt, Germany).

Analysis

A detailed description of the analytical procedures for determination of methanol, VFA, biogas composition, hydrogen, CH_4, VSS, total suspended solids (TSS) and ash has been presented in Chapter 2. The cobalt, nickel and iron content of the sludge were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS), Elom 6000, Perkim-Elmer, as described by Standard Methods [5]. The samples were dried at 40 °C, diluted with aqua-regia ($HCl:HNO_3$ – ratio 3:1) prior to digestion as described by Veeken [130].

For the enrichment culture, CH_4 and H_2 were determined in a Chrompack 9001 gas chromatograph. Injection volume was 200 µL. A PlotFlused 30 M* 0.53 mm silica column was used packed with Molsieve 5A (DF= 15 µm). The temperatures of the column, injection

port and thermal conductivity detector were 50, 60 and 130 °C, respectively. Argon was used as carrier gases (20 ml min^{-1}) and pressure was 150 kPa.

Results

Continuous experiment

The operation of the 3 reactors was divided into 3 phases according to the OLR applied, and the performance results are summarised in Table 1. The deprivation of cobalt or all metals in the medium did not show too much effect during the 136 days of the trial. The performance of reactor R3 (no metals supplied) was affected the most. The methanol removal efficiency decreased slightly in phase II while in phase III it was most pronounced.

Table 1 Performance of reactors R1, R2 and R3 operated during 136 days with methanol as substrate and different metal composition in the medium.

Period (days)		PHASE I 0 - 72			PHASE II 73 - 93			PHASE III 94 - 136		
		R1[a]	R2[b]	R3[c]	R1	R2	R3	R1	R2	R3
OLR	gCOD $L^{-1}.d^{-1}$	4.3	4.3	4.4	6.1	6.2	7.1	9.4	9.2	8.9
		(0.7)	(0.6)	(0.6)	(0.3)	(0.2)	(0.9)	(0.2)	(0.7)	(0.4)
[d]CH_4 formed	% total COD	90.2	92.3	90.2	89.1	89.3	81.4	88.5	85.1	76.5
		(5.4)	(2.9)	(5.6)	(7.7)	(7.8)	(4.3)	(7.2)	(4.5)	(4.2)
[e]Acetate formed	% total COD	3.8	3.3	3.1	4.2	2.8	2.7	1.0	0.5	0.2
		(1.3)	(1.2)	(1.2)	(1.5)	(0.3)	(0.2)	(0.6)	(0.4)	(0.4)
[f]Methanol degraded	% total COD	99.4	99.5	99.5	99.6	99.1	98.1	98.7	97.3	92.4
		(0.7)	(0.3)	(0.3)	(0.2)	(0.8)	(1.5)	(0.8)	(1.3)	(4.4)

[a] All metals supplied to the medium

[b] Medium was cobalt deprived

[c] Medium was all metals deprived

[d] CH_4 (%) = 100 *[methane produced (gCOD d^{-1})/ $Methanol_{in}$ (gCOD d^{-1})]

[e] Acetate (%) = 100 *[acetate produced (gCOD d^{-1})/ $Methanol_{in}$ (gCOD d^{-1})]

[f] Methanol removal (%) = 1- [100 * $Methanol_{ef}$ (gCOD d^{-1})/ $Methanol_{in}$ (gCOD d^{-1})]

All reactors were supplied with iron (Fe)

Standard deviation is given between brackets.

The methanol removal efficiency dropped 7% compared with the one for Phase I. The CH_4 formation dropped 15% compared with Phase I and 13.6% compared to the control reactor in Phase III. During the third phase, the performance of reactor R3 was characterised by

instability. Reactor R2 (all metals supplied except cobalt), had only a slight drop in the performance during Phase III. The concentration of acetate in the effluent dropped for all reactors throughout time. By the end of the experiment, the control reactor (R1) showed a higher concentration of acetate in the effluent, albeit still very low (0.4 mM).

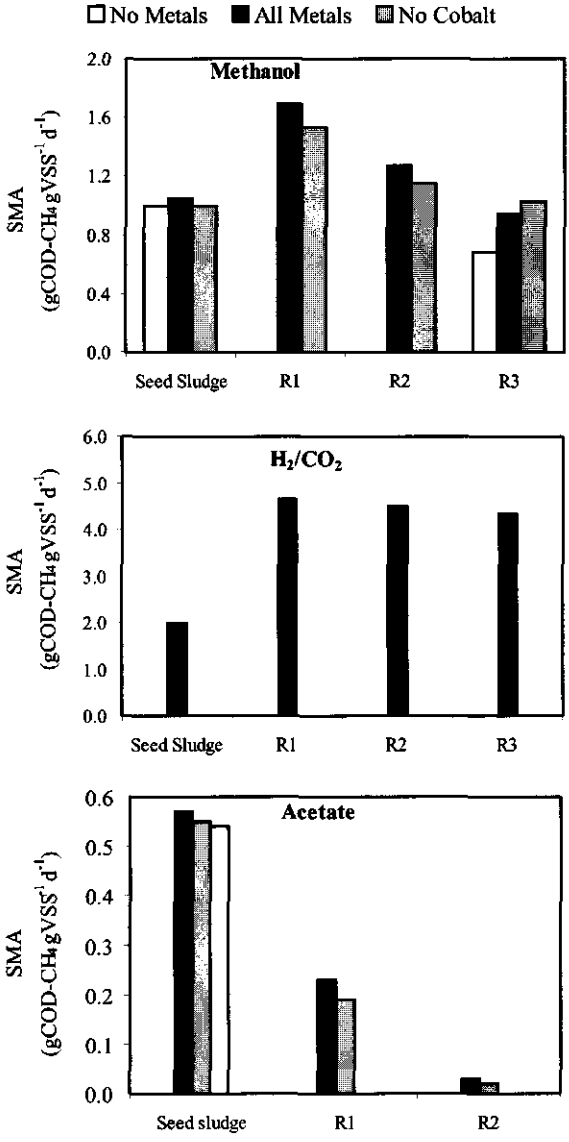


Figure 1 The specific methanogenic activity (SMA) of the seed sludge and the sludges from reactors R1, R2 and R3 at the end of the experiment for different substrates. **R1**: all metals supplied; **R2**: cobalt deprived; **R3**: all metals deprived. Results are mean of triplicate samples and standard deviation is not greater than 0.01 for methanol, 0.03 for acetate and 0.85 for H_2/CO_2.

Activity assays

The results of the SMA also did not show a significant difference when comparing the effects of addition or deprivation of the metals for each cultivated consortium tested with methanol,

H_2/CO_2 and acetate as substrate. Figure 1 shows the most relevant results. The SMA on cobalt deprived and all metals deprived medium are not shown for H_2/CO_2, and the SMA on acetate was not measured for reactor R3. The greater changes were observed when comparing the SMA of the seed sludge with the cultivated sludges. The SMA for reactor R1 sludge with

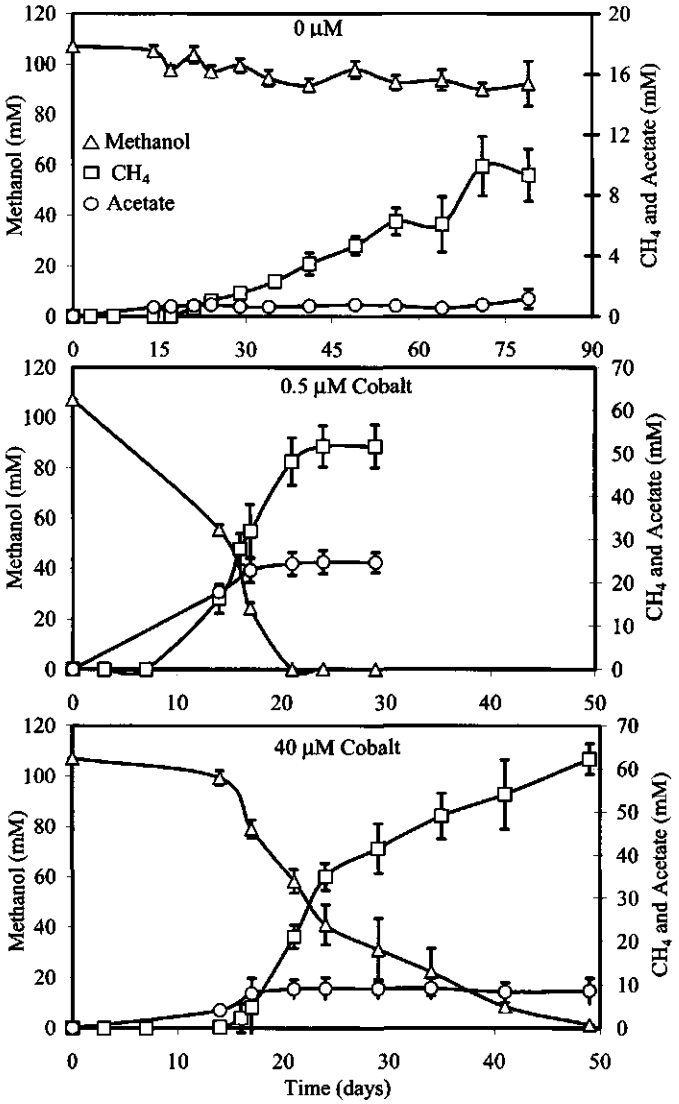


Figure 2 The course of methanol conversion and products formation by a cobalt-deprived enriched culture, when exposed to different concentrations of cobalt. Results are mean of at least triplicate samples.

methanol in a metals supplied medium, was 1.6 times higher than the one for the seed sludge. Even for reactor R2, which was cultivated in a cobalt deprived medium, it was 1.2 times higher than for the seed sludge. With H_2/CO_2, the SMA was about 2.3 times higher, for all 3 reactors, compared with the SMA on the seed sludge. Conversely, the activity with acetate

had dropped for all reactors when compared with the one of the seed sludge. For reactor R1, it dropped 60% and for reactor R2 almost no activity was detected.

The concentration of cobalt, nickel and iron was analysed for the seed sludge and for samples taken from all reactors at the end of the experiment (Table 2). The decrease in the metal contents of the sludges was about 40 – 45% of cobalt for both R2 and R3 reactors and 45% of nickel for reactor R3.

Table 2 Cobalt, nickel and iron content of the seed sludge and of the sludges cultivated in the 3 reactors during 136 days of operation with methanol as substrate.

Sludge Sample	Cobalt ($\mu g\ gTSS^{-1}$)	Nickel ($\mu g\ gTSS^{-1}$)	Iron ($\mu g\ gTSS^{-1}$)
Seed sludge	33.7 (0.8)	22.9 (0.7)	761.0 (0.5)
[a]R1	28.1 (1.2)	64.3 (1.0)	549.3 (14.0)
[b]R2	20.3 (0.7)	38.4 (1.4)	643.7 (26.3)
[c]R3	18.6 (0.5)	12.8 (0.6)	743.3 (6.2)

[a] all metals supplied to the medium

[b] medium was cobalt deprived

[c] medium was all metals deprived

Standard deviation is given between brackets.

Enrichment culture

Figure 2 depicts the effect of the different concentration of cobalt applied, on the rate of methanol consumption and on the products formed. When the medium was cobalt deprived, only 14.7 mM of methanol was consumed. 9.3 mM of CH_4 was formed and little acetate had accumulated after 79 days of experiment. When the medium was supplied with 0.5 μM of cobalt, which was the concentration normally used for the all metals medium, all methanol was converted within 24 days where 67% of the products formed (as COD) was methane and 33% was acetate. When cobalt was added in excess, metals precipitation was observed in the medium. In this case, it took more than 40 days to the complete methanol degradation. 88% of the methanol COD was converted to CH_4 and only 12% was converted to acetate. This experiment indicated that any concentration equal or over 0.5 μM would stimulate the growth of (homo)acetogens. Therefore, a second experiment was performed with much lower cobalt concentration as shown in Figure 3.

For all cobalt concentrations tested, no considerable lag phase took place. CH_4 concentration was higher than 70 mM in all tests performed. The differences were found on the reaction rate and acetate formation. When 0.01 μM of cobalt was added, methanol conversion was slow. No acetate was formed and H_2 pressure averaged 44 Pa during the whole trial. For 0.05 and 0.1 μM of cobalt added, acetate accumulated until around day 8, but it was completely consumed by the end of the trial. For 0.05 μM, the maximum H_2 pressure detected was 54 Pa

between days 7 and 9. For the 0.1 μM cobalt, H_2 reached 68 Pa at day 10. In the bottles where 0.2 μM of cobalt was added, less CH_4 was formed, comparing with all the other cases. The reaction rate was higher since methanol was completely consumed within 18 days. The maximum acetate accumulation took place at day 7 and 50% of that was consumed by the end of the experiment. Maximum H_2 pressure detected was 54 Pa, between days 4 and 7.

To check the importance of cobalt on the hydrogenotrophic group alone present in our cobalt-deprived enriched culture, we transferred the cobalt-deprived enriched culture to serum bottles containing either cobalt supplied (0.5 μM) or cobalt deprived medium, and H_2/CO_2

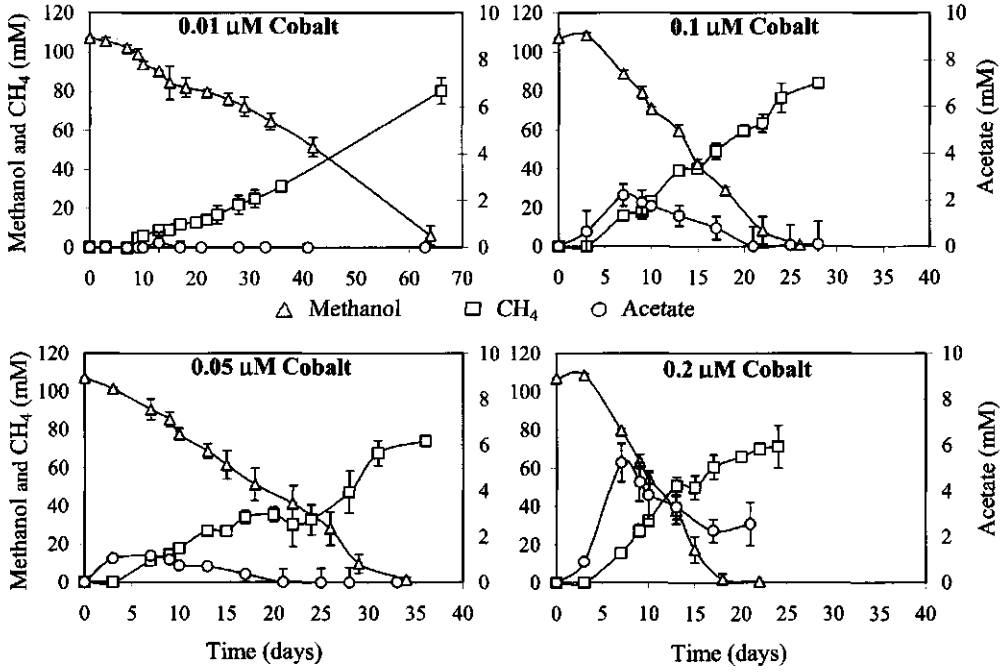


Figure 3 The course of methanol conversion and products formation by a cobalt-deprived enriched culture, when exposed to different concentrations of cobalt. Results are mean of at least triplicate samples.

was used as substrate. The absence of cobalt caused some delay in the reaction only in the beginning of the experiment. After 3 days, reaction rate was the same for both conditions and so it was the amount of methane accumulated (data not shown).

Discussion

The results obtained in this study indicate that our mixed consortium demands a proper amount of cobalt, even though low, to keep the trophic groups involved in the conversion of methanol operating properly. Therefore, the maximum activity of methanogens can be achieved and high loading rates can be applied. The decrease in the methane production rate

by the end of the continuous experiment for reactor R2 and reactor R3 was clearly caused due to metals limitation, as compared to the control reactor. It seems that the microorganisms were depending on the metal contents still available in the sludge, considering the decrease of those by the end of the experiment as compared to the seed sludge. It suggests that, in addition to bacterial growth, the specific methane conversion rate is affected by limiting concentrations of metals.

Cobalt requirement for the different involved trophic groups

For both cobalt-deprived enrichment culture and continuous experiment, the response on cobalt absence was not immediate. Previous results, indicate that for our cultivated consortium, about 50% of methanol is directly converted to methane by the methylotrophic methanogens and 50% is converted via H_2/CO_2 and acetate (Chapter 6), indicating that half of the methane formed in the system depends on the (homo)acetogenic step.

(Homo)acetogens

(Homo)acetogenesis have been shown to be enhanced by cobalt addition [61]. When studying the conversion of methanol under mesophilic conditions, Florencio et al. [31] observed that, on the course of their continuous experiments when using a cobalt deprived medium, the development of (homo)acetogenic bacteria decreased as compared to cobalt sufficient conditions. The latter was also observed in our continuous experiments, where the accumulation of acetate in the effluent dropped throughout time. The build up of pH_2 observed in the enrichment culture when methanol was used as substrate and different concentrations of cobalt were supplied (or cobalt was deprived), indicates that the lack of cobalt was hindering the (homo)acetogenic conversion of methanol to H_2/CO_2, thus delaying the reactions. Substantial methane production was always observed after the drop of the pH_2.

Methanogens

Methylotrophic methanogens The cultivated consortium maintained a good performance when exposed to a cobalt-deprived medium, as compared to a mesophilic methanol-fed UASB reactor with a predominantly methylotrophic consortium. Within about the same period of time (over 100 days) our reactor could sustain 97% of methanol removal, while the mesophilic reactor reached a maximum of 51% [31]. This is explained by the fact that the system does not depend completely on the methylotrophic methanogens, which demands a much higher concentration of cobalt than other methanogenic microorganisms [115] mainly when cells are grown on methanol [62].

Hydrogenotrophic methanogens The hydrogenotrophic methanogens seems to be present in high numbers in our sludge (Chapter 3). For the growth of *Methanothermobacter thermoautotrophicus* on H_2/CO_2 as sole carbon source, nickel requirement was found to be higher than cobalt requirement. Growth without the supplementation of cobalt in the medium

was observed, nevertheless, it was poor and 0.01 μM of cobalt was required [105]. The same was observed for our cobalt deprived enriched culture, when H_2/CO_2 was used as substrate. The hydrogenotrophic methanogens seemed to be more affected in the reactor R3, which was deprived of all metals. The drop of nickel concentration in the sludge and the methane production were more accentuated as compared to reactor R2.

Acetoclastic methanogens Cobalt seems to do not play an important role on the growth and activity of the acetoclastic methanogens [31, 75]. However, it is also found in literature that the addition of the combination of Ni^{2+} and Co^{2+}, increased the amount of coenzymes methyltransferase and methylreductase and accelerated the activity of a mesophilic-methanogenic mixed consortia [81] and it greatly enhanced the methanogenesis of acetic acid by a mixed methanogenic population from a fixed-film reactor [82]. As a matter of fact, it is not clear whether such stimulation is due to the combination of both metals or only by the addition of Ni itself, since Ni is present in a cofactor named F_{430}, which is the active site of an enzyme complex that catalyses the reduction of methyl-coenzyme M reductase. It seems that the cobalt-deprived cultivated consortium lost its ability to convert acetate to methane. When the test was performed with the cobalt deprived-sludge (reactor R2) the SMA on acetate was 87% lower than the one found for the cobalt-supplied reactor (control reactor R1). That indicates the reduction (or elimination) of acetotrophic microorganisms in our cobalt-deprived cultivated consortium. Considering the overall results, it seems that their disappearance was more due to the lack of acetate production by the (homo)acetogens, than for a cobalt-dependence itself as discussed below.

Acetate accumulation

The reintroduction of cobalt to the medium stimulated the formation of acetate. We have observed that, the competition for methanol between methanogenesis and (homo)acetogenesis takes place only when sufficient cobalt is supplied, confirming that acetogens does not seem to be as good scavenger for cobalt as methanogens does [29]. The acetate formed in such experiments was not further consumed. It might be that when acetate was not sufficiently produced, there was no substrate available for the acetoclastic methanogens to grow. When cobalt is supplied back to the medium, they cannot grow on acetate together with methanol, or they will grow in a much lower rate as observed by Smith and Mah [109]. It seems that, the growth of broth cultures on substrates that permit faster growth (i.e. H_2/CO_2 or methanol) than does acetate, favour the rapid development of cultures unable to utilise acetate. The small concentrations of acetate consumed during the experiments (maximum of 2.7 mM) suggests its syntrophic conversion to methane via H_2/CO_2 [102] or its consumption by other organisms present in the sludge such as *Methanothermobacter thermoautotrophicus*, which is shown to assimilate acetate when grown on CO_2 and H_2 in the presence of acetate [34].

Iron stimulation

It is also important to emphasise some changes that have occurred with the SMA of our cultivated consortium independent on the addition or deprivation of cobalt. The seed sludge used for the current experiments has been cultivated at very similar conditions for 2.5 years, except for iron concentration, which was increased from 0.3 to 24 μM in the last 6 months of experiments. The increase on the SMA on methanol and H_2/CO_2 indicates that the system was iron limited, since the SMA values had increased even to the reactors where cobalt was deprived.

"Optimal" cobalt concentration

For the cobalt-deprived enriched culture, 0.1 μM of cobalt was found to be the most appropriate concentration to be applied. Such concentration permits growth of methanogens without the competition with (homo)acetogens for methanol. Concentrations tested above 0.1 μM of cobalt, lead to the accumulation of acetate, which is not further consumed, as already discussed. Nevertheless, we recommend that cobalt should be always stepwise introduced to a system. Especially when concerning complex mixed consortium, where competition between methanogens and (homo)acetogens may take place. The full cobalt concentration required should be added just after being certain that methanogens are fully developed, avoiding then affecting the delicate balance between the microorganisms that compose the consortium.

Acknowledgements

This work was supported by "Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq" (Project n° 201055/97-0), an entity from the Brazilian Government for the development of Science and Technology.

8

Summary, general discussion and conclusions

The main objective of this thesis was to assess the feasibility of treating methanol-containing wastewaters under thermophilic conditions in a single-step upflow anaerobic sludge blanket (UASB)-reactor. We attempted to take into consideration all the drawbacks related to anaerobic conversion of methanol, especially, of thermophilic anaerobic treatment. The investigations were also focused on relevant microbiological and biotechnological aspects.

Methanol is a simple C1- compound that can potentially support a complex food chain under anaerobic conditions. Methanol can be the main pollutant in some specific wastewaters, but it is also a compound that may be formed under natural conditions as intermediate in the decomposition of organic matter [46, 103]. Methanol is the main organic pollutant in the kraft evaporator condensate from the pulp and paper industry [78], which comprises a wastewater discharged at temperatures suitable for thermophilic anaerobic treatment. Obviously, the thermophilic treatment option for hot types of wastewaters is attractive since then, pre-cooling which would be needed when applying mesophilic treatment can be avoided.

Thermophilic treatment also represents an attractive alternative for mesophilic digestion in the view of the higher metabolic rates of the bacteria involved and, consequently, the theoretical higher maximum specific methanogenic activities [124]. Nevertheless, so far, anaerobic treatment for industrial wastewaters is applied almost exclusively under mesophilic conditions. Very few full-scale thermophilic anaerobic systems have been installed to date.

When applying the anaerobic treatment of methanolic wastewaters, the accumulation of volatile fatty acids (VFA) has been found a problem of concern [14, 29, 70, 142-144] both under mesophilic and thermophilic conditions. Such an accumulation makes an effective chemical oxygen demand (COD) removal impossible and may even cause failure of the treatment process due to inhibition of the methanogens, especially at low pH-values.

As far as mesophilic conditions is concerned, the available information in literature suffices to achieve a satisfactory application of a stable high-rate methanogenic reactor system.

Any comprehensive studies concerning the application of high rate thermophilic reactor system with merely methanol as substrate so far have not been conducted. A number of

investigations have been conducted for kraft evaporator condensate under thermophilic conditions (53 °C) [77, 78, 80, 142], however, these results did not result in sufficiently good insight in the conversion of methanol itself, partially due to the complexity of the kraft evaporator condensate.

In the absence of nitrate, sulphate or oxidised metal ions like Fe^{3+} and Mn^{4+}, methanogens and acetogens are the expected predominant group of microorganisms in the anaerobic conversion of methanol [28]. Being a simple one carbon compound, methanol is considered to degrade just as easily as it is produced in nature. Methanol can be converted directly to methane by methylotrophic methanogens [122] but it also may be reduced to methane with H_2 [121]. Another possible transformation represents the conversion to acetic acid by (homo)acetogens, but this conversion is only possible in the presence of sufficient CO_2 [72]. Next, the acetic acid can be converted to methane by acetoclastic methanogens [88]. When the H_2 concentration is kept sufficiently low by syntrophic partnership, methanol can be oxidised to H_2 and CO_2 [46] followed by either methanogenesis performed by the hydrogenotrophic methanogens [23] or (homo)acetogenesis [102]. The low H_2 concentration in the system also enables the oxidation of acetic acid to H_2/CO_2 [45, 65, 152].

Substrate competition among the various microorganisms for available substrate(s) may be intense and the outcome of that will depend on a wide range of factors such as thermodynamics, nutrient uptake, metabolic rates, growth rates, and environmental conditions. These factors play a very important role and are of crucial importance concerning the bacterial population which ultimately will become predominant. The degradation route of methanol and its final fate in an anaerobic environment may alter significantly upon a change in the environmental conditions. These changes may cause a distinct shift in the microbial composition of mixed cultures, and may suppress specific microorganisms, if the imposed different environmental conditions persist.

Under mesophilic conditions, the factors found to be important for the anaerobic conversion of methanol are the presence of cobalt in the media, the concentration of methanol in the reactor, the pH inside the reactor, the level of bicarbonate and the concentration of undissociated VFA [28].

A good insight in the degradation pathway of methanol and understanding of the influence of relevant environmental conditions on its anaerobic transformation, obviously represent a powerful tool to optimise the thermophilic anaerobic treatment of methanol containing wastewaters. An undesirable accumulation of VFA then can be avoided whereas it also becomes possible to steer the system to the desired final product composition.

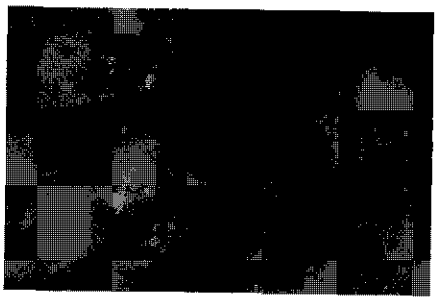
The feasibility of the thermophilic anaerobic treatment of methanol

The satisfactory reactor performance achieved in the investigations showed in Chapter 2, concerning the treatment of methanol at an organic loading rate (OLR) up to 47.3 $gCOD.L^{-1}.d^{-1}$ and a 3.2 h HRT, demonstrates the feasibility of the thermophilic treatment of methanol-containing wastewater by using a one stage UASB reactor. In contrast to the frequently reported VFA accumulation when treating methanolic wastewaters, a significant VFA accumulation was not detected in our experiments, even at bicarbonate concentrations exceeding 20 mM. This likely can be attributed to the relatively high specific methanogenic activity (SMA) of the sludge on acetate and also its affinity for acetate (Chapter 2). It indicates that acetic acid represents an intermediate, which is converted rapidly, once produced from methanol. Acetate was the main constituent of the VFA produced at relatively low OLR (below 20 $gCOD.L^{-1}.d^{-1}$), while at high OLR (above 30 $gCOD.L^{-1}.d^{-1}$), propionate and butyrate were the main VFA accumulating.

The overall reactor performance could be characterised as stable, even when exposed to non-optimal conditions such as a temperature drop (to 35 °C), overloading (to a value of 67 $gCOD.L^{-1}.d^{-1}$) and unfed conditions (during a period of 7 hours). The recovery from a feed interruption required more time than from the two other shock conditions, suggesting high maintenance energy requirement.

Chapter 3 deals with the physical characterisation of the seed and the cultivated sludge and the change in the sludge characteristics as well. The sludge properties were found to change very significantly during the 130 days of continuous reactor operation. The new 'granular' active biomass was of a good quality in terms of specific activity and settling characteristics. Biomass washout, which coincided with a high specific gas load, never appeared to be seriously detrimental for the stability of the system under the conditions tested. The average bacterial growth, as estimated on the basis of theoretical calculations, was 30% higher than the biomass washout, explaining the continuous increase in the sludge bed height. The fraction of sludge rinsed out from the system can be characterised as fluffy and spongy biomass.

The characteristics of the granular sludge formed are comparable to those found for the sludge cultivated under mesophilic conditions in UASB reactors, with methanol as the sole substrate and also for substrate mixtures containing methanol [11, 35, 70]. The results obtained, therefore, indicate that methanogenic-sludge granulation in thermophilic UASB reactors is very well possible with methanol as the sole organic substrate. Figure 1 illustrates some different types of sludge produced in some of the investigations conducted in the framework of this thesis study.



Sludge cultivated in a bicarbonate buffered system and used as seed sludge for all other experiments. (after 6 months of cultivation).

Sludge cultivated under acidic conditions. No bicarboante supplied. (after 4 months of cultivation).

Sludge cultivated in a bicarbonate deprived medium at neutral pH. Automatic pH controller, NaOH solution. (after 4 months of cultivation).

Fluffy biomass. Always present, no matter the conditions applied to the reactors.

Figure 1 Sludges cultivated during reactor operation at different conditions.

The effect of pH and inorganic carbon species ($\Sigma([HCO_3^-] + [CO_2])$)

The experiments in Chapter 4, concern the thermophilic anaerobic conversion of methanol in a non-pH buffered medium (at pH 4 ± 0.2) and in a phosphate buffered medium (pH 6.4 ± 0.1), in both situations without the supply of bicarbonate. Our sludge consortium was not able to degrade methanol under acidic conditions. During 160 days of continuous operation of the UASB reactor at an OLR of 6 $gCOD.L^{-1}.d^{-1}$, only 5% of the imposed methanol load was consumed and methane (CH_4) was not detected. Surprisingly, despite the absence of CH_4 formation, the hydrogenotrophic methanogens present in the sludge showed to be rather resistant to exposure to such severe pH-conditions. The SMA on H_2/CO_2 assessed at neutral pH, at the end of the UASB-experiment still amounted to 50% of the initial activity. On the other hand the acetate accumulation during the batch experiments indicates that the acetotrophic microorganisms did not resist such conditions and died or were washed out. Apparently, as compared to the other groups present in the sludge consortium, the hydrogenotrophic methanogens are the least affected group by the long term period exposed to the acidic conditions.

The results obtained in the phosphate buffered reactor demonstrate a poor performance of the system under these conditions, while the system also was found to be sensitive to any type of disturbance, even under conditions of low OLR. A clear competition manifested between methanogens and acetogens. At the end of the trial, the outcome of the competition was about 50% methanogenesis and 50% (homo)acetogenesis. The effect caused by the lack of bicarbonate is reflected in the overall reactor performance. The methanol-COD removal capacity of the system remained limited to a relatively low value of about 9.5 $gCOD.L^{-1}.d^{-1}$ viz. compared with the results obtained in Chapter 2.

It is clear that, the deprivation of bicarbonate in the system leads to a stress condition in the consortium, enabling, somehow, the (homo)acetogenic bacteria to compete with hydrogenotrophic methanogens for CO_2. The SMA on H_2/CO_2 measured by the completion of the continuous experiment remained unchanged, while, in the batch experiments conducted with methanol as substrate in a bicarbonate supplied medium, methanol was mostly converted to acetate, confirming that the methanogens only get the opportunity to compete with (homo)acetogens under reactor conditions due to bicarbonate limitation.

These studies strongly indicate that the addition of bicarbonate as a source of carbon dioxide is of crucial importance for the methanol conversion in our consortium. Indirectly, CO_2 acts as a H_2 sink, helping to keep the pH_2 value very low, so that the reaction becomes thermodynamically favourable and methanol can be converted to H_2/CO_2. The results indicate that the partnership with hydrogenotrophic methanogens does not suffice for the establishment of a high rate methanol conversion. Carbon dioxide is also used together with methanol for the acetate production, which contributes to the methane produced when operating the system under optimal conditions.

Acetate accumulation

Regarding the results we obtained in the long term-continuous experiment, there exists clear evidence that the use of 70 mM of phosphate buffer, negatively affected the acetotrophic microorganisms present in our consortium. Acetate accumulated in the system and, at the end of the experiment, any methanogenic activity on acetate could not be detected. For batch experiments where a bicarbonate-deprived medium was required we usually used phosphate buffer (72 mM). In the experiments conducted under these conditions, a distinct accumulation of acetate was observed. In an additional continuous UASB-experiment in a bicarbonate-deprived medium, the pH was maintained in neutral range by using an automatic pH controller and a NaOH solution instead of phosphate buffer. Although the poor performance and instability of the system were observed, the acetate accumulation did not occur while operating the system under optimum conditions (data not shown). Conrad et al. [20] reported the inhibition of acetotrophic methanogenesis by phosphate ($\geq$ 20 mM) in experiments

conducted with washed excised rice roots incubated in phosphate buffer under anaerobic conditions.

Recovery strategy

Our cultivated sludge appeared to be quite sensitive to pH shocks, both in the acidic and alkaline pH range, and a complete recovery of methanogenesis, was impossible when operating the system at the conditions normally applied. The results of the first tentative recovery experiments for the sludge exposed to an alkaline pH shock, together with the results of Chapter 4, indicate that, the mixed culture needs bicarbonate for achieving a full methanogenesis. However, if the proper environmental conditions are not met, the same bicarbonate addition stimulates the production of acetate. Based on this information we proposed a strategy to reactor recovery in Chapter 5.

We hypothesised that, in case acetogenesis would be restricted by the amount of added bicarbonate, the normal conversion would proceed, and the methanogens would be able to develop, once optimum conditions are established in the batch-mode system. Once methanogenesis has recovered and the proper environmental conditions could be maintained, (homo)acetogens would not outcompete methanogens for methanol and the formed acetate would be quickly consumed. The main point of the strategy was to operate the reactor in batch mode, until the complete depletion of the supplied amount of methanol was achieved. Thereafter the liquid phase was replaced prior to a new feeding. We believe that this feature is important in the strategy, since acetoclastic methanogens seems to be the most sensitive group present in the consortium (see conclusions section). According to Smith and Mah [109] a mesophilic strain of *Methanosarcina* is unable to metabolise acetate in the presence of methanol, or in case they do, they do in a much lower rate.

Of important practical interest is the fact that we were able to recover methanogenesis on methanol, even in case when (homo)acetogens were outcompeting methanogens. The time and the number of feedings required depend on the extent of deterioration of the sludge. Another important conclusion of the investigations presented in Chapters 4 and 5 is that caution needs to be taken when using $NaHCO_3$ for buffering methanol-containing wastewaters, since its introduction in the system will favour acetogenesis when proper conditions are not established.

The effect of cobalt

The investigations in Chapter 7, deal with the assessment of the importance of cobalt for the thermophilic cultivated consortium, and its effect on the competition between methanogens and acetogens. For this purpose, we conducted continuous UASB experiments and also batch experiments with a cobalt deprived-enriched culture. In both types of experiment, the response on cobalt absence was not immediate. The cobalt requirement of our cultivated

consortium was lower as compared to that of a mesophilic methylotrophic consortium [31]. The results indicated that our mixed consortium requires a proper amount of cobalt even though it is quite low, to keep the trophic groups involved in the conversion of methanol operating properly. Therefore, it is possible to attain the maximum activity of methanogens and, consequently, the application of high loading rates. For the cobalt-deprived enriched culture, 0.1 μM of cobalt was found to be the most appropriate concentration. It permits growth of methanogens without competition with (homo)acetogens for the methanol and methane is the sole end product. Concentrations exceeding 0.1 μM of cobalt, led to the accumulation of acetate. The cobalt deprivation does not seem to affect hydrogenotrophic methanogens, but it clear affects the (homo)acetogens as indicated by the observed decrease in acetate detected in the effluent reactors. The lack of acetate production in the system (both reactors and enrichment culture) led to the loss of the consortium ability to degrade acetate. This indicates that the acetotrophic microorganisms were washed-out. The addition of cobalt to the cobalt-deprived enrichment culture stimulated the formation of acetate. We observed that, the competition for methanol between methanogens and (homo)acetogens only manifested when sufficient cobalt was supplied, confirming that, apparently, methanogens are better scavengers for cobalt than acetogens [29].

Independent on whether cobalt is present or absent, clear changes have occurred in the SMA of the cultivated consortium. The seed sludge used for the investigations in Chapter 7 had been cultivated under very similar conditions during a period of 2 years. When conducting the metal experiments (last six months of experiments, see Chapter 7), we increased the iron concentration from 0.3 to 24 μM. The observed increase of the SMA on methanol and H_2/CO_2 indicates that the system was iron limited because, the SMA values increased even for the sludge in the reactors where cobalt was deprived. The SMA on acetate remained unaffected by the iron concentration.

The pathway elucidation

In Chapter 6, the pathway of methanol conversion by the mixed cultivated consortium was elucidated. The results of activity assays in the presence and absence of specific inhibitors indicated that, about 50% of methanol is directly converted to methane by the methylotrophic methanogens and 50% via the intermediates H_2/CO_2 and acetate. The high SMA of the sludge for H_2/CO_2, methanol and acetate confirms the involvement of the 3-methanogenic groups (hydrogenotrophic, methylotrophic and acetoclastic) in the conversion of methanol to methane.

As appeared from the experiments presented in Chapter 4, the deprivation of inorganic carbon species ($\Sigma([HCO_3^-] + [CO_2])$) in a phosphate buffered system, seriously reduces the rate of methanol conversion. It suggests that bicarbonate (CO_2) is required as an "electron"

(H_2) sink as well as a cosubstrate, for efficient and complete COD removal. The amount of bicarbonate supplied to the reactor during the sludge cultivation was 0.16 $gHCO_3^-$ for each 1gMeOH-COD while 0.64 $gHCO_3^-$ for each 1gMeOH-COD is necessary to the total conversion of methanol to acetate. The amount supplied to the reactor would be enough to sustain 25% of the total population as methylotrophic (homo)acetogens, without depending on the CO_2 formed during reactions. The results of the nuclear magnetic resonance (NMR) experiments showed that production of acetate is also interconnected to the oxidation of methanol to CO_2. Unfortunately, our results do not allow the quantification of the exact amount of methanol being converted to methane via acetate and via H_2/CO_2, but the results of the investigations conducted in the framework of this thesis suggest that, qualitatively, both of them play an equally important role.

The NMR spectroscopy technique was used to investigate the route of methanol conversion to acetate in bicarbonate sufficient and bicarbonate depleted environments. Results indicate that methanol is converted through a common pathway (Acetyl-CoA), on the one hand being reduced to the methyl group of acetate and on the other hand oxidised to CO_2, with CO_2 being incorporated as the carboxyl group of acetate. In addition, results show that the largest fraction of the acetate formed would be via a short biochemical pathway and a small fraction involves more enzymatic steps.

The conversion of H_2/CO_2 into acetate and vice-versa, although apparently possible, does not seem to play an important role on the final fate of methanol during the realisation of these experiments. Concerning the conversion of acetate to H_2/CO_2, it is important to emphasise some changes in the cultivated consortium, which occurred after the completion of all experiments. We observed that during the 2.5 years cultivating the sludge at very similar conditions (except for iron concentration as already mentioned above), the SMA on acetate declined drastically throughout the operation of the reactor. The SMA on acetate dropped from 0.84 to 0.54 $gCOD.gVSS^{-1}.d^{-1}$ after 2 years and to 0.23 $gCOD.gVSS^{-1}.d^{-1}$ after 2.5 years. This indicates that the acetoclastic methanogens are becoming less important over time, while the syntrophic conversion of acetate to methane via H_2/CO_2 is becoming more pronounced, considering the increase on the SMA on H_2/CO_2 (Chapter 7) and the fact that, we did not find serious acetate accumulation despite the fact that (homo)acetogens were still very active. Such a change in the character of the sludge looks very well possible. The syntrophic conversion of acetate to methane via H_2/CO_2 seems to represent an important metabolic pathway under thermophilic and extreme thermophilic conditions [148, 151, 152], once in high temperature habitats, the acetoclastic methanogenesis are presumed to become less significant.

It is clear that all routes (except for the conversion of H_2/CO_2 to acetate) play a role on the degradation of methanol for our mixed cultivated consortium. The balance among the various microorganisms present in the sludge is of paramount importance since it rules the reaction

that governs the pathway. On the one hand the alternative pathways act as an electron sink helping to keep the pH_2 sufficiently low, so that methanol can be converted to H_2/CO_2. On the other hand the alternative pathways convert the acetate to methane.

Conclusions and recommendations

The results of this study confirm that acetate accumulation represents the major bottleneck of the thermophilic-anaerobic treatment of methanol. However, despite that, the single step-UASB remains an attractive treatment option, since we had obtained sufficient evidences that the application of such a treatment can be successful.

The acetoclastic methanogens clearly is the most sensitive group of organisms present in the cultivated sludge. They are easily washed out/killed and apparently they are unable to grow in the presence of methanol, or at least the growth is significantly slower. The oxidation of acetate to H_2/CO_2 is also a sensitive process because it only proceeds if a H_2 sink is present in order to keep the pH_2 in the system sufficiently low. The hydrogenotrophic methanogens exert the highest SMA and they also have a higher resistance to adverse environmental changes, however, they depend on the (homo)acetogenic step.

As the contribution of direct conversion of methanol in our system is only about 50% of the total methane formed, the formation of the remaining 50% always will depend on the (homo)acetogenic step to take place. The involvement of the oxidation of methanol to H_2/CO_2 in the process implies in a very delicate system. It should be taken into account that small changes in the hydrogen partial pressure may have a strong impact on substrate conversion rates [60] while, when the microorganisms have alternative pathways, the spectrum of products may change [16, 63, 83].

The deprivation of cobalt and bicarbonate appear to exert a very similar effect on the cultivated consortium. In both cases, the (homo)acetogenic step is affected and it indirectly affects the methanogenic groups (except for the methylotrophic, which is directly affected by cobalt deprivation). Therefore, the addition/deprivation of cobalt or bicarbonate to stimulate/inhibit one or another group seems to be very risky, since the failure of (homo)acetogens will be detrimental to methanogens and affect the overall process.

Nevertheless, in order to develop a balanced consortium with methane as the target end product, we recommend that cobalt and bicarbonate should always be stepwise introduced to a system. The total amount required of cobalt or bicarbonate should be added only when it is for sure that methanogens are present, and sufficiently active, ensuring the required delicate balance among the microorganisms.

As one of the functions of bicarbonate is to buffer the system, its stepwise addition will not immediately provide the buffer capacity required by the system. Therefore, at the initial

stages the pH must be controlled in another way. Phosphate should not be considered as a pH-buffer, unless is it clear that is does not inhibit the subjected sludge consortium.

We recommend the use of bicarbonate for the treatment of methanol-containing wastewater where the syntrophic conversion via H_2/CO_2 is involved. The reasons are that *i*) a buffer is anyway required due to the dependency on a close-to-neutral pH and *ii*) the reactor performance with added bicarbonate is indubitable better and more stable. As shown in the continuous experiments, 0.16 gHCO_3^- per 1gMeOH-COD suffices for maintaining the pH in the range of 6.5 – 6.7 and a stable operating system.

8'

Samenvatting, discussie en conclusies

Het hoofddoel van het onderzoek, beschreven in dit proefschrift, was het vaststellen van de haalbaarheid van de behandeling van methanol houdende afvalwaters onder thermofiele condities in een eentraps UASB (upflow anaerobic sludge blanket) reactor. Daarbij is met name getracht alle nadelen die op dit moment aan de anaërobe omzetting, en dan met name onder thermofiele condities, van methanol verbonden zijn, in overweging te nemen. Het onderzoek was vooral ook gericht op relevante microbiologische en biotechnologische aspecten.

Methanol is een eenvoudige verbinding met slechts één koolstofatoom. Het staat aan de basis van een complex schema van metabole omzettingen welke plaats vinden onder anaërobe condities. Methanol kan de voornaamste verontreiniging zijn in enkele specifieke afvalwaters, maar het is ook een verbinding die onder natuurlijke omstandigheden gevormd kan worden als intermediair in de omzetting van organisch materiaal [46, 103]. Methanol is verder de voornaamste verontreiniging in het condensaat van de 'kraft'-verdamping in de pulp en papier industrie [78], een afvalstroom die wordt gevormd op temperaturen die bij uitstek geschikt zijn voor thermofiele anaërobe zuivering. Het is duidelijk dat de thermofiele behandeling een aantrekkelijk alternatief is voor de behandeling van dergelijke warme afvalwater stromen. In dit geval kan namelijk het koelen van het afvalwater, noodzakelijk om het te kunnen behandelen met een mesofiele behandelingstechniek, kan worden vermeden.

Thermofiele zuivering kan ook een aantrekkelijk alternatief zijn voor mesofiele behandeling in het licht van de hogere metabole omzettingssnelheden van de betrokken bacteriën, en de als gevolg daarvan in elk geval theoretisch hogere maximum specifieke methanogene activiteiten [124]. Desalniettemin wordt tot op heden anaërobe zuivering van industriële afvalwaters vrijwel uitsluitend onder mesofiele omstandigheden uitgevoerd. Slechts een handvol full-scale thermofiele anaërobe systemen is tot heden geïnstalleerd.

Bij toepassing van de anaërobe zuivering van methanol houdende afvalwaters is de ophoping van vluchtige vetzuren (VFA) een wezenlijk probleem gebleken, [14, 29, 70, 142-144] zowel onder mesofiele als onder thermofiele omstandigheden. Zo'n ophoping maakt een effectieve verwijdering van het chemisch zuurstof verbruik (CZV) onmogelijk, en kan zelfs het omzettingsproces lamleggen, door inhibitie van de methanogenen, met name bij lagere pH-waarden.

Voor zover het een proces onder mesofiele omstandigheden betreft, is de hoeveelheid in de literatuur beschikbare informatie afdoende voor het kunnen bereiken van een bevredigende toepassing van een stabiel high-rate methanogeen reactor systeem.

Overzichtelijke en eenduidige studies aangaande de toepassing van high-rate thermofiele reactor systemen met uitsluitend methanol als substraat zijn tot nog toe niet uitgevoerd. Een aantal studies is uitgevoerd naar de behandeling van 'kraft' verdamper condensaat onder thermofiele condities (53 °C) [77, 78, 80, 142], maar uit deze resultaten valt geen voldoende inzicht te verkrijgen in de omzetting van methanol zelf, ten dele vanwege de toch complexe samenstelling van 'kraft' verdampings condensaat.

In de afwezigheid van nitraat, sulfaat of geoxideerde metaalionen zoals Fe^{3+} en Mn^{4+} kan verwacht worden dat methanogenen en acetogenen de dominante groepen van micro-organismen zijn in de anaërobe omzetting van methanol [28]. Gezien het feit dat methanol een simpele, slechts één koolstofatoom tellende, verbinding is, wordt in het algemeen aangenomen dat methanol even gemakkelijk kan worden omgezet als het geproduceerd wordt in de natuur. Methanol kan direct worden omgezet in methaan door methylotrofe methanogenen [122], maar het kan ook met H_2 worden gereduceerd tot methaan [121]. Een andere mogelijke omzetting wordt gevormd door de conversie naar azijnzuur door (homo)acetogenen, maar deze omzetting vindt uitsluitend plaats in de aanwezigheid van voldoende CO_2 [72]. Vervolgens kan het azijnzuur omgezet worden in methaan door acetoclastische methanogenen [88]. Wanneer de H_2-concentratie voldoende laag wordt gehouden door een syntroof partnerschap kan methanol worden omgezet in H_2 en CO_2 [46], gevolgd door hetzij methanogenese door de hydrogenotrofe methanogenen [23], of door (homo)acetogenese [102]. De lage H_2 concentratie in het systeem maakt ook oxidatie van azijnzuur tot H_2/CO_2 mogelijk [45, 65, 152].

De substraat competitie tussen de verschillende micro-organismen voor beschikbaar substraat kan sterk zijn en het resultaat hangt af van een groot aantal factoren zoals thermodynamica, nutriënt opname, metabole snelheden, groeisnelheden, en omgevingsfactoren. Deze factoren spelen een uitermate belangrijke rol en zijn van cruciaal belang waar het gaat om het bepalen van welke bacteriepopulatie dominant wordt. De omzettingsroute van methanol en het uiteindelijke lot van deze verbinding in een anaëroob milieu kunnen wezenlijk veranderen als resultaat van een verandering van de omgevingsfactoren. Deze veranderingen kunnen een belangrijke verschuiving in de microbiële samenstelling van gemengde culturen bewerkstelligen, en kunnen specifieke micro-organismen doen verdwijnen, wanneer de opgelegde afwijkende milieufactoren lange tijd voorkomen.

De factoren die onder mesofiele omstandigheden van belang zijn voor de anaërobe omzetting van methanol zijn: de aanwezigheid van cobalt in het medium, de methanolconcentratie, de

pH in de reactor, de bicarbonaat concentratie en de concentratie ongedissocieerde vluchtige vetzuren (VFA) [28].

Een goed inzicht in de degradatieroute van methanol, en begrip van het effect van relevante procescondities op de anaërobe omzetting hiervan, is overduidelijk een belangrijk instrument om de thermofiele anaërobe zuivering van methanol houdende afvalwaters te kunnen optimaliseren. Een ongewenste ophoping van VFA kan dan worden vermeden terwijl het ook mogelijk wordt het systeem in de richting van de gewenste effluentsamenstelling te sturen.

De haalbaarheid van de thermofiele anaërobe verwijdering van methanol

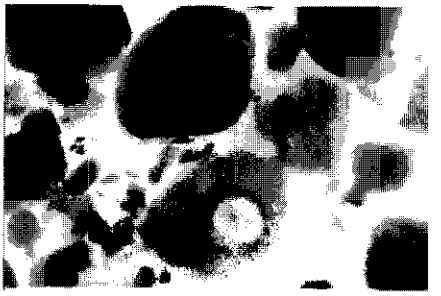
De bevredigende prestaties van de reactor in de experimenten beschouwd in Chapter 2, waarin de omzetting van methanol bij een organische belasting (OLR) van tot aan 47.3 g $COD.L^{-1}.d^{-1}$ bij een hydraulische verblijftijd (HRT) van 3.2 uur, demonstreren de haalbaarheid van thermofiele behandeling van methanol bevattende afvalwaters in een eentraps UASB reactor. In tegenstelling tot wat frequent in de literatuur aangaande de behandeling van methanol houdende afvalwaters wordt gemeld, werd een wezenlijke ophoping van VFA niet waargenomen in onze experimenten. Dit was zelfs niet het geval bij bicarbonaat concentraties ruim boven 20 mM. Dit kan waarschijnlijk worden toegeschreven aan de relatief hoge specifieke methanogene activiteit (SMA) van het slib in aanwezigheid van acetaat en aan de affiniteit van het slib voor acetaat (Chapter 2). Deze twee factoren duiden erop dat azijnzuur een intermediair is wat snel wordt omgezet, na uit methanol te zijn gevormd. Acetaat was ook het belangrijkste van de vluchtige vetzuren geproduceerd bij relatief lage organische belastingen (van minder dan 20 g $COD.L^{-1}.d^{-1}$), terwijl bij hoge organische belastingen (boven 30 g $COD.L^{-1}.d^{-1}$), propionaat en butyraat de voornaamste ophopende vetzuren waren.

De algehele prestaties van de reactor konden worden gekenschetst als stabiel, zelfs wanneer de reactor werd blootgesteld aan niet-optimale omstandigheden als een temperatuurdaling (tot 35 °C), overbelasting (tot een waarde van 67 g $COD.L^{-1}.d^{-1}$) en een periode zonder voeding (gedurende een tijd van 7 uur). Het herstel van de reactor van een periode zonder voeding kostte meer tijd dan herstel van de andere twee shock-condities, wat er op duid dat er een hoge energiebehoefte t.b.v. instandhouding (maintenance) is.

Chapter 3 behandelt de fysieke karakterisatie van het entslib (inoculum) en het gecultiveerde slib, en ook de veranderingen in de slibeigenschappen. De slibeigenschappen bleken gedurende de 130 dagen van continu reactorbedrijf die het experiment duurde zeer wezenlijk te veranderen. De nieuwe 'korrelige' actieve biomassa had een goede kwaliteit, wanneer beschreven in termen van specifieke activiteit en bezink karakteristieken. Uitspoeling van de biomassa, samenvallend met een hoge specifieke gasbelasting, bleek nooit een serieuze bedreiging voor de stabiliteit van het systeem onder de geteste condities. De gemiddelde

bacteriële groei, zoals geschat op basis van theoretische berekeningen, was 30% hoger dan de slibuitspoeling, wat ook verklaart waarom sprake was van een continue toename van de slibbedhoogte.

Slib gecultiveerd in een systeem gebufferd met bicarbonaat. Dit slib is ook gebruikt als entslib voor alle andere experimenten. (na 6 maanden cultivatie).



Slib gecultiveerd onder zure omstandigheden en in zonder toevoeging van bicarbonaat. (na 4 maanden cultivatie).

Slib gecultiveerd in een medium met een lage bicarbonaat concentratie bij een neutrale pH (gehandhaafd d.m.v. een automatische pH regeling, gebruik makend van een NaOH oplossing). (na 4 maanden cultivatie).

Sponzige biomassa. Altijd aanwezig, ongeacht de omstandigheden in de reactoren.

Figuur 1 Slib gecultiveerd in de reactor bij bedrijving hiervan onder verschillende condities.

Het deel van het slib wat uitspoelt kan worden gekarakteriseerd als zachte en sponsvormige biomassa. De karakteristieken van het korrelslib waren gelijk aan die gevonden voor slib gecultiveerd in UASB reactoren met methanol als enig substraat, en ook met methanol-houdende substraatmengsels, onder mesofiele omstandigheden [11, 35, 70]. De behaalde resultaten laten daarmee zien dat korrelvorming van methanogeen slib in thermofiele UASB reactoren met methanol als enig substraat zeer wel mogelijk is. Figuur 1 illustreert enkele verschillende typen slib geproduceerd bij enkele van de experimenten uitgevoerd in het kader van dit onderzoek.

Het effect van pH en anorganische koolstofverbindingen ($\Sigma([HCO_3^-] + [CO_2])$)

De experimenten in Chapter 4 betreffen de thermofiele anaërobe omzetting van methanol in een niet pH-gebufferd medium (bij een pH van 4 ± 0.2) en in een medium gebufferd met fosfaat (bij een pH van 6.4 ± 0.1). In beide situaties wordt geen bicarbonaat toegevoegd. Ons slib was niet in staat methanol onder zure omstandigheden om te zetten. Gedurende 160 dagen van continubedrijf van de UASB reactor bij een organische belasting van 6 g $COD.L^{-1}.d^{-1}$, was slechts 5% van de toegediende methanol geconsumeerd en methaan (CH_4) werd niet aangetroffen. Verrassend was dat, ondanks het niet optreden van methaanvorming, de hydrogenotrofe methanogenen aanwezig in het slib tamelijk resistent bleken te zijn tegen de blootstelling aan de tamelijk zware pH-omstandigheden. De specifieke methanogene activiteit op H_2/CO_2, vastgesteld bij een neutrale pH, bleek aan het eind van dit UASB experiment toch nog 50% van de initiële activiteit te bedragen. Aan de andere kant laat de acetaat ophoping in de batch experimenten zien dat de acetotrofe micro-organismen niet bestand waren tegen dergelijke condities. Deze micro-organismen stierven af of spoelden uit. Klaarblijkelijk, wanneer we vergelijken met de andere groepen organismen die aanwezig zijn in het slib, zijn de hydrogenotrofe methanogenen de groep die het minste last hebben van de langdurige blootstelling aan zure condities.

De resultaten verkregen met de fosfaat-gebufferde reactor tonen slechte prestaties van het systeem onder de gekozen omstandigheden. Tevens bleek het systeem gevoelig te zijn voor vele typen verstoring, zelfs indien sprake was van een lage organische belasting. Een duidelijke competitie bleek op te treden tussen methanogenen en acetogenen. Aan het eind van de testperiode was het resultaat van deze competitie circa 50% methanogenese en circa 50% (homo)acetogenese. Het gebrek aan bicarbonaat is weerspiegeld in de overall prestaties van de reactor. De verwijderingscapaciteit van het systeem voor methanol-CZV bleef beperkt tot een relatief lage waarde van rond 9.5 g $COD.L^{-1}.d^{-1}$, bijvoorbeeld te vergelijken met de resultaten zoals verkregen in Chapter 2.

Het is duidelijk dat het beperken van de bicarbonaat concentratie in het systeem leidt tot een stress conditie in het slib die, op een of andere wijze, de (homo)acetogene bacteriën in staat stelt met de hydrogenotrofe methanogenen te concurreren voor CO_2. De specifieke methanogene activiteit op H_2/CO_2, gemeten aan het eind van het continu-experiment, bleef onveranderd terwijl in de batch experimenten met methanol als substraat in een medium waaraan bicarbonaat werd toegevoegd, de methanol grotendeels werd omgezet in acetaat. Dit bevestigd dat de methanogenen alleen in de gelegenheid zijn te concurreren met de (homo)acetogenen wanneer een beperking van de bicarbonaat concentratie de reactorcondities kenmerkt.

Deze studies geven ook duidelijk aan dat de toediening van bicarbonaat als bron van CO_2 van doorslaggevend belang is voor de omzetting van methanol in ons slib. Indirect treedt het CO_2

op als een sink voor het aanwezige H_2, wat helpt om de pH laag te houden, waardoor de reactie thermodynamisch gunstig wordt en methanol omgezet kan worden in H_2/CO_2. Deze resultaten laten ook zien dat het partnerschap met de hydrogenotrofe methanogenen niet voldoende is voor een snelle methanol omzetting. Kooldioxide wordt, tezamen met methanol, ook gebruikt voor acetaatproductie, wat bijdraagt aan de productie van methaan wanneer het systeem onder optimale condities wordt bedreven.

Acetaat ophoping

Wat betreft de resultaten die we verkregen in het continue duurexperiment, bestaat er ook duidelijk bewijs dat het gebruik van de 70 mM fosfaatbuffer de acetotrofe micro-organismen in ons systeem negatief beïnvloedde. Acetaat hoopte op in het systeem, en aan het eind van het experiment kon geen methanogene activiteit op acetaat meer worden waargenomen. Voor de batchexperimenten waarin een carbonaatarm medium was vereist gebruikten we gewoonlijk een fosfaat buffer van 72 mM. In deze batchexperimenten werd dan ook een duidelijke ophoping van acetaat waargenomen. In een additioneel continue experiment met een UASB in een carbonaatarm medium werd de pH geregeld op een neutrale waarde door middel van een automatische pH-regelaar en een NaOH oplossing, in plaats van met een fosfaat buffer. Hoewel slechte prestaties en instabiliteit werden waargenomen in dit systeem, vond hierin geen acetaat ophoping plaats terwijl het systeem werd bedreven onder optimale condities (data niet weergegeven). Conrad *et al.* [20] berichtten over de inhibitie van acetotrofe methanogenen door fosfaat ($\geq$ 20 mM) in experimenten uitgevoerd met gewassen opgegraven rijstwortels geïncubeerd in een fosfaat buffer onder anaërobe omstandigheden.

Recovery strategie

Ons gecultiveerde slib scheen vrij gevoelig te zijn voor pH-schokken, zowel in gebieden van lage als hoge pH, en een compleet herstel van methanogenese bleek onmogelijk wanneer het systeem werd bedreven onder de normaal toegepaste condities. De resultaten van de eerste verkennende herstel experimenten met slib, blootgesteld aan een basische pH schok geven, samen met de resultaten in Chapter 4 aan dat de mengcultuur behoefte heeft aan bicarbonaat om ten volle in staat te zijn tot methanogenese. Echter, wanneer de goede omgevingsomstandigheden niet worden gerealiseerd, resulteert deze zelfde toevoeging van bicarbonaat in een stimulatie van de productie van acetaat. Gewapend met deze kennis stellen wij een in Chapter 5 een strategie voor reactorherstel voor.

Wij veronderstelden dat in het geval de acetogenese beperkt zou worden door de toegediende hoeveelheid bicarbonaat, de normale omzettingen doorgang zouden vinden, en de methanogenen in staat zouden zijn te ontwikkelen, wanneer de optimale condities zouden zijn ingesteld in het systeem in batchmode. Wanneer eenmaal de methanogenese zou zijn hersteld en de juiste omgevingscondities kunnen worden gehandhaafd, zullen de (homo)acetogenen

de methanogenen niet meer een te grote concurrentie aandoen voor het aanwezige methanol en kan het gevormde acetaat snel worden omgezet. Hoofdpunt van deze strategie is de reactor te bedrijven in batchmode, totdat complete uitputting van de aangeboden hoeveelheid methanol zou zijn bereikt. Daarna wordt de vloeistoffase vervangen, voorafgaand aan een hervatte voeding. Wij geloven dat deze stap van belang is in de strategie, aangezien de acetoclastische methanogenen de meest gevoelige groep in het slib schijnen te zijn (zie de sectie 'conclusies'). Volgens Smith en Mah [109] is een mesofiele strain van *Methanosarcina* niet goed in staat acetaat om te zetten in de aanwezigheid van methanol, of in elk geval op een veel lagere snelheid.

Van groot praktisch belang is het feit dat wij in staat waren tot herstel van de methanogenese op methanol, zelfs in het geval de (homo)acetogenen de methanogenen zware concurrentie aandeden. De tijdstippen en het aantal benodigde voedingen hingen hierbij samen met de mate van beschadiging van het slib. Een andere belangrijke conclusie van het werk gepresenteerd in de Chapters 4 en 5 is dat voorzichtigheid moet worden betracht wanneer $NaHCO_3$ wordt toegepast voor het bufferen van methanolhoudende afvalwaters, aangezien de introductie hiervan in het systeem de acetogenese zal stimuleren wanneer de juiste condities niet zijn ingesteld.

Het effect van cobalt

Het onderzoek in Chapter 7 heeft betrekking op de vaststelling van het belang van cobalt voor het gecultiveerde thermofiele slib, en het effect daarvan op de competitie tussen methanogenen en acetogenen. Voor dit doel hebben we continu-experimenten in een UASB uitgevoerd, naast batch experimenten met een cobalt-deficiënte cultuur. In beide typen experimenten was de respons op de afwezigheid van cobalt niet direct waarneembaar. De cobalt behoefte van ons gecultiveerde slib was, in vergelijking met een mesofiel methylotroof consortium lager [31]. De resultaten geven aan dat ons gemengde slib behoefte heeft aan een zekere, hoewel vrij lage, hoeveelheid cobalt om de activiteit van de trofische groepen die betrokken zijn bij de omzetting van methanol op peil te houden. Daardoor is het mogelijk de maximale activiteit van de methanogenen te bereiken en, daarmee, hoge belastingen. Voor de cobalt-deficiënte verrijkingscultuur werd een cobalt concentratie van 0.1 μM de meest optimale concentratie bevonden. Deze staat groei van de methanogenen toe zonder competitie met de (homo)acetogenen voor de aanwezige methanol, en methaan is het enige eindproduct. Cobalt concentraties boven 0.1 μM leidden tot een ophoping van acetaat. De cobalt onthouding lijkt de hydrogenotrofe methanogenen niet te hinderen, maar beïnvloedt duidelijk de (homo)acetogenen zoals aangegeven door de waargenomen afname van de acetaatconcentratie in het effluent van de reactoren. Het gebrek aan productie van acetaat in het systeem (zowel de reactoren als de verrijkingsculturen) leidde tot verlies van de cultuur om acetaat om te zetten. Dit geeft aan dat de acetotrofe micro-organismen werden

uitgespoeld. De dosering van cobalt aan de cobalt-deficiënte verrijkingscultuur stimuleerde de vorming van acetaat. Wij namen waar dat de competitie om methanol tussen de methanogenen en de (homo)acetogenen zich slechts dan manifesteerde wanneer voldoende cobalt werd toegediend. Dit bevestigd dat klaarblijkelijk methanogenen beter in staat zijn cobalt te benutten dan acetogenen [29].

Onafhankelijk van de vraag of cobalt aan- of afwezig is, hebben zich duidelijke veranderingen voorgedaan de specifieke methanogene activiteit van het gekweekte slib. Het entslib gebruikt voor de experimenten in Chapter 7 was gekweekt onder vrijwel constante condities gedurende een periode van twee jaar. Bij de uitvoering van de experimenten met de metalen (de laatste zes maanden van de experimenten, zie Chapter 7), voerden we de ijzer concentratie op van 0.3 tot 24 μM. De waargenomen toename van de specifieke methanogene activiteit (SMA) op methanol en H_2/CO_2 geeft aan dat het slib ijzer-gelimiteerd was omdat de SMA waarden zelfs toenamen voor het slib wat groeide in de reactoren gebruikt voor de cobalt-onthoudingsexperimenten. De specifieke methanogene activiteit op acetaat onderging geen effect van de ijzer concentratie.

De opheldering van de omzettingsroute

In Chapter 6 wordt de route van de methanol omzetting in de gemengde cultuur opgehelderd. De resultaten van activiteitsmetingen in de aanwezigheid en afwezigheid van specifieke remmers geeft aan dat rond 50% van de methanol direct wordt omgezet in methaan door de methylotrofe methanogenen en dat de overige 50% wordt omgezet via de intermediairen H_2/CO_2 en acetaat. De hoge SMA van het slib voor H_2/CO_2, methanol en acetaat bevestigt de betrokkenheid van de drie methanogene groepen (hydrogenotroof, methylotroof en acetoclastisch) bij de omzetting van methanol in methaan.

Zoals bleek in de experimenten gepresenteerd in Chapter 4, reduceert de onthouding van anorganisch koolstof ($\Sigma[(HCO_3^- + CO_2)]$) in een fosfaat gebufferd systeem de methanol omzetting in ernstige mate. Dit duidt erop dat bicarbonaat Dit duidt erop voor een efficiënte en complete verwijdering van CZV bicarbonaat (CO_2) zowel benodigd is als "electron" (H_2) sink en als co-substraat. De hoeveelheid bicarbonaat, toegevoegd aan de reactor gedurende de kweek van het slib was 0.16 g HCO_3^- voor elke 1 g MeOH-COD terwijl 0.64 g HCO_3^- per 1 g MeOH-COD benodigd is voor de totale omzetting van methanol in acetaat. De aan de reactor gedoseerde hoeveelheid is voldoende om 25% van de totale populatie als methylotrofe (homo)acetogenen in stand te houden, zonder afhankelijk te zijn van het CO_2 wat gedurende de omzettingen wordt gevormd. De resultaten van de NMR experimenten laten zien dat de productie van acetaat ook is gerelateerd aan de oxidatie van methanol tot CO_2. Helaas staan onze experimentele resultaten echter geen kwantificering toe van de exacte hoeveelheid methanol die via acetaat dan wel via H_2/CO_2 in methaan wordt omgezet, maar de

resultaten van de experimenten uitgevoerd in het kader van dit proefschrift suggereren dat, kwalitatief beschouwd, beide routes een even belangrijke rol spelen.

De NMR techniek werd gebruikt om de route van de omzetting van methanol in acetaat te onderzoeken in systemen met voldoende bicarbonaat en systemen met een tekort hieraan. De resultaten wijzen er op dat methanol wordt omgezet via een gemeenschappelijke route (Acetyl-CoA), waarbij aan de ene kant reductie plaatsvindt tot de methyl groep van acetaat en aan de andere kant oxidatie tot CO_2, plaatsvindt, waarbij de CO_2 wordt ingebouwd als de carboxyl groep van het acetaat. Daarnaast laten de resultaten zien dat de grootste factie van het gevormde acetaat gevormd wordt via een korte biochemische route. Slechts bij een klein deel is een meer-enzymatische route betrokken.

De omzetting van H_2/CO_2 in acetaat en vice-versa, hoewel ogenschijnlijk mogelijk, lijkt in de uitvoering van de experimenten geen belangrijke rol te spelen in de uiteindelijke bestemming van de methanol. Aangaande de omzetting van acetaat in H_2/CO_2 is het belangrijk enkele veranderingen in het gecultiveerde slib te benadrukken, die plaatsvonden na de afronding van de experimenten. Wij namen waar dat gedurende de 2.5 jaar van cultivering van dit slib onder vrijwel onveranderde omstandigheden (met uitzondering van de ijzerconcentratie zoals eerder genoemd) de specifieke methanogene activiteit op acetaat sterk afnam gedurende het bedrijven van de reactor. De specifieke methanogene activiteit op acetaat viel terug van 0.84 naar 0.54 g COD.g $VSS^{-1}.d^{-1}$ na 2 jaar en tot 0.23 g COD.g $VSS^{-1}.d^{-1}$ na 2.5 jaar. Dit duidt erop dat de acetoclastische methanogenen geleidelijk steeds minder belangrijk worden, terwijl de syntrofe omzetting van acetaat in methaan via H_2/CO_2 van groter belang wordt, getuige ook de toename van de specifiek methanogene activiteit op H_2/CO_2 (Chapter 7) en het feit dat wij geen significante acetaat ophoping aantroffen ondanks de nog steeds hoge activiteit van de (homo)acetogenen. Zo'n verandering in het karakter van het slib is niet onaannemelijk. De syntrofe omzetting van acetaat in methaan via H_2/CO_2 schijnt een belangrijke metabole route te vertegenwoordigen onder thermofiele en extreme thermofiele condities[148, 151, 152], terwijl in omgevingen met een hoge temperatuur de acetoclastische methanogenese verondersteld wordt in belang af te nemen.

Het is duidelijk dat alle aangegeven routes (behalve de omzetting van H_2/CO_2 in acetaat) een rol spelen in de afbraak van methanol in ons gemengde gecultiveerde slib. De balans tussen de verschillende micro-organismen die aanwezig zijn in het slib is van groot belang aangezien deze bepaalt wat de uiteindelijke omzettingsreacties worden. Aan de ene kant dient de alternatieve omzettingsroute als electronen-sink die helpt de pH_2 voldoende laag te houden zo dat methanol in H_2/CO_2 kan worden omgezet. Aan de andere kant wordt via de alternatieve omzettingsroutes acetaat in methaan omgezet.

Conclusies en aanbevelingen

De resultaten van dit onderzoek bevestigen dat de ophoping van acetaat een groot struikelblok vormt in de thermofiele anaërobe afbraak van methanol. Echter, ondanks dat, is de eentraps UASB nog steeds een aantrekkelijk alternatief is voor de zuivering van methanol-houdende afvalwaters. Wij hebben hier ook voldoende materiaal verzameld om te tonen dat de toepassing van zo'n behandeling succesvol kan zijn.

De acetoclastische methanogenen zijn overduidelijk de meest gevoelige groep organismen die aanwezig is in het gecultiveerde slib. Zij zijn gevoelig voor uitspoeling en afsterving en klaarblijkelijk niet, of slechts met zeer lage snelheid, in staat te groeien in de aanwezigheid van methanol. De oxidatie van acetaat tot H_2/CO_2 is ook een gevoelig proces aangezien het slechts dan verloopt wanneer een sink voor H_2 aanwezig is teneinde de pH_2 in het systeem voldoende laag te houden. De hydrogenotrofe methanogenen bezitten de hoogste specifieke methanogene activiteit en zij hebben ook een hogere bestendigheid tegen ongunstige veranderingen in de omgeving, maar zij zijn afhankelijk van de (homo)acetogene stap.

Aangezien de bijdrage van de directe omzetting van methanol aan de totale vorming van methaan in ons systeem slechts rond 50% bedraagt, is de vorming van de resterende 50% van het methaan afhankelijk van het plaatsvinden van de (homo)acetogene stap. De betrokkenheid van de oxidatie van methanol tot H_2/CO_2 in dit proces duidt erop dat sprake is van een zeer kwetsbaar systeem. Er moet rekening mee worden gehouden dat kleine veranderingen in de partiaalspanning van watersof een sterk effect kunnen hebben op de substraat-omzettings-snelheden [60] terwijl, wanneer alternatieve afbraakroutes beschikbaar zijn voor de micro-organismen, het spectrum van producten kan veranderen [16, 63, 83].

De onthouding van cobalt en van bicarbonaat schijnen zeer sterk vergelijkbare effecten te hebben op het gecultiveerde slib. In beide gevallen wordt de (homo)acetogene stap beïnvloedt en dit beïnvloedt indirect ook de methanogene groepen (behalve de methylotrofen, die direct beïnvloedt worden door cobalt tekorten). De dosering of onthouding van cobalt of bicarbonaat om de ene of de andere groep van micro-organismen te remmen of te stimuleren lijkt dan ook uitermate riskant, temeer daar problemen met de (homo)acetogenen desastreus zullen zijn voor de methanogenen en daarmee voor het gehele proces.

Desalniettemin bevelen wij toch aan dat cobalt en bicarbonaat altijd stapsgewijs worden geïntroduceerd in het systeem, teneinde de competitie tussen de (homo)acetogenen en de methanogenen voor methanol te vermijden, en teneinde een uitgebalanceerd slib te ontwikkelen met methaan als het bedoelde eindproduct. De totaal benodigde hoeveelheden cobalt en bicarbonaat mogen alleen worden gedoseerd wanneer zeker is dat methanogenen aanwezig, en voldoende actief, zijn, teneinde de delicate balans tussen de verschillende micro-organismen in stand te houden.

Daar een andere functie van bicarbonaat is het systeem te bufferen, betekent een stapsgewijze dosering van bicarbonaat dat niet direct voldoende buffercapaciteit in het systeem aanwezig zal zijn. Daarom moet in de opstartfase de pH op een andere manier geregeld worden. Fosfaat kan niet beschouwd worden als een geschikte buffer, tenzij duidelijk is dat het de activiteit van het slib niet remt.

Wij bevelen het gebruik van bicarbonaat aan voor de behandeling van methanol-bevattende afvalwaters voorzover hierbij een syntrofe omzetting via H_2/CO_2 betrokken is. De redenen hiervoor zijn dat *i*) een buffer sowieso noodzakelijk is vanwege de noodzaak van een vrijwel neutrale pH en *ii*) de prestaties van de reactor in geval van toevoeging van bicarbonaat overduidelijk beter zijn en de reactor ook stabieler is. Zoals aangetoond in de continu-experimenten, is 0.16 g HCO_3^- per 1 g MeOH-COD voldoende om de pH in het gebied van 6.5 – 6.7 te houden en een stabiel systeem te verkrijgen.

References

1. **Abram J. W. and D. B. Nedwell** (1978) Inhibition of methanogenesis by sulphate reducing bacteria competing for transferred hydrogen. Arch. Microbiol. **117**:89 - 92.

2. **Ahring B. K.** (1995) Methanogenesis in thermophilic biogas reactors. Antonie van Leeuwenhoek. **67**:91-102.

3. **Ahring B. K.** (1994) Status on science application of thermophilic anaerobic digestion. Water Sci. Technol. **30**(12):241-250.

4. **Ahring B. K., J. E. Schmidt, M. W-Nielsen, A. J. L. Macario and E. C. de Macario** (1993) Effect of medium composition and sludge removal on the production, composition, and architecture of thermophilic (55 °C) acetate-utilizing granules from an upflow anaerobic sludge blanket reactor. Appl. Environ. Microb. **59**(8):2538-2545.

5. **American P. H. A.** (1985) Standard methods for the examination of water and wastewater. 16th Edition. American Public Health Association, Washington D.C.

6. **Balk M., J. Weijma and A. J. M. Stams** (2002) *Thermotoga lettingae* sp. nov., a novel thermophilic, methanol degrading bacterium isolated from a thermophilic anaerobic reactor. Int. J. Syst. Evol. Micr. **52**:1361-1368.

7. **Basiliko N. and J. B. Yavitt** (2001) Influence of Ni, Co, Fe, and Na additions on methane production in *Sphagnum*-dominated northern American peatlands. Biogeochemistry Dordrecht. **52**(2):133-153.

8. **Becker U. and S. Peiffer** (1997) Heavy-metal ion complexation by particulate matter in the leachate of solid waste: A multi-method approach. J. Contam. Hydrol. **24**(3-4):313-344.

9. **Beliaeff B. and Y. Mary Jean** (1993) The "most probable number" estimate and its confidence limits. Water Res. **27**(5):799-805.

10. **Bhattacharya S. K., V. Uberoi, R. L. Madura and P. M. R. Haghighi** (1995) Effect of cobalt on methanogenesis. Environ. Technol. **16**(3):271-278.

11. **Bhatti Z. I., K. Furukawa and M. Fujita** (1995) Comparative composition and characteristics of methanogenic granular sludges treating industrial wastes under different conditions. J. Ferment. Bioeng. **79**(3):273-280.

12. **Bhatti Z. I., K. Furukawa and M. Fujita** (1996) Feasibility of methanolic waste treatment in UASB reactors. Water Res. **30**(11):2559-2568.

13. **Bhatti Z. I., K. Furukawa and M. Fujita** (1995) Methanogenic granular sludge formation in an upflow anaerobic sludge blanket reactor treating synthetic methanolic waste. World J. Microbiol. Biotechn. **11**(6):631-637.

14. **Bhatti Z. I., K. Furukawa and M. Fujita** (1993) Treatment performance and microbial structure of a granular consortium handling methanolic waste. J. Ferment. Bioeng. **76**(3):218-223.

15. **Brock** (1997) Biology of microorganisms. 8th Edition. Ed M.T. Madigan, J. M. Martinko and J. Parker. Prentice-Hall, New Jersey.

16. **Brosseau J. D., J. Y. Yan and K. V. Lo** (1986) The relationship between hydrogen gas and butanol production by *Clostridium saccharoperbutylacetonicum*. Biotechnol. Bioeng. **28**:305-310.

17. **Cayless S. M., D. M. L. da Motta Marques and J. N. Lester** (1990) A study of the effects of methanol in start-up of UASB reactors. Biological Wastes. **31**:123 - 135.

18. **Cheng R.** (1977) Physical chemistry with applications to biological systems. p. 538 . Macmillan Publishing Co., New York.

19. **Conrad R., F. Bak, H. J. Seitz, B. Thebrath, H. P. Mayer and H. Schutz** (1989) Hydrogen turnover by psychrotrophic homoacetogenic and mesophilic methanogenic bacteria in anoxic paddy soil and lake sediment. FEMS Microbiol. Ecol. **62**:285 -294.

20. **Conrad R., M. Klose and P. Claus** (2000) Phosphate inhibits acetotrophic methanogenesis on rice roots. Appl. Environ. Microb. **66**(2):828-831.

21. **Conrad R., B. Schink and T. J. Phelps** (1986) Thermodynamics of H_2-consuming and H_2-producing metabolic reactions in diverse methanogenic environments under *in situ* conditions. FEMS Microbiol. Ecol. **38**:353 - 360.

22. **Cord-Ruwisch R. and B. Ollivier** (1986) Interspecific hydrogen transfer during methanol degradation by *Sporomusa acidovorans* and hydrogenophilic anaerobes. Arch. Microbiol. **144**:163-165.

23. **Davidova I. A. and A. J. M. Stams** (1996) Sulfate reduction with methanol by a thermophilic consortium obtained from a methanogenic reactor. Appl Microbiol. Biotechnol. **46**:297-302.

24. **Dilworth M. J. and A. R. Glenn.** (1999) Problems of adverse pH and bacterial strategies to combat it. p. 4 - 18. *In* Bacterial responses to pH . Novartis Found Symp 221, Chichester.

25. **Driessen W., M. Tielbaard, L. Habets and P. Yspeert.** (2000) Anaerobic treatment of evaporator condensate from the chemical pulp industry. Proceedings of the VI

Latin-American Workshop and seminar on anaerobic digestion, Recife, Pernambuco, Brazil, p. 127-134.

26. **Fathepure B. Z.** (1987) Factors affecting the methanogenic activity of *Methanotrix soehngenii*. Appl. Environ. Microbiol. **53**:2978-2982.

27. **Field J.** (2002) Limits of anaerobic degradation. Water Sci. Technol. **45**(10):9-18.

28. **Florencio L.** (1994) The fate of methanol in anaerobic bioreactors. PhD. Thesis. Wageningen University. Wageningen, The Netherlands.

29. **Florencio L., J. A. Field and G. Lettinga** (1994) Importance of cobalt for individual trophic groups in an anaerobic methanol-degrading consortium. Appl. Environ. Microbiol. **60**(1):227-234.

30. **Florencio L., J. A. Field and G. Lettinga** (1995) Substrate competition between methanogens and acetogens during the degradation of methanol in UASB reactors. Water Res. **29**(3):915-922.

31. **Florencio L., P. Jenicek, J. A. Field and G. Lettinga** (1993) Effect of cobalt on the anaerobic degradation of methanol. J. Ferment. Bioeng. **75**(5):368-374.

32. **Florencio L., A. Nozhevnikova, A. van Langerak, A. J. M. Stams, J. A. Field and G. Lettinga** (1993) Acidophilic degradation of methanol by a methanogenic enrichment culture. FEMS Microb. Letters. **109**:1-6.

33. **Franklin M. J., W. J. Wiebe and W. B. Whitman** (1988) Populations of methanogenic bacteria in a Georgia salt marsh. Appl. Environ. Microb. **54**:1151 - 1157.

34. **Fuchs G., E. Stupperich and R. K. Thauer** (1978) Acetate assimilation and the synthesis of alanine, aspartate and glutamate in *Methanobacterium thermoautotrophicum*. Arch. Microbiol. **117**(1):61-66.

35. **Fukuzaki S. and N. Nishio** (1997) Methanogenic fermentation and growth of granular methanogenic sludge on a methanol-propionate mixture. J. Ferment. Bioeng. **84**(4):382-385.

36. **Fukuzaki S., N. Nishio and S. Nagai** (1990) Kinetics of the methanogenic fermentation of acetate. Appl. Environ. Microb. **56**(10):3158-3163.

37. **Fukuzaki S., N. Nishio, M. Shobayashi and S. Nagai** (1990) Inhibition of the fermentation of propionate to methane by hydrogen, acetate, and propionate. Appl. Environ. Microb. **56**(3):719-723.

38. **Garcia J. L., B. K. C. Patel and B. Ollivier** (2000) Taxonomic, Phylogenetic and ecological Diversity of Methanogenic Archaea. Anaerobe. **6**:205-226.

39. **Genthner B. R. S. and M. Bryant** (1987) Additional characteristics of one-carbon-compound utilisation by *Eubacterium limosum* and *Acetobacterium woodii*. Appl. Environ. Microb. **53**:471 - 476.

40. **Gonzalez-Gil G., R. Kleerebezem and G. Lettinga** (1999) Effects of nickel and cobalt on kinetics of methanol conversion by methanogenic sludge as assessed by on-line CH_4 monitoring. Appl. Environ. Microb. **65**(4):1789-1793.

41. **Gonzalez-Gil G., R. Kleerebezem, A. van Aelst, G. R. Zoutberg, A. I. Versprille and G. Lettinga** (1999) Toxicity effects of formaldehyde on methanol degrading sludge and its anaerobic conversion in Biobed expanded granular sludge bed (EGSB) reactors. Water Sci. Technol. **40**(8):195-202.

42. **Grotenhuis J. T. C.** (1992) Structure and stability of methanogenic granular sludge. PhD. Thesis. Wageningen University. Wageningen, The Netherlands.

43. **Grotenhuis J. T. C., J. B. Van Lier, C. M. Plugge, A. J. M. Stams and A. J. B. Zehnder** (1991) Effect of ethylene glycol-bis(beta-aminoethyl ether)-N,N-tetraacetic acid (EGTA) on stability and activity of methanogenic granular sludge. Appl. Microbiol. Biotechnol. **36**(1):109-114.

44. **Hall H. K., K. L. Karem and J. W. Foster** (1995) Molecular responses of microbes to environmental pH stress. Adv. Microb. Physiol. **37**:229 - 272.

45. **Hattori S., Y. Kamagata, S. Hanada and H. Shoun** (2000) *Thermacetogenium phaeum* gen. nov., sp. nov., a strictly anaerobic, thermophilic, syntrophic acetate-oxidizing bacterium. Int. J. Syst. Evol. Micr. **50**:1601-1609.

46. **Heijhuijsen J. H. F. G. and T. A. Hansen.** (1990) C_1- metabolism in anaerobic non-methanogenic bacteria. p. 163-191. *In* Autotrophic microbiology and one-carbon metabolism. G. A., Codd; L., Dijkhuizen; F.R., Tabita (Ed). Kluwer Academic Publishers, Boston.

47. **Hoban D. J. and L. van den Berg** (1979) Effect of iron on conversion of acetic acid to methane during methanogenic fermentations. J. Appl. Bacteriol. **47**(1):153-159.

48. **Hulshoff-Pol L.** (1989) The phenomenon of granulation of anaerobic sludge. PhD. Thesis. Wageningen University. Wageningen, The Netherlands.

49. **Hulshoff-Pol L., W. J. Zeeuw, Velzeboer C. T. M. and G. Lettinga** (1983) Granulation in UASB reactors. Water Sci. Technol. **14**:291-304.

50. **Jarvis A., A. Nordberg, T. Jarlsvik, B. Mathisen and B. H. Svensson** (1997) Improvement of a grass-clover silage-fed biogas process by the addition of cobalt. Biomass and Bioenergy. **12**(6):453-460.

51. **Jiang Y. L., M. S. Lim and D. H. Kim** (2001) Simulation studies of the hydrogen production from methanol partial oxidation steam reforming by a tubular packed-bed catalytic reactor. Chinese J. Chem. Eng. **9**(3):297-305.

52. **Jirka A. and M. J. Carter** (1975) Micro semi-automated analysis of surface and wastewater for chemical oxygen demand. Anal. Chem. **47**:1397-1410.

53. **Kanamaru T., S. Urano, M. Inoue, N. Nishiyama and C. Nishino** (1990) Development of SNG process from methanol. Kagaku Kogaku (in Japanese, abstract available in English). **54**(6):414 - 417.

54. **Kasali G. B., E. Senior and I. A. Watson Craik** (1989) Sodium bicarbonate effects on the anaerobic digestion of refuse. J. Chem. Technol. Biot. **45**(4):279-290.

55. **Kaszycki P., M. Tyszka, P. Malec and H. Koloczek** (2001) Formaldehyde and methanol biodegradation with the methylotrophic yeast *Hansenula polymorpha*. An application to real wastewater treatment. Biodegradation. **12**(3):169-177.

56. **Kerby R., W. Niemczura and J. G. Zeikus** (1983) Single-carbon catabolism in acetogens: analysis of carbon flow in *Acetobacterium woodii and Butyribacterium methylotrophicum* by fermentation and ^{13}C nuclear magnetic resonance measurement. J. Bacteriol. **155**(3):1208-1218.

57. **Kida K., T. Shigematsu, J. Kijima, M. Numaguchi, Y. Mochinaga, N. Abe and S. Morimura** (2001) Influence of Ni^{2+} and Co^{2+} on methanogenic activity and the amounts of coenzymes involved in methanogenesis. J. Biosci. Bioeng. **91**(6):590-595.

58. **Kim B.-K. and L. Daniels** (1991) Unexpected errors in gas chromatographic analysis of methane production by thermophilic bacteria. Appl. Environ. Microb. **57**(6):1866-1869.

59. **King G. M.** (1988) Methanogenesis from methylated amines in a hypersaline algal mat. Appl. Environ. Microb. **54**:130-136.

60. **Kleerebezem R. and A. J. M. Stams** (2000) Kinetics of syntrophic cultures: A theoretical treatise on butyrate fermentation. Biotechnol. Bioeng. **67**(5):529-543.

61. **Koesnandar, N. Nishio and S. Nagai** (1991) Effects of trace metal ions on the growth, homoacetogenesis and corrinoid production by *Clostridium thermoaceticum*. J. Ferm. Bioeng. **71**(3):181-185.

62. **Krzycki J. and J. G. Zeikus** (1980) Quantification of corrinoids in methanogenic bacteria. Curr. Microbiol. **3**:243-245.

63. **Lamed R. and J. G. Zeikus** (1980) Ethanol production by thermophilic bacteria: relationship between fermentation product yields and catabolic enzyme activities in

Clostridium thermocellum and *Thermoanaerobium brockii*. J. Bacteriol. **144**(2):569-578.

64. **LaPaglia C. and P. L. Hartzell** (1997) Stress-induced production of biofilm in the hyperthermophile *Archaeoglobus fulgidus*. Appl. Environ. Microb. **63**(8):3158-3163.

65. **Lee M. J. and S. H. Zinder** (1988) Isolation and characterisation of a thermophilic bacterium which oxidises acetate in syntrophic association with a methanogen and which grows acetogenically on H_2-CO_2. Appl. Environ. Microb. **54**:124-129.

66. **Lepisto R.** (1999) Extreme thermophilic anaerobic process for wastewater treatment. PhD. Thesis. Tampere University of Technology. Tampere, Finland.

67. **Lettinga G.** (1995) Anaerobic digestion and wastewater treatment systems. Antonie van Leeuwenhoek. **67**(1):3-28.

68. **Lettinga G., W. de Zeeuw and E. Ouborg** (1981) Anaerobic treatment of wastes containing methanol and higher alcohols. Water Res. **15**:172-182.

69. **Lettinga G., L. Hulshoff-Pol and G. Zeeman.** (1998) Anaerobic wastewater treatment. *In* Lecture notes biological wastewater treatment , Wageningen, The Netherlands.

70. **Lettinga G., A. T. Van der Geest, S. Hobma and J. Van der Laan** (1979) Anaerobic treatment of methanolic wastes. Water Res. **13**:725-737.

71. **Lindstrom B. and L. J. Pettersson** (2001) Hydrogen generation by steam reforming of methanol over copper-based catalysts for fuel cell applications. Int. J. Hydrogen Energy. **26**(9):923-933.

72. **Ljungdahl L. G.** (1986) The autotrophic pathway of acetate synthesis in acetogenic bacteria. Annu. Rev. Microbiol. **40**:415-450.

73. **Lustenhouwer J. W. A. and J. A. Hin** (1990) Sample digestion and ICP-AES analysis. Int. J. Anal. Chem. **39**:209-214.

74. **Lynd L. H. and J. G. Zeikus** (1983) Metabolism of H_2-CO_2, methanol and glucose by *Butyribacterium methylotrophicum*. J. Bacteriol. **153**:1415-1423.

75. **Mah R. A., M. R. Smith and L. Baresi** (1978) Studies on an acetate-fermenting strain of *Methanosarcina*. Appl. Environ. Microbiol. **35**(6):1174-1184.

76. **Mahoney E. M., L. K. Varangu, Cairns W. L., Kosaric N. and R. G. E. Murray** (1987) The effect of calcium on microbial aggregation during UASB reactor start-up. Water Sci. Technol. **19**:249-260.

77. **Minami K., T. Horiyama, M. Tasaki and Y. Tanimoto** (1986) Methane production using a bio-reactor packed with pumice stone and evaporator condensate of a kraft pulp mill. J. Ferment. Technol. **64**:523-532.

78. **Minami K., K. Okamura, S. Ogawa and T. Naritomi** (1991) Continuous anaerobic treatment of wastewater from kraft pulp mill. J. Ferment. Bioeng. **71**:270-274.

79. **Minami K., Y. Tanimoto, M. Tasaki, S. Ogawa and K. Okamura** (1988) Influence of pH on methane and sulphide production from methanol. J. Ferment. Technol. **66**:117-121.

80. **Minami K., Y. Tanimoto, M. Tasaki, S. Ogawa and K. Okamura** (1988) Influence of sulphate on methane fermentation in a defined medium. Water Sci. Technol. **20**(11-12):451-453.

81. **Mochinaga K., K., Y. Abe and S. Morimura.** (1997) Influence of Ni^{2+} and Co^{2+} on activity of microorganisms related to methane fermentation. Proceedings of the 8th International Conference on Anaerobic Digestion., Sendai, Japan, p. 27-30.

82. **Murray W. D. and L. v. d. Berg** (1981) Effects of nickel, cobalt and molybdenium on performance of methanogenic fixed-film reactors. Appl. Environ. Microb. **42**:502-505.

83. **Murray W. D. and A. W. Khan** (1983) Ethanol production by a newly isolated anaerobe, *Clostridium saccharolyticum*: effect of culture medium and growth conditions. Can J. Microbiol. **29**(3):342-347.

84. **Nanninga H. J. and J. C. Gottshal** (1987) Isolation of a sulphate-reducing bacterium growing with methanol. FEMS Microbiol. Ecol. **38**:125 -130.

85. **Nies D. H.** (1999) Microbial heavy-metal resistance. Appl. Microbiol. Biotechnol. **51**(6):730-750.

86. **Nikawa T., Y. Harata, S. Yamada and K. Yamazaki** (1998) Manufacture method of fuel gas from methanol or methanol-water mixture as raw material. Jpn. Kokai Tokkyo Koho (Patent written in Japanese, abstract available in English):11pp.

87. **Nishio N., R. G. Silveira, K. Hamato and S. Nagai** (1993) High rate methane production in a UASB reactor fed with methanol and acetate. J. Ferment. Bioeng. **75**(4):309-313.

88. **Nozhevnikova A. N. and V. I. Chudina** (1984) Morphology of the thermophilic acetate bacterium *Methanothrix thermoacetophila* sp. nov. Microbiology. **53**:618 - 624.

89. **Oleszkiewicz J. A. and A. Romanek** (1989) Granulation in anaerobic sludge bed reactors treating food industry wastes. Biological Wastes. **27:**217-235.

90. **Pacaud S., P. Loubiere and G. Goma** (1985) Methanol metabolism by *Eubacterium-Limosum B2* - effects of pH and Carbon-dioxide on Growth and organic-acid production. Curr. Microbiol. **12**(5):245-250.

91. **Pavlostathis G. S. and E. Giraldo-Gomez** (1991) Kinetics of anaerobic treatment: a critical review. Crit. Rev. Environ. Control. **21:**411-490.

92. **Perez M., L. I. Romero and D. Sales** (1998) Comparative performance of high rate anaerobic thermophilic technologies treating industrial wastewater. Water Res. **32**(3):559-564.

93. **Perski H. J., P. Schonheit and R. K. Thauer** (1982) Sodium dependence of methane formation in methanogenic bacteria. FEBS Letters. **142**(2):323-326.

94. **Pirnie M. I.** (1999) Evaluation of the fate and transport of methanol in the environment. p. 1-69. Institute of methanol. Report (electronic source), California.

95. **Ram M. S., L. Singh, M. V. S. Suryanarayana and S. I. Alam** (2000) Effect of iron, nickel and cobalt on bacterial activity and dynamics during anaerobic oxidation of organic matter. Water, Air, and Soil Poll. **117:**1-4.

96. **Rintala J. and G. Lettinga** (1992) Effects of temperature elevation from 37 °C to 55 °C on anaerobic treatment of sulphate rich acidified wastewaters. Environmental Technology. **13**(9):801-812.

97. **Robertson L. A. and J. G. Kuenen.** (1992) Nitrogen removal from water and waste. p. 227 - 267. *In* Microbial control of pollution . Cambridge University Press, Cambridge, England.

98. **Savage M. D. and H. L. Drake** (1986) Adaptation of the acetogen *Clostridium thermoautotrophicum* to minimal medium. J. Bacteriol. **165:**315 -318.

99. **Scherer P., H. Lippert and G. Wolff** (1983) Composition of the major elements and trace elements of 10 methanogenic bacteria determined by inductively coupled plasma emission spectrometry. Biol. Trace Element Res. **5:**149-163.

100. **Schink B.** (1994) Diversity, ecology and isolation of acetogenic bacteria. p. 197-235. *In* Acetogenesis . Chapman & Hall microbiology series, New York.

101. **Schink B.** (1987) Ecology of C_1-metabolising anaerobes. p. 81 - 85. *In* Microbial growth on C_1-compounds . Martinus Nijhoff Publishers, Dordrecht.

102. **Schink B.** (1997) Energetics of syntrophic cooperation in methanogenic degradation. Microbiol. Mol. Biol. Rev. **61**(2):262 -280.

103. **Schink B. and J. G. Zeikus** (1980) Microbial methanol formation: a major end product of pectin metabolism. Curr. Microbiol. **4**:387 - 390.

104. **Schmidt J. E. and B. K. Ahring** (1994) Extracellular polymers in granular sludge from different upflow anaerobic sludge blanket (UASB) reactors. Appl. Microbiol. Biotechnol. **42**(2-3):457-462.

105. **Schönheit P., J. Moll and R. K. Thauer** (1979) Nickel, cobalt and molybdenium requirement for growth of *methanobacterium thermoautotrophicum*. Arch. Microbiol. **123**:105-107.

106. **Seif H. A. A., S. G. Joshi and S. K. Gupta** (1992) Effect of organic load and reactor height on the performance of anaerobic mesophilic and thermophilic fixed film reactors in the treatment of pharmaceutical wastewaters. Environ. tech. **13**:1161-1168.

107. **Sharma J. and R. Singh** (2001) Effect of nutrients supplementation on anaerobic sludge development and activity for treating distillery effluent. Bioresource Technol. **79**(2)):203-206.

108. **Singh R. P., S. Kumar and C. S. P. Ojha** (1999) Nutrient requirement for UASB process: a review. Biochem. Eng. J. **3**:33-54.

109. **Smith M. R. and R. A. Mah** (1978) Growth and methanogenesis by *Methanosarcina strain 227* on acetate and methanol. Appl. Environ. Microb. **36**(6):870-879.

110. **Soto M., R. Méndez and L. J.M.** (1992) Characterisation and comparison of biomass from mesophilic and thermophilic fixed bed anaerobic digesters. Water Sci. Technol. **25**(7):203-212.

111. **Speece R. E.** (1996) Trace Metals. p. 221-224. *In* Anaerobic Biotechnology for Industrial Wastewaters . Archae Pr, Nashville, Tenesse, USA.

112. **Stams A., J. M.,, J. Van Dijk, B., C. Dijkema and M. Plugge C.** (1993) Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. Appl. Environ. Microb. **59**(4):1114-1119.

113. **Stams A. J. M., K. C. F. Grolle, C. T. M. J. Frijters and J. B. Van Lier** (1992) Enrichment of thermophilic propionate-oxidizing bacteria in syntrophy with *Methanobacterium thermoautotrophicum* or *Methanobacterium thermoformicicum*. Appl. Environ. Microb. **58**(1):346-352.

114. **Strayer R. F. and J. M. Tiedje** (1978) Kinetic parameters of the conversion of methane precursors to methane in hypereutrophic lake sediment. Appl. Environ. Microb. **36**:330 -340.

115. **Stupperich E., I. Steiner and J. Eisinger** (1987) Substitution of Coα-(5-hydroxybenzimidazolyl)cobamide (Factor III) by vitamin B_{12} in *Methanobacterium thermoautotrophicum*. J. Bacteriol. **169**:3076-3081.

116. **Syutsubo K., H. Harada and A. Ohashi** (1998) Granulation and sludge retainment during start-up of a thermophilic UASB reactor. Water Sci. Technol. **38**(8-9):349-357.

117. **Syutsubo K., N. Sinthurat, A. Ohashi and H. Harada** (2001) Population dynamics of anaerobic microbial consortia in thermophilic granular sludge in response to feed composition change. Water Sci. Technol. **43**(1):59-66.

118. **Takashima M. and R. E. Speece.** (1997) Competition for essential trace metals, Fe and Ni, between acetate-utilising methanogens. Proceedings of the 8th International Conference on Anaerobic Digestion, Sendai, Japan, p. 95-98.

119. **Takashima M. and R. E. Speece** (1990) Mineral requirements for methane fermentation. Crit. Rev. Env. Control. **19**(5):465-479.

120. **Taylor G. T. and S. J. Pirt** (1977) Nutrition and factors limiting the growth of a methanogenic bacterium (*Methanobacterium thermoautotrophicum*). Arch. Microbiol. **113**(1-2):17-22.

121. **Thauer R. K., K. Jungermann and K. Decker** (1977) Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. **41**:100 - 180.

122. **Touzel J. P., D. Petroff and G. Albagnac** (1985) Isolation and characterisation of a new thermophilic *Methanosarcina*, the strain CHTI 55. System Appl. Microbiol. **6**:66 -71.

123. **Van Lier J. B.** (1996) Limitations of thermophilic anaerobic waste-water treatment and the consequences for process design. Antonie van Leeuwenhoek. **69**:1 - 14.

124. **Van Lier J. B.** (1995) Thermophilic anaerobic wastewater treatment; temperature aspects and process stability. PhD. Thesis. Wageningen University. Wageningen, The Netherlands.

125. **Van Lier J. B., F. Boersma, M. M. W. H. Debets and Lettinga** (1994) High rate thermophilic anaerobic wastewater treatment in compartmentalised upflow reactors. Water Sci. Technol. **30**(12):251-261.

126. **Van Lier J. B., N. Groeneveld and G. Lettinga** (1996) Development of thermophilic methanogenic sludge in compartmentalized upflow reactors. Biotechnol. Bioeng. **50**(2):115-124.

127. **Van Lier J. B., C. F. Grolle K., T. M. J. Frijters C., J. M. Stams A. and G. Lettinga** (1993) Effects of acetate, propionate, and butyrate on the thermophilic anaerobic degradation of propionate by methanogenic sludge and defined cultures. Appl. Environ. Microb. **59**(4):1003-1011.

128. **Van Lier J. B., S. Rebac and G. Lettinga** (1997) High-rate anaerobic wastewater treatment under psychrophilic and thermophilic conditions. Water Sci. Technol. **35**(10):199-206.

129. **Vandermeijden P., C. Vanderdrift and G. D. Vogels** (1984) Methanol conversion in *Eubacterium-Limosum*. Arch. Microbiol. **138**(4):360-364.

130. **Veeken A.** (1998) Removal of heavy metals from biowaste. PhD. Thesis. Wageningen University. Wageningen, The Netherlands.

131. **Ventakaramani E. S., R. C. Ahlert and P. Corbo** (1984) Biological treatment of landfill leachates. CRC Critical reviews in Environmental Control. **14**(4):333-376.

132. **Verstraete W., D. deBeer, M. Pena, G. Lettinga and P. Lens** (1996) Anaerobic bioprocessing of organic wastes. World J. Microb. Biot. **12**(3):221-238.

133. **Voolapalli R., K. and C. Stuckey D.** (2001) Hydrogen production in anaerobic reactors during shock loads: Influence of formate production and H_2 kinetics. Water Res. **35**(7):1831-1841.

134. **Weijma J.** (2000) Methanol as electron donor for thermophilic biological sulphate and sulphite reduction. PhD Thesis. Wageningen University. Wageningen, The Netherlands.

135. **Weijma J., A. Stams, J. M., L. W. Hulshoff Pol and G. Lettinga** (2000) Thermophilic sulfate reduction and methanogenesis with methanol in a high rate anaerobic reactor. Biotechnol. Bioeng. **67**(3):354-363.

136. **Weijma J. and A. J. M. Stams** (2001) Methanol conversion in high-rate anaerobic reactors. Water Sci. Technol. **44**(8):7-14.

137. **Westermann P., B. K. Ahring and R. A. Mah** (1989) Acetate production by methanogenic bacteria. Appl. Environ. Microb. **55**(9):2257 - 2261.

138. **Wiegant W. M.** (1986) Thermophilic anerobic digestion for waste and wastewater treatment. PhD. Thesis. Wageningen University. Wageningen, The Netherlands.

139. **Wiegel J., M. Braun and G. Gottschalk** (1981) *Clostridium thermoautotrophicum* species novum, a thermophilic producing acetate from molecular hydrogen and carbon dyoxide. Curr. Microbiol. **5**:255-260.

140. **Winfrey M. R. and J. G. Zeikus** (1977) Effect of sulphate on carbon and electron flow during microbial methanogenesis in freshwater sediments. Appl. Environ. Microb. **33**:275 - 281.

141. **Wood H. G.** (1952) A study of carbon dioxide fixation by mass determination of the types of C^{13} acetate. J. Biol. Chem. **194**:905-931.

142. **Yamaguchi M., J. Hake, Y. Tanimoto, T. Naritomi, K. Okamura and K. Minami** (1991) Enzyme activity for monitoring the stability in a thermophilic anaerobic digestion of wastewater containing methanol. J. Ferment. Bioeng. **71(4)**:264-269.

143. **Yamaguchi M. and K. Minami** (1998) Production of acetic acid from methanol by thermophilic *Methanosarcina* sp.: acetate production as an index in abnormal methane fermentation. J. Ferment. Bioeng. **86**(2):239-242.

144. **Yamaguchi M., K. Minami, Y. Tanimoto and K. Okamura** (1989) Effects of volatile fatty acids on methanogenesis of methanol and of pregrowth with methanol on acetate utilization by methanogens. J. Ferment. Bioeng. **68**(6):428-432.

145. **Yu H. Q. and H. H. P. Fang** (2000) Thermophilic acidification of dairy wastewater. Appl. Microbiol. Biotechnol. **54**(3):439-444.

146. **Zeikus J. G., R. Kerby and J. A. Krzycki** (1985) Single-carbon chemistry of acetogenic and methanogenic bacteria. Science. **227**(4691):1167-1173.

147. **Zeikus J. G. and M. Winfrey** (1976) Temperatures limitation of methanogenesis in aquatic sediments. Appl. Environ. Microb. **31**:99-107.

148. **Zinder S. H.** (1990) Conversion of acetic acid to methane by thermophiles. Microbial. Rev. **75**:125 -138.

149. **Zinder S. H.** (1994) Syntrophic oxidation and "reversible acetogenesis". p. 386-415. *In* Acetogenesis . Chapman & Hall, New York.

150. **Zinder S. H.** (1986) Thermophilic waste treatment systems. p. 257-277. *In* Thermophiles: general, molecular and applied biology . Wiley-Interscience, New York.

151. **Zinder S. H., T. Anguish and S. C. Cardwell** (1984) Effects of temperature on methanogenesis in a thermophilic (58 °C) anaerobic digestor. Appl. Environ. Microb. **47**(4):808-813.

152. **Zinder S. H. and M. Koch** (1984) Non-aceticlastic methanogenisis from acetate: acetate oxidation by a thermophilic syntrophic coculture. Arch. Microbiol. **138**:263-272.

153. **Zinder S. H. and R. A. Mah** (1979) Isolation and characterisation of a thermophilic strain of *methanorsacina* unable to use H_2-CO_2 for methanogenesis. Appl. Environ. Microb. **38**(5):996-1008.

Acknowledgements

It is very difficult to translate feelings into words, but it is time to express 'formally' my gratitude to those who helped me during my period in Wageningen. I have to admit that writing this was as hard as to write the thesis abstract! How to be short when a simple 'thanks' is too little? Well, one extra page will not make the printing costs of this booklet raise too much!

I start it in a chronological order, and Carlos Nobuyoshi Ide made the " kick off" of this PhD adventure. Thanks Nobu, for your encouragement, inspiration and help with the procedures for applying for the CNPq's scholarship. Mario Kato is gratefully acknowledged, although I have to confess that several times during my first 6 months here I wondered: 'how the hell could I let Mario convince me that Wageningen would be better than Melbourne??' Well, it was not difficult to find out that he was right and now I'm sure there wouldn't be better choice. Thanks Mario, for all the tips and for opening the 'department's doors' for me.

Heleen Vos was the first person I met in Holland. Since my arrival at Schiphol airport, she has been a great support and is always ready to help, either with work or personal matters. Thanks for all Heleen, you are a guardian angel!

It is difficult to find a fair way to thank Gatze. I have to confess that I was scared to death when I knew that I would live in a shared flat where Prof. Lettinga used to live. Just after knowing him I started understanding why Mario sent me here. To meet Gatze was one of the greatest gifts I got in my life. Gatze, I thank you for introducing me to the mysteries of the methanol conversion! Your excitement about the topic and interest in each new result during the first year of my research were of great importance to keep me going (even when everything seemed hopeless). To read Dostoievski did not change my life as you said it would so, but still was a good reading suggestion. Very far from an 'exciting reading', it was as effective as sleeping pills in some of my sleepless nights...

To my co-promotor, Jules van Lier, after a very accurate quantitative-qualitative supervision analysis, I come to the conclusion that the 'net' balance of supervision was a positive number that can still be highly acknowledged, Gracias Jefe!

I also thank Look Hulshoff Pol, who was my co-promotor during the first year of my research.

My very special thanks to Jim Field, whose rescue came just in time. Jim, thanks for your brilliant advises, help and especially for your patience (supervision after supervision and by e-mail is not easy task!).

For Fons Stams I have no words to express my gratitude! Thanks Fons, first for allowing me to work at your lab. I'm especially thankful for the 'unofficial' supervision, nice discussions, review of papers and 'last minute' help, when I needed it the most.

The introduction to the anaerobic lab and the 'learning' period with Graciela Gonzalez were very important to the achievement of my goals. Thanks Graciela!! Salih Rebac was also there when I could not manage to build up the reactor set up by myself! His willingness to help is greatly appreciated. I wish you were still working here, thanks my friend!

Great part of this study would not have been possible without the good work of my students Bo Jiang, Gema Vila and Denise Cysneiros. Bo is specially acknowledged. He was my 'first victim' since I had just started when he became my student! The richness in details of our work together is mainly due to his high interest in knowing more and more...the partnership worked very well and it was a pleasure to work under his direct supervision at the lab of microbiology afterwards.

My officemates also deserve special thanks for some very nice times in daily life. Laura, Frank (I understand Frank, that your problem was with Bill Gates, not with me!), Johan (always so kind talking about Dutch habits and culture, and translating into English the department's e-mail's when important). Frank I have to thank twice, for 'giving' me the opportunity of moving to room 727 and enjoying a real friendly atmosphere with the always smiling Adrie, Paco and Iemke as well.

My Troelstraweg 'family' is not forgotten and for the very good times we spent together I thank my flatmates Jose, Gatze, Esnati, Elena, Sonja (my Dear Russina bacter!) and Renato.

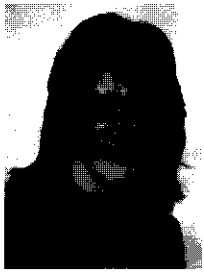
Some colleagues/friends I want to specially acknowledge: Geraldine, Jan Weijma, Jan Sipma (Naples streets will never be forgotten!), Marcus, Marcel, Grietje (what a nice summer I spent at your house!), Joost, Jaap, André, Piet, Marjo (how can you be sooooo nice?), Anita, Liesbeth, Geert, Ilse, Johannes, Sjoerd, Hillion, Kees, Caroline and Cor.

I was very lucky in meeting Liz and Marina. Before that, I used to think that the time to make 'friends forever' was gone...Spanish lessons were the best excuse to keep in touch con mi querida Liz! And Marina, as I already told you once, if there are 'twin souls' for friends, you are mine! It will be very difficult to go on without your company and our 'intermináveis pautas'.

To thank Marc without sounding like a romantic song or a 'pink movie' is a bit tricky... Anyway, here it goes: Marc, to share my life with you has been great! Your love, patience and care (despite the fact that sometimes I have the feeling that you care more for your CX and the newspaper...) were a great support in these last months, without you (and my yoga lessons) it would be impossible to keep serene.

A minha querida família, eu agradeço a paciência, suporte e o amor ilimitado. Sem o apoio de vocês (principalmente Mambi e Bimbo!), eu não teria chegado nem em Maringá, quanto mais aqui! Pit, minha bastante procuradora! Obrigada por tudo minha Pe-quenininha, quanto trabalho eu te dei!! Mãe, eu só fui reparar como o tempo voa quando outro dia você me disse que já faziam 17 anos que eu vivia inventando alguma coisa pra fazer longe de 'casa'. Nossa, isso soma metade do que eu vivi até agora... Mas acho que finalmente chegou a hora de sossegar num canto só (de preferencia pertinho de vocês). Dizem que para dizer que se viveu, é preciso escrever um livro, plantar uma arvore e ter um filho. Ao menos o livro tá pronto!!!

Last but not least, I gratefully acknowledge CNPq - (Conselho Nacional de Desenvolvimento Científico e Tecnológico), which financially supported this research.



Curriculum Vitae

The author of this thesis was born in Guarujá, São Paulo State, Brazil, on June 4th, 1967. In 1992 she obtained the Bachelor degree in Chemical Engineering at the State University of Maringá, Paraná. In 1993 she started working at the licensing and pollution control section at the Environmental Protection Agency in Campo Grande, Mato Grosso do Sul. In 1994 she spent 3 months in Yokkaichi, Japan, attending to a training course on Environmental Protection - Water Pollution Control, sponsored by the Japanese International Co-operation Agency (JICA). In 1997, she received her Master of Science degree on Tropical Public Health Engineering from the University of Leeds, England. In January 1999 she started her PhD research at the Department of Environmental Technology of Wageningen University. After accomplishing her PhD. studies, she will return to Brazil to resume her career.

Cover

Background: Caribbean See (Cancun, Mexico)

Illustration: View of partnership and a "friendly" substrate competition in the mixed consortium cultivated on methanol.