microbiological aspects of granular methanogenic sludge

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Proefschrift
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op gezag van de rector magnificus
dr. C.C. Oosterlee
in het openbaar te verdedigen op
dinsdag 12 mei 1987
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen.

STELLINGEN

1. Diffusie-limitatie speelt in het algemeen geen rol van betekenis in methanogeen zuiveringsslib.
   Dit proefschrift.

2. Het gehalte aan F_{420} is geen goede maat voor de potentiële methanogene activiteit van anaeroob slib.
   Dit proefschrift.

3. De stelling dat reductieve dechlorering van 3-chlorobenzoezuur geen energie levert is verwarrend en onjuist.
   D.R. Shelton and J.M. Tiedje (1984) Isolation and partial characterization of bacteria in an anaerobic consortium that mineralizes 3-chlorobenzoic acid. Appl. Environ. Microbiol. 49: 1530-1531.

4. De door Lovley gemeten drempelwaarde voor waterstofconsumptie in methaanbacteriën is een laboratoriumartefact.
   D.R. Lovley (1985) Minimum threshold for hydrogen metabolism in methanogenic bacteria. Appl. Environ. Microbiol. 49: 1530-1531.

5. Het idee om zuiveringsslib in een meer te dumpen met het doel de schadelijke effecten van zure depositie te bestrijden is slecht doordacht.
   W. Davison (1986) Sewage sludge as an acidity filter for groundwater-fed lakes. Nature 322: 820-822.

6. Zonder reductie-equivalenten geen reductieve dechlorering.
   J. Dolfing and J.M. Tiedje (1986) Hydrogen cycling in a three-tiered food web growing on the methanogenic conversion of 3-chlorobenzoate. FEMS Microbiol. Ecol. 38: 293-298.

7. Zure depositie is een potentiële bedreiging voor methanogene bacteriën in sedimenten en kan derhalve de koolstofcyclus beïnvloeden.
   D.W. Schindler, M.A. Turner, M.P. Stainton, and G.A. Linsey (1986) Natural sources of acid neutralizing capacity in low alkalinity lakes of the precambrian shield. Science 232: 844-847.

8. Het verschil in affiniteit voor waterstof tussen methaanbacteriën en sulfaat-reducerende bacteriën zoals gepresenteerd door Kristjansson et al. vertroebelt de discussie over de vraag waarom methaanproductie veelal onderdrukt is in de aanwezigheid van sulfaat.
   J.K. Kristjansson, P. Schönheit, and R.K. Thauer (1982) Different Ks values for hydrogen of methanogenic bacteria and sulfate reducing bacteria: an explanation for the apparent inhibition of methanogenesis by sulfate. Arch. Microbiol. 131: 278-282.

9. De hypothese dat er op aarde ecosystemen zijn die een goede kans maken een nucleaire holocaust te overleven doet niet ter zake.
   H.W. Jannasch and M.J. Mottl (1985) Geomicrobiology of deep-sea hydrothermal vents. Science 229: 717-725.

Stellingen behorende bij het proefschrift "Microbiological aspects of granular methanogenic sludge" van J. Dolfing.

Wageningen, 12 mei 1987

VOOR CARLA

Voorwoord

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Voor de medewerkers van de TFDL voor de fraaie electronenmicroscopie.
En voor Carla natuurlijk.

C O N T E N T S

CHAPTER 1

Introduction

1. Microbiology of anaerobic digestion.

Under anaerobic conditions in natural habitats microbial decomposition of organic material to inorganic products is predominantly accomplished by microbial oxidations, with protons, sulfur or carbon atoms as the exclusive electron sinks. In the absence of light and inorganic electron acceptors biodegradable organic material can be completely fermented to methane and carbon dioxide. Substrate conversion in such environments is thought to occur in a multistep process (Fig. 1; McInerney et al. 1980; Zeikus 1982). The reaction sequence can be divided into various distinct subprocesses (Gujer and Zehnder 1983), or it can be dealt with at the level of stages (McInerney et al. 1980), of metabolic groups (Zeikus 1982) or of individual microorganisms (Winter and Wolfe 1979). Precedents exist for the usage of the terms food chain and food web in this context (Winter and Wolfe 1979; Pfennig 1984; Shelton and Tiedje 1984), even though this terminology does not conform the classical concept of an organism at a lower trophic level being consumed by an organism at a higher trophic level (Dolfing and Tiedje 1986).

But no matter with what perspective anaerobic digestion is looked at, the effective conversion of organic matter into methane requires the coordinated metabolic activities of different microbial populations (Zehnder et al. 1982; Zeikus 1982). At least three different metabolic groups can be recognized on the basis of substrates degraded and metabolic end products formed (Zehnder et al. 1982): (i) hydrolytic and fermentative microorganisms, converting a variety of complex organic molecules into a broad spectrum of end products; (ii) hydrogen producing acetogenic bacteria converting organic acids and neutral compounds larger than methanol to hydrogen and acetate; and (iii) methanogenic bacteria converting H_2/CO_2, one carbon compounds or acetate into methane.

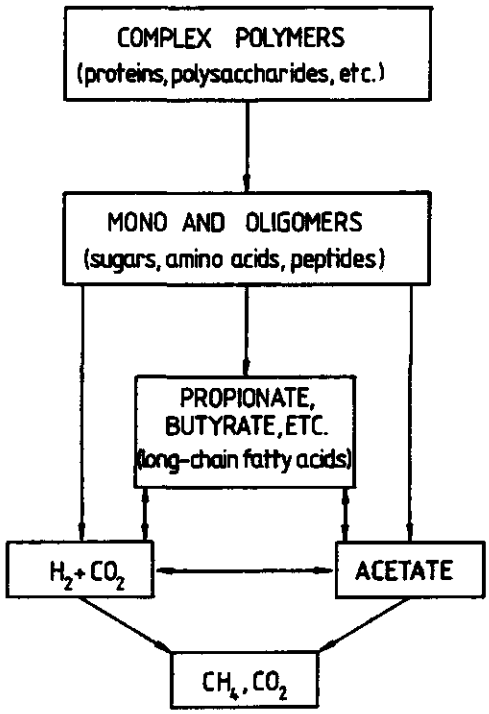

Fig. 1. Multistep scheme for the flow of carbon in the complete anaerobic conversion of organic matter to methane.

The methanogenic bacteria (Balch et al. 1979; Zehnder et al. 1982) perform a central role in anaerobic digestion by removing hydrogen and acetate and converting them to methane. The removal of acetate helps to prevent acidification of the system, and the removal of hydrogen directs the flow of electrons to the formation of more oxidized intermediates and enhances the thermodynamic efficiency of the metabolic reactions of many other bacteria.

Hungate (1966, 1967) was the first to express the idea that hydrogen production and utilization can profoundly influence the course of fermentations in anaerobic ecosystems. The regulatory role of hydrogen is effectuated by interspecies hydrogen transfer (Ianotti et al. 1973; Wolin and Miller 1982), i.e. a stimulation of hydrogenogenic organisms, induced by hydrogenotrophic organisms, to produce more hydrogen than would have been

produced in the absence of hydrogenotrophs. The special feature of interspecies hydrogen transfer is that hydrogenotrophs by consuming hydrogen can create conditions for obligate hydrogenogens to perform catabolic oxidations which would not have been energy yielding in the absence of hydrogenotrophs. The obligately hydrogen producing bacteria, or at least the reactions they catalyze, are extremely sensitive to hydrogen (Dolfing 1986). At slightly elevated hydrogen concentrations the reactions from which these organisms obtain their energy become endergonic. As a consequence the obligate hydrogen producers stop their activities at elevated hydrogen concentrations, while fermentative organisms under such conditions can alter their flow of electrons instead of towards hydrogen towards the formation of more reduced metabolites, which are generally not per se substrates for methanogenic bacteria. Hence, the cooperation between different metabolic groups mentioned above is a prerequisite for a complete conversion of biodegradable organic matter into methane and carbon dioxide. An extreme example of such a cooperation are the syntrophic relations between obligate hydrogen producers and hydrogen consumers which are fully interdependent in the anaerobic conversion of compounds like propionate, butyrate or benzoate. The thermodynamic rationale of syntrophic partnerships has been discussed by Wolin (1976) and Zehnder (1978), and the interactions between methanogens and non-methanogens have been extensively discussed by Mah (1982).

2. **Applied aspects of anaerobic digestion.**

Fermentation and methanogenesis are likely to occur if the supply of organic material is faster than the supply of inorganic electron acceptors like diatomic oxygen, nitrate or sulfate. To prevent nuisance caused by spontaneous uncontrolled fermentations in the open environment, engineers

have sought to apply methanogenic fermentations in closed systems, where waste is stabilized under controlled conditions with the concomittant production of energy-rich methane gas. The foundations for process control in anaerobic waste (water) treatment were laid in the thirties by the extensive studies of Buswell and coworkers (Buswell and Hatfield 1930; Buswell and Neave 1930). A subsequent significant process development occurred in the fifties when Stander (Stander 1950; Stander and Snyders 1950) recognized and demonstrated the value of retaining a large population of bacteria in the methane reactor. Various reactor types have since then been developed with the specific aim to retain biomass largely independently of the reigning hydraulic retention times (van den Berg and Kennedy 1983; Speece 1983). Two main types of reactors can be distinguished in which biomass retention is promoted either by the addition of solid support materials to which the biomass should adhere like e.g. upflow or downflow filters and fluidized bed reactors, or by selection for well settling biomass conglomerates. Stander (1950) achieved this selection in the clarigester reactor where he installed a settling tank for the return of bacterial solids, which was located in the top of the digester. The upflow anaerobic sludge blanket (UASB) process (Lettinga et al. 1980) is a more sophisticated form of the upflow sludge blanket concept. The main improvement of Lettinga's UASB process compared to the conventional USB process was the installation of an improved gas solids separation system in the upper part of the reactor. The basic idea underlying the UASB process is that under optimal conditions a well flocculated biomass with superior settling characteristics would be obtained. Development of well-settling biomass makes it unnecessary to install support material for bacterial film formation in the reactor, so that no reactor space has to be sacrificed. The present study concentrates on the microbiology of well-settling biomass in UASB reactors.

3. Formation of bacterial conglomerates during anaerobic digestion.

The formation of well-settling biomass is a widespread phenomenon in USB reactors. Well-settling conglomerates have been observed not only in methanogenic USB reactors, but also in denitrifying systems (Klapwijk et al. 1981) and in USB systems operated as acidification systems (Zoetemeyer 1982). These observations indicate that the U(A)SB system selects for well-settling biomass, apparently in the form of conglomerates (macroscopically visible clusters of bacteria). Three types of conglomerates may be distinguished; they are briefly described below.

- Flocs are conglomerates with a loose structure. After settling, a layer of flocs forms macroscopically one layer.
- Pellets are conglomerates with a more dense structure than flocs. After settling these conglomerates are still visible as separate entities.
- Granules are dense pellets; they have a granular appearance and their shapes do not rely on the presence of water. They can withstand a certain amount of pressure.

Methanogenic sludge granules as described above have been observed in UASB reactors treating waste waters from sugar production and from potato processing. Until now the expression granular sludge has been used, however, indiscriminately for various types of well-settling sludges, in particular as a synonym for pelletized sludge (Hulshoff Pol et al. 1983). Though the general descriptions as given above are somewhat hazy, they may serve as a framework for the classification of various types of sludge. At present various macroscopically different (methanogenic) sludge conglomerates (apart from flocs) have been reported for UASB reactors. Some of their major features are shown in Fig. 2.

(i) Methanogenic sludge pellets. These conglomerates mainly consist of filamentous organisms that are strongly intertwined to form spaghetti like structures. They are more or less spherical in shape and can be up to 1 cm in diameter.

(ii) Methanogenic sludge granules. These conglomerates consist mainly of coc and rod-shaped bacteria occurring as single cells or in short chains. The particles are spherical and may be up to 5 mm in diameter.

(iii) Spiky methanogenic sludge granules. These conglomerates have been observed in a full scale UASB reactor treating waste water of a maiz starch factory. The $CaCO_3$ content of these aggregates was 60%, and filamentous organisms predominated. Settling properties were excellent.

(iv) Methanosarcina granules. The predominant organisms in these granules are Methanosarcina.

The factors that trigger the formation of microbial conglomerates are currently not known (Marshall 1984). Two concepts have been advocated to study the mechanisms by which microorganisms achieve adhesion and aggregation: the reductionist approach that regards a single mechanism as of paramount importance, and the holistic (or integrated) approach that bacteria use various mechanisms simultaneously to achieve and maintain attachment (Costerton 1984). The reductionists try to bring down the complicated series of processes and events to single, ordered steps that lead to the formation and preservation of aggregates and biofilms. Three stages may be discerned in the development of such conglomerates (after Marshall 1976): (1) the adsorption step in which organisms are deposited on a substratum, or on each other; this is generally a reversible process; (2) permanent attachment: this often involves polymers acting as bridges between two surfaces; (3) colonization by development of microcolonies.

The initial adhesion step is dependent upon the surface properties of the

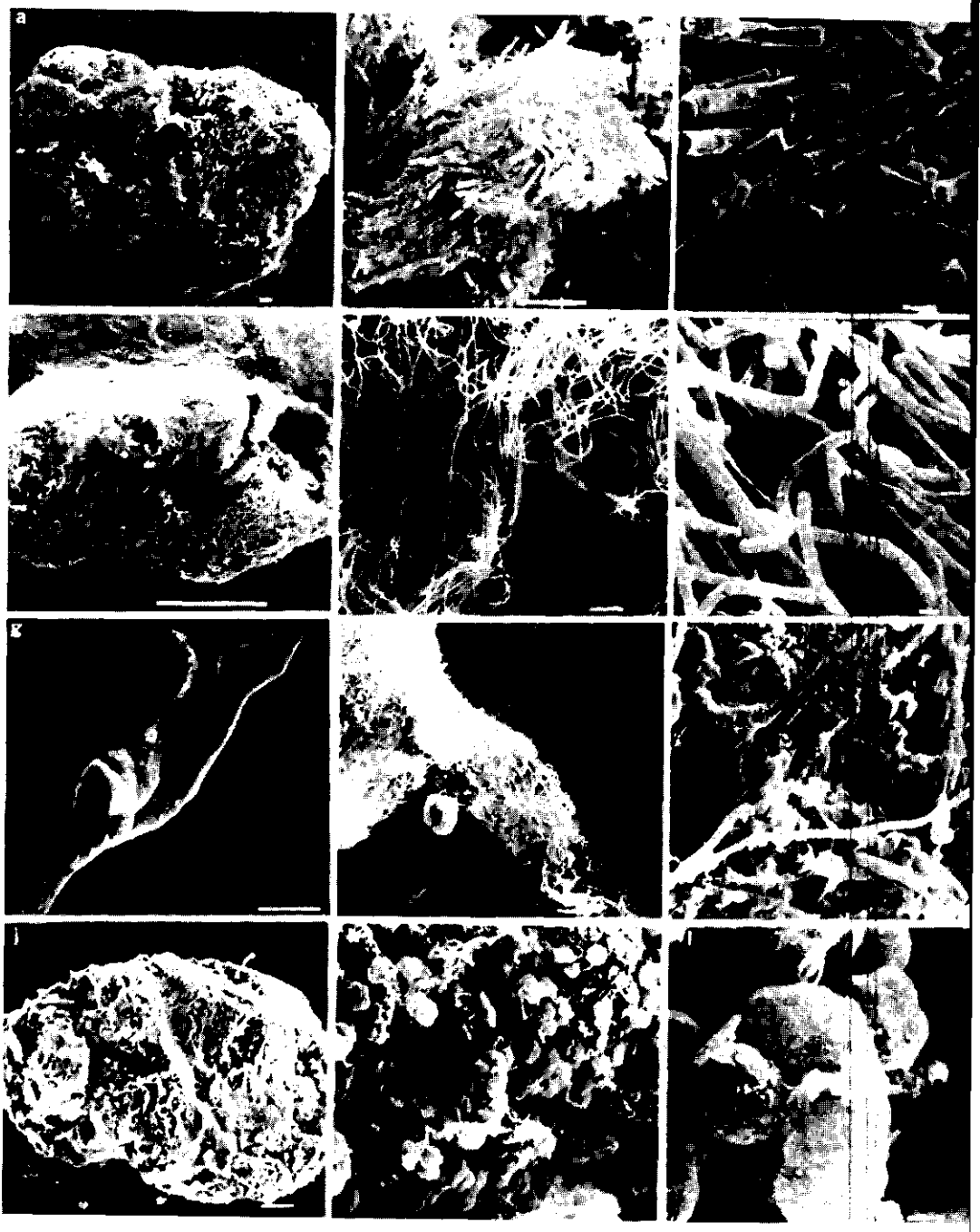

Fig. 2. Scanning electron micrographs of various well settling methanogenic sludg grown in UASB reactors. A-C: granular sludge grown on a mixture of acetat and propionate at pH=7.2; D-F: spaghetti-like pellets grown on a mixture acetate and propionate at pH=7.2; G-I: spikey granules grown on wastewate of a maize-starch factory; J-L: Methanosarcina-dominated granules grown c a mixture of acetate and propionate at pH=6. The bars indicate 100 µm for panels a,g,j; 10 µm for panels b,e,h,k; 1 µm for panels c,f,i,l; and 100C µm for panel d.

bacteria (Fletcher and McEldowney 1984). As a result of adhesion, physiological activity may lead to changes in cell surface components, resulting in the accumulation of extracellular polymers, in firm attachment without accumulation of polymers, or in detachment. the attachment of a given bacterium may vary with culture conditions, and various organisms may show different attachment responses to similar culture conditions. It is important to note that it is virtually impossible to generalize about the influence of particular factors on bacterial attachment. A number of complex adhesive interactions is likely to be involved in bacterial adhesion and bacterial strains probably differ considerably in their attachment process (Fletcher and McEldowney 1984). Thus studies of bacterial adhesion and analysis of the surface composition of bacteria involved in aggregation and biofilm formation are best conducted in natural environments. Bacteria that are removed from natural environments may loose both their exopolymers and their pili upon subculturing in vitro (Costerton 1984). Observations made with such altered strains do not necessarily yield ecologically relevant information on the behaviour of these organisms in their natural environment.

4. Scope of the present study.

At the time this study was initiated granular methanogenic sludge had only been found in industrial reactors treating complex waste streams. The complexity of these wastes, however, results in the formation of a diverse microbial population in the sludge conglomerates, which complicates a study of the interrelationships between the various groups of microorganisms and the factor(s) that trigger the expression of their ability to form granular sludge. Hence the first objective in this study was to develop a laboratory scale set-up to grow granular methanogenic sludge under simple, well

defined conditions. Various mixtures of organic compounds were tested in UASB reactors with a volume of one liter and the end result was that we were able to grow granules on either propionate or ethanol as the sole carbon and energy source in these reactors (chapter 2).

The second objective of this study was to characterize the chemical and bacteriological composition of granular methanogenic sludge. Results on this subject are presented in chapter 3. Since culture counts of different metabolic groups of bacteria present in granular sludge were in disagreement with results from direct light and electron microscopic observations, we developed and used activity tests as a tool to characterize the microbial composition of methanogenic environments. This approach is described in chapter 4. The results obtained with these activity tests were compared with estimates obtained via measurements of F_{420}, a previously advocated method to estimate the methanogenic potential of anaerobic environments (chapter 5). Methanogenic activity tests also proved a valuable tool to evaluate the importance of mass transfer limitation on the rate of methanogenesis in granular sludge at low substrate concentrations (chapter 6).

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CHAPTER 2

Production of Granular Methanogenic Sludge on Laboratory Scale

Submitted for publication

PRODUCTION OF GRANULAR METHANOGENIC SLUDGE ON LABORATORY SCALE

Jan Dolfing, Lucia Luijten, Wim G.B.M. Bloemen, and Alexander J.B. Zehnder

Abstract

Cultivation of granular methanogenic sludge was studied in bench scale upflow anaerobic sludge blanket (UASB) reactors and batch culture experiments. Since the first observation of granular methanogenic sludge had been made with a full scale UASB reactor treating waste water of a sugar refinery, our first series of experiments was done with waste water of this sugar refinery as the feed solution. In a bench scale UASB reactor inoculated with digested sewage sludge, the sludge formed fluffy pellets. However, inoculation with disintegrated sludge granules orginating from the above-mentioned full scale UASB reactor resulted in restoration and growth of methanogenic sludge granules. Cultivation of granular methanogenic sludge was also feasible in bench scale UASB reactors using ethanol as a substrate. Thus, a well defined bench scale system is now available in which the mechanism of granulation can be studied in more detail.

Cultivation of granular methanogenic sludge in batch culture was only possible in experiments where propionate was stoichiometrically converted to methane and acetate (and probably carbon dioxide). In this case acetate was not further converted into methane. This reinforces the hypothesis that acetoclastic Methanothrix-like organisms play an important role in determining the morphological development of anaerobic sludge under methanogenic conditions.

Introduction

Increasing environmental problems with waste disposal and rising energy costs stimulated the research in the field of anaerobic waste water treatment in the last decade. As result new treatment processes have been introduced (23). One of them, the upflow anaerobic sludge blanket (UASB) process has proved to be promising (18) and is now successfully applied on full scale (19). In UASB reactors a granular type of sludge may develop (11). Granular sludge has two attractive characteristics, namely excellent settling properties (11,18) and a high specific activity (8,9,10,18). The settling properties are important to efficiently retain the active biomass in the reactor. The high specific activity allows reduced contact time between waste and the active biomass, an economically significant feature.

It is not known which factors govern the formation of granular sludge (1,7). In order to study the mechanism of granulation we started a systematic investigation on the factors which govern formation and development of the granules. The present paper deals with the feasibility of cultivating the active microbial biomass which forms this granular methanogenic sludge, using bench scale reactors. Batch cultivation was compared with cultivation in UASB reactors, and the influence of the seed sludge was investigated.

Materials and methods

The seed sludge

Active methanogenic biomass which consisted of granular methanogenic sludge was kindly provided by the CSM-sugar refinery at Breda, The Netherlands. It had been cultivated in a 30 m^3 UASB reactor on a waste stream of a plant which produced liquid sugar (19). Before using this

sludge for experiments, it was washed in an upflow column with a anaerobic buffer containing (in grams per liter): $NaHCO_3$, 10; K_2HPO_4, 0.4; KH_2PO_4, 0.3. The flow rate of the buffer was chosen such as to allow the granules to stay in the column, while flocs and dispersed material were washed out. Disintegrated granular sludge was made by milling washed granules with a cream maker (Jubilee Model, Royston Sales Ltd, G.B.) under strict anaerobic conditions (8).

Digested sewage sludge was obtained from the municipal waste water treatment plant of Ede, The Netherlands. The dry matter contained 64% volatile suspended solids.

Experimental set up for upflow experiments

Upflow experiments were performed in perspex reactor columns, made in our own workshop with a working volume of one liter. The internal diameter was 5.5 cm, the height 40 cm. Separation between gas, fluid and liquid was obtained in the reactor with an inverted funnel. The experimental set-up was as depicted in Fig. 1. Substrate was pumped into the bottom of the column with a peristaltic pump (1200 Varioperspex, LKB-Sweden). The spent medium left the reactor at the top, and was discharged into the sink unless

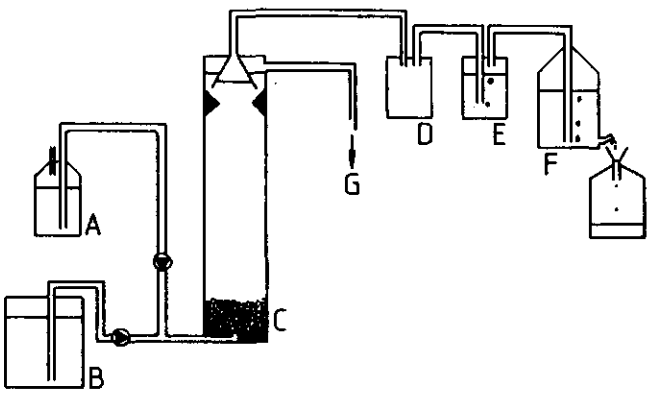

Fig. 1. Experimental set-up of a bench scale upflow anaerobic sludge blanket system for the cultivation of granular methanogenic sludge. A, feed solution; B, dilution water; C, upflow anaerobic sludge blanket (UASB) reactor; D, empty bottle to prevent back flow of the solution from E; E, CO_2-trap filled with 5 M NaOH which was used at the same time to produce a slight overpressure; F, bottle of Mariotte (A liquid displacement system) to determine the amount of methane formed; G, effluent.

stated otherwise. Gas produced in the column was separated from the mixed liquor with the use of an inverted funnel. To remove all CO_2 the gas was filtered through a NaOH solution (5 M). The amount of methane produced was measured by collecting the rest of the gas in a liquid displacement system. The gas consisted of 99.5% methane. This percentage was determined by gas chromatography. All experiments were done in temperature controlled rooms or cabinets at 30°C.

Media composition

Upflow experiments: Medium 1. The first series of upflow experiments were performed with waste water from the sugar plant of CSM at Breda. The waste water (pH = 3.4) had a COD (chemical oxygen demand) value of 13 g l^{-1} and contained in mmol l^{-1}: ethanol, 54: acetate, 9; propionate, 1.5; butyrate, 1.0; valerate, 1.5; lactate, 20; lactate, 20; sucrose, 1.2.

The waste water was stored in barrels at ambient outdoor temperatures. Its composition did not significantly change during a storage of nine months. Prior to feeding the waste to the reactors, nitrogen and phosphate were added to mimic procedures used at CSM. The desired ratio of COD:N:P (350:5:1) was obtained by adding 170 mg $(NH_4)_2HPO_4$ and 336 mg urea per liter. $NaHCO_3$ (5.6 g l^{-1}) was added to raise the pH to 6.0. This nutrient enriched waste water was prepared in 10 liter batches and stored at 4°C for at most three days before feeding it to the reactors.

Upflow experiments: Medium 2. A second series of experiments was performed with artificially prepared waste water. Its composition was based on the waste water of the sugar plant. Starting from this composition a series of different modifications was prepared (R_1-R_5) by successively omitting different compounds from the mixtures (Table 1). The ratio between

the acids and their sodium salts was chosen such as to obtain a theoretical pH = 7.2. The calculation was based on the ratio between HCO_3^- and CO_2. The final COD of each medium was 64 g l^{-1}. All media were supplied per liter with 42 ml of a trace elements solution containing the following components (in milligrams per liter): $FeCl_2$, 500; $MnCl_2.4H_2O$, 100; $CoCl_2.6H_2O$, 170 $ZnCl_2$, 100; $CaCl_2$, 20; H_3BO_3, 19; Na_2MoO_4, 10; Na_2SeO_3, 28; $NiCl_2.6H_2O$, 50. The filter sterilized media were diluted with various amounts of tap water prior to feeding it to the columns by means of a multichannel pump. The final COD of the column influent varied between 2 and 6.5 g l^{-1}, depending on the loading rate. As a function of the final COD the dilution water was buffered with 1-4 grams sodium biocarbonate.

Medium for batch experiments. The following basal medium was used for batch culture experiments (components in grams per liter tap water): $NaHCO_3$, 10; NH_4Cl, 0.5; K_2HPO_4, 0.4; KH_2PO_4, 0.3; $MgCl_2.6H_2O$, 0.2; and 20 ml l^{-1} trace elements solution (composition see above). The medium was prepared anaerobically under carbon dioxide (final pH = 7.0) by boiling, and cooling under continuous flushing with O_2-free carbon dioxide and was subsequently dispersed in flushed serum bottles with a volume of 1200 ml. The last traces of oxygen were removed by the addition of one ml of a concentrated FeS-solution prepared according to Brock and O'Dea (3) to 500 ml of medium. Sodium formate, sodium acetate, and sodium propionate were added from 2.5 M stock solutions to final concentrations of 20 mM (unless otherwise indicated).

Inoculation of the upflow reactors.

In the first series of experiments the reactors were inoculated with 320 ml of disintegrated granular sludge (20 gram dry weight, % ash 15) or 1 liter of digested sewage sludge (22.4 gram dry weight, % ash 36). In the second series of experiments the reactors were inoculated with 100 ml of granular sludge (9.8 gram dry weight, % ash 19).

Definitions of various types of methanogenic conglomerates.

In the following we distinguish between three types of sludge conglomerates namely: (i) Granules, very dense conglomerates which have a granular apperance and are readily settled. Their surface is macroscopically smooth and with a dissection microscope no filamentous bacteria can be seen on the surface; (ii) Pellets, well settling conglomerates of irregluar shape that clearly form separate entities; the surface as seen with the naked eye is smooth but with the use of a dissection microscope, filamentous extrusions can be distinguished. (iii) Flocs, loose aggregates that cannot be distinguished any more as separate entities after settling.

Electron microscopy. Material for scanning electron microscopy was fixed in a 0.025 M cacodylate buffer (pH = 6.8) containing 2% glutaraldehyde for three hours at room temperature and post-fixed for two hours at room temperature in a 0.025 M cacodylate buffer (pH = 6.8) containing 1% OsO_4. Dehydration occurred through a graded series of water-acetone mixtures. The dehydrated material was quickly frozen in liquid freon, cleaved, dried following the method of critical point drying (13) coated with gold, and examined in a scanning electron microscope operated at an acclerating voltage of 15 kV.

Analytical methods

Methane and hydrogen were determined with a Packard-Becker 406 gas chromatograph equipped with a thermal conductivity detector and a molecular-sieve column (60-80 mesh), operated at 50°C with argon as carrier gas at a flow rate of 20 ml min^{-1}.

Volatile fatty acids (VFA) were determined with a Varian 2400 gas chromatograph at 180°C equipped with a chromosorb 101 column (80-100 mesh). Carrier gas was nitrogen saturated with formic acid at a flow rate of 30 ml

min^{-1}. Before injection, the samples were filtered through a 0.45 µm membrane filter and acidified with cation exchange resin [Amberlite IR-120(H)].

Ethanol was determined with a Packard-Becker 417 gas chromatograph at 185°C equipped with a Poropak R column (80-100 mesh). The carrier gas was nitrogen at a flow rate of 30 ml min^{-1}.

Results

Experiments in UASB System

a. Sugar refinery waste water

Waste water of a sugar refinery, on which granular methanogenic sludge had been cultivated in a full scale reactor (19), was used to test whether granular methanogenic sludge can be obtained in small reactors from digested sewage sludge. Two reactors were seeded with digested sewage sludge and disintegrated granular sludge, respectively. To obtain good mixing and to provide enough alkalinity, 75-95% of the effluent was recycled. In the ractor seeded with disintegrated granular sludge restoration of granules was observed after about two months and further growth of active granular biomass was achieved upon prolonged incubation. In the reactor seeded with digested sewage sludge fluffy pellets developed.

Representative micrographs of both types of aggregates are presented in Fig. 2. Whereas the reactors seeded with disintegrated granular sludge gave granules closely resembling the original granules, though somewhat weaker of consistency, the pellets obtained from digested sewage sludge remained fluffy. Although this reactor was run for 7 months, granules were never observed.

b. Artificially prepared waste waters

Having shown that our experimental set up was adequate to cultivate

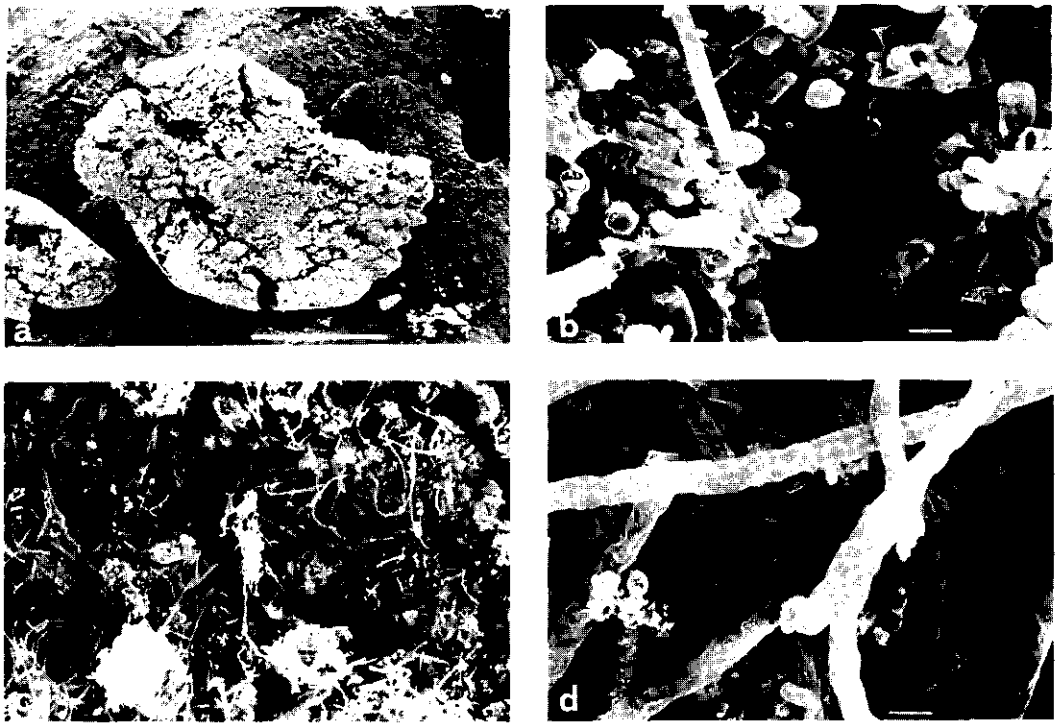

Fig. 2. Scanning electron micrographs of methanogenic sludge conglomerates cultivated in bench scale UASB reactors on waste water of a sugar refinery. (a) and (b): Granules present 4 months after inoculation with disintegrated sludge granules. (a) Cleaved granules; Marker bar = 1 mm. (b) Mixed flora of the center of the cleaved granule as shown in (a); Marker bar = 1 µm. (c) and (d): Details of fluffy pellets present 7 months after inoculation with digested sewage sludge. Note the filamentous colonies of network and the entrapped microcolonies of rod and coc-shaped bacteria. (c) Marker bar = 10 µm. (d) Marker bar = 1 µm.

granular sludge, a second series of experiments was started. In these experiments the influence of different carbon and energy sources on the growth of granular methanogenic sludge was tested. The effluent was not recirculated in these experiments. The composition of the media (Table 1) was chosen such as to obtain a pH = 7.2 at 100% conversion of the influent COD to methane and CO_2/HCO_3^-. The columns were inoculated with washed granular sludge from the CSM sugar refinery. The time courses of the loading rates for the various substrates in these experiments are depicted

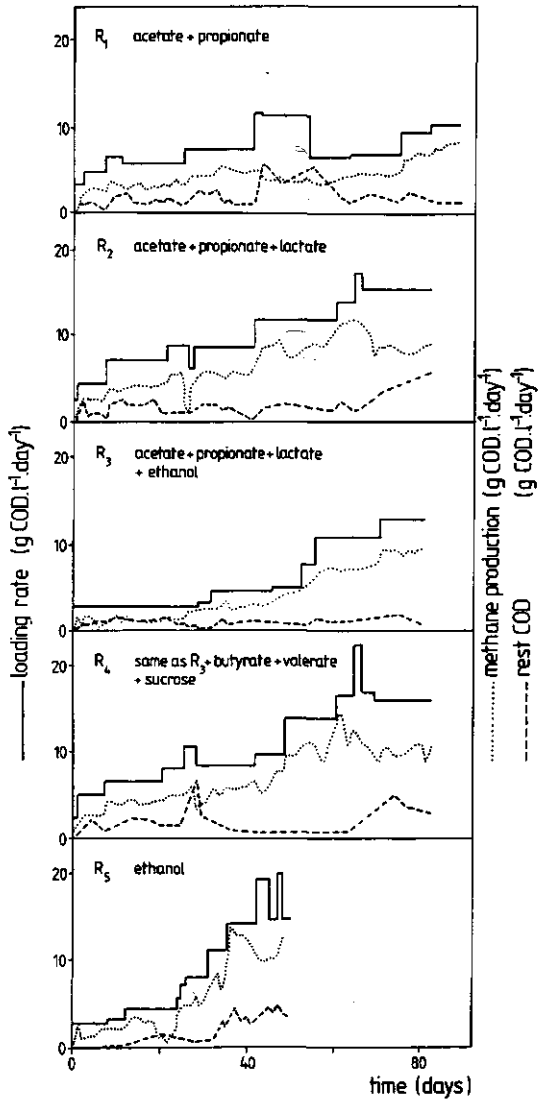

Fig. 3. Time course of performance in upflow experiments inoculated with granular methanogenic sludge. All parameters are given as gram COD per liter of reactor volume per day.

in Fig. 3. Whenever the VFA concentrations were below 3 mM the loading rate was increased. Fig. 3 shows that the increase in the loading rates was fastest with the reactor fed on ethanol. Until day 27 no significant amounts of VFA (i.e. conc. < 1 mM) were found in the effluent of reactor R_5

Table 1. Composition of concentrated media used to test the influence of the substrate on the growth of granular methanogenic sludge in UASB reactors inoculated with washed granular sludge[a].

Constituent	Reactor number				
	R_1	R_2	R_3	R_4	R_5
Acetic acid	51.0	22.3	8.3	5.0	-
Na-acetate.$3H_2O$	40.3	9.5	-	-	-
Propionic acid	-	-	1.7	3.1	-
Na-propionate	28.0	21.2	4.4	-	-
Na-lactate (60%)	-	32.5	10.2	6.2	-
Ethanol (96%)	-	-	23.0	13.8	34.9
Butyric acid	-	-	-	9.2	-
Na-valerate	-	-	-	5.4	-
Sucrose	-	-	-	2.1	-

a In g l^{-1}; in addition all media contained (in g l^{-1} tap water) $FeSO_4$, 0.085; yeast extract, 0.425; $(NH_4)_2 HPO_4$, 0.708; ureum, 1.40; and 42 ml of the trace elements solution described in the text.

Table 2. Comparison of sludge volume index and growth yield of sludges grown in bench scale UASB reactors on various substrates.

Reactor number	Substrate[a]	SVI[b]		Y
		ml g^{-1} sludge	ml g^{-1} VSS	gVSS $mol^{-1}CH_4$
R_1	Acetate + propionate	16.4	18.2	0.4
R_2	Acetate + propionate + lactate	14.9	16.7	0.3
R_3	Acetate + propionate + lactate + ethanol	16.2	17.3	0.3
R_4	Same as R_3 + butyrate + valerate + sucrose	15.7	17.6	0.4
R_5	Ethanol	10.2	12.3	0.9

a For details see materials and methods, Table 1. The seed sludge had been grown on waste water of a liquid-sugar factory plant and had a sludge volume index of 9.8 ml/gram sludge or 12.5 ml/gram VSS.

b SVI = sludge volume index after settling for 15 minutes.

though the effluent still contained some ethanol. After day 27 at a loading rate of 8 gram COD $l^{-1}day^{-1}$ methanogenesis from VFA was the rate-limiting step for this reactor, analogical to the other reactors.

Good growth of granular methanogenic sludge was achieved with ethanol as energy source. On the other media tested the conglomerates that developed contained locally more filamentous bacteria of the type shown in Fig. 2 (c) and (d). This fact seems to make the conglomerates somewhat weaker as could be observed after vigorous shaking. In the reactor fed with acetate plus propionate and acetate plus propionate plus lactate some very large (d ≈ 1 cm) pellets developed. The sludge volume index on the other four substrate mixutres tested was significantly higher than when ethanol was the sole energy source (Table 2). This might be due to the formation of a significant amount of floccular, non-granular sludge. The growth yield on ethanol was significantly higher than that on the other substrate mixtures.

Representative micrographs of sludge granules cultivated on ethanol (Fig. 4) show different morphotypes of rod and coc-shaped bacteria. No filamentous organisms were observed in these granules.

The core of the granules appeared to consist of the original (seed) granule, while the outer layer was made up by the newly developed flora, specialized in the conversion of ethanol to CH_4 and CO_2.

Growth experiments in batch culture.

Methanogenic sludge granules were incubated in serum bottles with the addition of various substrates (formate, acetate, and proprionate), and methane formation was monitored. The rate of methane formation was dependent on the substrates (Fig. 5). The kinetic parameters listed in Table 3 are derived from these experiments.

The morphology of the granules changed when cultivated in batch systems. The original granules consisted of a heterogenous microflora composed of different bacterial morphotypes (see e.g. the core of the granules

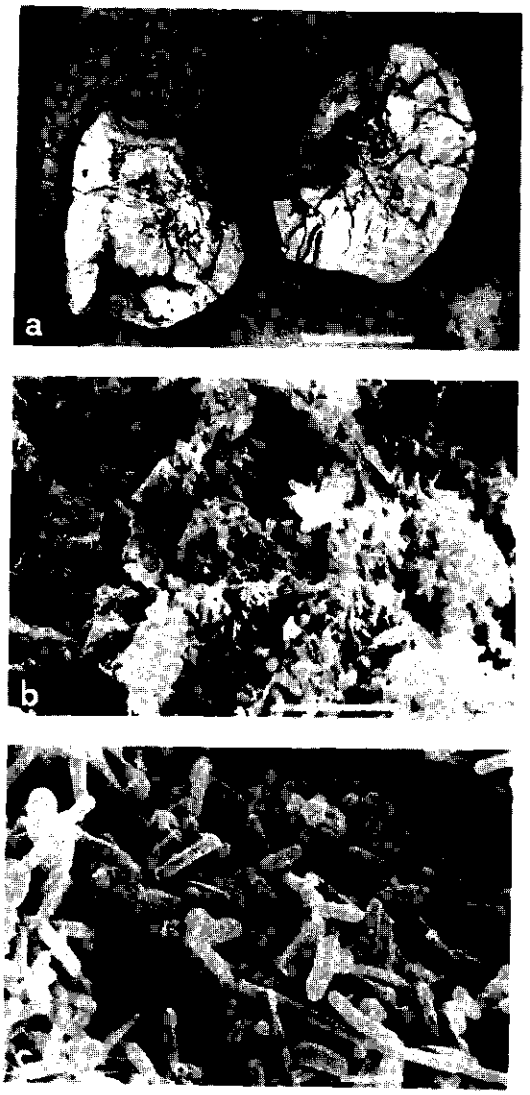

Fig. 4. Scanning electron micrographs of granular methanogenic sludge cultivated on ethanol in a bench scale UASB reactor. (a) Overview of a cleaved granule. Note the apparent presence of the seed granule in the center, enclosed by a distinct layer of newly formed biomass. Marker bar = 1 mm. (b) Detail of the center of a granule. Marker bar = 10 µm. (c) Detail of the outer layer of a granule. Marker bar = 1 µm.

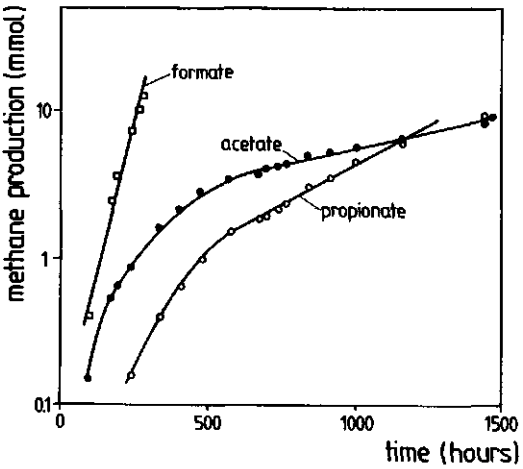

Fig. 5. Methane production from volatile fatty acids by methanogenic sludge granules in batch culture under growth supporting conditions.

Table 3. Comparison of kinetic parameters of granular methanogenic sludge in batch culture with data from the literature.

Substrate	Organism/Sludge	μ (d^{-1})	y^a (g mol^{-1})	q^b (mol g^{-1} d^{-1})
Formate	present investigation	0.34	0.6	0.52
	Methanobacterium formicicum[c]	1.32	1.2	1.20
Acetate	present investigation	0.04	0.8	0.04
	Methanothrix soehngenii[d]	0.21	1.3	0.04
	Methanosarcina barkeri[e]	1.27	2.0	0.13
Propionate	present investigation[f]	0.06	2.7	0.02
	enrichment culture[g]	0.16	1.2	0.13
	Syntrophobacter wolinii[h]	0.10	-	-

[a] Expressed as gram biomass formed per mol substrate consumed
[b] Expressed as mol substrate converted per gram biomass per day
[c] Data from Schauer and Ferry (20); T = 37°C; pH = 7.6
[d] Data from Huser et al. (17); T = 37°C; pH = 7.4
[e] Data from Smith and Mah (21); T = 35°C
[f] Data are given for the conversion of propionate to acetate plus methane according to propionate → acetate + 3/4 CH_4
[g] Data from Koch cited in Gujer and Zehnder (12); T = 33°C
[h] Data from Boone and Bryant (2) for a methanogenic coculture with *Methanospirillum hungatei*; T = 35°C

cultivated in an UASB reactor on ethanol as depicted in Fig. 4). Cultivation on formate resulted in a disintegration of the granules. The remnants of the granules grew out to flocs. Table 3 compares the kinetic parameters of these flocs with those for Methanobacterium formicicum. The organism that formed the flocs was a brightly fluorescing irregular rod, closely resembling M. formicicum when viewed under epifluorescence microscopy (6).

The growth rates of methanogenic pellets on acetate decreased in time as estimated from the logarithm of the amount of methane formed. Addition of fresh nutrients or vitamins did not restore the initial growth rates. The granules grew out to fluffy pellets consisting of long, sometimes intertwined filaments of bacteria, that closely resembled Methanothrix soehngenii. Incubation with acetate plus propionate also resulted in the formation of such fluffy pellets, as shown in Fig. 6. In some experiments with propionate as the sole substrate, this compound was only partly converted to methane, while the intermediates acetate and probably CO_2 accumulated. The resulting granules were not fluffy. From these experiments, with relatively insignificant methanogenesis from acetate (Table 4) the kinetic parameters listed in Table 3 were obtained.

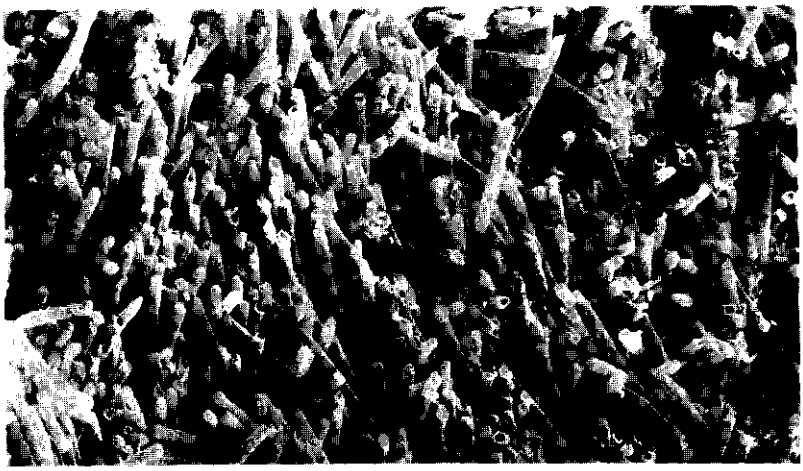

Fig. 6. Scanning electron micrograph of a methanogenic sludge pellet developed in batch culture on acetate plus propionate. 3 Months after inoculation with granular sludge.

Table 4. Products formed in the fermentation of propionate by methanogenic sludge granules in batch cultures under growth supporting conditions.

exp. no.	propionate fermented (mmol)	products (mmol) acetate	CH_4[a]	CH_4[b]	cells (mg)	$Y_{propionate}$ (g mol^{-1})
1	7.78	8.23	4.22	5.38	29.8	3.8
2	8.90	6.54	9.19	9.04	29.6	3.1
3	8.46	8.37	6.24	6.43	22.1	2.6

[a] Amount of methane actually measured
[b] Calculated methane production assuming: propionate → acetate + $3H_2$ + CO_2; acetate → CO_2 + CH_4; $4H_2$ + CO_2 → CH_4 + $2H_2O$

Discussion

Cultivation of granular methanogenic sludge was possible in batch systems as well as in bench scale UASB reactors. Both modes of cultivation gave rise to further growth of sludge granules. Cultivation in batch systems, however, generally resulted in the development of pellets more fluffy than the original granular inoculum. The granules generally were eventually overgrown by filamentous organism showing much resemblance to Methanothrix soehngenii a non-fluorescing obligately acetoclastic methanogen (17,24) or when formate was the substrate, by an organism showing much resemblance to M. formicium (4). The only case in which batch-cultivated granules did not become fluffy was in experiments with propionate as only substrate, when acetate was not significantly converted to methane, i.e. when growth of M. soehngenii-like organisms was virtually absent. These results indicate that the mode of growth of M. soehngenii-like organisms is important for the structure of the methanogenic conglomerates that develop. M. soehgnenii-like bacteria occur as single organism or in short chains in granular methanogenic sludge from the reactor of the

sugar refinery. In our upflow experiments with waste water of that sugar refinery seeded with disintegrated methanogenic sludge granules resulted in formation and growth of sludge granules. In a parallel experiment, inoculated with digested sewage sludge, however, fluffy pellets developed. This had been observed previously in UASB reactors inoculated with digested sewage sludge and fed with a mixture of acetate and propionate (14,15,22). These pellets contained a large population of M. soehngenii-like organisms occurring in long intertwined filaments, which appeared to serve as a matrix, enclosing micro-colonies of other groups of bacteria. Apparently the composition of the seed sludge is one of the factors that determined which kind of conglomerates will eventually develop, i.e. which mode of growth the M. soehngenii-like organism will adopt. De Zeeuw and Lettinga (5) have argued that the biomass retention time plays a crucial role in the development of the two types of conglomerates. Another important factor is the substrate on which the sludge is cultivated. Granular methanogenic sludge can be cultivated on ethanol. On the other media tested the granules grew out to less dense conglomerates. This suggests that non-acetoclastic bacteria influence either directly or indirectly the acetoclastic M. soehgnenii-like organisms, directly by overgrowing and enclosing the latter, thereby making it impossible for them to form long filaments, or indirectly by producing substances that trigger M. soehngenii-like organisms to grow singly or in short chains. Hulshoff Pol et al. (14,16) have reported that the addition of crushed granular methanogenic sludge to digested sewage in an UASB reactor fed with acetate plus propionate may give rise to the development of methanogenic sludge granules with a diameter of 1-2 mm. In our upflow reactor experiments with acetate plus propionate granules consisting of short chains of M. soehngenii-like organisms were observed too, but apart from these conglomerates, a large amount of the newly formed biomass consisted of long filaments. The reason

for this discrepancy is not clear.

The observation that two different types of sludges developed on the same medium depending on the source of the inoculum, made in parallel experiments indicates that the formation of well settling conglomerates (i.e. granulation and pelletization) initially is a purely biological phenomenon. This conclusion is corroborated by the observation that growth of granular sludge was dependent on the composition of the substrate. The accumulation of granular or at least well settling biomass in UASB systems depends on selection of granular sludge in the upflow reactor. Without selection the microflora will not express its potential ability to form well settling aggregates. In an upflow reactor organisms that are unable to enhance their retention in the reactor will be washed out of the system. The mechanism of the formation of different types of well settling conglomerates must be viewed separately from this selection.

The biomass yield in batch experiments agreed well with data previously reported for pure cultures (Table 3) except the high growth yield on propionate, accompanied by a low specific propionate-converting activity of the presumed syntrophic association of propionate degraders and hydrogenotrophic methanogens (12). Generally in batch culture the sludge granules were overgrown by dispersed growing bacteria, a fraction that is permanently washed out of the upflow system. Therefore, it should be noted that the growth yields reported here for UASB experiments are net values. Preliminary results indicate that the sludge wash out under upflow conditions amounted to 80% of the overall growth yield, resulting in a net growth yield of 20% of the true growth yield. These observations indicate that, though growth of sludge granules in batch systems is possible, cultivation of granular methanogenic sludge can best be done in upflow systems. Ethanol seems the substrate of choice to study the mechanisms of granulation and pelletization of methanogenic biomass.

Acknowledgements

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CHAPTER 3

Chemical and Bacteriological Composition of Granular Methanogenic Sludge

Can. J. Microbiol. 31: 744-750

Chemical and bacteriological composition of granular methanogenic sludge

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Methanogenic sludge granules grown on waste water from a sugar refinery consisted of several bacterial morphotypes embedded in a matrix of extracellular material. Comparison of critical point drying and freeze-drying methods for preparing samples for scanning electron microscopy to determine the presence of extracellular material indicated that the former method permitted observations of extracellular material and intact cells. The effects of different extraction methods used for isolation of these extracellular polymers was also investigated by scanning electron microscopy. Of the various extraction procedures (EDTA, NaOH, autoclaving, water–phenol), water–phenol left most of the cells intact and was found to be a very efficient method of extraction. Extracellular polymers equivalent to 10–20 mg hexose/g of granules were extracted. The high resistance of the granules against disintegration by various chemical methods suggested that different extracellular polymers and probably different groups of organisms contributed to the matrix in which the bacteria were embedded. The chemical composition of the granules did not differ from the composition of bacteria in general. The buoyant density of 1.00–1.05 $g \cdot g^{-1}$ of the granules indicated that a simple agglomeration was the mechanism by which these methanogenic consortia improved their settling characteristics.

DOLFING, J., A. GRIFFIOEN, A. R. W. VAN NEERVEN et L. P. T. M. ZEVENHUIZEN. 1985. Chemical and bacteriological composition of granular methanogenic sludge. Can. J. Microbiol. **31**: 744–750.

Des granules de boues méthanogènes croissant sur les eaux usées d'une rafinerie sucrière se sont avérées constituées de plusieurs morphotypes bactériens enrobés dans une matrice de matériel extracellulaire. Une comparaison des méthodes de séchage au point critique et de lyophilisation, pour préparer les échantillons en vue de déterminer la présence de matériel extracellulaire par microscopie à balayage, a permis d'établir que la première méthode était propice aux observations de matériel extracellulaire et de cellules intactes. Les effets de différentes méthodes d'extraction employées pour l'isolation des polymères extracellulaires ont aussi été étudiés par microscopie électronique à balayage. Des divers procédés d'extraction, l'EDTA, le NaOH, l'autoclavage, et l'eau–phénol, c'est l'eau phénolée qui a laissé la plupart des cellules intactes et s'est avérée être une méthode d'extraction très efficace. Des polymères extracellulaires équivalents à 10–20 mg d'hexoses par gramme de granules ont été extraits. La résistance élevée des granules à la désintégration par diverses méthodes chimiques fut l'indice que des polymères extracellulaires différents contribuaient au matériel matriciel enrobant les bactéries. En général, la composition chimique des granules ne fut pas différente de celle en bactéries. La densité de flottaison des granules, de 1,00–1,05 $g \cdot g^{-1}$, démontre que le mécanisme par lequel les consortiums méthanogènes améliorent leur formation caractéristique, relève de la simple agglomération.

[Traduit par le journal]

Introduction

Upflow anaerobic sludge blanket (UASB) reactors can be successfully applied for the anaerobic treatment of waste water if well-settling sludges with high methanogenic activities develop (Lettinga et al. 1980). The formation of a granular type of sludge which possessed these characteristics has been observed in a UASB reactor which treats waste water of a sugar factory (Pette and Versprille 1982). Study of these granules may help to understand how they are formed and may give an insight into how to manipulate reactor conditions to maximize their formation. In the present paper we report on the chemical and bacteriological composition of these granules.

Materials and methods

Source of the sludge

Granular methanogenic sludge was obtained from the Centrale Suiker Maatschappij sugar factory at Breda, The Netherlands. It had been cultivated in a 30-m^3 upflow anaerobic sludge blanket reactor operated at 32°C on a waste stream of a liquid sugar plant (Pette and Versprille 1982). The sludge age of this particular sludge is not known, but the sludge residence time in upflow anaerobic sludge blanket reactors may exceed 200 days (W. J. de Zeeuw. 1984. Ph.D. thesis, Agricultural University, Wageningen, The Netherlands). Sludge samples were stored anaerobically at 4°C until chemical and bacteriological analysis.

Procedure for bacterial counts

The medium for most-probable-number (MPN) determination of various groups of bacteria contained the following per litre of demineralized water (in grams): KH_2PO_4, 0.75; K_2HPO_4, 1.45; NH_4Cl, 0.9; cysteine-HCl, 0.5; $MgCO_3$, 0.15; $NaHCO_3$, 10; 100 mL of rumen fluid; and 9 mL of a trace elements solution. The trace elements solution contained the following (in milligrams per litre of demineralized water(: $FeSO_4$, 500; $MnCl_2 \cdot 4H_2O$, 100; $CoCl_2 \cdot 6H_2O$, 170; $ZnCl_2$, 100; $CaCl_2 \cdot 2H_2O$, 20; H_3BO_3, 19; Na_2MoO_4, 10; Na_2SeO_3, 28; $NiCl_2 \cdot 6H_2O$, 50. Sterile anaerobic techniques were followed as described by Balch et al. (1979). The medium was dispensed in 5-mL amounts into 20-mL glass tubes (Bellco Glass, Inc., Vineland, NJ) which contained carbon dioxide as gas phase. Before autoclaving 0.04 mL of a FeS solution prepared according to Brock and O'Dea (1977) was added per tube as a reducing agent. The autoclaved tubes (pH = 7) were supplied with 0.02 mL of a vitamin solution, which was sterilized by filtration through a 0.22-μm Milipore filter. The vitamin solution contained the following per litre of demineralized water (in milligrams): biotin, 2; folic acid, 2; pyridoxine hydrochloride, 10; thiamine hydrochloride, 5; riboflavin, 5; nicotinic acid, 5; DL-calcium pantothenate, 5; vitamin B_{12}, 0.1; *p*-aminobenzoic acid, 5; lipoic acid, 5; 2-mercaptoethanesulfonic acid, 20; menadione, 4; haemine hydrochloride, 40. Carbon sources as indicated in Table 3 were added from sterile concentrated stock solutions to reach final concentrations of 20 m*M* for volatile fatty acids or 8 m*M* for sucrose. For estimation of hydrogenotrophic methanogens, hydrogen was added to the headspace to reach a final composition of 80% H_2 and 20% CO_2. Washed and resuspended granular sludge was disintegrated with the use of a cream maker (Royston Ltd., England) under vigorous gassing with carbon dioxide (O_2 free). There were no indications of O_2 contamination or cell lysis owing to this disintegration procedure as evident from the

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TABLE 1. Composition of the neutral sugars fraction (as percent of total neutral sugars) of the extracellular polymers of granular methanogenic sludge grown on liquid waste water from a sugar factory

Component	Treatment method	
	Autoclaving[a]	EDTA[b]
Rhamnose	20.5	20.3
Ribose	6.0	0.0
Arabinose	1.5	2.8
Xylose	0.8	0.0
Mannose	11.9	11.5
Galactose	28.1	37.8
Glucose	31.1	27.7

[a] Autoclaving for 10 min at 120°C.
[b] Incubation with 1% EDTA for 5 h at 4°C.

TABLE 2. Chemical composition of various granular methanogenic sludges

Component	% of dry weight
Ash	10–23
Protein	35–60
Carbohydrate[a]	
Total	6–7
Extra cellular	1–2
TOC[b]	41–47
Nitrogen (Kjeldahl)	10–15

NOTE: Range of data of granular sludge grown on waste water of a liquid sugar factory and of laboratory-grown granular sludges (J. Dolfing, L. Luÿten, W. B. M. Bloemen, and A. J. B. Zehnder, to be published).
[a] As anthrone-positive material (hexose).
[b] TOC, total organic carbon.

sence of differences between the potential methanogenic activities f intact and disintegrated granules (Dolfing 1985). Subsequently the cterial suspension was diluted according to the MPN technique. fter 8 months of incubation at 30°C in the dark, methane formation Dolfing and Mulder 1985) and substrate depletion (Adamse 1980) ere determined by gas chromatography and tubes were examined by icroscopy.

xtraction procedures

With EDTA

Six millilitres of washed granular sludge (approximately 0.3 g dry eight) was placed in 130-mL serum bottles, and 44 mL of demin-alized water plus 50 mL of 2% tetrasodium ethylenediamine tetra etic acid (Na_4 EDTA) (final concentration, 1% EDTA), or 50 mL f 4% Na_4 EDTA (final concentration, 2% EDTA), was added per ottle. After sealing, the bottles were incubated at 4°C for 23 h on a tating wheel at a speed of 13 rotations per minute.

With 0.1 N NaOH

Granular sludge (6 mL) was added to 94 mL of 0.1 *N* NaOH ontained in a serum bottle of 130 mL capacity and incubated at 20°C r 23 h as above.

Autoclaving

Three millilitres of washed granular sludge (approximately 0.15 g) as added to 44 mL of demineralized water contained in an lenmeyer flask which was then covered with a copper head and aced in an autoclave. After pressurizing the autoclave to 1 atm = 120°C) (1 atm = 101.325 kPa), extraction times between 0 and 60 in were tested. Upon completion of the extraction procedures, the anules were allowed to settle, and samples were taken from the uid. The samples were centrifuged for 30 min at 3000 × *g* and the pernatant was analyzed. The amount of sludge was determined by ntrifuging the remaining mixed liquor for 30 min at 10 000 × *g* and ying the pellet overnight at 105°C.

Water–phenol

Granular sludge (10 mL) was disintegrated with the use of a cream aker and added to 10 mL of 90% phenol (v/v) in water, incubated a water bath at 70°C for 20 min, cooled on ice, and centrifuged at C (3000 × *g*, 30 min). The supernatant was dialyzed overnight to move the remaining phenol.

nalytical methods

Hexoses were measured by the anthrone method as described by erbert et al. (1971) with glucose as the standard. The Dische car-zole method (Bitter and Muir 1962) was used to measure hexuronic ids with D-glucouronic acid as the standard. Protein was determined ith the method of Lowry et al. (1951) with bovine serum albumin as e standard. Appropriate blanks for sodium hydroxide and EDTA ere included in the analytical procedures. For the analysis of the mino acids, 10 mL of 2% sulphosalicyclic acid (v/v) in water was dded to samples containing approximately 2 mg protein. The mixtures were cooled on ice for 20 min, and centrifuged for 20 min at 3000 × *g*; 10 mL of 2% sulphosalicyclic acid solution was added again to the pellet and the procedure was repeated four times. The pellet was subsequently hydrolyzed and analyzed as described by Slijkhuis (1983).

Total organic carbon content was determined in a Beckman model 915A total organic carbon analyzer by substracting the amount of inorganic carbon from the total amount of carbon. A solution of $NaHCO_3$ and an acidified solution of phthalic acid were used as standards.

To determine total nitrogen, samples of washed granules were digested by the Kjeldahl procedure (Kjeldahl 1883). Ammonia in these samples was distilled off and determined with Nesslers's reagent (Herbert et al. 1971).

Transmission electron microscopy

Granules were fixed in 2.5% glutaraldehyde + 0.15% of ruthenium red in 0.1 *M* cacodylate buffer (pH 7.2) for 12 h and postfixed for 2 h in 2% OsO_4 in the same buffer containing ruthenium red. Dehydration occurred with a graded series of water–acetone mixtures. These solutions contained the ruthenium red–cacodylate buffer until the 50% stage, after which the stain became insoluble. The material was finally dehydrated in propylene oxide and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate as described by Reynolds (1963) and examined using a Jeol electron microscope at an accelerating voltage of 60 kV.

Preparation of freeze-dried samples for scanning electron microscopy

Granules were fixed in 25 m*M* cacodylate buffer (pH 6.8) containing 2% glutaraldehyde for 3 h at room temperature, washed three times with cacodylate buffer (25 m*M*; pH 6.8), and postfixed for 2 h in cacodylate buffer containing 1% OsO_4. The samples were washed twice in double-distilled water and lightly blotted to remove excess surface water before freeze-drying. The samples were frozen in supercooled freon and transferred to the cold stage of a freeze dryer, held at −70°C. The dried samples were sputter-coated with carbon and examined in a Philips scanning electron microscope using 15 kV accelerating voltage.

Preparation of critical point-dried samples

The samples were fixed as described above and washed prior to dehydration in a graded water–acetone series (10–100%, 15 min in each). The granules were frozen in supercooled freon, carved, dried following the method of critical point drying (Horridge and Tamm 1969), coated with gold, and examined in a Philips scanning electron microscope using 15 kV accelerating voltage.

Results

Chemical composition of the granules

None of the following treatment methods resulted in a complete disintegration of the granules: HCl (pH 1), NaOH (pH 14), 5% EDTA, boiling, autoclaving, and incubation with cellulase. However, these procedures invariably weakened the

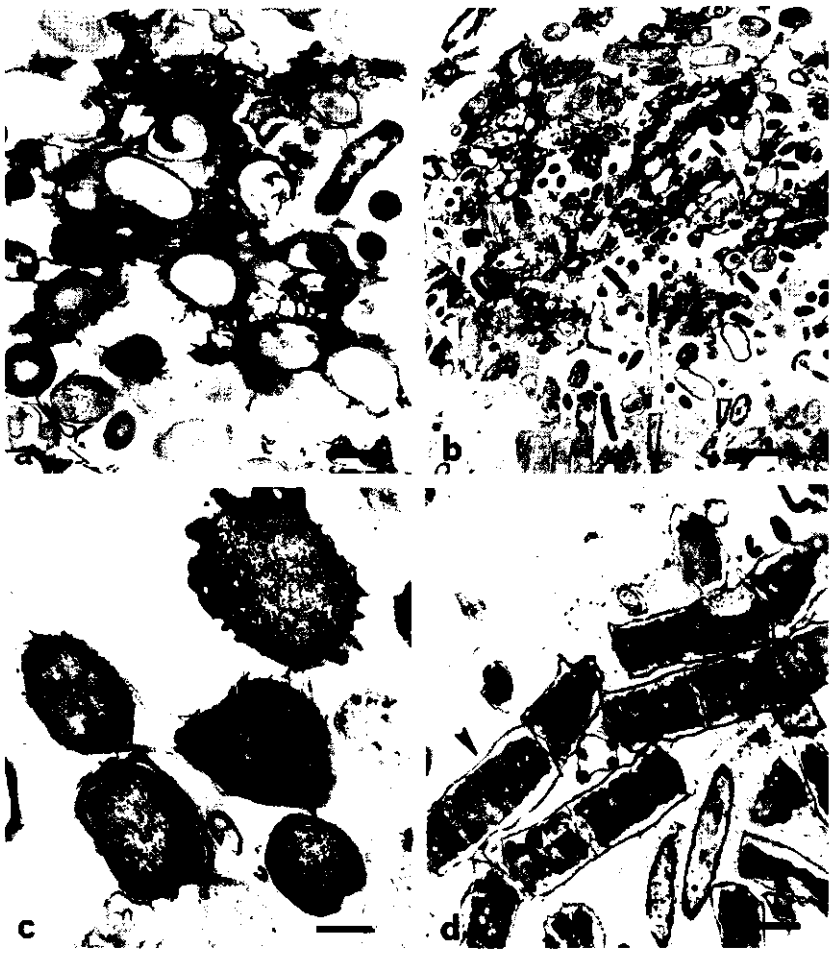

FIG. 1. Transmission electron micrograph of a section of ruthenium red stained granular methanogenic sludge grown on waste water of a sugar refinery. (*a*) Large amounts of locally occurring extracellular material. Bar = 0.55 μm. (*b*) Large number of different bacterial morphotypes and patchiness in the occurrence of extracellular material. Bar = 1.5 μm. (*c*) Extreme condensation of extracellular material surrounding individual cells. Bar = 0.27 μm. (*d*) Absence of extracellular material around *Methanothrix*-like cells (arrow). Bar = 0.55 μm.

granules, e.g., after autoclaving, 33% of the granular dry weight could be suspended by sonification compared with only 9% for untreated granules.

Autoclaving for 10 min resulted in the extraction of 10 mg of anthrone-positive material (i.e., carbohydrates) per gram of granule dry weight. Prolonged autoclaving resulted in the release of about 20 mg carbohydrate per gram of granules. The amount of liberated protein (Lowry-positive material) increased during autoclaving to 45 mg · g sludge^{-1}. The liberated protein may have originated in part from protein-containing cell envelopes.

Extraction with EDTA (1 or 2%), 0.1 *N* NaOH, or water–phenol also resulted in the release of 10–20 mg of carbohydrates per gram of granules. The amount of uronic acids in the extracellular carbohydrate fraction as determined after water–phenol extraction was 13%. The carbohydrate composition of the extracellular polymers is given in Table 1. The chemical composition of the granules is given in Table 2. The ash content of samples taken at various occasions from the same reactor varied between 10 and 20%. About 30% of the ash fraction consisted of FeS, which was responsible for the black colour of the granules and the smell of H_2S that was released after exposure of the granules to concentrated HCl. The protein fraction as determined with the Lowry method was about 52% of the ash-free matter. Calculating the protein fraction from the sum of the amino acids as determined with an amino acid analyzer gave a value of 43 as percent of ash-free dry weight.

By weighing and volumetric determinations, buoyant densities of the granules were estimated (to be 1.00–1.05 g · cm^{-3}). The settling properties of the granules varied between 0.5 and 3 cm · s^{-1} for granules of different sizes.

Microbial composition of the granules

The results of serial dilutions of disintegrated granular methanogenic sludge incubated during 8 months in anaerobic media

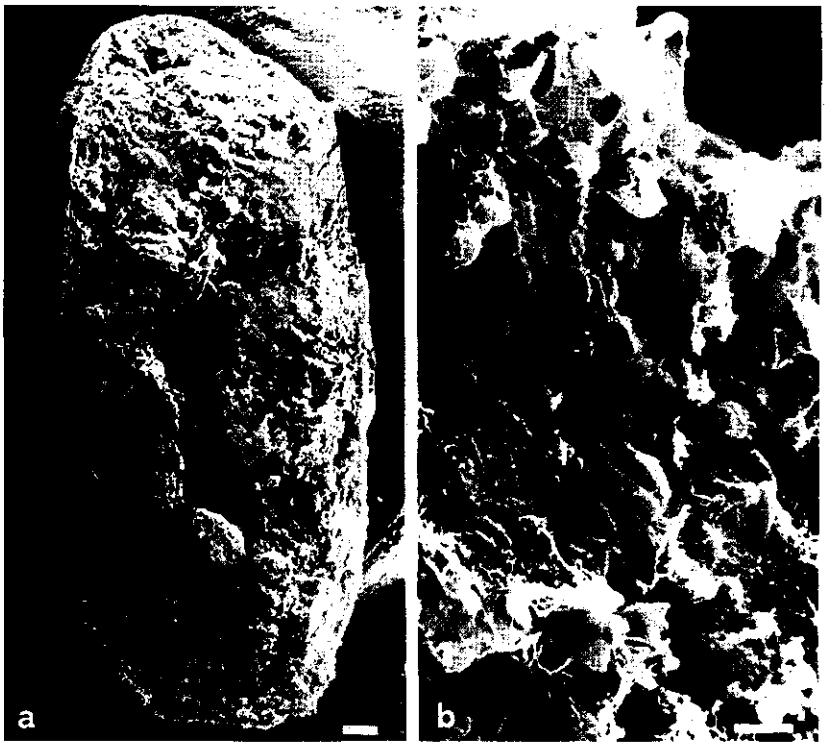

FIG. 2. Scanning electron micrograph of the inside of cleaved granular methanogenic sludge grown on waste water of a sugar refinery. (*a*) Overview. Bar = 100 μm. (*b*) Cobweblike material surrounding the cells. Bar = 1 μm.

containing various energy sources are presented in Table 3. The data show that hydrogenotrophic ($H_2 + CO_2 \rightarrow CH_4$) methanogens made up about 10% of the total culturable population, whereas acetotrophic ($CH_3COOH \rightarrow CH_4 + CO_2$) methanogens amounted to only 1%. Direct microscopic counts of disintegrated granules, however, gave different results: about 20% of the total population was found to consist of autofluorescent methanogens (Doddema and Vogels 1978). Another 20–30% showed much resemblance to the nonautofluorescing obligately aceticlastic methanogen *Methanothrix soehngenii* (Zehnder et al. 1980; Huser et al. 1982).

Electron microscopy

Transmission electron micrographs (Fig. 1) corroborated the conclusion that significant numbers of *Methanothrix*-like organisms were present in granular sludge grown on waste water of a sugar refinery. The micrographs show that the granules tested consisted of a wide variety of bacterial morphotypes which occur in microcolonies and are distributed at random throughout the granules. Ruthenium red was included in the preparation procedures for transmission electron microscopy, but it generally failed to stain the extracellular material that was visible if granules were examined by scanning electron microscopy (Fig. 2). Locally, however, significant amounts of extracellular material were shown to occur in transmission electron micrographs, but at other places this material was not observed. Condensation of extracellular material surrounding the cells was sometimes detected in transmission electron micrographs. In comparison with nonruthenium red stained control samples, ruthenium red stained granules showed no increased amount of electron-dense extracellular material.

TABLE 3. Culture counts of different metabolic groups of bacteria present in granular methanogenic sludge grown on the waste water of a liquid sugar factory

Metabolic group	No. of organisms[a] (mL^{-1})
Sucrose degraders	10^{10}
Hydrogenotrophic methanogens	10^{9}
Acetotrophic methanogens	10^{8}
Propionate degraders	10^{7}
Butyrate degraders	10^{7}
Valerate degraders	10^{7}

[a] According to the most-probable-number technique with three tubes per dilution in each experiment. The numbers have been rounded off to the next exponential factor.

Two different methods of drying, an obligate step in sample preparation for scanning electron microscopy, were compared for their ability to preserve extracellular material, viz., freeze-drying and critical point drying. Freeze-drying resulted in images that showed bacteria covered with extracellular material. This view was confirmed by comparing freeze-dried granules with a culture of a bacterium that produced copious amounts of slime (*Agrobacterium* A8) and a mutant of this strain that produced only insignificant amounts of slime (Fig. 3). Generally individual cells could hardly be detected in freeze-dried preparations of granule sludge. Examination of

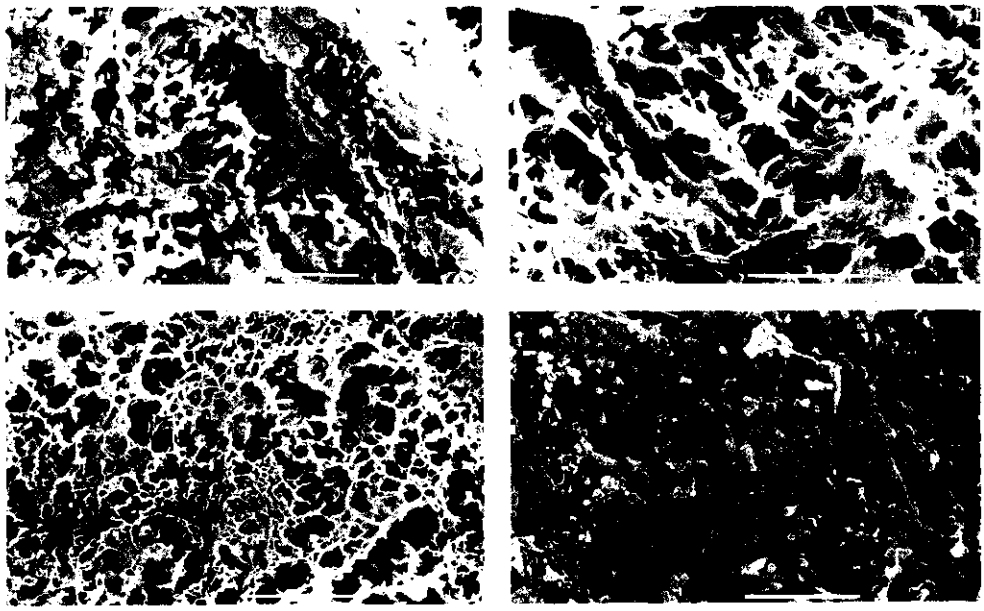

FIG. 3. Scanning electron micrographs of (*a*) a mutant of *Agrobacterium* A8 which produced only insignificant amounts of extracellular aterial, after freeze-drying; (*b*) parent strain of *Agrobacterium* A8 which produced copious amounts of extracellular material, after freeze-ying; (*c*) granular methanogenic sludge grown on waste water of a sugar refinery, after freeze-drying; (*d*) granular methanogenic sludge grown ı waste water of a sugar refinery, after critical point drying. Bar = 10 μm.

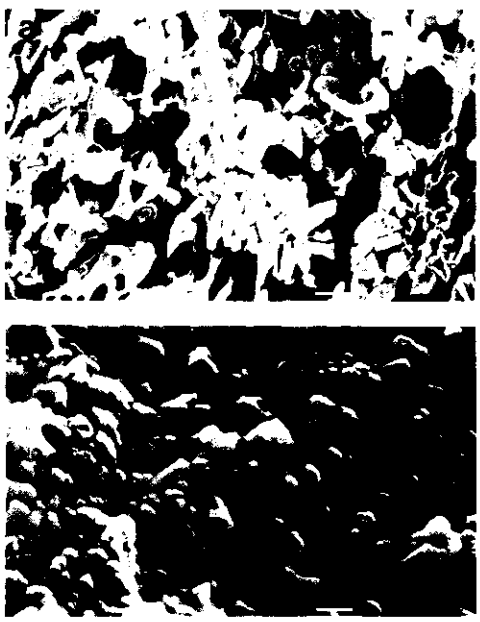

FIG. 4. Scanning electron micrographs of water–phenol treated granular methanogenic sludge grown on waste water of a sugar refinery. (*a*) After freeze-drying. (*b*) After critical point drying. Bar = 1 μm.

critical point dried granules sludge showed that this method resulted in the condensation of extracellular material that becomes visible as fibrils which surround the bacteria. Thus critical point drying allowed detection of condensed extracellular material and simultaneously allowed observation of the bacteria which made up the granules. Therefore, this method was used for the preparation of samples in experiments aimed at the evaluation of (*i*) the efficiency of various treatment methods to extract extracellular material from the granules, and (*ii*) the effects of these methods on the integrity of the cells.

Autoclaving for 10 min was the only treatment method tested that did not significantly affect the cells. The other methods induced visible disruption of the cells. Water–phenol extracted granules appeared as completely distorted aggregates after critical point drying. Examination of these granules after freeze-drying showed that the distortion was probably due to the dehydration step with acetone which damaged the cells that were denuded by the water–phenol extraction (Fig. 4). The critical point drying method results in a significant shrinkage of the granules: after dehydration with acetone the mean diameter of the granules was diminished to 67% of the original diameter, and after critical point drying the mean diameter was diminished further to 58% of its original magnitude.

Discussion

It is apparent from the present study that granular sludge grown on waste water from a sugar refinery consists of several bacterial morphotypes. This is not surprising since the composition of such waste water is very complex (Pette and Versprille 1982). The groups of various types of bacteria are quite randomly distributed throughout the granule and an inter-

nal organization is not obvious. This corroborates observations of methane-producing biofilms from fixed-bed reactors (Robinson et al. 1984), and indicates that granules (and biofilms alike) are formed by the activities of various groups of microorganisms rather than by the action of a single type of bacterium.

It is of distinct advantage for a bacterium in an UASB reactor to be part of a large particle since it will, according to Stokes' law, sink much faster than discrete cells, and therefore, improve its chance to remain in the reactor. Buoyant densities of the granules of 1.00−1.05 $g \cdot cm^{-3}$ were equal to densities for dispersed bacterial cells, as reported by Woldringh et al. (1981), which shows that the excellent settling properties of the granules are mainly due to aggregation of the microorganisms.

Bacterial cells in natural environments are often surrounded by capsules. Fletcher and Floodgate (1973) have shown that many aquatic bacteria are surrounded by a capsule of acidic polysaccharides, which mediates their adhesion to solid surfaces, and Bae et al. (1972) found that most bacterial cells examined directly from soil are surrounded by thick capsules. Ruthenium red has been shown to be a specific stain for mucopolysaccharides and polysaccharides (Luft 1971) and hyaluronic acid (Huet and Herzberg 1973). We used it as a stain to determine if extracellular polysaccharides are involved in embedding the cells in a presumed glycocalyxlike matrix (Costerton et al. 1981). However, contrary to observations made with scanning electron microscopy (Figs. 2 and 3), transmission electron microscopy (Fig. 1) did not reveal significant amounts of extracellular material. In theory this could be due to pronounced condensation during the dehydration steps of preparation for electron microscopy. However, condensation is prevented by the attachment of fibers to more than one solid structure. If the presumed cementing substance really occurs throughout the whole granules (as is suggested by Fig. 2) and does stain with ruthenium red, it should have been visible in our granules because its structure would at least have been partially preserved by its multiple attachment points with the embedded bacteria. Dehydration procedures did not result in disintegration of the granules; thus at least partial preservation of the presumed extracellular matrix was to be expected. Indeed local extracellular material could be observed which was condensed to different fibrous patterns (Fig. 1). The latter observations suggest that chemically different extracellular polymers were condensed to different degrees. The occurrence of different extracellular polymers is also suggested by the analysis of the carbohydrate composition of the extracellular polymers that are obtained with various extraction methods (Table 1), although the observed differences in Table 1 may be more a reflection of differences in extraction procedures rather than an indication of diversity of origin.

The results presented here (Table 2) indicate that granular methanogenic consortia are chemically comparable with bacteria in general. Their protein and carbohydrate contents are comparable with values given by Herbert (1975). The ash content was somewhat higher. The extracellular polysaccharide fraction of the consortia was only 1−2%. This relatively small fraction is apparently able to form a strong matrix for the embedment of the cells. Presumably various extracellular polymers, most likely produced by more than one group of bacteria, participate in this matrix.

Values of 1−2% for the extracellular polymer fraction compare well with literature data reported for aerobic activitated sludges (Brown and Lester 1980). However, as the total quantities of polymers present are generally not known, it is impossible to make a valid comparison between various sludges. Frequently, different extraction procedures have different efficiencies in removing (different fractions of) extracellular material and remove, for example, specifically slime polymers, as distinct from adherent capsular material (Wilkinson 1958). The ratio between different extracellular polymers may vary with culture conditions, and consequently the efficiency of extraction procedures will also vary. Therefore, it was recently suggested that acid hydrolysis be used as a method for the quantification of the total polymer fraction (Rudd et al. 1982). Our observations of water−phenol treated granular sludge suggest that this method is very efficient in extracting extracellular material without disrupting the cells and is at least equally well suited for estimating the total amount of extracellular material. In constrast the relatively crude acid hydrolysis method is only applicable for pure cultures and only if noncapsulated controls are available to correct for intracellular polysaccharides.

The MPN viable counting technique proved to be inadequate for the quantitative assessment of the physiological groups of bacteria that occur in granular methanogenic consortia. Possibly, many of the metabolically active organisms are not cultivable in artificial culture media, especially in a reactor where the biomass retention time is very high and especially with organisms with a very low maximum growth rate such as the fatty acid degrading bacteria. Furthermore, the clump- and chain-forming bacteria may have been incompletely dispersed and therefore gave a lower bacterial count by the MPN method. Therefore, methanogenic activity tests have been performed on various substrates with these granules. The data thus obtained were compared with potential specific activities of pure cultures. The results corroborate microscopic observations that methanogens, especially acetotrophic methanogens, form a significant part of the granular consortia grown on waste water of a sugar factor (Dolfing and Bloemen 1985).

Acknowledgement

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CHAPTER 4

Activity Measurements as a Tool to Characterize the Microbial Composition of Methanogenic Environments

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Activity measurements as a tool to characterize the microbial composition of methanogenic environments

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Summary

A rapid and reliable method to assess the potential specific activity of methanogenic sludge is presented. The method is based on the gas chromatographic analysis for methane in the headspace of closed vials. Gas is sampled with a pressure lock syringe, which allows quantification independent of the pressure prevailing in the vials.

The influence of various parameters as substrates, pH, NaCl and NH_4Cl concentrations on the activity of methanogenic sludge was investigated with this method. Data on the methanogenic activities on different substrates are discussed in terms of the different physiological groups present in the sludge.

Key words: *Acetoclastic methanogens - Community structure - Formate - Hydrogenotrophic methanogens - Potential methanogenic activity*

Introduction

Methanogenic activity of an anaerobic system depends on the presence of methanogenic bacteria and of substrates converted by these bacteria to methane. Such substrates may be available in adequate amounts or they may be derived from other compounds by non-methanogenic bacteria which are often living in a nutritional relationship with the methanogens [1].

To estimate the potential methanogenic activity of a habitat like anaerobic sludge, an aliquot from this ecosystem should be supplied with an excess of substrate and subsequently the methane produced should be measured. Methods of this type have been used in activated sludge for carbohydrate-decomposing bacteria [2], in soil for methane-

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oxidizing bacteria [3], and for ammonium-oxidizing bacteria [4]. Belser and Mays [4] have shown that activity measurements are more suitable than MPN counts for the quantification of nitrifying biomass. Substrate conversion activities in methanogenic environments have been determined in various habitats. For example, actual activities of sediments at in situ concentrations of substrates were estimated by using tracer methods [5]. Potential activities were estimated in anaerobic reactors by adding excess substrate [6]. These activities (expressed per liter rather than per gram) were used to assess the carbon and electron flow rather than to estimate the actual and potential methanogenic activities of the biomass involved in the anaerobic conversion of various substrates.

In addition to estimating total methanogenic activity, the method to be introduced here aims to differentiate the various physiological groups of methanogenic bacteria in the biomass and the non-methanogenic organisms as far as they act as producers of methanogenic substrates. To give an estimate of the size of the biomass connected with various metabolic groups of methanogenic bacteria in the sample to be tested, a comparison has to be done between the methane production of the sample as a response to specific substrates and methane production of pure cultures. Specific activities of various metabolic groups of anaerobic bacteria, derived from literature data, are compiled in Table 1, where the methanogenic bacteria have been divided into three major metabolic subgroups: (1) organisms that utilize only H_2-CO_2 (or formate) for methane production, (2) organisms that produce methane from acetate only, and (3) organisms that produce methane from both H_2-CO_2 and acetate. The specific methanogenic activities of the hydrogenotrophic methanogens are generally a factor of 10 higher than the specific activities of the acetoclastic methanogens (Table 1). This means that ten times more cell material is required to produce methane from acetate at the same rate as methane from hydrogen.

Therefore, to bring the acetoclastic and hydrogenotrophic methanogens on a comparable level as to weight of biomass, the production rates on hydrogen should be divided by a factor of ten. The proportion of the mixed function methanogenic biomass, i.e., *Methanosarcina* spp, can be estimated by comparing the potential methanogenic activities on a mixture of hydrogen plus acetate with the activities on hydrogen and on acetate separately. The presence of *Methanosarcina* spp will give a lower activity on hydrogen plus acetate than the sum of the activities on hydrogen and on acetate, if it is assumed that (i) *Methanosarcina* spp in the biomass are adapted to methane formation from acetate and consequently are able to produce methane from both acetate and hydrogen, and (ii) the presence of elevated hydrogen concentrations inhibits methane formation from acetate in *Methanosarcina* spp. The effect of H_2-CO_2 on methanogenesis from acetate in *Methanosarcina* spp is controversial [12, 15, 16]. The results presented by Traore et al. [17], however, show conclusively that both substrates can be used simultaneously by *Methanosarcina* spp at low dilution rates, i.e., low substrate concentrations. However, at high substrate concentrations, as used in the present tests, no methane production should take place from acetate when a mixture of both substrates is present in excess.

The basic assumptions for the estimations outlined above are that (1) acetate formation from H_2-CO_2 [18, 19] and (2) hydrogen formation from acetate [20] play no significant role in the test samples.

TABLE 1

SPECIFIC ACTIVITIES OF VARIOUS METABOLIC GROUPS OF MESOPHILIC ANAEROBIC BACTERIA OF IMPORTANCE IN METHANOGENIC ECOSYSTEMS

Metabolic group	Species	Substrate	Specific activity[a] (μmol $CH_4 \cdot g^{-1} \cdot min^{-1}$)[b]	Ref.
Hydrogenotrophic methanogens	*Methanobacterium formicicum*	H_2	350–550[b]	7
	Methanobacterium bryantii	H_2	300[c]	8
	Methanobrevibacter arboriphilus	H_2	600[c]	8
	Methanobrevibacter arboriphilus	H_2	116–490–1180[b,d]	9
	Methanospirillum hungatei	H_2	260[c]	8
Acetotrophic methanogens	*Methanothrix soehngenii*	acetate	24[b]	10
Mixed function methanogens	*Methanosarcina barkeri* (Fusaro)	H_2	200[c]	8
	Methanosarcina barkeri (DM)	H_2	95	11
	Methanosarcina barkeri (Fusaro)	acetate	30[c]	8
	Methanosarcina barkeri (Ms)	acetate	60	12
	Methanosarcina barkeri (227)	acetate	170[b]	13
	Methanosarcina barkeri (227)	acetate	23–29[b]	14

[a] Specific activities are expressed as μmol CH_4 per g cells (dry weight) per min.

[b] Data obtained under growth supporting conditions.

[c] Data obtained in activity tests.

[d] Data obtained on various media.

In the present paper a test for methanogenic activity is described, based on gas chromatographic analysis of the headspace of closed anaerobic vials containing small samples of sludges or biomass from various habitats which were supplied with test substrates. The results of these tests were used to assess the microbial composition of biomass grown in reactors treating anaerobically various waste waters.

Materials and Methods

Sources of the sludges

Granular sludge was a gift from the sugar factory CSM at Breda, The Netherlands. It had been taken from a 30 m^3 upflow anaerobic sludge blanket reactor [21] treating the waste stream from a liquid-sugar factory [22]. A different anaerobic sludge was obtained from a 500 m^3 upflow anaerobic sludge blanket reactor treating waste water from ZBB-de Bijenkorf, a maize starch factory at Koog aan de Zaan, The Netherlands. Part of this sludge was further cultivated for 3 months in the laboratory in a 12 l upflow anaerobic sludge blanket reactor on an artificial waste water containing as carbon sources (on COD basis): acetate (46%) plus propionate (54%). A third type of sludge was a gift of L. W. Hulshoff Pol. It had been cultivated in 30 l upflow anaerobic sludge

blanket reactors on artificial waste waters counting as carbon sources (on COD basis): (a) acetate (46%) plus propionate (54%); (b) acetate (42%) plus propionate (48%) and sucrose (10%); (c) acetate (2%) plus propionate (3%) and sucrose (95%). The mode of cultivation has been described previously [23].

Activity tests

The anaerobic techniques used were essentially those of Hungate [24]. Serum vials with a volume of 130 ml were made anaerobic by flushing with O_2-free carbon dioxide, and subsequently filled with 40 ml of an anaerobic buffer solution. The carbon dioxide was made free from residual O_2 by passing it over hot copper coils at 350° C. The buffer was made anaerobic by boiling, followed by cooling to room temperature under continuous gassing with O_2-free carbon dioxide. Unless stated otherwise, the activity tests were performed under a headspace of carbon dioxide, in a buffer solution containing per liter demineralized water: NH_4Cl (0.5 g), K_2HPO_4, $3H_2O$ (0.4 g), KH_2PO_4 (0.4 g), $NaCHO_3$ (10 g) (pH, 7.0). Methanogenic activity-pH relationships were established in 0.2 M potassium phosphate buffers (pH values as indicated) containing 0.5 g NH_4Cl per liter demineralized water under a headspace of nitrogen.

Fresh sludge was anaerobically distributed into the vials in portions of 100–1000 mg. Substrate was added to the mixed liquid from concentrated stock solutions to reach final concentrations of 20–50 mM. The vials were closed with serum bottle caps and incubated in a shaking water bath at 30° C. In the case of hydrogen as test substrate 80 ml of this gas was supplied. When stored (at 4° C) sludge was tested, the biomass was completely acclimatized by incubating it overnight at 30° C in the presence of small amounts of substrates that would not significantly alter the composition of the biomass. After an overnight reacclimatization the headspace was flushed with O_2-free carbon dioxide prior to the determination of the methane production rate, and new substrate was added. The measurement of the methane production rate usually took a few hours. At the end of the activity tests the amount of sludge present in the vials was determined by weighing the pellet after centrifuging the mixed liquid and discarding the supernatant. The pellet was dried to constant weight overnight at 105° C. The percentage ash was determined after reheating for 2 h at 600° C.

Analysis of gases

Samples were taken from the headspace with a gas pressure lock syringe (Unimetrics, Anaheim, CA). Methane and hydrogen were determined by using a Packard-Becker 406 gas chromatograph equipped with a thermal conductivity detector and a molecular sieve column, operated at 50° C. The carrier gas was argon at a flow rate of 20 ml · min^{-1}.

Results

Optimalization of the test conditions

In preliminary experiments with anaerobic-sludge samples, acetate, propionate and hydrogen were each stoichiometrically converted to methane without detectable formation of intermediary products. Therefore, methane production was used as a measure of the potential conversion capacity. The determination of increasing amounts of methane

is much easier and more accurate than the measurement of decreasing concentrations of substrate.

In order to function as a reliable method for measuring the potential specific methanogenic activity of biomass, methane production should be dependent on the amount of sludge over a wide range of sludge concentrations. This prerequisite was fulfilled as shown in Fig. 1. The methanogenic activity of granular sludge grown on waste water of a sugar refinery (Fig. 1A) was 3.3 μmol $CH_4 \cdot g^{-1} \cdot min^{-1}$; the methanogenic activities on H_2-CO_2 and acetate of sludge from a maize starch factory were 3.0 μmol $CH_4 \cdot g^{-1} \cdot min^{-1}$ and 0.6 μmol $CH_4 \cdot g^{-1} \cdot min^{-1}$, respectively (Fig. 1B). These linear relationships between amount of sludge and methanogenic activity were easily obtained with acetate as the substrate. With H_2-CO_2, however, vigorous shaking was necessary for reliable results, especially at higher potential activities of the sludge.

To overcome errors due to phase transfer limitation we tested to see if the methanogenic activity on H_2-CO_2 was comparable with the activity on formate. It was found that these activities were generally of the same order of magnitude for the systems studied. This indicates that formate can be generally used instead of H_2-CO_2 to estimate the hydrogenotrophic methanogenic potential of digester sludge.

The composition of the buffer solution for the activity tests under carbon dioxide was a simplification of the basal medium used by Adamse [25], trace elements and vitamins were omitted. These omissions are justified since the potential methanogenic activity of anaerobic sludge is determined in short-term experiments during which no significant growth should occur. Carbon dioxide was used as gas phase. Using this gas had two advantages, namely carbon dioxide could be used in combination with hydrogen as substrate and together with the biocarbonate in the medium it provided a reliable pH buffer system [26].

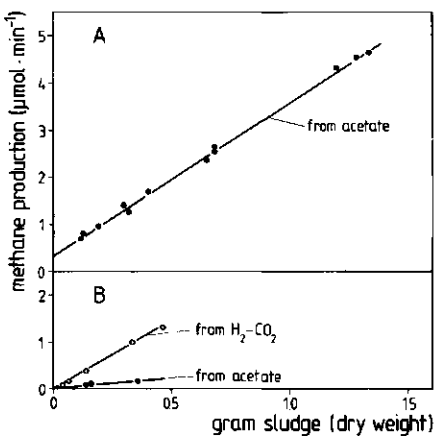

Fig. 1. Effect of different amounts of biomass on the methane production rate from acetate and H_2-CO_2. Sludge was added to 40 ml buffer in quantities indicated. The potential methanogenic activities of the sludges can be derived from the slopes of the lines. (A) Sludge grown in an anaerobic reactor treating waste water from a liquid sugar factory. (B) Sludge grown in an anaerobic reactor treating waste water from a maize starch factory.

TABLE 2

INFLUENCE OF NH_4Cl ON THE POTENTIAL METHANOGENIC ACTIVITY[a] OF GRANULAR SLUDGE

NH_4Cl (mg/l)	Rate of CH_4 production (μmol $CH_4 \cdot g^{-1} \cdot min^{-1}$)
0	3.13[b] ± 0.29
500	5.33 ± 0.50
1500	5.47 ± 0.40
5000	4.43 ± 0.21

[a] The potential methanogenic activity was determined with a mixture of sodium acetate and sodium propionate (20 mM each) as substrates at 30 °C and pH = 7.0.

[b] Mean ± standard deviation of experiments in triplicate.

The basal medium serving as a starting point for the composition of the buffer solution contained 0.5 g · l^{-1} NH_4Cl. Table 2 shows that the optimum NH_4Cl concentration for granular methanogenic sludge is between 0.5 and 1.5 g · l^{-1} NH_4Cl. Thus, all further activity tests were done at 0.5 g · l^{-1} NH_4Cl.

Performance of the test

High salt concentrations may affect methane production. Figure 2 gives an example of the effect of NaCl on the acetoclastic methanogenic activity of granular sludge: methanogenesis was inhibited by 50% at a concentration of NaCl of about 150 mM.

The effect of pH on the methanogenic activity of granular methanogenic sludge is shown in Fig. 3. The experiments were performed under a nitrogen atmosphere in 0.2 M phosphate buffer.

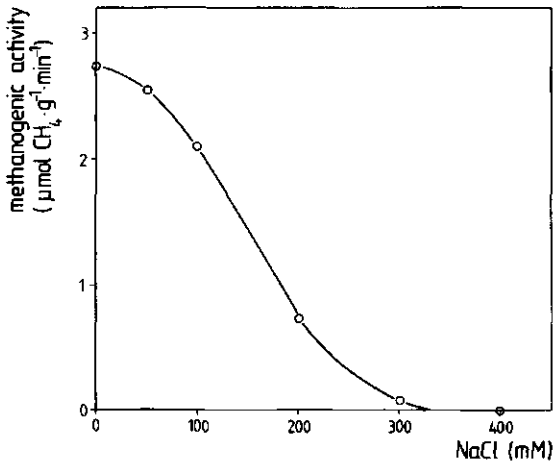

Fig. 2. Influence of the NaCl content of the CO_2-HCO_3 buffer on the potential acetoclastic methanogenic activity of granular sludge grown in an anaerobic reactor treating waste water from a liquid sugar factory.

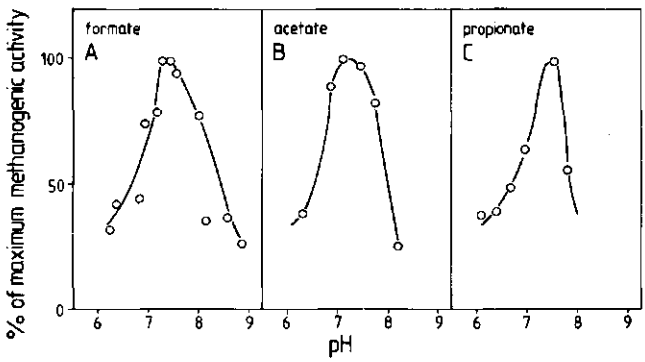

Fig. 3. The effect of pH on the methanogenic activity of granular methanogenic sludge grown in an anaerobic reactor treating waste water from a liquid sugar factory. The following substrates were used: (A) formate; (B) acetate; (C) propionate.

The potential methanogenic activities of digested sewage sludge were low compared to the activities of biomass cultivated in UASB reactors on soluble substrates (Table 3). The composition of the growth medium influenced the potential methanogenic activity of the biomass that developed. The presence of 10% sucrose (on COD-basis) in the growth medium reduced the potential methanogenic activity of the biomass by 30–70% as compared to the activity of the biomass cultivated on a mixture of acetate and

TABLE 3

POTENTIAL METHANOGENIC ACTIVITIES OF BIOMASS GROWN IN VARIOUS ANAEROBIC WASTE WATER TREATMENT SYSTEMS

Origin of biomass	Growth substrate[a]	Potential methanogenic activity (μmol $CH_4 \cdot g^{-1}$ VSS $\cdot$ min^{-1})[b]			
	Test substrates:	H_2	Formate	Acetate	Propionate
Sewage digester	Sewage	N.D.	0.3	0.3	0.1
	46% acetate + 54% propionate[c]	15.5[d] ± 3.5	20.0 ± 4.0	14.9 ± 1.9	6.9 ± 1.2
	42% acetate + 48% propionate + 10% sucrose	10.5 ± 2.1	8.5 ± 2.0	6.9 ± 0.4	4.4 ± 1.3
	2% acetate + 3% propionate + 95% sucrose	10.5 ± 1.9	6.3 ± 0.6	5.0 ± 0.8	2.0 ± 0.9
Maize starch factory	Maize starch waste water	5	8	1.0	1.3
	46% acetate + 54% propionate	14.1 ± 2.4	16.7 ± 1.1	16.4 ± 0.5	4.7 ± 0.5
Liquid sugar factory	Liquid sugar waste water	10	11	10	4.5

[a] Cultivation time was 3 months or more.

[b] VSS: volatile suspended solids, i.e., the organic fraction of the sludge, taken as indicative for the biomass fraction of the sludge; ND, not determined.

[c] Percentages of the total amount of COD present in the growth medium.

[d] Mean ± standard deviation of three rate measurements.

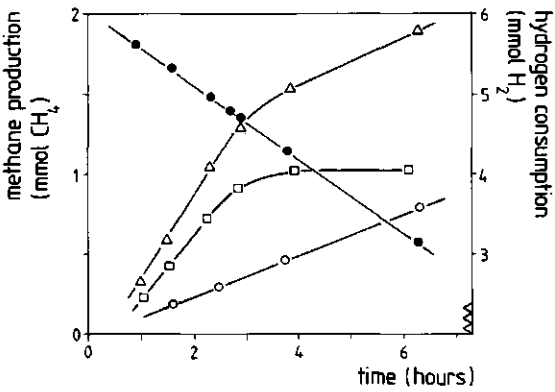

Fig.4. Methanogenic activities of granular methanogenic sludge on hydrogen (○), acetate (□) and hydrogen plus acetate (△). After about 200 min all acetate had been consumed, while hydrogen consumption (●) continued at the same rate.

propionate. A further increase of the sucrose fraction in the growth substrate to 90% gave only a further minor reduction in potential methanogenic activity.

A comparison between the potential methanogenic activities of acetate- plus propionate-grown biomass originating from sludge of either a sewage sludge digester or a maize starch factory indicates that the potential methanogenic activities of biomass that had been developed after 3 months of cultivation reflected the composition of the growth substrate rather than the potential activities of the inoculum.

Application of the test

In order to obtain information on the metabolic groups of the methanogenic population, activity tests were performed with acetate, hydrogen and acetate plus hydrogen (Fig. 4 and Table 4). Table 4 summarizes the results with various sludges and shows that the methanogenic activities on hydrogen plus acetate were equal to the sum of the activities on hydrogen plus acetate separately. This demonstrates that *Methanosarcina-*

TABLE 4

COMPARISON OF THE METHANOGENIC ACTIVITIES ON HYDROGEN, ACETATE AND HYDROGEN PLUS ACETATE OF BIOMASS CULTIVATED ON VARIOUS WASTE STREAMS

Growth substrate	Potential methanogenic activity (μmol $CH_4 \cdot g^{-1}$ VSS $\cdot$ min^{-1})[a]		
Test substrates:	H_2	Acetate	H_2 + Acetate
Liquid sugar waste water	10	10	20
Maize starch waste water	5	1	7
Acetate plus propionate	13	10	24

[a] VSS, volatile suspended solids.

like organisms formed no significant part of the acetotrophic methanogenic population in these sludges. In that case the sum of the activity on hydrogen and that on acetate would have been higher than that on a mixture of hydrogen plus acetate since the *Methanosarcina* spp would not produce methane from acetate.

Discussion

For a reliable estimation of a specific biochemical activity the active agent, be it biomass or enzyme, should be present in the test vial in such amounts that it is the limiting factor in the reaction to be studied. This means in our case that, within a certain limit, methane production should increase linearly with an increasing amount of methanogenic biomass employed. This demand has been fulfilled for the method presented here over a wide range of biomass concentrations (Fig. 1).

Three aspects of this method should be emphasized.

(1) The measured activity represents a potential activity; i.e., conditions in the environment from which the sample was obtained may not be as conducive to methanogenesis as the assay conditions, where ample supply of substrates is provided and possibly inhibitory compounds are absent or present only in low concentrations.

(2) The production rate measured reflects the activity of biomass which is not specifically enriched on the test substrate. Incubations are conducted over a short period, normally less than 24 h, so little or no growth will occur.

(3) Low amounts of biomass prevent negative effects of mass transfer resistance or phase transfer limitation. The method described here enables the rapid and reliable determination of the potential methanogenic activity. The basic difference from methods presented previously [27, 28], is that the biomass is tested with a range of substrates so that the proportions of various metabolic groups can be estimated. Practical advantages are that this method is fast, does not require expensive equipment and is very straightforward. The most serious drawback encountered is that at high specific activities for hydrogen, mass transfer from headspace to liquid may become rate-limiting at relatively low sludge concentrations. Vigorous shaking may impede phase transfer limitation of hydrogen, but frequently gave technical problems. However, since a clear correlation was observed between the methanogenic activities on H_2-CO_2 and on formate, formate can generally be used to test the hydrogenotrophic methanogenic potential of biomass, in spite of the fact that about 50% of the hydrogenotrophic methanogens known to date cannot use formate [29]. The presence of formate-splitting organisms in the tested biomass that did not consume (all of the) formed hydrogen, e.g., *Methanothrix soehngenii* [30], and/or a higher specific methanogenic activity on formate than on hydrogen of formate-utilizing methanogens may explain these observations.

This method may be useful to assess potential targets of toxic or inhibitory compounds to the level of the metabolic groups affected by these compounds. Furthermore, the influence of various environmental factors can be easily evaluated. It must be stressed that this technique provides no information on actual conversion rates in the environment where supply of substrate may be suboptimal.

The main reason for developing this technique was to obtain a fast and reliable method to assess the presence of various metabolic groups of methanogenic bacteria in

methanogenic environments (viz., waste water treatment systems), and to estimate their relative proportions in the total biomass present. The most probable number (MPN) viable counting technique is one of the most commonly used methods to obtain this information. It led, however, to confusing and clearly wrong results with the sludges tested here as was clear from a comparison with microscopic observations (data not shown). Possible explanations for these wrong results are (i) suboptimal culture conditions (especially flaws in composition of the media), (ii) not all metabolically active cells are culturable, e.g., obligate proton-reducing acetogens which can only grow in presence of hydrogenotrophs, and (iii) incomplete dispersion of bacteria naturally occurring in conglomerates (flocs, pellets, granules). In view of possible erratic results and long incubation periods (up to several months) which are needed to obtain growth at high dilutions, it was decided to employ potential activity measurements for estimating the proportions of various metabolic groups in anaerobic biomass.

The most sensitive step in this approach is certainly the choice of the specific activity of the reference population. As shown in Table 1 the specific activities of the various bacteria of one physiological group may vary by a factor of ten, but generally it is a factor of two or three. Assuming mean specific activities of 250 μmol $CH_4 \cdot g^{-1} \cdot min^{-1}$ for hydrogenotrophic methanogens, and of 25 μmol $CH_4 \cdot g^{-1} \cdot min^{-1}$ for acetoclastic methanogens, it can be concluded that a significant portion of the biomass in anaerobic waste water treatment systems consists of methanogenic bacteria. Thus, the fraction of acetoclastic methanogens must be high. In fact, microscopic observations revealed high densities of bacteria showing much resemblance to *Methanothrix soehngenii*, a fat rod with characteristic morphology [30].

The presence in granular sludges of large fractions of *M. soehngenii*-like organisms has been confirmed by our observation that hydrogen did not significantly influence methanogenesis from acetate (Fig. 4 and Table 4), in contrast to observations with *Methanosarcina* [16]. Methanogenesis from acetate by *M. soehngenii* is not affected by the presence of hydrogen in the medium [30]. The discussion as to inhibition by hydrogen of methanogenesis from acetate in *Methanosarcina* seems to be solved by the experiments of Traore et al. [17]. These authors showed that at low dilution rates (and low substrate concentrations) hydrogen and acetate are converted simultaneously to methane. At high growth rates, however, when sufficient hydrogen was available for *Methanosarcina* virtually no methanogenesis from acetate occurred. These results indicate that in *Methanosarcina* methanogenesis from acetate is inhibited under non-hydrogen-limited conditions. Therefore the results of the present experiments as shown in Fig. 4, and Tables 3 and 4 indicate that *M. soehngenii*-like organisms represented the main acetoclastic population in the biomass.

The potential methanogenic activities of the biomass were shown to reflect the composition of the culture medium (Table 3) and consequently the type of waste water on which the organism had been grown. Therefore, the potential methanogenic activities on a range of substrates give information on the extent to which different physiological groups of bacteria, involved in methane production, contribute to the total biomass. These activities may be used to obtain some insight into the relative importance of various intermediates in the flow of carbon during the conversion of complex compounds on which the biomass was cultivated. Obviously, for a clear interpretation of the

substrate conversion activity of biomass on non-direct methanogenic substrates, the conversion to substrates for methanogenic bacteria should limit the rate of methanogenesis. This is true for the results with anaerobic sludges of the present investigation, where the methanogenic activity on propionate was clearly lower than on H_2-CO_2 and acetate (Table 3). The specific activities of non-methanogenic bacteria are assumed to be generally higher than the activities of methanogenic bacteria: e.g., succinate converter 1300 μmol succinate $g^{-1} \cdot min^{-1}$ [31], sludge from an anaerobic acidification reactor 240 μmol glucose $g^{-1} \cdot min^{-1}$ [32]. This would imply that the presence of relatively small numbers of such organisms in methanogenic consortia is sufficient to make methanogenesis the rate-limiting step in reactions catalyzed by such consortia.

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CHAPTER 5

Comparison of Methane Production Rate and Coenzyme F_{420} Content of Methanogenic Consortia in Anaerobic Granular Sludge

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Comparison of Methane Production Rate and Coenzyme F_{420} Content of Methanogenic Consortia in Anaerobic Granular Sludge†

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The coenzyme F_{420} content of granular sludge grown on various substrates and substrate combinations was measured, and the potential of the sludge to form methane (maximum specific methane production rate) from hydrogen, formate, acetate, propionate, and ethanol was determined. The F_{420} content varied between 55 nmol g of volatile suspended solids (VSS)$^{-1}$ for sludge grown on acetate and 796 nmol g of VSS^{-1} for sludge grown on propionate. The best correlation was found between the F_{420} content and the potential activity for methane formation from formate; almost no correlation, however, was found with acetate as the test substrate. The ratio between the potential methanogenic activities (q_{CH_4}) of sludges grown on various substrates and their F_{420} content was in general highest for formate (48.2 μmol of CH_4 μmol of F_{420}^{-1} min^{-1}) and lowest for propionate (6.9 μmol of CH_4 μmol of F_{420}^{-1} min^{-1}) as test substrates. However, acetate-grown granular sludge with acetate as test substrate showed the highest ratio, namely, 229 μmol of CH_4 μmol of F_{420}^{-1} min^{-1}. The data presented indicate that the F_{420} content of methanogenic consortia can be misleading for the assessment of their potential acetoclastic methanogenic activity.

Attempts have long been made to quantify the potential methanogenic activity (q_{CH_4}) of anaerobic sludge by the use of a simple method(s) (19, 21). This potential activity (maximum specific methane production rate) can be measured by determining the rate of methane production of a sludge sample with one of the methanogenic substrates (e.g., hydrogen, acetate, etc.) or any precursor of these substrates (e.g., fatty acids, alcohols, etc.)

The coenzyme F_{420} content of sludge has been suggested recently as a means for the assessment of potential methanogenic activity (3). Coenzyme F_{420}, a 5-deazaflavin analog (4), plays a key metabolic role in methanogenic bacteria as an electron carrier in both anabolic and catabolic redox reactions (10, 17, 18, 24). Different types of F_{420} have been described, all consisting of a 7,8-dimethyl-8-hydroxy-5-deazariboflavin chromophore with an extended side chain composed of lactic and glutamic acid residues (20). Various amounts of F_{420} have been found in all methanogenic bacteria tested (Table 1). With one exception (*Streptomyces griseus* [6]), this coenzyme is only found in methanogens (22). Thus, in anaerobic sludges methanogens are the only organisms containing this cofactor, which in its oxidized form shows a strong absorbancy at 420 nm (1). It therefore was logical to use F_{420} concentrations as a measure of the total amount of methanogens in an anaerobic habitat or as a value to express the potential methanogenic activity of the biomass in the habitat. The utilization of the F_{420} content as a parameter has several advantages, e.g., sampling can be done without maintaining anaerobic conditions, no time-consuming incubation tests have to be carried out, and the analysis of F_{420} is relatively simple. However, there are also some difficulties. The predictive value of F_{420} depends on knowledge of the relationship between the F_{420} content of a biomass and its potential methanogenic activity. A comparison of literature data on specific methanogenic activities of methanogens and on their F_{420} contents (Table 1) suggests that the maximum specific activities of the methanogens, expressed per mole of F_{420}, vary over the different metabolic subgroups. The paucity of data, especially on acetoclastic methanogens, makes it difficult to draw any definite conclusions. This uncertainty is augmented by the fact that it is often impossible to compare the data reported in the literature. These data have often been obtained by different analytical techniques and have been reported in different units that cannot always be converted unambiguously.

In this paper we report the F_{420} contents of a number of granular methanogenic sludges grown on different substrates and the potential methanogenic activities of these sludges on hydrogen, formate, acetate, propionate, and methanol.

MATERIALS AND METHODS

Seed sludge, media, and growth conditions. Granular methanogenic sludge (J. Dolfing et al., submitted for publication) was kindly provided by the CSM sugar refinery at Breda, The Netherlands. It had been cultivated in a 30-m^3 upflow anaerobic sludge blanket reactor treating wastewater from this sugar refinery and occasionally functioning as the second phase of a two-step system for the anaerobic conversion of solid wastes.

The sludges were grown in media with various energy sources. The media were prepared in concentrated stock solutions and diluted just before being fed to the reactors. The diluent was composed such as to supply the following nutrients (in grams per gram of chemical oxygen demand fed to the reactor): $(NH_4)_2HPO_4$, 0.01; urea, 0.02; KCl, 5; and trace element solution, 0.6. The trace element solution contained (in milligrams liter of demineralized water^{-1}): $FeSO_4$, 500; $MnCl_2 \cdot 4H_2O$, 100; $CoCl_2 \cdot 6H_2O$, 170; $ZnCl_2$, 100; $CaCl_2 \cdot 2H_2O$, 20; H_3BO_3, 19; Na_2MoO_4, 10; Na_2SeO_3, 28; and $NiCl_2 \cdot 6H_2O$, 50. $NaHCO_3$ was added in various amounts so as to keep the pH in the reactor at 7.0 to 7.5. The concentrated stock solutions contained (in milligrams per gram of substrate chemical oxygen demand): $FeSO_4$, 1.2

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TABLE 1. Summary of previously reported data on the levels of F_{420} present in various mesophilic methanogenic bacteria and their potential specific methanogenic activities

Bacterium	F_{420}[a,b] (μmol g^{-1})	Sp act[b] (μmol of CH_4 g [dry wt] of $cells^{-1}$ min^{-1})	$q_{CH_4(F_{420})}$ (μmol of CH_4 μmol of F_{420}^{-1} min^{-1})
Hydrogen-utilizing methanogens			
Methanobacterium formicicum	1.2[c] (5)	350–550 (14)	290–460
Methanobacterium bryantii	1.4[c]–2.4[c] (5)	300 (13)	125–214
Methanobrevibacter ruminantium	0.04[c] (5)	—[d]	—
Methanobrevibacter arboriphilus	1.8[c] (5)	600 (13)	335
Methanogenium marisnigri	0.7[c] (5)	—	—
Methanospirillum hungatei	1.9[c] (5)	260 (13)	135
Formate-utilizing methanogen			
Methanobacterium formicicum	3.0–3.6[e] (2)	8,000–14,000 (2)	40–80
Acetate-utilizing methanogens			
Methanosarcina acetivorans	0.027[e] (16)	200 (16)	7,400
Methanothrix soehngenii	0.33[e] (23)	25 (23)	75

[a] These data have been normalized (if necessary) to micromoles gram (dry weight) of $cells^{-1}$, assuming an F_{420} molecular weight of 840, a protein content of 0.5 g g (dry weight)$^{-1}$, and a dry weight/wet weight ratio of 0.2.
[b] Numbers in parentheses are reference numbers.
[c] Originally reported in milligrams of F_{420} gram (wet weight) of $cells^{-1}$.
[d] —, Not reported.
[e] Originally reported in micrograms of F_{420} milligram of $protein^{-1}$.

(except in the reactor fed with ethanol) and yeast extract, 6.1. The energy sources were (in grams $liter^{-1}$): acetic acid, 116.0, plus sodium acetate · $3H_2O$, 34.8; propionic acid, 77.0, plus sodium propionate, 40.2; ethanol, 69.8; acetic acid, 34.0, plus sodium acetate · $3H_2O$, 80.6, plus sodium propionate, 56.0; or acetic acid, 12.4, plus sodium acetate · $3H_2O$, 20.2, plus ethanol, 58.6.

After the seed sludge was washed with anaerobic buffer solution (containing [in grams $liter^{-1}$] $NaHCO_3$, 10; KH_2PO_4, 0.3; and K_2HPO_4, 0.4), five 1-liter upflow anaerobic sludge blanket reactors (Dolfing et al., submitted) were each inoculated with 16.8 g (dry weight) of sludge. Substrate was continuously supplied from concentrated stock solutions in such quantities that the concentrations of volatile fatty acids (mainly acetate and propionate) in the effluents varied between 1 and 5 mM. The concentrated stock solutions were pumped into the reactors with a flow rate of 10 to 100 ml day^{-1}. The flow rate of the diluent (2 to 8 g of $NaHCO_3$ of tap $water^{-1}$) varied between 1.5 and 3 liters day^{-1}. This resulted in influent chemical oxygen demands of 2 to 15 g $liter^{-1}$.

Analytic procedures. Methane was measured with a gas chromatograph (model 406, Becker) equipped with a molecular sieve (60-80 mesh) column connected to a thermal conductivity detector. The column temperature was isothermic at 50°C, the carrier gas was argon, and the flow rate was 20 ml min^{-1}.

Determination of F_{420}. Two milliliters of homogenized sludge (homogenization with a Potter tube) was diluted five times with 8 ml of glycine buffer (10 mM glycine plus 5 mM Mg-EDTA, pH 7.0), boiled for 14 min, and immediately cooled on ice to room temperature. After centrifugation for 10 min at 10,000 × *g*, 5 ml of supernatant was vigorously mixed with 10 ml of isopropanol and centrifuged again for 10 min at 10,000 × *g*. After adjustment of the pH to 8.8 with a minimum amount of concentrated KOH, the amount of F_{420} was determined from excitation spectra by comparing the peak surface area from 430 to 460 nm with that of a standard series of pure F_{420} (in 67% isopropanol at pH 8.8). F_{420} was a gift from G. Vogels, Nijmegen, The Netherlands. Spectra were run on an Aminco SPF-500 ratio spectrofluorimeter equipped with an HP 9815A calculator and an HP 7225A graphic plotter. The accuracy with this method was ±10%. This method has been described in detail by W. de Zeeuw (Ph.D. thesis, Agricultural University, Wageningen, The Netherlands, 1984).

Determination of potential methanogenic activities. Serum vials of 130-ml capacity containing an atmosphere of carbon dioxide were supplied with 1 to 5 ml of sludge, 40 ml of an anaerobic buffer solution, and 30 mM test substrate. When hydrogen was the test substrate, 80 ml of the gas was supplied. The anaerobic buffer (pH 7.0) was composed of (in grams liter of demineralized $water^{-1}$): $NaHCO_3$, 10; NH_4Cl, 0.5; KH_2PO_4, 0.3; and K_2HPO_4, 0.4. Incubation took place with vigorous shaking. Gas samples for measuring methane were taken from the serum vials with a pressure lock syringe. The dry weight of the samples was determined at the end of the experiments. The sludge was centrifuged at 10,000 × *g* for 15 min, and the pellets were dried overnight at 105°C. Ash-free dry weights were obtained by keeping the pellets at 600°C until the weight was constant.

RESULTS

Cultivation of granular methanogenic sludge on various substrates resulted in a profound change in the bacterial composition of the granules (data not shown). This transformation was paralleled by a change in the potential methanogenic activities for the different substrates. After 3 to 4 months of cultivation, the potential methanogenic activities for the biomass reflected the composition of the growth substrates (rather than that of the seed culture) (Table 2). The granules cultivated on acetate showed a significant increase in their potential for methanogenesis from acetate compared with the seed culture. On the other substrates tested, the potential methanogenic activities were significantly lower than those of the seed sludge. Microscopic observations indicated that the newly formed biomass consisted almost completely of an organism that showed much resemblance to *Methanothrix soehngenii*, an obligately ace-

TABLE 2. Potential methanogenic activities and coenzyme F_{420} contents of anaerobic biomass cultivated on various substrates[a]

Growth substrate	F_{420} content (μmol g of VSS^{-1})	Potential methanogenic activity (μmol of CH_4 g of VSS^{-1} min^{-1}) on test substrate[b]:				
		H_2	Formate	Acetate	Propionate	Ethanol
Sugar wastewater	0.152	8.5 (3.1)	11.0 (0.9)	4.1 (0.2)	2.8 (0.1)	7.1 (0.7)
Acetate	0.055	2.0 (0.5)	2.0 (0.5)	12.6 (4.5)	0.06	1.9 (0.1)
Propionate	0.796	12.0 (2.7)	28.2	6.0 (0.9)	5.6 (0.6)	2.4 (0.4)
Ethanol	0.658	39.4 (12.0)	34.8 (4.5)	8.1 (0.9)	0.04	14.9 (1.2)
Acetate + propionate	0.440	9.8 (7.0)	23.6 (5.2)	11.5 (1.5)	3.7 (0.7)	4.9 (0.9)
Acetate + ethanol	0.583	25.6 (7.2)	19.2 (2.2)	4.6 (1.3)	0.02	25.2 (1.8)

[a] Inoculation was carried out with methanogenic biomass grown on wastewater from a sugar factory. The values listed were obtained after 3 to 4 months of cultivation. The characteristics of the inoculum are the values for sugar wastewater as the growth substrate.
[b] Numbers in parentheses are the standard deviation of three rate measurements.

toclastic methanogenic "fat rod" with flat ends (8, 23). Significant methanogenic activities with propionate were only observed with granules that had been cultivated in the presence of propionate (Table 2). Similar results were obtained with ethanol. Interestingly, all granular sludges except the acetate-grown granules showed considerable potential methanogenic activities on formate as the test substrate. These activities were generally the highest observed in this investigation.

A correlation between the F_{420} content and the potential methanogenic activity of the granules was only found with formate as the test substrate (Fig. 1). With all other compounds, no clear-cut relationship could be found. The slope in Fig. 1 gives a potential methanogenic activity on formate of 40 μmol of CH_4 μmol of F_{420}^{-1} min^{-1}. The individual values varied between 32.9 and 62.3, with an average of 48.2 μmol of CH_4 μmol of F_{420}^{-1} min^{-1}. The range of the potential methanogenic activities, in relation to the F_{420} content of the granular sludges, was as follows for the other test substrates (in micromoles of CH_4 micromole of F_{420}^{-1} $minute^{-1}$): hydrogen, 15.1 to 59.9 (average, 37.4); propionate, 0.1 to 18.3 (average, 6.9); and ethanol, 3.0 to 46.4 (average, 19.2). The activity on acetate as a test substrate ranged in general from 7.5 to 26.8, with an average of 16.9. The sludge cultivated on acetate as sole energy and carbon source represented an exception; its ratio was 229. These acetate-grown granules still contained some hydrogen- and formate-oxidizing activities (Table 2) which probably were responsible for the F_{420} content. Without these activities, the ratio might even have been considerably higher.

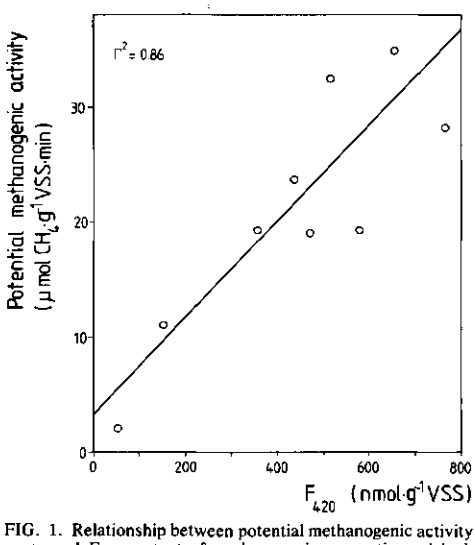

FIG. 1. Relationship between potential methanogenic activity on formate and F_{420} content of methanogenic consortia enriched on various substrates (see Table 2).

DISCUSSION

If the potential methanogenic activity of a biomass was proportional to the amount of F_{420} present, then this activity could be derived from the amount of F_{420} in the biomass. However, the results presented here show that it is generally not possible to estimate the potential methanogenic activity of a biomass in this way. A good correlation between F_{420} content and potential methanogenic activity was found only for methanogenic activities with formate as the substrate. For the other direct methanogenic substrates, H_2-CO_2 and acetate, no correlation between F_{420} content and methanogenic activity was observed (Table 2). This limited predictive value of the F_{420} content can be explained by the relatively large amount of F_{420} required for the transduction of electrons from formate in formate-utilizing methanogens (14), which is reflected in the fact that the $q_{CH_4(F_{420})}$ of formate-utilizing methanogens is lower than that of hydrogen- or acetate-utilizing methanogens (Table 1). Therefore, a significant methanogenic activity of the sludge on formate is accompanied by a relatively large amount of F_{420} that will obscure the relationship between F_{420} content and potential methanogenic activity of biomass on acetate, especially as the $q_{CH_4(F_{420})}$ of acetoclastic methanogens is relatively high and the potential methanogenic activity of the biomass is generally lower on acetate than on formate.

The results presented here on the F_{420} content of granules which had been cultivated on acetate, and where the newly formed biomass consisted almost completely of *M. soehngenii*-like organisms, are in agreement with values previously reported (de Zeeuw, thesis) of 20 to 90 nmol of F_{420} g of volatile suspended solids $(VSS)^{-1}$ for acetate-grown sludges. These values are, however, considerably lower than the value of 55 μg of F_{420} mg of $protein^{-1}$ (i.e., about 330 nmol of F_{420} g [dry weight]$^{-1}$ *A*) reported for *M. soehngenii* (23). The latter value was determined with a bioassay based on that of Eirich et al. (5). Apart from differences in the analytical techniques used, the difference between the F_{420} content of acetoclastic biomass reported here and that of *M. soehngenii* may have been caused by variations due to strain differences and the influence of growth conditions or

growth rates of the microorganisms. Pause and Switzenbaum (12) found for mixed cultures of acetotrophic methanogens that an increase of the biomass retention time from 12 to 16 days resulted in a decrease of the F_{420} content from 4.4 to 1.3 μmol of F_{420} g of VSS^{-1} and an increase of the $q_{CH_4(F_{420})}$ from 8 to 18 μmol of CH_4 μmol of F_{420}^{-1} min^{-1}. However, these findings may have been influenced by population shifts in the reactor. The only data available for pure cultures, viz., for *Methanobacterium formicicum*, show that the F_{420} content of this organism when grown on formate was hardly influenced by its growth rate (2). The specific methanogenic activity of *M. formicicum* decreased with decreasing dilution rate (as determined under growth-supporting conditions) (2), and as a result the $q_{CH_4(F_{420})}$ of *M. formicicum* on formate varied between 40 and 80 μmol of CH_4 μmol of F_{420}^{-1} min^{-1} (calculated from data in reference 2). From the data of Zehnder et al. (23), a $q_{CH_4(F_{420})}$ of 75 μmol of CH_4 μmol of F_{420}^{-1} min^{-1} can be calculated. This is lower than our value of 229 μmol of CH_4 μmol^{-1} for acetoclastic biomass, which is probably still an underestimation as part of the F_{420} in the acetate-grown granules can be attributed to the presence of formate-utilizing methanogens originating from the seed sludge. The high F_{420} contents of the sludges analyzed here and the positive correlation between the F_{420} content of these sludges and their potential methanogenic activities on formate indicate that a significant fraction of these sludges consisted of formate-utilizing methanogens.

The parameter $q_{CH_4(F_{420})}$ was originally introduced to determine the potential methanogenic activity of mixed microbial communities of anaerobic digesters (3). This parameter, however, cannot be used for accurate estimation of the potential methanogenic activity of biomass on acetate. In many ecosystems and anaerobic wastewater treatment systems most methane is formed from acetate (9, 11, 15). Therefore, the data presented here strongly suggest that in general it is still more appropriate to measure directly the potential methanogenic activity of a biomass, preferably on well-defined simple substrates, than to use an indirect method like determination of the concentration of coenzyme F_{420}. The quantitative application of the F_{420} content as a parameter to evaluate changes in the performance of an anaerobic digestion system is only useful if operation conditions and wastewater are well defined and $q_{CH_4(F_{420})}$ has been determined under optimal circumstances.

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CHAPTER 6

Kinetics of Methane Formation by Granular Sludge at Low Substrate Concentrations

The influence of mass transfer limitation

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Kinetics of methane formation by granular sludge at low substrate concentrations

The influence of mass transfer limitation

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Summary. Apparent K_m values for methanogenesis from volatile fatty acids by various sludge granules were determined from progress curve experiments. The influence of mass transfer resistance on the kinetics of methane formation in biofilms was demonstrated with the apparent K_m values thus obtained. It was shown that the effects of mass transfer resistance depend on the maximum specific activity of the biolayers and on their thickness. The data indicate that mass transfer resistance in methanogenic biolayers becomes only of significance at low substrate concentrations and in thick biolayers with high methanogenic activities.

Introduction

Highly efficient bioreactors have been developed recently for the anaerobic treatment of waste water (McCarty 1982; van den Berg and Kennedy 1983). In these systems the solids retention time is separated from the hydraulic retention time, i.e., the active bacterial biomass is retained in the reactor independently of the flow velocity of the waste water. The retention of the biomass can be obtained along different lines, e.g., by immobilizing the microorganisms on filter material or by selecting for well settling aggregates. In all these cases densely packed biolayers are formed. In thick biolayers the substrate might not reach deeper zones because of mass transfer resistance. This effect can be of great significance to the overall reaction rate in a reactor system. The concentration gradient in a biolayer can be estimated from Fick's first law:

$$F = -\varphi D \cdot dC/dx,$$

where F is the flux, φ is the porosity of the biolayer, D is the diffusion coefficient, and dC/dx is the gradient of the substrate concentration in the biolayer. Fick's law predicts that a substrate flux through a biolayer is accompanied by a concentration gradient. The magnitude of the gradient increases with increasing fluxes. The flux depends on the specific activity and on the dimensions of the biolayer. Based on the concentration gradient, the bacteria inside the biolayer are exposed to lower substrate concentrations as compared to the bacteria at the surface. Therefore they will show a lower activity. The relationship between activity and substrate concentration generally follows Monod-kinetics. The Monod-equation predicts that the velocity of an enzymatically catalyzed reaction depends linearly on the amount of substrate present at concentrations around the K_m value and below. Therefore mass transfer resistance becomes of significance only if the bulk substrate concentration is around or below the K_m value or if the concentration gradient in the biolayer reaches this range. This implies that the effects of mass transfer resistance depend on (i) the bulk substrate concentration, (ii) the K_m value of the bacteria for the substrate, (iii) the thickness of the biolayer, and (iv) the maximum specific activity of the biolayer. The latter factor determines the flux and thereby influences the concentration gradient in the biolayer.

Granular methanogenic sludge, grown in an upflow anaerobic sludge blanket (UASB) reactor (Lettinga et al. 1980) forms a nice model system to

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investigate the effect of mass transfer resistance on the methanogenic activity of densely packed biolayers at low substrate concentrations. Ngian et al. (1977) have demonstrated that mass transfer resistance can significantly increase apparent K_m values for organisms that occur in aggregates or biofilms. Therefore, apparent K_m values for various sludge granules were determined. This parameter was used to test the theoretical prediction of Henze and Harremoës (1983) that mass transfer resistance in methanogenic biofilms is not significant at a layer thickness below 1 mm.

Materials and methods

Source of granular sludge. Granular sludge was obtained from the CSM-sugar factory at Breda, The Netherlands. It was grown in an UASB reactor with a volume of 30 m^3 treating liquid sugar waste which contains a considerable amount of dissolved sugar (Pette and Versprille 1982). A part of the granular sludge was also cultivated in the laboratory in an UASB reactor with a volume of one liter on a medium containing either ethanol or propionate as the only carbon and energy source. Granular sludge from the CSM-sugar factory served as seed material. After three months these laboratory cultures were harvested and the granules were analyzed for their specific methanogenic activities.

Buffer solution. It had the following composition (in $g \cdot l^{-1}$): $NaHCO_3$, 10; NH_4Cl, 0.5; K_2HPO_4, 0.4; KH_2PO_4, 0.3. The buffer was prepared anaerobically under carbon dioxide (final pH = 7.0).

Disintegration of granular sludge. Granular sludge was disintegrated with a cream maker (Royston BEL Ltd, England) under strictly anaerobic conditions using O_2-free carbon dioxide.

Acitivity tests. CSM-sludge granules, intact or disintegrated, were transferred under strictly anaerobic conditions to preflushed serum vials with a volume of 130 ml or 500 ml (experiments with hydrogen) containing 50 ml of buffer solution. Acetate and propionate were added from concentrated stock solutions to reach final concentrations of 20 mM. Hydrogen was added to the CO_2-headspace to obtain hydrogen partial pressures of 1 atmosphere unless indicated otherwise. Incubations were in a water bath at 30° C under vigorous shaking.

Progress curve experiments. Ethanol or propionate-grown granules were transferred under strictly anaerobic conditions to preflushed screw cap tubes (Bellco Glass) of 20 ml capacitiy containing 7 ml of buffer solution. The granules were distributed over the tubes in such a way that each tube contained 1 to 5 granules of the same size. These granules were washed twice by adding fresh buffer solution after settling of the granules and removal of the old buffer. Washed granules were incubated under saturating substrate concentrations (20 mM) in 7 ml of buffer solution. When the methanogenic activity was maximal, part of the buffer (plus substrate) was withdrawn from the opened tube under continuous gassing with O_2-free carbon dioxide. The tubes were closed again and further incubated. This withdrawal of substrate was done to accelerate the exhaustion of the medium so that the final methane production was reached earlier and at a lower methane concentration thus diminishing the absolute error in the determination of the methane production as much as possible. This enlarges the accuracy of the estimation of the progress curve and consequently that of the apparent K_m value.

All incubations were performed in a water bath at 30° C under vigorous shaking.

Analyticial procedures. Gas samples were withdrawn with a pressure-lock syringe and analyzed for methane and hydrogen with a Packard-Becker 406 gas chromatograph equipped with a molecular sieve column (60—80 mesh) and a thermal conductivity detector. The column was operated at 50° C. The carrier gas was argon at a flow rate of 20 $ml \cdot min^{-1}$.

After the activity tests had been performed, the tubes were weighed. The volume of the gas phase was calculated from the difference in weight of the tubes as incubated and as filled completely with water. The volume of the liquid phase was calculated from the difference in weight of the tubes as incubated and as emptied and dried. The size of the granules was measured by using a binocular microscope. The dry weight was determined after drying the granules overnight at 105° C. Ash content was determined after drying the granules to constant weight at 600° C. The difference between dry weight and ash content was defined as volatile suspended solids (vss).

Results

Activity tests

Activity tests were performed with acetate, propionate, and hydrogen. Table 1 summarizes the

Table 1. Comparison of the potential methanogenic activities of granular sludge[a] and disintegrated[b] granular sludge supplied with different substrates

Biomass	Potential methanogenic activity ($\mu moles\ CH_4 \cdot g^{-1}\ vss \cdot min^{-1}$)		
	Acetate	Propionate	H_2
Intact granular sludge	6.3	2.5	1.4
Disintegrated granular sludge	6.8	2.2	4.5

[a] Cultivated in an upflow anaerobic sludge blanket reactor with a volume of 30 m^3 on waste water of a sugar refinery. The mean diameter of the granules was 1.5—2.0 mm

[b] Disintegrated by treating the sludge in a cream maker under continuous gassing with CO_2

specific potential methanogenic activities of intact and disintegrated sludge granules from the sugar refinery. The data show that the methanogenic activities with acetate and propionate were not influenced by the size of the granules. However, methanogenesis from hydrogen was clearly enhanced after disintegration of the granules. The methanogenic activity of granular sludge on H_2-CO_2 revealed a greatly reduced specific activity of intact granules as compared to disintegrated granules at partial pressures of hydrogen (p_{H2}) below 1 atm. Increased partial pressures of hydrogen enhanced the methanogenic activities of the granules; however, the activities of the disintegrated granules remained relatively unaffected (data not shown). The Lineweaver-Burk plot of the inverse of the specific methane production rate ($1/q_{CH4}$) versus the inverse of the hydrogen partial pressure ($1/p_{H2}$) gave a straight line for both intact and disintegrated sludge granules (Fig. 1). The extrapolation of the line representing the data for intact granules yields a negative intercept with the ordinate. This indicates that hydrogen transfer is limited in this kind of granules at low partial pressures of hydrogen.

Progress curve experiments

The maximum specific methane production rates of the granules used in this study are listed in Table 2. Formate, acetate, and propionate were stoichiometrically converted to methane and carbon dioxide by washed granules. Hydrogen or acetate have not been detected as free intermediates. Since methane formation is much easier to determine than the disappearance of volatile fatty acids, especially formate, methane production rates were used to calculate substrate depletion rates. The specific methanogenic activities of the sludge granules cultivated on ethanol or propionate (Table 2) differed markedly from the original granules with which the reactors had been seeded (Table 1).

Figure 2 gives a typical progress curve for methane formation from which the apparent K_m val-

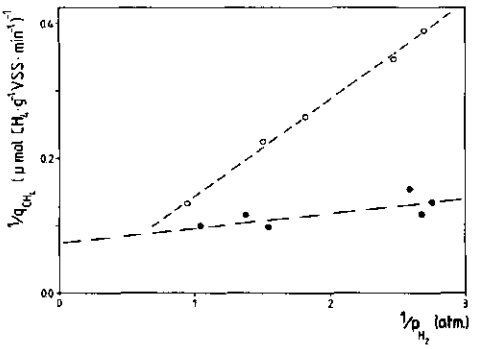

Fig. 1. Effect of the granular structure of methanogenic sludge on the specific hydrogen conversion rate, in a Lineweaver-Burk plot. The experiments were performed with 0.61 g sludge·bottle^{-1}; ○: intact granular sludge; ●: disintegrated granular sludge

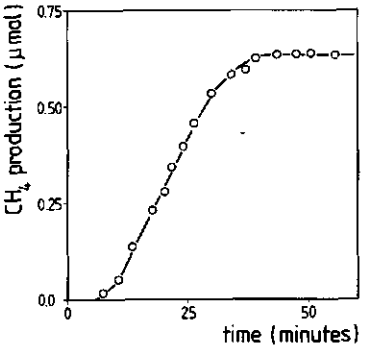

Fig. 2. A typical example of the progress of methane formation by ethanol-grown granular methanogenic sludge incubated with 3 mM formate at 30°C

Table 2. Maximum specific activities of intact granules used for the determination of apparent K_m values

Growth substrate	Test substrate	Specific activity[a] (μmol·g^{-1} vss·min^{-1})
Ethanol	Formate	72—88[b]
Propionate	Acetate	5—19
Propionate	Propionate	6— 7

[a] Specific activities are expressed as micromole substrate converted to methane per gram volatile suspended solids per minute

[b] Range of the specific activities of different granules

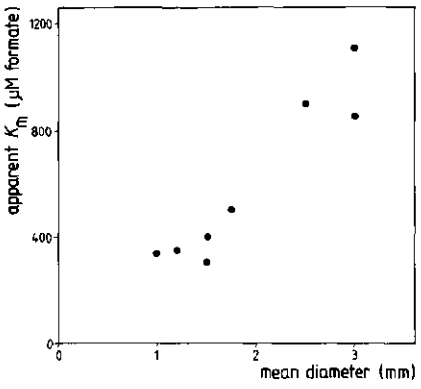

Fig. 3. Comparison of the apparent K_m values for methane formation from formate of granular methanogenic sludge with different granule sizes. The sludge had been grown on ethanol

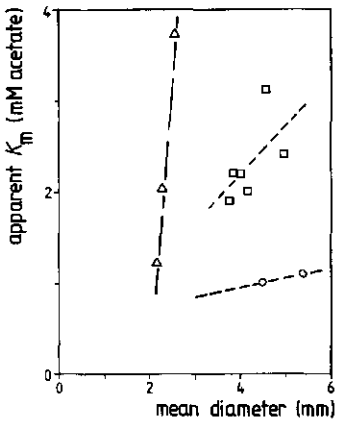

Fig. 4. Effect of granule diameter on the apparent K_m values for acetate conversion at various specific methanogenic activities; V_0 in µmol acetate. g^{-1} vss $\cdot min^{-1}$: ○: 5—7; □: 10—12; △ 15—19

Table 3. Summary of kinetic parameters for propionate conversion by various methanogenic sludge granules[a]

Granule diameter (mm)	Initial conversion potential[b] (µmol propionate $\cdot g^{-1}$ vss $\cdot min^{-1}$)	Apparent K_m (mM)
2.3	6.53	0.88
2.3	7.25	1.07
2.5	6.92	0.82
3.0	7.27	1.36
4.6	6.16	1.33
4.6	6.30	0.90
4.6	6.30	1.36
Mean —	6.7 ± 0.5[c]	1.10 ± 0.24[c]

[a] Cultivated in an upflow anaerobic sludge blanket reactor with propionate as carbon and energy source for 3 months

[b] Calculated from the initial methane production rate at saturating propionate concentrations, assuming Propionate $+1/2\ H_2O \rightarrow 7/4\ CH_4 + 5/4\ CO_2$. Neither hydrogen nor acetate were detected as free intermediates

[c] Mean ± standard deviation

ues were derived by estimating the substrate concentration at which the tangent of the curve equalled half the tangent of the maximum production rate. The K_m values thus obtained for methane formation from formate on ethanol-grown sludge were plotted against the diameter of the granules which were responsible for the methane formation (Fig. 3). The data show a positive correlation between the diameter of the granules and the apparent K_m value for formate. Analogically, propionate-grown granules tested with acetate as substrate yielded a positive correlation between the diameter of the sludge granules and the apparent K_m value for acetate. This correlation was influenced by the maximum specific activity of the granules (Fig. 4). No increase in the apparent K_m value for propionate could be detected with increasing granule diameter of propionate-grown granules (Table 3).

Discussion

The data presented here show that mass transfer resistance becomes only of significance at low substrate concentrations in granules (biolayers) with high methanogenic activities and large diameters. In our experiments with methanogenic

granules from an industrial reactor, no mass transfer limitations for volatile fatty acids could be detected. This can be ascribed to the relatively small size of the granules (mean diameter $\leqslant 2$ mm) and their relatively low specific methanogenic activities for hydrogen, acetate, and propionate (Table 1). Effects of mass transfer resistance could be detected in some cases if methanogenic sludge granules were cultivated on simple substrates, such as ethanol and propionate. This cultivation resulted in the growth of an uniform population with a high specific activity for the methanogenic conversion of the growth substrate. Interestingly, in all this material the apparent K_m value for propionate conversion remained constant, whereas the diameter of the granules influenced clearly the apparent rate constant for acetate conversion. This is not surprising since the specific activity for acetate conversion was significantly higher than for propionate. Mass transfer limitation in the conversion of formate was already detectable at small diameters of the granules, probably because of the high specific activity for this substrate. Published estimates of formate K_m values for methanogens range from 0.22 mM for *Methanospirillum hungatei* to 0.58 mM for *Methanobacterium formicicum* (Schauer et al. 1982). Comparison of the data presented in Fig. 3 with these values suggests that mass transfer limitation hardly influences the apparent K_m values for formate or ethanol-grown granules at diameters below 2 mm. Assuming that the intrinsic apparent K_m value of the populations that convert the substrate can be estimated from our data (Fig. 3, 4; Table 3) this would result in apparent K_m values of 0.15—0.30 mM for formate, 0.5—1.0 mM for acetate and 0.9—1.3 mM for propionate. These values are significantly lower than the half velocity constant of 0.2 kg COD·m^{-3} (3 mM acetate) used by Henze and Harremoës (1983) for their estimation of the significance of mass transfer resistance in anaerobic waste water treatment systems. The fact that our results still corroborate the prediction that mass transfer does not become of significance until the biofilm thickness reaches values above approximately 1 mm may be due to a significant difference between the assumption of Henze and Harremoës (1983) for the density and the conversion capacity of the biofilm and the actual values of the granules used in this study. Our results indicate that mass transfer resistance will not be of much influence in industrial reactors which usually convert more complex substrates, resulting in a relatively low specific activity of the sludge for simple substrates such as hydrogen, formate, acetate and propionate. The most profound effects in this study were found for hydrogen and formate, substrates that are hardly ever fed as such to anaerobic reactors, but are intermediates that are produced and consumed during the biomethanation process, probably by adjacent bacteria in the same granule.

The fact that we have been able to demonstrate a size-apparent K_m value relationship for formate and acetate is an indication that a significant portion of the bacteria inside the granules is metabolically active, evenso they seem to be extremely substrate-limited.

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CHAPTER 7

Summary

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Samenvatting

Summary

The settling characteristics of anaerobic sludge are enhanced by the formation of microbial conglomerates. Various types of conglomerates having different structures, were distinguished in the present study, viz. granules, pellets and flocs (chapter 1). Granular methanogenic sludge, often developing in upflow anaerobic sludge blanket reactors, is optimal for an economic application of the system. One of the hypotheses to explain the formation of these granules is that bacteria would excrete extracellular polymers, responsible for the formation of stable, well settling aggregates. Scanning electron micrographs showed that indeed extracellular material can be detected in granular methanogenic sludge grown on waste water of a liquid sugar plant (chapter 3). Various methods were used to quantify the fraction of this material and to elucidate its chemical composition: 1 - 2% of the dry weight of the granules appeared to consist of extracellular sugars. The relative proportions of these sugars varied somewhat with the extraction methods used and the batch of granules analysed.

Granular methanogenic sludge grown on waste water of a sugar refinery consisted of a diverse microflora composed of various types of bacteria (chapter 3). As these granules had been formed on a complex waste water the growth of granular methanogenic sludge was studied under better defined conditions with respect to substrate supply. In chapter 2 it was shown that growth of methanogenic sludge granules is possible in bench scale UASB reactors with a volume of one liter. The first series of experiments was carried out with waste water of the sugar factory on which granular sludge had been found to develop in a full scale UASB reactor. Development and growth of granular methanogenic sludge was possible when a UASB reactor with a volume of one liter had been seeded with disintegrated granular

sludge. However, when the reactor had been seeded with digested sewage sludge more fluffy pellets developed. Subsequent experiments showed that various compounds could be omitted from artificially prepared waste waters that originally reflected the composition of the waste water of the sugar refinery. Cultivation of methanogenic sludge granules proved possible on ethanol or propionate as most simple substrates. Feeding granular methanogenic sludge for prolonged periods with acetate as sole energy source resulted in weaker, more fluffy pellets.

Incubation of granular methanogenic sludge in batch culture under growth-supporting conditions generally resulted in the overgrowth of the original seed granules by dispersed growing bacteria and development of fluffy pellets. Cultivation of granular methanogenic sludge in batch culture was only possible in experiments where propionate was stoichiometrically converted to methane and acetate (and probably carbon dioxide).

By studying the potential methanogenic activities of granular methanogenic sludges (chapter 4) it was shown that the biomass consisted for a significant part of acetoclastic methanogens. These potential specific activities showed that obligately acetoclastic methanogens were quantitatively more important than hydrogenotrophic methanogens. These conclusions were drawn from a comparison between the potential methanogenic activities measured in the present experiments, and literature data on the potential activities of various groups of anaerobic bacteria. This approach provided a reliable tool for the estimation of the microbial composition of methanogenic consortia and was faster and more accurate than MPN counts. The resulting estimates of the acetoclastic biomass with respect to the occurrence of obligately and facultatively acetoclastic methanogens were corroborated by microscopic observations and indicated that Methanothrix soehngenii-like organisms are the most numerous acetoclastic methanogens in granular sludge.

An indirect method for the determination of the potential methanogenic activity of anaerobic biomass as suggested by others, viz. the estimation of the amount of F_{420} present in methanogenic consortia was tested with granular sludge in the present study. It was shown (chapter 5) that a good correlation existed between the F_{420} content of different granular consortia and their potential methanogenic activity on formate. No correlation, however, was observed between the F_{420} content and the potential acetoclastic methanogenic activity of such consortia. This indicates that F_{420} content is not a reliable parameter for assessing the overall potential methanogenic activity of anaerobic biomass.

In densely packed biolayers mass transfer limitation will influence the conversion capacity of the biomass at low substrate concentrations. The results reported in chapter 6 show that the effect of mass transfer limitation in granular methanogenic sludge is dependent on the composition of the biolayer, viz. its potential specific activity, the thickness, and on the type of substrate. To detect significant effects methanogenic granules had to be cultivated on specific substrates which resulted in the development of uniform biolayers. Even with this material mass transfer resistance was of no significance unless biolayer thickness reached values above 1 mm. These results indicate that mass transfer resistance will generally not be effective in industrial reactors. They furthermore show that not only the outer parts, but also the inner parts of the granules consisted of actively catabolizing bacteria.

Samenvatting

Bij de anaërobe zuivering van afvalwater speelt de vorming van methaan door methanogene bacteriën een belangrijke rol. Deze organismen, die vaak ten nauwste samenleven met andere bacteriën die nodig zijn voor de afbraak van afvalstoffen, dienen binnen de reactor te worden gehouden om het continu toegevoerde afwalwater te zuiveren. Bij het zgn. UASB proces, waarbij het afvalwater de reactor van beneden naar boven doorstroomt, zijn de bacteriën aanwezig in conglomeraten die zich als een soort slib-bed onder in de reactor bevinden. Hoe beter deze conglomeraten bezinken hoe minder zij de kans lopen met het gezuiverde water te worden afgevoerd zoals dat met slecht bezinkende afzonderlijk levende bacteriën het geval is. Vorming van goed bezinkbare conglomeraten van bacteriën is dus een eerste eis bij het goed functioneren van een UASB reactor en dus voor de economische toepasbaarheid van dit anaërobe zuiveringssysteem. Afhangende van de structuur van deze bacterieconglomeraten worden onderscheiden: korrels, pellets en vlokken (hoofdstuk 1). Korrels zijn meer afgerond dan pellets en hebben een vastere structuur waardoor ze meer bestand zijn tegen mechanische invloeden. Vlokken zijn minder afgerond dan pellets en hebben een lossere structuur.

Het in dit proefschrift beschreven onderzoek was vooral gericht op de microbiologische aspecten van methanogeen korrelslib. Een van de mogelijke verklaringen voor de wijze van korrelvorming betreft de eventuele uitscheiding door bepaalde bacteriën van verbindingen die verantwoordelijk zijn voor het ontstaan van stabiele goed bezinkende conglomeraten. Extracellulair materiaal is inderdaad zichtbaar in opnamen gemaakt met een rasterelectrononmicroscoop van methanogeen korrelslib gegroeid op afvalwater van een suikerfabriek. Verschillende methoden werden toegepast om de grootte en de chemische samenstelling van deze slibfractie te bepalen; het bleek dat 1

ã 2% van het drooggewicht van de korrels uit extracellulair materiaal bestond. De onderlinge verhoudingen van deze suikers varieerden afhankelijk van de toegepaste extractiemethode en van het tijdstip waarop het slib uit de reactor verkregen was.

Methanogeen korrelslib gegroeid op afvalwater van de bovengenoemde suikerfabriek bestond uit een gevarieerde bacterieflora (hoofdstuk 3). Omdat deze korrels zich ontwikkeld hadden in afvalwater dat verschillende componenten bevatte, werd de groei van methanogeen korrelslib in het laboratorium bestudeerd onder beter gedefiniëerde omstandigheden, waarbij vooral aandacht werd besteed aan het energiesubstraat. Kweken van methanogeen korrelslib bleek mogelijk in UASB reactoren van 1 liter (hoofdstuk 2), geënt met fijngemaakt korrelslib en gevoed met afvalwater van de suikerfabriek waarop korrelslib was ontstaan in een UASB reactor op praktijkschaal. Werd een dergelijke laboratoriumreactor geënt met slijkgistingsslib dan ontwikkelden zich slappere pellets. Verder onderzoek leerde dat verscheidene componenten van het complexe voedingsmedium konden worden weggelaten zonder dat de groei van methanogeen korrelslib nadelig werd beïnvloed. Ethanol of propionzuur bleken de meest eenvoudige substraten om dit doel te bereiken. Voortgezette voeding met azijnzuur als energiebron leidde tot het ontstaan van minder stevige methanogene pellets.

Incubatie van methanogeen korrelslib in niet continu doorstroomde reactoren, namelijk in batch cultures, onder omstandigheden waarbij groei mogelijk was leidde in het algemeen tot de uitgroei van de oorspronkelijke korrels tot slappe pellets en dispers groeiende bacteriën. Het kweken van methanogeen korrelslib in batch culture was alleen mogelijk bij voeding met propionzuur dat stoichiometrisch werd omgezet in methaan en azijnzuur (en waarschijnlijk koolzuur).

Door meting van de potentiële activiteit van korrelslib voorzien van verschillende specifieke testsubstraten werd aangetoond dat de biomassa

voor een belangrijk deel bestond uit azijnzuursplitsende methanogene bacteriën (hoofdstuk 4). Azijnzuursplitsende methanogene bacteriën kwamen in grotere aantallen voor dan waterstofgebruikende methanogenen. Dit werd geconcludeerd door vergelijking van de potentiële methanogene activiteiten gemeten in dit onderzoek met gegevens uit de literatuur betreffende de potentiële activiteiten van verschillende groepen anaërobe bacteriën. Deze aanpak maakte een betrouwbare schatting mogelijk van de microbiële samenstelling van methanogene consortie en was sneller en nauwkeuriger dan tellingen volgens de MPN methode. De met behulp van eerstgenoemde methode gemaakte schattingen van obligaat- en facultatief azijnzuursplitsende methanogene bacteriën werden bevestigd door microscopische waarnemingen: bacteriën van het type Methanothrix soehngenii zijn de meest voorkomende azijnzuursplitsende methanogenen in het hier onderzochte korrelslib.

In de literatuur is voorgesteld om de potentiële methanogene activiteit van anaërobe biomassa te schatten door bepaling van de hoeveelheid F_{420}, een coenzym specifiek aanwezig in methanogene bacteriën. Deze indirecte methode is getest in hoofdstuk 5. Een goede correlatie bleek te bestaan tussen het F_{420}-gehalte van verschillende soorten korrelslib en de potentiële methanogene activiteit met mierezuur als testsubstraat. Geen verband werd echter gevonden tussen het F_{420}-gehalte van deze consortia en hun potentiële methanogene activiteit met azijnzuur. Dit betekent dat het F_{420}-gehalte geen betrouwbare maatstaf is voor de "overall" potentiële methanogene activiteit van een anaërobe biomassa.

Bij lage substraatconcentraties wordt de omzettingscapaciteit van de biomassa in volgepakte biolagen beïnvloed door diffusie van de beschikbare substraten. Het effect van diffusielimitatie in korrelslib hangt af van de samenstelling van de biolaag, namelijk van de potentiële specifieke activiteit en de dikte van deze laag, en van het type substraat (hoofdstuk 6). Alleen met korrelslib gekweekt op eenvoudige substraten, en dus bestaande

uit een uniforme populatie, kon enig effect van diffusielimitatie worden vastgesteld; deze effecten waren pas waarneembaar bij biolagen dikker dan 1 mm. Deze resultaten geven aan dat diffusielimitatie in het algemeen niet van belang zal zijn in industriële UASB reactoren. Bovendien wijzen ze erop dat niet alleen de bacteriën aan de buitenkant van de korrel maar ook die in de korrel metabolisch actief zullen zijn.

Curriculum vitae

De auteur is geboren op 29 augustus 1955 te Middelburg.

In 1973 behaalde hij het diploma gymnasium aan het Eerste Christelijk Lyceum te Haarlem en begon met de studie in de richting Milieuhygiëne aan de Landbouwhogeschool te Wageningen. Praktijkervaring werd opgedaan bij de Deltadienst van Rijkswaterstaat te Middelburg, het Technion in Haifa, Israel, en de Eidgenössische Anstalt für Wasserversorgung und Abwasser-reinigung in Dübendorf, Zwitserland. Het doctoraalexamen, afgelegd in maart 1980, omvatte de vakken waterzuivering en microbiologie. Van juni 1980 tot juni 1984 is het onderzoek voor dit proefschrift verricht bij de vakgroep microbiologie van de Landbouwhogeschool, waaraan hij was verbonden als promotie-assistent.

Van september 1984 tot mei 1987 was de auteur verbonden aan de vakgroep microbiologie van Michigan State University in East Lansing, USA, waar hij als research associate onderzoek verrichtte met betrekking tot de reductieve dechlorering van gechloreerde aromatische verbindingen.