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Volume I

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ABSTRACT

TECHNOLOGICAL AND MICROBIOLOGICAL ASPECTS OF ANAEROBIC DIGESTION GRANULES

By

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The process of anaerobic granule formation was characterized in three laboratory-scale upflow anaerobic sludge blanket (UASB) reactors (35 °C) fed glucose molasses solution or brewery wastewater, using both digested sewage sludge and aerobic activated sludge as inocula. The granulation process in this study was basically the same as that reported using volatile fatty acid (VFA) mixtures. An important parameter affecting the granulation process was specific COD loading rate.

Two types of granules developed on VFA mixtures containing low sulfate concentrations (<0.15 mM) were compared systematically as to their microbial population, operational performance, granular structure, and kinetic parameters. The R-granules which had rod-type *Methanothrix* species as predominant acetate-utilizing methanogens showed higher granule density and better operational performance than the F-granules which contained filamentous *Methanothrix* species as predominant species.

Granules from an industrial UASB reactor treating brewery wastewater containing a moderate sulfate concentrations (0.6 to 1.3 mM) were characterized in terms of the role of sulfate reducing bacteria (SRB) in substrate metabolism and

granule structure. Syntrophic propionate and ethanol degradation was performed by SRB.

Five methanogenic strains, two propionate-degrading strains, and two butyrate-degrading strains were isolated from the R-granules and identified as prevalent organisms. VFA degradation was characterized by comparing these isolated species versus the granules in terms of the role of different species in VFA metabolism, including the synthesis of valerate and 2-methylbutyrate, isomerization between isobutyrate and butyrate, synthesis of formate from H_2 plus HCO_3^-, and interspecies electron transfer.

Methanobacterium formicicum T1N was a predominant species in the R-granules and played an important role in granule formation and it formed aggregates in an UASB reactor (35 °C) fed with formate as substrate. The aggregate formation by the isolated methanogenic and VFA-degrading cultures from the R-granules was investigated. Defined granules were formed in a laboratory-UASB reactor (35 °C) fed with a medium containing acetate, propionate and butyrate mixture using these species as inoculum: *Methanobacterium formicicum* T1N, *Methanothrix* M7, *Methanosarcina mazei* T18, propionate degrading strain PT and butyrate degrading strain BH. *Methanobacterium formicicum* and *Methanothrix* species were demonstrated to be adhesive organisms important for granule formation.

Dedicated to my late father, De-Rang Wu, my mother, Bo-Hua Dai,

and my wife, Ping Xing,

whom I love.

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KEY TO ABBREVIATIONS

BOD	Biological oxygen demand
ca.	Calculated
CF	Cationized ferritin
COD	Chemical oxygen demand
DD-water	Double-distilled water
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
EGTA	Ethylene glycol-bis (β-aminoethyl ether)-N,N,-tetraacetic acid
F-granules	Filament-type granules
FID	Flame ionization detector
GC	Gas chromatography
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
Mb.	*Methanobacterium*
Mbv.	*Methanobrevibacter*
Mc.	*Methanococcus*
MPN	Most probable number
Ms.	*Methanosarcina*
Msp.	*Methanosprillum*
Mst.	*Methanosaeta*
Mtrx.	*Methanothrix*
NMR	Nuclear magnetic resonance
OD	Optical density
PHB	Poly-β-hydroxybutyrate
PBB	Phosphate buffured basal medium
R-granules	Rod-type granules
SEM	Scanning electron microscopy
SIA	Slide immunoenzymatic assay
sp.	species
SRB	Sulfate-reducing bacteria
SS	Suspended solids
STP	Standard pressure and temperature
SVI	Sludge volume index
TCD	Thermal conductivity detector
TEM	Transmision electron microscopy

UASB	**Upflow anaerobic sludge blanket**
VFA	**Volatile fatty acid**
VSS	**Volatile suspended solids**

INTRODUCTION

In recent years, anaerobic treatment has received considerable attention from environmental engineers and microbiologists. The most widely used process configuration is the upflow anaerobic sludge blanket (UASB) process. The development of anaerobic digestion granules with good settling properties is essential for the start-up and operation of UASB system. To date, most UASB systems are starting using granules obtained from existing reactors, although this is not always successful and is not always a practical and economical proposition. Therefore, understanding how to cultivate granules from non-granular inoculum materials and determining the underlying mechanisms of the granulation process is attractive.

The objectives of this study were to investigate: (i) the empirical principles for the cultivation of granules, (ii) the influence of microbial composition on operational, kinetic, and metabolic performance of granules, and (iii) the role of select bacterial species in granulation. Therefore, this study is a comprehensive investigation of the granulation process and the characterization of granules, including:

- investigating and generalizing the empirical principles involved in the development of granules from non-granular inoculum materials,
- characterizing the performance of granules from different sources,
- isolating prevalent species from these granules and
- characterizing their roles in substrate metabolism and granule formation.

This work was initiated at Tsinghua University, Beijing, P. R. China, and

completed at Michigan State University and Michigan Biotechnology Institute, Lansing, Michigan.

The major contents in Chapter 2 have been published in the Proceedings of Fourth International Symposium on Anaerobic digestion, Anaerobic Digestion 1985 (Wu *et al.*, 1985), Acta. Scient. Circumstant (Wu *et al.*, 1986; 1987a) and Water Research (Wu *et al.*, 1987b). A portion of the materials in Chapter 3 and 6 was presented as posters at the Annual Meeting of the American Society of Microbiology in 1988 (Thiele *et al.*, 1988b), 1990 (Wu and Jain, 1990) and 1991 (Wu *et al.*, 1991a), respectively. A portion of the contents in Chapter 4 was presented at the 46th Industrial Waste Conference, Purdue University (Wu and Hickey, 1991) and will be published in Applied and Environmental Microbiology (Wu *et al.*, 1991b).

CHAPTER 1

GENERAL LITERATURE REVIEW

1.1 HISTORIC BACKGROUND OF ANAEROBIC DIGESTION

Alexandre Volta (1776) is considered to be the first to realize the relation between decaying vegetation and inflammable gas, although "natural gas" from underground was utilized by ancient chinese people about 2,000 years ago. Since Louis Mouras (1881) invented the first septic tank, anaerobic digestion has been applied for organic waste and wastewater treatment (McCarty, 1981).

Before the 1950's, anaerobic digestion was utilized mainly for the treatment of solid organic waste (anaerobic sludge digestors) and for primary treatment of sewage (Imhoff tanks). During the last twenty years anaerobic biological wastewater treatment processes have been developed for industrial wastewater treatment. The application of anaerobic digestion for industrial wastewater was initiated when Schroepfer *et al.*(1955) proposed an anaerobic contact process. Since then, new conceptual process configurations have been introduced one after another, including the anaerobic filter (Young and McCarty, 1967), upflow anaerobic sludge blanket (UASB) reactor (Lettinga *et al.*, 1980), downflow anaerobic fixed film reactor (van den Berg and Lentz,

1979), anaerobic fluidized bed reactor (Hickey and Owens, 1981), anaerobic attached microbial film expanded bed reactor (Jewell *et al.*, 1981), anaerobic baffled reactor (McCarty, 1982), upflow anaerobic reactor combining a sludge blanket and a filter (Guiot *et al.*, 1985; Guiot and van den Berg, 1985) or upflow anaerobic hybrid reactors (Crawford and Teletzke, 1986; Oleszkiewicz *et al.*, 1986), and many others.

Mathematical modeling of anaerobic digestion process was initiated in the late 1960's (Andrews, 1968; Lawrence and McCarty, 1969; O'Rourke, 1969) and was developed by many researchers. During this period, a better and better understanding of methanogenic reactions was obtained (Zeikus, 1977, 1979; Balch *et al.*, 1979; Bryant, 1979; Wolfe and Higgins, 1979; Zehnder *et al.*, 1981). It is difficult to single out results of microbiological research that specifically contributed to the development of the current anaerobic systems, but microbiological research has indeed provided the knowledge and ideas that the scientists and engineers have put to use in the development of operational strategies for anaerobic processes. Conversely, research of the high-rate processes has also stimulated fundamental research on the microbiology of these systems.

The advantages of anaerobic biological wastewater treatment over conventional aerobic biological treatment include:

- low production of excess sludge;
- low nutrient requirement (nitrogen, phosphorus, etc.);
- no energy requirement for aeration;
- high volumetric organic loading rates can be tolerated;
- potential recovery of energy in the formed biogas;
- degradation of some toxic compounds (such as halogenated compounds) which are recalcitrant to aerobic degradation; and

- anaerobic sludge can be preserved under unfed conditions for a long time without a serious reduction of activity.

Anaerobic treatment also has certain drawbacks and unsolved problems:

- slow growth of anaerobic bacteria especially methanogenic and syntrophic acetogenic bacteria and resulting slow start-up of the process;
- sensitivity of methanogenic bacteria and syntrophic acetogens to environmental factors (pH, temperature etc.) and toxic compounds; and
- the effluent from an anaerobic reactor normally requires post-treatment for removal of remaining BOD (biological oxygen demand) or COD (chemical oxygen demand), ammonia and malodorous compounds.

1.2 MICROBIOLOGICAL PRINCIPLES OF ANAEROBIC DIGESTION

Anaerobic digestion or the decomposition of organic material to inorganic products by microbial ecosystems involves biological oxidations with protons, sulfur or carbon atoms serving as the electron sinks. In the absence of light and inorganic electron acceptors (such as sulfate, nitrate), the end products of the biodegradation of organic materials are methane and carbon dioxide. A certain amount of sulfide is also produced through sulfate reduction in anaerobic digestors, but this is generally insignificant in terms of carbon flow.

1.2.1 Trophic groups for anaerobic degradation

The complete anaerobic digestion of complex organic compounds (polysaccharides, proteins and lipids) requires at least three trophic groups of

microorganisms, which form a microbial food chain or web (Bryant, 1979; McInerney *et al.*, 1979a; Zeikus, 1979, 1983; Thiele and Zeikus, 1988a):

- Hydrolytic-fermentative microorganisms;
- Syntrophic acetogenic bacteria; and
- Methanogenic bacteria (Figure 1.1).

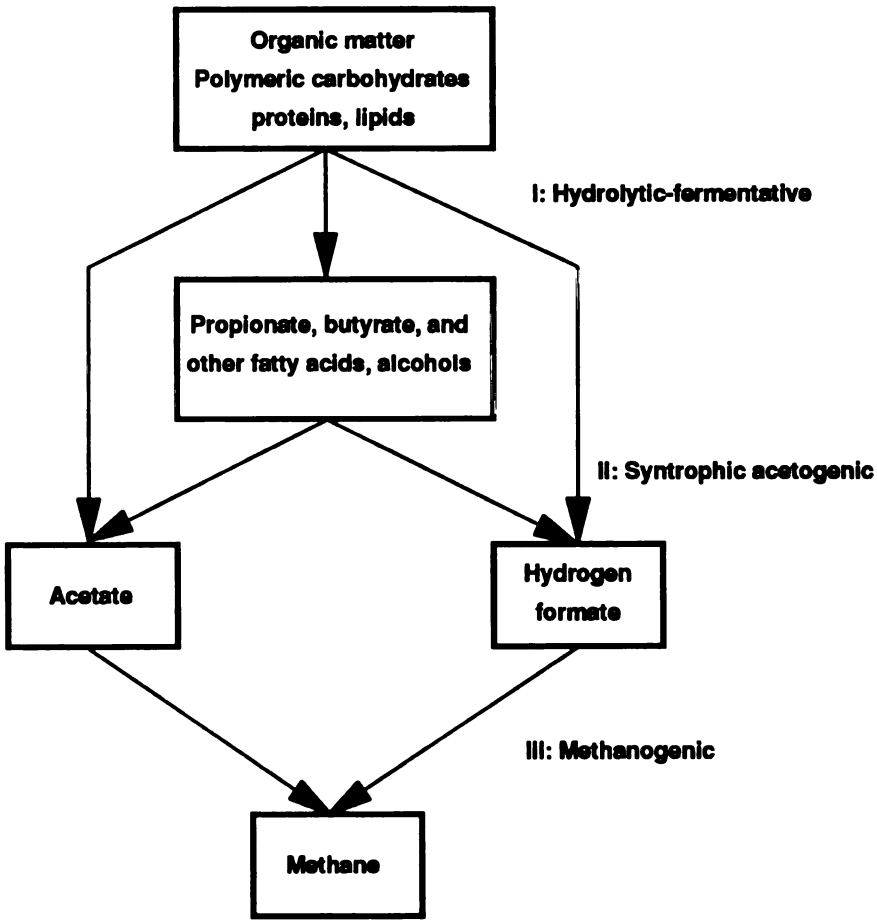


Figure 1.1 Carbon flow scheme in complete mineralization of organic matter to methane and carbon dioxide

The hydrolytic-fermentative organisms convert a variety of complex organic matter into simple organic compounds (formate, acetate, propionate, butyrate, ethanol etc.), hydrogen and carbon dioxide. Eubacteria are prevalent hydrolytic-fermentative microorganisms, although fungi and protozoa also play a role in some anaerobic ecosystems (Hungate, 1966; Bryant, 1977; Williams, 1986; Mountfort, 1987).

The syntrophic acetogens then convert the metabolic products from the first group, such as ethanol, propionate, butyrate, valerate, isovalerate, benzoate etc., into direct methanogenic precursor (acetate and hydrogen or formate). These bacteria are called "syntrophic bacteria" because they can only convert the above substrates in the association with a hydrogen-utilizing partner, usually a methanogen (or sulfate reducer if sulfate is available) to prevent the accumulation of the intermediate product H_2 or formate and, as a result, to keep the conversion thermodynamically favorable (Bryant *et al.* 1967; McInerney *et al.*, 1979a; Zeikus, 1979; Thiele and Zeikus, 1988a).

The third trophic group is the methanogenic bacteria. Methanogens utilize the acetate, H_2-CO_2 and formate produced by other trophic groups to produce methane and CO_2.

In addition to these three major trophic groups mentioned above, homoacetogens are an additional trophic group involved in anaerobic digestion (Zeikus, 1983; Zehnder *et al.*, 1981). Homoacetogens, which include several species of *Acetobacterium, Acetogenenium*, and *Sporomusa* use glucose, lactose, ethanol, formate and H_2-CO_2 to produce acetate (Balch *et al.*, 1977; Leigh *et al.*,1981; Moller *et al.*, 1983; Ljungdahl, 1986; Breznak *et al.*, 1988). A thermophilic isolate was observed to oxidize acetate into H_2-CO_2 together with a hydrogen-utilizing methanogen (Lee *et al.*, 1988). Markie and Bryant (1981) estimated that only 1 to 2% of total acetate was synthesized from H_2-CO_2 in a 40 °C cattle waste digestor.

Sulfate reducing bacteria are a diverse group of bacteria that exist in anaerobic ecosystems. Using sulfate as electron acceptor, they can degrade almost all intermediates produced fermentatively under anaerobic conditions (Widdel, 1988). Some strains can also grow syntrophically with H_2-utilizing methanogens on substrates such as ethanol and lactate (Bryant *et al.*, 1977). The role of sulfate reducing bacteria in anaerobic digestion of industrial wastewater has not been well emphasized.

1.2.2 Methanogenesis

Methanogenesis by pure cultures of methanogens has been investigated comprehensively during the last two decades. All methanogens belong to the archaebacterial kingdom and differ from eubacteria in their RNA polymerase, oligonucleotide catalogs for 16SrRNA, membrane lipids, and wall components. Systematic reviews of the taxonomy and physiology of methanogens are given by several authors (Zeikus, 1977; Balch *et al.*, 1979; Woese and Wolfe, 1985; Jones *et al.*, 1987; Woese, 1987; Jain *et al.*, 1988). Methanogens generally cannot utilize the intermediatory products with three or more carbon atoms for growth or methane production. They can use only simple energy sources such as formate, acetate, methanol, methylamines, CO, and H_2. Recently, however, it was reported that several strains of *Methanococcus, Methanospirillum* and *Methanobacterium* can partially oxidize alcohols (ethanol, isopropanol and isobutanol) into acetate and acetone (Widdel, 1986; Zellner and Winter, 1987). The prevalent methanogens in anaerobic waste digestors are acetate-utilizing methanogens which use acetate as an energy source, hydrogen-utilizing methanogens which use H_2-CO_2 or both H_2-CO_2 and formate as energy sources, and mixed function methanogens, which use H_2-CO_2 and acetate as well as methanol and methylamines. Some important methanogens in anaerobic

digestors are summarized in Table 1.1. *Methanobacterium, Methanococcus, Methanospirillum, Methanobrevibacter, Methanothrix* (*Methanosaeta*), and *Methanosarcina* are frequently found in anaerobic sewage digestors and anaerobic wastewater treatment plants.

Using ^{14}C as a tracer, it was determined that about 60 to 75% of methane is produced from acetate in anaerobic digestors (Jeris and McCarty, 1965; Smith and Mah, 1966; Zeikus, 1979; Mackie and Bryant, 1981). Acetate-utilizing methanogens perform the important function of carbon and electron removal (i.e. they remove at least 50 to 60% of the total COD in digestors). Hydrogen and formate-utilizing methanogens are also essential for anaerobic conversion of organic matter since H_2 and formate accumulation would retard and eventually stop the syntrophic acetogenic reactions. Methanogenesis occurs over a wide temperature range, ca. 0°C to 97 °C (Zehnder *et al.*, 1981). Most pure cultures of methanogens isolated to date have been mesophilic (optimum temperature at 37 to 40 °C) although some thermophilic strains have been isolated (optimum temperature at 55 to 60 °C). Methanogens are able to grow at high temperatures, up to 90 to 97°C, have also been isolated (Stetter *et al.*, 1981; Lauerer *et al.*, 1986). No psychrophilic methanogens have been isolated to date. All methanogens tested show a marked pH optimum somewhere between 6.0 and 8.0 (Jain *et al.*, 1988). Methanogenesis is observed to be most active in habitats with a pH around 6.8 to 7.2 (Zehnder *et al.*,1981).

Table 1.1 Methanogenic bacteria in anaerobic ecosystems

Family	Morphology	Substrates	Opt. temp. (°C)	Opt. pH
Methanobacteriacea				
Methanobacterium formicicum	rod	H_2, formate	37	7.0
Mb. bryantii	rod	H_2	38	7.0
Mb. thermoautotrophicum	rod	H_2	65-70	7.2-7.6
Mb. wolfei	rod	H_2	55-65	7.0-7.5
Mb. thermoaggregans	rod	H_2	65	7.0-7.5
Mb. smithii	rod	H_2, formate	38	6.9-7.4
Methanobrevibacter ruminantium	rod	H_2, formate	38	7.2
Mbv. arboriphilus	rod	H_2	30-37	7.5-8.0
Methanosphera stadtmaniae	coccus	H_2, methanol	36-40	6.5-6.9
Methnococcaceae				
Methanococcus vannielii	coccus	H_2, formate	36-40	7.0-9.0
Mc. voltae	coccus	H_2, formate	32-40	6.7-7.4
Mc. maripaludis	coccus	H_2, formate	38	6.8-7.2
Mc. deltae	coccus	H_2, formate	37	ND
Mc. thermolithotrophicus	coccus	H_2, formate	65	6.5-7.5
Mc. jannaschii	coccus	H_2	85	6.0
Methanomicrobiaceae				
Methanomicrobium mobile	rod	H_2, formate	40	6.1-6.9
Mm. limicola	planes	H_2, formate	40	7.0
Methanospirillum hungatei	spirillum	H_2, formate	30-37	6.6-7.4
Methanosarcinaceae				
Methanosarcina barkeri	sarcina	H_2, methanol, methylamines, acetate	35	7.0
Ms. mazei	sarcina or coccus	H_2, methanol, methylamines, acetate	40	6.0-7.0
Methanosaeta concilii	filament	acetate	35-40	7.1-7.5
Mts. thermoacetophila	filament	acetate	60	6.5

Source: Jones *et al.* (1987); Patel and Sprott (1990). ND: not determined.

1.2.3 Syntrophic acetogenesis

The term of "syntrophic acetogen" is used to describe those anaerobic bacteria that oxidize saturated fatty acids, ethanol and benzoate and produce acetate together with a hydrogen or formate utilizing partner (methanogens or sulfate reducers). The syntrophic acetogens isolated to date can be conveniently classified as propionate degraders, butyrate degraders, isovalerate degraders and benzoate degraders. The syntrophic acetogenic bacteria that have been isolated to date are listed in Table 1.2.

i) Propionate degraders

During complete degradation of complex organic materials under methanogenic conditions, about 15% of the total carbon is degraded via propionate as an intermediate (Gujer and Zehnder 1983). To date, only two defined syntrophic cultures have been isolated. One is a coculture consisting syntrophic propionate degrader (*Syntrophobacter wolinii*) and *Desulfovibrio* G11 (Boone and Bryant, 1980). Another is a thermophilic methanogenic consortium T13, consisting of fluorescent methanogenic rods, *Methanosarcina* sp., conical tapered rods with central endospores, and filamentous rods with terminal endospores (Mucha *et al.*,1988). Using a kinetic analysis of results from a continuously fed, mixed digestor, Heyes and Hall (1983) suggested that there were at least two sub-groups of propionate-utilizing organisms: one, a slower growing group with a maximum specific growth rate (μ_{max}) of 0.0054 h^{-1} and a half velocity coefficient (K_s) of 11 mg/L, similar to *Syntrophobacter wolinii* (μ_{max} of 0.004 h^{-1}) and another faster growing group with a μ_{max} of 0.05 h^{-1} and a K_s of 330 mg/L. These fast growing propionate utilizing bacteria have not been isolated.

Table 1.2 Syntrophic acetogenic bacteria isolated to date

Organisms	Syntrophic partner	Temp (°C)	Substrate	Reference
Syntrophobacter wolinii	*Desulfovibrio* G11 or *Msp. hungatei* JF1 plus *D.* G11	37	propionate	(1)
Syntrophomonas wolfei	*Desulfovibrio* G11, *Msp. hungatei* JF1	35	monocarboxylic saturated fatty acids (4 to 8 carbons)	(2)
Syntrophospora bryantii	*Desulfovibrio* E70, *Msp. hungatei* M1h	28	monocarboxylic saturated fatty acids (4 to 11 carbons)	(3)
Strain NSF-2	*Msp. hungatei* or *Desulfovibrio* sp.	37	monocarboxylic saturated fatty acids (4 to 6 carbons)	(4)
Strain SF-1	*Msp. hungatei* or *Desulfovibrio* sp.	37	monocarboxylic saturated fatty acids (4 to 6 carbons)	(4)
Syntrophomonas sapovarans	*Msp. hungatei* or *Desulfovibrio*	35	monocarboxylic saturated fatty acids	(5)
Strain RotseeSO4	*Desulfovibrio*.sp	30	butyrate	(6)
Thermophilic bacterium	*Methanobacterium thermoautotrophicum*	55	butyrate	(7)
Thermophilic bacterium	*Methanobacterium thermoautotrophicum*	55	butyrate	(8)
Strain Gralval	*Desulfovibrio* E70	28	isovalerate	(9)
Syntrophus buswellii	*Desulfovibrio* G11 or	37	benzoate	(10)
Strain BZ-2	*Desulfovibrio* PS-1 *D.* PS-1 plus *Methanospirillum* PM-1	37	benzoate	(4)

(1) Boone and Bryant, 1980. (2) McInerney *et al.*, 1979, 1981. (3) Stieb and Schink, 1985; Zhao *et al.*, 1990.
(4) Shelton *et al.*, 1984; Dwyer *et al.*, 1988. (5) Roy *et al*, 1985; Lorowitz *et al.* 1989
(6) Tomei *et al.*, 1985. (7) Henson and Smith, 1985. (8) Ahring and Westermann 1987a, 1987b.
(9) Stieb and Schink, 1986. (10) Mountfort and Bryant, 1982; Mountfort *et al.*, 1984.

ii) Butyrate degraders.

Butyrate is an important intermediate product in anaerobic digestors. All syntrophic butyrate degrading acetogens isolated and identified to date are either *Syntrophomonas* sp. or *Syntrophospora* sp. Butyrate degraders can syntrophically metabolize even numbered fatty acids with up to 18 carbon atoms (McInerney *et al.*, 1979b, 1981; Roy *et al.*, 1985, 1986; Beaty *et al.*, 1990; Zhao *et al.*, 1990) and grow as monocultures on crotonate (Beaty and McInerney, 1987; Lorowitz *et al.*, 1989; Zhao *et al.*, 1990). Syntrophic butyrate degraders also degrade fatty acids with 5 or more odd-numbered carbon atoms. The degradation products by *Syntrophomonas wolfei* are consistent with β-oxidation (i.e. even-carbon-numbered fatty acids are converted into acetate and H_2; odd-carbon-numbered fatty acids into acetate, propionate and H_2; longer branched-chain acid (isoheptanoate) into acetate, shorter branched-chain acid (isovalerate) and H_2), as is shown in Table 1.3. Enzyme analysis indicated that *Syntrophomonas wolfei* contained high levels of β-oxidation enzymes (Beaty *et al.*, 1986). The degradation of saturated monocarboxylic fatty acids by *Syntrophospora bryantii* ("*Clostridium bryantii*") also appears to occur via β-oxidation, based on the end products (Stieb and Schink, 1985). Butyrate degraders do not degrade propionate or isovalerate. Butyrate-degrading *Syntrophospora* sp. oxidize 2-methylbutyrate into H_2 , acetate and propionate (Stieb and Schink., 1985; Roy *et al.*, 1985) but do not utilize isobutyrate.

Table 1.3 β-oxidation of normal saturated monocarboxylic fatty acids by *Syntrophomonas wolfei*

Acid	Carbon	Reaction
Even-numbered acids		
Butyrate	4	$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + 2H_2 + H^+$
Caproate	6	$CH_3CH_2CH_2CH_2CH_2COO^- + 4H_2O \rightarrow 3CH_3COO^- + 4H_2 + 2H^+$
Caprylate	8	$CH_3CH_2CH_2CH_2CH_2CH_2CH_2COO^- + 6H_2O \rightarrow 4CH_3COO^- + 6H_2 + 3H^+$
Odd-numbered acids		
Valerate	5	$CH_3CH_2CH_2CH_2COO^- + 2H_2O \rightarrow CH_3CH_2COO^- + CH_3COO^- + 2H_2 + H^+$
Heptanoate	7	$CH_3CH_2CH_2CH_2CH_2CH_2COO^- + 4H_2O \rightarrow CH_3CH_2COO^- + 2CH_3COO^- + 4H_2 + 2H^+$

Note: data from McInerney *et al.* (1981).

iii) Isovalerate degraders

Isoveralerate, a commonly observed branched-fatty acid found in anaerobic digestors, originates primarily from protein fermentation. Only two cocultures of syntrophic isovalerate-degrading bacteria have been isolated, strain Gra I val with a *Desulfovibrio* sp. and strain Go I val with a *Desulfovibrio* sp. (Stieb and Schink, 1986). Isovalerate (1 mol) is syntrophically converted to acetate (3 mol).

iv) Isomerization between butyrate and isobutyrate.

Isobutyrate is mainly produced from protein fermentation (McInerney, 1988). Isomerization between butyrate and isobutyrate was observed during both syntrophic butyrate degradation (Lovley and Klug, 1982; Gourdon *et al.*, 1988; Lema *et al.*, 1988) and isobutyrate degradation using digested sludge (Zinder *et al.* 1984a; Lema *et al.*, 1988). Using nuclear magnetic resonance (NMR) experiments with ^{13}C-labelled butyrate, Tholozan *et al.* (1988a) demonstrated that isomerization between butyrate and isobutyrate was reversible and butyrate was an intermediate of syntrophic isobutyrate degradation. The experimental data of syntrophic butyrate and isobutyrate degradation by sludge were found to fit a kinetic model developed on this mechanism (Aguilar *et al.*, 1990). To date, it is not clear whether the isomerization is coupled with syntrophic butyrate degradation and what controls the direction of the isomerization. It is not possible to further investigate the mechanism of isomerization and syntrophic isobutyrate degradation using anaerobic digested sludge containing both syntrophic isobutyrate degraders and syntrophic butyrate degraders which do not utilize isobutyrate. To date, no defined syntrophic isobutyrate-degrading culture has been isolated (Stieb and Schink, 1989). A spore-forming rod, strain SF-1, reported to be syntrophic butyrate-degrading bacteria that used isobutyrate (Shelton and Tiedje, 1984), was later reported not to be capable of this reaction (Stieb and Schink, 1989). Isolation

of a syntrophic isobutyrate degrader is necessary to study the metabolism of isobutyrate.

Table 1.4 Possible reactions during syntrophic acetogenesis

Reaction	Free Energy kJ/reaction
1. Interspecies H_2 transfer	
$EtOH + H_2O \rightarrow Ac^- + H^+ + 2H_2$	+9.6
$Pr^- + 3H_2O \rightarrow Ac^- + H^+ + 3H_2 + HCO_3^-$	+76.1
$Bt^- + 2H_2O \rightarrow 2Ac^- + H^+ + 2H_2$	+48.3
2. Interspecies formate transfer	
$EtOH + 2HCO_3^- \rightarrow Ac^- + H^+ + 2HCOO^- + H_2O$	+6.9
$Pr^- + 2HCO_3^- \rightarrow Ac^- + H^+ + 3HCOO^-$	+72.4
$Bt^- + 2HCO_3^- \rightarrow 2Ac^- + H^+ + 2HCOO^-$	+45.6
3. Methanogenesis	
$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3\ H_2O$	-135.6
$4HCOO^- + H_2O + H^+ \rightarrow CH_4 + 3HCO_3^-$	-130.1
4. Sulfate reduction	
$4H_2 + SO_4^{2-} + H^+ \rightarrow 4H_2O + HS^-$	-152.2
$4HCOO^- + SO_4^{2-} + H^+ \rightarrow 4HCO_3^- + HS^-$	-146.7

EtOH: ethanol; Ac: acetate; Pr: propionate; Bt: butyrate.
Free energy values under standard conditions from Thauer *et al.*, (1977)

V) Interspecies electron transfer

Based on energetic analysis, the free energy available for acetogenesis from ethanol,propionate and butyrate with either hydrogen or formate as an intermediate is not thermodynamically favorable under standard conditions (Table 1.4). The levels of hydrogen and formate have to be maintained at low levels (10^{-3} to 10^{-5} atm H_2) via

methanogenesis or sulfate reduction during acetogenesis to allow the reaction to proceed. Hungate was the first to express the idea that hydrogen production and utilization can profoundly influence the course of fermentations in anaerobic ecosystems (Hungate, 1967). In fact, low hydrogen partial pressures were observed in all syntrophic acetogenic cultures (Bryant, 1979; McInerney and Bryant, 1980; Beaty *et al.*, 1986). Experiments also demonstrated that perturbation with high partial pressures of H_2 inhibited syntrophic butyrate degradation until hydrogen was consumed to a low level (Ahring *et al.*, 1987b; Dwyer *et al.* 1988).

In addition to hydrogen, formate was recently suggested to be an intermediate in syntrophic ethanol degradation and fatty acid degradation (Thiele and Zeikus, 1988a, 1988b; Ozturk *et al.*, 1989; Boone *et al.*, 1989). The concept of interspecies electron transfer using formate as the electron carrying intermediate requires that formate be synthesized from bicarbonate by syntrophic acetogens and oxidized back to bicarbonate by formate-utilizing methanogens or sulfate reducers to maintain a low formate concentration (Thiele and Zeikus, 1988a, 1988b). This concept appears to be possible in the case where syntrophic acetogenic isolates are associated with a hydrogen and formate-utilizing syntrophic partners, either *Methanobacterium* sp., *Methanospirillum* sp. or *Desulforvibrio* sp. (see Table 1.2). In addition, hydrogen production was observed when formate was added to a culture of *Syntrophomonas wolfei* sp., indicating that this syntrophic butyrate degrading organism appeared to have formate dehydrogenase activity (Boone *et al.*, 1989). However, there are some exceptions to this proposed suggestion. For example, syntrophic butyrate degraders were isolated with *Methanobacterium thermoautotrophicum*, which does not utilize formate (Henson and Smith, 1985; Ahring *et al.*, 1987a). In the latter case, formate cannot serve as an intermediate during interspecies electron transfer. Furthermore, the mechanism of

inhibition of H_2 has not been well explained by present interspecies formate transfer models. Absolute evidence of formate as an intermediate and the relationship between formate and hydrogen during syntrophic acetogenesis requires further evaluation.

1.2.4 Sulfate reducing bacteria

Sulfate reducing bacteria (SRB) are quite diverse, in terms of metabolic activities, morphotypes, trophic properties and substrate affinities. In the presence of sulfate, acetate can be oxidized to CO_2 by some pure SRB cultures; propionate, butyrate, and other normal fatty acids can be oxidized completely to CO_2 or converted to acetate or acetate plus propionate (in the case of odd-numbered long chain acids with 5 or more carbon atoms); branch-fatty acids such as isobutyrate, isovalerate and 2-methylbutyrate can also be oxidized completely to CO_2 or to acetate (Widdel and Pfennig, 1984; Widdel, 1988). Hydrogen and formate can be utilized by many SRB as electron donors for sulfate reduction (Widdel, 1988). A few strains of SRB also utilize benzoate and phenyl compounds as electron donors. Some SRB and the substrates which they can use as electron donors for sulfate reduction are presented in Table 1.5.

Other than reduce sulfate in monoculture, SRB can also grow in syntrophic associations in the presence of sulfate. SRB utilize H_2 and formate as electron donors to reduce sulfate when grown together with syntrophic acetogens that degrade benzoate (Mountfort and Bryant, 1982; Mountfort *et al.*, 1984) and fatty acids with 3 or more carbons (Boone and Bryant, 1980; McInerney *et al.*, 1981; Stieb and Schinck, 1985). Degradation of acetate and methanol via sulfate reduction was also demonstrated using a coculture consisting of *Desulfovibrio vulgaris* and *Methanosarcina barkeri* (Phelps *et al.*, 1985). Methanol can be degraded to CO_2 via sulfate reduction by a coculture

consisting of *Desulfovibrio vulgaris* and homoacetogen, *Sporomusa acidovorans* (Cord-Ruwisch and Ollivier, 1986).

In the absence of sulfate, certain SRB, such as *Desulfovibrio* sp., can grow together with H_2-utilizing methanogens to convert ethanol or lactate to acetate syntrophically (Bryant *et al.*, 1977; McInerney and Bryant, 1981; Traore *et al.*, 1983). Syntrophic conversion of formate to methane was also observed in syntrophic association consisting a H_2-utilizing methanogen, *Methanobacterium bryantii* and *Desulfovibrio vulgaris* which can split formate to hydrogen and CO_2 (Guyot and Brauman, 1986). It was proposed that sytrophic hydrogen production by SRB in an association with hydrogen-dependent methanogens occurred in the sediments from Lake Mendota (Conrad *et al.*, 1987). No mention of SRB capable of syntrophically catabolizing volatile fatty acids (VFAs) has been reported. VFAs such as propionate and butyrate, are thought to be converted only by syntrophic acetogens in concert with H_2-utilizing methanogens (Boone and Bryant, 1980; McInerney *et al.*, 1979, 1981; Stieb and Schink, 1985; Widdel, 1988).

Table 1.5 Representative sulfate reducing bacteria

Organism	Optimum temp. (°C)	Substrate used as electron donors
Desulfotomaculum *acetoxidans*	35	acetate, butyrate, valerate, ethanol, butanol
D. sapomandens	38	formate, acetate, fatty acids (4 to 18 carbons) ethanol, isovalerate, 3-methylbutyrate, benzoate
D. nigrificans	55	H_2, formate, lactate, ethanol, fructose
Desulfovibrio vulgaris	30-36	H_2, formate, lactate, ethanol, fumarate, malate
D. desulfuricans	30-36	H_2, formate, lactate, ethanol, fumarate, malate
D. sapovorans	34	fatty acids (4 to 16 carbon), 2-methylbutyrate, lactate
D. baarsii	35-39	formate, acetate, fatty acids (3 to 18 carbon), isovalerate, 2-methylbutyrate, 3-methylbutyrate
Desulfomonas pigra	37	H_2, lactate, ethanol
Desulfobulbus propionicus	28-39	H_2, propionate, lactate, ethanol
D. elongatus	35	H_2, propionate, lactate, ethanol
Desulfobacter postgatei	28-32	acetate
D. hydrogenophilus	28-32	H_2, acetate, ethanol
D. latus	28-32	acetate
D. curvatus	28-30	H_2, acetate, ethanol
Desulfococcus multivorans	35	formate, acetate, fatty acids (3 to 16 carbon), isovalerate, 2-methylbutyrate, 3-methylbutyrate lactate, ethanol, benzoate, phenylacetate, phenylpropionate
D. niacini	29	H_2, formate, acetate, fatty acids (3 to 16 carbon), isovalerate, 2-methylbutyrate, 3-methylbutyrate ethanol, nicotinate, succinate, glutarate, pimelate
Desulfosarcina variabilis	33	H_2, formate, acetate, fatty acids (3 to 14 carbon), 2-methylbutyrate, 3-methylbutyrate, lactate, ethanol, benzoate, phenylacetate, phenylpropionate, fumarate
Desulfobacterium autotrophicum	20-26	H_2, formate, acetate, fatty acids (3 to 16 carbon), isovalerate, 2-methylbutyrate, lactate, succinate, ethanol, fumarate
D. vacuolatum	25-30	H_2, formate, acetate, fatty acids (3 to 16 carbon), isovalerate, 2-methylbutyrate, 3-methylbutyrate, lactate, succinate, fumarate, malate, ethanol
D. phenolicum	28	formate, acetate, butyrate, fumarate, malate, benzoate, ethanol, phenol, p-cresol, glutarate

Note: Fatty acids: molocarboxylic saturated fatty acids.
Data from Widdel (1988).

1.3 ANAEROBIC GRANULES

1.3.1 Upflow Anaerobic Sludge Blanket (UASB) System

The upflow anaerobic sludge blanket (UASB) system, developed by Lettinga and his coworkers in 1970's (Lettinga *et al.*, 1980, 1984; de Zeeuw, 1988), has received widespread acceptance and been successfully used to treat industrial wastewaters. Although the reversed Dorr Oliver Clarigester, used successfully to treat several industrial wastes in 1960's (Hemens *et al.*, 1962; Stander *et al.*, 1967), can be considered to be a precursor of the UASB process as it is now employed, the first upflow sludge blanket concept was actually used by Winslow and Phelps (1911) and referred to as the "biolytic tank" . A typical UASB plant design consists of a biological reaction zone and a sedimentation zone. In the reaction zone, the organic compounds in influent are mineralized to methane and CO_2 as they pass through the sludge bed. The gas produced and the sludge buoyed by entrapped or attached gas bubbles are separated from liquid effluent via a gas-solid-liquid separator installed at the top of the reactor. In well-operated UASB reactors, the anaerobic sludge has high settleability and is often granular in nature. High concentrations of biomass retained in the UASB system allows the process to achieve high removal efficiencies at high volumetric COD loading rates. Several modifications of the conventional UASB process have been developed and currently being marketed. One such modification, the "combined UASB-AF process" utilizes filter-type packing media at the top part of settler section to prevent suspended solids from escaping (Lettinga *et al.*, 1981; Wang *et al.*, 1985). The packed media material can be also installed at the front of the gas-solid-liquid separator to enhance biomass retention and granulation. This modification led to development of the "UASB filter process" (Guiot *et al.*, 1985;

Guiot and van den Berg, 1985) or "hybrid anaerobic reactor" (Crawford and Teletzke, 1986; Oleszkiewicz *et al.*, 1986) in which the filter media serves as the sole solid-liquid-gas separation device. The hybrid upflow anaerobic reactor is also considered to be a modification of upflow anaerobic filters (Young, 1990; Tilche, 1990). These modified process configurations have not yet enjoyed large-scale application as has the conventional UASB process.

To date, over a hundred full-scale UASB plants have been commissioned in the Netherlands and other Western European countries, Canada, the U.S.A., China, etc. Various food processing wastewaters (sugar beet, liquid sugar, potato starch, potato processing, yeast, coffee, dairy, corn starch, vegetable canning, slaughter house, soft drinking, rendering plant, mussel conning, fish processing), fermentation wastewaters (distillery, brewery, citrate manufacture, acetone-butanol fermentation), industrial wastewaters (paper mill, teraphthalate production, wool washing, fibre board manufacturing), landfill leachate, and sewage have been successfully treated at mesophilic temperatures (Lettinga *et al.*, 1983; 1984; 1985; Wang *et al.*, 1985; de Zeeuw, 1988; Hulshoff Pol, 1989). Thermophilic treatment of wastewater using the UASB reactors has also been demonstrated to be possible via laboratory-scale experiments (Wiegant *et al.*, 1983, 1985; Wiegant and Lettinga, 1985) and pilot-scale test (Lanting *et al.*, 1989).

Selection of a suitable inoculum material is essential to successfully start-up a UASB reactor. Inoculum material should possess microorganisms that can degrade the organic compounds in the target wastewater, and induce granule formation (or develop sludge with good-settling properties). The inoculum can be classified as granular or non-granular sludge.

Granular sludge from existing UASB reactors is widely used as inoculum

material to start-up new UASB reactors, mainly in Western Europe, Canada and the U.S.A. As more UASB reactors are put into operation, the surplus granular sludge from these operating reactors can be stored and used to inoculate new reactors. Utilization of granular sludge as inoculum has the advantage of being able to achieve high COD removal rate within a short start-up period.

When granular sludge is not available for the start-up of reactors, non- granular inoculum materials can be used. Over the last ten years, much research has been concentrated towards understanding and improving the start-up of UASB reactors and formation of granules from non-granular materials. Non-granular materials such as anaerobic digested sludge (Hulshoff Pol *et al.*, 1982, 1983; de Zeeuw, 1984; Wu, 1984; Wu *et al.*, 1985; Manjunath *et al.*, 1989), and cow manure (Wiegant *et al.*, 1985; Wiegant and de Man, 1986) have been used as inoculum to start-up the UASB reactors and cultivate granular sludges. Investigation and generalization of empirical principles for cultivation of granules from non-granular materials are of importance in application of UASB reactors (as well as upflow anaerobic hybrid reactors).

1.3.2 Granulation in anaerobic reactors

The formation of biomass with good-settling is a widespread phenomenon in the UASB and other anaerobic reactors, and it is due to the formation of conglomerates by microorganisms themselves. These conglomerates can be classified as flocs, pellets, or granules (Dolfing, 1987):

- Flocs are conglomerates with a loose structure. After settling, a layer of flocs forms a macroscopic layer;
- Pellets are conglomerates with a more dense structure than flocs. After settling they 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.

Anaerobic granules are formed in the UASB reactor and other plug flow pattern anaerobic reactors. Granules that contain methanogenic bacteria as predominant species can remain in a fixed granular shape for long periods of time (several years). Granules were also observed formed in an UASB reactor acidifying glucose. The granules formed were about 1 mm in diameter but were unstable under unfed conditions (Zoetemeijer *et al.*, 1981). Granules were also reported formed in upflow sludge blanket denitrification reactors fed with methanol as the carbon source. These granules contained calcium precipitates and were not stable (Klapwijk *et al.*, 1981). They disaggregated when methanol was replaced with another carbon source such as ethanol or glucose (Van der Hoek, 1988).

i) Feasibility of granulation

To date, granular sludge has been developed from a number of wastewaters using non-granular inoculum materials at mesophilic, thermophilic and ambient temperatures. Anaerobic methanogenic granules have been primarily observed in the UASB reactors treating sugar beet waste at mesophilic temperatures (Heertjes *et al.*, 1978; van der Meer, 1979; Lettinga *et al.*, 1980). The development of granular sludge from digested sewage sludge was also reported in mesophilic UASB reactors treating other wastewaters such as, potato processing wastewater (Lettinga *et al.*, 1980), paper mill wastewater (Haberts *et al.*, 1985), brewery wastewater (Wu *et al.*, 1985; Hu *et al.*, 1985), thermomechanical pulping effluent (Vouriranta *et al.*, 1985), alcohol distillery wastewater (Shen, 1985), sugar manufacture wastewater (Wang *et al.*, 1985), slaughter house wastewater and teraphthalate production wastewater (Wu, W.-G., 1989), whey

(Clark, 1988; Killilia and Colleran, 1988), a sucrose solution (Sierra-Alvarez *et al.*, 1988; Harada *et al.*, 1988), night soil (Endo and Tohay, 1985), and VFA mixtures (Hulshoff Poll *et al.*, 1982, 1983; de Zeeuw, 1984; Narada *et al.*, 1988). Granules have also been obtained using activated sludge as inoculum in UASB reactors treating glucose molasses (Wu *et al.*, 1987b), citrate fermentation wastewater (Wu *et al.*, 1987b) and a VFA mixture (Guyot *et al.*, 1990). The formation of granular sludge (granulation) was also reported in thermophilic lab-scale plants inoculated with digested sewage sludge and cow manure and fed with vinasse (Wiegant *et al.*, 1985), a sugar solution (Wiegant and Lettinga, 1985), acetate and an acetate-butyrate mixture (Wiegant and de Man, 1986). At ambient temperatures, granulation was also observed to occur in the reactors treating brewery wastewater (Jiang, Q., 1989) and slaughterhouse wastewater (Wu, W.-G., 1989). Granular sludge up to 3-5 mm in size was also obtained in lab-UASB reactor treating sewage without addition of inoculum material (Barbosa and Sant'Anna, 1989).

In addition to UASB reactors, anaerobic granules have also been observed to form in anaerobic filters (Young and Dahab, 1983), upflow anaerobic reactors combining a sludge blanket and a filter (UBF) treating sugar solution (Guiot *et al.*, 1985, 1988), upflow anaerobic hybrid reactor treating thermal sludge conditioning liquor (Kimata *et al.*, 1990), sludge bed anaerobic baffled reactors treating molasses stillage evaporator condensates (Tilche and Yang, 1988) and sewage (Orozco, 1988), and at the top above the carrier media in anaerobic fluidized bed reactors fed with either acetate, propionate, butyrate or a mixture of these acids (Gorris and van Deursen, 1988) and fed with beet sugar wastewater (Iza *et al.*, 1988). A summary of granulation from non-granular inocula with various wastewaters in UASB and other upflow-type reactors is presented in Table 1.6.

Table 1.6 Granulation test using non-granular inoculum materials[a]

Wastewater	Reactor volume (m^3)	Temp. (°C)	Inoculum	Granulation period (months)	COD Load achieved (kg/m^3-d)	Reference[b]
VFA mixture	0.030	30	DS	>3.0	60	(1)
Acetate+YE	0.00575	55	DS	3.6	31	(2)
Acetate+YE	0.00575	55	CM	3.6	51	(2)
Distillery	24	30	DS	6	12-24	(5)
Slaughterhouse	21	15-27	DS	9	3-4	(5)
Teraphthalate production	20	35	DS	6	12-20	(5)
Sugar molasses	0.0114	30	DS	1.5	13	(6)
VFA mixture	0.0045	35	AS	NR	NR	(7)
Sludge thermal conditioning liquor	6.0	34-37	DS	>6	30	(8)
Glucose molasses	0.02	35	DS	5.0	14	(10)
Citrate	0.048	35	AS	4	22	(11)
Brewery	0.02	35	DS	1.5	32	(10)
Citrate	6	35	AS	4	12-15	(10)
Brewery	10	19-23	DS+AS	12	3-8	(12)
Glucose molasses	0.03	35	AS	1.5	12	(11)

a) DS: Digested sludge; AS: Activated sludge; CM: Cow manure; NR: Not reported; YE: yeast extract.
b) (1) Hulshoff Pol *et al.* (1983); (2) Wiegant and de Man, 1986; (5) Wu, W.-G. (1989); (6) Manjunath *et al.*,1989; (7) Guyot *et al.*, 1990; (8) Kimata *et al.*, 1990; (9) Gu, G.-G. (1986); (10) Wu *et al.*, 1985; (11) Wu *et al.*, 1987b; (12) Jiang, Q. (1989).

ii) Process of granulation in UASB reactors

The granulation progress has been investigated using lab-scale UASB reactors under mesophilic conditions (Hulshoff Pol *et al.*, 1982, 1983; Hulshoff Pol, 1989; de Zeeuw, 1984; Brummeler *et al.*, 1985; Wu, 1984; Wu *et al.*, 1985, 1986; 1987b; Manjunath *et al.*, 1989). Hulshoff Pol *et al.*(1982, 1983) indicated that when digested sewage sludge was used as inoculum and a VFA mixture (acetate plus propionate) was used as feed, three different phases could be distinguished during the granulation progress. Each phase was characterized according to the sludge concentration profiles in reactors and the nature of the sludge. In phase I, the sludge bed expanded and the sludge concentration in sludge blanket increased. During phase II, the sludge concentration in the blanket continued to increase and the wash-out of light fraction of sludge started; consequently the total amount of the sludge in the reactor dropped to the minimum level. At the same time, granules were gradually formed in the sludge bed. In phase III, the growth of granules exceeded the rate of sludge wash-out and, therefore, the total biomass in the reactor increased again. De Zeeuw (1984, 1988) found that the amount of sludge that washed-out and level of sludge remaining inreactor depended heavily on the density of inoculated sludge (i.e. high density sludge led to less wash-out). The results on granulation, however, were all obtained using VFA mixtures and anaerobic digested sludge from the same sources. Therefore, general principles for granulation cannot be drawn until experiments with other inoculum and substrate are performed.

Wiegant and de Man (1986) described granulation in thermophilic UASB reactors using a sugar solution and acetate as feed and with digested sewage sludge or cow manure as inoculum. The granulation process under thermophilic temperature conditions is essentially the same as that under mesophilic conditions (Lettinga *et al.*,

1985; Wiegant and de Man, 1986).

Addition of a small amount of crushed granules to non-granular inoculum can stimulate the granulation process (Hulshoff Pol *et al.*, 1983; Hulshoff Pol, 1989; Alibhai and Forster, 1986). This is probably the result of supplying microorganisms which are responsible for granulation and supplying bio-nuclei sites for bacterial attached growth. Results of adding inert materials to the inoculum sludge in order to enhance granulation have not produced clear conclusions. No difference was found for granulation under thermophilic conditions with or without the addition of sand (50-100 μm) along with the inoculated digested sludge, although the granules formed in the former case had sand particles within them. By contrast, addition of hydroanthracite particles (100 μm in size) significantly reduced the time needed for granulation under mesophilic conditions (Hulshoff Pol, 1989).

1.3.3 Characteristics of granules.

Characterization of anaerobic granules has been performed by several researchers, based on aspects of physical-chemical properties, granule type, metabolic activity and microbial composition.

i) Physical and chemical characteristics

The specific gravity of anaerobic digestion granules from UASB reactors were estimated to be generally in a range between 1.00 to 1.09 (Van der Meer, 1979; Dolfing *et al.*, 1985; Wu, 1984; Wu *et al.*, 1985; Hulshoff Pol, 1989). The size of granules varies from 0.1 to 8 mm in diameter. The granule size distribution also depends on the microbial population, feed composition and biogas flux rate in reactors. Granules containing more filamentous *Methanothrix* tend to grow to a larger size than those granules containing mainly rod-type *Methanothrix* (Hulshoff Pol *et al.*, 1983).

More carbohydrates in the feed stimulate the formation of large-sized but lower-density granules (Sierra-Alvarez *et al.*, 1988).

The mineral composition of granules varies widely; the ash contents in granules vary according the mineral composition in feeds. Generally, in lab-scale UASB reactor experiments with synthetic feed (such as glucose solutions, VFA mixtures), the ash content of the sludge bed can be as low as 12% (Thiele *et al.*, 1990) to 15 to 16% (Wu, 1984; Wu *et al.*, 1985). This is because these synthetic feeds contained low concentrations of calcium and iron salts. When the feed contains more inorganic matter, the ash content will be higher. Granules in a laboratory-scale reactor fed with a lactate solution containing relatively high salt concentrations had ash contents as high as 41.5% (Switzenbaum and Eimstad, 1987). Granules from reactors treating industrial wastewaters generally have higher ash contents, varying from 19% to 46% (Alibhai and Forster, 1986). The higher ash levels are due principally to the presence of higher concentrations of calcium and/or iron salts in the wastewaters or more suspended solids in the feed. For example, in the granules developed on citrate fermentation wastewater, which contained soluble calcium salt at a concentration of about 800 mg/L, ash contents were as high as 26% (Wu *et al.*, 1987). Dubourguier *et al.* (1988a) reported that the sludge from lower part of an UASB reactor contained higher ash levels than that from the upper part of the bed. Research on the correlation between the ash content and the granule strength did not yield positive results (Hulshoff Pol, 1989), suggesting that the strength of granules probably relies upon other factors. Chemical precipitates may, however, be important in the granulation process. Lettinga *et al.* (1985) pointed out that granulation was enhanced by supplying carrier materials such as anthracite or gravel and that mineral precipitates or crystals inside the granules served as carriers or nuclei sites that helped initiate granulation.

Table 1.7 Specific COD removal rates of anaerobic granules

Substrate source	Temp. °C	Specific COD removal rate gCOD/gVSS-d	Volumetric load of reactor gCOD/m^3-d	Reference
VFA-mixture	30	2.2	60	(1)
Yeast	30-35	0.7-0.9	7-14	(1)(2)
Sugar beet	30	1.3	14	(3)
Potato	35	1.0-1.4	30-40	(1)(2)
Sucrose	30	0.99	20	(4)
Acetate	35	2.1	17	(5)
Acetate	55	4.2-7.3	162	(6)
VFA-mixture	30	1.3-1.8	10	(7)
Sludge thermal conditioning liquor	34-37	1.1	30	(8)
Brewery	35	1.9-2.2	32	(9)
Glucose molasses	35	1.2	14	(9)
VFA mixture	35	7-9	90-100	(10)
Sucrose	27-29	1.1	11	(11)
Sucrose	27-29	2.6	34	(11)
Sucrose+ethanol	27-29	2.6	17	(11)

(1) Hulshoff Pol *et al.*, 1983; (2) Lettinga *et al.*, 1984; (3) Patte *et al.*, 1980;
(4) Sierra-Alvarez *et al.*, 1988; (5) Harada *et al.*, 1988; (6) Wiegant and Man, 1986;
(7) Brummeler *et al.*, 1985; (8) Kimata *et al.*, 1990; (9) Wu *et al.*, 1985;
(10) Thiele *et al.*, 1990; (11) Guiot *et al.*, 1988.

ii) Metabolic activity

Metabolic activities of granules can be expressed in terms of specific methanogenic activity, specific COD removal rate and specific substrate conversion rate, depending on the objectives of the assay. COD removal rate can be determined directly by COD measurements or indirectly by methane production (0.35 L (STP) methane per g COD). To date, most researchers have used these assays to characterize the activity of granules (Lettinga *et al.*, 1980, 1984, 1985; Hulshoff Pol *et al.*, 1983; de Zeeuw 1984; Dolfing and Blomen, 1985; Harada *et al.*, 1988). At mesophilic conditions, the activity of granules can be as high as 7 to 8 g COD/gVSS-day for granules developed on VFA mixtures (Thiele *et al.*, 1990). Granules from full-scale UASB reactors treating complex wastewaters usually possess activities ranging from 0.5 to 1.0 g COD/gVSS-d. A comparison of reported activities of granules from different wastes in terms of specific COD removal rate is provided in Table 1.7. Methane production rate and COD removal rate, however, do not describe the activity of individual microbial trophic (sub-trophic) groups in anaerobic sludge. Therefore, for investigation of biodegradability and toxicity of wastewater, application of specificsubstrate conversion rate rather than COD removal rate is recommended (Wu *et al.*, 1989).

iii) Type of granules

Depending on the predominant acetate utilizing methanogens observed, anaerobic granules developed on VFA mixtures (acetate, propionate and butyrate) under mesophilic temperature conditions have been classified into three types (Hulshoff Pol *et al.*, 1983; Lettinga *et al.*, 1984; de Zeeuw 1984, 1988), i.e.:

- "Rod-type" granules, which are mainly composed of rod-shaped bacteria resembling *Methanothrix* sp. in fragments of about 4 to 6 cells. This type of

granule originated from full-scale plants treating potato-processing waste and sugar-beet wastes, and developed in a lab-scale reactor started using a small amount of crushed granules and digested sewage sludge. These granules are compact and spherical;

- "Filament-type" granules, which consist predominantly of long multicellular rod-shaped bacteria. These granules were developed on VFA substrates using a digested sewage sludge as inoculum. The prevailing bacteria resembled the *Methanothrix soehngenii* which grows as long filaments. These granules appeared to be less dense;
- "Sarcina type" granules, which were developed in the presence of a high concentration of acetic acid in a reactor. The prevailing bacteria were *Methanosarcina*-like cells. These granules have a compact spherical shape.

To date, the reason why rod-type or filament-type granules are formed remains a question (Hulshoff Pol *et al.*, 1983; de Zeeuw, 1988) although several hypothesis were presented (Wiegant, 1988; Colleran, 1988). The original inocula (digested sewage sludges) of rod-type and filament-type granules were from two different sources (Lettinga and Hulshoff Pol, 1990). Three mesophilic strains of *Methanothrix* have been identified to be *Methanothrix soehngenii* which grow as long filaments (Touzel *et al.*, 1988; Kamagata and Mikami, 1990). The rod-type *Methanothrix* sp. has not been thoroughly investigated.

In UASB reactors fed with complex organic wastewaters, the granules formed are not reported to be distinctly identified into the three types mentioned above, except for the rod-type granules developed on potato-processing and sugar-beet wastewaters, respectively (Lettinga *et al.*, 1985).

iv) Microbial composition of granules

Direct count of cells using microscopy indicates that granular sludges contain cell counts between 1 x 10^{12} and 4 x 10^{12} cells per g VSS (Dubourguier *et al.*, 1988a, 1988c). Granular sludges, developed either on complex substrates or VFA mixtures, have been observed to contain all the microbial trophic groups typically found in anaerobic digestors, i.e. hydrolytic-fermentative, syntrophic acetogenic, and methanogenic bacteria as well as sulfate reducing bacteria. Methanogens, particularly acetate utilizers, were found to be the major microorganisms in all the granules examined. The most probable numbers (MPN) of different trophic groups in the granules treating starch industry wastewater were reported that the population of different trophic groups (cells/mL) were glucose and lactose utilizers of 10^9, H_2-CO_2-utilizing methanogens of 10^9, acetate-utilizing methanogens of 10^9, ethanol utilizing syntrophs 10^8 to 10^9, propionate and butyrate utilizing syntrophs of 10^8, SRB utilizing lactate of 10^8 to 10^9, SRB utilizing ethanol of 10^7, SRB propionate-utilizing of 10^7 and SRB butyrate-utilizing of 10^7 (Dubourguier *et al.*, 1988a). Dolfing *et al.* (1985) obtained 10^{10} cells/mL of sucrose degraders, 10^9 of hydrogen utilizing methanogens, 10^8 of acetate-utilizing methanogens, 10^7 of syntrophic propionate or butyrate utilizers in the granules treating liquid sugar wastewater.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have also been used to study the microbial composition and structure of granules. *Methanothrix* sp., which is easy to identify based on their bamboo-shape, is normally the predominant organism observed (Dolfing *et al.*, 1985; Dolfing, 1987; Hulshoff Pol *et al.*, 1983; Hulshoff Pol, 1989; Lettinga *et al.*, 1984, 1985; Wu *et al.*, 1985, 1987; Wiegant and de Man, 1986; Kimata *et al.*, 1990). *Methanobacterium* sp. and *Methanobrevibacter* sp. can be identified by their autofluorescence under

fluorescence microscopy or by TEM based on their morphotypes. Rod morphotype *Methanobacterium* or *Methanobrevibacter*-like bacteria rather than *Methanospirillum*-like bacteria were found, in granules from different sources, to be the major hydrogen utilizing methanogens (Dubourguier *et al.*, 1988b; Wu *et al.*, 1985). Using SEM and TEM presumptive identification and immunological probes, Dubourguier *et al.* (1988a, 1988b) and Prensier *et al.* (1988) reported that *Methanobrevibacter* sp. were the main H_2-utilizing methanogens in granules treating sugar beet waste.

Immunological techniques have also been used to characterize methanogenic species in granules (Koornneef *et al.*, 1990). Using antibody probes and antigenic fingerprinting methods, methanogens antigenically related to *Methanobacterium formicicum* strain MF, *Methanobrevibacter arboriphilicus* strain AZ, and *Methanothrix soehngenii* strain Opfikon were found in all granules from five UASB reactors maintained on different substrates. The other methanogens found were antigenically related to: *Methanospirillum hungatei* JF1 and *Methanobrevibacter smithii* ALI in four of the granules; *Methanobacterium bryantii* MoH in three; *Methanobrevibacter arboriphilus* DH1 in two; *Methanobrevibacter ruminantium* M1, *Methanogenium marisnigri* JR1 and *Methanosarcina thermophila* TM1 in one each. A trend was observed towards a wider diversity of methanogenic subpopulations parallelling with an increase in the complexity of substrate.

Microcolonies were found in granular sludges treating starch wastewater (Dubourguier *et al.*, 1988a, 1988c). *Syntrophobacter* sp. and *Syntrophomonas* sp. were identified as the propionate degrader and the butyrate degrader in these granules, respectively. These two syntrophs were associated with *Methanobrevibacter arboriphilicus* as microcolonies inside the granules and the distances between syntrophs

and the methanogens were very short. In these microcolonies, the ratio between methanogens and syntrophs was 2.33 to 2.46 for *Syntrophobacter* and 0.48 to 0.71 for *Syntrophomonas*.

Isolation and characterization of predominant bacteria in the granules is perhaps the best technique to aid in understanding the ecophysiology and the interaction among the different trophic groups, as well as the contribution of individual organism in the granulation process. Novaes *et al.*(1988) reported that three acetogens, *Butyrivibrio fibrisolvens*, *Clostridium* sp. and *Desulfovibrio desulfuricans*, and two methanogens, a hydrogen-utilizing *Methanobacterium* sp. and filament-forming *Methanothrix* sp. were isolated from the granules treating sewage.

1.3.4 Mechanism of granule formation

To date, the formation of the granules from non-granular materials and the growth of the granules are still not well understood at the microbial level. However, several hypothetical reasons for granulation have been discussed and summarized as follows.

i) The agglutination of *Methanothrix*

Based on direct microscopic and SEM examination, *Methanothrix* are the predominant bacteria in granules from various sources. This organism forms a network in the granules which may play an important role in the stabilization of the granular structure (Wiegant and de Man, 1986; Wiegant, 1988; Dubourguier *et al.*, 1988a, 1988c). In the formation of granules from non-granular material, the initial aggregation or initial granulation is a decisive step. It was proposed that the initial granulation might be attributed to the growth of *Methanothrix* sp. on small inert particles from the inoculum (Hulshoff Pol *et al.*, 1988), the growth of *Methanothrix*

sp. on clumps of *Methanosarcina* sp. (de Zeeuw, 1988), the bridge effect of *Methanothrix* sp. on microflocs originating from the inoculated digestor sludge (Dubourguier *et al.*, 1988a), or the formation of small aggregates of *Methanothrix* caused by biogas turbulence (Wiegant, 1988).

Granules, mainly consisting of *Methanothrix* sp., were obtained using acetate (2.8 to 3.0 g/L) as major carbon and energy source under thermophilic temperatures (Wiegant and de Man, 1986) and mesophilic temperatures (Narada *et al.* 1988). This fact supports the importance of *Methanothrix* in the granulation process. However, the role of other organisms can not be ignored since yeast extract (0.15 to 0.3 g/L) was added to the feed used in these experiments. For example, the presence of methanogenic activity from sucrose, propionate, formate and H_2-CO_2 was detected at levels as high as 10 to 5% of that from acetate (Narada *et al.* 1988). Therefore, further study still needs to be conducted to better establish the role of *Methanothrix* in the granulation process.

ii) Syntrophic microcolonies

It was proposed that syntrophic relationships in mixed cultures might eventually lead to the formation of stable microcolonies or consortia (Hirsch, 1984). Schink and Thauer (1988) calculated the diffusion distance for transfer metabolites (H_2 and acetate) and indicated that aggregation of bacteria of different trophic groups was important for energetics and kinetics of substrate conversion. Dubourguier *et al.* (1988a, 1988b, 1988c) observed syntrophic microcolonies in the granules and emphasized their importance in interspecies hydrogen transfer. However, no evidence has been presented to prove that the formation of syntrophic microcolonies proceeds formation of granules. The role of the syntrophic growth in the granulation process is, however, worth investigating.

iii) Extracellular polymer producing bacteria

Organisms that produce extracellular polymer and form clumps are also considered to play an important role in granulation. This is because polymers have been clearly observed on the surface of granules under SEM (Dolfing *et al.*, 1985; Dubourguier *et al.*, 1988a). The nature or role of these extracellular polymers and the organisms responsible for their production are not known. Further research is needed in this area.

Some hydrolytic-fermentation bacteria such as *Selenomans ruminantium, Clostridium butyricum* produce polysaccharides and form aggregates when grown on carbohydrate-containing media in anaerobic gas-lift reactors (Zoutberg *et al.*, 1988). It was reported that granulation on carbohydrate-containing wastewater was faster than on a VFA mixture (Lettinga *et al.*, 1984; de Zeeuw, 1988). However, it is not reasonable to draw any conclusion that the polysaccharides produced by fermentative bacteria play an essential role in granulation. These bacteria do not exist in a large numbers in the granules developed on VFA mixtures or even in granules fed with carbohydrate-containing wastewater. The fraction of extracellular polysaccharids in granules is only 1 to 2% based on dry weight (Dolfing *et al.*, 1985). This is contrast to approximately 30% in aggregates of *Clostridium butyricum* (Zoutberg *et al.*, 1988). Polymers other than polysaccharides may play an important role in binding bacteria together. Clearly more research is needed.

Hydrogen utilizing methanogens exist at a great number in most granules. Sam-Soon *et al.* (1988) proposed that the pelletization in anaerobic reactors was due to the action of *Methanobacterium* strain AZ (i.e. *Methanobrevibacter arboriphilicus* AZ). This methanogen uses hydrogen as its only energy source and produces all of the amino acids (except cysteine) required for cell synthesis (Zehnder and Wuhrmann,

1977). At a high hydrogen partial pressure and limited cysteine supply, overproduction of amino acids occurs. These acids induce formation of extracellular long-chain polypeptides which bind this microorganism and other bacteria to form the pellets or granules. This hypothesis, however, cannot explain why granulation occurs in low hydrogen partial environments, when VFA mixtures are used as substrates (Hulshoff Pol *et al.*, 1983; de Zeeuw, 1984) or acetate plus a small amount of yeast extract is used as substrate (Wiegant and de Man, 1986; Narada *et al.*, 1988). The role of hydrogen-formate utilizing methanogens in granule formation needs to be evaluated further.

iv) Mineral precipitates and small inorganic particles

Small sand and other inert particles are frequently observed in granules (Lettinga *et al.*, 1984; de Zeeuw, 1984; Dubourguier *et al.*, 1988a). The attachment of cells to the surfaces of inert matter has been proposed as the initiating step for granulation (Archer, 1988). Granules be developed using powdered zeolite (100 μm in size) as carrier (Yoda *et al.*, 1989). Hulshoff Pol (1989) reported that when the inert particles (40 to 100 μm in size) were removed from inoculated sewage sludge, granulation was not observed within the period of time that was usually required for the formation of granules. However, granules can be developed without the addition of any inert materials (Lettinga *et al.*, 1985; Thiele *et al.*, 1990).

Mineral precipitates, such as calcium phosphate, calcium carbonate and ferrous sulfide, are formed as a consequence of metabolic activities and accumulate inside granules (Wu *et al.*, 1987; Dubourguier *et al.*, 1988a, 1988b). Ferrous sulfide was observed attached firmly to the sheath of *Methanothrix* sp. and, therefore, was thought to stabilize bacterial aggregation within granules (Dubourguier *et al.*, 1988a). Low concentrations of Ca^{2+} (100 to 200 mg/L) was found to have a positive effect on

granulation (Hulshoff Pol *et al.*, 1983). High concentrations of Ca^{2+} (>600 mg/L) has a negative influence on granule formation (Hulshoff Pol *et al.*, 1983; Guiot *et al.*, 1988). EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,-tetraacetic acid), a Ca^{2+} chelating agent was used to treat several granular sludges from a laboratory-scale reactor and full-scale reactors (Grothenthuis *et al.*, 1988b). In a EGTA/Ca^{2+} solution with a ratio of 1:3 (mol:mol), the granules disintegrated or became weaker. Based on this, Grothenthuis *et al.*(1988b) suggested that calcium might play an important role in granule structure as calcium phosphate precipitates for adhesion of bacteria or/and a constituent of extracellular polymers. However, no conclusive evidence has been reported that indicates mineral precipitates are essential for granulation.

Granulation is undoubtedly a complex process which includes several individual steps and involves a number of different bacteria, some of which may not be essential but do accelerate the process. Identifying and characterizing the key organisms in granules is one way to study the granulation process.

1.4 GOALS AND OBJECTIVES OF THIS STUDY

The objectives of this study were to characterize the principles for the cultivation of anaerobic granules, the effect of microbial composition in granules on the physical, operational, kinetic and metabolic performance of granules, and the prevalent bacterial species in granules and their roles in granule formation.

In order to examine and generalize the empirical principles for granulation obtained from previous experiments on anaerobic digested sludges with VFA mixtures, both anaerobic digested sewage sludge and aerobic activated sludge were used as

inoculum for the cultivation of granules using complex organic wastewaters (glucose molasses solution and brewery wastewater) (Chapter 2).

Two typical types of granules (rod-type and filamentous-type) were compared with regard to operational performance, physical and chemical characteristics, substrate kinetics, and microbial populations. The role of microbial populations was investigated for granules developed on a VFA mixture with low levels of sulfate (Chapter 3).

Granules developed on a brewery wastewater containing moderate concentrations of sulfate (0.6 to 1.3 mM) were characterized with respect to their metabolic performance and granular structure. The role of sulfate reducing bacteria was investigated (Chapter 4).

The prevalent microbial species in the rod-type granules were isolated and characterized in order to understand the role of individual species in granular structure (Chapter 5).

The metabolism of fatty acids (with 1 to 5 carbon atoms) and H_2-CO_2 in the isolated species was characterized and compared with the rod-type granules to understand the role of these species in fatty acid degradation by the granules. (Chapter 6).

Based on the isolation and characterization of the prevalent species in granules, the microbial and physicochemical factors affecting granulation were investigated. Defined bacterial species were used to develop granules to determine their role in aggregation and granule formation (Chapter 7).

CHAPTER 2

CULTIVATION OF GRANULES IN LAB-SCALE UASB REACTORS FED WITH COMPLEX SUBSTRATES USING NON-GRANULAR INOCULUM

2.1 INTRODUCTION

Obtaining anaerobic granules from non-granular inoculum materials is a difficult start-up problem for the application of the UASB process. To date, detail studies on granulation have been performed using volatile fatty acid (VFA) mixtures as feed and anaerobic digested sewage sludge as inoculum (Hulshoff Pol *et al.*, 1983; Hulshoff Pol, 1989; de Zeeuw 1984). The granulation process, when complex organic compounds are used as feed, has not been studied in detail. Other inocula besides anaerobic digested sludge and cow manure have not been evaluated for granulation under mesophilic temperature conditions.

In this chapter, granulation with anaerobic digested sludge as inoculum on a glucose molasses solution and brewery wastewater was investigated to characterize granulation on complex substrates. The results are compared with the results obtained using VFA mixtures. Significant levels of methanogens were observed in aerobic activated sludges and, therefore, activated sludge was used as inoculum to cultivate

anaerobic granules and study granulation process. The granules formed were primarily characterized in terms of physical characteristics, chemical composition, metabolic activity and microbial structure. Some empirical parameters for the cultivation of granules are discussed and summarized.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals

All chemicals used were of analytical grade and obtained from Beijing Analytical Chemical Company (Beijing, China) except glucose molasses (containing 70% glucose) (Wuhan Sugar Company, Wuhan, Hubei, China).

2.2.2 Activated sludge samples for enumeration of methanogens

Four activated sludges were utilized as the samples for the enumeration of methanogenic bacteria. Sludge No.1 was obtained from an aeration tank (dissolved oxygen of 4.0 mg/L) at the Gaobeidian Sewage Treatment Plant, Beijing; No.2 from a secondary sedimentation tank of an activated sludge plant in the Changzhou Dongfanhong Textile Company, Changzhou; No.3 from an activated sludge aeration tank treating chemical wastewater at the Xiayoung Petrochemistry Company, Beijing; and No.4 from a secondary sedimentation tank in the 1st National Textile Company of Sichuan Province, Chengdu, China. These activated sludges are representative of the activated sludges treating sewage and industrial wastewater.

2.2.3 Enumeration of total methanogenic bacteria

The enumeration experiments of total methanogenic bacteria in different aerobic

activated sludge and anaerobic granules were conducted using the Hungate technique (Bryant, 1972). Preparation and sterilization of medium and dilution of samples were performed under oxygen-free nitrogen atmosphere. Glass pressure tubes (20 ml in volume, Tiangjing Glass Instrument Company, Tiangjing, China) with butyl rubber stoppers were used in these experiments.

The enumeration medium contained following components (per liter): NH_4Cl, 1.0 g; $MgCl_2 \cdot 6H_2O$, 0.1 g; $CaCl_2$, 0.1 g; K_2HPO_4, 0.4 g; KH_2PO_4, 0.4 g; $Na_2S \cdot 9H_2O$, 0.1 g; $NaHCO_3$, 1.0 g; yeast extract 2.0 g; trypticase peptone, 2.0 g; L-cysteine-HCl, 0.5 g; sodium acetate, 2.0 g; sodium formate 2.0 g; resazurin, 0.001 g; trace element solution (Zeikus and Wolfe, 1973), 10 mL and vitamin solution (Zeikus and Wolfe, 1973), 10 mL. The final pH was 7.0 to 7.2. After inoculation, each anaerobic tube was pressurized with H_2-CO_2 (80:20) gas mixture by 0.2 atm. Each tube contained 4.5 mL of the medium.

A serum bottle (50 mL in volume), containing about 10 glass beads (3-4 mm diameter), was made anaerobic by flushing with O_2-free nitrogen, then filled with 30 mL of activated sludge or anaerobic granules plus the effluent liquid from a laboratory-scale UASB reactor (described below), and subsequently sealed with a black rubber stopper. The sample in the bottle was dispersed using a vibrator and inoculated into the tubes. Serial dilutions were made from 10^{-1} to 10^{-8} for the activated sludges and from 10^{-1} to 10^{-11} for the granules. Three or five tubes per dilution were used. The inoculated tubes were incubated at 35 °C for 25 days. Methane production was checked by gas chromatography to establish whether or not the tubes were positive for methanogens. After one week , methane was checked again. The Most Probable Number (MPN) value in the sample and 95% confidence limits were calculated by using a microcomputer program for the computation of MPN (MacDonell *et al.*, 1984).

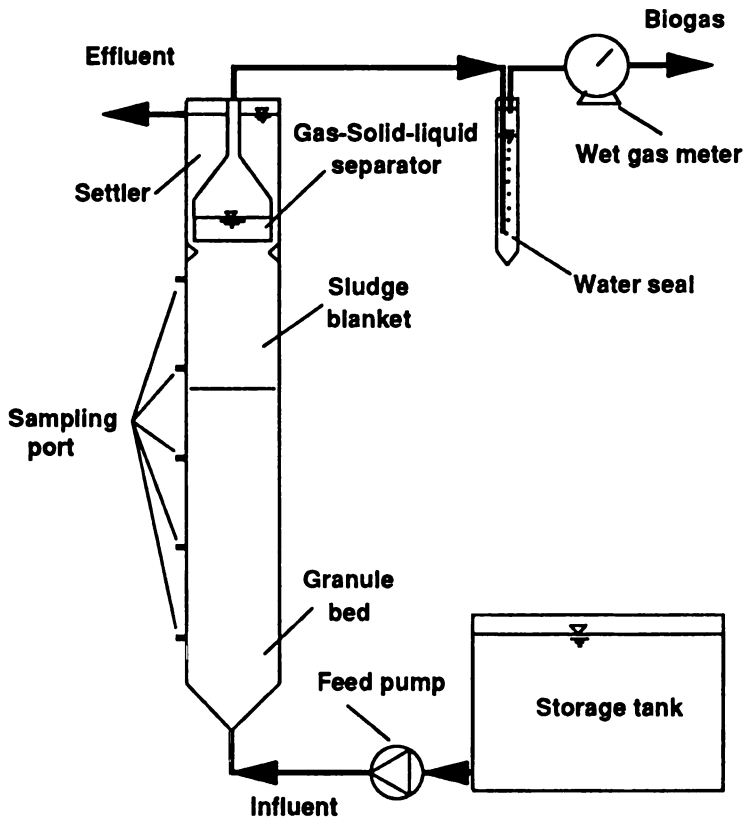


Figure 2.1 Schematic diagram of laboratory-UASB reactors used in granulation experiments

2.2.4 Laboratory-scale UASB reactors

Three laboratory scale-UASB reactors (A, B and C) made of plexiglas tubes were used for the cultivation of anaerobic granules. A schematic diagram of the three reactors is presented in Figure 2.1. The feed was stored in a polyvinyl chloride (PVC) plastic tank (200 liter in volume) and delivered to the reactors on a continuous basis using a IZJT replacement pump (Shijiazhoung Chemical Engineering Institute, Shijiazhoung, China). The volume of biogas produced was measured using a DSD-0.5 wet gas meter (Shanghai Gas Meter Company, Shanghai, China). Reactors A and B were identical with an inner diameter of 10.4 cm and a height of 2.6 m. Their total volume was 21 liters (reaction volume 17.8 L). The total volume of reactor C was 29 L (reaction volume 23 L) with an inner diameter of 14 cm and a height of 2.0 m. The sampling ports were placed along the profile (height) of the three reactors. All the reactors were installed in a 35 °C constant temperature chamber.

2.2.5 Inoculum materials

Two different inocula were used for the reactor experiments. The inoculum of reactors A and B was an anaerobic digested sludge obtained from an Imhoff tank at the Jiuxianqiao Sewage Treatment Plant, Beijing. The inoculum of reactor C was activated sludge from an aeration tank at the Gaobeidian Sewage treatment Plant, Beijing.

2.2.6 Feed composition

Two feed solutions were used in this study. Reactors A and C were fed with a glucose molasses solution while reactor B was fed with a brewery wastewater (Table 2.1). The glucose molasses solution was prepared with glucose molasses every other

day. Nickel (as $NiSO_4 \cdot 6H_2O$), cobalt (as $CoCl_2 \cdot 6H_2O$), and molybdenum (as $Na_2MoO_4 \cdot 2H_2O$) were added to this solution to achieve concentrations of 0.1, 0.05, and 0.05 mg/L, respectively. The brewery wastewater (15,000 to 20,000 mg COD/L) was collected from the saccharification and yeast processing sections at Beijing Brewery Company. The wastewaters from the two sections were mixed (1:1, vol:vol) and diluted with tap water to achieve an influent COD concentration between 1,000 and 4,000 mg/L, simulating the concentration of the total sewered brewery wastewater for this plant.

Table 2.1 Composition of feeds used in granulation experiments

Reactor	A and C	B
Feed	Glucose	Brewery
COD(mg/L)	1000-4000	1000-4000
BOD_5/COD	0.8	0.74
BOD_5/Kjeldahl nitrogen(as N)	30	34-56
BOD_5/phosphates(as P)	150	ND
Ca(mg/L)	80-90	80-90
Na_2CO_3(mg/L)	250-600	250-600
Acetate(mg/L)	ND	200-500
Propionate (mg/L)	ND	100-250
Butyrate (mg/L)	ND	30-80
Ethanol (mg/L)	ND	140-280
pH	5.5-6.0	5.5-6.0
Trace element supplemented		
Ni^{2+} (mg/L)	0.1	0.1
Co^{2+} (mg/L)	0.05	0.05
Mo^{2+} (mg/L)	0.05	0.05
$FeCl_3$ (mg/L)	10	NA

(1) ND: not determined. NA: not added.
(2) $NiSO_4$, $CoCl_2$ and Na_2MoO_4 were used as trace element sources for nickel, cobalt and molybdenum, respectively.

2.2.7 Analysis of mineral composition in granules

Granules (10 mL) were washed 5 times with 30 mL of double distilled water, to remove any wastewater from the reactors, and then centrifuged at 3,000 xg for 5 min. The pellet was then transferred to a pre-weighed porcelain evaporating dish. The dry weight (103 °C) and the ash weight (550 °C) of the pellet were then determined. The ash was then dissolved into a mixture (3 mL) of concentrated HNO_3, H_2SO_4 and HCl (1:1:1). The supernatant of the acidified solution, after settling, was diluted with double distilled water by 5 to 20 times prior to analysis. Metals, including Fe, Ca, Mg, Mn, Ni, Co, Na and K, in the diluted sample were determined using a Hitachi 180-80 Polarized Zeeman Atomic Spectrophotometer.

2.2.8 Light and electronic microscopic examination

Sludges and granules were examined using a Leitz Orthoplan Microscope. Sample preparation of granular sludges for scanning electron microscopy (SEM) is described in Appendix D. The SEM graphs were taken on a Hitachi S520 and a JEOL JSM 35C at 25 kV. They were equipped with a EDA x 9100 X-ray spectrometer (Hitachi Donshi Ltd., Japan).

2.2.9 Analytical Methods

Analysis for the determination of COD, BOD_5, suspended solids (SS), volatile suspended solids (VSS), NH_4-N, Kjeldahl nitrogen, and alkalinity as well specific gravity of granular sludge were made in accordance with Standard Methods (APHA *et al.*, 1980). Sludge volume index (SVI) was measured using a 100 mL graduated cylinder.

Volatile fatty acids (VFA) were determined using a 102G gas chromatograph (Shanghai Analytic Instrument Company, Shanghai, China) with a flame ionization detector (FID) and nitrogen as carrier gas. Methane and CO_2 were measured by a 102G gas chromatograph with thermal conductivity detector (TCD) and N_2 as carrier gas. The pH was measured using an PHS-2 precision pH meter (Shanghai Analytical Instrument Co., Shanghai, China).

2.3 MOST PROBABLE NUMBER (MPN) ENUMERATION OF METHANOGENS IN ACTIVATED SLUDGES

To identify whether the activated sludge samples contained methanogens, three kinds of substrates (i.e. acetate, formate and H_2-CO_2) were added to the enumeration medium. These substrates can be utilized by the known methanogenic bacteria. Therefore, the counting results should accurately indicate the number of total methanogens. The results of the MPN enumeration demonstrated that a significant and consistent population of methanogens was present in each of the activated sludges at levels of ca. 10^8/gSS (Table 2.2).

Microscopic examination indicated that, in these activated sludges, some clumps of rods and cocci exhibiting blue-green autofluorescence were dispersed inside the sludge flocs. These bacteria were likely hydrogen-utilizing methanogens. After incubation of for over a month, *Methanococcus*-like cocci and *Methanobacterium*-like rods exhibiting autofluorescence were observed very clearly in the 10^{-5} and/or 10^{-6} MPN tubes while *Methanosarcina*-like aggregates were observed in lower dilution MPN tubes (10^{-2} to 10^{-3}). *Methanothrix*-type cells could not be identified in the MPN tubes, however, since this species does not exhibit autofluorescence.

This results indicated that activated sludge contains enough methanogens to serve as an inoculum for an anaerobic reactor.

Table 2.2 MPN values of methanogenic bacteria in activated sludges from four treatment plants

Sample	SS conc.	Tubes per	MPN		95% confidence limits $x10^8$/gSS	
	gSS/L	dilution	$x10^5$/mL	$x10^8$/gSS	lower	upper
No.1	4.0	3	4.6	1.1	0.25	5.0
No.2	10.9	3	15	1.4	0.38	4.9
No.3	11.1	5	9.2	5.8	2.6	26.5
No.4	11.9	5	1.7	1.3	0.55	3.9

No.1: sludge treating sewage, VSS/SS=0.58.
No.2: sludge treating textile wastewater, VSS/SS=0.67.
No.3: sludge treating petro-chemistry wastewater, VSS/SS=0.63.
No.4: sludge treating textile wastewater,VSS/SS=0.75.

2.4 GRANULATION PROCESS IN LABORATORY SCALE UASB REACTOR

2.4.1 Anaerobic digested sludge as inoculum fed a glucose molasses solution

Reactor A was inoculated with an anaerobic digested sludge and fed a glucose molasses solution to investigate the process of granulation on a complex substrate and the effect of granulation on sludge behavior in a sludge blanket.

Before start-up, reactor A was filled with 10 L of medium containing glucose

molasses (COD 1,000 mg/L) and 10 L of a dilute anaerobic digested sewage sludge containing 1080 g SS (26% volatile). After inoculation, the reactor was incubated at 35 °C without feed for 14 hours and subsequently fed continuously at the feed rate of 0.26 L/hr. It required 160 days to complete granulation in the reactor. Operational data including COD concentrations in influent and effluent , volumetric COD loading rates, COD removal percentages, total biomass content (in terms of SS and VSS), as well as specific COD loading rates and specific COD removal rates, are illustrated in Figure 2.2a and 2.2b. The operational data obtained from this reactor is summarized in Table 2.3. The pH in the effluent was maintained between 6.4 and 6.9 with the alkalinity ranging from 600 to 700 mg/L (as $CaCO_3$). In most cases, effluent COD concentrations were lower than 300 mg/L with acetate and propionate lower than 1.0 mM, and 0.5 mM, respectively. Butyrate was not detected. Granulation in this reactor was similar to that on a VFA mixture using anaerobic digested sludge as inoculum reported by Hulshoff Pol *et al.* (1983). It can be described as occurring in three phases:

i) Phase I: start-up (day 1 to day 77)

In this phase, the sludge in the reactor developed from a poorly aggregated form to relatively dense flocs. No granules were observed in the reactor. The initial volumetric COD loading rate was 1 g/L-d and the specific COD loading rate less than 0.1 g/gVSS-d. The reactor loading rate was gradually increased to a specific COD loading rate of 0.3 g/ gVSS-d (Figure 2.2b and Table 2.3). At the end of this phase, expansion of sludge bed was observed. The solid concentration in sludge blanket increased from 1.0 to 3.0 gSS/L to 22.5 gSS/L over this period.

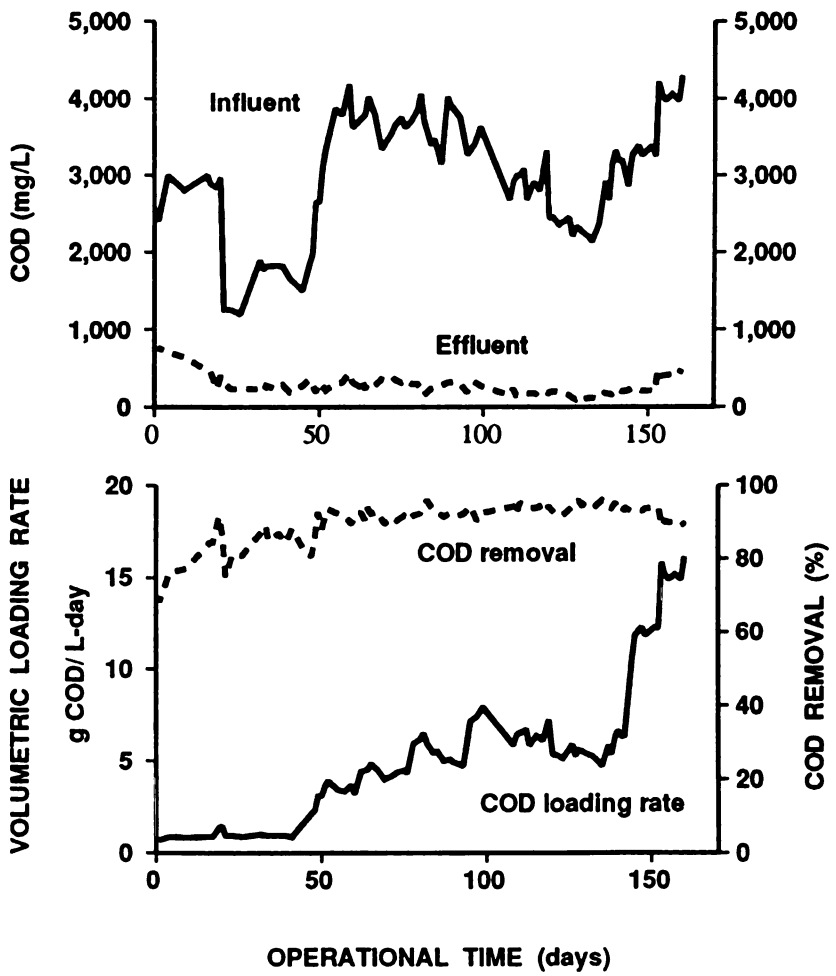


Figure 2.2a Operational results of reactor inoculated with digested sludge and fed with glucose-molasses (reactor A).

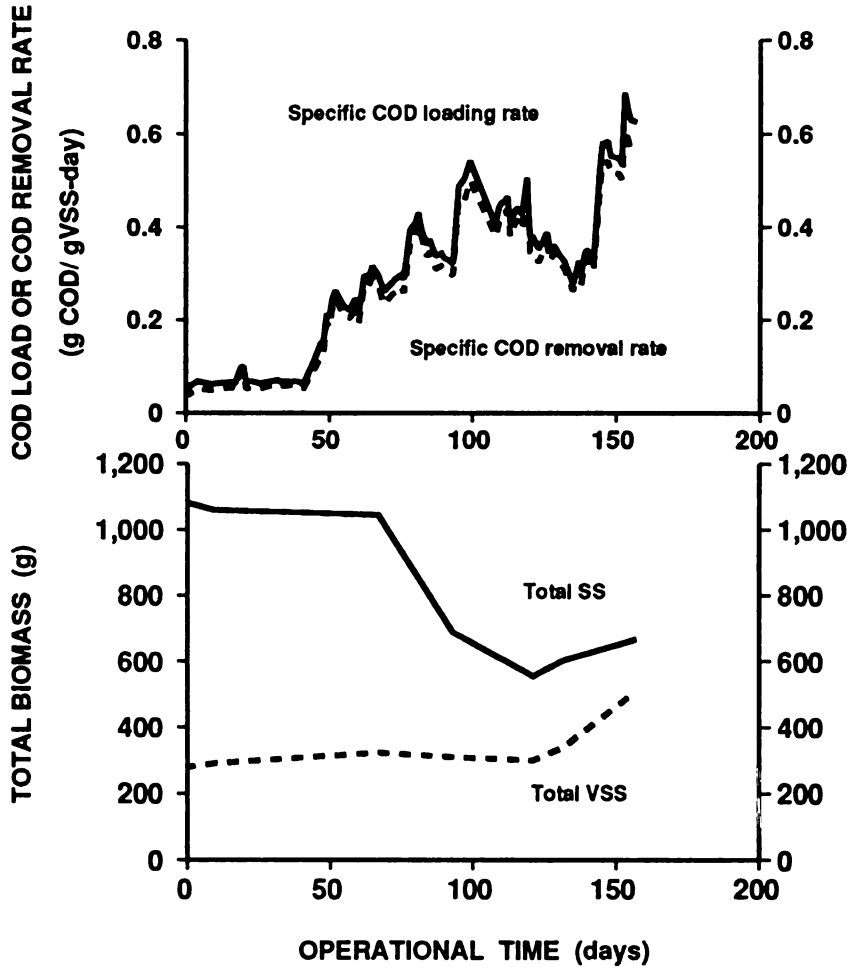


Figure 2.2b Operational results of reactor inoculated with digested sludge and fed with glucose-molasses (reactor A).

Table 2.3 Operational results of reactor inoculated with digested sludge and fed with glucose-molasses during granulation (reactor A)

Date day	Inf. COD g/L	COD removal %	COD loading rate g/L-d	COD loading rate g/gVSS-d	Gas production L(STP)/L-d	CH_4 %	HRT hr
Phase I							
1-16	3.5-3.8	81-88	1.0-1.1	0.073-0.076	0.22-0.28	ND	80
17-20	2.8-2.9	87-89	1.3-1.4	0.1	0.48-0.49	ND	50
21-27	1.2-1.3	78-83	0.9-1.0	0.07	0.39-0.4	ND	32
28-41	1.6-1.8	88-91	0.9-1.0	0.07	0.35-0.42	ND	45
42-54	1.5-3.3	82-92	1.7-3.9	0.15-0.24	1.0-1.8	60	20
55-60	3.1-4.2	89-92	2.8-3.7	0.18-0.29	1.3-2.1	61	17
61-77	3.6-4.0	89-93	4.3-4.8	0.28-0.31	2.6-3.0	65	20
Phase II							
78-88	3.6-4.7	90-94	5.8-7.5	0.38-0.44	2.7-3.8	60	15
89-94	3.8-4.0	93-94	4.8-5.1	0.33-0.35	2.5-2.8	60	19
95-125	2.4-3.2	93-95	5.2-7.2	0.43-0.52	2.8-3.5	60	11
126-134	2.2-2.9	93-95	5.3-7.0	0.35-0.40	2.6-3.2	62	10
Phase III							
135-142	2.7-3.4	93-95	5.4-6.8	0.35-0.40	3.0-3.5	62	12
143-151	3.2-3.4	95-96	11.6-12.4	0.61-0.64	4.9-5.5	60	6.5
152-160	3.8-4.3	87-95	14.3-16.1	0.62-0.70	7.1-7.9	60	6.5

(1) The date sequence begins on the first day of the continuous feed.
(2) ND: not determined.

ii) Phase II: granule formation and floc wash-out (day 78 to day 134)

In this phase, the volumetric COD loading rate was maintained at about 5 g/L-d with a specific loading rate of approximate 0.3 to 0.4 g/gVSS-d. A small amount of granules were observed in the sludge from the bottom of the sludge bed between day 80 and day 90. Subsequently, granules developed through whole sludge bed. Thediameter of the granules initially formed was about 0.1 to 0.2 mm. In this phase, sludge concentration in sludge blanket was maintained as high as 23 gSS/L, and the light fraction of the sludge (consisting mainly of flocs) was washed out through the top settler of the reactor. At the end of this phase, the total mass of sludge (SS) in the reactor was reduced to a minimum level (Figure 2.2b).

iii) Phase III: granule growth (day 135 to day 160)

In this phase the flocculent sludge in sludge blanket was almost completely washed-out. Granules developed quickly and filled the sludge bed. The total mass of sludge began to increase since the granules formed were not washed-out. Consequently, a volumetric COD loading rate up to 16 kg/m^3-d and hydraulic retention time of 6.5 hours were achieved with a COD removal of more than 90%. The sludge concentration in the sludge blanket eventually dropped to 6.4 gSS/L (Table 2.4).

During the granulation process, the concentration and nature of sludge in the sludge blanket changed (Table 2.4). Initially, sludge was in a flocculent form with low concentrations (1 to 3 gSS/L) in the sludge blanket. At the end of phase I and during phase II, the solids concentration increased while the flocculent sludge washed out. Eventually, the solids concentration declined to a low level as small granules became the major components of the sludge. The settling property and sludge volume index (SVI) improved as granules developed. The results of settling tests of the sludge taken

from sludge blanket during granulation are illustrated in Figure 2.3. When flocculent sludge was predominant, zone settling prevailed, and a clear interface occurred between the top clarified liquid and the sludge blanket in the cylinder. When both granules and floc were present (Phase II), settling began as flocculent settling without an interface and, after 2 minutes, became the zone settling. When granules or distinct particles prevailed (Phase III), no interface formed during the settling tests. Free settling was observed. Flocculent sludge had a SVI of 29 mL/gSS while granular sludge had a SVI of 21 mL/gSS.

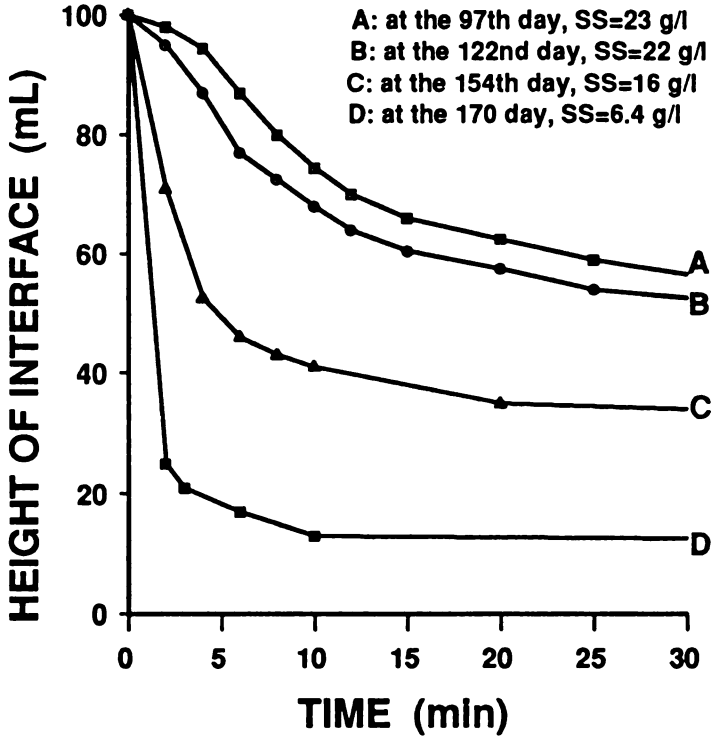


Figure 2.3 Results of settling tests conducted in a 100 mL cylinder with sludge from reactor A at day 97 (A), day 121 (B), day 154 (C), and day 170 (D), respectively.

Table 2.4 Composition and settleability of sludge from sludge blanket region during granulation in reactor A

Date day	SS g/L	v m/h	SVI mL/g	Sludge composition
Phase I				
2	0.5	ND	ND	tiny floc
50	3.0	ND	ND	floc
60	4.0	ND	ND	floc
68	22.5	ND	ND	floc
Phase II				
90	23.0	ND	ND	floc
97	23.3	0.30	24.3	floc
108	16.1	0.54	29.3	floc
119	19.1	0.53	28.6	floc, few granules
122	22.1	0.40	24.4	floc, a few granules
123	21.1	0.44	22.4	floc plus granules
Phase III				
148	14.3	1.37	21.5	granules, a few flocs
154	10.9	1.52	21.0	granules, few flocs
170	6.4	ND	21.0	granules

(1) v: The maximum settling velocity of sludge interface was measured in a 100 mL graduated cylinder. Sludge samples were withdrawn from the sampling port at 2 m height of reactor A.
(2) ND: Not determined.

2.4.2 Anaerobic digested sludge as inoculum for the brewery wastewater fed reactor

Reactor B was inoculated with anaerobic digested sludge and fed a brewery wastewater. This experiment was designed to investigate the feasibility of obtaining granular sludge using brewery wastewater and examine the effect of COD loading rate on granulation.

Reactor B was inoculated with 10 liters of dilute anaerobic digested sludge with a higher volatile fraction (42%) than that in reactor A (20%). After inoculation, a brewery wastewater (diluted to approximately 1,000 mg COD /L) was fed intermittently (for 18 hours per day) at a flow rate of 0.5 L/hr. After 10 days, the reactor was fed continuously at 0.5 L/hr.

Continuous operation lasted 75 days. Granulation occurred more quickly in reactor B than in reactor A. The pH in the effluent ranged from 6.9 to 7.2 during the operational period. The results of continuous operation are illustrated in Figure 2.4 and summarized in Table 2.5. The three phases of granule formation observed in reactor A, were also observed in reactor B. The length of each phase, however, was reduced compared with reactor A.

During Phase I (day 1 through day 18), reactor B received higher volumetric COD loading and higher specific COD loading rates than reactor A. After continuous feeding of reactor B for 10 days, the volumetric COD loading rate was increased from 2-3 to 4.0-5.0 g/L-d (0.2 to 0.35-0.44 g/g VSS-d). After 10 days, COD removal increased from 58% to 75%. By day 18, COD removal reached 92% with a loading rate of 5.5 g/L-d. During this period, the sludge appeared flocculent, much like that observed in reactor A during phase I. No granules were observed, and expansion of

the sludge bed was observed.

Phase II in reactor B lasted 24 days (day 19 through day 42). The volumetric COD loading rate exceeded 6 g/L-d (Figure 2.4a), resulting in a specific loading rate greater than 0.4 g/g VSS-d (Figure 2.4b). On day 19 a few small granules, 0.1 to 0.2 mm in diameter, were observed at the bottom of the sludge bed. Upon increasing the COD loading rate (> 0.6 g/gVSS-d) and the hydraulic loading rate (0.37 to 0.49 m/hr), granulation progressed rapidly. The sludge blanket composition changed from mostly floc to primarily granules within 10 days. On day 42 the total amount of sludge in the reactor reached a minimum level due to continuous wash-out of flocs. By this time, most of the sludge had become granular in nature.

A dense granule bed developed by the beginning of Phase III (day 43 to day 75). The granule bed increased in height continuously as the volumetric COD loading rate was increased from 11 to 32 g/L-d (0.9 to 2.2 gCOD/gVSS-d). The sludge blanket region was had a solids concentration of 3 to 4 gSS/L, and the sludge bed had a concentration of 70 gSS/L. Biogas production ranged from 7 to 17 L/L-d in the reactor (biogas flux of 0.28 to 0.96 m/hr). COD removal was about 90% at a HRT of 3.2 hours with an influent COD of 4,250 mg/L.

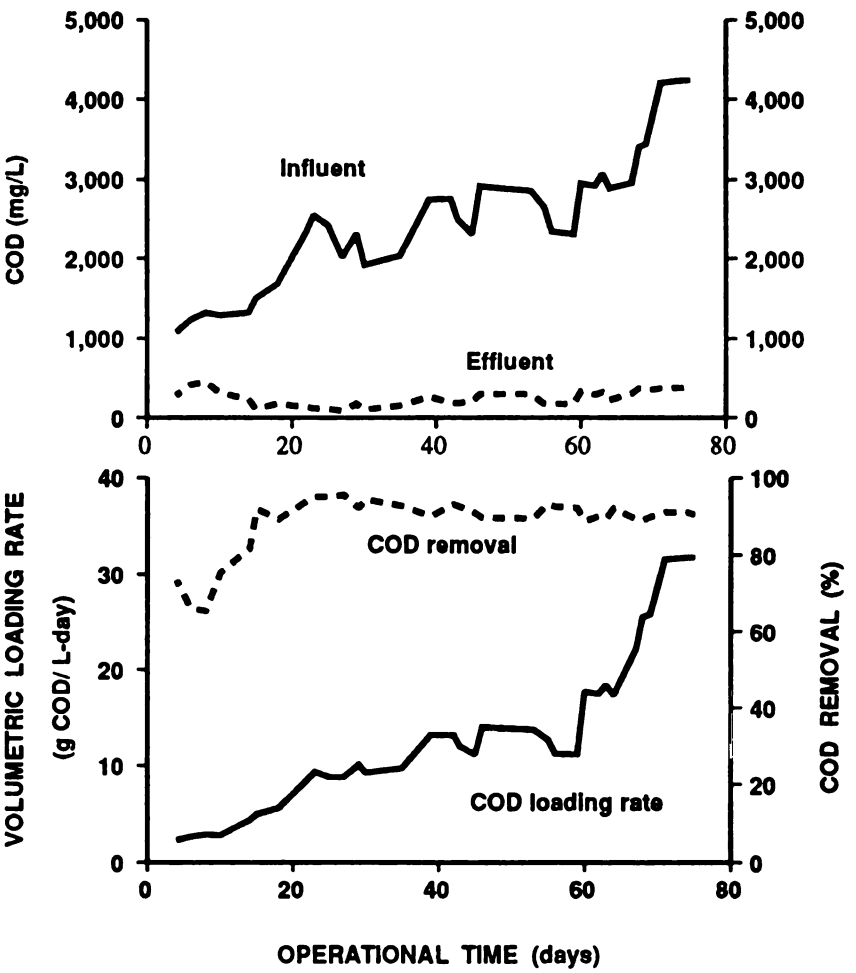


Figure 2.4a Operational results of reactor inoculated with digested sludge and fed with brewery wastewater (reactor B)

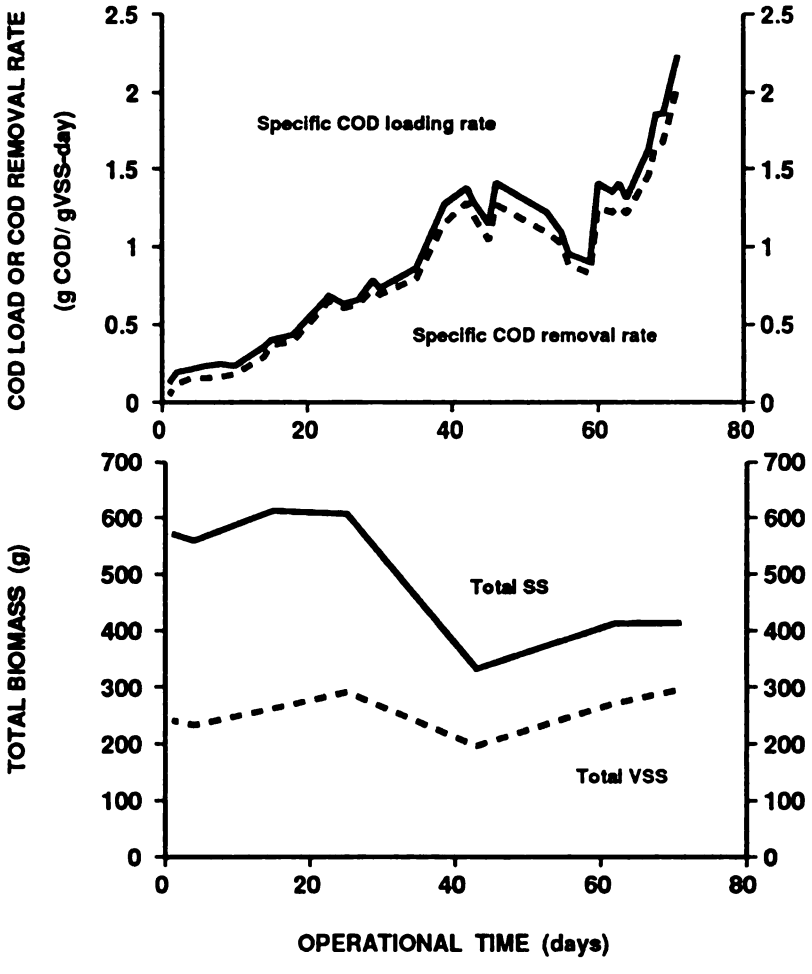


Figure 2.4b Operational results of reactor inoculated with digested sludge and fed with brewery wastewater (reactor B)

Table 2.5 Operational results of reactor inoculated with digested sludge and fed with brewery wastewater (reactor B)

Date day	Inf. COD g/L	COD removal %	COD loading rate g/L-d	COD loading rate g/gVSS-d	Gas production L(STP)/L-d	CH_4 %	HRT hr
Phase I							
1-10	1.0-1.3	58-75	2.0-3.0	0.18-0.24	0.56-1.22	79	11
11-18	1.3-1.7	82-92	4.3-5.5	0.35-0.44	2.0-2.6	80	7.2
Phase II							
19-25	2.0-3.0	94-96	6.1-9.0	0.60-0.63	3.3-4.8	80	6.6
26-29	2.0-2.5	94-96	8.7-9.0	0.61-0.68	4.1-4.8	80	5.5
30-42	1.9-2.7	92-94	9.1-12.9	0.76-1.3	4.2-6.2	80	5.0
Phase III							
43-59	2.3-2.8	89-92	11.0-13.7	0.9-1.40	5.7-6.9	80	5.0
60-66	2.9-3.0	86-92	17.0-18.3	1.3-1.40	7.1-8.6	79	4.0
67-75	2.7-4.25	89-92	22.5-31.7	1.5-2.2	10-17.2	79	3.2

The date sequence begins on the first day of the continuous feed.

2.4.3 Activated sludge as inoculum fed a glucose molasses solution

Reactor C was used to investigate the granulation process using activated sludge as the inoculum.

After thickening, activated sludge of 22 liters, containing 179 g VSS (volatile fraction of 68%), was added to reactor C. This resulted in an average VSS concentration of 7.8 g/L in the reaction region. The reactor was fed intermittently for 14 days prior to the start of continuous feed. For the first 5 days, glucose molasses (20 g) and $NaHCO_3$ (6 g) were added daily to reactor C; thereafter, a glucose molasses solution (1000 mg COD/L) was fed intermittently at a flow of 1 liter/hr for 10 to 12 hr each day.

The continuous feed operation lasted 70 days. Changes in the volumetric and specific COD loading rate, COD removal, COD concentration in influent and effluent, and total biomass concentrations (SS and VSS) are summarized in Figure 2.5. Operational data are summarized in Table 2.6. At the beginning of the continuous feed period, the volumetric COD loading rate was set at 1.0 g/L-day. COD removal was only 65% at that time. After a week, the COD loading rate was increased to 1.5 g/L-day and COD removals of 70% were observed. As the operation continued, COD loading rate was increased to 6.0 g/L-day by day 27 and to ca. 12.0 g/L-d (with a COD removal of 80 to 85%) by day 60. A higher specific COD loading rate was applied to reactor C compared with reactor A. After 20 days of continuous operation reactor C was loaded to more than 0.5 gCOD/gVSS-d.

Table 2.6 Operational results of reactor started with activated sludge and fed with glucose-molasses (reactor C)

Date day	Inf. COD g/L	COD removal %	COD loading rate g/L-d	COD loading rate g/gVSS-d	Gas production L(STP)/L-d	CH_4 %	HRT hr
Phase I + Phase II							
1-6	0.9-1.0	64-67	1.0-1.2	0.18-0.20	0.30-0.37	70	23
7-14	1.2-1.5	70-74	1.5-1.9	0.32-0.35	0.49-0.60	67-70	20
15-19	1.2-1.4	67-70	2.0-2.5	0.36-0.46	0.66-0.78	67	15
20-23	1.3-1.6	68-73	3.4-3.8	0.48-0.63	0.82-0.92	68	10
24-38	1.5-1.8	66-77	5.3-6.8	0.67-0.99	1.3-2.2	66	7
Phase III							
39-60	2.1-3.0	78-90	7.5-9.9	0.95-1.1	2.5-3.7	68	7
61-70	3.6-3.8	80-85	11.8-13.2	1.2-1.4	4.2-5.1	67	7

The date sequence begins on the first day of the continuous feed.

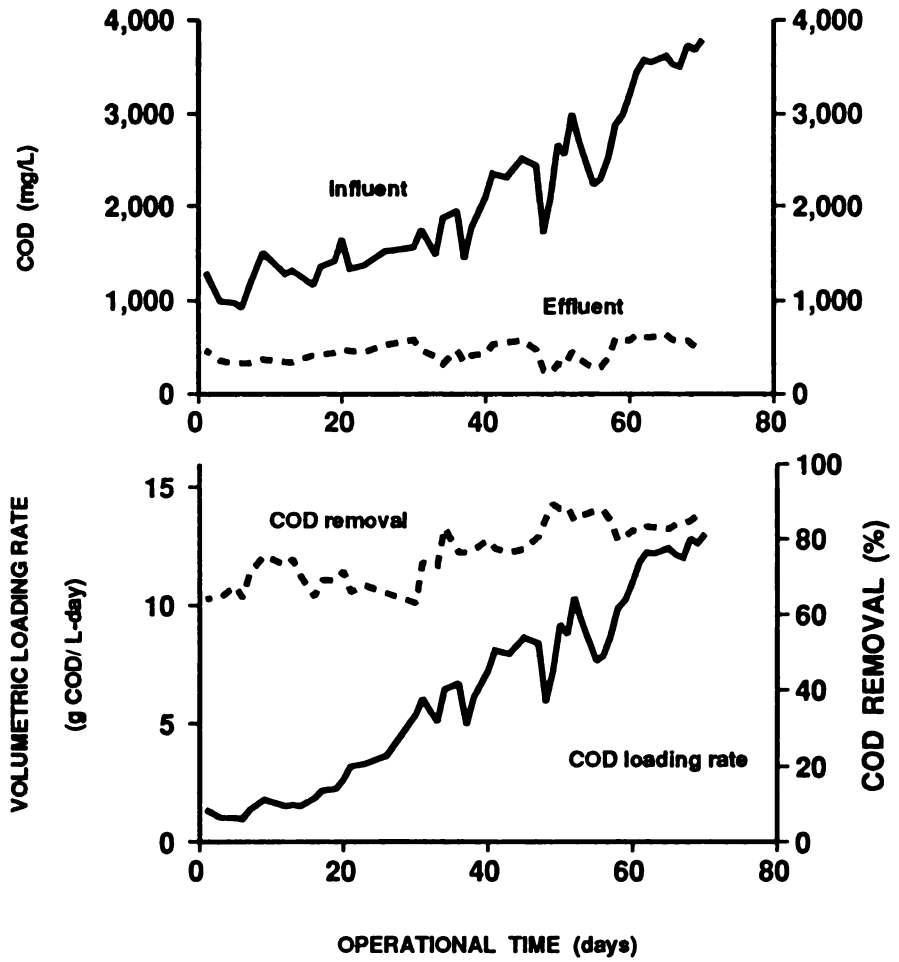


Figure 2.5a Operational results of reactor inoculated with activated sludge and fed with glucose-molasses (reactor C)

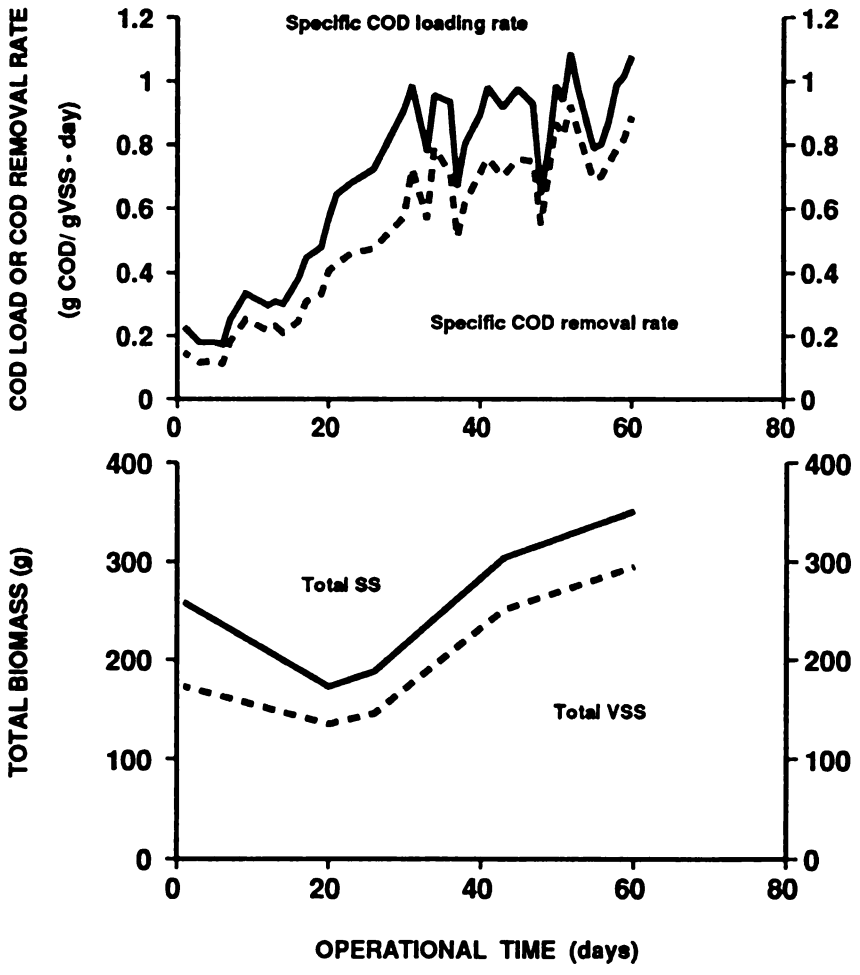


Figure 2.5b Operational results of reactor inoculated with activated sludge and fed with glucose-molasses (reactor C)

Granules started to form on day 33. The COD loading rate was between 0.8 to 0.96 g/gVSS-d at that time. The pH value of the effluent was maintained at 7.0 to 7.3, but the alkalinity in the effluent varied from 400 mg/L to 800 mg/L (as $CaCO_3$) depending upon the Na_2CO_3 addition rate.

The granulation process was somewhat similar to that observed in reactors A and B with some exceptions. The changes of total biomass (as SS and VSS) appeared to be the same as in reactors A and B. The granulation process followed the three phases as before (Table 2.6). However, phase I and phase II (day 1 to day 38) can be combined since a significant sludge bed expansion did not occur. At the beginning of continuous operation, the sludge bed volume had stabilized due to the 14 days of pre-incubation of activated sludge under anaerobic conditions. After initiating continuous operation, the light fraction of sludge in reactor C washed-out. On day 20, the total amount of sludge (or SS) retained in the reactor was reduced to a minimum level, 67% of the original level (Figure 2.5b). At that time, granules were not observed in the sludge bed. Granules were observed prior to reaching the minimum solid level in reactors A and B. This was also observed by Hulshoff Pol *et al.* (1983) and de Zeeuw (1984) in their work. It was not until day 33 that granular sludge began to appear in the lower portion of reactor C. The sludge bed region in reactor C filled-up with granules during the following week. Subsequently (phase III), the total amount of sludge increased significantly as granules grew in size.

2.4.4 Sludge profile during granulation process

Typical sludge profiles in reactors A, B and C during granulation are illustrated in Figure 2.6. The sludge profiles of the different phases of granulation process in reactors A and B were similar to those reported by Hulshoff Pol *et al.* (1983) and de

Zeeuw (1984). But changes in the sludge profiles of reactor C were slightly different.

In both reactors A and B, three distinct sludge profiles were observed during the different phases of granulation. At the beginning (phase I), sludge was not active and biogas production was lower than 1 L/L-day; thus, the sludge concentration in the sludge blanket region was low and most sludge accumulated at the bottom (day 67 and day 2 in Figure 2.6a and 2.6b, respectively). As volumetric COD loading rate and the activity of sludge increased (phase II), the concentration of sludge in sludge blanket also increased up to 20 gSS/L or more and the concentration along the reactor profile became more uniform (day 93 and day 25 in Figures 2.6a and 2.6b, respectively). At the end of the phase I or in phase II, the interface between the sludge bed and the sludge blanket became difficult to observe. As the reactors became filled with granules (phase III), the difference between sludge bed and sludge blanket region again became visible, with low solids concentrations in the sludge blanket and high concentrations in the sludge bed (day 157 and day 62 in Figures 2.6a and 2.6b, respectively).

In reactor C, the concentration in the sludge bed was lower than in reactors A and B throughout (Figure 2.6c). High concentrations of solids in the sludge blanket were not observed.

2.4.5 Growth rate of granules and sludge yield

Growth rate of granular bed is strongly dependent on the specific COD loading rate applied and feed substrate. The growth rate of the granule bed was estimated based on the change in granule bed height. The rate was approximately 0.015 day^{-1} in reactor A when the specific COD loading rate was about 0.7 g/g VSS-day, 0.0094 day^{-1} in reactor B at a COD loading rate of 1.5 to 2.2 g/gVSS-d, and 0.0095 day^{-1} in reactor C at COD loading rate of 0.8 g/gVSS-d (Table 2.7).

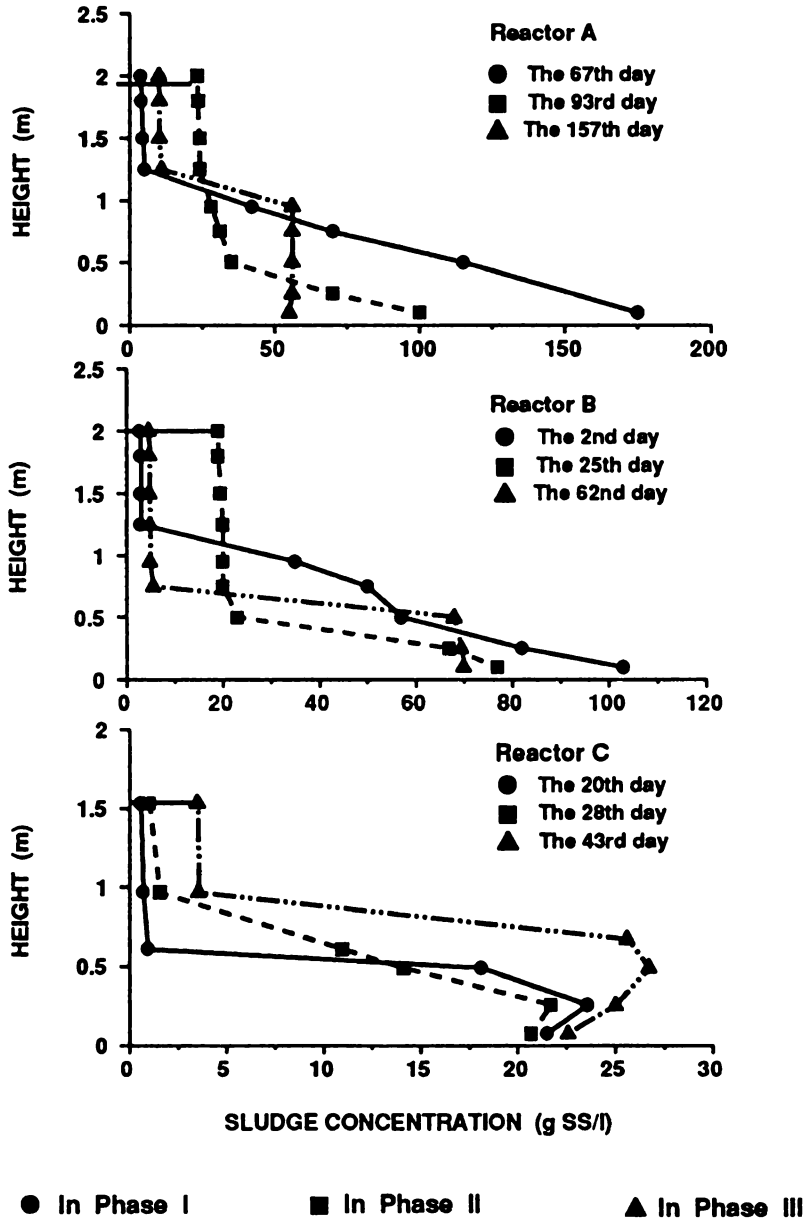


Figure 2.6 Comparison of the sludge concentration profiles in reactors A, B and C during granulation.

The apparent sludge yield coefficient was estimated by comparing the quantity of the sludge increase in the reactor plus solids in the effluent versus the quantity of COD removed. The apparent sludge yield coefficients were 0.074, 0.049, and 0.08 gVSS/g COD for reactors A, B and C, respectively (Table 2.7).

Table 2.7 Comparison of performance of granules from reactors A, B and C (35 °C)

Reactor	A	B	C
Feed	glucose	brewery	glucose
Inoculum			
COD removal rate g/gVSS-d	0.06	0.12	0.1
Granules			
COD removal rate			
g/gVSS-d	0.62	2.0	1.0
g/L(granules)-d	35.9	132.0	36.0
CH_4 production rate L(STP)/L(granules)-d	10.8	47.5	11.0
Granule growth rate (1/d)	0.015	0.0094	0.0095
Sludge yield (gVSS/gCOD removed)	0.074	0.049	0.080
Total methanogens MPN (10^{11} cells/gSS)	4.5	5.4	2.9

(1) Activity of inoculum sludge was calculated based on operational data.
(2) Granular bed volume was operational volume, calculated based on the measured bed height.
(3) MPN number of methanogens was enumerated using 3 tubes per dilution.
(4) The COD removal rate of inoculum was determined after pre-incubation period.

2.5 CHARACTERIZATION OF GRANULES DEVELOPED ON COMPLEX SUBSTRATES

2.5.1 Physical characteristics and chemical composition

The size distribution of the granules taken from the upper portion of reactors A and B is shown in Figure 2.7. Although these samples might not contain some of the larger size granules (> 3.0 mm in diameter) which accumulated at the reactor bottom, they are reasonably representative samples since the granules were well mixed by the high rate of biogas production. The major fraction of granules was observed to be between 0.3 and 0.7 mm in diameter in the two reactors. Reactor A, however, contained more granules with a large size than reactor B. In reactor A, 90% of total granule volume was contributed by the granules with a diameter of 1.0 mm or more compared to reactor B (48.7 %). The size of granules in reactor C appeared to be larger than reactor A and B, ranging from 0.2 to 2.0 mm in diameter. Granules up to 4 mm in size were observed.

The physical characteristics and chemical composition of the granules from reactors A, B, and C were summarized in Table 2.7. The specific gravity of the granules in reactor A, B and C ranged from 1.02 to 1.04. The granules originating from digested sludge had a higher specific gravity and density than those obtained from activated sludge. The content of Kjeldahl-nitrogen and the ratio between VSS and SS in the three lab-scale UASB reactors were approximately the same. All the granules contained significant amounts of precipitated calcium, magnesium and iron.

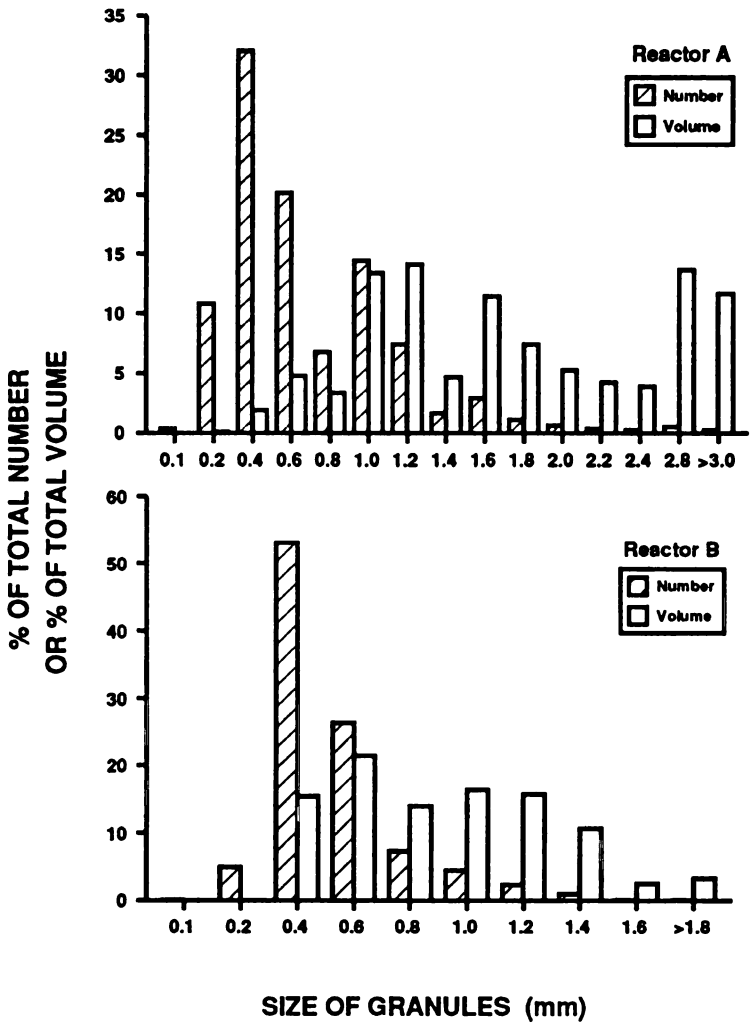


Figure 2.7 Size distribution of granules from reactors A and B. Granules from reactor A (total number 782) were withdrawn from the sampling port at 0.7 m height (granule bed height was about 1 m at that time) after 180 days of operation. Granules from reactor B (total number 587) were from the sampling port at 0.4 m (granule bed height was 0.6 m) after 60 days of operation.

Table 2.8 Comparison of physical and chemical properties of granules in reactors A, B and C

Reactor	A	B	C
Specific gravity	1.04	1.03	1.02
Density (g SS/L)	110	112	70.6
VSS/SS	0.84	0.86	0.84
Kjeldahl-N (g/gVSS)	0.26	0.27	0.29
Ash(%)	16	14	16
Metal content (mg/gSS)			
Calcium	19.0	21.0	8.9
Magnesium	3.9	2.7	15
Iron	3.0	1.9	10.6
Nickel	0.33	0.27	0.26
Cobalt	0.04	0.03	0.035
Zinc	0.36	0.85	0.89
Sodium	22.8	21.9	22.1
Potassium	0.61	0.76	0.86

Manganese in all granules was below detection limit (<0.01 mg/gSS).

2.5.2 Operational performance of granules

Operational performance of the granular sludges produced in reactors A, B and C is also summarized in Table 2.7. In reactor A, inoculated anaerobic digested sewage sludge had a specific COD removal rate of 0.06 gCOD/gVSS-d. After granulation, the activity increased to about 0.6 g COD/gVSS-d with the glucose molasses solution as feed. The COD concentration in the effluent was maintained low (380 to 400 mg/L) and included some acetate (1 to 1.2 mM) and propionate (0.4 to 0.6 mM).

The initial specific COD removal rate in reactor B was 0.1 g COD/gVSS-d and a final specific COD removal rate of 2.0 g COD/gVSS-day was achieved when the volumetric loading rate was 32 g/L-day (COD removal of 90%). At that time the effluent contained 1 mM acetate and 0.26 mM propionate.

As shown in Figure 2.5b, the specific COD removal rate of reactor C was initially 0.1 g/gVSS-d at the beginning of continuous feed. At the end of the experiment, the maximum specific COD removal rate was estimated to be 1.0 g COD/gVSS-d, with an effluent COD of 500 mg/L.

Comparing the data of the three reactors, the granules fed with brewery wastewater had the highest activity among the three granules. It should be noted that the COD concentration in the three reactors was normally maintained lower than 300 mg/L and did not exceed 600 mg/L. The fatty acid concentrations in the reactors were always controlled at lower than 1 to 2 mM acetate and 1.0 mM propionate. If the effluent fatty acid concentrations were maintained at a higher level, the specific activity would likely be enhanced.

The MPN enumeration indicates that the total numbers of methanogens in the granules produced in the three reactors were at the same level (10^{11}/gSS) (Table 2.7).

2.5.3 Microscopic and scanning electron microscopic examination.

i) Microscopic examination

In granules taken from reactors A, B, and C, a large number of bacteria autofluorescing blue-green under UV light were observed. Most of these bacteria were rods, with a few sarcina and cocci. These methanogen-like bacteria may had been *Methanobacterium*, *Methanosarcina* and *Methanococcus* respectively. In addition, a few spiral filaments with autofluorescence were also observed and appeared morphologically similar to *Methanospirillum* sp. A large number of bamboo-shaped, *Methanothrix*-like filamentous bacteria, which did not show autofluorescence were the dominant bacterial morphotype in the granules.

ii) SEM examination.

The SEM photographs of the granules from reactors A, B and C are shown in Figure 2.8. The overall surface of the granules was rough and uneven (Figure 2.8A, B and C). Some crystals and precipitates were embedded in the granules. These precipitates contained Ca or Si as major components as determined when analyzed by energy dispersive X-ray spectrometry. In addition, iron was dispersed uniformly on the surface of the granules and through the cross section of granules, most likely as ferrous sulfide precipitates.

Figure 2.8 SEMs graphs of granules from reactors A (A, D and G), B (B, E and H) and C (C, F and I). A, B, and C: Outlines of granules. D, E, and F: *Methanaothrix* sp. on the surface of granules. G, H and I: Aggregates of *Methanosarcina* sp. on the surface of granules.

A
B
C
D
E
F
G
H
I

A heterogeneous bacterial population was observed on the surface of the granules. Most of the bacteria could not be definitively identified using only their morphology. However, some methanogenic bacteria having distinct morphotypes were easy to recognize. Long filamentous rods were observed on the granules from reactors A and B (Figure 2.8 D and E), while rod chains consisting of 2 to 5 cells were observed frequently in the granules of reactor C (Figure 2.8F). Both these rods (0.7 to 0.8 μm in diameter) showed the typical morphotype (bamboo-like) of *Methanothrix* species (Zehnder *et al.* 1980; Patel, 1984; Zinder *et al.* 1984a; Touzel *et al.* 1988). The long filaments are presumably *Methanothrix soehngenii* while the short chained rods might be an unidentified *Methanothrix* sp. In addition, bacteria resembling *Methanosarcina* sp. were dispersed over the surface of the granules from the three reactors (Figure 2.8G, H and I).

2.6 DISCUSSION

2.6.1 Feasibility of using activated sludge as inoculum

MPN enumeration and reactor experiments demonstrated that activated sludge is a viable alternative inoculum to digested sewage sludge for the cultivation of anaerobic granules. It is essential that the inoculum material for an anaerobic reactor contain a modest microbial ecosystem involved in methanogenesis, and this ecosystem should contain a considerable variety of methanogenic bacteria including hydrogen utilizing and acetate utilizing methanogens. Microscopic examination and SEM observation showed the existence of a variety of methanogen-like bacteria resembling *Methanobacterium*, *Methanococcus* and *Methanosarcina* in the activated sludges

enumerated by MPN technique, and in the granules cultivated using this inoculum. *Methanospirillum*-like bacteria and *Methanothrix*-like bacteria found in the granules, probably also originated from the activated sludge. Similar observations were made for other anaerobic granules cultivated using activated sludge from a textile wastewater facility (Wu *et al.*, 1987b) and a VFA mixture (Guyout *et al.*, 1990).

Although other important anaerobes (i.e. hydrolysis-fermentative bacteria and syntrophic acetogens) were not investigated, the successful start-up of reactor C with activated sludge as inoculum demonstrated that activated sludges possess those bacteria. Methane production from glucose and other carbohydrates should pass through several intermediates such as propionate, butyrate and ethanol etc. (Cohen 1982; Cohen *et al.*, 1984; Chartrain and Zeikus, 1986a, 1986b).

The results of bacterial enumeration revealed that the four activated sludges, which appear typical of most activated sludges, contained almost identical numbers of methanogens, up to 10^8 cells/gSS (Table 2.2) . This value is larger than that observed in freshwater sediments i.e. 10^2 to 10^6 /gSS (Zeikus and Winfrey, 1976), and approximately equal to that determined in a lotus pond mud, i.e. $4x10^7$ /g mud. The latter source was successfully used as the inoculum for a digestor (Oi *et al.*, 1985). This means that activated sludge has a sufficient population of methanogens to be utilized as the inoculum for anaerobic processes. The MPN values for methanogens in activated sludges were, however, smaller than those of anaerobic digested sludge. For example, Zeikus (1979) reported 10^8/mL (about 10^{10} cells/gSS) in a digested sludge, and Zhao *et al.* (1985) found 10^7/mL (10^9 to 10^{10} cells/gSS) in a digested rural waste sludge. The MPN values for methanogens in activated sludge will be one or two magnitudes smaller than those in the digested sludges if the digested sludges contain 30 to 100 gSS/L. Thus, start-up of an anaerobic reactor using activated sludge may

require a longer period than digested sludge in order to accumulate a sufficient population of methanogens and syntrophic acetogens which grow slowly.

The existence of methanogens in the activated sludge is likely due to some anaerobic nuclei inside the sludge flocs. The nuclei protects the methanogens from oxygen or high redox potential, and the metabolic products of aerobes or other anaerobes provide them with substrates (Wu *et al.*, 1987a, 1987b).

2.6.2 Characteristics of the granulation progress

Both glucose molasses and brewery wastewater are carbohydrate-containing but not composition-defined substrates. The granulation progress using these substrates is of significance in starting-up a reactor treating similar organic wastewater. In the experiments with reactors A and B, the same three phases of granulation and sludge profiles were observed, as described by Hulshoff Pol *et al.* (1983) and de Zeeuw (1984) who studied granulation on VFA (acetate, propionate, and butyrate) mixtures using digested sludge as inoculum. This demonstrates that the granulation progress on complex organic substrate is basically the same as that on VFA mixture when digested sewage sludge serves as inoculum, although the microbial composition may be different due to different sources. The granulation process using activated sludge in reactor C was similar to that using digested sludge except for somewhat different sludge profiles. Therefore, cultivation of granules on carbohydrate-containing feed with either anaerobic digested sludge or aerobic activated sludge as the inoculum can be conducted in essentially the same manner as systems started on VFA feed.

2.6.3 Parameters for cultivation of granules

The parameters affecting granulation and the performance of the developed

granules include type of inoculum, amount of inoculum, composition of feed, operational variables (specific COD loading rate, hydraulic loading rate, etc.) and environmental factors (pH, temperature, and nutrient requirements). Some of these parameters have been discussed previously (Lettinga *et al.*, 1984, 1985; Hulshoff Pol *et al.*, 1983, 1988; Hulshoff Pol, 1989; de Zeeuw, 1984, 1988).

i) Type of inoculum

The experiments in this study confirmed that anaerobic digested sewage sludge can serve as inoculum to cultivate granules, as reported previously (Hulshoff Pol *et al.*, 1982, 1983; de Zeeuw, 1984; Manjunath *et al.*, 1989), and demonstrated that activated sludge can also be used. Several additional inocula have been reported for the cultivation of granules. A sewer slurry was used to start a mesophilic UASB reactor treating a starch processing wastewater (Lui *et al.*, 1985). Wiegant and de Man (1986) successfully cultivated thermophilic anaerobic granules using cow manure as inoculum. Thus, it appears that the inoculum materials for the cultivation of granules can be quite diverse and its choice will depend in part on availability. The key factor for any inoculum material is that it should contain a modest microbial ecosystem involved in methanogenesis from complex organic compounds.

Based on microscopic examination and SEM observation, the predominant methanogens in the granules from reactors A, B and C appeared similar, i.e. *Methanothrix*-like, and *Methanobacterium*-like bacteria were most common. The anaerobic granules cultivated from activated sludge and from digested sewage sludge possessed the same order of magnitude of methanogens i.e. 10^{11} cells/g SS. This demonstrates that after long term incubation, there is no significant difference in the total amount of the methanogens in the granules cultivated from different inocula under similar conditions. Wiegant and de Man (1986) also reported that no significant

difference was observed in the thermophilic granules originating from digested sludge and from cow manure. Therefore, cultivation of granules in an anaerobic digestor does not appear to be significantly affected by the type of inoculum used, provided it contains an ecosystem capable of methanogenesis.

The type of inoculum may, however, affect the physical characteristics and operational performance of the granules. Granules from digested sewage sludge had a greater specific gravity and were denser than those from the activated sludge (Table 2.8) although they were fed with same glucose molasses solution. The reason for this observation is not clear. It may be because the digested sludge contained more inorganic or inert materials than the activated sludge, or microbial composition of the two inocula may differ in a manner that was not observed.

ii) Amount of inoculum

In this chapter, the initial average sludge concentrations in reactors A, B and C were 13.2, 11.4 and 6.0 g VSS/L and initial specific COD removal rates were 0.05, 0.12 and 0.1 gCOD/gVSS-d, respectively. The minimum amount of sludge retained was 51% of the amount of the inoculum (as SS) in reactor A, 58% in reactor B, and 67% in reactor C. These data were similar to other system studied with digested sludge i.e. initial activity was 0.12 gCOD/gVSS-d and about 50% retained in the reactor (de Zeeuw, 1984). Therefore, a minimum amount of inoculum of about 10 gVSS/L in the reactor is advisable.

iii) Specific COD loading rate

The specific organic loading rate obviously effects the rate of granule formation and subsequent growth. In reactor A, after start-up, the loading rate was maintained at lower than 0.3 g COD/gVSS-d for 77 days. Granules were not observed until 2 weeks after a higher COD loading rate was applied. In reactor B, ten days after

start-up, the COD loading rate was increased to over 0.3 g COD/gVSS-d. Consequently, granules were observed by day 19. In reactor C, after 10 days of continuous feed, the COD loading rate was increased to over 0.4 gCOD/gVSS-d. Granules were observed after 33 days. Thus, a COD loading rate in excess of 0.3 gCOD/gVSS-d appears important for granule formation. In all three reactors the progress of granulation, once initiated, was so fast that the sludge bed in the reactors filled with granules within one or two weeks once a COD loading rate higher than 0.6 g COD/gVSS-d was applied. The same observation has also been reported by other researchers. Hulshoff Pol *et al.* (1983) tested two UASB reactors, inoculated with digested sludge, and fed with acetate-propionate mixture at 30 °C. One reactor was fed at a loading rate of 0.3 gCOD/gVSS-d for more than 13 weeks without granulation occurring. Another reactor loaded at 0.9 g COD/gVSS-d had rapid granulation. Manjunath *et al.* (1989) observed that granulation in lab-scale reactors fed a sugar molasses solution was possible only at a volumetric COD loading rates above 5 g/L-d (COD loading rate of more than 0.7 g/gVSS-d).

The positive effect of a high specific loading rate on granulation may possibly be attributed to: (1) the enhanced growth rate of some aggregate-forming methanogenic bacteria such as *Methanobacterium formicicum* and *Methanothrix* sp. or some syntrophic acetogens which utilize butyrate and other volatile fatty acids (see Chapters 5 and 7); or (2) enhanced growth rate of some aggregate-forming fermentative bacteria. For example, Zoutberg *et al.* (1988) reported that *Selenomonas ruminantium* and *Clostridium butyricum* produce polysaccharides when they grow on glucose. More work will be required to determine the true reason for the dramatic effect loading rate exerts on the granulation process.

iv) Hydraulic loading rate and biogas flux

The selective retention of pre-formed aggregates and concurrent wash-out of flocculent sludge is important in cultivation of granules (Hulshoff Pol *et al.*, 1983; Lettinga *et al.*,1984, 1985; de Zeeuw 1984; 1988). Clearly, the hydraulic flux and biogas flux, resulting from CH_4 and CO_2 production, in the reactor play an important role in the granulation process. The same forces also play an essential role in the reduction of formation of channels and dead space in the sludge bed region (van der Meer, 1979). In these experiments, the hydraulic loading rate was controlled between 0.25 and 0.5 m/hr. This was sufficient to wash-out the light fraction of sludge from the reactors with a concurrent biogas flux of 0.18 m/hr (reactor C) to 0.24 m/hr (reactor A). The optimal hydraulic loading rate and biogas flux will, obviously, vary with the nature of inoculum material and the reactor design. The range used here, however, appears to work quite well and can be used as a guide.

v) Wastewater composition

The composition of wastewater will have a dramatic effect on the granulation process as well as the type and performance of the granules formed.

Up to now, granulation under mesophilic temperature conditions has been reported in UASB reactors treating organic wastewaters such as sugar-beet (Heertjes *et al.*, 1978; van der Meer, 1979; Lettinga *et al.*, 1980), potato processing (Lettinga *et al.*, 1980), paper mill (Haberts *et al.*, 1985), alcohol distillery (Shen 1985), sucrose solutions (Sierra-Alvarez *et al.*, 1988; Harada *et al.*, 1988), night soil (Endo and Tohay, 1985), citrate fermentation (Wu *et al.*, 1987), and pulping wastewaters (Vuorianta *et al.*, 1985). Granulation was also reported to occur in the reactors fed with VFA mixtures consisting of acetate-propionate, or acetate-propionate-butyrate (Hulshoff Pol *et al.*, 1983; de Zeeuw, 1984). This indicates that granulation can occur for most

starch and sugar based moderate to high-strength organic wastewaters.

Granulation did not occur in reactors fed a VFA mixture or sucrose solution, which contained high ammonium concentrations (1000 mg NH_4-N/L) (Hulshoff Pol *et al.*, 1983; Harada *et al*, 1988). No granulation was observed in a reactor fed with thermal sludge conditioning liquor containing high total nitrogen (NH_4-N and total Kjeldahl nitrogen>1100 mg/L)(Hall and Jovanovic, 1982; Crawford and Teletzke, 1986). The reason for the absence of granule formation in the presence of high ammonium or total nitrogen concentrations is unknown and requires further elucidation.

Obviously, wastewaters containing significant concentration of carbohydrates will develop granules consisting of more fermentative microorganisms, while a wastewater composed mainly of fatty acids and alcohols will produce granules containing a higher percentage of methanogens and syntrophic acetogens. The latter will normally have a higher specific methanogenic activity. This has been demonstrated by the experiments conducted here. As is shown in Table 2.7, the granules developed on brewery wastewater (mainly composed of ethanol, acetate, propionate and butyrate) had a three-fold higher specific COD removal rate compared to those developed on the glucose molasses solution. This is despite the fact that the COD concentrations in the two reactor were almost equal.

The wastewater composition also appears to affect the size distribution of the granules. Granules developed on glucose molasses had more granules of a larger size than those developed with brewery wastewater (Figure 2.7). The fact that carbohydrates, such as sucrose, induced large sized granules was also reported by Sierra-Alvarez *et al.* (1988).

vi) pH and nutrients

A wide pH range (6.0 to 7.5) may be suitable for the cultivation of granules although the optimal pH for mesophilic anaerobic digestors is considered to be between 6.7 and 7.4 (Zehnder *et al.*, 1981). In these experiments, the granules were formed at pH between 6.5 and 6.9 in reactor A, and between 7.0 and 7.2 in reactors B and C. Actually, the pH inside the granules is different from the pH in the liquid bulk because the metabolism of substrates occurs inside the granules and bacterial aggregates which may eventually develop into granules. When methanogenesis from acetate or propionate occurs within granules, the pH inside granules will increase to levels above that in the liquid bulk. In fact, granules have developed on an acetate-propionate mixture at pH 6 (Brummeler *et al.*, 1985).

The nutrient levels supplied in this study provided a modest carbon/nitrogen ratio as well as supplementing trace elements Ni, Co and Fe. To date, there are several reports on the trace elements enhancing methanogenic activity in anaerobic reactors and granular formation (Murray *et al.*, 1981; Takashima and Speece, 1988; Guiot *et al.*, 1988). Therefore, ensuring that wastewaters are supplemented with the proper nutrients is advisable.

2.7 SUMMARY

1) Aerobic activated sludges contain considerable amounts of methanogenic bacteria (i.e. approximately 10^8 cells/g VSS) and can be used as inoculum material for the cultivation of granules.

2) Anaerobic granules can be developed from both anaerobic digested sewage sludge and aerobic activated sludge on carbohydrate-containing wastewater, glucose

molasses solution and brewery wastewater under mesophilic temperature conditions.

3) The changes in total biomass during the granulation progress with non-granular inoculum materials was basically similar to those reported using VFA mixtures as substrate and digested sludge as inoculum (Hulshoff Pol *et al.*, 1983; de Zeeuw, 1985). The three phases of granulation with three typical sludge concentration profiles were observed during granulation with digested sludge. The granulation process and sludge profiles with activated sludge were somewhat different.

4) Based upon microscopic and SEM examinations, the prevalent acetate-utilizing methanogens in the granules were *Methanothrix* sp. and prevalent hydrogen-utilizing methanogens were *Methanobacterium* sp.

5) Specific organic loading rate was an important parameter for initiation of granulation.

CHAPTER 3

COMPARISON OF TWO TYPES OF GRANULES DEVELOPED ON FATTY ACID CONTAINING FEEDS

3.1 INTRODUCTION

To date, at least three distinct types of mesophilic granules have been found in laboratory UASB reactors fed with VFA mixtures: (1) "rod-type" granules which contain rod-shaped *Methanothrix*-like cells growing as aggregates with 3 to 5 cells as the predominant acetate utilizing species; (2) "filament-type" granules which contain long filamentous *Methanothrix soehngenii*-like cells as the prevalent species; and (3) "sarcina-type" granules which contain *Methanosarcina* sp. as the prevalent species (Lettinga *et al.*, 1984, 1985; de Zeeuw, 1988). In general during wastewater treatment, high levels of acetate such as 2 to 3 times the half velocity coefficient, K_m (ca. 5 mM acetate) for *Methanosarcina barkeri* are not maintained in the reactor. These type granules, therefore, are uncommon. Most granules from wastewater treatment systems contain *Methanothrix*-like bacteria as predominant acetate utilizing methanogens (Gorris *et al.*, 1988; Dubourguier *et al.*, 1988b; Tilche and Yang, 1988; see Chapter 2). No detailed study has been done, however, to compare and contrast the rod-type and

filament-type granules.

Recently, a type of high-performance anaerobic granules was developed on a VFA mixture (Thiele *et al.*, 1990). Based on the morphotype of predominant *Methanothrix* sp. in these granules, they can be classified to be the rod-type granules (R-granules for short). The granules developed from a laboratory-UASB reactor treating brewery wastewater (reactor B in Chapter 2) were subsequently grown on a VFA mixture plus some glucose and ethanol. The granules were observed to be the filament-type granules (F-granules for short). The purpose of this chapter is to compare these two types of granules in relation to operational performance, physical-chemical properties, substrate kinetics, metabolic performance and overall microbial composition.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals, analytic methods and media

Chemicals, gases, analytical methods and media used in this and following chapters are described in Appendix A. Observations using light microscope, scanning electron microscope (SEM) and transmission electron microscope (TEM), the granule size distribution, and microbial cell density analysis are described in Appendix D.

3.2.2 Granule sources

Two types of anaerobic granules were investigated in this experiment. The R-granules were initially obtained from an UASB reactor treating potato-processing wastewater. They were shipped to Michigan Biotechnology Institute (MBI) from

France by Dr. Dubourguier. They were subsequently starved and then fed a VFA mixture to improve their operational and metabolic performance (Thiele *et al.*, 1990). The F-granules were transferred from a laboratory-scale UASB reactor treating brewery wastewater (reactor B in chapter 2) to MBI.

Table 3.1 Feed composition for laboratory reactors

Feed	A	B
ethanol (mM)	0	6.5
acetic acid (mM)	100	130
propionic acid (mM)	50	40
butyric acid (mM)	50	40
glucose (mM)	0	2
COD (g/L)[a]	20	21
KH_2PO_4 (mM)	3	3
Na_2SO_4 (mM)	0.1	0.1
$FeSO_4$ (mM)	0.05	0.05
$Na_2S \cdot 9H_2O$ (mM)	0.15	0.15
NH_4Cl (mM)	6.6	0
NaOH (mM)	100	100
urea (mM)	0	6.6
NaCl (g/L)	1.0	1.0
$MgCl_2$ (g/L)	0.2	0.2
$CaCl_2$ (g/L)	0.1	0.1
Resazurin (g/L)	0.001	0.001
Trace element solution[b] (mL/L)	10	10

a: COD was calculated based on stoichiometry of full oxidation to CO_2.
The ratios (g COD/mole) are 96, 64, 112, 160 and 192 for ethanol, acetate, propionate, butyrate and glucose respectively.

b: Trace element solution is the same as used in the basal medium (see Appendix A)

3.2.3 Reactor feed composition

The feed composition for the reactors is listed in Table 3.1. Two different feeds were used for the F-granules. Both had an influent COD of 20 to 21 g/L. The R-granules were supplied with feed A throughout the duration of the experiments. Feed A contained 100 mM acetic acid, 50 mM propionic acid, and 50 mM butyric acid as major organic substrates. Feed B contained 110 mM acetic acid, 40 mM propionic acid, 40 mM butyric acid, 0.5 g/L glucose, and 8 mM ethanol. Both media were supplemented with essential nutrients and trace elements.

3.2.4 Laboratory-scale reactors

Two glass column reactors, 2.1 cm in diameter and 28 cm in length, were used. The reactor volume was 100 mL and settler volume was 150 mL. All parts were either of glass (column and settler), stainless steel tubing or butyl rubber tubing (American Scientific Products, McGaw Park, IL). A schematic diagram of the reactor systems is presented in Figure 3.1. The reactors were operated at 35 °C with continuous feed supplied by a Minipuls peristaltic pump (Gilson Medical Electronics, Middleton, WI). Recycle of treated effluent from top settler to the reactor inlet (recycle flow of 1 L/hr) was accomplished using another peristaltic pump. Gas samples for methane and hydrogen analysis and liquid samples for fatty acids were withdrawn from the sampling ports on the reactor (Figure 3.1). Gas production was measured by liquid displacement of an HCl solution (0.1 N) in an inverted graduated cylinder.

3.2.5 Reactor operation

The two reactors were operated side by side under the same environmental

conditions. Reactor R was started with feed A and inoculated with the R-granules. Reactor F was inoculated with the F-granules and started using feed B. Initially, the reactors were autoclaved, flushed with oxygen-free N_2 gas, and filled with the basal medium (Appendix A) buffered with 30 mM $NaHCO_3$ and 30 mM potassium phosphate (pH 7.0 to 7.1) and pre-reduced with 0.3 mM Na_2S. After the redox potential indicator, resazurin, became colorless (indicating reduced conditions), about 10 mL of granules were inoculated into each reactor through a sampling port using a sterile syringe. After inoculation, the reactors were sealed and the continuous feed started. The reactors were monitored daily for VFA concentrations, biogas production, methane content, flow rate, and pH. Growth of the granule bed height was also recorded. After over one year of operation, the granules in both reactors were examined to determine biomass concentration, predominant morphologies, granule density, size distribution and methanogenic activity. Data were recorded with regard to physical characteristics, biochemical and microbial composition, and metabolic performance to help compare performance.

The feed supplied to the F-granules was changed from feed B to feed A after one and half years operation to investigate if there was any effect of medium composition on performance. The reactor was then operated for four months to collect additional data for performance comparison with R-granules.

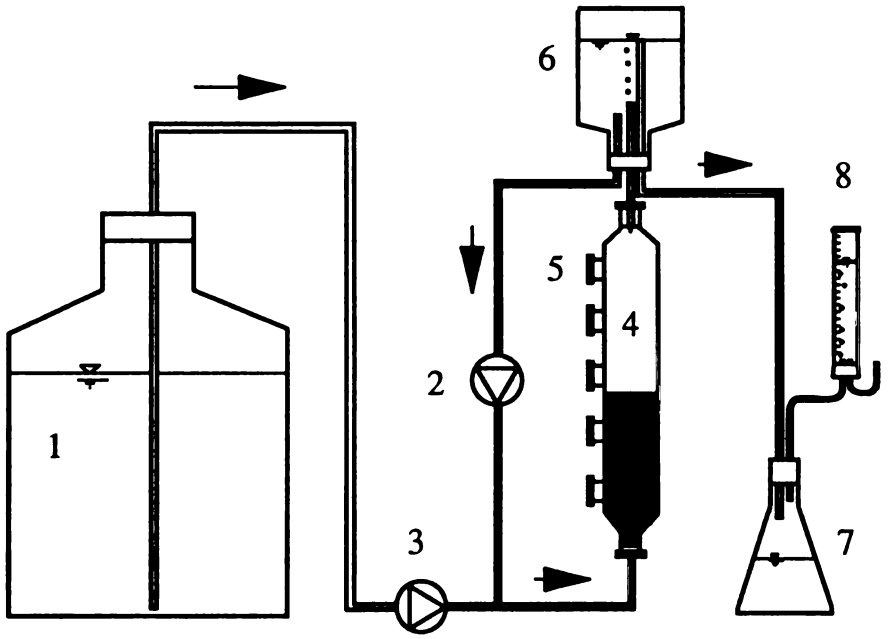


Figure 3.1 Schematic diagram of lab-upflow reactor system used for comparison of rod-type and filament-type anaerobic granules. 1: medium reservoir; 2: recycle pump; 3: feed pump; 4: column reactor; 5: sampling port with a butyl rubber stopper; 6: settler; 7: effluent collecting flask; 8: biogas volume measurement.

3.2.6 Determination of maximum substrate conversion rates, K_m and specific growth rates

Eight substrates (methanol, ethanol, formate, acetate, propionate, butyrate, isobutyrate, valerate, and glucose) were tested for determination of maximum conversion rates by both R- and F-granules. The rates were determined by pulse-addition of the respective substrates into the reactors and tracking their removal over time (Appendix B). Apparent half velocity coefficients (K_m) for acetate, propionate, isobutyrate, butyrate and valerate were determined (in triplicate) by time-course experiments using granules which were disrupted to eliminate diffusion resistance (Appendix B). Specific growth rates of acetate utilizing methanogens, propionate degraders and butyrate degraders were determined in operating reactors (Appendix B).

3.2.7 Temperature and pH effects

The effect of temperature on conversion of acetate, propionate, butyrate, formate and H_2-CO_2 was tested separately in 58 mL serum bottles containing 20 mL of the basal medium (Appendix A) which was reduced by Na_2S (0.4 mM) and buffered with 90 mM $NaHCO_3$ (pH 6.8). The bottles were pressurized by addition of 0.3 atm of CO_2 gas into headspace except samples incubated with H_2-CO_2 (80:20). For samples incubated with H_2-CO_2, the bottles were pressurized to 1.4 atm. Incubation temperatures of 22, 30, 35, 37, 40, 42, 45, 50, 55 and 60 °C were examined using triplicate samples for each substrate at each temperature. The initial concentrations of formate, acetate, propionate and butyrate were 70, 10, 10 and 10 mM, respectively. Granules were withdrawn from reactor using a glass syringe with an 18-gauge needle and placed into bottles containing the basal medium (pH 6.8) without addition of any substrate. Subsequently, granules (0.5 mL) were transferred to each test bottle. After

5 hours of incubation at the respective temperatures, 0.6 mL liquid samples and/or 0.4 mL gas samples were withdrawn from each bottle for VFA and methane analysis, respectively. The conversion rate of acetate, propionate and butyrate were calculated from VFA analysis and the amount of biomass added. Formate and H_2-CO_2 conversion rates were based on methane production. Substrate conversion and CH_4 production rates were normalized based on the highest rates.

The effect of pH on substrate conversion rates by the R- and F-granules was also examined using acetate, propionate, butyrate, formate and H_2-CO_2 as substrate. Medium conditions were similar to the temperature studies. The pH of the medium was adjusted to 5.5, 6.0, 6.5, 7.0, 7.5, 7.9 and 8.4 by adding different amounts of sodium bicarbonate into liquid phase and CO_2 gas into the headspace. Bottles were incubated in a shaking water bath (37 °C). Results reported were averages of triplicate samples.

3.2.8 Microbial population analysis

Microbial populations for both type granules were enumerated using the most probable number (MPN) technique in sealed pressure tubes. All the tubes contained the basal medium (9 mL) buffered with 20 mM phosphate and 20 mM bicarbonate, respective substrate and 1.0 mM sodium sulfide as reductant (medium for acetate utilizing methanogens contained 0.3 mM Na_2S). The headspace was pressurized to 40 kPa using an N_2-CO_2 (95:5) gas mixture except for H_2-utilizing methanogens. The tubes for methanogens were supplemented with 0.01% yeast extract, plus 15 mM acetate, 80 mM formate, 80 mM methanol, or 202 kPa (2.0 atm) of H_2-CO_2 (80:20). Syntrophic bacterial populations utilizing propionate or butyrate were enumerated in the tubes containing the basal medium with 15 mM of the respective energy source

and pre-inoculated with 0.5 ml of a H_2 and formate-utilizing methanogen, *Methanobacterium formicicum* strain T1N (isolated from the R-granules, see Chapter 5) as the syntrophic H_2-utilizing partner. A series of controls were prepared, which contained the same components with the exception of the energy source. Hydrolytic-fermentative bacteria were enumerated using glucose (2 g/L) or lactose (1 g/L) as substrates.

The granule samples were withdrawn from the reactors into anaerobic tubes, dispersed by repeatedly pressing granules back and forth through a 23-gauge needle and glass syringe, and re-injecting the disrupted granules into the tubes. These disrupted or dispersed samples were then diluted serially and inoculated into the MPN tubes by injecting 1 mL of sample into 9 mL medium. The samples were diluted serially from 10^{-1} to 10^{-12} using 5 tubes for each dilution. One mL of undiluted sample was taken for each granule sample to determine suspended solids (SS).

For the enumeration of methanogens, the inoculated tubes were incubated at 37°C for 2 months, and the results were recorded to be positive on the basis of the presence of methane (>2 mM) in the headspace of the tubes. The MPN numbers of syntrophic populations were assayed after 3 months of incubation at 37 °C. Methane formation(>0.5 mM CH_4) was used to establish whether or not the tube was positive. Hydrolytic bacterial populations were enumerated in the tubes containing the basal medium with 2 g/L of glucose or lactose. The tubes were incubated at 37°C for 2 weeks. The results for positive tubes were recorded on the basis of increase in optical density (>0.2) at 660 nm and production of volatile fatty acids (>5mM total VFA), and compared with non-inoculated control tubes.

The MPN values and 95% confidence limits were calculated using a MPN microcomputer program (MacDonell *et al.*, 1984).

3.3 OPERATIONAL PERFORMANCE

3.3.1 Substrate utilization and COD removal performance

Growth and operational conditions maintained during the comparison of the reactors incubated with the R- and F-granules are presented in Table 3.2. The environmental conditions (pH, temperature, and hydraulic loading rate) were maintained approximately the same for both reactors. The operational conditions in both reactors were controlled to achieve approximately the same effluent composition (i.e. 2 to 5 mM of acetate, 2 to 5 mM of propionate and 0.1 to 0.3 mM of butyrate). Trace amounts of isobutyrate, 2-methylbutyrate and valerate were also detected in the effluents.

The operational performances of both reactors after one year is summarized in Table 3.3. Both systems had relatively high VFA degradation and COD removal rates. The R-granules had almost double the specific volumetric methane production rate (150 to 180 L CH_4/ L granules-day) compared with F-granules (70 to 90 CH_4/ L granules-day), on the basis of granular bed volume. After reactor F was fed the same medium as that of reactor R for four months, the system did not exhibit any significant increase in VFA degradation or COD removal rates except perhaps a slight increase in methanogenic activity levels. Hydrogen partial pressure in biogas for reactor F decreased from 4-7 Pa to 1-3 Pa (the same as in reactor R) after changing the feed. The volumetric COD removal rate, based on the reactor volume (100 mL) was as high as 225 to 250 g COD/L-d and 125 to 145 g COD/L-d for reactors R and F, respectively. The reactor with R-granules had significantly higher COD removal capacity.

During almost two years of operation with feed A, the R-granules maintained

a dense structure and a constant yellowish-blue color. Original F-granules appeared greyish-black in color. However, after 4 months of operation with feed B, the granules changed to greyish-yellow spherical granules of a size larger than the R-granules. After one and a half years of operation, the influent of the F-granules was switched to feed A (no glucose and ethanol). There was no discernible change in the size or color of F-granules after 4 months.

3.3.2 Growth rate of granule beds

After inoculation, the granule beds in both reactors grew continuously until they attained a maximum height. The bed levels did not exceed a certain height regardless of the amount of room available for bed expansion. At high organic loading rates for reactor R (400 to 450 g COD/L granules-d) and for reactor F (200 to 250 g COD/L granules-d), the maximum height attained appeared to be strongly dependent on biogas flux and the physical characteristics of the granules. The maximum bed height for reactor R was 16 cm when the biogas flux rate was 1.2 to 1.4 m^3/m^2-hr, and 19 to 20 cm at a biogas flux of 0.8 to 0.9 m^3/m^2-hr for reactor F. The R-granules occupied over 55% of reactor volume when biogas production was about ca. 120 volume per reactor volume per day. The F-granules occupied 65% of reactor volume at a biogas production rate of 70 vol/vol-d.

The granule bed growth rate of the F-granules (0.045 to 0.048 /day) was almost twice that of the R-granules (0.022/day). This was apparently due these granules being less dense. Cell growth yields of both types of granules were approximately the same (0.02 to 0.025 g SS/g COD removed). Switching the feed for the F-granule reactor did not significantly change the granule bed growth rate or the bed density.

Table 3.2 Comparison of growth and operational conditions of the R-granules and F-granules

Conditions	Granules rod-type	filament-type	
Feed	A	B	A
ethanol (mM)	0	6.5	0
acetate (mM)	100	130	100
propionate (mM)	50	40	50
butyrate (mM)	50	40	50
glucose (mM)	0	2	0
Operational condition			
hydraulic load (m/h)[a]	4.4	4.4	4.4
COD loading rate (g COD/L granule-d)	400-450	200-250	200-250
COD removal (%)	>97%	>97%	>97%
Effluent composition:			
ethanol (mM)	0	0	0
acetate (mM)	2-5	2-5	2-5
propionate (mM)	2-5	2-5	2-4
butyrate (mM)	0.1-0.3	0.1-0.3	0.1-0.3
isobutyrate (mM)	0.05-0.1	0.05-0.1	0.05-0.1
2-methylbutyrate (mM)	<0.05	<0.05	<0.05
valerate (mM)	<0.05	<0.05	<0.05
glucose (mM)	0	0	0
biomass (mg SS/L)	<20-50	<30-50	<20-50
pH	7.3-7.5	7.2-7.4	7.3-7.7

a: Hydraulic load was calculated based on the upflow velocity caused by recycle plus feed flow.

Table 3.3 Operational results of the R-granules and F-granules

Parameter	Granules: rod-type feed A	Granules: filament-type feed B	Granules: filament-type feed A
volumetric methane production rate (L/L granules-d)			
operational[a]	150-180	70-90	80-90
maximum[b]	230-250	110	120-130
gas composition			
methane (%)	70-75	68-70	70-75
hydrogen (Pa)	1-3	4-7	1-3
operational biogas loading rate[c] (m^3 gas/m^2-hr)	1.3-1.4	0.8-0.9	0.8-0.9
volumetric COD removal rate (g COD/L-d)			
reactor volume	225-250	125-145	135-150
total system	90-100	50-54	50-58
specific COD removal rate (g COD/g VSS-d)			
operational[a]	6.9-7.6	5.6-6.9	5.5-6.9
growth			
granule growth rate (1/d)[c]	0.022±0.002	0.045±0.003	0.048
growth yield (g SS/g COD)[d]	0.022	0.026	0.024

a: Long-term average over more than four months to one year.
b: Measured after increasing feed rate or injecting VFA stock solution to achieve 10-15 mM acetate, 10-15 mM propionate, and 5-10 mM butyrate inside reactors.
c: Calculated based on the cross section of reactor column. At these loading rates, the maximum bed height was 15.5 cm in reactor R and 19 cm in reactor F fed with both feed A and B.
d: Calculated based on the total COD removed vs. the increased biomass of granule bed plus the washed-out biomass in effluent.

3.4 PHYSICAL AND CHEMICAL CHARACTERISTICS

The major physical characteristics and biochemical composition of the R- and F-granules after one year of operation and the composition of the F-granules fed with feed A for four months are compared in Table 3.4. The R-granules had a higher specific gravity (ca. 1.03) and biomass per unit of granule bed volume (65 to 70 g SS/L granules) compared with the F-granules (specific gravity of 1.01 and ca. 40 g SS/L granules). The ratio of VSS/SS and protein contents of the two granules were approximately equal. The total carbonate content of the R-granules (about 0.08 mmol/g SS) was higher than in F-granules (0.05 mmol/g SS), indicating that R-granules contained more than twice amount of carbonate salts, probably calcium and magnesium carbonates, per unit volume.

Table 3.4 Comparison of the Physical and chemical characteristics of the R-granules and F-granules

Parameter	Granule Type		
	rod	filament	
	feed A	feed B	feed A
specific gravity	1.025-1.03	1.01	1.01
density, g SS/L granules	65-70	39-40	40-43
VSS/SS	0.87	0.88	088
protein, g protein/g SS	0.77	0.78	ND
internal carbonate, mmol/g SS	0.07-0.08	0.05	ND

ND: Not determined.

The size distribution of the R- and F-granules after one year of operation is shown in Figure 3.2. Over 80% of total number of R-granules were less than 1.2 mm in diameter and 74% of total granule bed volume was composed of granules not larger than 1.4 mm in diameter. By contrast, only 40% of total number of F-granules had diameters less than 1.2 mm and 85% of total granule bed volume was comprised of granules with a diameter larger than 1.4 mm. After the F-granules received a change in feed, no significant change in size distribution was observed. Most granules were still relatively large, ranging from 1.2 to 2.0 mm in diameter or more.

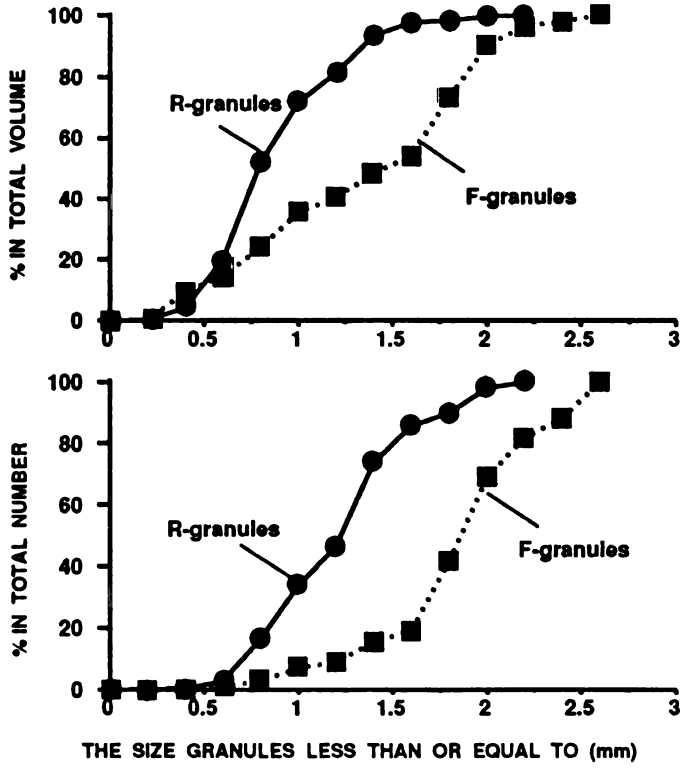


Figure 3.2 Size distribution of the R- and F-granules. The F-granules were fed with feed B. Total number of granule samples were 343 for the R- and 146 for F-granules, respectively.

3.5 KINETICS FOR GROWTH AND METABOLISM ON DIFFERENT SUBSTRATES

3.5.1 Specific growth rate

Hydrogen and formate-utilizing methanogens have relatively high specific growth rates compared to acetate-utilizing methanogens and syntrophic acetogens. Growth of the latter bacterial groups, especially of propionate degraders, may be a rate limiting step in the development of granules.

The conversion rates, per reactor volume, of acetate, propionate and butyrate increased exponentially as the function of operational time for both reactors (Figure 3.3). The specific growth rates of different trophic groups and respective fatty acid concentration in the reactors is summarized in Table 3.5. These data demonstrate that the specific growth rates of acetate, propionate and butyrate degraders in both R-granules and F-granules were quite similar (0.1 to 0.13/day) when subject to similar operational condition regardless of which feed was used. These data also reveal that the growth of syntrophic propionate degraders is likely the rate limiting reaction in both granules because the propionate concentration in the reactors was much higher than the apparent K_m for propionate degradation (Table 3.6).

An attempt was made to determine the threshold concentrations of different fatty acids below which substrate conversion ceases. To do this, the feed to the reactors was stopped for one hour while recycle was continued. Acetate, propionate, isobutyrate, butyrate, 2-methylbutyrate and valerate in both reactors were consumed to concentrations below detection limits of the GC analysis (acetate <0.01 mM, other fatty acids <0.005 mM).

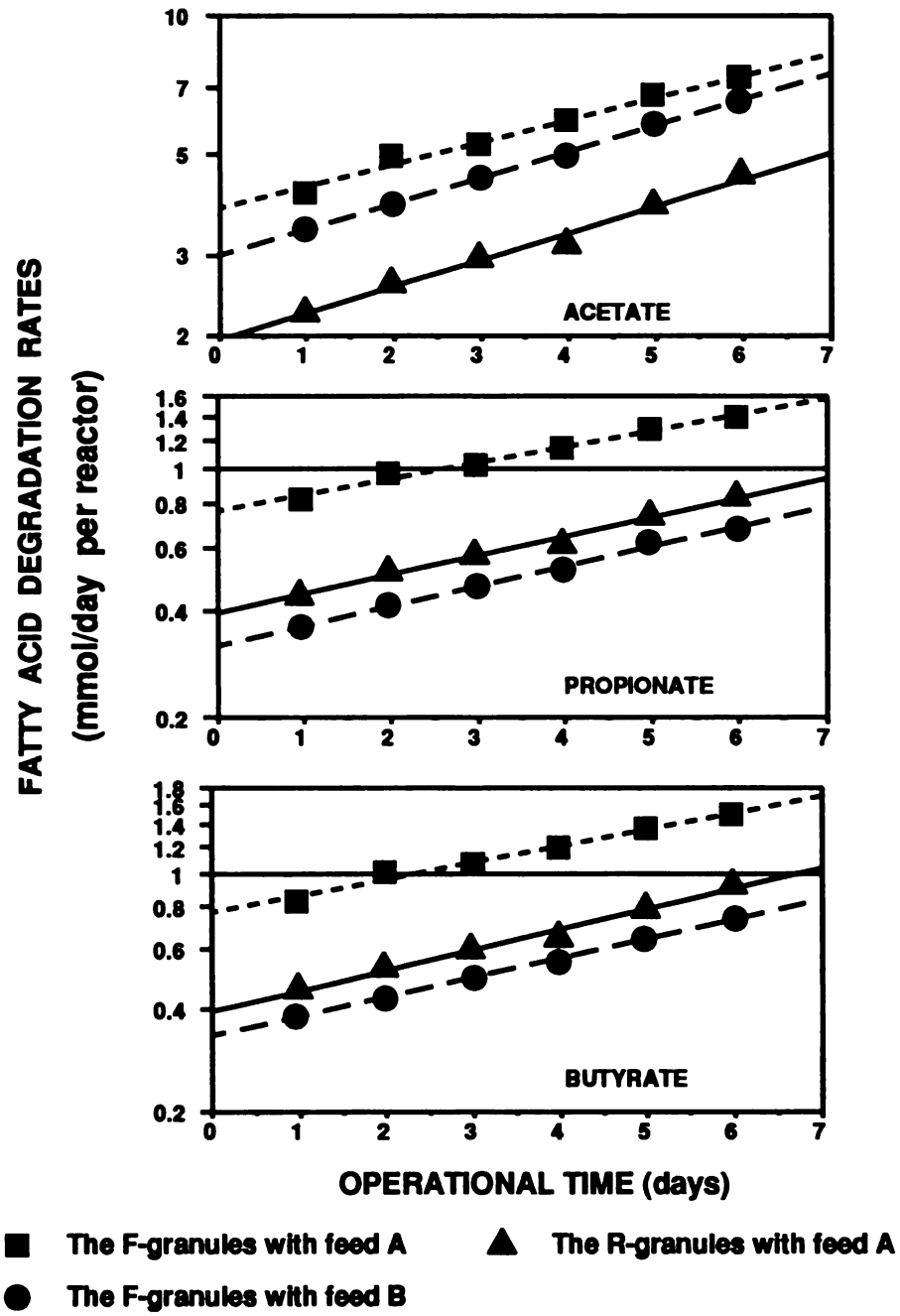


Figure 3.3 Increase of fatty acid conversion rates of the R and F-granules in reactors versus operational period

Table 3.5 Comparison of growth rate of metabolic trophic groups of the R- and F-granules

Parameter	Granules		
	rod-type	filament-type	
	feed A	feed B	feed A
VFA concentration in reactors[a]			
acetate (mM)	2.74 ±0.30	4.28 ±0.35	2.58 ±0.24
propionate (mM)	1.98 ±0.53	1.70 ±0.33	1.83 ±0.48
butyrate (mM)	0.15 ±0.015	0.16 ±0.05	0.07 ±0.01
Growth rate[b]			
Acetate degraders			
growth rate (1/d)	0.126	0.129	0.111
regression coefficient	0.984	0.998	0.992
Propionate degraders			
growth rate (1/d)	0.120	0.130	0.104
regression coefficient	0.987	0.996	0.992
Butyrate degraders			
growth rate (1/d)	0.128	0.129	0.113
regression coefficient	0.982	0.999	0.992

a: Average concentrations in one week of operation.
b: Obtained from logarithmic linear regression of fatty acid degradation rate per reactor versus operational period (day).

3.5.2 Maximum specific substrate conversion rate and half velocity coefficient (K_m)

The maximum substrate conversion rates for different substrates are summarized in Table 3.6. The maximum rates were determined directly in the reactors by injecting concentrated stock solutions of different substrates to obtain a high substrate concentration in the systems. After pulse addition of formate, high hydrogen levels were observed. Observed hydrogen concentrations in biogas reached as high as 0.14% (vol/vol) in reactor R (CH_4 content was 44 to 45% at that time) and up to 0.66% in reactor F (CH_4 content 42 to 43%). The total formate conversion rates and the rates of methane and hydrogen production are presented in Table 3.6. The ratio of methane to hydrogen produced was ca. 1600 for the R- and 270 for the F-granules.

After pulse addition of butyrate (6 to 8 mM), acetate and isobutyrate accumulated. In reactor R, acetate and isobutyrate concentrations accumulated to 1.5 and 0.2 mM, respectively. In reactor F, acetate and isobutyrate levels of 1.2 mM and 0.05 mM, respectively, were observed. The total conversion rate of butyrate, the rate of butyrate degradation to acetate plus CH_4, and the rate of butyrate isomerization to isobutyrate are presented in Table 3.6. The ratio between conversion of butyrate into acetate and CH_4 and isomerization of butyrate to isobutyrate was 20 and 47 for R-granules and F-granules, respectively. Pulse addition of isobutyrate to either type of granules produced only trace amounts (near detectable levels) of acetate and butyrate. This indicated that butyrate produced from isobutyrate was rapidly converted to CH_4 via acetate, because the conversion rates of butyrate are higher than the conversion rates of isobutyrate to butyrate in both systems (Table 3.6). The ratio between the rate of butyrate to acetate and CH_4 and the rate of isobutyrate to acetate and CH_4 was 2.6 and 3.5 for the R- and F-granules, respectively. This indicates that

conversion of isobutyrate to butyrate is likely the rate limiting step in the degradation of isobutyrate. Higher conversion rates of isobutyrate and isomerization rates were observed for the R-granules compared to the F-granules, indicating that the R-granules contained more isobutyrate-butyrate degraders than the F-granules.

Table 3.6 Comparison of maximum of rates and half velocity coefficients (K_m) for major fermentation and intermediate products by R- and F-granules

Substrate	Maximum Conversion Rates				K_m (mM)	
	Volumetric (mol/L-day)		Specific (mmol/g VSS-hr)			
	R	F	R	F	R	F
methanol	0.93	0.2	0.69	0.24	ND	ND
ethanol	0.23	2.2	0.17	2.7	ND	ND
formate	55.9	29.6	41.3	35.9	ND	ND
to CH_4	55.9	29.5	41.3	35.8	ND	ND
to H_2	0.035	0.11	0.025	0.13	ND	ND
acetate	7.1	3.2	5.3	5.4	0.43±0.01	0.42±016
propionate	1.8	0.76	1.3	0.92	0.048±0.02	0.037±0.005
butyrate	4.1	1.8	3.0	2.2	0.15±0.014	0.19±0.006
to acetate	3.9	1.76	2.88	2.14	ND	ND
to isobutyrate	0.20	0.04	0.15	0.05	ND	ND
isobutyrate	1.5	0.5	1.11	0.61	0.15±0.034	0.15±0.056
valerate	2.0	1.2	1.70	1.45	0.13±0.013	0.10±0.009
glucose	0.11	0.5	0.08	0.61	ND	ND

(1) ND: Not determined.
(2) The granule bed densities used for the determination of maximum conversion rates were 64 and 39 g SS/L granules for the R- and F-granules, respectively.
(3) Temperature: 35 °C.

After pulse addition of valerate, propionate accumulated to levels of 1.2 mM and 1.5 mM in reactor R and F, respectively. Trace amounts of butyrate and 2-methylbutyrate were also observed. Propionate is a product of valerate degradation by syntrophic butyrate degraders (McInerney *et al.*, 1981; Stieb and Schink, 1985; Shelton and Tiedje, 1984; Roy *et al.*, 1985; Ahring and Westermann, 1987a). The measured conversion rates of propionate were 27% and 58% lower than those of valerate on a molar basis for the R- and F-granules respectively (Table 3.6). This is likely the reason for the observed accumulation of propionate. The accumulation of butyrate and 2-methylbutyrate may be due to o the activity of syntrophic isobutyrate degraders (see Chapter 6).

When reactors were subject to pulse addition of ethanol, methanol, acetate and glucose, no accumulation of fatty acids or other organic compounds was observed. The conversion rates for methanol and all the fatty acids tested were higher in the R-granules than those in the F-granules, based on volumetric conversion rates (mol/L granules-day). The conversion rates were approximately 2-fold higher for formate, propionate, butyrate and valerate, approximately 3-fold greater for acetate and isobutyrate, and 4.5-times greater for methanol for the R-granules. When the specific rates were calculated in terms of substrate converted by unit of biomass (i.e. mmol substrate/g VSS-hr), however, the specific activities of the F-granules were only slightly less than the R-granules for volatile fatty acids and methanol. The conversion rate for ethanol and glucose were approximately 10- and 5-fold higher in the F-granules compared to the R-granules.

The maximum hydrogen conversion rates could not be determined directly in the reactors. They were measured indirectly in pH controlled batch experiments. These results were compared with formate conversion rates. The R- and F-granules

were observed to have different ratios of the conversion rates of H_2-CO_2 and formate, depending on the pH. At pH 7.0, the ratio was approximately 1.03 for both the R- and F-granules. At pH 7.5, however, the ratio was 0.85 for the R-granules and 1.4 for the F-granules.

Determination of apparent K_m demonstrated that the disrupted R- and F-granules had a similar range of K_m values i.e. 0.43 and 0.42 mM for acetate, 0.048 and 0.037 mM for propionate, 0.15 and 0.15 mM for isobutyrate, 0.15 and 0.19 mM for butyrate, and 0.13 and 0.10 mM for valerate, respectively (Table 3.6).

3.5.3 Temperature and pH effects

The effect of temperatures on the substrate conversion rates for H_2-CO_2, formate, acetate, propionate, and butyrate is illustrated in Figure 3.4. Both R- and F-granules showed the same temperature maximums. Temperature maximums were observed to be ca.45 °C for H_2-CO_2 and formate, and 40 °C for acetate, propionate and butyrate. The R-granules but not F-granules showed significant butyrate conversion activity even at 50 °C. These results only represent the effect of temperature on the substrate metabolic activities over a short term (within one day) and do not show the effect of temperature on microbial growth. Long-term effects may be quite different.

The effect of pH on the conversion rates of H_2-CO_2, formate, acetate, propionate, and butyrate is presented in Figure 3.5. Both type of granules had similar pH optima for conversion of formate, acetate and butyrate. However, they had different optimum pH for maximum conversion rates of H_2-CO_2 and propionate. The optimum pH for H_2-CO_2 utilization by the R-granules was 7.0, compared to 7.5 for the F-granules. By contrast, the highest propionate conversion rate was at pH 7.4 for the R-granules compared to 7.8 for the F-granules.

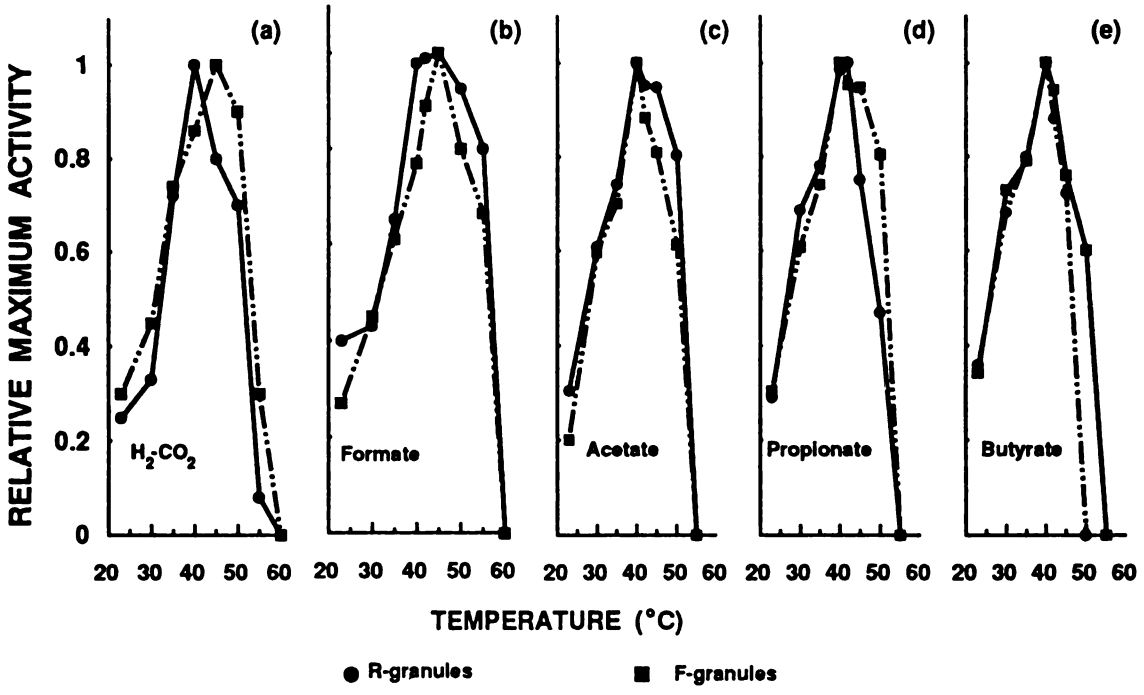


Figure 3.4 Temperature effect on substrate conversion rates at pH 6.8. (a) H_2-CO_2; (b) formate; (c) acetate; (d) propionate; (e) butyrate.

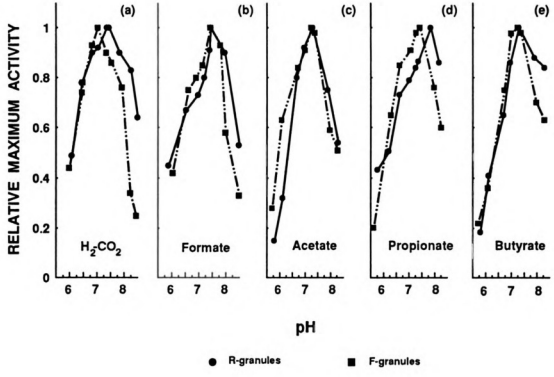


Figure 3.5 pH effects on substrate conversion rates at 37°C. (a) H_2-CO_2; (b) formate; (c) acetate; (d) propionate; (e) butyrate.

3.6 MICROBIAL COMPOSITION AND STRUCTURE OF GRANULES

3.6.1 Most probable number (MPN) enumeration

The results of MPN enumeration for various microbial trophic groups in the two types of granules are presented in Table 3.7. Both type of granules had approximately the same population levels for H_2-, formate-, and acetate-utilizing methanogens per gram of SS. Similar results were obtained for syntrophic propionate- and butyrate-degrading bacteria. The F-granules contained 10 to 100-fold higher numbers of hydrolytic-fermentative bacteria than R-granules when lactose or glucose were used as substrates as would be expected based on the respective feeds used.

Based on the MPN values, the major microbial populations in both types of granules were H_2-, formate-, and acetate-utilizing methanogens. These organisms contributed about 90% of the total culturable microbial populations. Syntrophic acetogens contributed about 10% of population recorded via the MPN test. The population of hydrolytic-fermentative microorganisms was not large even in the F-granules, which contained a higher level of hydrolytic bacteria (10^{11} cells/g VSS) compared to the R-granules (10^9). These MPN results are consistent with the substrate conversion rates of the two types of granules.

Based on the MPN results, the number of total bacteria in the granules is estimated to be between 10^{12} to 10^{13} cells/g SS.

Table 3.7 Trophic group populations in the R- and F-granules

Bacterial group	MPN x 10^{12}/g SS		95% confidence limits x 10^{12} cells/g SS			
			R		F	
	R	F	lower	upper	lower	upper
I. Hydrolytic						
Lactose-utilizing	0.0027	0.12	0.0010	0.069	0.044	0.36
Glucose-utilizing	0.0056	0.19	0.0019	0.16	0.047	0.64
II. Syntrophic						
Propionate-utilizing	0.066	0.034	0.016	0.22	0.010	0.090
Butyrate-utilizing	0.42	0.68	0.15	1.2	0.12	1.9
III. Methanogenic						
H_2-utilizing	3.5	3.1	1.2	10.0	1.1	9.4
Formate-utilizing	2.0	4.7	0.62	6.3	1.7	13.0
Acetate-utilizing	1.3	0.66	0.46	3.9	0.23	1.9
Methanol-utilizing	0.0043	0.031	0.0017	0.012	0.010	0.093

(1) MPN enumeration used 5 tubes per dilution level. The bacterial concentrations in the samples were 2.6 to 8.2 mg SS/mL.
(2) For all MPN enumeration results, the assumption tests were accepted.

3.6.2 Microscopic and electron microscopic examination

Significant differences in the microbial composition of different granules were easily observed by microscopic and electron microscopic examinations. Comparison of disrupted granules under phase-contrast microscopy is presented in Figure 3-6. The most significant difference between the R- and F-granules was the predominant acetate-utilizing methanogens observed. In the R-granules, a large number of bamboo-shaped rods in chains of 3 to 5 cells were observed (Figure 3.6A). In the F-granules, long-filamentous, bamboo-shaped rods in chains of ten or more cells were observed (Figure 3.6B and C). Neither of these cells had significant epifluorescence, a characteristic which associates these with *Methanothrix* species. *Methanobacterium*-like rods were found to be the predominant methanogens in both granules that fluoresced, when observed under ultra-violent light. *Methanosarcina* aggregates and a spirillum-shaped methanogen, resembling *Methanospirillum* sp., were also observed under epifluorescent microscopy.

The structures of the R- and F-granules were observed using SEM and TEM photomicrographs. Both the surface and inside sections of the R-and F-granules are presented in Figure 3.7. Under low magnification, both granules were observed to have an uneven surface (Figure 3.7 A and B). Under higher magnification, dense microbial structures, both on the surface and inside the granules, were observed for the R-granules. The rod-shaped, *Methanothrix*-like and other bacteria were packed tightly together to form dense granules (Figure 3.7C and E). On the surface of the F-granules, at least two types of long-filamentous bacteria were observed to occupy much of the space. These filaments formed a loose structure compared to the tightly packed R-granules (Figure 3.7D). One of these organisms that grew as *Methanothrix soehngenii*-like filaments, was observed both outside and inside the F-granules (Figure 3-7F). The

other filamentous type cells, which were much thinner (about 0.2 μm in diameter) than *Methanothrix* sp. (0.7 to 0.8 μm diameter), were observed mainly on the surface of the F-granules. The same organisms were also observed in the washed-out flocs from the F-granules reactor. These type of cells disappeared from the F-granules when feed B (glucose containing) was replaced with feed A.

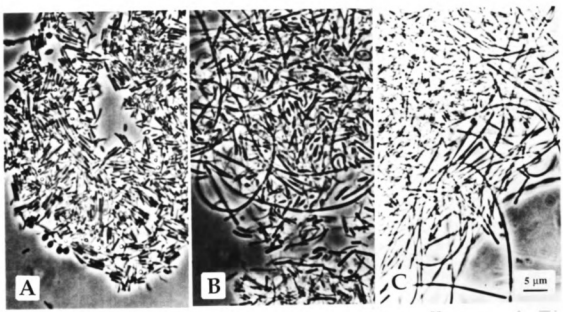


Figure 3.6 Microscopic comparison between the R-granules and F-granules. (A) Disrupted R-granules showing rod-type *Methanothrix* as predominant bacteria in the granules. (B) Disrupted F-granules showing *Methanothrix soehngenii*-like filaments as predominant bacteria when fed with VFA mixture plus ethanol and glucose. (C) Long filamentous *Methanothrix soehngenii*-like bacteria were still predominant bacteria in the F-granules after fed with VFA mixture for more than 4 months.

Figure 3.7 SEM photos of the R-granules fed with VFA mixture and the F-granules fed with VFA plus glucose and ethanol. (A) Overview of the R-granules. (B) Overview of the F-granules . (C) Densely packed rod-type *Methanothrix* sp. on the surface of the R-granules. (D) The surface of the F-granules shows filamentous *Methanothrix soehngenii*-like bacteria and other filaments form a loose network. (E) The cross section of the R-granules showing dense structure packed by *Methanothrix* sp. and other bacteria. (F) The cross section of the F-granules showing the net work formed mainly by *Mtrx. soehngenii*-like filaments.

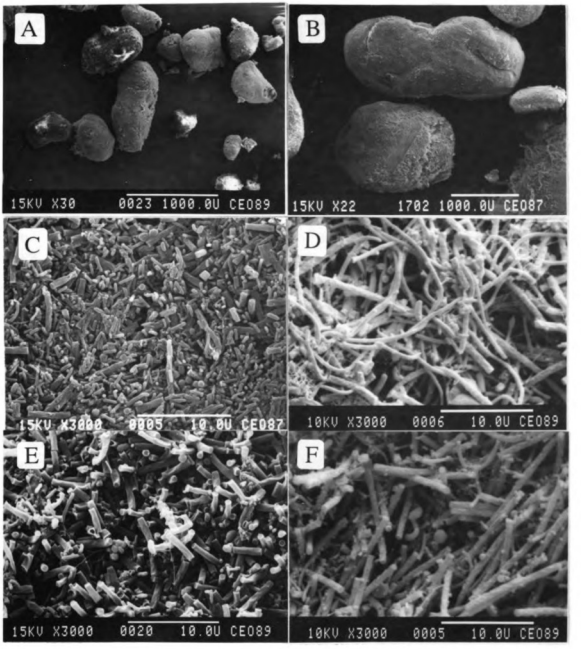
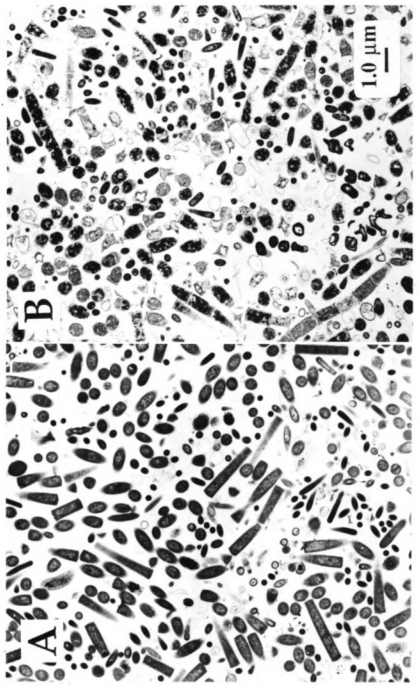

A
15KV X30 0023 1000.0U CE089
B
15KV X22 1702 1000.0U CE087
C
15KV X3000 0005 10.0U CE087
D
10KV X3000 0006 10.0U CE089
E
15KV X3000 0020 10.0U CE089
F
10KV X3000 0005 10.0U CE089

Figure 3.8 TEM photos of the R-granules and F-granules. (A) Higher cell density of the R-granules. (B) TEM thin section showing lower cell density of the F-granules.


A
B
1.0 µm

TEM examination revealed that the R-granules had a higher microbial density than the F-granules. *Methanothrix*-morphotype cells were prevalent in both types of granules (Figure 3.8A and B). Using cross sections of the granules, *Methanothrix*-like bacteria were counted. Nearly $2.9x10^7$ cells/cm^2 were observed in the R-granules, and 2.0×10^7 cells/cm^2 were found present in the F-granules. Other bacteria and empty cells were observed to be present at 3.7×10^7 and 1.4×10^7 cells/cm^2, and 3.6×10^7 and 0.4×10^7 cells/cm^2 in the R- and F-granules, respectively (Table 3.8). Viable cell density can be estimated by considering that the granules have three dimensions instead of two. Cell densities were calculated to be 7×10^{11}/cm^3 and 4.6×10^{11} cells/cm^3 for the R- and F-granules, respectively. These values compare favorably with the results for the MPN analysis.

Table 3.8 Comparison of microbial cell densities of the R- and F-granulesm (10^7 cells per cm^2)

Granules	Rodtype	Filament-type
Methanothrix-like	2.9	2.0
Other bacteria	3.7	3.6
empty cells	1.4	0.4
total cells	9.0	6.0

3.7 DISCUSSION

3.7.1 Similarities and differences between the R- and F-granules

A summary of the similarities and differences between the R-granules and F-granules is presented in Table 3.9. The two types of granules had significant differences in physical characteristics (density, specific gravity, and granule size), volumetric operational performance, specific metabolic activities for longer chain (more than two carbons) VFAs, and microbial composition. Similarities between the two granule types were observed in relation to biomass fraction, cell yield, specific operational activity, specific metabolic activities for H_2-CO_2, formate and acetate, apparent K_m for VFA degradation, and specific growth rates. The temperature and pH effects were somewhat different for the different granules. Both the R- and F-granules exhibited similar specific activities and achieved high specific COD removal rates (up to 6.9 to 7.6 and 5.5 to 6.9 gCOD/gVSS-d, respectively). This activity level is much higher than reported for other high-rate mesophilic granules fed with VFA mixtures. Hulshoff Pol (1989) reported that the activity of granules developed on an acetate-propionate mixture were never exceeded 4.0 gCOD/gVSS-d at 38 °C. Wiegant and de Man (1986) reported that their thermophilic granules (55 °C) had activities of 4.2 to 7.3 g COD/gVSS-d. It is likely that the high specific activities obtained is due to several factors including feeding the R- and F-granules with VFA mixture (acetate, propionate, and butyrate), maintaining a relatively high substrate concentration in the effluent, and supplying a low level of sulfate (0.15 mM sodium salt and ferric salts) in the feed (Thiele *et al.*, 1990). These factors probably are also the reason for the increase in the specific COD removal rate of F-granules which increased by about 2-fold over their original activity of 2.0 gCOD/gVSS-d when developed on brewery

wastewater (Chapter 2). The COD removal rate observed for the R-granules (based on rate per mass of biomass) were only slightly higher (10-20%) than observed for the F-granules.

The specific volumetric COD removal rate and maximum specific volumetric conversion rates of acetate, propionate, and butyrate of the R-granules were approximately twice those of F-granules. This can be attributed to the difference in granule densities (i.e. the R-granules contained ca. 60% more biomass than the F-granules per unit volume). Higher density and higher specific gravity of the R-granules allowed more biomass to be retained in reactor R per unit volume. Greater than 1.5 times of the biomass was stably maintained in reactor R compared to reactor F, even with a 1.5-fold greater biogas flux rate. This resulted in higher volumetric COD removal and higher volumetric methane production rates.

The growth rates of granule bed for the two types of granules were different. The F-granules grew more rapidly on a volumetric basis than the R-granules. Both type of granules had a similar cell growth yield i.e. 0.022 to 0.026 g SS/gCOD. The difference in bed growth rate is because the denser granules require less volume for accumulation of the same amount of biomass.

The two types of granules were initially supplied with different feed compositions. The observed metabolic activities and microbial populations developed appeared to be directly related to the feed supplied to the different reactors. Feed A used for the R-granules had 25% more propionate and butyrate than feed B which was supplied to the F-granules. Accordingly, higher propionate and butyrate conversion rates were observed for the R-granules. The F-granules had higher specific conversion rates for glucose and ethanol. Again this is because these granules were fed with glucose and ethanol while the R-granules were not.

Table 3.9 Similarities and differences between the R-granules and F-granules

Parameters	Similarity	Difference
Physical		
density (gSS/ml granules)		R > F (65-70 vs. 40)
specific gravity		R > F (1.03 vs. 1.01)
size		F had more large sized granules (>1.2 mm).
Chemical		
protein content (g/gSS)	same (0.77-0.78)	
VSS/SS	same (0.88)	
Operational		
volumetric activity (gCOD/L granules-d)		R > F (112-250 vs. 125-150)
specific activity (gCOD/gVSS-d)	similar values	
cell yield (gSS/g COD removed)	similar values	
granule growth rate (1/d)		R < F (0.022 vs. 0.045-0.048)
Metabolic		
specific growth rates	similar for VFA degraders	
specific activity (mmole/gVSS-d)	similar values for H_2-CO_2 C_1 and C_2	R had higher values for methanol, C_3, C_4, i-C_4, C_5; and lower values for ethanol and glucose.
apparent K_m for VFAs	similar values	
pH effect	similar for C_1, C_2 and C_4	R-granules had higher optima for C_3 and H_2-CO_2.
temperature effect	similar for C_1, C_2 and C_3	F-granules had higher tolerance for C_4 and H_2-CO_2.
Microbial		
MPN enumeration (cell/gSS)	same level for methanogens and syntrophic acetogens	F-granules had higher number of fermentative bacteria.
SEM observation		R had rod-*Methanothrix* as predominant species; F had filament-*Methanothrix* as predominant species.
TEM observation		R-granules had higher cell density.

Note: C_1: formate; C_2: acetate; C_3: propionate; C_4: butyrate; i-C_4: isobutyrate; C_5: valerate.

The differences in pH effect on H_2-CO_2 conversion rates and the ratio of H_2-CO_2 and formate conversion as results of various pH also indicated that the R- and F-granules probably had different hydrogen utilizing methanogens. The former exhibited low pH optima and a high affinity for formate. Other observed differences in the granules include VFA metabolism. The propionate degraders in the F-granules preferred more alkaline conditions and butyrate degraders in the R-granules had greater tolerance to higher temperatures (45 to 50°C). Finally, based on substrate conversion rates, the R-granules contained more isobutyrate-butyrate degraders and more *Methanosarcina* sp.

MPN enumeration results revealed that both types of granules had 10^{12} cells/g SS. This is consistent with the cell count results of TEM (7×10^{11} for the R-granules and 4.6×10^{11} cells/cm^3 for the F-granules) and $1\text{-}4 \times 10^{12}$ cells/g SS of total cells in other anaerobic granules (Dubourguier *et al.*, 1988c). The MPN values of acetate-utilizing methanogens were of the same magnitude as hydrogen- or formate-utilizing methanogens. The values of syntrophic acetogens were about 10% of total cell counts for both granule types. This indicates that the MPN technique used in this study did not likely underestimate population levels of the different trophic groups. This is in contrast to the results published elsewhere (Dolfing *et al.*, 1985; Dubourguier *et al.*, 1988b). The MPN results for the different trophic groups were consistent with the maximum substrate conversion rates observed.

The results of microscopic examinations revealed that the two types of granules had different microbial compositions. Microscopic and SEM examination indicated the major difference in the microbial composition between the two types of granules was the acetate-utilizing methanogens which predominated. Short rod type acetate utilizing methanogens were prevalent in the R-granules while typical *Methanothrix soehngenii-*

like filamentous cells were dominant in the F- granules.

3.7.2 Factors affecting granule density

There are at least two factors that have a significant influence on the granule density (or specific gravity): (i) mineral composition, and (ii) microbial cell density. The former is dependent primarily on the feed composition, and the latter on the microbial population developed. The latter factor is a function of both the operational conditions and the feed composition. Some granules treating industrial wastewater contain a significant amount of mineral precipitates and crystals (VSS/SS less than 0.7). This tends to increase the specific gravity. But these granules do not generally, however, have high metabolic activity. The R-and F-granules in this work were basically microbial aggregates and contained very few mineral precipitates (ca. 0.88 g VSS/g SS). The differences in the specific gravity between the two granule types are mainly due to differences in the microbial cell densities.

SEM examination of the granules revealed that *Methanothrix*-type cells appeared to play an important role in determining the type of granules formed. These methanogens appear to form a network that helps bind the other bacteria together. The morphotype of the methanogen that predominates effects the structure of granules. The short rod-shaped acetate-utilizing methanogen grows compactly and, subsequently, leads to a dense structure as observed for the R-granules. Filamentous *Methanothrix*-like cells build loose granule structure with more space between the organisms. The fact, that denser granules consist mainly of short rod-shaped cells and loose granules are composed of filamentous *Methanothrix* sp., was observed in earlier studies (Hulshoff Pol *et al.*, 1983; de Zeeuw, 1984, 1988). The formation of a loose granular structure may also be partly due to the presence of filamentous hydrolytic bacteria

found in the F-granules. Thin-filaments grew on the surface of the F-granules when these granules received a feed containing glucose. These filaments disappeared when glucose was removed from the feed. The change in feed from glucose-containing medium to non-glucose medium did not, however, improve the density or result in decrease of the size of the F-granules. It can, therefore, be assumed that these thin filamentous cells may be of less importance in the maintenance of granular structure than the filamentous *Methanothrix*-type cells. Whether they have a role in the development of low density granules is not clear. Filamentous hydrolytic bacteria of thin diameter (0.2 μM) have been observed in other anaerobic reactors (Endo and Tohya, 1988).

Besides acetate-utilizing methanogens, syntrophic acetogens and other methanogens also contribute to the microbial cell density, because they are present in considerable numbers in these granules. Kinetic analysis, temperature and pH effects indicated that there were some differences in the microbial population of hydrogen-utilizing methanogens, and propionate and butyrate degraders. The roles of these organisms in determining granular structure and density are not known.

A higher percentage of large sized granules was observed in the reactor fed a glucose molasses feed solution compared to a brewery wastewater (Chapter 2). It has been reported elsewhere that glucose or sucrose induced the formation of large granules (up to 3-5 mm) (Guiot *et al.*, 1985) or low strength granules (3-6 times less than those grown in partly acidified waste) (Sierra-Alvarez *et al.*, 1988). These effects have been generally attributed to hydrolytic-fermentative bacterial growth. The results obtained here cast some doubt on these being the only factors affecting granule density. Determination if glucose or other carbohydrates provide any stimulus to the growth of long filamentous *Methanothrix*-type methanogens is an area of research that could help

answer these questions.

3.7.3 Kinetic parameters for VFA degradation

Both disrupted R- and F-granules had the same K_m for acetate degradation. Comparing the apparent K_m with data in the literature, the K_m values for acetate are close to the K_s (0.46 mM) reported for *Methanothrix soehngenii* Opfikon (Zehnder *et al.*, 1980; Huser *et al.*, 1982), and slightly lower than the K_s (1.2 mM) for *Methanothrix concilii* GP6 (Patel, 1984) and a thermophilic acetate-utilizing methanogenic bacterium (0.8 mM) which showed a similar morphotype to *Methanothrix* sp. (Ahring and Westermann, 1985). The specific growth rates observed (ca. 0.11 to 0.13 /d) are within the range (0.11 to 0.29/d) reported for *Methanothrix soehngenii* (Zehnder *et al.*, 1980; Huser *et al.*, 1980; Touzel *et al.*, 1988). These facts further support the microscopic observation that the predominant acetate-utilizing methanogens in both granules were *Methanothrix*-type. High MPN values for methanol-utilizing methanogens would have indicated the presence of a large population of *Methanosarcina*. This was not observed, indicating that *Methanosarcina* did not play a significant role in acetate catabolism. In addition, the low residual acetate concentration (<0.01 mM) observed when the feed was stopped for one hour, was much less than the reported threshold acetate concentrations (0.4 to 1 mM) for *Methanosarcina* (Min and Zinder, 1989; Westermann *et al.*, 1989a).

The apparent K_ms observed for propionate degradation in both the granules (0.048 mM for the R- and 0.037 mM for F-granules) are within a range (0.044 to 0.191 mM) similar to that reported for sludge from a municipal waste digester (Kaspar and Wuhrmann 1978) but different from other reported values 0.43 mM (Lawrence and McCarty, 1969) and 0.15 mM and 4.5 mM (Heyes and Hall, 1983) for other

methanogenic sludges. This difference may be due to the existence of different propionate degraders in these systems or it may represent the effect of mass transfer limitations in these latter referenced studies. The specific growth rates of propionate degraders in the R- and F-granules (0.12 to 0.13/d) were close to that reported for *Syntrophobacter wolinii* (0.103/d), the only mesophilic syntrophic propionate degrader isolated to date (Boone and Bryant, 1980). This organism is also considered to be dominant syntrophic acetogen in granules (Dubourguier *et al.*, 1988a, 1988c). One of propionate degraders observed in both the R- and F-granules, however, is a spore-former, and thus different from *Syntrophobacter wolinii* (see Chapter 5).

The butyrate degraders in both granules had almost the same specific growth rate (0.13 /d) at a butyrate concentration of 0.15 to 0.16 mM. This is close to half the maximum specific growth rates reported for methanogenic cocultures of *Syntrophomonas* sp. (0.19 to 0.42/d) (McInerney *et al.*, 1979, 1981: Tomei *et al.*, 1985; Dwyer *et al.*, 1988) and essentially equal to that of *Syntrophospora bryantii* (0.17 to 0.23/d) (Stieb and Schink, 1985).

3.8 SUMMARY

1) Two types of anaerobic granules capable of high COD removal rates (≥7 gCOD/gVSS-day) were developed in laboratory-scale UASB reactors at 35 °C. One granule type had a rod-type *Methanothrix* sp. as predominant species (the R-granules) while the other had a filament-type *Methanothrix soehngenii*-like acetate utilizers as predominant species (the F-granules).

2) The R-granules had a higher density (65 to 70 vs 39 to 40 gSS/L), specific gravity (1.03 vs 1.01) and specific volumetric methane production rate (180 vs 120 L CH_4 /L granules-day) than the F-granules. Switching a feed containing glucose,

ethanol and VFAs to a feed containing only VFAs did not change the size or density of the F-granules. The differences in operational performance were mainly due to the different microbial composition, especially the predominant acetate-utilizing methanogens in the granules. The long filamentous *Methanothrix* sp. appeared to induce lower density and large sized granules.

3) Acetate, propionate and butyrate degraders from both types of granules had similar specific growth rates and substrate affinities.

4) Both types of granules had the same optimum temperatures for H_2-CO_2 and formate utilization (45 °C) and for acetate, propionate and butyrate utilization (40 °C), and showed basically the same optimum pH for H_2-CO_2, formate, acetate, propionate and butyrate utilization.

5) Results of most probable number (MPN) enumerations indicated that both types of granules had the same numbers of cells per gram of SS in the terms of methanogens (10^{12} for H_2-CO_2, formate, and acetate utilizing) and syntrophic acetogens ($10^{10\text{-}11}$ for propionate and butyrate utilizing). Hydrolytic fermentative bacteria were present in greater numbers in the F-granules (10^{11}) than in the R-granules (10^{9}) as would be expected based upon the respective feed used for the two reactors.

6) Observations made using scanning and transmission electron microscopy showed higher cell densities in the R-granules (which did not contain filamentous type of *Methanothrix* sp.) compared with the F-granules which contained *Methanothrix soehngenii*-like long filaments as the predominant acetate-utilizers.

CHAPTER 4

CHARACTERIZATION OF METABOLIC PERFORMANCE AND MICROBIAL STRUCTURE OF GRANULES GROWN IN THE PRESENCE OF SULFATE: ROLE OF SULFATE-REDUCING BACTERIA

INTRODUCTION

In Chapter 3, a characterization and comparison of two types of granules developed on the feed containing a very low level of sulfate (0.1 to 0.15 mM) was made. The role of sulfate reducing bacteria (SRB) in substrate metabolism and the granular structure was not considered for the R- and F-granules since sulfate reducing bacteria did not appear to play a significant role in substrate metabolism. To date, published work on the granule-formation, microbial composition, the substrate conversion potentials, and microbial structures of granules has focused on methanogens and syntrophic acetogens. (Hulshoff Pol *et al.*, 1983; Hulshoff Pol, 1989; Wu *et al.*, 1985, 1987b; Dolfing, 1985; Dolfing and Blomen, 1985; Dolfing and Mulder, 1985; Dolfing *et al.*, 1985; Dubourguier *et al.*, 1988a, 1988b, 1988c; Prensier *et al.*, 1988; Harada *et al.*, 1988). This is likely due to the observation that sulfate reducing bacteria were found to be present at much lower levels than syntrophic acetogens in granules treating sugar wastewater using the most probable number (MPN) enumeration (Dubourguier *et al.*, 1988a, 1988c).

In this Chapter, anaerobic granules obtained from a full-scale UASB plant treating brewery wastewater which contained ethanol, acetate and propionate as major carbon sources were characterized for their physical and chemical characteristics, substrate metabolism and microbial composition. These granules were grown in the presence of a significant level of sulfate (0.6 to 1.3 mM). The important role that SRB played in syntrophic methanogenesis for these granules was examined.

4.2 MATERIALS AND METHODS

Chemicals, gases, media, and analytical methods are described in Appendix A. Microscopic and electron microscopic examination is presented in Appendix D. The calculation of free energy available for syntrophic acetogenesis from ethanol is described in Appendix E.

4.2.1 Source of methanogenic granules

Brewery granules were obtained from a full-scale upflow anaerobic sludge blanket (UASB) reactor operated at G. Heileman Brewery Co. (LaCrosse, WI). This system was commissioned in 1982 using 20,000 kg of anaerobic granular sludge obtained from a UASB reactor treating sugar beet wastewater at the sugar factory CSM, Breda, the Netherlands and 2,000 kg of municipal digestor sludge from the LaCrosse treatment plant (Sax, 1985). The operational volumetric COD loading rate was about 10 kgCOD/m^3-d with a COD removal of 80 to 90%. The reactors were maintained at an operating temperature between 28 and 30°C. Influent sulfate concentrations in raw wastewater ranged from 0.6 to 1.3 mM. Sulfate removal of between 20 and 60% was normally observed in this system. The major chemical composition of the brewery wastewater fed to the UASB reactor and in the effluent is

summarized in Table 4.1. The majority of COD in the influent was due to incoming ethanol, acetate, and propionate.

Table 4.1 Composition of the influent brewery wastewater and effluent of UASB reactor

Parameters	Influent	Effluent
COD (mg/L)	1,200-2,000	170-300
Acetate (mM)	2.0-3.2	0.2-0.4
Propionate (mM)	1.0-2.1	0.06-0.2
Ethanol (mM)	7.3-13.0	<0.05
SO_4^{2-}(mM)	0.6-1.3	0.4-1.0
Cl^- (mM)	2.5-4.8	2.5-4.8
pH	6.0-7.0	7.0-7.2

(1) Trace amounts (<0.2 mM) of glucose, butyrate, lactate and succinate were also found in the influent.
(2) Detection limit for ethanol was 0.05 mM.

Granules were anaerobically prepared, shipped, and immediately transferred into 158 mL serum bottles under an N_2 atmosphere. The granules were stored at room temperature (about 20°C). The maximum substrate conversion rates and the maximum methane production rates were determined within 72 hours.

4.2.2 Substrate degradation and maximum specific activity assays

Substrate degradation assays were performed using ethanol and a mixture of acetate, propionate and butyrate. Eight substrates (acetate, propionate, butyrate,

methanol, ethanol, formate, H_2-CO_2, and glucose) were tested to determine maximum substrate conversion rates and/or maximum methane production rates of the granules at 35°C.

The maximum substrate conversion rates were determined for acetate, propionate, butyrate, methanol, ethanol, and glucose, based on substrate consumption per gram of VSS per day using the data within the initial several hours of the assay when the substrate conversion could be approximated by zero order kinetics. The maximum methane production rates were calculated based on the moles of methane produced per gram of VSS per day. Both substrate conversion and methane production were conducted in the presence and in the absence of added $FeSO_4$ (8 to 9 mM initial concentration) except for glucose conversion. For H_2-CO_2 and formate, both intact and disrupted granules were used as inocula to assay the effect of substrate diffusion. Disrupted granules were prepared from a 30 gSS/L cell suspension using a cell homogenizer inside an anaerobic glovebag (Coy Laboratory Products Inc., Ann Arbor, MI).

Before the media were inoculated with granules, the basal medium (50 mL per bottle) was reduced with an $Na_2S \cdot 9H_2O$ stock solution (2.5%). Subsequently, the intact granules (about 2 mL) were transferred to each bottle inside the anaerobic glovebag. The headspace in the bottles was flushed with an N_2-CO_2 mixture (70:30) to remove the H_2 gas from the glovebag. For inoculation of disrupted granules, the cell suspension (5 mL) was added by syringe directly to the bottles. Inoculated bottles were then supplied with small amount (about 1 mM for fatty acids and ethanol, and 0.1 g/L for glucose) of the respective substrates and incubated in a 35 °C shaking water bath for 2 to 3 hours to activate the respective trophic bacteria and thereby eliminate any lag in substrate consumption. The serum bottles were subsequently supplied with

the respective substrates. The concentrations used were about 9 mM for acetate or propionate, 5 mM for butyrate, 60 mM for formate, 22 mM for methanol, 17 mM for ethanol, 2 g/L for glucose, and 1.0 atm for H_2-CO_2 (80:20). These concentrations were much higher than the known values of K_m for the respective substrates during anaerobic digestion and allowed determination of maximum substrate conversion rates. The water bath was shaken at 125 strokes per min to reduce mass transfer resistance from bulk liquid to the surface of the granules. During the experiments, gas samples for methane and H_2 and liquid samples for VFA, ethanol and glucose were taken every 1 to 3 hours, depending on observed substrate conversion rates. After an experiment was concluded, the contents of serum bottles were centrifuged at 20,000 x g for 6 to 10 min and the pellet was collected for solids analysis.

4.2.3 Mass balance experiments for methanogenesis and sulfate reduction

Mass balance experiments for H_2-CO_2, formate, ethanol, acetate, and propionate were performed in 158-mL serum bottles as described above with the following exception. Intact granules (1mL) were transferred into the bottles and then incubated in a 35 °C water bath for about 12 hours to consume any residual substrate and, therefore, reduce potential errors in methane production calculation. Subsequently, gas samples were withdrawn from the bottles for the determination of starting methane and H_2 levels. The respective substrate stock solutions or H_2-CO_2 gas mixture was then added to the bottles. Bottles without substrate added were used as controls to estimate the methane production from any endogenous metabolism. The bottles containing the respective substrates were prepared as two groups: with and without addition of $FeSO_4$ (9.0 mM). After 96 hours of incubation (120 hours for methanol) at 35 °C, substrate consumption (including sulfate) and methane formation were quantified for mass

balance calculations.

The calculation of percentages of substrate converted via sulfate reduction is described in Appendix F.

4.2.4 Molybdate effect experiments

The effects of molybdate on the conversion of acetate, propionate and ethanol and related methane production rates were determined as follows. Intact granules were transferred into serum bottles containing 50 mL medium reduced by sodium sulfide and then supplied with 1 mM of respective substrates. An Na_2MoO_4 stock solution was then added to the bottles to obtain the desired starting concentrations of molybdate. The bottles were incubated in a 30 °C shaking water bath for about 12 hours to permit molybdate induced inhibition levels to stabilize. The temperature of the water bath was then increased to 35 °C, and the respective substrates were added to the bottles. The experiments designed to measure the maximum substrate conversion rates and the maximum methane production rates were then begun.

4.2.5 Enrichments of special trophic groups

Acetate (20 mM), propionate (20 mM), and ethanol (20 mM) were used as the substrates to enrich the prevalent species in the granules. One set of the enrichments was supplied with sodium sulfate (5 mM) while another was not. Disrupted granules (0.5 mL) with a concentration of 30 gVSS/L were inoculated into the anaerobic bottles containing 50 mL of the respective enrichment media. These bottles were then incubated at 35 °C. The growth of different trophic species was monitored by examining methane production and substrate consumption. The enrichments were transferred at least 5 times at a 1% (vol/vol) inoculum. The final enrichments were

examined by microscopy and electron microscopy.

4.3 PHYSICAL, CHEMICAL AND MICROBIAL CHARACTERIZATION OF GRANULES

4.3.1 Physical and chemical characteristics

The granules were black, and their size varied from 0.3 to 4.5 mm in diameter (most less than 3 mm). The physical and chemical characteristics of the granules are summarized in Table 4.2. The granules had a relatively high specific gravity (1.046) and granule bed density (91.7 gSS/L). The ash content was 18% based on the ratio of VSS/SS. Based on X-ray analysis, the major mineral solids in the granules apparently were ferrous sulfide, calcium phosphate as well as silicon compounds. Carbonate precipitates were insignificant. Assuming all the carbonate in the granules were present as calcium salts, a maximum of 2.4 mg $CaCO_3$/gSS would have been present.

4.3.2 Microscopic and electron microscopic examination

Observation of disrupted granules using phase-contrast epifluorescence microscopy revealed that the granules contained a number of morphologically distinct bacteria. *Methanobacterium*-like rods were identified as the prevalent H_2-CO_2 and formate-utilizing methanogens while *Methanothrix*-like bamboo-shaped rods appeared to be the prevalent acetate utilizing methanogenic bacteria. *Methanospirillum*-like spirilla were occasionally observed. *Methanosarcina*-type aggregates were not observed. When granules were incubated with a methanol containing (initial concentration of 22 mM) medium at 35 °C for 5 days, some *Methanosarcina*-like

aggregates that auto- fluorescenced (indicating the presence of coenzyme F_{420}) were observed. In addition to methanogens, several morphotypes of non-fluorescent, unidentified short rods were observed as the predominant cells. These bacteria were normally found in small clusters surrounded by the *Methanobacterium*-like rods.

Table 4.2 Physical, chemical and microbial characteristics of brewery granules

Specific gravity	1.046
Granule bed density (g SS/L granules)	91.7
Dry weight/wet weight (g/g)	0.13
VSS/SS (g/g)	0.82
Internal carbonate (as CO_2 μmol/g SS)	24.4
Mineral composition (%)[a]	
Silicon	2.43
Sulfur	22.6
Phosphorus	31.0
Potassium	6.95
Sodium	13.2
Calcium	10.9
Iron	12.9
Microbial morphotype density ($x10^7$ cells/cm^2)[b]	
Methanothrix-like	1.31
Methanobacterium-like	2.22
others	1.49
empty cells	0.76
total cells	5.78

a) Mineral composition was analysis by EDX, based on the average values of different sites on the surface and cross section of granules.
b) The total numbers of cells counted on TEM thin cross section samples were 6769.

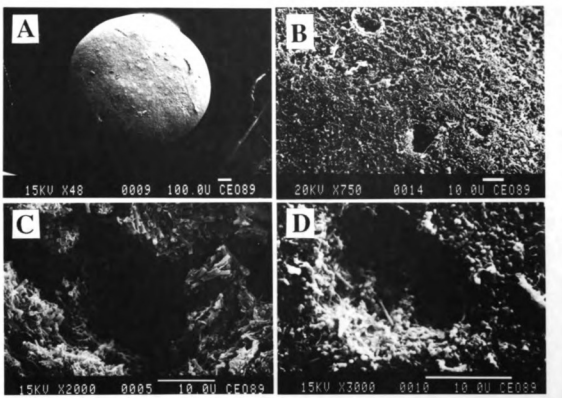
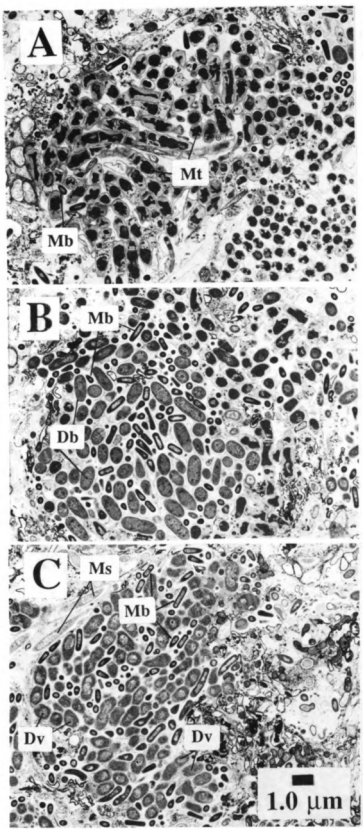


Figure 4.1 SEM microphotographs of brewery granules. (A) Low magnification SEM micrograph of a granule shows that small holes exist on the surface of granules. (B) Outline of vents or holes on granule surface. (C) Opening of a vent hole surrounded by *Methanothrix*-like bacteria. (D) Another opening surrounded by *Methanobacterium*-like rods and other bacteria.

Figure 4.2 TEM micrographs of thin cross section of granules that show the presence of different microcolonies. (A) Type I microcolony of primarily *Methanothrix*-like bacteria (Mt) and low levels of *Methanobacterium*-like bacteria (Mb). (B) Microcolony type II is homogeneously composed of *Methanobacterium*-like rods (Mb) and regularly shaped, *Desulfobulbus*-like rods (Db). (C) Microcolony consists of irregularly-shaped, *Desulfovibrio*-like rods (Dv) and *Methanobacterium*-like rods (Mb). *Methanospirillum*-like cells (Ms) are also observed outside the type III colony.


A
Mt
Mb
B
Mb
Db
C
Ms
Mb
Dv
Dv
1.0 μm

The granules were generally spheroidal in shape. A large number of small holes or vents were observed on the surface of the granules (Figure 4.1A). These holes appeared to be randomly distributed on the granule surface with openings of 10 to 20 μm (Figure 4.1B). Some of the openings were surrounded by bamboo-shaped *Methanothrix*-like rods (Figure 4.1C), while others were surrounded by *Methanobacterium*-like rods together with other fat rods. (Figure 4.1D).

Thin-section analysis of the granules revealed that the distribution of different bacterial morphotypes was not homogeneous and that low- and high-cell density areas existed within the granules. Although it is impossible to say with any certainty that the low-density areas were not simply artifacts of sample preparation, it appears that these areas may be internal gas channels. This is supported by the observation that these low-density areas were generally void of cells or dead cells and were approximately the same diameter as the vent holes on the granule surface. The high cell density areas were mainly composed of microcolonies or clusters which can be characterized into three distinct types. The first type of microcolony consisted almost entirely of *Methanothrix*-like bacteria, while *Methanobacterium*-like rods were occasionally observed within the microcolonies (Figure 4.2A). These microcolonies contributed about 30 to 40% of the total area of the thin sections. The remaining two types of microcolonies were more heterogeneous with respect to morphotypes. They consisted predominantly of *Methanobacterium*-like rods in close association with other rod-shaped bacteria presumed to be involved in acetogenesis. One type of microcolony was composed of gram-negative-like regular rods which grow rather heterogeneously with the rod-shaped methanogens (Figure 4.2B). The final type of microcolonies were composed of irregular shaped, gram-negative rods and rod-shaped methanogens. They also grew quite heterogeneously (Figure 4.2C). The two

non-methanogenic rods were the prevalent non-methanogenic bacteria found in the thin-sections of the granules. Around these cells, a polymer matrix structure was easily visible (Figure 4.2B and C). Whether this polymer matrix is in part responsible for maintaining the integrity of the granules or strengthening the granular structure is not clear. Other than the above morphotypes, *Methanospirillum*-like cells were occasionally observed (Figure 4.2C). The approximate concentration of the different morphotypes observed in the TEM thin-sections is summarized in Table 4.2. The total microbial density of the granule cross section was ca. 6×10^7 cells/cm^2. Among these cells, ca. 23% were the *Methanothrix*-like bacteria, 38% were the *Methanobacterium*-like bacteria, 26% were other rod-shaped bacteria while 13% were empty cells. Based on these data, the granules would have about 4.4×10^{11} cells/cm^3 or 4.0×10^{12} cells/gVSS, based on the density and VSS/SS ratio in Table 4.2.

4.3.3 Predominant species in enrichments

Six enrichments were obtained: two acetate enrichments (plus and minus sulfate), two ethanol enrichments (plus and minus sulfate) and two propionate enrichments (plus and minus sulfate).

Methanothrix soehngenii-like long filamentous rods were predominant species in the two acetate enrichments. These cells grew as flocs. *Methanosarcina*-like cells were not observed. Non-auto-fluorescencent, irregularly-shaped rods, resembling *Desulfovibrio* sp., and short rods with auto-fluorescence, presumably *Methanobacterium* sp. or *Methanobrevibacter* sp., were occasionally observed during microscopic examination of the enrichment not receiving sulfate (Figure 4.3A). These two rod-shaped methanogens were also observed in the enrichment receiving sulfate; however, many more *Desulfovibrio*-like cells were observed.

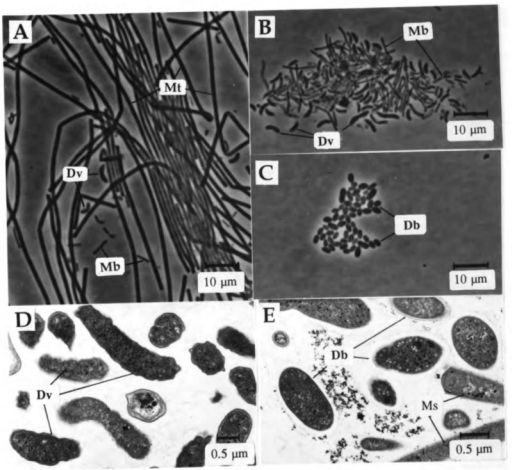


Figure 4.3 Microbial species observed in acetate, ethanol and propionate enrichments. (A) *Methanothrix soehngenii*-like long filaments (Mt) were predominant species in the acetate enrichment minus sulfate. *Methanobacterium* (Mb) and *Desulfovibrio* (Dv)-like cells were also found. (B) Syntrophic growth of *Desulfovibrio* sp (Dv). and *Methanobacterium* sp. (Mb) in ethanol enrichment minus sulfate. (C) Clump of *Desulfobulbus*-like (Db), regular fat rods in propionate enrichment (plus sulfate). (D) TEM thin section of *Desulfovibrio* sp. (Dv) in ethanol enrichment showed the same morphology as the irregular rods in granules (Figure 4.2D). (E) TEM micrograph of syntrophic growth on propionate of *Desulfobulbus*-like (Db) and *Methanospirillum*-like (Ms) cells in propionate enrichment minus sulfate.

In the two ethanol enrichments, *Desulfovibrio*-like cells were the predominantorganisms. In the absence of sulfate, these cells grew together with *Methanobacterium*-like cells (Figure 4.3B), and methane production was observed during ethanol degradation. In the sulfate added enrichment, no methanogens were observed. This result indicates that the *Desulfovibrio*-like cells can grow on ethanol either syntrophically with methanogens or alone if sulfate is present as an electron acceptor. Propionate was not utilized by these cultures. Thin section TEM micrographs (Figure 4.3D) revealed that the *Desulfovibrio*-like cells had the same morphotype as the irregular rods identified in the syntrophic microcolonies inside the granules as shown in Figure 4.2C.

In the propionate enrichments, the predominant cells were fat rods which were observed to form large aggregates (Figure 4.3C). *Methanosprillum*-like, *Methanobacterium*-like, and *Mtrx. soehngenii*-like cells were found in the enrichment not receiving sulfate. In the presence of sulfate added, only *Methanospirillum*-like cells were observed occasionally. A polymer structure around the sulfate reducing rods can be observed easily (Figure 4.3E). Both the propionate enrichments were able to utilize ethanol. Based upon TEM microscopy, these rods were similar in appearance to *Desulfobulbus propionicus* and the non-methanogenic rods in Figure 4.2C.

4.4 SUBSTRATE METABOLIC PERFORMANCE

4.4.1 Time-course of substrate degradation

Substrate degradation of some major intermediates was tested with essentially no sulfate (ca. 3.7 μM of sulfate added with the trace element solution) to examine

syntrophic methanogenic reactions in the granules. The results of syntrophic ethanol conversion is illustrated in Figure 4.4 and summarized in Table 4.3. As ethanol was consumed, acetate accumulated and then declined upon depletion of ethanol. As would be anticipated, H_2 accumulated rapidly during ethanol conversion (up to 97 Pa) and was then consumed to low levels. Formate (up to 70 μM) was observed to accumulate and then be subsequently consumed as ethanol levels decreased. Propionate also accumulated to a maximum level of 0.26 mM and was then degraded. The values of free energy available for interspecies hydrogen transfer and formate transfer were basically the same (Table 4.3). Free energy available for synthesis of formate from hydrogen was near zero, indicating these two electron carrying intermediates were at equilibrium.

Simultaneous degradation of acetate, propionate and butyrate was examined to determine the relative rates of conversion of these three VFAs (Figure 4.5). Acetate was consumed much quicker than the other VFAs. Butyrate consumption was extremely slow, indicating that this acid is not a normal substrate for these granules as would be expected based on the composition of brewery wastewater. During butyrate degradation, a considerable amount of isobutyrate (0.7 mM) accumulated and a trace amount of 2-methylbutyrate (0.05 mM) was formed. Butyrate disappeared faster than isobutyrate under these conditions. This result indicates the presence of butyrate- and isobutyrate-butyrate-degrading microorganisms in the granules (see Chapter 6). The H_2 partial pressure observed during this experiment was much lower than previously observed during ethanol consumption. Formate was not detected (<5 μM).

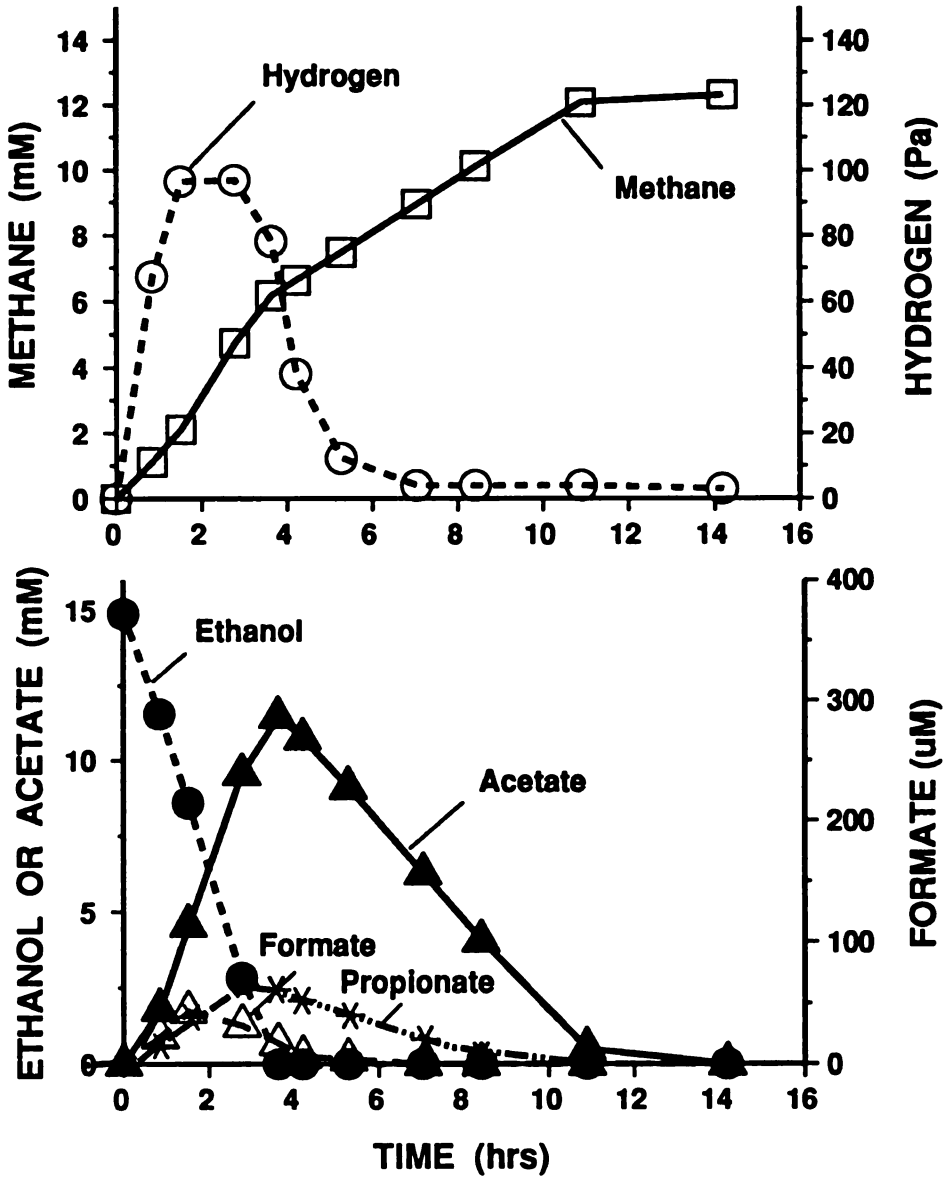


Figure 4.4 Syntrophic ethanol degradation and intermediate product formation by brewery granules in the absence of sulfate at 35°C.

Table 4.3 Intermediates formed during syntrophic ethanol degradation by brewery granule and energetic analysis of interspecies electron transfer and formate synthesis

Time	ethanol	acetate	propionate	H_2	formate	ΔG' (kJ/reaction)		
						interspecies transfer		Formate
(hr)	(mM)	(mM)	(mM)	(Pa)	(μM)	H_2	Formate	Synthesis
0	14.9	0.1	<0.02	1	<5	-62	<-54	<+4.2
0.85	11.5	1.8	<0.02	68	36	-33	-35	-1.5
1.5	8.6	4.5	0.045	97	70	-28	-29	-0.7
2.8	2.8	9.5	0.15	97	55	-23	-26	-1.1
3.6	<0.05	11.5	0.26	78	30	NC	NC	-2.4
5.3	<0.05	9.1	0.19	12	<5	NC	NC	NC

(1) Calculation of ΔG'values is described in Appendix E.
(2) NC: Not calculated. Detection limits were 0.02 mM, 0.05 mM and 5 μM for propionate, ethanol and formate, respectively in this assay.
(3) Test conditions: initial pH 7.0, HCO_3^- 59 mM, and 35 °C.

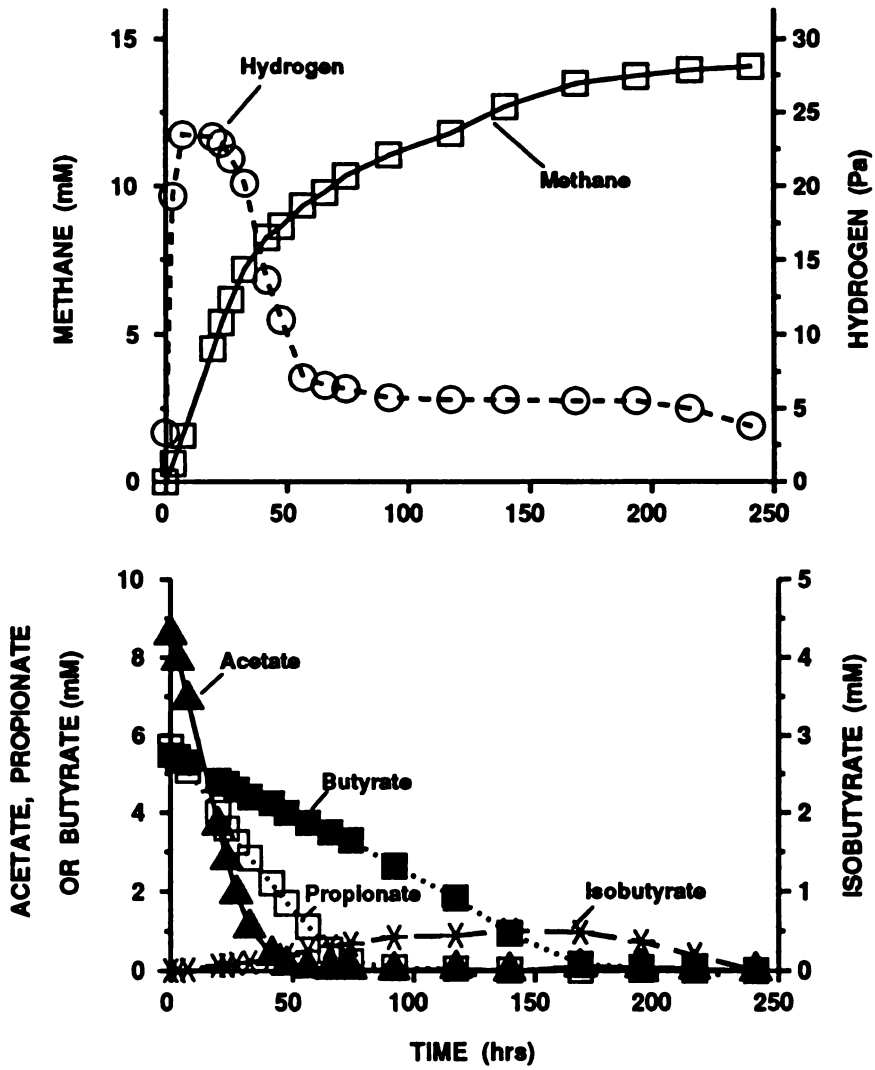


Figure 4.5 VFA (acetate, propionate and butyrate) degradation in the absence of sulfate by brewery granules at 35 °C.

4.4.2 Substrate conversion rates and maximum methane production rates

Although the experiments conducted above provided some information as to the capacity of the granules to degrade common substrates, the assays were performed with only trace sulfate present. As indicated in the composition of brewery wastewater (Table 4.1), moderate quantities of sulfate were normally present in the wastewater these granules had become acclimated to. Accordingly, some major intermediates were tested to determine whether the presence of sulfate influenced their metabolism. The maximum substrate conversion rates and/or maximum methane production rates for the eight substrates examined (with/without sulfate) are summarized in Table 4.4.

In the absence of sulfate, the granules demonstrated relatively high maximum specific conversion rates (mmol/gVSS-d) for ethanol (45.1), acetate (8.0), propionate (4.1) and glucose (5.7), and low rates for methanol (1.2) and butyrate (0.85). The maximum specific methane production rates from formate and H_2-CO_2 were essentially the same. Although granules physically disrupted to reduce mass transfer resistance had much higher specific methane production rates than the intact granules, the methane production rates were essentially the same for both substrates for both intact and disrupted granules. The addition of sulfate did not significantly affect the methane production rates from H_2-CO_2 or formate. When formate was used as the substrate to determine the maximum methane production rate, significant concentrations of hydrogen accumulated, up to more than 150 Pa, at the beginning of the experiment and was subsequently consumed. The initial maximum H_2 accumulation rates from added formate were the same in the absence and presence of sulfate (Table 4.4). Comparison of the maximum methane production rates with the initial maximum hydrogen accumulation rate, ca. 1 to 2% of the formate was converted and released into the headspace as hydrogen gas.

Table 4.4 Maximum substrate conversion rates and maximum methane production rates by the brewery granules in the presence and the absence of sulfate (35 °C)[a]

Substrate	Max. substrate conversion rate (mmol/gVSS-d)		Max. methane production rate (mmol/gVSS-d)		Granules
	$-SO_4^{2-}$	$+SO_4^{2-}$	$-SO_4^{2-}$	$+SO_4^{2-}$	
1. glucose	5.7	ND	8.8	ND	intact
2. methanol	1.2	1.2	0.84	0.56	intact
3. ethanol	45.1	44.5	29.8	26.7	intact
4. H_2-CO_2	ND[b]	ND	13.7	13.6	intact
H_2-CO_2	ND	ND	42.3	42.9	disrupted
5. formate	ND	ND	13.5[c]	13.4[c]	intact
formate	ND	ND	36.8[d]	37.3[d]	disrupted
6. acetate	8.0	8.1	8.0	7.9	intact
7. propionate	4.1	5.4	6.6	7.0	intact
8. butyrate	0.85	0.84	1.9	1.5	intact

a) $FeSO_4 \cdot 5H_2O$ was used as the sulfate source.
b) ND: not determined.
c) The initial H_2 accumulation rates from formate were 1.04 and 1.08 mmol/gVSS-d, respectively.
d) The initial H_2 accumulation rates from formate were 1.61 and 1.41 mmol/gVSS-d, respectively.

In the presence of sulfate, the maximum substrate conversion rates for methanol, ethanol, acetate, and butyrate were essentially equal to those samples without added sulfate. The addition of sulfate did not change the maximum methane production rates from acetate, but did reduce methane production rates from methanol and ethanol

indicating involvement of SRB in degradation of these compounds. The maximum propionate conversion rate increased by 30% due to addition of sulfate. The maximum methane production rate from propionate was also observed to increase as a consequence of sulfate addition.

4.4.3 Effect of sulfate on methane yields

Mass balances were conducted with various substrates (H_2, formate, acetate, propionate, and ethanol) in the absence or presence of added sulfate to determine substrate consumption, methane production and sulfate consumption (Table 4.5). Sulfate was supplemented to obtain initial SO_4^{2-} concentrations ranging from 8.2 to 9.4 mM. In the experiments conducted with added sulfate, a minimum of 1.6 mM and, in general, much higher levels of sulfate were present at the conclusion of the experiments. The amount of methane formed in the presence of added sulfate indicates a limited role for sulfate reduction in the complete conversion of these substrates. The major exception to this was propionate. The methane yield in the presence of sulfate was reduced by 28% compared to samples converting propionate without added sulfate. Based on the data obtained, the percentages of sulfate reduction in the conversion of different substrates are shown in Table 4.5 (Calculation is described in Appendix F). Only a small amount of H_2, formate and acetate was converted by sulfate reduction. The methane yield from propionate and ethanol in the presence of sulfate was reduced by 28 and 12%, respectively, compared with samples converting propionate and ethanol without added sulfate. Based on methane produced, ca. 60% of propionate and 30% of ethanol were converted to sulfide and acetate when a high level of sulfate was supplied.

Table 4.5 Substrate conversion by brewery granules in the presence and the absence of sulfate

Substrate	Substrate consumed (mmol)		SO_4^{2-} consumed (mmol)	CH_4 formed (mmol)		CH_4 formed $+SO_4^{2-}/-SO_4^{2-}$	Substrate converted via sulfate reduction (+ sulfate)	Granule
	$-SO_4^{2-}$	$+SO_4^{2-}$	$+SO_4^{2-}$	$-SO_4^{2-}$	$+SO_4^{2-}$	(mmol:mmol)	(%)	
1. H_2	1.83	1.83	0.02	0.46	0.43	0.94	6	intact
H_2	4.97	4.97	0.011	1.24	1.22	0.98	2	disrupted
2. formate	4.50	4.50	0.06	1.09	1.03	0.95	5	intact
formate	3.35	3.35	0.0058	0.834	0.826	0.99	1	disrupted
3. acetate	1.00	1.00	0.007	0.99	0.95	0.96	4	intact
4. propionate	1.00	1.00	0.41	1.67	1.20	0.72	60	intact
5. ethanol	1.00	1.00	0.18	1.45	1.27	0.88	28	intact

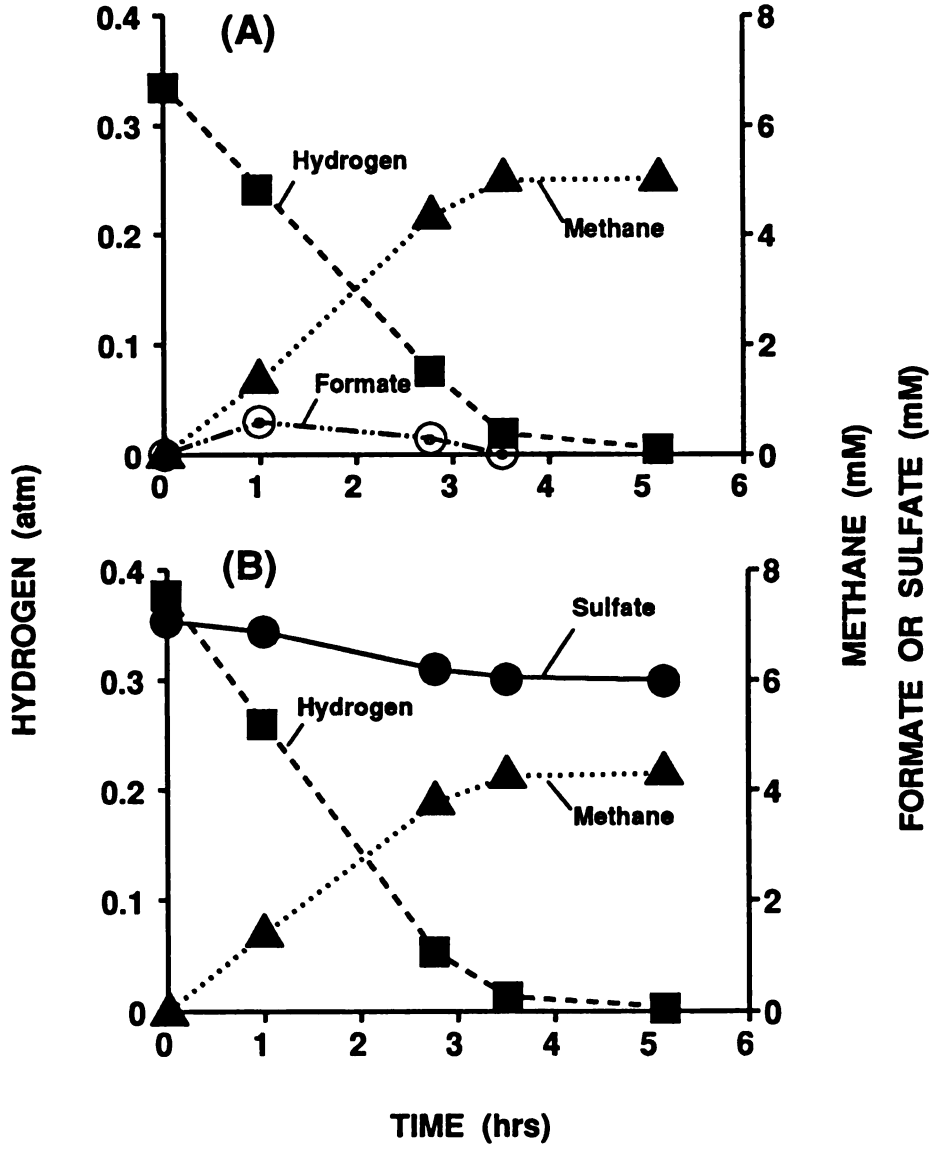


Figure 4.6 Methanogenesis from H_2-CO_2 by the brewery granules (initial pH 7.10). (A) Initial H_2 partial pressure was 0.34 atm, and CO_2 pressure was 0.32 atm. Formate accumulated and then consumed in the absence of sulfate. (B) Initial H_2 partial pressure was 0.38 atm, CO_2 pressure was 0.30 atm, and $FeSO_4$ concentration was 7.5 mM. Formate did not accumulated to a detectable level (>5 μM), and sulfate was converted to a small extent.

4.4.4 Formate from H_2-CO_2 and the effect of sulfate

As described above, significant concentration of hydrogen accumulated during formate conversion by the granules in the presence and absence of sulfate. Formate production was also observed during methanogenesis from H_2-CO_2. The concentrations of formate that accumulated were influenced by hydrogen pressure supplied and addition of sulfate. When moderate hydrogen pressure (0.34 atm) was supplied in the absence of sulfate, significant amounts of formate (0.6 mM) accumulated within 1 hour and were then consumed concurrent with hydrogen consumption (Figure 4.6a). When sulfate was exceeded, formate concentration was as low as the detectable level (5 μM) during the methanogenesis from H_2-CO_2 (Figure 4.6b). When higher hydrogen pressure (0.9 atm) was supplied, formate accumulated to 0.8 and 0.2 mM in the absence and presence of sulfate, respectively, and was then degraded. This indicates that a reversible reaction between formate synthesis and hydrogen production can be performed by the granules.

4.4.5 Effect of molybdate on substrate metabolism

Molybdate is known to be an effective and relatively selective inhibitor of SRB (Taylor and Oremland, 1979; Banat *et al.*, 1981; Smith and Klug, 1981). Experiments using different concentrations of sodium molybdate were conducted to determined more clearly if SRB were in fact involved in the conversion of propionate, acetate and ethanol. Acetate conversion was not sensitive to MoO_4^{2-} (Table 4.6). The addition of 10 mM molybdate reduced the maximum conversion rate of acetate by only 17%. Syntrophic propionate conversion, however, was extremely sensitive to molybdate addition. Only 0.1 mM molybdate was required to decrease the maximum propionate

degradation rate by 67%. The inhibition of propionate conversion increased in proportion to the concentration of MoO_4^{2-} (Figure 4.7). Syntrophic conversion of ethanol was moderately sensitive to molybdate addition. In the presence of 0.1 mM molybdate, ethanol conversion was reduced ca. 6%. When molybdate concentration was increased between 2.0 and 10.0 mM, the maximum conversion rate of ethanol was reduced by 30%.

Table 4.6 Molybdate effects on the maximum substrate conversion rates for acetate, propionate and ethanol

Molybdate (mM)	0	0.1	0.2	0.5	1.0	2.0	10.0
1. acetate							
rate, mmol/gVSS-d	8.16	7.60	ND	ND	ND	7.14	6.74
inhibition (%)	0	6.9	ND	ND	ND	12.6	17.4
2. propionate							
rate, mmol/gVSS-d	4.17	1.37	0.41	0.20	0.20	0.14	ND
inhibition (%)	0	67.1	90.2	95.2	95.2	96.6	ND
3. ethanol							
rate, mmol/gVSS-d	39.0	36.6	ND	ND	ND	27.7	27.0
inhibition (%)	0	6.3	ND	ND	ND	29.1	30.9

ND: not determined.

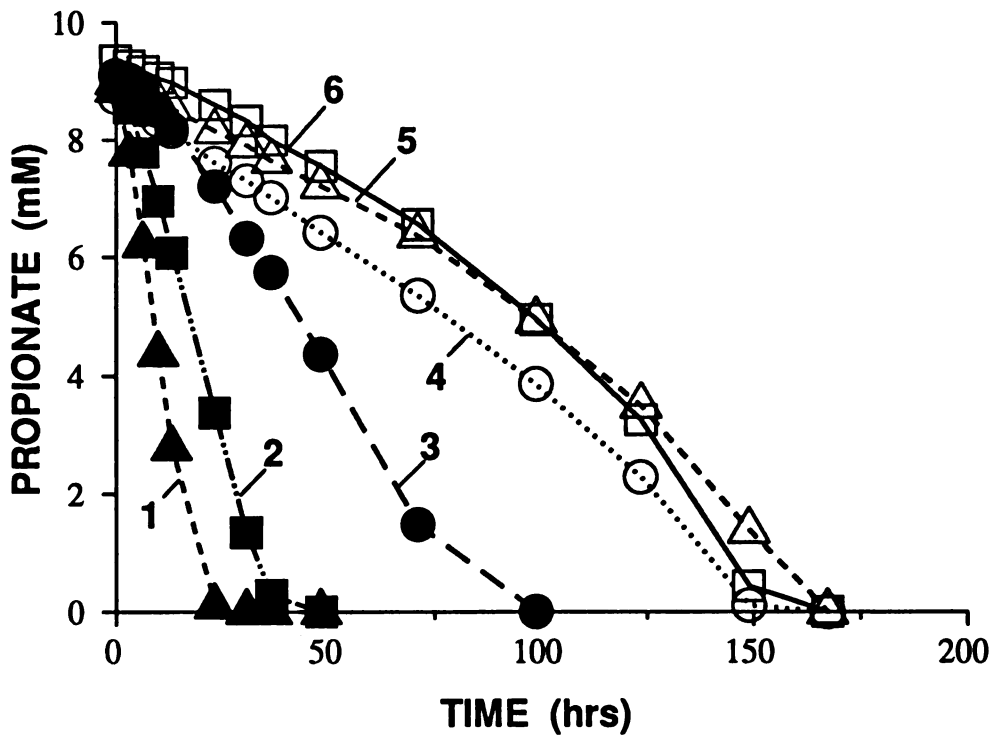


Figure 4.7 The effect of different Na_2MoO_4 concentrations on the propionate conversion by the granules in the absence of sulfate at 35 °C. Molybdate concentrations (mM) were 0, 0.1, 0.2, 0.5, 1.0 and 2.0 and granule biomass (gVSS) were 0.23, 0.24, 0.29, 0.24, 0.26 and 0.33 for sample No.1 through No.6, respectively.

4.5 DISCUSSION

4.5.1 Microbial population of granules

Substrate composition has a strong effect on determination of microbial composition in an ecosystem. Characterization of the influent revealed that the major substrates consistently available to the granules used in this study were ethanol, acetate and propionate (65 to 90% of total COD). Therefore, the prevalent microbial trophic groups in the granules would be expected to consist of ethanol-, propionate-, acetate-, and H_2 and formate-utilizing bacteria. This was, in fact, borne out by subsequent substrate degradation experiments. Based on the maximum specific conversion rates for the various substrates tested, these granules contained all three microbial trophic groups described in Chapter 1: hydrolytic-fermentative bacteria, syntrophic acetogenic bacteria (ethanol-, propionate-, butyrate and isobutyrate-utilizing), and methanogenic bacteria. However, the majority of trophic groups appeared to be ethanol, propionate, acetate, and H_2 and formate-utilizing bacteria as would be anticipated based on the wastewater composition. Hydrolytic-fermentative bacteria constituted a limited portion of the overall population in the granules, based on the relatively low glucose conversion rate. Methanol-utilizing bacteria and butyrate (or butyrate-isobutyrate) degraders also did not appear to be present in great numbers since poor substrate conversion and methane production rates were observed using these substrates. TEM examination also supports this. The majority of the bacteria in the granules were *Methanothrix*-like and *Methanobacterium*-like methanogens. The remaining cells were apparently mainly bacteria involved in ethanol and propionate degradation. The level of sulfate (0.6 to 1.3 mM) in the influent provided favorable conditions for sulfate

reducing bacteria to proliferate.

4.5.2 Syntrophic ethanol degradation and interspecies electron transfer

Ethanol was the major carbon source present in the brewery wastewater. Experiments indicated that during syntrophic ethanol degradation, acetate, formate, H_2 and propionate were formed as intermediates. All were subsequently converted to methane. Acetate accumulation is a consequence of maximum rate of acetate consumption being much lower than the rate of ethanol consumption (Table 4.3). The accumulation of both H_2 and formate, to relatively high concentrations, during syntrophic ethanol conversion is interesting. It appears likely both may be involved in interspecies electron transfer during syntrophic ethanol oxidation to acetate. Energetic analysis indicated that the free energy available for both interspecies formate and hydrogen transfer during syntrophic ethanol degradation was basically the same and that the concentration of formate and the partial pressure of hydrogen were at an equilibrium level ($\Delta G'$ for formate synthesis from H_2 plus HCO_3^- was near zero, as shown in Table 4.3). The levels of formate and H_2 suggested that the reversible conversion ($formate^- + H_2O \rightleftharpoons H_2 + HCO_3^-$) was at equilibrium during syntrophic ethanol degradation. This indicates that both formate and hydrogen serve as intermediates during syntrophic ethanol degradation by the brewery granules regardless of which (or both) are actually produced by ethanol-degrading organism(s). The concentrations of formate and hydrogen are likely regulated by the environmental conditions such as HCO_3^-, pH etc.

The accumulation of propionate probably is due to the action of some SRB such as *Desulfobulbus propionicus*, which can ferment ethanol to propionate or produce propionate from ethanol, hydrogen and HCO_3^-, or from acetate, hydrogen and HCO_3^-

in the absence of sulfate (Laanbroek *et al.*, 1982). In the batch experiments and in the full-scale UASB reactor, the production of propionate by the granules is likely associated with ethanol fermentation since the hydrogen partial pressure did not reach high levels in any cases of the experiments. The production of propionate by the propionate-utilizing population may be a survival strategy that allow these organisms to compete for common substrate (such as ethanol) and simultaneously "store" substrate that only a limited population, including themselves as predominant microbes, can use. This reaction, however, may not be important for ethanol degradation by the granules since the molar ratio between propionate formed versus ethanol converted was less than 2% (Table 4.3), and the propionate conversion rate was much slower than the ethanol conversion rate.

4.5.3 Hydrogen and formate metabolism

Hydrogen production from formate was observed in pure cultures of *Methanobacterium formicicum* (Schauer and Ferry, 1980; Schauer *et al.*, 1982). The reversible reaction between hydrogen production from formate and formate synthesis from hydrogen and bicarbonate (or CO_2) was described using a formate hydrogenlyase system from *Mb. formicicum* (Baron and Ferry, 1989) and pure cultures (Chapter 6). The production of hydrogen and formate synthesis were observed during experiments using the brewery granules. This may be due to the presence of H_2-CO_2 and formate-utilizing methanogens such as *Mb. formicicum* and *Msp. hungatei* in the granules. These methanogens can synthesize formate from hydrogen plus bicarbonate or CO_2 (see Chapter 6). However, formate was produced at much lower quantities in the presence of sulfate than in the absence of sulfate. This indicates that the synthesis of formate from H_2 and HCO_3^- may involve SRB, which utilize hydrogen as electron donor to

reduce sulfate when sulfate is available and synthesize formate from hydrogen when sulfate is limiting.

The observation that the methane production rates from both formate and H_2-CO_2 increased over three-fold when the granules were disrupted reveals the presence of mass transfer limitation for both formate and H_2-CO_2 within intact granules. The observation that methane production rates from these two substrates were essentially identical for both intact and disrupted granules is somewhat perplexing and contrary to what would be anticipated based upon mass transport calculation for microbial flocs (Ozuturk *et al.*, 1988) and individual cells (Boone *et al.*, 1989). The catabolism of formate should be more favorable as mass transfer limitations become greater because of the higher diffusivity of formate compared with hydrogen in the intact granules. The production of methane from formate would be expected to be greater than that from hydrogen. The reason this was not observed is not clear. There are several plausible explanations. It is possible that the gas channels or vents observed in the granules not only serve as conduits for release of gas formed within the granules but also exchange dissolved and gaseous substrates into the interior portions of the granules, effectively reducing mass transfer limitations. The observation of such channels or vents is not new. Similar, although considerably larger vents (100 to 500 μm) with a smooth microbial lining were observed in thick biofilms from anaerobic filters, while small vents (10 to 20 μm) were observed in thinner films from the walls of the reactors (Robinson *et al.*, 1984). These authors suggested these channels might be conduits for nutrients as well as gas exit. Internal gas channels in individual cysts of *Ms. barkeri* have been observed (Bochem *et al.*, 1982). Holes and channels were also observed in biofilms and granules from anaerobic filters, UASB reactors and fluidized bed reactors (Switzenbaum and Eimstad, 1987). The presence of these

channels could be, in part, responsible for the high effectiveness factors observed for granules degrading acetate and other fatty acids (Tramper *et al.*, 1984). Although these channels may reduce mass transfer resistance, they do not altogether eliminate it as demonstrated by the differences in methane production rates from formate and H_2-CO_2 by intact and disrupted granules.

An alternate explanation for the same reaction rates of formate and H_2 could be that a reversible conversion between formate and hydrogen occurred in the granules. Significant hydrogen accumulation was observed at the beginning of methane production from formate (Table 4.3). Formate formation up to 0.6 to 0.8 mM was also observed during methane production from H_2-CO_2. In fact, the formate and hydrogen concentrations were observed to remain close to their calculated thermodynamic equilibrium. If formate is rapidly converted to H_2 and vice versa in granules to maintain close to thermodynamically equilibrium, little difference in methane production rates via formate and H_2 would be expected if the reversible conversion between hydrogen and formate is rapid, as was observed. These two explanations are not mutually exclusive and, in fact, both could contribute to the observed phenomena.

4.5.4 The role of sulfate reducing bacteria in substrate metabolism

Based on the relative amounts of methane formed in the presence and in the absence of sulfate during the conversion of H_2-CO_2 and formate (Table 4.4), SRB played a minor role in the consumption of these electron transfer intermediates. Only 6 and 5 percent of the total amount of hydrogen and formate were, respectively, used for sulfate reduction. Similarly, only about 4 percent of available acetate was converted via sulfate reduction. This is consistent with the observation that the majority of the morphotypes observed in the thin sections of the granules were acetate-

utilizing *Methanothrix*-like and hydrogen/formate-utilizing *Methanobacterium*-like cells (Table 4.2).

Consumption of sulfate during degradation of ethanol and propionate, however, indicates some involvement of SRB in the conversion of the substrates. The degradation of ethanol, acetate and propionate by pure cultures of SRB via sulfate reduction has been reported (Widdel, 1988). In this study, the role of SRB in syntrophic acetogenesis of ethanol and propionate was investigated by adding molybdate in the absence of sulfate. Molybdate is well established as an inhibitor of SRB (Taylor and Oremland, 1979; Banat *et al.*, 1981; Smith and Klug, 1981; Yadav and Archer, 1988). The concentration of molybdate required for essentially complete inhibition of sulfate reduction was found to be 0.2 mM for a lake sediment (Smith and Klug, 1981). A molybdate concentration of 0.1 mM completely inhibits the growth of *Desulfovibrio desulfuricans* on ethanol in a coculture with *Mb. formicicum* MF (Yadav and Archer, 1988). The inhibition level of molybdate for methanogens appeared to be much higher than that for SRB. Molybdate at a concentration of 10 mM did not inhibit the growth of *Mb. formicicum* (Yadav and Archer, 1988). In lake sediments, a high level (20 mM) of molybdate inhibited methanogenesis (Smith and Klug, 1981). A decrease of methanogenesis and the accumulation of acetate in an anaerobic filter was reported to be due to a concentration of 10 mM molybdate (Hilton and Archer, 1988). Our data indicated that in the absence of sulfate, the maximum acetate conversion rate was only slightly decreased by the addition of molybdate (0.1 to 10 mM).

Mass balance experiments revealed that ca. 30% of ethanol was converted via sulfate reduction if sufficient sulfate was present. The molybdate inhibition experiments showed that in the absence of sulfate, syntrophic ethanol conversion was

inhibited by 29% by 2.0 mM of molybdate. These results strongly suggested that SRB were involved in ethanol conversion whether sulfate was available or not. It is well known that in the absence of sulfate, *Desulfovibrio* sp. can syntrophically catabolize ethanol together with H_2-utilizing methanogens (Bryant *et al.*, 1977; McInerney and Bryant, 1981; Traore *et al.*,1983). Results from ethanol enrichment studies indicated that *Desulfovibrio* sp. may be one of SRB capable of syntrophic ethanol degradation in the granules. Molybdate severely inhibited the syntrophic conversion of propionate at low levels (0.1 to 0.2 mM) with essentially complete inhibition (95 to 97%) at higher molybdate concentrations (0.5 to 2.0 mM). Comparison experiments were performed using the rod-type granules described in Chapter 3, which were grown on a volatile fatty acid mixture (acetate, propionate and butyrate) medium containing low sulfate (0.15 mM). The same level of molybdate addition (0.1 to 2.0 mM) did not affect syntrophic propionate conversion by the rod-type granules to any where near this extent (see Chapter 5). This suggests that the syntrophic conversion of propionate in the brewery granules was mainly performed by SRB. To date, several strains of propionate-degrading SRB have been reported (Widdel and Pfennig, 1982; Widdel and Pfnennig, 1984; Samain *et al.*, 1984; Widdel, 1988), but the syntrophic conversion of propionate by SRB associated with methanogens has not been reported. Results suggest that the propionate-degrading SRB in the brewery granules are able to couple propionate oxidation with sulfate reduction if sulfate is available, or to complete propionate oxidation syntrophically with another partner that is able to remove H_2 or formate formed during propionate oxidation if sulfate is not available. This represents a considerable ecological advantage for this type of SRB over obligately syntrophic propionate degraders in ecosystems where sulfate is continuously or intermittently available. The results also suggest that these ethanol- and propionate-degrading SRB

cannot effectively compete with methanogens for hydrogen or formate. Otherwise, a considerably greater portion of the hydrogen and formate would have been used for sulfate reduction during the mass balance experiments.

4.5.5 The role of sulfate reducing bacteria in granular structure

Microscopic observations, especially TEM examination indicated that SRB played an important role in the granular structure. One distinct characteristic of the brewery granules was that they were mainly composed of microcolonies and clusters. Microcolonies or clusters composed of the syntrophic acetogens, *Syntrophomonas* sp. or *Syntrophobacter* sp. and H_2-utilizing *Methanobrevibacter* sp., were observed inside granules examined by Dubourguier *et al.* (1988a, 1988c). TEM examination revealed that syntrophic microcolonies also existed in the granules treating brewery wastewater. In addition, it was observed that *Methanothrix* sp. formed their own microcolonies. The prevalent species in the other microcolonies were preliminarily identified by using series dilution with different substrates and microscopic and TEM observation of the cells from highly-diluted enrichments. *Mtrx. soehngenii*-like long filaments were obtained in acetate enrichment (Figure 4.3A), revealing that this species probably is the predominant acetate utilizer in the granules. The irregular rods in Figure 4.2C were presumably identified to be a *Desulfovibrio* sp. which can grow alone on ethanol plus sulfate and can also grow syntrophically on ethanol with *Methanobacterium* sp. in the absence of sulfate. The regularly-shaped, non-methanogenic rods were found in high dilutions of the propionate enrichments obtained both in the presence and absence of sulfate. When sulfate was present, both sulfate and propionate were consumed and no methane was produced. These enrichments could also utilize ethanol in the presence of sulfate. This indicates that these unknown rods are sulfate reducers.

Morphotypically, these rods are quite similar to *Desulfobulbus propionicus* as reported by Widdel and Pfennig (1982). However, *Desulfobulbus propionicus* cannot grow syntrophically with H_2-utilizing methanogens (Laanbroek *et al.*, 1982; Widdel, 1988). A polymer-like structure was observed around the SRB cells in the microcolonies or clusters in the TEM micrographs of the granules (Figure 4.2B and C) and in the enrichments (Figure 4.3D and E). This circumstantially indicates that the polymers produced by SRB may be at least in part responsible for the formation and/or stabilization of the clusters in these granules.

4.5.6 Proposed electron flow in the presence of sufficient amount of sulfate

Based upon mass balance experiments, an electron flow scheme for the anaerobic degradation of the major compounds (ethanol, acetate and propionate) in the brewery wastewater can be constructed (Figure 4.8). This scheme illustrates that in the presence of high sulfate concentrations (8 to 9 mM), approximately 87% of the energy associated with the soluble organic matter is channeled to methane with the remainder used for sulfate reduction. The percentage of organic matter converted to methane in the UASB system was somewhat higher (ca. 97%) than that calculated based upon the mass balance experiments. Part of the explanation for this is that the sulfate concentration in the brewery wastewater was considerably lower (0.6 to 1.3 mM) than that used in the mass balance experiments. A maximum of between 5 and 6 percent of the COD in brewery wastewater could be used for sulfate reduction based on the relation of COD and sulfate concentration in the wastewater. Mass transfer limitations within the granules may have limited sulfate reduction below this maximum of 5 to 6 percent.

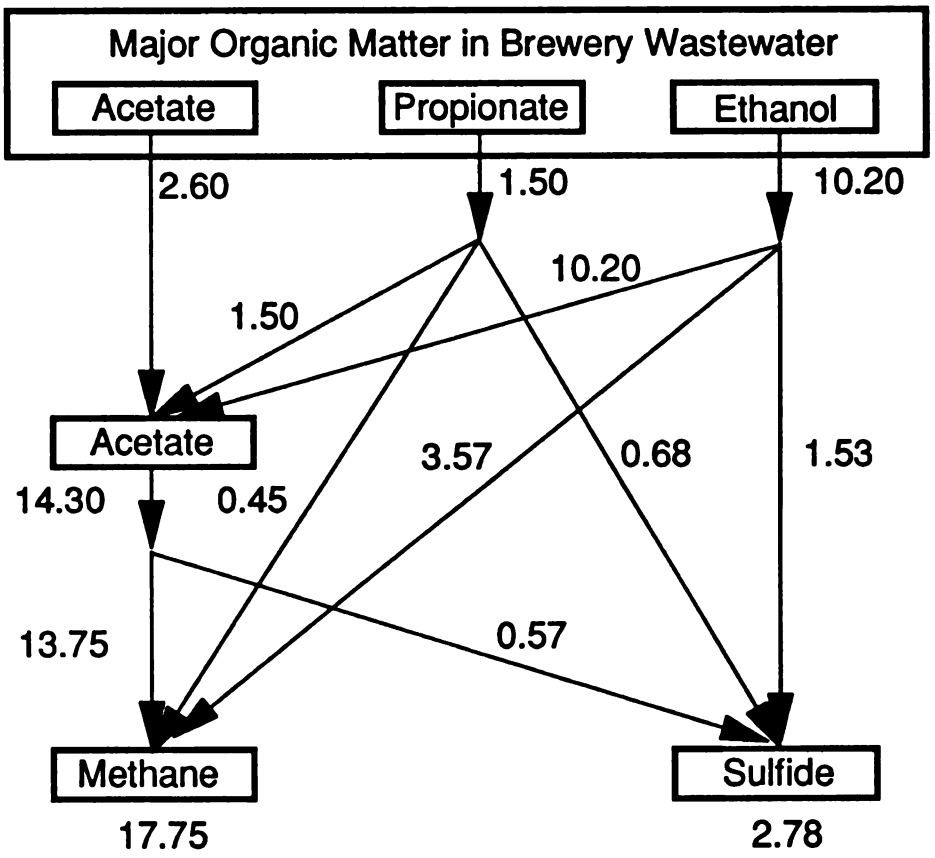


Figure 4.8 Substrate and electron flow scheme proposed for the anaerobic digestion of major organic matter in one liter of brewery wastewater in the presence sufficient sulfate. The respective concentrations of acetate, propionate, and ethanol are assumed to be 2.6, 1.5, and 10.2 mM, respectively. The ratio between methanogenesis and sulfate reduction is estimated from results in Table 4.5 and equations F-1 to F-10 in Appendix F.

4.6 SUMMARY

1) The granules from an industrial UASB plant treating brewery wastewater which contained a moderate level of sulfate (0.6 to 1.3 mM) had high specific gravity (1.046) and contained ferrous sulfide as the major mineral precipitate. A large number of small holes or vents (10 to 20 μm in diameter) was found on the surface of granules. These holes may serve as conduits of gas produced within the granules and to deliver substrates to the interior of the granules.

2) The maximum substrate conversion rates at 35 ℃ in absence of sulfate were 1.2, 45.1, 8.04, 4.14, 0.85, and 5.75 mmole/gVSS-d for methanol, ethanol, acetate, propionate, butyrate, and glucose, respectively. The maximum methane production rates (mmol/gVSS-d) from H_2-CO_2 and formate were essentially equal (13.7 and 13.5 for intact granules, and 42 and 37 for physically disrupted granules, respectively).

3) The presence of sulfate (8 to 9 mM) did not change the maximum conversion rates of major substrates except for propionate conversion rate which was increased by 30%. Mass balance calculation indicated that significant parts of ethanol (30%) and propionate (60%) were converted through sulfate reduction while only small parts (<5%) of H_2-CO_2, formate, and acetate were converted through sulfate reduction.

4) A reversible reaction between hydrogen production from formate and formate synthesis from H_2-CO_2 was observed to occur with these granules. Addition of sulfate reduced the concentration of formate produced. This indicates that SRB may have been involved in formate synthesis. This may be one reason for observed approximate equivalent maximum substrate conversion rates for both formate and H_2-CO_2 in the granules.

5) Hydrogen and formate accumulated at an equilibrium level during syntrophic ethanol degradation. Energetic analysis suggests both are intermediates of syntrophic ethanol degradation.

6) In the absence of sulfate, addition of 0.1 to 2.0 mM molybdate inhibited syntrophic propionate conversion by 67 to 97%. Molybdate at 2 to 10.0 mM was observed to inhibit syntrophic ethanol conversion by 29%. This indicates that the syntrophic propionate conversion was performed primarily by sulfate reducing bacteria and the syntrophic ethanol conversion partially by SRB. This conclusion was supported by tests using enrichments of the predominant species.

7) At least four types of microcolonies were observed inside the granules. One of these microcolonies consisted of *Methanothrix*-like rods, while the remaining types were mainly composed of non-methanogenic rods together with *Methanobacterium*-like rods. One of the non-methanogenic rods that had an irregular morphotype was, tentatively, identified as a *Desulfovibrio* sp. The remaining rods were observed to be propionate-degrading, sulfate-reducing bacteria. These SRB could also convert ethanol to propionate, allowing a potentially large ecological advantage for these organisms. These SRB, based upon TEM and SEM observation, may play an important role in granular structure for these granules.

TECHNOLOGICAL AND MICROBIOLOGICAL ASPECTS OF ANAEROBIC DIGESTION GRANULES

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CHAPTER 5

MICROBIAL COMPOSITIONS AND CHARACTERIZATION OF PREVALENT SPECIES IN VOLATILE FATTY ACID FED GRANULES

5.1 INTRODUCTION

Knowledge of the microbial composition of granules and the role of individual species in metabolism, adhesion and granulation is of critical importance to understand how high-rate anaerobic digestion systems work. Several recent studies have attempted to investigate and understand the microbial population levels in granules (Dolfing *et al.*, 1985; Dubourguier *et al.*, 1988a, 1988b, 1988c). These studies, however, did not define the prevalent species composition or their number. Novaes *et al.* (1988) reported on the species composition and a number of prevalent hydrolytic-fermentative and methanogenic bacteria in anaerobic granules from a system treating sewage sludge. More recently, methanogenic subpopulations have been immunologically identified and quantified in five different granular consortia from USAB reactors maintained on different substrates (Kooenneef *et al.*, 1990). Other important fatty acid degrading acetogens, however, that may have been present in these granules were not investigated. Isolation and characterization of the prevalent species in granules is

essential to aid in understanding the ecophysiology and interaction among the different trophic bacteria, as well as identifying the contribution of individual organisms in substrate metabolism and the granulation process.

Chapter 3 describes studies on two types (R-type and F-type) of anaerobic granules developed on a VFA feed consisting of acetate, propionate and butyrate. The R-granules exhibited better operational performance than the F-granules. The results of substrate conversion experiments and MPN enumerations revealed that the R-granules were mainly composed of methanogens, which utilize acetate, H_2-CO_2, and formate, and syntrophic propionate- and butyrate-utilizing acetogens. The purpose of this chapter was to investigate, isolate and characterize the prevalent syntrophic acetogenic and methanogenic bacteria in the R-granules and examine their role in granular structure.

5.2 MATERIALS AND METHODS

5.2.1 Granules and microbial sources

The R-granules were maintained in upflow anaerobic reactors by continuously feeding a VFA mixture consisting of 100 mM acetate, 50 mM propionate and 50 mM butyrate in the basal medium (see Chapter 3). *Methanobacterium formicicum* strain MF (DSM 1535) was obtained from the Deutsche Sammlung von Mikroorganismen.

5.2.2 Role of sulfate-reducing bacteria in fatty acid degradation

The assays for substrate degradation by the granules were performed using a mixture of acetate, propionate and butyrate in 158 ml serum bottles with 50 mL of

basal medium (Appendix A) at 35 °C. The concentration of granules was approximately 5 gSS/L. The test procedures were the same as described in Chapter 4 (4.2.2 and 4.2.4). Ferrous sulfate (5mM) and sodium molybdate (0.5 to 2 mM) were added to check if sulfate-reducing bacteria (SRB) played a role in substrate metabolism.

Mass balance experiments for H_2, formate, acetate, propionate, and butyrate were performed to determine percentage of substrate converted via sulfate reduction. The method employed was the same as described in Chapter 4 (4.2.3). The concentration of granules was approximately 2.0 gVSS/L. The calculation of percentages of substrate converted via sulfate reduction is described in Appendix F.

5.2.3 Enumeration of prevalent species

The enumeration of prevalent species of methanogens and syntrophic acetogens were carried out by comparing microbial morphotypes found in serially diluted MPN tubes with the morphotypes of isolated strains from the granules. The procedures for MPN enumeration were described in Chapter 3 (3.2.8).

5.2.4 Isolation and characterization of methanogens

All the manipulations were performed under strictly anaerobic conditions either inside an anaerobic glove bag (M/S Coy Manufacturing Co., Ann Arbor, MI) or by using sterile syringes and needles for all transfers in pressure tubes (27 mL) and serum bottles (158 mL).

The isolation of methanogens was done by enrichment in the basal medium (Appendix A) containing 1.5 atm (151 kPa) of H_2-CO_2 (80:20) gas mixture in headspace plus 2 mM sodium acetate, or 40 mM formate plus 2 mM acetate for

hydrogen-formate utilizing methanogens, and 15 mM sodium acetate for acetate-utilizing methanogens. Penicillin-G (1 mg/mL) was added to the hydrogen, or formate enrichments while vancomycin (0.2 mg/mL) was used for acetate enrichments. Approximately 0.3 mL of granules were anaerobically removed from the reactor, disrupted by passage through a syringe with a 23-gauge needle, and transferred into 158 mL anaerobic bottles containing 50 mL of the enrichment medium. These enrichments were replenished with substrates at least three times before being transferred into fresh enrichment medium. Growth of methanogens was monitored by microscopy and production of methane.

To obtain colonies of pure methanogenic isolates from H_2-CO_2 and formate enrichments, H_2-CO_2 was used as substrate for the growth of cells on agar medium. After three transfers, enriched cultures were serially diluted and then plated on the appropriate enrichment medium solidified with 1.65% Difco purified agar. These plates were incubated inside pressure paint tanks as described by Jain and Zeikus (1987). An H_2-CO_2 (80:20) gas mixture was pressurized to 1 atm as substrate. During the incubation period (1 to 2 months), production of methane in the paint tanks was periodically monitored and when necessary, the paint tanks were re-pressurized with H_2-CO_2. The colonies were picked from the plates with sterile toothpicks and placed into 10 mL of liquid media contained in 27 mL pressure tubes. After turbid growth was observed, the cultures were purified by streaking on agar medium plates. A single colony was then picked and placed into the liquid medium and, after growth was observed, re-checked on agar plates for purity. Isolated colonies were again picked as before, and the purity of cultures was confirmed by microscopic examination.

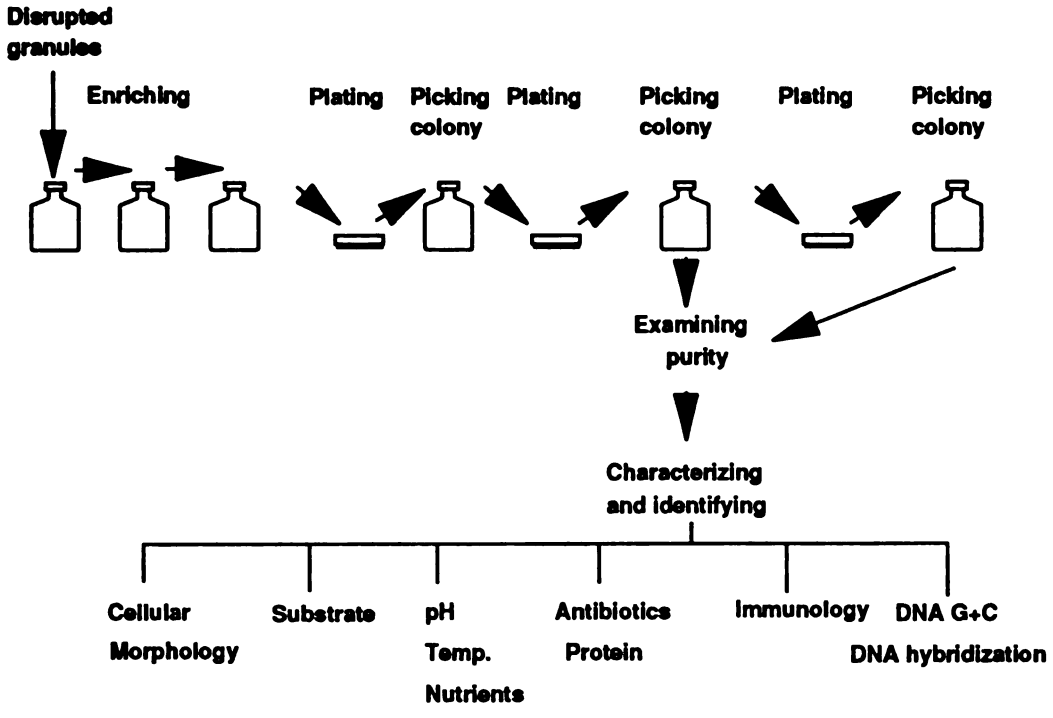


Figure 5.1 Procedures for the isolation and characterization of methanogens from H_2-CO_2 or formate enrichements

For the isolation of acetate-utilizing methanogens, direct tube dilutions were used. This procedure was repeated several times with end dilutions until a purified culture was obtained and it was verified by observation that no other morphotype cells grew on acetate, glucose (5g/L) or yeast extract (5g/L)-containing medium. Methanogens were identified in accordance with established methods (Boone and Whitman 1988; Jain *et al.*, 1988; Jones *et al.*, 1987). The general tests employed for identification included morphological characteristics, substrate range, pH and temperature optima, sensitivity to antibiotics and/or antigenic analysis. The procedures for isolation and characterization of methanogens using H_2-CO_2 or formate and then H_2-CO_2 are illustrated in Figure 5.1.

5.2.5 Isolation and characterization of fatty acid degrading acetogens

Syntrophic butyrate, isobutyrate, and propionate degraders were initially enriched in 158 mL serum bottles containing 50 mL of the basal medium buffered with 20 mM potassium phosphate and 40 mM $NaHCO_3$ (pH 7.1 to 7.2). Substrates were added to obtain 15 mM of propionate, isobutyrate and butyrate, respectively. The bottles were inoculated with disrupted granules as described above. Enrichments were transferred at least three times (2 to 3 months) with 2% inoculum (v/v). The growth of syntrophic acetogens was monitored by substrate disappearance and methane production in the bottles. Subsequently, enrichments were serially diluted and inoculated into bottles containing a pre-grown H_2-utilizing methanogen, *Methanobacterium formicicum* strain T1N or *Methanospirillum hungatei* strain BD, which was also isolated from the R-granules. The cultures in the positive end-dilution tubes were used for plating on the appropriate agar medium in an anaerobic glove bag. An H_2-CO_2-utilizing methanogen was first plated as an agar lawn and these plates

were kept in between the plates onto which the end dilutions were plated. The plates were kept in the paint tanks with 0.8 atm (80.8 kPa) of an N_2-CO_2 (95:5) gas mixture under 37°C for 3 to 6 months. Prior to incubation, the paint tanks were flushed and evacuated with the N_2-CO_2 mixture at least 5 times, in order to remove maximum possible H_2 coming from the glove bag. The growth of syntrophic VFA degraders was monitored by methane production in the paint tanks. The isolated colonies of acetogens were picked, with *Mb. formicicum* or *Msp. hungatei* and placed into tubes containing the appropriate fatty acid.

A propionate degrading, sulfate reducing strain PW was initially isolated as a syntrophic coculture together with *Msp. hungatei* strain BD. The further purification was performed using serial dilution (up to 10^{-9}) and then streaking on agar medium plates until pure colonies were isolated. The medium (containing 20 mM $NaSO_4$ and 20 mM sodium propionate) used for purification and maintenance of strain PW is described in Appendix A.

Purity of the syntrophic acetogens was checked after growth in liquid medium by microscopy and by streaking on a plate of the appropriate medium and observing colony homogeneity. The general procedures for isolation and characterization of syntrophic acetogens are illustrated in Figure 5.2.

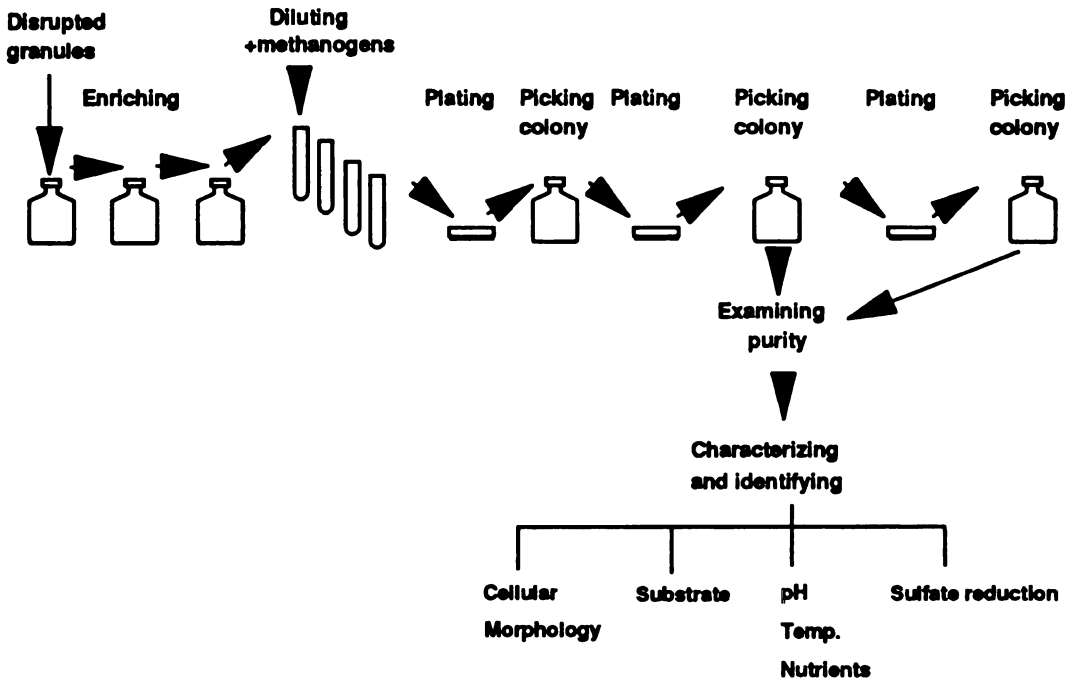


Figure 5.2 Procedures for the isolation and characterization of syntrophic VFA-degrading bacteria

5.2.6 Immunological assays

The antigenic fingerprints of methanogen strains T1N, T18 and M7 were determined with a panel of calibrated antibody probes using a battery of complementary procedures that included indirect immunofluorescence and quantitative slide immunoenzymatic assay (SIA), as described previously by Macario and de Macario (1983). Antigenic relatedness was measured by comparing the strains' fingerprints with those of reference organisms (Macario and de Macario, 1985, 1988).

5.2.7 Sensitivity to antibiotics

Sensitivity of isolated methanogens to various antibiotics were assayed in triplicate in 27 mL pressure tubes containing 10 mL of the basal medium. A gas mixture of H_2-CO_2 (80:20, 2.0 atm) was used as substrate for strains T1N, RF, BD and T18. Acetate was used for the growth of strain M7. Aqueous solutions of antibiotics were filter-sterilized for soluble antibiotics. Insoluble antibiotics were dissolved in a 30% methanol aqueous solution in 58 mL serum bottles. The bottles were then sealed with butyl rubber stoppers and flushed with N_2 for 30 min. The antibiotics were added at a 1.0 mg/mL concentration for all cultures except for strain M7 (0.1 mg/mL). Controls with and without methanol in the absence of antibiotics were kept to examine the effect of methanol on growth except for strain T18. The methanol concentration used here did not affect cell growth or methane production.

5.2.8 Medium, microscopic examinations and analytical methods

The medium composition for the isolation and maintenance of methanogens and VFA-degrading acetogens is described in Appendix A. Techniques of microscopic, scanning electron microscopic (SEM), and transmission electron microscopic (TEM)

examination are described in Appendix D. Total cellular proteins of *Mb. formicicum* strain MF and isolated methanogens strains T1N and RF were analyzed as previously described by Jain *et al.* (1987). Cells for the analysis were grown on H_2-CO_2. VFAs, H_2 and methane were detected by using gas chromatography (Appendix A).

The half velocity coefficient (K_m) for acetate utilization by strain M7, isolated from the R-granules, was determined using time-course experiment (see Appendix C).

5.3 SULFATE REDUCING BACTERIA IN THE R-GRANULES

In Chapter 3, the major trophic groups in the R-granules were characterized based on the MPN enumeration and the maximum specific substrate conversion rates. Acetate-utilizing, H_2-CO_2 and formate-utilizing methanogens were the predominant bacteria. Propionate- and butyrate-degrading bacteria were also prevalent.

To further examine the role of sulfate reducing bacteria (SRB) in the metabolism of acetate, propionate and butyrate, the maximum substrate degradation rates for acetate, propionate and butyrate were determined from batch experiments (35 °C) in the presence and absence of sulfate (5 mM) or molybdate (0.5 to 2.0 mM) (Table 5.1). Addition of sulfate or molybdate did not significantly influence the degradation rates of acetate and butyrate, indicating that SRB may not be involved in the metabolism of these two fatty acids. Addition of sulfate slightly increased the maximum specific propionate degradation rate (by ca. 10%) while addition of molybdate reduced the rate by ca. 22%, indicating that SRB may have been partially involved in the metabolism of propionate.

The results of mass balance experiments (Table 5.2) showed that SRB did not

play a visible role in conversion of H_2, formate and acetate by the R-granules when sulfate was added to 9.0 mM. The portion of butyrate degraded via sulfate reduction was low (ca. 5.8%) while a significant fraction of propionate (ca. 12%) was degraded via sulfate reduction.

To further investigate the microbial ecology of the R-granules, isolation of prevalent methanogens was performed using acetate, H_2-CO_2 and formate as substrate. Propionate and butyrate were used as substrates to enrich syntrophic VFA degrading organisms. The isolated syntrophic VFA degraders were examined to determine whether they were sulfate-reducing bacteria.

Table 5.1 Effect of sulfate and molybdate on the maximum specific fatty acid degradation rates (mmole/gVSS-hr) by the R-granules

Treatment	Specific VFA degradation rate		
	acetate	propionate	butyrate
Control	6.1	1.59	3.70
$FeSO_4$ (5mM)	6.1	1.75	3.70
Na_2MoO_4 (0.5 mM)	6.0	1.36	3.70
Na_2MoO_4 (1.0 mM)	5.7	1.33	3.50
Na_2MoO_4 (2.0 mM)	5.7	1.23	3.58

Table 5.2 Mass balance for substrate conversion in the presence and the absence of sulfate by the R-granules

Substrate	Substrate consumed (mmol)		SO_4^{2-} consumed (mmol)	CH_4 formed (mmol)		CH_4 formed	Substrate converted via sulfate reduction
	$-SO_4^{2-}$	$+SO_4^{2-}$	$+SO_4^{2-}$	$-SO_4^{2-}$	$+SO_4^{2-}$	$+SO_4^{2-}/-SO_4^{2-}$ (mmol:mmol)	(%, +sulfate)
1. H_2	2.30	2.30	0.0026	0.563	0.561	0.996	0.4
2. formate	4.50	4.50	0.0016	0.881	0.880	0.999	0.1
3. acetate	0.90	0.90	0.0030	0.822	0.820	0.997	0.3
4. propionate	0.62	0.62	0.0558	1.05	0.994	0.947	12.0
5. butyrate	0.58	0.58	0.0147	1.40	1.38	0.986	5.8

5.4 ISOLATION AND CHARACTERIZATION OF METHANOGENS

Five prevalent strains of methanogenic bacteria were isolated from the R-granules (Figures 5.3 and 5.4). Among them, three strains (strains T1N, RF and BD) were H_2-CO_2 and formate-utilizing methanogens; strain T18 was mixed function methanogen (using acetate, H_2-CO_2 and methanol), and strain M7 was an acetate-utilizing methanogen. Their general properties are summarized in Table 5.3. The effects of pH and temperature on growth are illustrated in Figure 5.5.

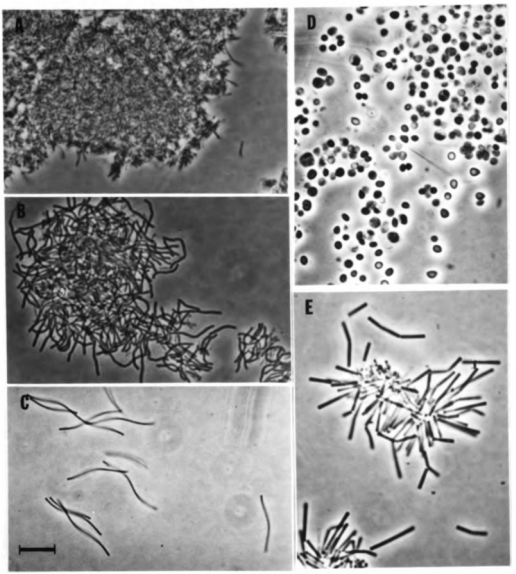


Figure 5.3 Phase-contrast microphotographs of the prevalent methanogens isolated from the R-granules. (A) Aggregate of *Methanobacterium formicicum* strain T1N. (B) Small clumps formed by *Methanobacterium formicicum* strain RF. (C) *Methanospirillum hungatei* strain BD. (D) Disaggregated *Methanosarcina mazei* strain T18. (E) Single cells and rosette clumps of *Methanothrix* strain M7. Bar represents 10 μm.

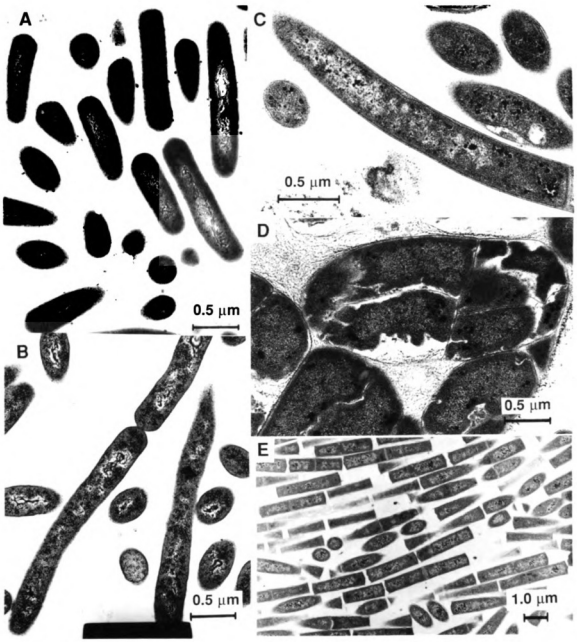


Figure 5.4 Transmission electron photographs of methanogens isolated from the R-granules. (A) *Methanobacterium formicicum* strain T1N. (B) *Methanobacterium formicicum* strain RF. (C) *Methanospirillum hungatei* strain BD. (D) *Methanosarcina mazei* strain T18. (E) *Methanothrix* strain M7.

Table 5.3 General properties of prevalent methanogens isolated from the R-granules

Properties	Methanogenic Strain T1N	RF	BD	T18	M7
Cellular					
cell shape	rod	rod	spirillum	sarcina	rod
cell wall, gram	+	+	-	+	-
Motility[a]	-	-	+	-	-
growth[b]					
optimum temp. (°C)	37-40	38-40	45	35-37	40
no growth at (°C)	>45	45	>50	>45	>45
optimum pH	6.5-7.0	6.7-7.4	7.0-7.5	6.0-6.5	7.0
pH range	5.3-8.5	5.9-8.2	6.0-8.2	5.5-8.5	5.6-8.5
doubling time (hr)	6-8	6-8	6-7	10-12	30-36
colony color	yellow	yellow	brown	yellow-brown	NF
colony size (mm)	1-2	1-2	2-3	3-5	NF
Substrate[c]					
H_2-CO_2	+	+	+	+	-
formate	+	+	+	-	-
acetate	-	-	-	+	+
methanol	-	-	-	+	-
monomethylamine	-	-	-	+	-
dimethylamine	-	-	-	+	-
trimethylamine	-	-	-	+	-

a: +, motile; -, not motile.

b: Growth parameters were determined by using H_2-CO_2 as substrate except by using acetate for strain M7. pH effect and doubling time were determined at 37°C.

NF: No colony formed and isolated by serial dilution.

c: +, growth; -, no growth. None of the strains used ethanol, 2-propanol, 2-butanol, and glucose as growth substrates.

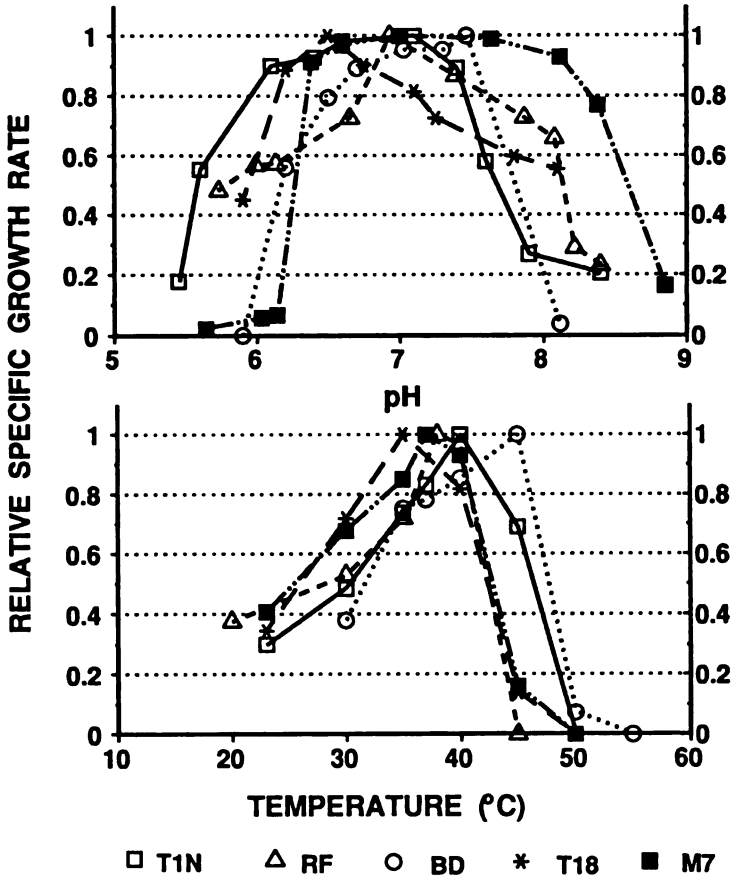


Figure 5.5 Effects of pH and temperature on the growth of methanogens isolated from the R-granules. *Methanobacterium formicicum* trains T1N and RF, *Methanospirillum hungatei* BD, and *Methanosarcina mazei* T18 were grown on H_2-CO_2. *Methanothrix* strain M7 was grown on acetate.

5.4.1 *Methanobacterium formicicum* strain T1N

Strain T1N, isolated from a H_2-CO_2 enrichment, displayed a rod morphotype. This strain grew on agar medium as yellowish, round colonies with 1 to 1.5 mm in diameter. Its cells were slender, cylindrical, and irregularly crooked, with blunt, rounded ends and gram-positive-like cell walls (Figure 5.4A). The average cell length and width was 1.2 to 3 by 0.35 μm when grown at 37°C. Strain T1N was very adhesive and formed large aggregates (up to 2 to 3 mm) during growth on H_2-CO_2 or formate (Figure 5.3A) at temperatures of 37°C or lower. Cells of strain T1N grew very slowly at 45 °C and formed long filaments (50 to 100 μm) which formed thin and loose flocs. When the temperature was decreased back to 37 °C or lower, the cells returned to their original morphotype and again formed large dense aggregates. The optimum temperature and optimum pH range for growth were 37 to 40 °C and pH 6.5 to 7.5, respectively (Figure 5.5).

Quantitative comparison of the antigenic fingerprints of strain T1N with characterized methanogen strains indicated that it was not antigenically related to referenced strains (Table 5.4). However, the effect of antibiotics on growth (Table 5.5) and total cellular protein patterns (Figure 5.6) of strain T1N were the same as those of *Mb. formicicum* strain MF (neotype strain). Further identification comparing the ratio of guanine plus cytosine and DNA-DNA hybrization with strain MF (relatedness >88%) showed that these two strains were genetically related (Table 5.6). Strain T1N was identified as a member of *Methanobacterium formicicum*, based on its similar cellular and physiological characteristics to the neotype strain (Bryant and Boone 1987; Boone and Whitman, 1988), although strain T1N showed antigenically no-relatedness to the neotype strain.

Table 5.4 Antigenic fingerprint of methanogen isolates

Strain	Reaction with S probe from antiserum No.*																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
T1N		0		0	0					0	0							0		
T18			0			2						0	4	2	4					
M7	0	0		0	0		0	2	0	0	0					0	0		0	0

(1) The determination was performed by Dr. E. Conway de Macario.
(2) The immunizing strain for the antisera as follows: 1 *Methanobrevibacter smithii* PS; 2 *Methanobacterium formicicum* MF; 3 *Methanosarcina barkeri* MS; 4 *Methanobacterium bryantii* MoH; 5 *Methanobacterium bryantii* MoHG; 6 *Methanosarcina barkeri* R1M3; 7 *Methanobrevibacter ruminantium* M1; 8 *Methanobrevibacter arboriphilus* DH1; 9 *Methanobrevibacter smithii* AL1; 10 *Methanobacterium thermoautotrophicum* GC1; 11 *Methanobacterium thermoautotrophicum* G; 12 *Methanosarcina barkeri* 227; 13 *Methanosarcina mazei* S6; 14 *Methanosarcina barkeri* W; 15 *Methanosarcina thermophila* TM1; 16 *Methanobrevibacter arboriphilus* AZ; 17 *Methanobrevibacter arboriphilus* DC; 18 *Methanothermus fervidus* V24S; 19 *Methanothrix soehngenii* Opfikon; 20 *Methanothrix* CALS-1. Blanks mean not done.

Table 5.5 Comparison of sensitivity to various antibiotics (1 mg/mL) among methanogen strains T1N and RF and *Methanobacterium formicicum* strain MF

strain	T1N	RF	MF
Cycloserine	-	-	-
Vancomycin	-	-	-
Penicillin-G	-	-	-
Bacitracin	+	+	+
Rifampicin	-	-	-
Chloramphenicol	+	+	+
Tetracycline	+	-	+*
Erythromycin	-	-	-
Kanamycin	-	-	-
Gramicidin S	+	+	+
Gramicidin D	-	-	-
Monensin	-	ND	-*
Neomycin	+	ND	NR
Novobiocin	+	ND	NR
Streptomycin	-	-	-*
Ampicillin	-	-	-*

(1) Data for *Mb. formicicum* strain MF from Hilpert *et al.*(1981) in exception of "*" which was obtained in this study.
(2) ND: Not determined; NR: Not reported.
(3) +: Sensitive; -: not sensitive.

Table 5.6 Guanine-plus-cytosine contents and DNA-DNA hybridization between strain T1N and *Methanobacterium formicicum* strain MF

strain	T1N	MF
G+C, %	45.8	44.6
% similarity with		
DNA of T1N	100	88
DNA of MF	96	100

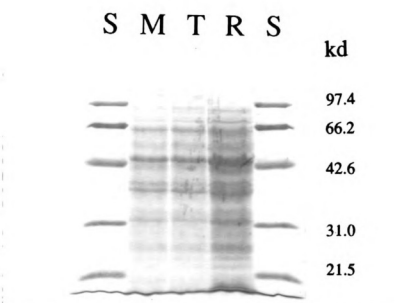


Figure 5.6 Sodium dodecyl sulfate polyacrylamide (SDS)-gel electrophoresis of total cellular proteins of strains T1N, RF and *Methanobacterium formicicum* strain MF. Lanes S shows the standard protein with molecular weight markers, while lanes T, R and M represent strains T1N, RF and MF, respectively.

5.4.2 *Methanobacterium formicicum* strain RF

Strain RF, isolated from a formate enrichment, displayed a rod-morphotype. It utilized H_2-CO_2 and formate as energy sources. It formed long filaments during growth that associated into small, loose clumps in liquid medium (Figure 5.3B). Cells of strain RF were 3 to 15 μm in length and 0.4 μm in diameter and had a gram-positive- like cell wall (Figure 5.4B). The optimum pH and temperature range for growth were 7.0 to 7.5 and 37 to 40 °C, respectively (Figure 5.5). The effects of various antibiotics on growth was essentially the same as for *Mb. formicicum* strain MF (and strain T1N) except that strain RF was not inhibited by tetracycline (Table 5.4). The electrophoretic protein patterns of *Mb. formicicum* strain MF (neotype strain) and RF were identical (Figure 5.6). Therefore, strain RF was identified as a member of *Methanobacterium formicicum*, based on similar cellular and physiological characteristics to the neotype strain (Bryant and Boone 1987; Boone and Whitman, 1988).

The major difference among strains T1N, RF and MF was observed in the character of aggregate formation. Cells of strain T1N can form aggregates after disaggregation by washing with distilled water and disruption on sonication while the cells of strains RF and MF cannot (Chapter 7).

5.4.3 *Methanospirillum hungatei* strain BD

Strain BD which displayed a spirillum morphotype, was isolated from a formate enrichment. Strain BD grew as a short spirillum at the beginning of the logarithmic phase but later formed long thin spiral filaments typical of *Methanospirillum* (Figure 5.3C). The cells of strain BD were 0.4 by 4 to 15 μm in size and had gram-negative cell wall (Figure 5.4C).

Table 5.7 Comparison of sensitivity to various antibiotics (1 mg/mL) between methanogen strain BD and *Methanospirillum hungatei* strain JF1

strain	BD	JF1
Cycloserine	-	-
Vancomycin	-	-
Penicillin-G	-	-
Bacitracin	ND	-
Rifampicin	-	-
Chloramphenicol	+	+
Tetracycline	-	-
Erythromycin	ND	-
Kanamycin	-	-
Gramicidin S	+	+
Gramicidin D	-	-
Monensin	ND	+
Streptomycin	-	-
Ampicillin	-	-

(1) Data for *Msp. hungatei* strain JF1 from Hilpert *et al.* (1981).
(2) +: Sensitive; -: not sensitive.
(3) ND: Not determined.

Strain BD utilized H_2-CO_2 and formate, but did not utilize 2-propanol and 2-butanol. Strain BD grew poorly at 50°C and formed long filaments up to 200 to 300 μm. The optimum temperature for growth was 45 °C and the optimum pH range was 6.8 and 7.5 The effect of the antibiotics on the growth of strain BD was the same as that reported for *Msp. hungatei* JF1 (Hipert *et al.*, 1981) as shown in Table 5.7. Strain BD has been identified as *Methanospirillum hungatei* on the basis of identical morphological and physiological characters with *Methanospirillum hungatei* (Boone and Whitman 1988;

Jain *et al.*, 1988; Patel *et al.*, 1976; Zellner and Winter 1987). This strain is different from *Methanospirillum* strains which utilize 2-propanol and 2-butanol (Widdel, 1986).

5.4.4 *Methanosarcina mazei* strain T18

Strain T18 was isolated from a H_2-CO_2 enrichment but it could also grow on acetate, methanol and methylamines. It formed spherical packets during early growth in liquid medium but it disaggregated into single cells during late growth, showing a typical *Methanosarcina mazei* morphotype (Figure 5.3D). The size of cells varied from 1.2 to 3.0 μm in diameter and a polymer-like structure was observed (Figure 5.4D). The doubling time of strain T18 on methanol and methylamine was much shorter (6 to 7 hours) than on H_2-CO_2 (10 to 12 hours). The doubling time for growth on acetate was 32 to 34 hours. Addition of H_2 (more than 500 Pa) was required to initiate growth on acetate, although H_2 partial pressure was low (about 20 Pa) during its growth on acetate. The threshold concentration for acetate was observed at about 0.3 to 0.4 mM. The requirement of H_2 to initiate growth on acetate was similar to that reported for *Ms. barkeri* 227 and *Ms. mazei* S6 pre-grown on H_2 (Boone *et al.*, 1987). Optimum growth conditions on H_2-CO_2 were at pH 6.3 to 6.5, and at a temperature of 35 to 37 ℃ (Figure 5.5). Quantitative comparison of antigenic fingerprints of strain T18 with characterized methanogen strains indicated that it was closer to *Ms. mazei* strain S6 and *Ms. thermoautotrophicum* strain GC1 than *Ms. barkeri* strains R1M3 and 227 (Table 5.5). Therefore, strain T18 was identified as *Methanosarcina mazei* since it displayed strong antigenic resemblance and had typical morphological and physiological characteristics of this species described in literatures (Lui *et al.*, 1985; Mah, 1980; Touzel *et al.*, 1983).

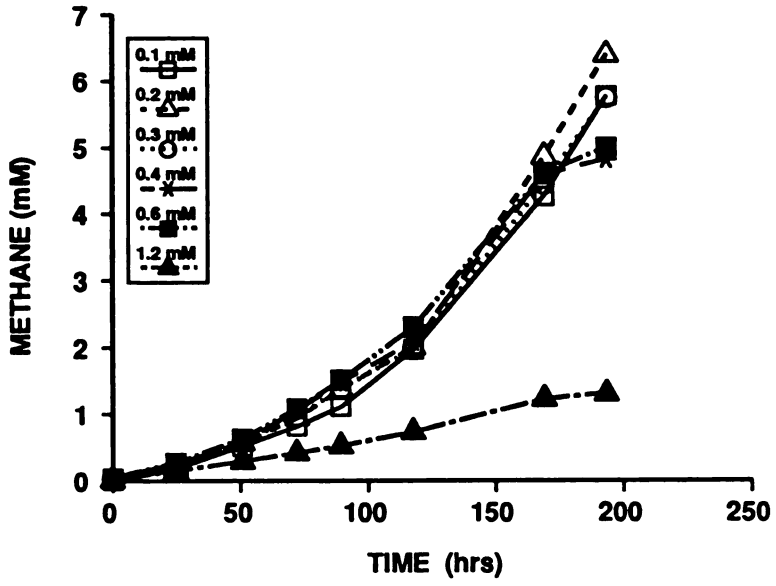


Figure 5.7 Effect of sulfide concentration on methanogenesis from acetate by strain M7.

5.4.5 *Methanothrix* strain M7

Strain M7 was a poorly fluorescent, bamboo-shaped rod with square ends that was isolated from an acetate end dilution enrichment tube (10^{-9}). Attempts to obtain colony growth on either purified agar or gelrite (0.6, 0.7 and 0.8%) solidified medium plates were not successful. Strain M7 grew as rods consisting of 1 to 4 cells but did not form long filaments in the growth media tested. Notably, the cells are adhesive and in liquid medium, and the cell ends aggregate to form a rosette clumps (Figure

5.3E). The cells were 0.7 by 2.2 μm in size and had gram-negative cell wall structure (Figure 5.4E). The morphotype of single cell of strain M7 appeared to be the same as that of *Methanothrix soehngenii* or *Methanoseata concilli* in literaturs (Zehnder *et al.*, 1980; Huser *et al.*, 1982; Patel, 1984; Touzel *et al.*, 1988; Patel and Sprott, 1990). Strain M7 used acetate as a substrate for growth but did not use H_2-CO_2, formate, methanol, or methylamine. The K_m value for acetate degradation was 0.41 mM (see Appendix C).

Strain M7 was extremely sensitive to sulfide compared with other acetate-utilizing methanogens isolated to date. Sodium sulfide of 1.2 mM significantly inhibited the growth when pH was 7.4 (Figure 5.7). The effect of various antibiotics on growth was similar to that for *Methanothrix soehngenii* strain Opfikon (neotype strain) and *Methanoseata concilli* strain GP6 (Table 5.8) except that strain M7 was more sensitive to penicillin-G (Strain M7 was inhibited by a concentration of 1.0 mg/mL while strain GP6 was not). Quantitative comparison of the antigenic fingerprints of strain M7 with reference strains indicated it was an immunotype distinct from *Mtrx. soehngenii* but showed a very weak reaction to *Methanobrevibacter arboriphilus* (Table 5-6).

Based on its morphological and physiological characteristics, strain M7 was identified as *Methanothrix* strain M7.

Table 5.8 Comparison of sensitivity to various antibiotics (0.1 mg/mL) among methanogen strain M7, *Methanothrix soehngenii* strain Opfikon and *Methanosaeta concillii* strain GP6

strain	M7	Opfikon	GP6
Cycloserine	+	+	+
Vancomycin	-	-	-
Penicillin-G*	+	-	-
Bacitracin	-	NR	-
Rifampicin	-	NR	NR
Chloramphenicol	+	NR	NR
Tetracycline	+	NR	NR
Erythromycin	+	NR	NR
Kanamycin	+	NR	NR
Gramicidin S	+	NR	NR
Gramicidin D	+	NR	NR
Monensin	+	NR	NR
Neomycin	+	NR	NR
Novobiocin	+	NR	NR
Streptomycin	-	NR	NR
Ampicillin	-	-	-

(1) Data for *Methanothrix soehngenii* Opfikon and *Methanosaeta concillii* GP6 from Touzel *et al.* (1988), and Patel and Sprott (1990).
(2) *: Strains M7 and Opfiko were inhibited by penicillin-G (1.0 mg/mL) while strain GP6 was not inhibited.
(3) +: Sensitive; -: not sensitive.
(4) NR: Not reported.

5.5 ISOLATION AND CHARACTERIZATION OF FATTY ACID DEGRADING ACETOGENS

Four different fatty acid degrading strains were also isolated from the R-granules (Figure 5.8). Two of the strains (strains PT and BH) were isolated as co-cultures with H_2-CO_2 and formate-utilizing methanogenic strains. One strain (strain PW) was isolated as a monoculture. The fourth (strain IB) was isolated as a tri-culture with a H_2-CO_2 and formate-utilizing methanogen, and a mixed function methanogen present to consume H_2-CO_2 and formate. Characterization of these VFA-degrading organisms except strain PW was not possible in mono-culture because of lack of growth under test conditions without a methanogen. The general properties of the fatty acid degrading acetogens isolated are shown in Tables 5.9 and 5.10.

5.5.1 Propionate degrading strain PT

Syntrophic acetogen strain PT, which degraded propionate, was isolated as a co-culture with *Mb. formicicum* strain T1N (Figure 5.8A). Vegetative cells were lemon-oval shaped and formed round central endospores in the late stationary phase of growth. The size of cells were 0.85 to 0.95 by 0.9 to 1.2 μm and the cell wall showed gram-positive structure (Figure 5.9B). Strain PT degraded propionate but not butyrate, and did not reduce sulfate in the presence of propionate. The doubling time of strain PT during growth on propionate in co-culture with strain T1N was 7 days at 37°C. Under these conditions, propionate was converted to CH_4 and acetate as end products of the co-culture.

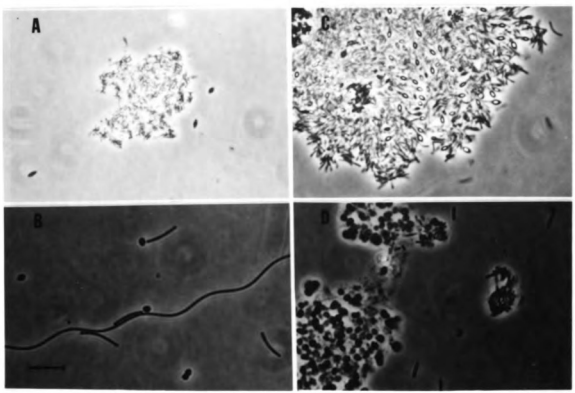


Figure 5.8 Phase-contrast microphotographs of fatty acid degrading acetogens isolated from the R-granules. (A) Spore-forming, propionate degrading acetogen strain PT and a clump of *Methanobacterium formicicum* strain T1N. (B) Non-spore-forming, propionate degrading acetogen strain PW with *Methanospirillum hungatei* strain BD. (C) Spore- forming, butyrate degrading acetogen BH with *Methanobacterium formicicum* strain T1N. (D) Isobutyrate-butyrate degrading acetogen strain IB with aggregate of *Methanosarcina mazei* strain T18 and clump of *Methanobacterium formicicum* strain T1N. Bar represents 10 μm.

Figure 5.9 Transmission electron photographs of fatty acid degraders. (A) strain PW, (B) strain PT and (C) strain IB isolated from the R-granules.

A
0.5 µm
B
0.5 µm
C
0.5 µm

Table 5.9 General properties of prevalent propionate degraders isolated from the R-granules

Property	Strain PT	PW
Cellular		
cell wall	gram-negative	gram-negative
cell shape	oval	short rod
endospores	central	none
Doubling time (days)	7	5
Methanogen partner	strain T1N	strain BD
Syntrophic acetogenesis		
propionate	+	+
Sulfate reduction		
propionate	-	+
formate	-	+
H_2	-	+
pyruvate	-	+

Note: Both strains PT and PW did not utilize glucose, butyrate, isobutyrate, succinate, lactate, benzoate, ethanol and butanol.

5.5.2 Propionate degrading strain PW

Propionate degrading strain PW was initially isolated as a co-culture with *Msp. hungatei* strain BD in the absence of sulfate (Figure 5.8B). Subsequently, it was found that this strain PW oxidized propionate to acetate and reduced sulfate to sulfide. Pure colonies of strain PW were obtained on solidified agar medium containing sodium sulfate (20 mM) and sodium propionate (20 mM) after 1.5 months of incubation. Colonies of strain PW were smooth, round in shape with dark-brown color and a diameter of approximately 1.0 mm. This strain was a short rod with rounded ends (0.76 to 0.90 by 1.0 to 1.2 μm in size) and showed gram-negative cell wall structure (Figure 5.9A). Spore formation was not detected. In the presence of sulfate strain PW could utilize H_2, formate, propionate, and pyruvate as electron donors to reduce sulfate. Strain PW could

also grow syntrophically in the absence of sulfate when *Msp. hungatei* strain BD or *Mb. formicicum* species (strain T1N or strain RF or strain MF) was added to the pure culture of strain PW. The doubling time of strain PW in co-culture with *Msp. hungatei* strain BD on propionate (no sulfate present) was 5 days at 37°C. A three-day doubling time was observed in pure culture with sulfate present. The co-culture formed CH_4 and acetate from propionate while the pure culture oxidized propionate to acetate and reduced sulfate to sulfide.

5.5.3 Butyrate degrading strain BH

Syntrophic acetogen strain BH degraded butyrate and was isolated as a co-culture with *Mb. formicicum* strain T1N (Figure 5.8C). Strain BH was rod-shaped with a size of 0.40 to 0.56 by 2.6 to 4.7 μm. This organism formed terminal endospores (Figure 5.10). Based on TEM observation, the wall of the cells appeared gram-positive. Poly-β-hydroxybutyrate (PHB), which has been found inside *Syntrophomonas* sp. (Dubourguier *et al.*, 1988a), was not observed inside the cells of strain BH. The doubling time of strain BH on butyrate in co-culture with strain T1N was 66 h at 37°C. The co-culture converted butyrate into CH_4 and acetate. The coculture could utilize monocarboxylic saturated fatty acids with 4 to 9 carbon atoms to methane and acetate or methane, acetate and propionate. The coculture also converted 2-methylbutyrate to CH_4, acetate, and propionate (Table 5.10). However, this culture did not use propionate and isobutyrate and could not use butyrate as an electron donor to reduce sulfate. Strain BH displayed morphological and physiological properties similar to spore-forming *Syntrophospora bryantii* (Stieb and Schink, 1985; Zhao *et al.*, 1990) and was likely identified as a strain of this species. This strain appears unrelated to *Syntrophomonas wolfei* (McInerney *et al.*, 1981) and *Syntrophomonas sapovarans* (Roy *et al.*, 1986), which both do not form spores.

Figure 5.10 llustration of different growth stages during sporulation by sytrophic butyrate degrader strain BH presumably being *Syntrophospora bryantii*

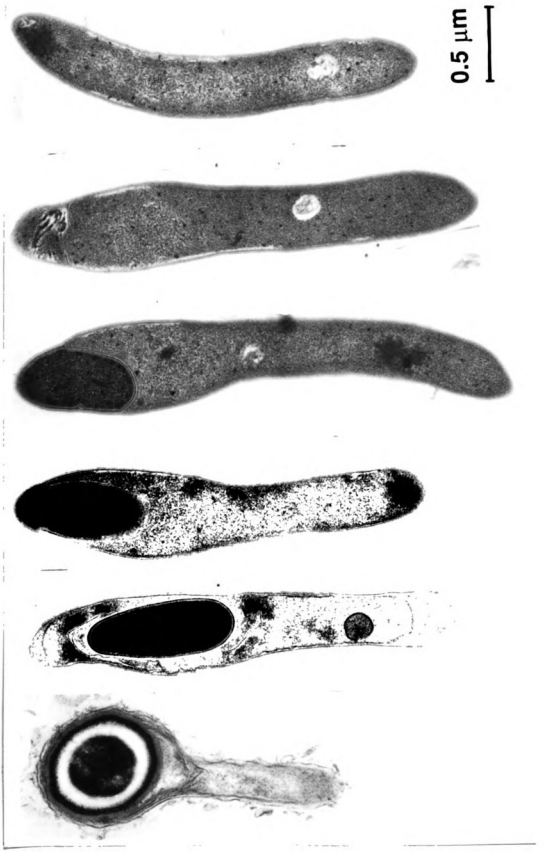

0.5 µm

Table 5.10 General properties of prevalent syntrophic butyrate and isobutyrate-butyrate degraders isolated from R-granules

Property	Strain BH	Strain IB
Cellular		
cell-wall	gram-positive	gram-positive
cell shape	medium rod	medium rod
endospores	terminal	none
Methanogen partner	strain T1N	strain T1N and T18
Doubling time (hrs)	66	60
Substrates degraded		
butyrate	+	+
isobutyrate	-	+
valerate	+	+
2-methylbutyrate	+	+
caproate	+	+
4-methylvalerate	ND	+
heptanoate	+	+
2-methylhexanoate	ND	+
caprylate	+	+
pelargoate	+	+

(1) Strains BH and IB do not use ethanol, propionate, isovalerate, trimethylacetate and 3-methylvalerate.
(2) ND: Not determined.

5.5.4 Isobutyrate-butyrate degrading strain IB

Syntrophic acetogen strain IB was grown as a tri-culture with methanogens *Mb. fomicicum* strain T1N and *Ms. mazei* strain T18 (Figure 5.8D). Strain IB was isolated as a tri-culture from an isobutyrate enrichment culture by adding strain T1N and then plating serial dilutions onto agar plates. The cells of strain IB were rod-shaped with a size of 0.45 by 2.0 to 3.5 μm. Strain IB had gram-positive cell wall structure (Figure

5.9C). PHB was not observed inside the cells of this strain. The doubling time of strain IB in tri-culture on isobutyrate was 60 h at 37°C. The tri-culture can utilize monocarboxylic saturated fatty acids with 4 to 9 carbon atoms, and also use branched-fatty acids isobutyrate, 2-methylbutyrate, 4-methylvalerate, and 2-methylhexanoate (Table 5.10). The culture did not use butyrate as electron donor to reduce sulfate. When strain IB degraded either isobutyrate or butyrate in the tri-culture, isomerization always occurred resulting in both fatty acids being present (Chapter 6).

5.6 IDENTIFICATION OF PREVALENT SPECIES

The prevalent species in the R-granules were identified by microscopic and electron microscopic examination and compared to the isolated strains.

Under a microscope, four prevalent cell morphotypes of methanogens were observed in disrupted granules: a short, bamboo-shaped rod with square ends (*Methanothrix* strain M7-like), a spirillum (*Msp. hungatei* strain BD-like), a thin rod (*Mb. formicicum*-like), and a sarcina (*Ms. mazei*-like). All of these cell types (except the fat, *Methanothrix*-like rod) actively fluoresced under UV light which is a diagnostic for co-enzyme F_{420} (Mink and Dugan, 1977). The same morphotypes of syntrophic acetogens that degraded propionate (strains PT and PW) and butyrate (strains BH and IB) were also observed. To examine the population levels of individual isolated strains in the granules, the microbial morphotypes in various dilutions of MPN tubes were examined compared with the morphotypes of isolated strains (Table 5.11). The same morphotypes as *Mb. formicicum* (strain T1N or strain RF) were observed in the

highest positive MPN tubes (10^{12}/gSS) using H_2-CO_2 or formate as the substrate. *Methanothrix* strain M7-like rods, consisting of 2 to 4 cells, were observed as predominant morphotype in the highest positive MPN tube (10^{12}/gSS) using acetate. A few of *Methanobacterium*-like cells, presumably, strain T1N or RF, were also found in the highest acetate tubes (10^{12}/gSS). *Ms. mazei*-like, and *Methanospirillum*-like cells were observed at levels of 10^8 and 10^9/gSS, respectively. Spore-forming, propionate-degrading strain PT was observed in all the highest positive MPN tubes using propionate as substrate (10^{11}/gSS) while strain PW-like cells were observed in lower positive tubes (10^{10}/gSS or lower). Spore-forming, butyrate degrading strain BH and non-spore forming isobutyrate-butyrate-degrading strain IB were observed in the highest positive MPN tubes (10^{11}/gSS) containing butyrate as substrate.

Table 5.11 Prevalent morphotypes observed in MPN tubes

Substrate for MPN	morphtype observed	level (cell/g SS)
1. propionate	strain PT-like	10^{11}
	strain PW-like	10^{10}
2. butyrate	strains BH and IB-like	10^{11}
3. H_2-CO_2	*Methanobacterium*-like	10^{12}
	Methanosarcina-like	10^9
	Methanospirillum-like	10^{10}
4. formate	*Methanobacterium*-like	10^{11}
	Methanospirillum-like	10^9
5. acetate	*Methanothrix*-like	10^{12}
	Methanosarcina-like	10^8
	Methanobacterium-like	10^{12}

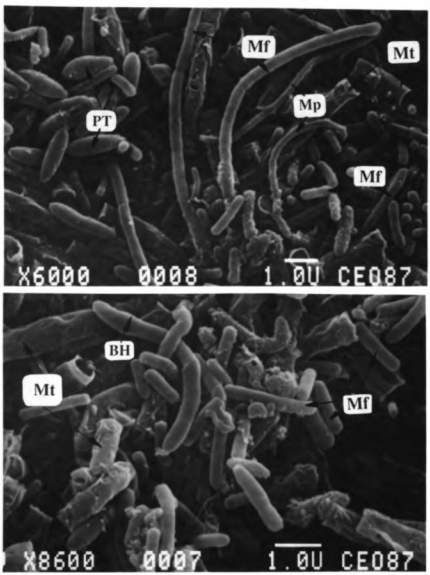


Figure 5.11 Scanning electron photomicrographs of the R-granules. Mf: *Methanobacterium formicicum*-like rods; Mt: *Methanothrix*-like rods: Mp: *Methanospirillum hungatei*-like filaments; BH: *Syntrophospora bryantii*-like rods; PT: spore-forming, syntrophic propionate degrader.

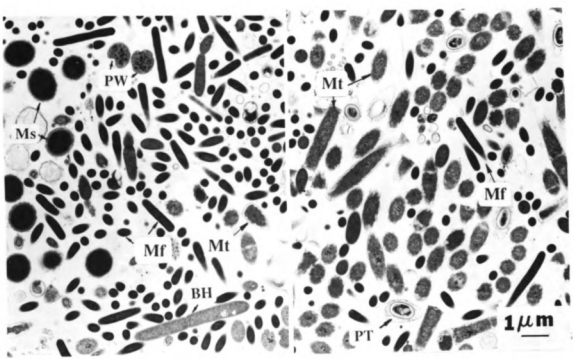


Figure 5.12 Transmission electron photomicrographs of the R-granules. Mf: *Methanobacterium formicicum*-like rods; Mt: *Methanothrix*-like rods; Ms: *Methanosarcina mazei*-like cocci; BH: spore-forming, *Syntrophospora bryantii*-like rods; PT: spore-forming syntrophic propionate degrader; PW: non-spore-forming propionate degrader.

The role of the isolated strains in the microbial composition of the granules was investigated using SEM and TEM examination of granules in comparison with the morphotypes of the isolates. The same morphotypes as those of *Mb. formicicum* (strain T1N or RF) and *Methanothrix* strain M7 were observed as predominant species on the surface and cross sections of the granules (Figure 5.11 and Figure 5.12). Fatty acid

degrading strains PT, PW, BH and IB-like morphotypes were also frequently observed on the surface and granule cross sections (Figure 5.11 and 5.12). The several cell morphotypes were closely juxtaposed but did not appear as microcolonies. Aside from *Methanospirillum*-like cells, which were only observed on the surface of the granules under SEM (Figure 5.11), all other methanogen-like, and syntrophic acetogen-like cells were found inside and on the surface of the granules. Small aggregates of *Methanosarcina*-like cells were scattered on the surface and inside of the granules (Figure 5.12) and were not evenly juxtaposed to the putative syntrophic acetogenic cells. The same morphotypes as either *Syntrophomonas* sp. or *Syntrophobacter* sp., described by Dubouguier *et al.* (1988a, 1988c), were not observed on the thin cross section of the granules. This reveals that these two species, at least, may not be prevalent species in the R-granules.

5.7 DISCUSSION

5.7.1 Identification of isolated methanogens

Five strains of methanogens were isolated from the R-granules. Except strain M7, all of the prevalent methanogenic strains isolated are previously described species: *Mb. formicicum*, *Msp. hungatei* and *Ms. mazei* (Table 1.1 in Chapter 1). Strain M7 is identified as being a *Methanothrix* sp., based on its morphological and physiological characteristics. However, it may be a new species different from *Mtrx. soehngenii* since this strain grew as non-filamentous, bamboo-shaped rods consisting of 1-4 cells, formed rosette clumps and was not antigenically related to *Mtrx. soehngenii* strains which form long filaments (Zehnder *et al.*, 1980; Huser *et al.*, 1982; Touzel *et al.*,

1988). Strain M7 was more sensitive to sulfide than *Mtrx. soehngenii* or *Methanosaeta concilli* (Patel 1984; Patel and Sprott, 1990) on whose growth was not inhibited by 1.0 mM sulfide as was strain M7. The results of DNA-DNA hybrization showing less relatedness (<10%) between strain M7 and *Mtrx. soehngenii* strain Opfiko also support that strain M7 may be a new species (Touzel, 1989).

5.7.2 Identification of isolated syntrophic acetogens

Among the four fatty acid degraders isolated from the R-granules, only strain BH appeared to be a described species, *Syntrophospora bryantii* sp. Syntrophic acetogen strain IB appears to be a new, non-spore-forming isobutyrate-butyrate degrading species that grows with methanogens in the absence of sulfate. It differs morphologically and metabolically from *Syntrophomonas* sp. and *Syntrophospora* sp. which do not utilize isobutyrate, and also from *Desulfococcus multivorans* which grows on isobutyrate via sulfate reduction (Stieb and Schink, 1989) and other SRB (Widdel, 1988). It is also morphologically different from the major non-methanogenic bacteria found in a syntrophic isobutyrate degrading mesophilic enrichment culture (Stieb and Schink, 1989).

Propionate-degrading strains PW and PT appear as new species since they are both morphologically different from non-spore forming *Syntrophobacter wolinii*, which grows with short chains and filaments (Boone and Bryant, 1980) and physiologically different from two reported spore-forming, thermophilic propionate degrading-bacteria (Mucha *et al.* 1988). Strain PW reduced sulfate and was morphologically similar to *Desulfobulbus propionicus* (Widdel and Pfennig, 1982, 1984). However, *Desulfobulbus propionicus* and other propionate-utilizing SRB species isolated to date could not grow syntrophically with a H_2-utilizing methanogen (Laanbreoek *et al.*, 1982; Widdel, 1988).

This strain, presumably, is a classic example of a sulfate reducer which grows syntrophically on propionate. The isolation of strain PW confirmed the presence of SRB which can also degrade propionate syntrophically in the absence of sulfate as suggested in Chapter 4.

5.7.3 The role of prevalent species in granules

In Chapter 3, it was concluded that the granules developed on the VFA mixture contained methanogens and syntrophic acetogens as prevalent species and an insignificant number of hydrolytic-fermentative bacteria, based on the maximum substrate conversion rates (Table 3.5 in Chapter 3) and MPN enumeration data (Table 3.7 in Chapter 3). Based on SEM, TEM and microscopic examination, the predominant bacteria observed in the R-granules were H_2-CO_2 and formate-utilizing *Mb. formicicum* (including strains T1N and RF), and acetate-utilizing *Methanothrix* strain M7. The predominance of *Mb. formicicum* species is also supported by the same levels of H_2-CO_2 and formate utilizing methanogens in MPN enumeration (Table 3.7 in Chapter 3). The prevalence of *Methanothrix* strain M7 was verified by cell counts on cross sections of the R-granules (2.9×10^7 *Methanothrix*-like cell/cm^2 vs. 9×10^7 total cell/cm^2 in Table 3.8) and the similar affinity for acetate by strain M7 and the granules (both strain M7 and the disrupted R-granules had K_m of ca. 0.4 mM). These methanogens are adhesive species and may be important for the stability of granular structure.

The combination of syntrophic acetogens and methanogens isolated from the granules can mineralize all fatty acids from reactor feed and metabolic intermediates formed in the reactors, including acetate, propionate, isobutyrate, butyrate, valerate and 2-methylbutyrate. All the isolated strains appear to be representative metabolic species

in the R-granules. The microbial composition of the granules appears to be strongly dependent on the feed composition. The MPN enumerations suggested that the population levels of the syntrophic acetogenic and methanogenic strains in these granules were as high or higher than the total number of anaerobes reported in the granules developed on industrial wastewater, which contained more hydrolytic-fermentative bacteria (Dolfing *et al.*, 1985; Dubourguier *et al.*, 1988a, 1988c).

In MPN enumeration experiments, H_2-CO_2-utilizing, *Methanobacterium*-like cells were observed in the highest acetate tubes (Table 5.11) for both the R- and F-granules. It is interesting how these bacteria got energy source in these tubes where acetate was supplied as sole substrate. It may be due to a small amount hydrogen produced by acetate-utilizing methanogens, which was utilized as energy source by the H_2-CO_2-utilizing methanogens.

5.8 SUMMARY

1) The R-granules, developed on a VFA mixture, were mainly composed of methanogens and syntrophic propionate and butyrate degraders. Sulfate-reducing bacteria did not play a significant role in metabolism of H_2, formate, acetate, and butyrate in the presence of sulfate but they were involved, to a limited extent, in syntrophic propionate degradation.

2) The prevalent methanogens in the granules were isolated and identified as *Methanobacterium formicicum* strains T1N and RF, *Methanospirillum hungatei* strain BD, *Methanothrix* strain M7 and *Methanosarcina mazei* strain T18. *Mb. formicicum* stain T1N, *Mb. formicicum* strain RF, and *Methanothrix* strain M7 were the

predominant species in the granules. These organisms appeared to play an important role in granular structure.

3) A spore-forming, syntrophic propionate degrader strain PT and sulfate-reducing, propionate degrader, strain PW (which can also degrade propionate syntrophically) were isolated as the prevalent propionate degraders. Both strains are different from other propionate degraders isolated to date.

4) A spore-forming, syntrophic butyrate degrader, strain BH, was isolated and identified as a prevalent syntrophic butyrate degrader. A rod-shaped, syntrophic isobutyrate-butyrate degrader, strain IB, was also isolated as another prevalent butyrate degrader in the granules.

5) Microscopic and electron microscopic examination confirmed that above species represented the major organisms contained in the granules. The combination of all isolated strains can completely mineralize all fatty acids from reactor feed and metabolic intermediates formed in the reactors.

CHAPTER 6

CHARACTERIZATION OF FATTY ACID METABOLISM BY DEFINED CULTURES ISOLATED FROM GRANULES

6.1 INTRODUCTION

Acetate, propionate, butyrate and valerate are common metabolic products of hydrolysis-fermentation. Isobutyrate and 2-methylbutyrate are intermediates of the anaerobic digestion of proteins (McInerney, 1988). Isobutyrate, valerate and 2-methylbutyrate were observed in laboratory-scale UASB reactors containing either the R-granules or F-granules (Chapter 3) and during batch bottle experiments using granules from a full-scale UASB reactor treating brewery wastewater (Chapter 4), when a VFA mixture consisting of acetate, propionate and butyrate was used as the only added substrates. The appearance of isobutyrate during syntrophic butyrate degradation is believed to be the presence of a reversible isomerization reaction between butyrate and isobutyrate (Tholozan *et al.*, 1988a; Aguilar *et al.*, 1990). However, the mechanism of the isomerization is poorly understood. How valerate and 2-methylbutyrate are formed from acetate, propionate and butyrate is not clear.

In Chapter 4, both H_2 and formate were observed as intermediate products

during syntrophic ethanol degradation. The role of hydrogen and formate in interspecies electron transfer during VFA degradation and the relationship between hydrogen and formate, however, requires further evaluation.

In previous studies (Chapter 5), five methanogens and four syntrophic VFA-degrading cultures were isolated from the R-granules developed on an VFA mixture. These cultures could utilize all the fatty acids mentioned above. Among five methanogens isolated from granules developed on the VFA mixture, four strains could utilize H_2-CO_2 and three strains could convert formate to methane (Chapter 5). In this chapter, characterization of the metabolism of fatty acids with three to five carbon atoms (which were feed components or observed in the previous reactor experiments) by these defined cultures and comparison of results from the granules was performed. This comparison allowed tentative identification of the species responsible for the different metabolic steps and possible pathways involved. The isomerization between butyrate and isobutyrate, the interspecies electron transfer, and formate synthesis from H_2 plus HCO_3^- are also discussed.

6.2 MATERIALS AND METHODS

Medium and analytical methods are described in Appendix A. Free energy calculations are presented in Appendix E.

6.2.1 Sources of cultures

The R-granules were developed and maintained in lab-scale UASB reactors, as described in Chapter 3. Defined cultures used for the different assays are described in

Table 6.1. These cultures were isolated from the R-granules or obtained from commercial culture collections (Chapter 5).

Table 6.1 Defined cultures used for characterization of substrate metabolism

Culture	Objective of the assay
1. Monoculture	
a) *Mb. formicicum* MF	H_2 and formate metabolism
b) *Mb. formicicum* T1N	H_2 and formate metabolism
c) *Mb. formicicum* RF	H_2 and formate metabolism
d) *Msp. hungatei* BD	H_2 and formate metabolism
e) *Ms. mazei* T18	H_2 and acetate metabolism
2. Coculture	
a) Strain PT plus *Mb. formicicum* T1N	Propionate degradation
b) Strain PW plus *Msp. hungatei* BD	Propionate degradation
c) Strain BH plus *Mb. formicicum* T1N	VFA (C_4 and C_5) degradation
3. Triculture	
Strain IB, *Ms. mazei* T18 and *Mb. formicicum* T1N	VFA degradation, isomerization, Interspecies electron transfer

6.2.2 Experimental procedures

All experiments were performed using 158 mL serum bottles containing 50 mL media at 35 °C and 37 °C for the anaerobic granules and defined cultures, respectively. The bottles were placed horizontally in a New Brunswick shaking water bath operated at 125 strokes/min to reduce mass transfer resistance for H_2. Prior to inoculation, all the bottles were brought to the desired temperature by placing the bottles in the water bath. The growth of methanogens and syntrophic cultures was monitored by determining methane production, substrate consumption, and intermediate or end

product formation.

6.2.3 Calculation of conversion rates for isobutyrate-butyrate degradation

Syntrophic degradation of butyrate and isobutyrate involves three reactions: i) isomerization of butyrate to isobutyrate, ii) isomerization of isobutyrate to butyrate, and iii) β-oxidation of butyrate to acetate and formate or H_2. This model can be expressed as:

$$\text{isobutyrate} \underset{v_2}{\overset{v_1}{\rightleftharpoons}} \text{butyrate} \xrightarrow{v_3} 2 \text{ acetate} + 2 \text{ formate (or } H_2) \qquad (6\text{-}1)$$

where, v_1 is the rate of butyrate to isobutyrate, v_2 is the rate of isobutyrate to butyrate, and v_3 is the rate of butyrate to acetate and formate or H_2. Calculation of the rates for v_1 and v_2 is difficult when the difference between the concentrations of butyrate and isobutyrate is not significantly high. When $S_b \gg S_{ib}$, the isomerization of isobutyrate to butyrate is thermodynamically unfavorable (see 6.4.3) and the apparent rate for butyrate to isobutyrate (v_1) can be estimated as:

$$v_1 = (S_{ib2}-S_{ib1})/(t_2-t_1) \qquad (6\text{-}2)$$

Similarly, when $S_{ib} \gg S_b$, the apparent rate for isobutyrate to butyrate (v_2) can be approximately calculated as:

$$v_2 = (S_{ib1}-S_{ib2})/(t_2-t_1) \qquad (6\text{-}3)$$

The rate for v_3 can be calculated as:

$$v_3 = (S_{ib1}-S_{ib2}+S_{b1}-S_{b2})/(t_2-t_1) \qquad (6\text{-}4)$$

where, S_{ib1} and S_{ib2}, or S_{b1} and S_{b2} are the concentrations of isobutyrate or butyrate at time t_2 and t_1, respectively.

6.2.4 Perturbation with formate and H_2

Perturbation with formate or hydrogen was used to investigate the effect of formate or H_2 on syntrophic butyrate and isobutyrate degradation. Well-grown

syntrophic VFA-degrading cultures were used. At the beginning of experiments, the headspace of the bottles was replaced by an N_2-CO_2 (70:30) gas mixture to obtain a pH of about 7.0, and then a substrate (butyrate or isobutyrate) stock solution was added to the bottles to obtain a final concentration of about 2 mM. The bottles were then incubated in the water bath for about 8 to 12 hours to activate cells. Subsequently, substrate (stock solution (1M)) was added to the bottles by syringe injection to obtain a substrate concentration of 5 to 10 mM. At the same time, liquid and gas samples were withdrawn for initial determination of the pH and fatty acids in liquid phase, and CO_2, CH_4 and H_2 in gas phase. After several hours of incubation, anaerobic sodium formate stock solution (4 M) was added into the cultures to provide a concentration of 15 to 16 mM to examine the effect of formate on the syntrophic VFA conversion. To examine the effect of H_2, H_2 gas was added by syringe injection into the headspace of the bottles. The pH, fatty acids, CH_4 and H_2 were monitored continuously throughout the experiments. CO_2 was determined at the end of each experiment.

The bicarbonate concentration in the liquid phase was estimated from pH and CO_2 partial pressure in gas phase according to Henrry's law and bicarbonate-CO_2 equilibrium at 37 °C (Snoeyink and Jenkins, 1980).

6.3 SYNTROPHIC METABOLISM OF FATTY ACIDS

Fatty acid degradation was characterized using both the R-granules and defined syntrophic cultures in order to identify the role of individual acetogens in fatty acid metabolism by the granule ecosystem. Propionate, butyrate, isobutyrate, valerate and 2-methylbutyrate were used as substrates since they were feed components for the granules or they were found in the reactor effluent (Chapter 3).

6.3.1 Syntrophic degradation of fatty acids by granules

Simultaneous degradation of acetate, propionate, and butyrate by the R-granules is illustrated in Figure 6.1. Butyrate was consumed much quicker than propionate since these granules had a higher specific activity for butyrate (Table 3.5 in Chapter 3). At the beginning of the assay, acetate accumulated since the total rate of acetogenesis from propionate plus butyrate was much higher than that of acetate utilization. Within the initial 34 hours, isobutyrate, valerate and 2-methylbutyrate accumulated to the highest levels each attained and then decreased as the butyrate concentration decreased to less than 1 mM. The hydrogen partial pressure observed was proportional to the butyrate plus propionate concentrations. Higher butyrate and propionate levels resulted in higher hydrogen levels. Formate was not detected (<5 μM).

When butyrate and isobutyrate were used as substrates, a reversible isomerization was observed. Isobutyrate was produced during butyrate degradation and vice versa (Figure 6.2A and B). The level of isobutyrate formed during butyrate degradation, however, was much higher than the concentration of butyrate that accumulated during isobutyrate degradation. In the both cases isobutyrate persisted longer. Hydrogen and acetate were intermediates. Neither valerate nor 2-methylbutyrate were observed.

When propionate was used as the primary substrate, acetate concentrations remained low (<0.3 mM). This is because the granules had a higher maximum specific conversion rate of acetate than that of propionate (Table 3.5 in Chapter 3). The hydrogen pressure remained low (<7 Pa) during propionate conversion and no other fatty acids were detected.

These results indicate that the isomerization between butyrate and isobutyrate was performed by some organisms during butyrate degradation. No formation of valerate and 2-methylbutyrate during either propionate or butyrate degradation indicates that the formation of these acids require butyrate together with propionate.

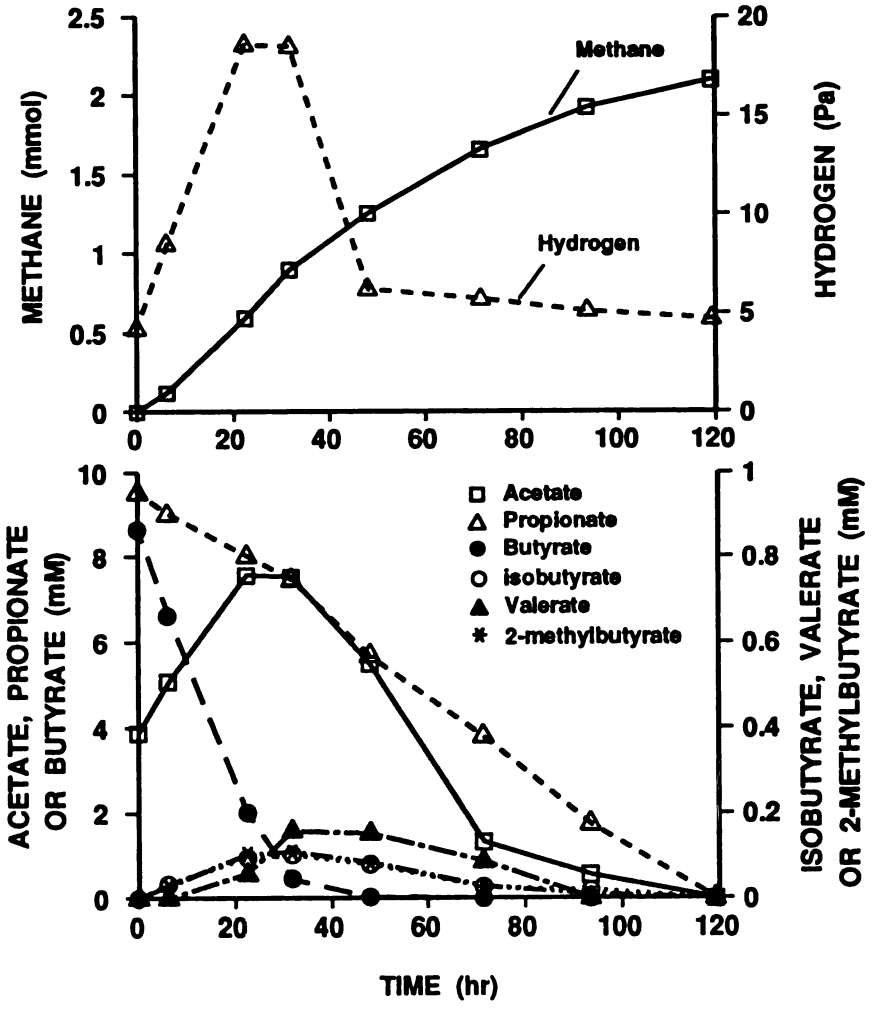


Figure 6.1 Simultaneous degradation of a mixture consisting acetate, propionate and butyrate by the R-granules. Isobutyrate, valerate, 2-methylbutyrate and hydrogen accumulated during initial portion of the assay and were then consumed.

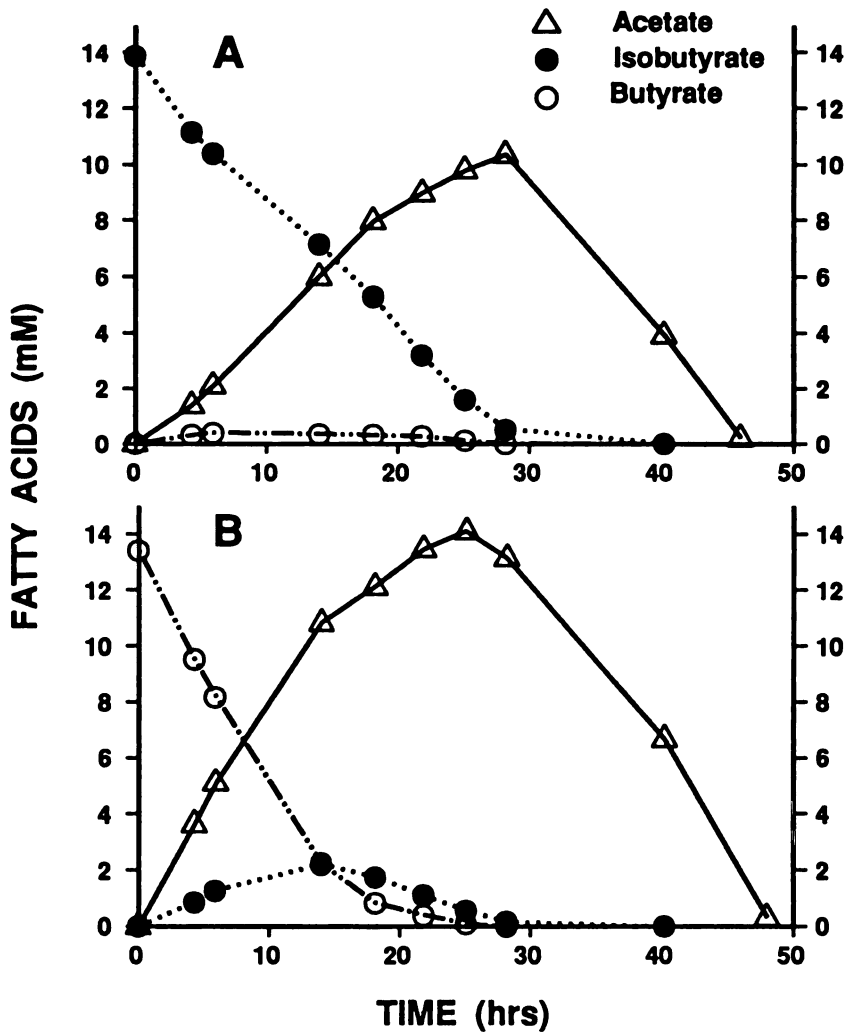


Figure 6.2 Butyrate and isobutyrate degradation by the R-granules. (A) Butyrate formation during syntrophic isobutyrate degradation. (B) Isobutyrate formation during syntrophic butyrate degradation.

6.3.2 End products of syntrophic fatty acid degradation by defined cultures

i) Syntrophic propionate degraders

The coculture of syntrophic propionate degrader strain PT plus *Mb. formicicum* strain T1N and the coculture of propionate degrader strain PW and *Msp. hungatei* strain BD had the same end products for propionate degradation (Table 6.2). The mole ratio for propionate consumed, methane produced, and acetate produced is ca. 1:0.75:1. Hydrogen (from an initial ca. 8 Pa to final level of 2 Pa) was observed during syntrophic propionate degradation. Formate was not detected (lower than 5 μM).

Table 6.2 End products of propionate degradation by syntrophic cocultures (mmole per mmole propionate consumed)

Culture	CH_4	acetate
strain PT plus strain T1N	0.74	0.97
strain PW plus strain BD	0.73	0.96

These cocultures did not utilize butyrate and other fatty acids. Butyrate and other fatty acids were also not detected during propionate degradation. This indicates that the degradation of propionate by these cocultures may follow the methylmalonyl-CoA path way (Koch *et al.*, 1983; Mucha *et al.*, 1988) rather than the reductive carboxylation-acetogenesis pathway in which butyrate is an intermediate

(Tholozan *et al.*, 1988b, 1990). Neither valerate nor 2-methylbutyrate was detected during the degradation of propionate in the presence of butyrate and isobutyrate. This indicates that these two propionate degraders are not involved in the formation of valerate and 2-methylbutyrate.

Table 6.3 End products of VFA degradation by defined butyrate degrading cultures (mmole per mmole substrate consumed)

Substrate	Number of carbons	Cultures: BH plus T1N			Cultures: IB, T1N and T18	
		CH_4	acetate	propionate	CH_4	propionate
butyrate	4	0.47	2.01	0	2.46	0
isobutyrate	4	ND			2.44	0
valerate	5	1.02	0.96	0.46	1.52	0.91
2-methylbutyrate	5	1.01	0.97	0.47	1.49	0.92
caproate	6	0.88	2.82	0	3.84	0
4-methylvalerate	6	ND			3.82	0
heptanoate	7	0.99	2.0	0.98	2.46	0.99
2-methylhexanoate	7	ND			2.49	0.97
caprylate	8	1.45	3.82	0	4.65	0
pelargoate	9	0.73	2.97	0.98	3.72	0.90

(1) Experiment was performed at 35°C. Initial VFA concentration was 10 mM for the acids with 4 to 6 carbon atoms, 5 mM for those with 7 atoms, and 2.5 mM for those with 8 to 9 atoms, respectively. Initial pH was 7.0 to 7.1.
(2) ND: not determined.
(3) T1N: *Mb. formicicum* strain T1N; T18: *Ms. mazei* strain T18; BH:butyrate degrader strain BH; IB: isobutyrate-butyrate degrader strain IB.

ii) Syntrophic butyrate and isobutyrate degraders

The end products produced per mmole of substrate for the coculture consisting of strain BH and *Mb. formicicum* strain T1N and the triculture consisting of isobutyrate-butyrate degrader strain IB, *Mb. formicicum* strain T1N and *Ms. mazei* strain T18 are summarized in Table 6.3. Considering that one mmole of acetate produced from syntrophic acetogenesis should be converted to ca. one mmole of methane by *Ms. mazei* T18, the end products of both cultures were basically the same for the degradation of normal carboxylic saturated fatty acids with four or more carbon atoms. Based on the nature of the end products, the degradation appears to follow β-oxidation, as described in Table 1.3 in Chapter 1. As far as branched fatty acids are concerned, both cultures were able to degrade 2-methylbutyrate. A significant difference is that strain IB was able to degrade isobutyrate while strain BH did not.

6.3.3 Metabolism of VFA by a coculture of strain BH plus *Methanobacterium formicicum* strain T1N

In the following experiments, the respective time course for degradation of butyrate, valerate, and 2-methylbutyrate by a coculture of strain BH and *Mb. formicicum* strain T1N was monitored to investigate the metabolic pathways involved. The degradation of butyrate in the presence of propionate was also examined to identify whether this coculture could synthesize valerate and 2-methylbutyrate.

i) Growth on butyrate

The growth of the coculture on butyrate is illustrated in Figure 6.3. Acetate concentration increased from an initial level of 2.3 mM (added with inoculum) at a molar ratio of approximately 1.9 (acetate produced per butyrate consumed). No isobutyrate was observed, indicating this coculture does not carry out isomerization

between butyrate and isobutyrate. Hydrogen was observed produced during the syntrophic degradation. The maximum observed partial pressure of hydrogen was less than 20 Pa. Formate was not detected (<5 μM).

ii) Growth on valerate and 2-methylbutyrate

When this coculture was grown on either valerate or 2-methylbutyrate, the amounts of acetate and propionate produced were proportional on a molar basis to the amount of valerate or 2-methylbutyrate consumed (0.97 or 0.98).

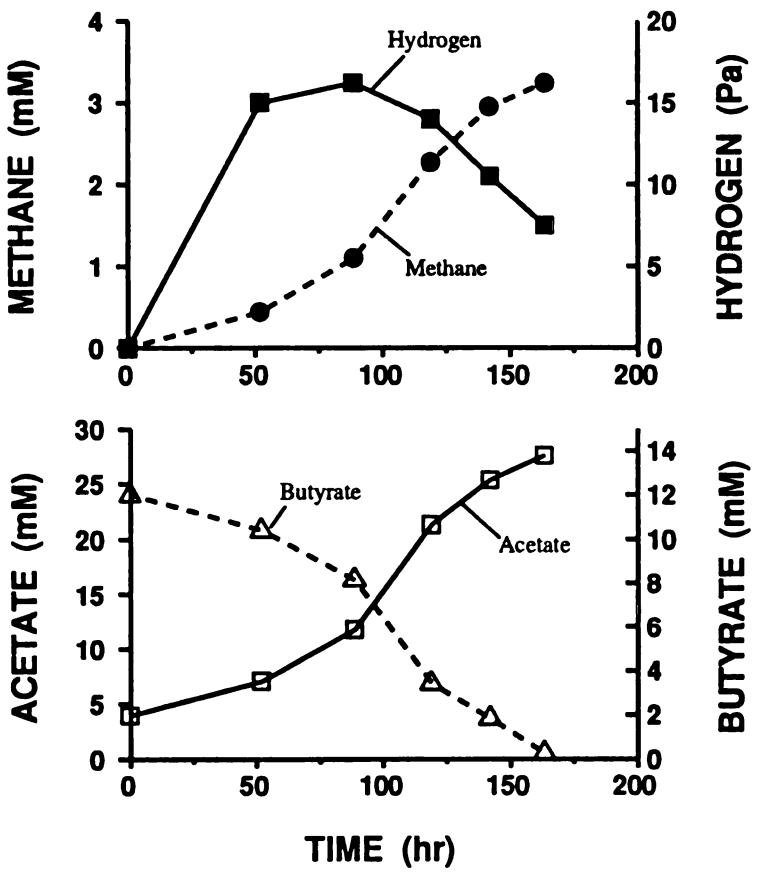


Figure 6.3 The growth of the coculture consisting of strain BH and *Mb. formicicum* strain T1N on butyrate. Acetate and methane were end products. Hydrogen gas was observed during butyrate degradation.

iii) Growth on butyrate in the presence of propionate

The growth of this coculture on butyrate in the presence of propionate is shown in Figure 6.4. The initial acetate (from inoculum), propionate and butyrate concentrations were ca. 2.4, 8.8 and 12.3 mM, respectively. Acetate, methane, hydrogen and valerate were produced during syntrophic oxidation of butyrate. Valerate accumulated up to a peak concentration of ca. 0.11 mM and was subsequently degraded when the butyrate concentrations decreased.

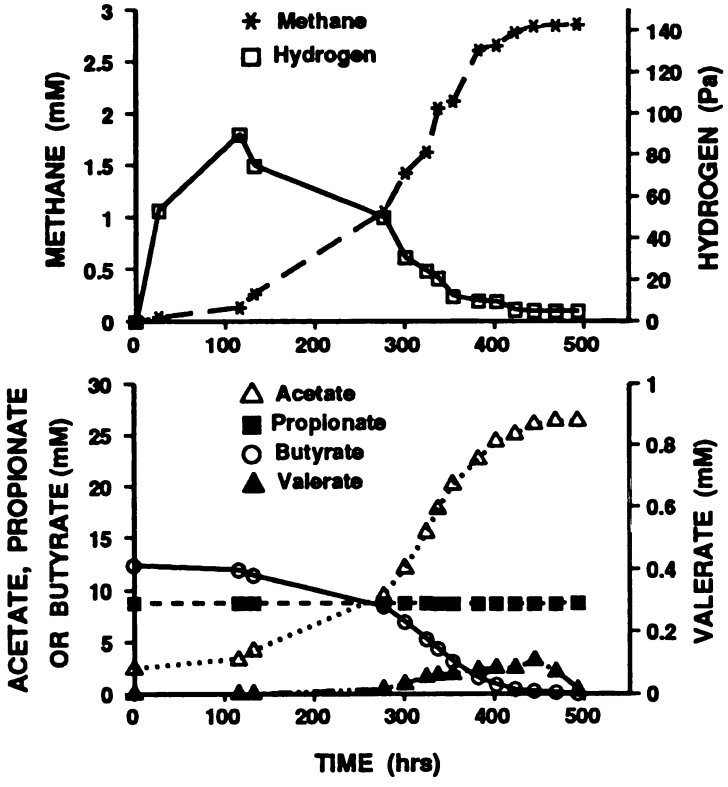


Figure 6.4 The growth of the coculture consisting of strain BH and *Mb. formicicum* strain T1N on butyrate in the presence of propionate. Acetate and methane were end products. Hydrogen and valerate were observed during butyrate degradation.

To test if propionate degradation was performed by the coculture and if valerate could be formed in the absence of butyrate, a control experiment was carried out by inoculating the coculture into a medium containing 10 mM acetate and 10 mM propionate. After 3 months of incubation, neither propionate nor acetate was consumed, and no other fatty acids were formed.

To further identify if the formation of valerate was coupled with syntrophic butyrate degradation, two additional experiments were performed. Propionate (8 mM) or propionate (8 mM) plus butyrate (10 mM) were added to a pre-grown coculture in which butyrate had been consumed to a very low level (<0.1 mM) and acetate (20 mM) had accumulated. Hydrogen (1.3 atm) was added to inhibit syntrophic butyrate degradation. In the experiment using propionate plus butyrate, valerate was detected only after hydrogen was consumed to a low level (<4 Pa) and butyrate degradation had began. No valerate was formed in the experiment in the absence of butyrate.

These results reveal that valerate was synthesized from propionate and the synthesis reaction was coupled to active syntrophic butyrate oxidation.

6.3.4 Metabolism of VFA by a triculture containing strain IB, *Mb. formicicum* strain T1N and *Ms. mazei* strain T18

The degradation processes of butyrate, isobutyrate, valerate, and 2-methylbutyrate by a triculture consisting of strain IB, *Mb. formicicum* strain T1N and *Ms. mazei* strain T18 were monitored to investigate what intermediates were produced. The degradation of butyrate in the presence of propionate was also examined to determine whether this triculture were able to synthesize valerate and 2-methylbutyrate.

i) Growth on butyrate and isobutyrate

The growth of the triculture on butyrate is presented in Figure 6.5. After

inoculation, butyrate was degraded with concurrent production of isobutyrate. The highest concentration of isobutyrate detected was 0.75 mM. This occurred as the butyrate concentration decreased form 7.2 to 3.1 mM. Subsequently, both were simultaneously degraded with concurrent production and consumption of acetate. The hydrogen partial pressure in the bottle reached a peak level of 18.5 Pa at 48 hours of incubation and eventually decreased to the initial level of 4.8 Pa.

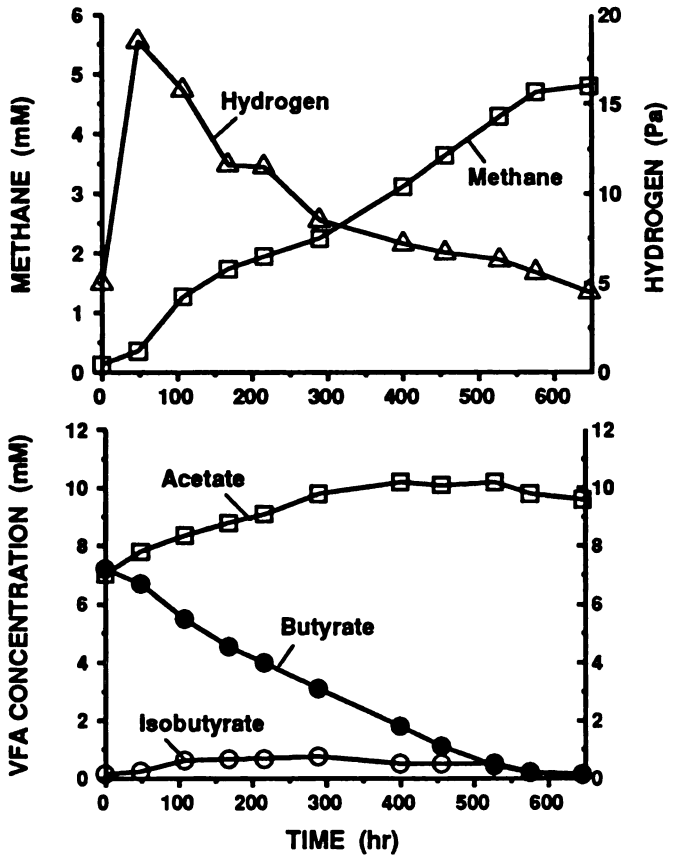


Figure 6.5 The growth of the triculture consisting of strain IB, *Mb. formicicum* strain T1N and *Ms. mazei* strain T18 on butyrate. Acetate, which was then converted to methane by *Ms. mazei* T18, and methane were end products of syntrophic butyrate degradation. Hydrogen and isobutyrate were observed during butyrate degradation.

In the assay using isobutyrate as the substrate, isomerization of isobutyrate to butyrate occurred immediately (Figure 6.6). The concentration of butyrate rose to 2.4 mM as the isobutyrate concentration decreased from 8.0 to 2.7 mM. Thereafter, the concentrations of both acids decreased at ca. the same rate. The change in hydrogen partial pressure in the headspace and the acetate production and consumption was similar to the time-course profile observed for butyrate degradation. The highest H_2 concentration observed was 23 Pa.

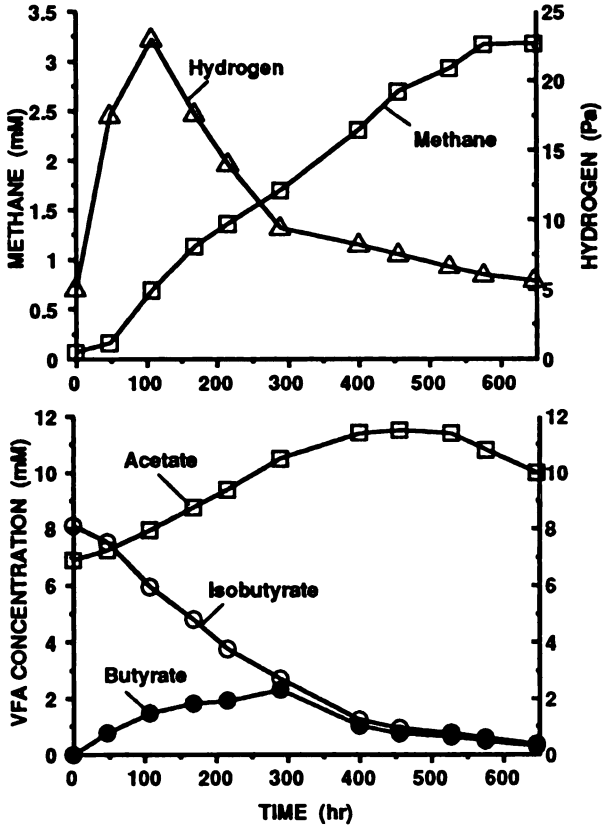


Figure 6.6 The growth of the triculture consisting of strain IB, *Mb. formicicum* strain T1N and *Ms. mazei* strain T18 on isobutyrate. Acetate, which was then converted to methane by *Ms. mazei* T18, and methane were end products of syntrophic isobutyrate degradation. Hydrogen and butyrate were observed during isobutyrate oxidation.

When the triculture was inoculated in the medium containing almost the same levels of butyrate and isobutyrate (4.4 and 4.1 mM, respectively), the concentrations of the two acids decreased at approximately the same rate until both were completely consumed (Figure 6.7). The peak H_2 concentration observed during this assay was 16 Pa. Formate was not detected in these or any of the previous experiments.

These results demonstrate that strain IB carries out a reversible isomerization between isobutyrate and butyrate during syntrophic oxidation of butyrate or isobutyrate.

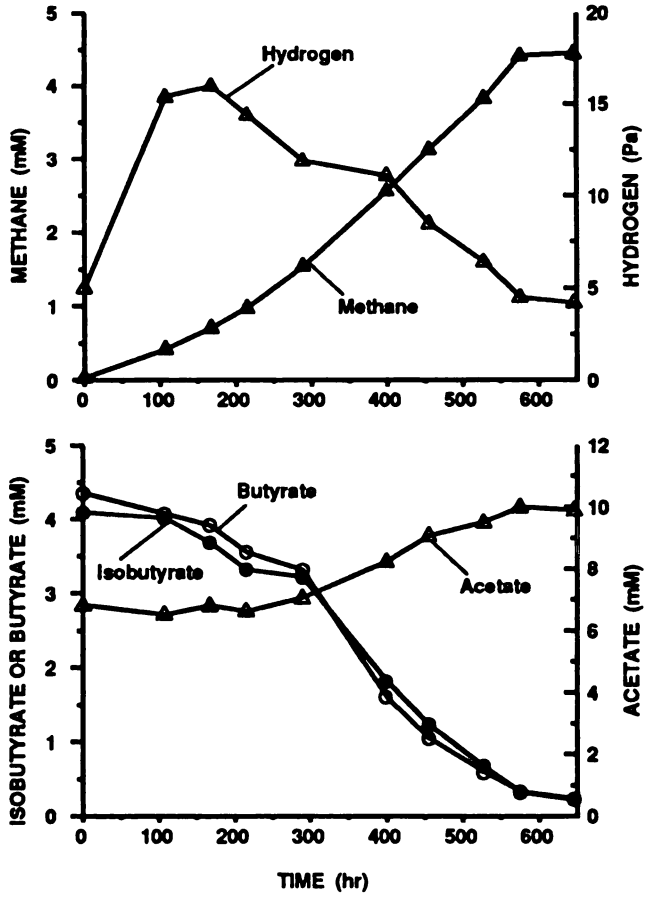


Figure 6.7 The growth of the triculture consisting of strain IB, *Mb. formicicum* strain T1N and *Ms. mazei* strain T18 on an equal molar concentrations of isobutyrate and butyrate.

ii) Kinetic analysis of syntrophic degradation of isobutyrate and butyrate

A fed batch experiment was performed to estimate the relative rates of syntrophic butyrate degradation and the isomerization reaction.

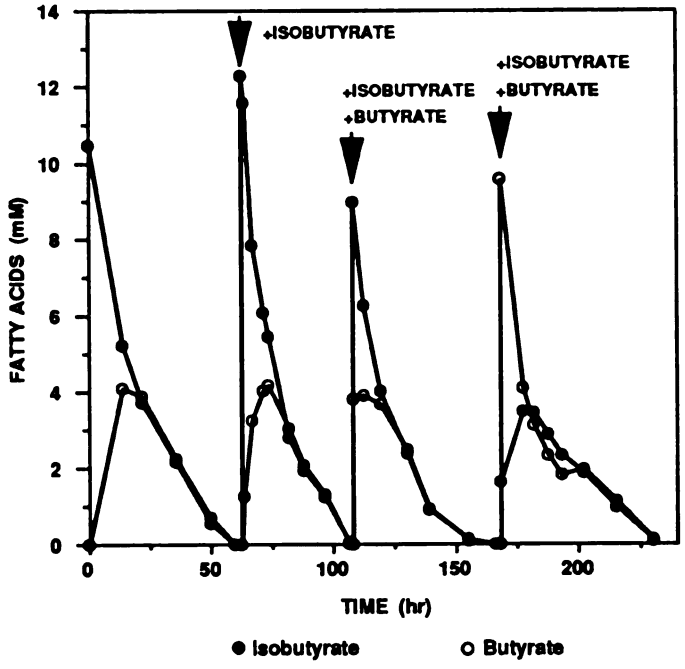


Figure 6.8 Batch-fed experiment on isobutyrate and butyrate degradation by the triculture consisting of strain IB, *Mb. formicicum* strain T1N and *Ms. mazei* strain T18. An equal level of isobutyrate and butyrate occurred after each addition of substrate.

The results of syntrophic isobutyrate and butyrate degradation in this fed batch experiment is presented in Figure 6.8. Substrate (isobutyrate or isobutyrate plus butyrate) was added four times. Initially, only isobutyrate (10.5 mM) was provided in the medium. Isobutyrate was isomerized to butyrate until an equal level

(approximate 4 mM of each butyrate and isobutyrate) was reached. Subsequently, butyrate and isobutyrate disappeared at approximately the same rate, as observed previously. Adding another 12.3 mM of isobutyrate to the culture led to the same result. The third addition of substrate was performed using 9.0 mM of isobutyrate and 3.8 mM of butyrate. The isobutyrate concentration decreased immediately while little change was observed in the butyrate concentration. When the concentrations of both acids reached approximately equal concentration they subsequently disappeared at the same rate. Finally, 1.6 mM isobutyrate and 9.6 mM butyrate were supplemented into the culture. The concentration of butyrate decreased while the concentration of isobutyrate increased. When the concentrations of the two acids were equal, they disappeared at the same rate.

Results of the isomerization and conversion of butyrate and isobutyrate when isobutyrate or butyrate was added as substrate and the calculated net degradation rates are presented in Table 6.4. When only isobutyrate was added to the culture (No.1 in Table 6.4), the apparent rate of isobutyrate to butyrate (v_2) was about 4 times of the degradation rate of butyrate (v_3). As butyrate accumulated (from 0 to 4.1 mM), the butyrate degradation rate (v_3) increased from 0.09 to 0.22 mM/hr. Conversely, when the system was supplied with a higher starting butyrate concentration (9.6 mM), the highest butyrate degradation rate (v_3 = 0.40 mM/hr) was observed at the beginning of the experiment. This rate was twice the apparent rate of butyrate to isobutyrate (v_1).

Table 6.4 Isomerization and conversion rates of butyrate and isobutyrate by the syntrophic triculture of *Mb. formicicum* T1N, *Ms. mazei* T18 and strain IB.

No.	time hr	iC_4 mM	C_4 mM	conversion rate (mM/hr) v_1	v_2	v_3
1	0	10.5	0			
	13.7	5.2	4.1	ND	0.39	0.09
	21.6	3.7	3.9	ND	ND	0.22
	35.6	2.2	2.3	ND	ND	0.23
	49.8	0.55	0.7	ND	ND	0.22
	62.4	0	0	ND	ND	0.13
2	0	1.6	9.6			
	9.2	3.5	4.1	0.20	ND	0.40
	13.6	3.4	3.1	ND	ND	0.23
	19.3	2.9	2.3	ND	ND	0.23
	24.8	2.3	1.8	ND	ND	0.19
	47.0	1.0	1.1	ND	ND	0.13
	85.7	0.11	0.12	ND	ND	0.05

(1) The sequence of time begins up on addition of substrate.
(2) The apparent rates for the formation of isobutyrate from butyrate (v_1), the formation of butyrate from isobutyrate (v_2), and butyrate degradation (v_3) were calculated from equations (6-2), (6-3) and (6-4), respectively.
(3) ND: not determined.

The experimental data presented in Table 6.4 shows that when isobutyrate was used as the sole substrate, the apparent conversion rate of butyrate to acetate and methane (v_3= 0.09 mM/hr) was much lower than when isobutyrate and butyrate were both present (v_3=0.22 mM/hr). This confirmed that butyrate was an intermediate in the degradation of isobutyrate (i.e. isobutyrate was isomerized to butyrate and then degraded via β-oxidation). Thus, the syntrophic conversion of isobutyrate and butyrate by strain IB in the triculture involves at least three reactions: (1) isomerization of butyrate to isobutyrate, (2) isomerization of isobutyrate to butyrate, and (3) β-oxidation of butyrate to acetate and H_2 or formate. When the concentration of isobutyrate or butyrate is much higher than the K_m, the reaction rates for v_1, v_2 and v_3 in Table 6.4 can be used to estimate the relations among these three reactions, assuming that the K_m of the three reactions were close to the apparent K_m of isobutyrate and butyrate degradation by anaerobic granules (0.12 and 0.15 mM, respectively) as described in Table 3.6 of Chapter 3,. When the concentration of isobutyrate is much higher than butyrate (case No.1), the value of v_2 (0.39 mM/hr) could be estimated to be the maximum rate of isobutyrate to butyrate (V_{max2}). V_{max3} could be estimated as 0.22 mM/hr. When the concentration of butyrate was higher (case No.2), V_{max1} could be estimated to be 0.20 mM/hr, and V_{max3} was 0.40 mM/hr. Thus, the ratio among V_{max1}, V_{max2} and V_{max3} could be roughly estimated as 0.5:1.77:1.0.

iii) Growth on valerate and 2-methylbutyrate

During the time-course for degradation of valerate (initial concentration of 7.4 mM), propionate increased to a concentration of 6.8 mM (Figure 6.9). The molar ratio between propionate produced and valerate consumed was 0.92. During the assay, isobutyrate, butyrate and 2-methylbutyrate were also produced. Isobutyrate, butyrate

and 2-methylbutyrate appeared almost simultaneously after 45 hours of growth. The concentration of butyrate formed was slightly higher than the concentration of isobutyrate formed. Butyrate and isobutyrate disappeared with the consumption of valerate prior to the disappearance of 2-methylbutyrate. The highest hydrogen partial pressure (5.6 Pa) observed during valerate conversion was much lower than that observed during butyrate or isobutyrate conversion.

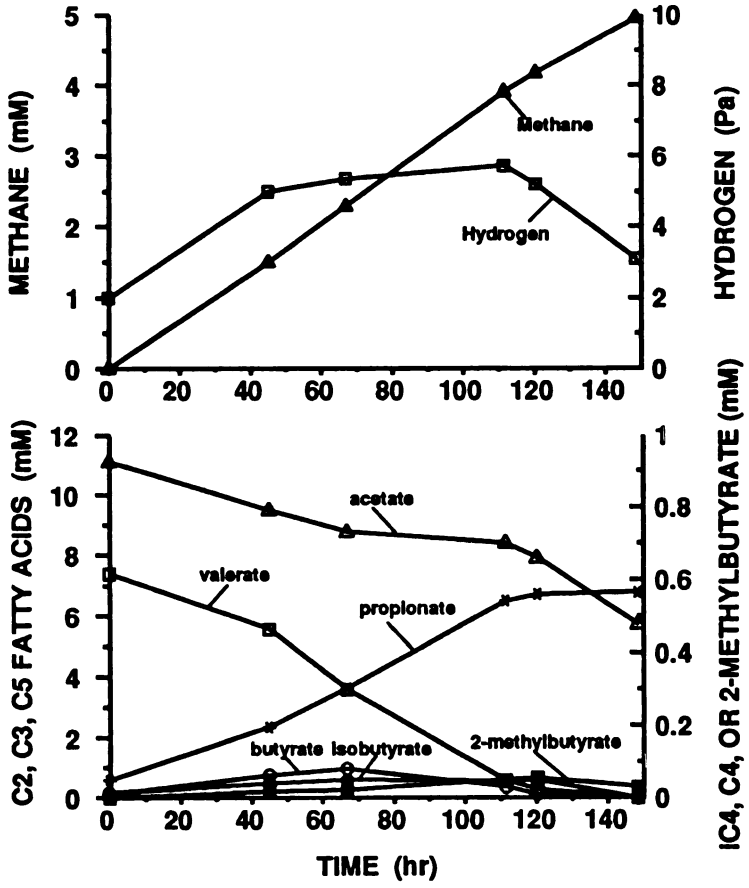


Figure 6.9 The growth of the triculture consisting of strain IB, *Mb. formicicum* strain T1N and *Ms. mazei* strain T18 on valerate. Acetate, which was then converted to methane by *Ms. mazei* T18, propionate and methane were end products of valerate degradation. Butyrate, isobutyrate and 2-methylbutyrate were observed during valerate degradation.

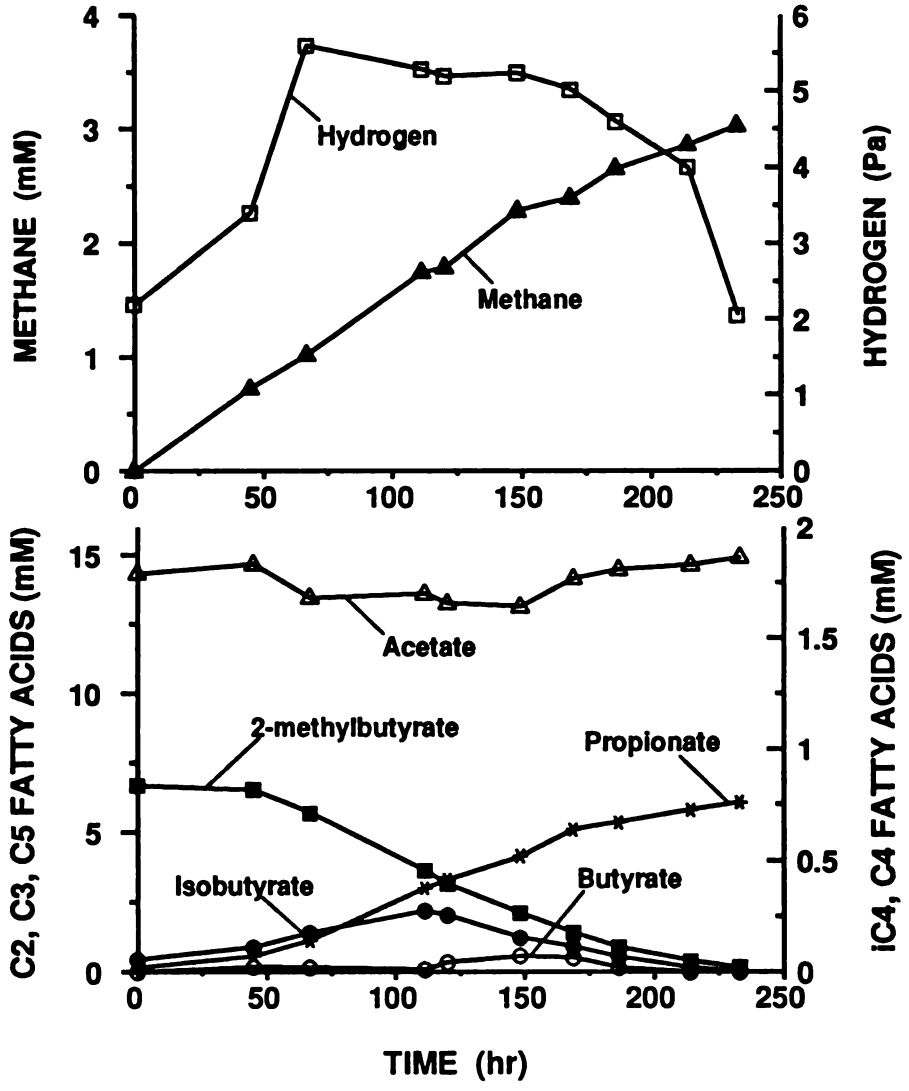


Figure 6.10 The growth of the triculture consisting of strain IB, *Mb. formicicum* strain T1N and *Ms. mazei* strain T18 on 2-methylbutyrate. Acetate, which was then converted to methane by *Ms. mazei* T18, propionate and methane were end products of 2-methylbutyrate degradation. Butyrate and isobutyrate were observed during the degradation.

The initial concentration of 2-methylbutyrate applied to the triculture was 6.7 mM (Figure 6.10). Propionate was produced as 2-methylbutyrate was converted at a molar ratio of 0.91 (propionate formed versus 2-methylbutyrate consumed). In addition to propionate, isobutyrate reached a maximum concentration of 0.27 mM after 116 hours. Butyrate, which was observed only after isobutyrate, reached a maximum level (0.06 mM) after 168 hours. Isobutyrate probably isomerized to butyrate rather than butyrate being produced directly. Both butyrate and isobutyrate were consumed prior to the complete consumption of 2-methylbutyrate. No valerate was detected during the assay. The maximum hydrogen partial pressure (5.3 Pa) detected was similar to that detected during valerate degradation. Formate was not detected in the triculture containing either valerate or 2-methylbutyrate during their degradation.

iv) Syntrophic butyrate degradation in the presence of propionate

In this experiment, butyrate and propionate were added into a well grown triculture to achieve starting concentrations of 6.0 mM and 6.1 mM, respectively. As is shown in Figure 6.11, the isomerization of butyrate to isobutyrate occurred immediately. A significant amount of 2-methylbutyrate was also produced. This appeared to be coupled with the disappearance of propionate. After 55 hours, the butyrate concentration decreased to a low level (0.1 mM). The highest concentration of 2-methylbutyrate accumulated, 0.91 mM, corresponded to a decrease in propionate from 6.1 mM to 5.2 mM. When 2-methylbutyrate was consumed, the concentration of propionate rose to its original concentration. The trend of the change in hydrogen partial pressure in the headspace was similar to other experiments, with the highest concentration being 9 Pa. The acetate concentration in the culture increased from initial 14.9 mM to 24.9 mM during the assay due to the slow conversion of acetate to methane.

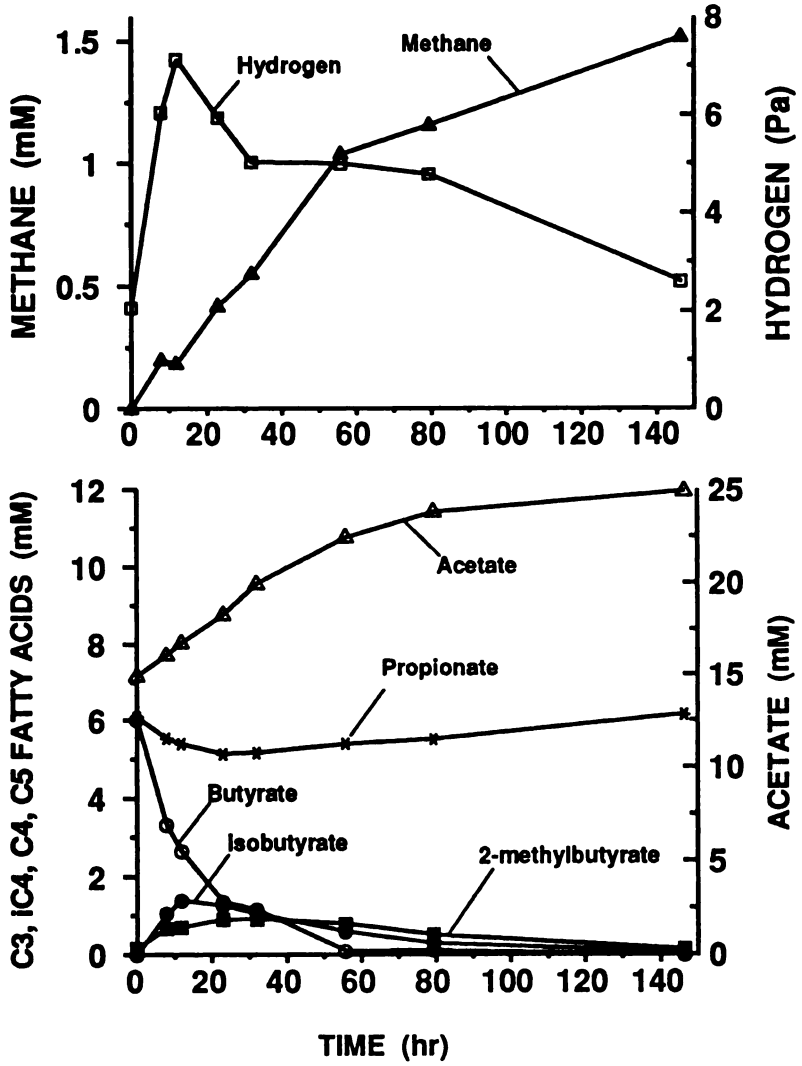


Figure 6.11 The growth of the triculture consisting of strain IB, *Mb. formicicum* strain T1N and *Ms. mazei* strain T18 on butyrate in the presence propionate. Acetate, which was then converted to methane by *Ms. mazei* T18, and methane were end products of butyrate degradation. Isobutyrate and 2-methylbutyrate were observed during the degradation.

To test if propionate degradation was carried out by strain IB and if 2-methylbutyrate could be formed in the absence of isobutyrate or butyrate, a control experiment was performed by inoculating the triculture in a medium containing 10 mM acetate and 10 mM propionate. After 3 months incubation, acetate was degraded and methane was produced. No propionate was degraded and no other fatty acids were formed.

6.4 CHARACTERIZATION OF THE MECHANISM OF ISOMERIZATION BETWEEN BUTYRATE AND ISOBUTYRATE

The perturbation of syntrophic fatty acid degradation with high concentrations of either formate or H_2 was used to determine if isomerization between butyrate and isobutyrate was coupled with butyrate or isobutyrate degradation. The triculture of strain IB, *Mb. formicicum* T1N and *Ms. mazei* T18 was utilized.

6.4.1 Perturbation with formate

Results of perturbation of isobutyrate degradation with formate is presented in Figure 6.12 and Table 6.5. Prior to the addition of formate, the hydrogen partial pressure was low (4 Pa) and no detectable level of formate (<5 μM) was observed in the triculture. After 4 hour of incubation, formate (15.7 mM) was added. The addition of formate immediately caused complete inhibition of both the isomerization and syntrophic oxidation reactions. The concentrations of isobutyrate and butyrate remained constant for about 12 hours until the formate concentration decreased to a low level (<0.43 mM). Methane was produced continuously at a higher rate than that prior to the addition of formate, indicating that formate was converted to methane.

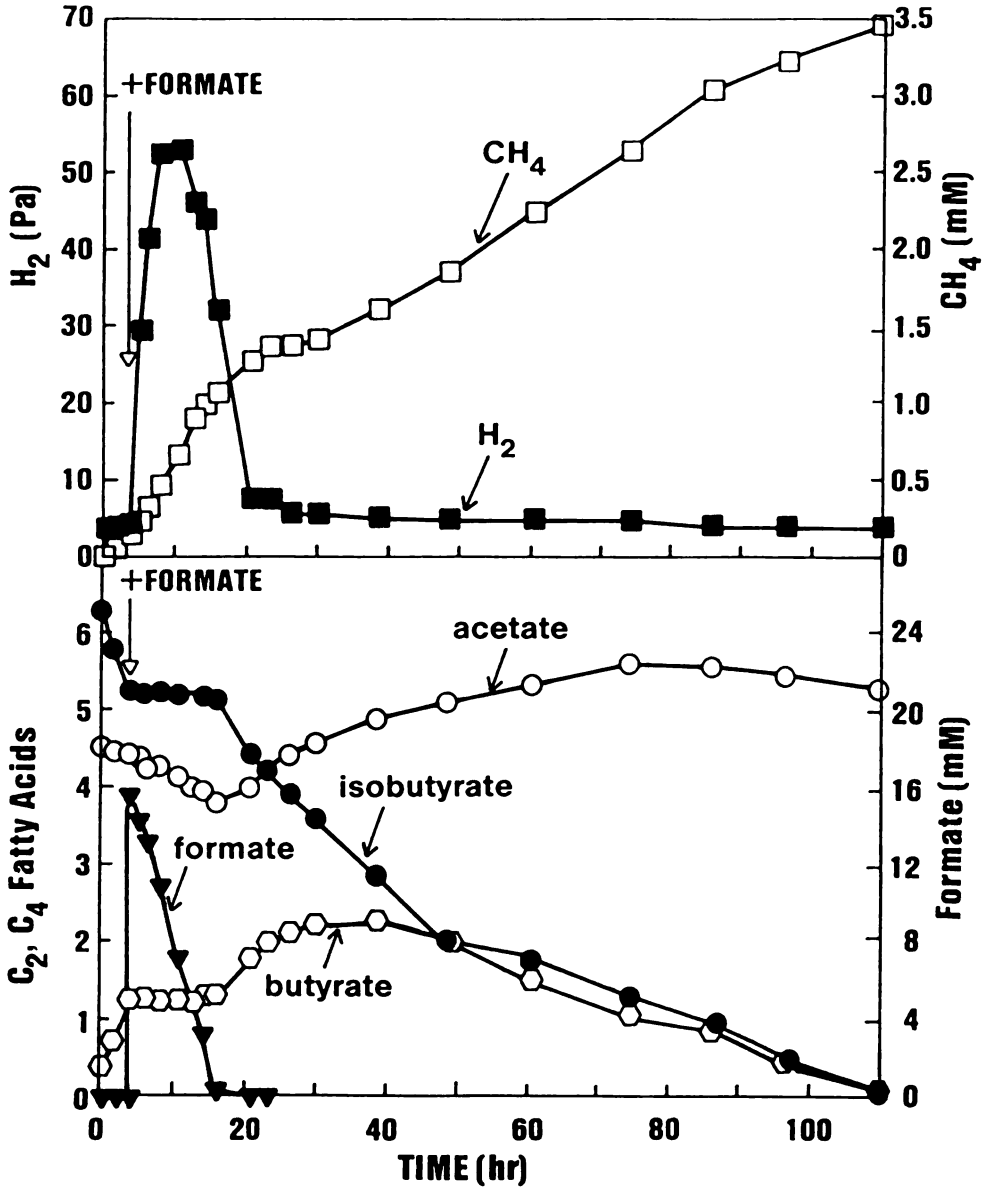


Figure 6.12 Perturbation of syntrophic isobutyrate degradation by the triculture consisting of strain IB, *Mb. formicicum* strain T1N and *Ms. mazei* strain T18 of with formate. The isomerization from isobutyrate to butyrate and syntrophic degradation was inhibited in the presence of formate with detectable level.

Table 6.5 Experimental results of isobutyrate conversion in the presence of the perturbation with formate

Time	Fatty acids (mM)				H_2	Free energy (kJ/reaction)
(hr)	C_1	C_2	i-C_4	C_4	(Pa)	Formate transfer
0	<0.005	4.54	6.31	0.40	3.6	<-31.6
1.63	<0.005	4.48	5.78	0.71	4.3	<-33.1
3.97	<0.005	4.45	5.24	1.25	4.3	<-34.3
3.97	15.7	4.45	5.24	1.25	4.3	+58.2
5.13	14.4	4.43	5.23	1.26	29.5	+57.3
6.35	13.25	4.26	5.21	1.28	41.6	+56.0
8.03	11.0	4.28	5.23	1.28	52.5	+54.1
10.63	7.25	4.14	5.21	1.25	52.8	+49.6
12.83	5.0	4.02	5.22	1.26	46.0	+45.4
14.12	3.36	3.97	5.19	1.29	44.0	+41.1
15.9	0.43	3.82	5.17	1.30	32.0	+19.5
20.8	<0.005	4.0	4.43	1.79	7.6	<-35.9
23.3	<0.005	4.22	4.21	1.99	7.6	<-35.5
26.4	<0.005	4.43	3.90	2.11	5.7	<-34.9
30.0	ND	4.57	3.59	2.22	5.5	NC
38.5	ND	4.88	2.87	2.26	5.1	NC
48.5	ND	5.10	1.99	2.01	4.9	NC
60.4	ND	5.34	1.76	1.51	4.7	NC
74.2	ND	5.61	1.26	1.10	4.5	NC
85.9	ND	5.57	0.95	0.87	4.1	NC
96.3	ND	5.46	0.51	0.43	4.0	NC
109.6	ND	5.29	0.09	0.08	3.0	NC

(1) Free energy calculation is described in Appendix E.
(2) HCO_3^- in the culture varied from 46 to 37 mM as hydrogen was converted. A concentration of 40 mM was chosen for free energy calculation.
(3) C_1: formate; C_2: acetate; i-C_4: isobutyrate; C_4: butyrate.
(4) ND: not determined. NC: not calculated.

As soon as formate was added, the hydrogen partial pressure in the headspace of the cultures increased from 4.3 Pa to 29.5 Pa in about one hour and finally peaked at 52.8 Pa before declining as formate was consumed. By the time formate reached the undetectable level (<5 μM), the H_2 partial pressure had decreased to 7.6 Pa. The rate of methane production decreased to approximately the rate observed before the addition of formate. Unlike isobutyrate and butyrate, the acetate concentration decreased from 4.45 mM to 3.82 mM during the perturbation with formate, indicating that the addition of formate did not stop acetate conversion to methane. When formate was consumed, isomerization of isobutyrate to butyrate resumed. The isobutyrate concentration decreased from 5.2 mM at hour 15.9 to 2.0 mM at hour 48.5. The butyrate level increased from 1.3 mM to 2.0 mM during the same period. These two fatty acids then decreased at approximately the same rate.

Butyrate degradation perturbed with formate addition produced similar results to those obtained during isobutyrate degradation (Figure 6.13 and Table 6.6). The addition of formate immediately induced complete inhibition of butyrate degradation and the isomerization reaction. The hydrogen partial pressure in headspace increased rapidly due to formate addition. The isomerization of butyrate to isobutyrate and butyrate oxidation resumed when the formate concentration decreased to a low level (<0.86 mM). Addition of formate did not inhibit acetate degradation.

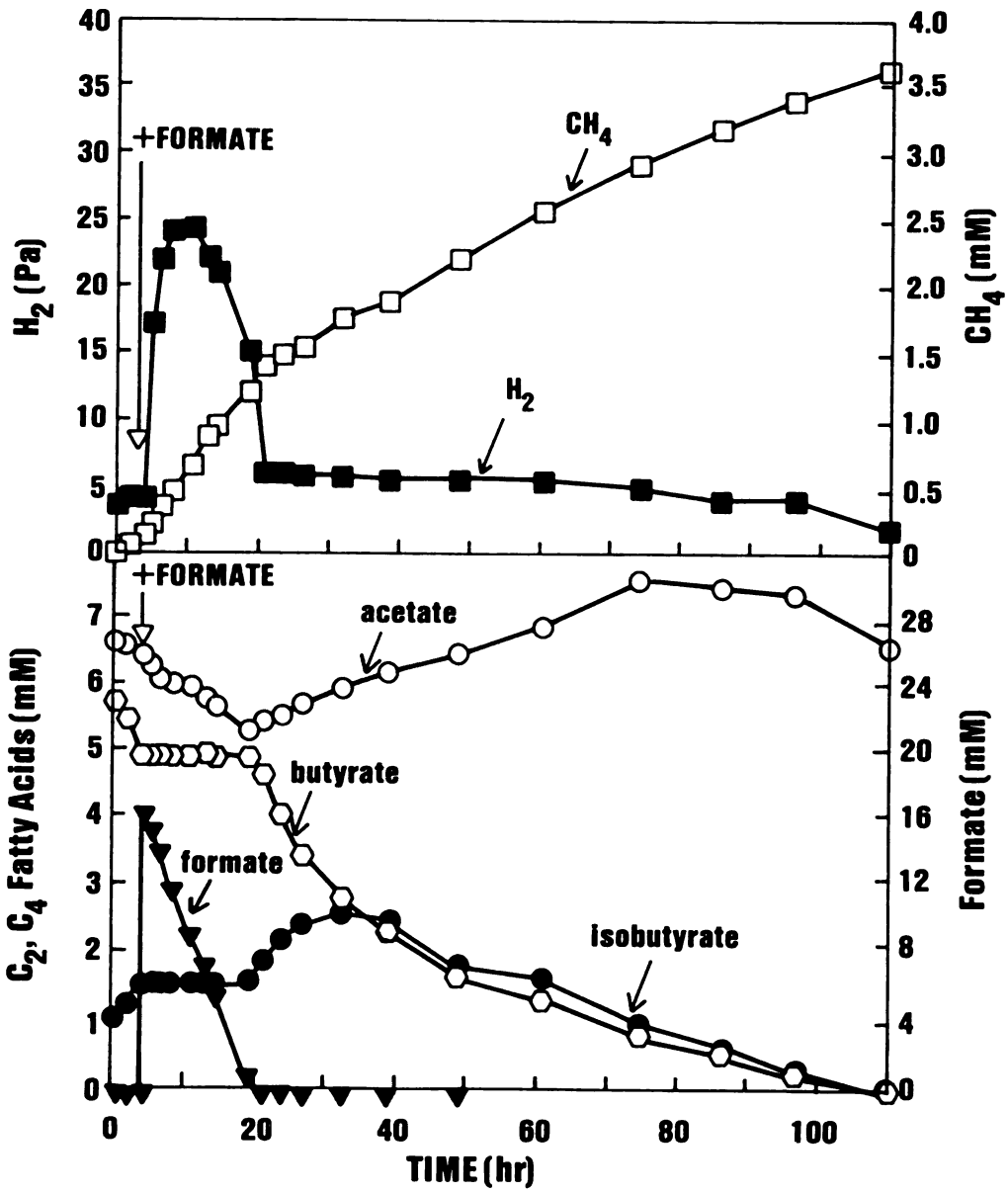


Figure 6.13 Perturbation of syntrophic butyrate degradation by the triculture consisting of strain IB, *Mb. formicicum* strain T1N and *Ms. mazei* strain T18 with formate. The isomerization from butyrate to isobutyrate and syntrophic degradation was inhibited in the presence of formate with detectable level.

Table 6.6 Experimental results of butyrate conversion in the presence of the perturbation with formate

Time	Fatty acids (mM)				H_2	Free energy (kJ/reaction)
(hr)	C_1	C_2	i-C_4	C_4	(Pa)	formate transfer
0	<0.005	6.67	1.06	5.82	3.6	<-30.0
1.63	<0.005	6.64	1.27	5.52	4.3	<-30.3
3.97	<0.005	6.48	1.61	4.98	4.3	<-30.9
3.97	16.6	6.48	1.61	4.98	4.3	+62.1
5.13	15.6	6.34	1.60	4.98	17.2	+61.4
6.35	14.2	6.13	1.61	4.99	21.9	+60.0
8.03	12.0	6.06	1.61	4.98	24.1	+58.1
10.63	9.3	6.01	1.61	4.98	24.4	+55.3
12.83	7.5	5.85	1.61	4.98	22.0	+52.9
14.12	5.8	5.72	1.60	4.99	21.0	+50.1
18.8	0.86	5.35	1.61	4.97	15.0	+29.6
20.8	0.006	5.50	1.95	4.72	6.0	-21.6
23.3	<0.005	5.60	2.26	4.12	6.0	<-32.8
26.4	<0.005	5.75	2.48	3.50	5.8	<-32.4
32.0	<0.002	6.00	2.67	2.87	5.7	<-31.6
38.5	<0.002	6.23	2.54	2.40	5.5	<-30.6
48.5	ND	6.50	1.86	1.73	5.3	NC
60.4	ND	6.92	1.68	1.40	5.1	NC
74.2	ND	7.61	1.01	0.85	4.9	NC
85.9	ND	7.51	0.69	0.58	4.0	NC
96.3	ND	7.41	0.31	0.26	3.8	NC
109.7	ND	6.65	0	0	1.8	NC

1) Free energy calculation is described in Appendix E.
2) HCO_3^- in the culture varied from 46 to 37 mM as hydrogen was converted. A concentration of 40 mM was chosen for free energy calculation.
3) C_1: formate; C_2: acetate; i-C_4: isobutyrate; C_4: butyrate.
4) ND: not determined. NC: not calculated.

6.4.2 Perturbation with hydrogen

Hydrogen gas was added to the triculture growing on isobutyrate (Figure 6.14 and Table 6.7). Before addition of H_2, the concentration of isobutyrate decreased from 5.23 to 3.97 mM while the butyrate concentration rose from 1.43 to 2.27 mM and acetate concentration increased from 10.78 to 11.40 mM, indicating that isobutyrate was continuously isomerized to butyrate and then degraded to CH_4 via acetate. The H_2 partial pressure was about 20 Pa and formate was below the detectable level. About 0.17 atm (17 kPa) H_2 was added to perturb isomerization and degradation of isobutyrate and butyrate. The addition of H_2 resulted in measurable quantities of formate accumulating. The formate concentration reached a peak level of 1.32 mM. Isomerization of isobutyrate to butyrate and subsequent degradation of butyrate were inhibited for at least 13 hours until the H_2 partial pressure in the headspace and the formate level in the liquid phase decreased to less than 2.72 kPa and 0.025 mM, respectively. Unlike the perturbation with formate, acetate degradation was inhibited during this assay. This may be because high partial pressure of H_2 inhibited acetate degradation by *Ms. mazei* species, as reported by Ferguson and Mah (1983).

The effect of addition of hydrogen on the isomerization of butyrate and its degradation by the triculture is presented in Figure 6.15 and Table 6.8. The addition of H_2 (ca. 0.4 atm or 41.3 kPa) resulted in the significant formation of formate. The highest formate level detected was 1.92 mM. The addition of hydrogen inhibited the isomerization and degradation of butyrate as well as acetate degradation. The isomerization and degradation of butyrate resumed after the H_2 partial pressure and the formate level decreased to less than 1.7 kPa and 0.01 mM, respectively.

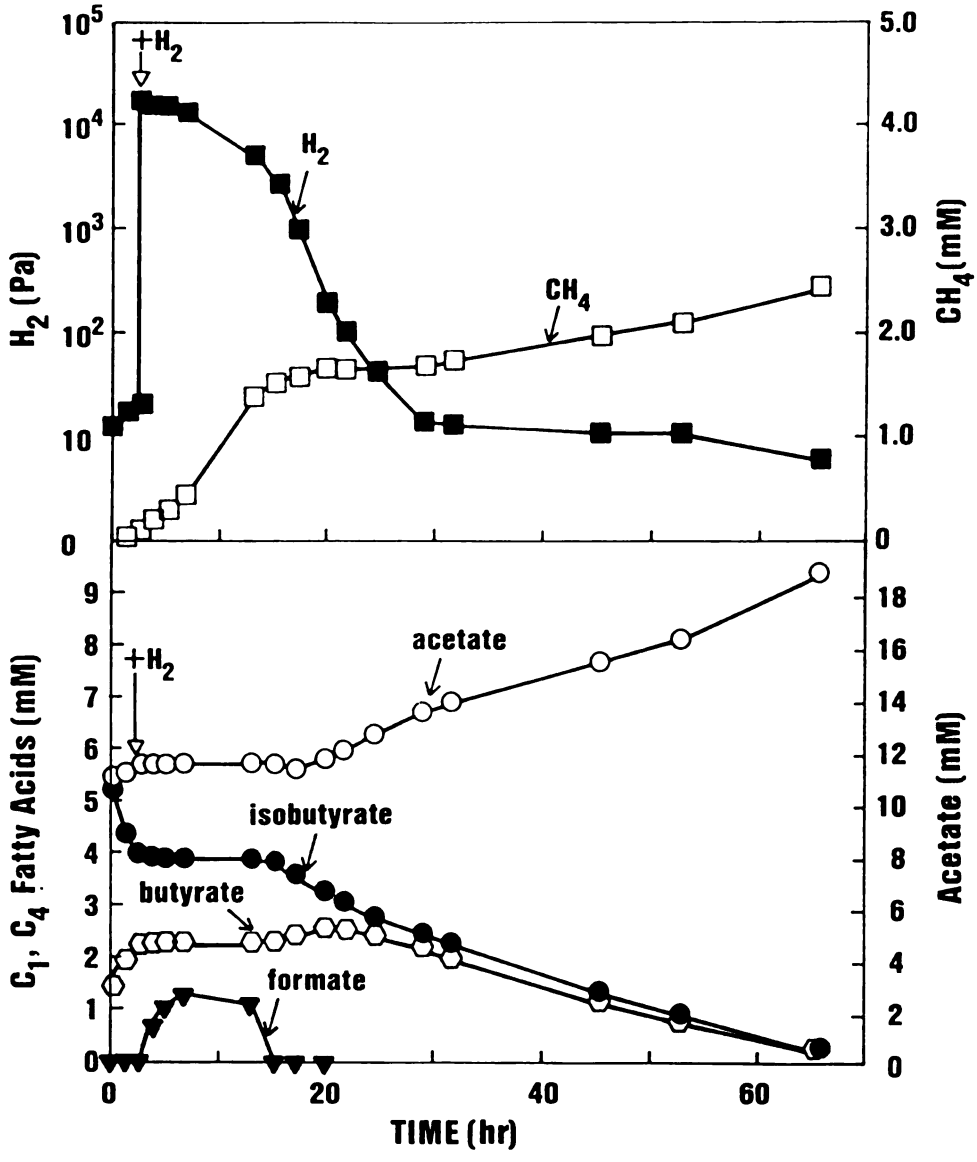


Figure 6.14 Perturbation of syntrophic isobutyrate degradation by the triculture consisting of strain IB, *Mb. formicicum* strain T1N and *Ms. mazei* strain T18 with hydrogen. The isomerization from isobutyrate to butyrate and syntrophic degradation was inhibited in the presence of higher partial pressure of hydrogen. Formate was synthesized during the perturbation with hydrogen.

Table 6.7 Experimental results of isobutyrate degradation in the presence of the perturbation with hydrogen gas

Time	Fatty acids (mM)				H_2	Free energy (kJ/reaction)
(hr)	C_1	C_2	i-C_4	C_4	(Pa)	formate transfer
0	<0.005	10.78	5.23	1.43	12.7	<-25.5
1.43	<0.005	11.07	4.35	1.99	17.1	<-25.6
2.5	<0.005	11.40	3.97	2.27	20.1	<-25.4
2.5	<0.005	11.40	3.97	2.27	17076	<-25.4
3.77	0.70	11.43	3.91	2.30	16200	+35.1
4.95	1.05	11.42	3.90	2.30	15100	+39.3
6.7	1.32	11.42	3.89	2.29	13130	+41.6
13.05	1.10	11.42	3.89	2.29	4968	+39.7
15.23	0.025	11.42	3.83	2.28	2720	+0.75
17.2	<0.005	11.22	3.58	2.39	952	<-25.4
19.92	<0.005	11.60	3.28	2.57	193	<-25.1
21.68	<0.005	11.92	3.07	2.55	102	<-24.6
24.5	<0.005	12,57	2.76	2.42	43	<-23.6
28.9	<0.005	13.43	2.45	2.18	14	<-22.4
31.6	ND	13.80	2.25	1.99	13	NC
45.3	ND	15.39	1.33	1.15	10	NC
52.7	ND	16.25	0.91	0.79	8	NC
65.6	ND	18.78	0.23	0.21	4	NC

(1) Free energy calculation is described in Appendix E.
(2) HCO_3^- in the culture varied from 46 to 37 mM as hydrogen was converted. A concentration of 40 mM was chosen for free energy calculation.
(3) C_1: formate; C_2: acetate; i-C_4: isobutyrate; C_4: butyrate.
(4) ND: not determined. NC: not calculated.

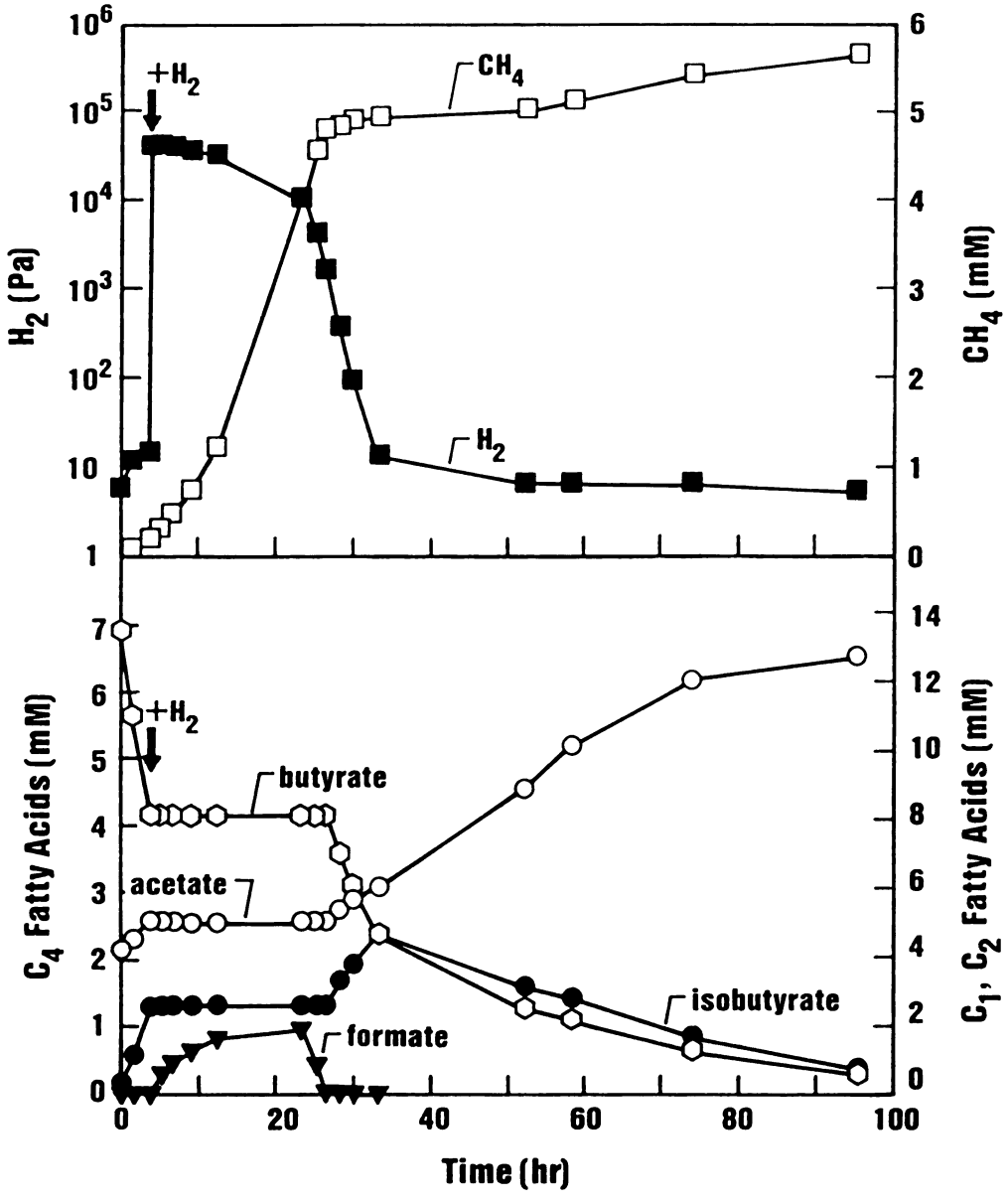


Figure 6.15 Perturbation of syntrophic butyrate degradation by the triculture consisting of strain IB, *Mb. formicicum* strain T1N and *Ms. mazei* strain T18 with hydrogen. The isomerization from butyrate to isobutyrate and syntrophic degradation was inhibited in the presence of higher partial pressure of hydrogen. Formate was synthesized during the perturbation with hydrogen.

Table 6.8 Experimental results of butyrate degradation in the presence of the perturbation with hydrogen gas

Time	Fatty acids (mM)				H_2	Free energy (kJ/reaction)
(hr)	C_1	C_2	i-C_4	C_4	(Pa)	formate transfer
0	<0.005	4.33	0.19	6.92	5.8	<-30.4
1.6	<0.005	4.65	0.58	5.64	12.2	<-32.1
3.7	<0.005	5.19	1.30	4.40	14.7	<-32.4
3.7	<0.005	5.19	1.30	4.40	41300	+32.4
5.2	0.60	5.14	1.33	4.17	40070	+26.5
6.5	0.94	5.14	1.32	4.17	38740	+31.1
8.9	1.25	5.14	1.33	4.16	36360	+34.0
12.2	1.67	5.14	1.33	4.17	32160	+36.8
23.2	1.92	5.14	1.33	4.17	10810	+38.3
25.2	0.90	5.13	1.32	4.17	4123	+30.5
26.4	0.01	5.14	1.33	4.17	1700	-16.0
28.2	<0.005	5.51	1.70	3.59	386	<-31.9
29.8	<0.005	5.80	1.93	3.08	97.2	<-31.3
33.3	<0.005	6.16	2.41	2.36	13.5	<-30.6
52.2	ND	9.11	1.60	1.30	6.8	NC
58.4	ND	10.43	1.43	1.11	5.5	NC
74.1	ND	12.40	0.86	0.64	4.8	NC
95.5	ND	13.08	0.34	0.30	4.5	NC

(1) Free energy calculation is described in Appendix E.
(2) HCO_3^- in the culture varied from 40 to 48 mM as hydrogen was converted. A concentration of 40 mM was chosen for free energy calculation.
(3) C_1: formate; C_2: acetate; i-C_4: isobutyrate; C_4: butyrate.
(4) ND: not determined. NC: not calculated.

6.4.3 Energetic analysis of butyrate and isobutyrate isomerization

During the experiments when butyrate degradation was perturbed by adding formate and hydrogen, the available free energy for conversion of butyrate to isobutyrate and vice versa was still thermodynamically favorable (Table 6.9), although close to equilibrium. Apparently, the isomerization reaction was completely inhibited by high concentrations of formate or H_2. The isomerization reaction occurred only when degradation of butyrate was observed. When butyrate degradation was inhibited, the isomerization reaction did not occur. This indicates that isomerization of butyrate to isobutyrate or isobutyrate to butyrate is coupled to butyrate degradation and is not an independent reaction.

Table 6.9 Free energy values for butyrate formation from isobutyrate and isobutyrate formation from butyrate when syntrophic butyrate or isobutyrate degradation was completely inhibited by high level of formate or H_2.

No.	Concentration (mM)		Driving force, kJ/reaction	
	iC_4	C_4	from iC_4 to C_4	from C_4 to iC_4
1	5.24	1.25	-1.5	
2	1.61	4.98		-4.7
3	3.90	2.30	-0.7	
4	1.33	4.17		-4.9

(1) Free energy calculation is described in Appendix E.
(2) iC_4: isobutyrate. C_4: butyrate.
(3) No.1: isobutyrate degradation inhibited by formate.
No.2: butyrate degradation inhibited by formate.
No.3: isobutyrate degradation inhibited by H_2.
No.4: butyrate degradation inhibited by H_2.

During the degradation of butyrate and isobutyrate by the defined triculture, isobutyrate was isomerized to butyrate until near equal concentrations were reached. Subsequently, both were converted to methane via acetate. The following thermodynamic analysis is used to explain how the ratio between isobutyrate and butyrate controls the direction of isomerization and why the two fatty acids tend to reach equal concentrations. In the following simulation, conditions were pH=7.0, the total concentration of an isobutyrate-butyrate mixture was 10 mM, the concentrations of formate, acetate and bicarbonate were assumed to be 5 mM, 2 μM and 50 mM, respectively, and formate was assumed to be the electron carrier during syntrophic butyrate oxidation.

i) When the butyrate concentration was much higher than isobutyrate, butyrate was simultaneously isomerized to isobutyratye and degraded to acetate and formate (or H_2), and isobutyrate accumulation occurred:

$$p \text{ butyrate}^- \rightarrow p \text{ isobutyrate}^- \qquad (6\text{-}5)$$

and

$$\text{butyrate}^- + 2\ HCO_3^- \rightarrow 2\ \text{acetate}^- + H^+ + 2\ \text{formate}^- \qquad (6\text{-}6)$$

The sum of these two reactions is:

$$(1+p)\ \text{butyrate} + 2\ HCO_3^- \rightarrow 2\ \text{acetate}^- + H^+ + 2\ \text{formate}^- + p\ \text{isobutyrate}^- \qquad (6\text{-}7)$$

where p is a stoichiometric coefficient for butyrate that is isomerized to isobutyrate when one molecule of butyrate is degradaded to acetate and formate (or H_2). The value of p is ≥ 0 because when butyrate is not isomerized to isobutyrate, p is equal to zero. The change in free energy available for reactions (6-7) vs. the change of percentage of butyrate in the butyrate-isobutyrate mixture, using various vales of p, is presented in Figure 6-16a. More free energy was available when the value of p was

larger than zero at higher butyrate percentages. This is consistent with the observation that rapid isobutyrtate formation occurred when the butyrate concentation was much higher than isobutyrate (Figure 6.5, 6.13 and 6.15). As the percentage of butyrate was reduced (as result of isobutyrate accumulation) to less 50%, the most energetically favorable condition was observed when p approached to zero. Based on the data of experiment No.2 in Table 6.4, when the initial concentrations of isobutyrate and butyrate were 1.6 and 9.6 mM, respectively, the apparent conversion rates of butyrate to isobutyrate and butyrate to acetate and formate (or H_2) were 0.20 and 0.40 mM/hr, respectively. The p value here, therefore, can be estimated to be larger than 0.5.

ii) When the isobutyrate concentration was much higher than butyrate, isobutyrate was isomerized to butyrate, butyrate was then degraded to acetate and formate (or H_2), and butyrate accumulation occurred:

$$q \text{ isobutyrate}^- \rightarrow q \text{ butyrate}^- \qquad (6\text{-}8)$$

and

$$\text{butyrate}^- + 2\ HCO_3^- \rightarrow 2 \text{ acetate}^- + H^+ + 2 \text{ formate}^- \qquad (6\text{-}9)$$

The sum of reactions (6-8) and (6-9) is

$$q \text{ isobutyrate}^- + 2\ HCO_3^- \rightarrow 2 \text{ acetate}^- + H^+ + 2 \text{ formate}^- + (q\text{-}1) \text{ butyrate}^- \qquad (6\text{-}10)$$

where q is a stoichiometric coefficient for isobutyrate that is isomerized to butyrate when one molecule of butyrate is degraded. The value of q is ≥ 1 because when butyrate does not accumulate, q is equal to 1. The change in free energy available for reaction (6-10) vs the change of percentage of butyrate in the butyrate-isobutyrate mixture, using various vales of q, is presented in Figure 6-16b. Higher driving force can be obtained with value of q larger than 1 at lower butyrate percentage (more isobutyrate). This is consistent with the observation that fast butyrate accumulation

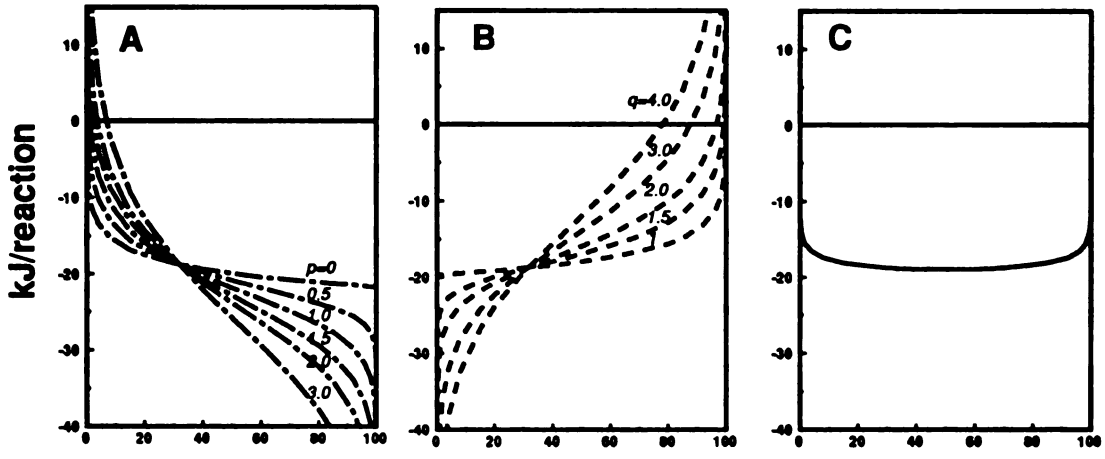
occurred when isobutyrate concentration was much higher than butyrate (Figures 6.6, 6.8, 6.12 and 6.14). As the percentage of butyrate increased (as result of butyrate accumulation) to more than 30%, the most energetically favorable condition was observed when q approached to zero. Based on the data of experiment No.1 in Table 6.4, when the initial concentrations of isobutyrate and butyrate were 10.5 and 0 mM, respectively, the apparent conversion rates of isobutyrate to butyrate and butyrate to acetate and formate (or H_2) were 0.39 and 0.09 mM/hr, respectively. The q value here, therefore, can be estimated to be larger than 4.3.

iii) Butyrate and isobutyrate were at near equal concentrations and were degraded to acetate and formate (or H_2) simultaneously. Based on above analysis, when the ratio between butyrate and isobutyrate was near equal, the most energetically favorable conditions are at a range where p value in equation (6-7) approached to zero and q value in equation (6-10) was near 1. Both reactions (6-7) and (6-10) could be performed at p=0 and q=1 and became:

$$1/2\ \text{butyrate}^- + 1/2\ \text{isobutyrate} + 2\ HCO_3^- \rightarrow 2\ \text{acetate}^- + H^+ + 2\ \text{formate}^- \qquad (6\text{-}11)$$

Calculation of free energy available for reaction (6-11) indicates that the most thermodynamically favorable condition is obtained at equal concentrations of isobutyrate and butyrate (Figure 6-16c). Experimental results are consistent with this analysis (Figures 6.5, 6.7, 6.8, 6.12 to 6.15).

The same simulation can also be obtained by changing the concentrations of acetate, isobutyrate-butyrate mixture and formate, or assuming H_2 as electron carrier within normal ranges found in anaerobic digesters.



A: (1+p) butyrate$^-$ + 2 HCO_3^- → 2 acetate$^-$ + H^+ + 2 formate$^-$ + p isobutyrate$^-$
($p \geq 0$)

B: q isobutyrate$^-$ + 2 HCO_3^- → 2 acetate$^-$ + H^+ + 2 formate$^-$ + (q-1) butyrate$^-$
($q \geq 1$)

C: 1/2 butyrate$^-$ + 1/2 isobutyrate + 2 HCO_3^- → 2 acetate$^-$ + H^+ + 2 formate$^-$

Figure 6.16 Free energy change of isomerization coupled with butyrate oxidation vs. the percentage of butyrate in a butyrate-isobutyrate mixture, based on interspecies formate transfer. Simulation was conducted at pH 7.0, acetate 5 mM, formate 2 μM, bicarbonate 50 mM and isobutyrate plus butyrate 10 mM.

6.5 CHARACTERIZATION OF HYDROGEN-FORMATE METABOLISM

In a previous study, formate was observed during methanogenesis from H_2-CO_2 by the granules developed on a brewery wastewater (Chapter 4), and also observed during the perturbation of fatty acid degradation by hydrogen (Figure 6.14 and 6.15). Methanogenesis from H_2-CO_2 was examined using the R-granules and several pure methanogenic cultures to identify if the formation of formate was also performed by other anaerobic granules and to identify the organisms responsible for formate synthesis.

6.5.1 Formate synthesis by the R-granules

Formate formation during methanogenesis from H_2-CO_2 by the R-granules (35 ℃) is presented in Figure 6.17. The initial experimental conditions were: pH 7.05, hydrogen partial pressure 0.95 atm, and HCO_3^- 50 mM. Inoculated granules were 0.8 gVSS/L. Formate (0.042 mM) was observed after ca. 5 minutes of inoculation and was synthesized rapidly to reach a maximum level of 3.5 mM within 6 hours. Formate then gradually declined as H_2 was consumed. During the initial one hour, the rate for methane production and net formate synthesis (assuming that formate was not concurrently degraded) were 50 mmole/hr and 101 mmole/hr, respectively. This indicates that a minimum of one third of total hydrogen consumed was used to synthesize formate. Formate formation from hydrogen was a common characteristic of various granules. It was also produced by the F-granules and brewery granules (Chapter 4).

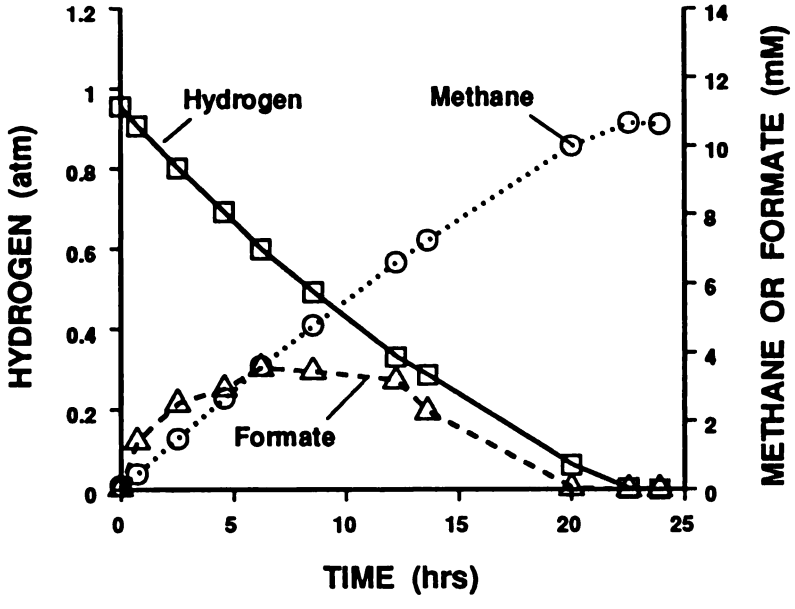


Figure 6.17 Formate formation during methanogenesis from H_2-CO_2 by the R-granules.

6.5.2 Formate synthesis by pure methanogen cultures

Five strains of methanogens were examined to determine if they could synthesize formate from H_2 plus HCO_3^- or CO_2. Four of these strains, *Mb. formicicum* strain MF (type strain), *Mb. formicicum* strain T1N, *Mb. formicicum* strain RF and *Msp. hungatei* strain BD can use both H_2-CO_2 and formate to produce methane. One strain, *Ms. mazei* T18 can use H_2-CO_2 and acetate but does not use formate to produce methane.

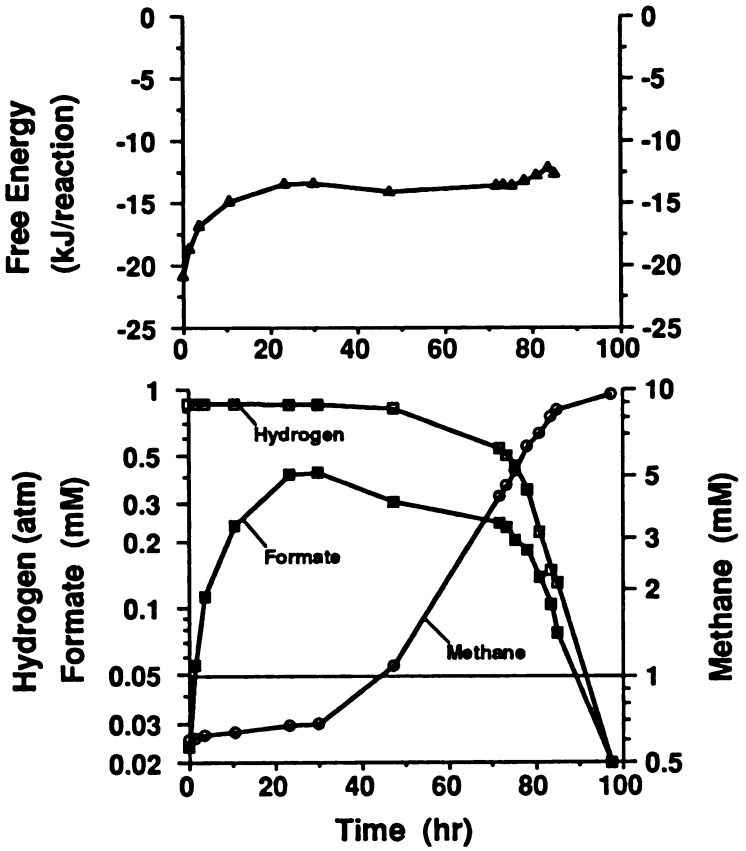


Figure 6.18 Formate formation during the growth of *Mb. formicicum* strain MF on H_2-CO_2. Free energy calculation is described in Appendix E.

i) *Methanobacterium formicicum* sp.

Formate was observed during the growth of three strains of *Mb. formicicum* tested. Formate synthesis by *Mb. formicicum* strain MF grown on H_2-CO_2 and the changes in free energy ($\Delta G'$) for formate synthesis from H_2 plus bicarbonate at 37 °C is presented in Figure 6.18. Initial conditions were pH of 6.90, H_2 of 0.86 atm., and 50 mM HCO_3^-. After inoculation (2%, vol/vol), formate (0.024 mM) was observed within a few minutes. The formate level increased quickly during the initial 24 hours,

reaching a maximum concentration of 0.4 mM. The available free energy for formate synthesis changed from H_2 plus HCO_3^- from -21 to -14 kJ/reaction. As hydrogen was further consumed, the formate concentration gradually declined from 0.40 mM to 0.18 mM. Finally, formate was consumed to an undetectable level (<5 μM) as the H_2 partial pressure decreased to below 10^{-3} atm. During this period, a near steady-state value for ΔG' of approximately -14 kJ/reaction was observed.

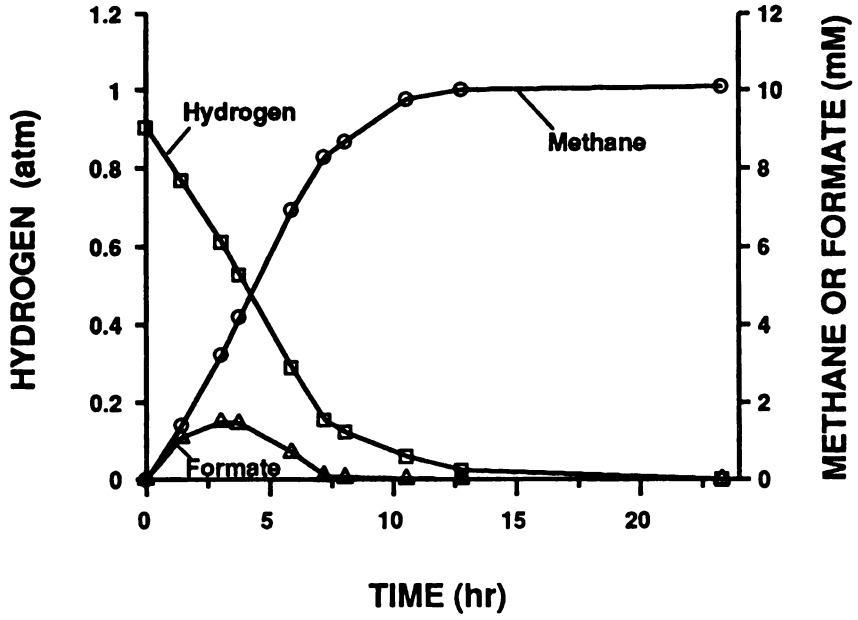


Figure 6.19 Formate formation during the growth of *Mb. formicicum* strain T1N on H_2-CO_2.

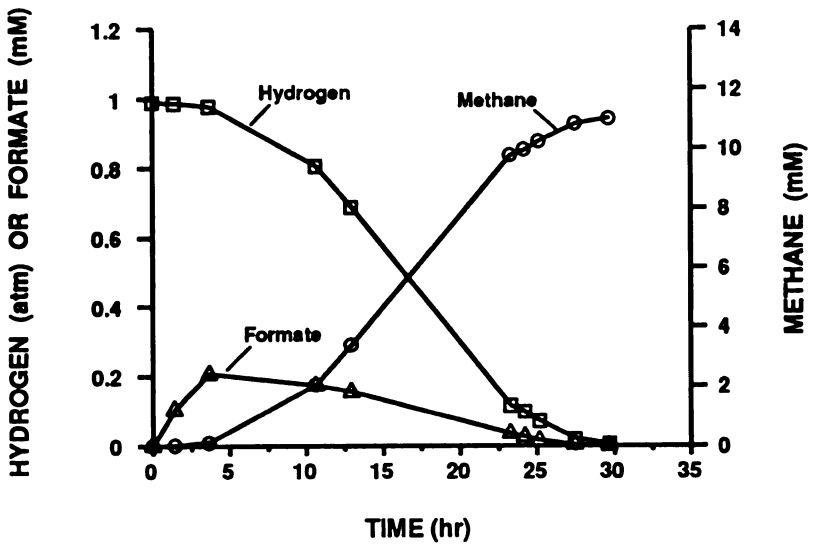


Figure 6.20 Formate formation during the growth of *Mb. formicicum* strain RF on H_2-CO_2.

Similar patterns of formate synthesis were observed during the growth of *Mb. formicicum* strain T1N (Figure 6.19) and *Mb. formicicum* strain RF (Figure 6.20) on H_2-CO_2. Energetic calculations indicated that the free energy values for the synthesis of formate from H_2 plus HCO_3^- were favorable. These results indicate that synthesis of formate is a common physiological characteristics of *Mb. formicicum* species.

ii) *Methanospirillum hungatei* strain BD

Formate was synthesized by *Msp. hungatei* strain BD grown on H_2-CO_2, as illustrated in Figure 6.21. The initial growth conditions were pH 7.04, H_2 partial pressure 1.36 atm, and 60 mM HCO_3^-. The culture was incubated in the shaking water bath (37 °C). A trace amount of formate was observed within a half hour after

inoculation. The level of formate continuously increased to 1.5 mM until the hydrogen partial pressure had decreased to 0.17 atm. The formate concentration then concurrently decreased as H_2 was consumed. The free energy available for formate synthesis remained favorable during the entire assay.

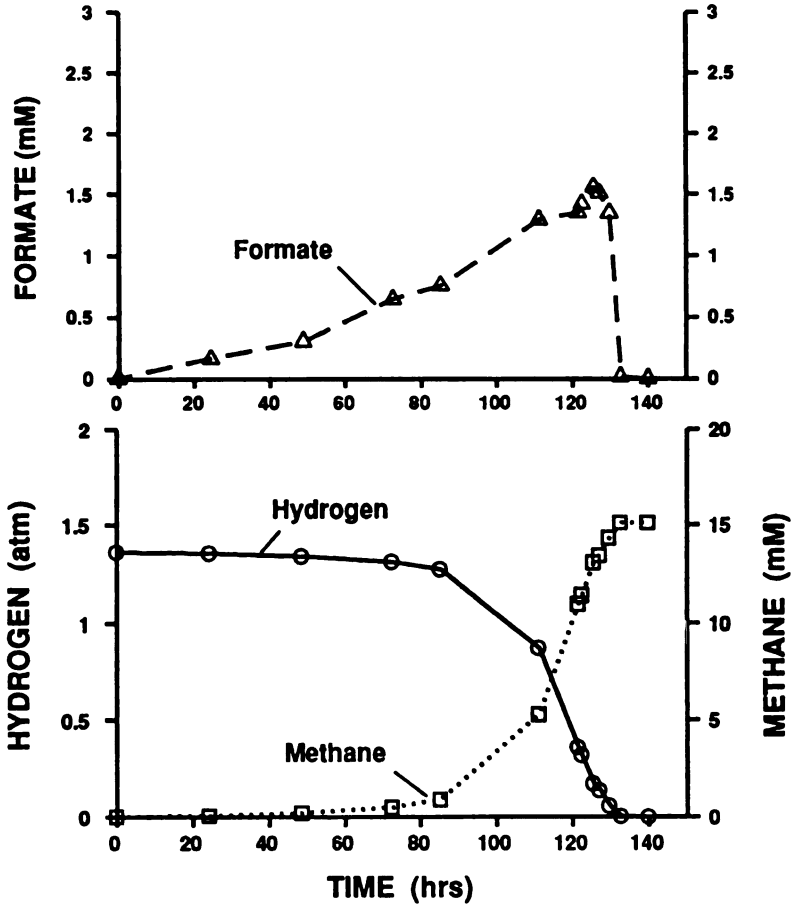


Figure 6.21 Formate formation during the growth of *Msp. hungatei* strain BD on H_2-CO_2.

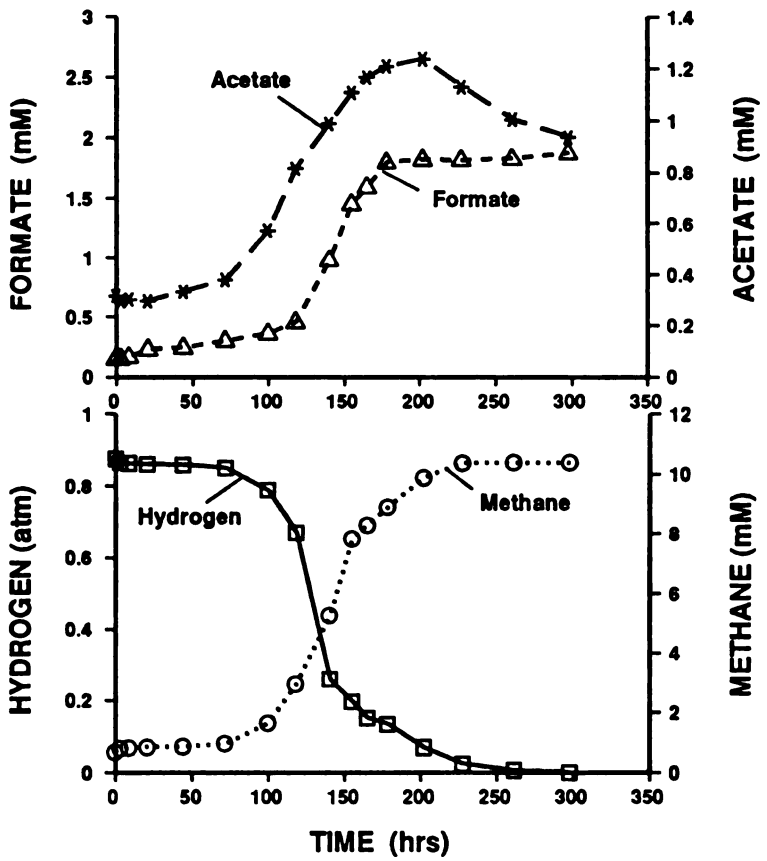


Figure 6.22 Formate and acetate formation during the growth of *Ms. mazei* strain T18 on H_2-CO_2. Formate was synthesized but was not consumed.

iii) *Methanosarcina mazei* strain T18

Formate was also synthesized by *Ms. mazei* strain T18 when grown on H_2-CO_2 (Figure 6.22). The initial growth conditions were pH 6.8, 0.88 atm H_2 and 50 mM HCO_3^-. A low initial level of formate (0.15 mM) was present as a consequence of inoculation. The formate level continuously increased until hydrogen was consumed.

Unlike the situation during the growth of *Mb. formicicum* and *Msp. hungatei*, the formate synthesized was not subsequently consumed. Acetate formation was also observed during the growth. The acetate concentration increased from an initial level of 0.32 mM to a peak level (1.24 mM) and then decreased to a final concentration of 0.95 mM.

6.6 DISCUSSION

6.6.1 The role of different species in VFA degradation

Four syntrophic acetogens and five methanogens were isolated from the R-granules. The characterization of substrate utilization for these bacteria reveals how these organisms work together in the granule ecosystem to completely mineralize acetate, propionate and butyrate in the reactor feed to methane and CO_2. Together with *Mb. formicicum* strain T1N, *Mb. formicicum* strain RF, and *Msp. hungatei* strain BD, propionate is syntrophically degraded to acetate and methane by strains PW and PT. Likewise, butyrate is syntrophically degraded to acetate and methane by strains BH and IB, respectively in conjunction with H_2 and formate-utilizing methanogens that work as syntrophic partners to maintain thermodynamically favorable conditions for VFA degradation.

The formation of isobutyrate, 2-methylbutyrate and valerate was observed during the degradation of acetate, propionate and butyrate by the R-granules. Within the R-granules, isobutyrate was isomerized from butyrate by strain IB and 2-methylbutyrate was synthesized from propionate during syntrophic butyrate degradation by strain IB. Valerate was synthesized from propionate during syntrophic butyrate degradation by

strain BH. The isobutyrate formed is also degraded by strain IB, while 2-methylbutyrate and valerate were degraded by strains IB and BH.

6.6.2 Isomerization in complex anaerobic ecosystems

Results from Chapter 4 demonstrated that isobutyrate was formed from butyrate and subsequently isomerized back to butyrate and degraded by the granules developed on a brewery wastewater. The formation of isobutyrate from butyrate and vice versa were also observed in the R- and F-granules (Chapter 3). These facts confirmed the results of Tholozan *et al.* (1988a) with digested sludge (i.e. the isomerization between butyrate and isobutyrate was reversible). In the R-granules, the metabolic function was performed by an isobutyrate-butyrate degrader, strain IB. The remarkable characteristic of syntrophic degradation of isobutyrate or butyrate by strain IB (and, probably, other syntrophic isobutyrate-butyrate degraders) was that equal concentrations of isobutyrate and butyrate were eventually achieved and then both the fatty acids disappeared at the same rates. This was not observed during the isobutyrate or butyrate degradation in anaerobic digested sludges or anaerobic granules. In the latter case, butyrate always disappeared at a much higher rates than isobutyrate (e.g. Figures 4.5, 6.1 and 6.2) because other butyrate degraders, such as *Syntrophospora* species (Stieb and Shinck, 1985; Zhao *et al.*, 1990) or/and *Syntrophomonas* species (McInerney *et al.*, 1981) that use butyrate but do not utilize isobutyrate, are present.

The physiological reason for isomerization of butyrate to isobutyrate is not clear since isobutyrate formed has to be re-isomerized to butyrate for further degradation. One possible explanation may be that the reversible function of an isomerase system allows the bacteria to "store" butyrate in a form that only a limited number of organisms can utilize. This is a type of extracellular storage mechanism rather than

storage depot for carbon and energy as inclusion within cells such as PHB which was observed inside the cells of *Syntrophomonas* sp. (Dubourguier *et al.*, 1988a).

6.6.3 Pathways of syntrophic degradation of 2-methylbutyrate and valerate

Valerate and 2-methylbutyrate are by-products of butyrate degradation that were observed formed only in the presence of propionate. These compounds can then be degraded by strain BH and strain IB.

i) By strain BH

The pathway of syntrophic valerate degradation by coculture of strain BH and *Mb. formicicum* strain T1N appeared to be β-oxidation since propionate and acetate were observed during the degradation and the molar ratio among these acids produced and methane formed was also consistent with that expected from a β-oxidation reaction (Table 1.3 in Chapter 1). The end products (methane, acetate and propionate) of 2-methylbutyrate degradation were basically the same as those of valerate degradation. The pathway of 2-methylbutyrate degradation by strain BH was not clear since propionate can not be produced directly from β-oxidation of 2-methylbutyrate and no other fatty acids were observed.

ii) By strain IB

Syntrophic degradation of 2-methylbutyrate and valerate by strain IB appeared more complicated than degradation of acids with four carbon atoms. Although these two fatty acids have a similar molecular structure as in butyrate and isobutyrate, the presence of a reversible isomerization between 2-methylbutyrate and valerate was not observed. Isomerization from 2-methylbutyrate to valerate could not detected during 2-methylbutyrate conversion (Figure 6.9). However, isomerization of valerate to 2-methylbutyrate did indeed occur. A trace amount of 2-methylbutyrate was

repeatedly detected during syntrophic degradation of valerate (Figure 6.10).

During degradation of 2-methylbutyrate, propionate was formed at a molar ratio of 0.91 (propionate produced per 2-methylbutyrate consumed). However, isobutyrate and butyrate also were formed and then degraded. This shows that conversion of 2-methylbutyrate is more complicated than a simple β-oxidation reaction.

The results of butyrate conversion in the presence of propionate provided useful information to examine the syntrophic conversion of 2-methylbutyrate. In addition to isobutyrate, 2-methylbutyrate was also formed during butyrate degradation in the presence of propionate (Figure 6.11). It appeared that 2-methylbutyrate may had been synthesized from propionate and isobutyrate, because (i) the increase in the 2-methylbutyrate concentration was directly proportional to the decrease in propionate concentration, and the propionate concentration was restored to its original level when the 2-methylbutyrate formed was subsequently consumed; (ii) no 2-methylbutyrate was formed in the presence of acetate plus propionate without isobutyrate or butyrate present; and (iii) isobutyrate was detected prior to the detection of butyrate during 2-methylbutyrate conversion. Presumably, the formation of 2-methylbutyrate may involve the exchange of the methyl-group in isobutyrate with ethyl-group in propionate:

$$\underset{\text{(propionate}^-\text{)}}{CH_3CH_2COO^-} + \underset{\text{(isobutyrate}^-\text{)}}{CH_3\overset{\overset{CH_3}{|}}{C}HCOO^-} \rightleftharpoons \underset{\text{(2-methylbutyrate}^-\text{)}}{CH_3CH_2\overset{\overset{CH_3}{|}}{C}HCOO^-} + \underset{\text{(acetate}^-\text{)}}{CH_3COO^-}$$

$\Delta G^{\circ\prime} = -0.03$ kJ/reaction (6-12)

In this proposed reaction, 2-methylbutyrate and acetate are produced in the presence of higher concentrations of propionate and isobutyrate and vice versa since the reaction

appears to be reversible. This properly explains the syntrophic degradation of 2-methylbutyrate: initially, a molecule of 2-methylbutyrate exchanges its ethyl-group for the methyl-group of a molecule of acetate to form a molecule of isobutyrate and a molecule of propionate; and then isobutyrate is isomerized to butyrate. Eventually, the butyrate is converted to acetate and formate or H_2 via β-oxidation. Thus, during the syntrophic conversion of 2-methylbutyrate in the presence of acetate, the sequence of detectable fatty acids other than 2-methylbutyrate and acetate are expected to be propionate and isobutyrate followed by butyrate. These assumptions have been supported by the experimental observations (Figure 6.10).

On the other hand, the isomerization of 2-methylbutyrate to valerate and then degradation via β-oxidation was not supported by the experiments performed.

When the triculture was grown on valerate, propionate was produced at a molar ratio of 0.92 (propionate produced/valerate consumed). The conversion of valerate to methane and propionate probably followed β-oxidation as has been shown for other syntrophic butyrate-degrading cultures (McInerney *et al.*, 1981; Stieb and Schink, 1985). However, the formation of isobutyrate, butyrate and 2-methylbutyrate during valerate conversion (Figure 6.9) indicates that other pathways also exist. One possible pathway may be degradation of valerate through 2-methylbutyrate since 2-methylbutyrate was detected during the conversion of valerate. Another possible pathway may be entail exchange of the ethyl-group of valerate with the methyl-group in acetate to form butyrate and propionate:

$$\underset{\text{(valerate}^-\text{)}}{CH_3CH_2CH_2CH_2COO^-} + \underset{\text{(acetate}^-\text{)}}{CH_3COO^-} \rightleftharpoons \underset{\text{(propionate}^-\text{)}}{CH_3CH_2COO^-} + \underset{\text{(butyrate}^-\text{)}}{CH_3CH_2CH_2COO^-}$$

$$\Delta G^{\circ\prime} = +0.04 \text{ kJ/reaction} \qquad (6\text{-}13)$$

Butyrate formed in the above manner could be converted via further β-oxidation. At the same time, butyrate is also concurrently isomerized to isobutyrate since a lower concentration of isobutyrate (compared with butyrate) was detected in the experiment. The latter two pathways may not be the major metabolic reactions for valerate conversion, however, since the levels of butyrate, isobutyrate and 2-methylbutyrate observed were low. More work is needed to define the actual pathways and the significance of the isomerization reaction(s).

6.6.4 Formate synthesis from H_2 plus HCO_3^- or CO_2

All H_2-CO_2 and formate utilizing methanogens (*Mb. formicicum* and *Msp. hungatei*) in the R-granules were able to synthesize formate from H_2 plus HCO_3^- (or CO_2) and eventually catabolize it to methane. The mixed function methanogen (*Ms. mazei*), can synthesize formate from H_2 plus HCO_3^- (or CO_2) but cannot catabolize it. The synthesis of formate by *Mb. formicicum* is likely performed by the formate hydrogenlyase system consisting of hydrogenase and formate dehydrogenase which can perform a reversible conversion between H_2 plus HCO_3^- and formate (Baron and Ferry, 1989). The same enzyme system probably exists in *Msp. hungatei.* Formate hydrogenlyase, which was involved in formate synthesis from H_2-CO_2, was also found in *Methanosarcina barkeri* (Nagai and Nishio, 1989). A similar enzyme may exist in *Ms. mazei.*

Whether syntrophic acetogens are also able to synthesize formate from H_2 plus HCO_3^- (or CO_2) is not clear. Hydrogen production from formate has been found in a monoculture of *Syntrophomonas wolfei* (Boone *et al.*, 1989). This suggests that formate synthesis by this syntrophic acetogen may also be possible. Further research should be performed to identify whether syntrophic propionate and butyrate degraders

can synthesize formate from H_2 plus HCO_3^- (or CO_2) and vice versa.

6.6.5 Interspecies electron transfer

In Chapter 4, both H_2 and formate occurred as intermediate products during syntrophic ethanol degradation. The level of H_2 and formate was equilibrium for synthesis of formate from H_2 plus HCO_3^-. If both H_2 and formate are also intermediate products during syntrophic butyrate degradation at near equalibrium concentrations, the formate concentration will be too low to detect (<5 μM). The role of formate and H_2 serve as electron carriers, therefore, can be evaluated only indirectly.

The perturbation of the triculture consisting of strain IB, *Mb. formicicum* strain T1N and *Ms. mazei* strain T18 with formate demonstrated that addition of formate completely inhibited butyrate and isobutyrate oxidation. The hydrogen partial pressure accumulated concurrently with formate addition. Free energy calculations, based on interspecies formate transfer indicated that during the perturbation with formate, extremely unfavorable energetics occurred. The syntrophic butyrate degradation restarted only when formate and H_2 concentrations became very low and the free energy values became thermodynamically favorable (Table 6.5 to 6.6). Perturbation with H_2 also caused complete inhibition of syntrophic butyrate oxidation. Significant amounts of formate accumulated in the triculture and induced unfavorable energetics for syntrophic butyrate degradation. Syntrophic butyrate degradation also restarted when H_2 and formate concentrations became very low (Table 6.7 to 6.8). These results indicated that both formate and H_2 were an intermediate during syntrophic butyrate degradation.

High level of hydrogen caused complete inhibition of syntrophic butyrate degradation during hydrogen perturbation (Table 6.8 and 6.9). During formate

perturbation, only a limited level of hydrogen accumulated. Because of mass transfer resistance of hydrogen between gas and liquid phases, the dissolved hydrogen concentrations could not be accurately estimated from the hydrogen partial pressures measured. As a result, the actual $\Delta G'$ values for interspecies hydrogen transfer are difficult to calculate from the data of hydrogen partial pressures in Tables 6-6 to 6-9, especially when the hydrogen concentration was low. Based on the observation of the reversible conversion between hydrogen and formate in granules and pure methanogenic cultures, however, both formate and hydrogen are possible intermediates during syntrophic isobutyrate-butyrate degradation by this triculture.

In anaerobic digesters, both formate and hydrogen can be considered as intermediates during syntrophic acetogenesis. Anaerobic digesters are bicarbonate-rich ecosystems (usually, with alkalinities ranging from 800 to 3000 mg/L as $CaCO_3$). As described above, H_2-CO_2 and formate-utilizing methanogens such as *Mb. formicicum* and *Msp. hungatei* (which are prevalent species in most anaerobic digesters) can perform reversible conversion between H_2 plus HCO_3^- and formate. It is also possible that other organisms in the ecosystems such as syntrophic acetogens can perform this reversible reaction. Therefore, both hydrogen and formate are likely converted from one to another continuously. It is virtually impossible, therefore, to identify which of these serves as the predominant electron carrier during syntrophic acetogenesis.

6.7 SUMMARY

1) Syntrophic propionate degradation in the R-granules was performed by strains PT and PW together with H_2-formate-utilizing methanogens. Syntrophic butyrate

degradation was performed by strains BH and IB.

2) Reversible isomerization between butyrate and isobutyrate occurred during syntrophic butyrate and isobutyrate degradation by anaerobic granules. In the R-granules, this reaction was performed by syntrophic isobutyrate-butyrate degrading strain IB. The reversible isomerization was coupled with butyrate oxidation. The direction of the isomerization was controlled by the ratio between isobutyrate and butyrate. The most thermodynamically favorable condition for the syntrophic degradation of butyrate or isobutyrate in conjunction with the isomerization was at equal concentrations of isobutyrate and butyrate.

3) Valerate and 2-methylbutyrate were produced during syntrophic butyrate degradation by anaerobic granules when propionate was present. Strain BH synthesized valerate from propionate only during syntrophic butyrate degradation. Isobutyrate degrading strain IB was responsible for the synthesis of 2-methylbutyrate. The metabolic pathway for syntrophic valerate degradation by strains IB and BH appeared to follow β-oxidation while the pathways for the degradation of 2-methylbutyrate appeared to be more complicated.

4) The syntrophic degradation of isobutyrate and butyrate by the triculture consisting of strain IB, *Mb. formicicum* strain T1N and *Ms. mazei* T18 was completely inhibited in the presence of a high hydrogen partial pressure (>2000 Pa) or a measurable level of formate (>10 to 200 μM). Significant level of formate (more than 1 mM) was detected during the perturbation with hydrogen (17 to 40 kPa). Both hydrogen and formate are likely electron carriers in syntrophic degradation.

5) Formate synthesis from hydrogen plus bicarbonate or CO_2 was observed during the growth of H_2-CO_2 and formate utilizing methanogens including *Mb. formicicum* species (strains MF, T1N and RF) and *Msp. hungatei* strain BD. Formate

synthesis was also performed by *Ms. mazei* strain T18, unlike other strains, which was not able to catabolize this formate to methane. These methanogens are, at least partially, responsible for the formate synthesis during methanogenesis from H_2-CO_2 by anaerobic granules and defined syntrophic fatty acid degrading cultures. The formation of formate from hydrogen plus bicarbonate and vice versa reveals that both formate and hydrogen are electron carriers in interspecies electron transfer during syntrophic fatty acid degradation.

CHAPTER 7

ECOPHYSIOLOGICAL ROLE OF DEFINED SPECIES ISOLATED FROM GRANULES IN GRANULE FORMATION

7.1 INTRODUCTION

At present, factors controlling the granulation process are still poorly understood (Archer, 1988; Colleran, 1988). The granulation process is believed to be substantially microbial self-immobilization. Identification of the bacterial species that play an essential role in granule formation is the key to understanding the phenomenon of granule formation. The key species may be defined as ones which i) form dense aggregates by themselves in an anaerobic reactor; and/or ii) provide a binding surface for other bacteria, which cannot form aggregates by themselves, to attach and form granules. Therefore, these species should have the ability to aggregate and will likely be a prevalent microorganism in the granules.

Methanogens have already been proposed as key species in granule formation. Several proposals on microbial mechanisms of granule formation (see Chapter 1) can be summarized as follows: i) *Methanothrix* sp. plays an essential role in granulation (Wiegant and de Man, 1986; Dubourguier *et al.*, 1988a, 1988c; Hulshoff Pol *et al.*,

1988; Hulshoff Pol, 1989); ii) hydrogen-utilizing methanogen *Methanobrevibacter arboriphilus* strain AZ produces extracellular polypeptides to induce granule formation under high H_2 partial pressure condition (Sam-Soon *et al.*, 1988); iii) *Methanosarcina* produces initial aggregates as nuclei to form granules (de Zeeuw, 1988). These proposals and mechanisms, however, have not been validated experimentally.

Microcolonies of syntrophic acetogens in granular structures have been observed within anaerobic granules (Dubourguier *et al.*, 1988a, 1988b). In Chapter 4, syntrophic microcolonies consisting of acetogens and methanogens were major structural components of granules developed on brewery wastewater. The role of syntrophic acetogens in granule formation has not been clearly elucidated.

In Chapters 5 and 6, methanogens and syntrophic acetogens isolated from the R-granules as prevalent species were characterized according to their taxonomy and role in fatty acid metabolism. These organisms include H_2-CO_2 and formate-utilizing methanogens, acetate-utilizing methanogens, syntrophic propionate degraders and syntrophic butyrate degraders. Study of these isolated organisms provides a convenient method to directly study the microbial mechanism of granulation with volatile fatty acids as substrates. One of the prevalent H_2-CO_2 and formate-utilizing methanogens isolated was *Methanobacterium formicicum* strain T1N, which readily formed strong aggregates.

In this chapter, *Mb. formicicum* strain T1N was examined to determine if it played a key role in the formation of granules. The aggregate forming behavior among various defined methanogenic and syntrophic fatty acid degrading cultures was also compared to screen for potential granule-forming syntrophic associations. Finally, anaerobic granules using defined cultures were developed and characterized according to their performance.

7.2 MATERIALS AND METHODS

7.2.1 Bacterial strains and analytical methods

All cultures used in this chapter were isolated from the R-granules (Chapter 4) except *Methanobacterium formicicum* strain MF obtained from the Deutsche Sammlung von Mikroorganismen. The R-granules were maintained in upflow anaerobic reactors (see Chapter 3). Chemicals, gases and media for growth of methanogens and syntrophic acetogens are described in Appendix A. The microscopic examination and electron microscopic examinations (including the sample preparation and cell density account) are provided in Appendix D. Analytical methods except formate analysis are described Appendix A. Liquid samples for formate determination were acidified with 12 N HCl (10:1, v/v) and centrifuged. Formate was determinate by a HPLC Water 600 (Millipore Corp., Water Chromatography Div., Milford, MA) fitted with a Biorad 87H column at 85 °C and with 0.012 N H_2SO_4 as mobile phase (flow rate 0.6 mL/min). The sensitivity of this method for formate determination was 2 mM.

7.2.2 Hydrophobicity measurement

The hydrophobicity of the cell surface of *Mb. formicicum* strains MF, T1N and RF, *Methanospirillum hungatei* strain BD and *Methanothrix* strain M7 was measured using the method of Rosenberg et al. (1980). Cells were dispersed in a phosphate buffer (pH 7.1) containing (g per liter): $K_2HPO_4 \cdot 3H_2O$, 22.2; KH_2PO_4, 7.26; urea, 1.8; and $MgSO_4 \cdot 7H_2O$, 0.2. Hexadecane, xylene or hexane (0.4 mL) was added as an apolar phase into cell suspensions (2.4 mL). Following 10 min pre-incubation at 30

°C, the mixtures were agitated uniformly on a vortex mixer for 120 seconds. After allowing 15 min for the apolar phase to rise completely, light absorbance of aqueous phase was measured at 400 nm. The change in absorbance of aqueous phase and the adsorption of cells to the apolar phase were used to estimate hydrophobicity.

7.2.3 Re-aggregation test

Cells (about 1.0 g wet weight) of *Mb. formicicum* strains T1N, MF and RF or the R-granules were washed in double-distilled (DD) water, disrupted with a glass rod in 15 mL DD-water, and then centrifuged for 10 min at 3,000 x g. The pellet was then washed in DD-water at least 6 additional times. Subsequently, the suspensions of cells were sonicated with an ultrasonic cell disrupter (Kontes Glass Corp., Vineland, NJ) for two seconds, and diluted using DD-water to an optical density (OD) of 0.3 to 0.6 at 660 nm. At this stage, the cells in suspension appeared to be completely dispersed (when checked by microscopy) and did not re-aggregate after they were vortexed for 2 min. Addition of sodium chloride solution (0.04 M, 0.2 mL) to the diluted suspensions (2.0 mL) was used to test the effect of the concentration of saline on re-aggregation of cells of strain T1N, strain MF and disrupted granules. In addition to the saline, acetic acid-sodium acetate buffers (0.04 M) with pH ranging from 3.6 to 5.8, KH_2PO_4-NaOH buffers (0.04 M) with pH from 6.0 to 8.5, NaOH-Na_2CO_3 buffers (0.04 M) with pH 11 and 12 were also used to test pH effect on re-aggregation. The above buffers (0.2 mL) were added into the 2 mL of cell suspensions.

7.2.4 Sensitivity to tetracycline

In order to estimate the population level of tetracycline sensitive, H_2-CO_2-utilizing methanogens, the R-type granules (0.3 mL) were withdrawn from an operating

lab-scale upflow reactor and transferred into an anaerobic pressure tube containing 10 mL of the basal medium, buffered with 30 mM potassium phosphate and 20 mM sodium bicarbonate (pH 7.1), and pre-reduced by addition of an Na_2S solution (2.5%) to achieve a concentration of 1.0 mM. The granules were disrupted by passing them through a 1 mL glass syringe with a 23 gauge needle several times until the cells were completely dispersed in the medium. Disrupted granule suspensions (0.5 mL) were transferred into 158 mL serum bottles containing 25 mL of the basal medium (pH 6.9) with 2 mM acetate. The bottles were then pressurized with 180 kPa H_2-CO_2. Duplicates were used. Tetracycline was added to one set (1 mg/mL) while another set that did not received the antibiotics was maintained as a control. Tetracycline was prepared in an anaerobic water as a suspension (50 mg/mL) due to its low solubility. The suspension (0.5 mL) was then injected to the serum bottles. The serum bottles were inoculated with the granule suspension only after all tetracycline had dissolved. These bottles were then incubated in a 35 °C shaking water bath (125 strokes per min). Methane production was determined every 8 to 12 hours to monitor growth.

7.2.5 Aggregate formation by defined cultures

Various pure methanogenic cultures and defined syntrophic cultures were incubated to determine if they formed aggregates. A total of three methanogenic cultures (*Mb. formicicum* strains T1N, MF and RF), two syntrophic butyrate-degrading cocultures (butyrate degrader strain BH plus *Mb. formicicum* strain T1N, and strain BH plus *Msp. hungatei* strain BD), one syntrophic propionate degrading coculture (propionate degrader strain PT plus *Mb. formicicum* strain T1N), and one syntrophic propionate degrading triculture (strain PT, strain T1N plus *Methanothrix* strain M7) were examined. The growth conditions used for these cultures are described in

Appendix A. To prepare the triculture of strain PT, strain T1N and strain M7, a bottle containing well-grown coculture of strains PT plus T1N, isolated from the R-granules (Chapter 5), was inoculated with 2% of *Methanothrix* strain M7 culture as the third partner to utilize acetate. To obtain the coculture of strain BH and *Msp. hungatei* strain BD, the coculture of strain BH plus *Mb. formicicum* strain T1N, isolated from the R-granules (Chapter 5), was heat-treated at 80 °C for 20 min to kill the cells of strain T1N. The treated culture was then inoculated (2%, v/v) into a bottle containing fresh medium. A 2% inoculum of well grown *Msp. hungatei* strain BD was added.

All the defined cultures were incubated at 37 °C. Initially, culture transfer was done using syringes equipped with 22 gauge needles. When aggregates or clumps were formed in the bottles, culture transfer was performed using syringes equipped with 18 gauge needles in order to protect the structure of the aggregates or clumps from any damage during transfer.

7.2.6 Upflow reactors

Laboratory-scale upflow reactors, made of a glass column and a glass bottle, were operated to investigate the development of aggregates using *Mb. formicicum* strain T1N and cultivation of granules using defined syntrophic fatty acid degrading cultures as inoculum. Total volume of each reactor was 220 mL including a 120 mL settler. All tubing used was either stainless steel or black butyl rubber of 0.25-in (0.64 cm) thickness. A schematic diagram of reactor system is presented in Figure 7.1. The reactors were operated at 35 °C. During continuous operation, oxygen-free N_2 (at 40 kPa passed through a sterilized glass wool filter) was bubbled into the medium reservoir to keep a constant positive pressure in the headspace and maintain anaerobiosis. The medium was pumped into the bottom of the reactor using a

peristaltic pump (Gilson Medical Electronics Inc., Middleton, WI). After gas-liquid-solid separation in settler, the effluent was allowed to leave the reactor through a water sealer made of a 5 mL glass tube installed inside the effluent collecting flask in order to keep the reactor free of oxygen. Some of the liquid in the settler was recycled back to the reactor using another peristaltic pump, at a flow rate of 1 L/hr, to neutralize the feed and to maintain the desired hydraulic loading rate (ca.2.8 m/hr). Biogas samples for methane and hydrogen determinations were withdrawn under sterile conditions from the glass "T" tube on effluent tubing using a 1 mL glass syringe. Liquid samples were withdrawn aseptically through the sampling port on the reactor.

7.2.7 Feed composition for reactor experiments

For the growth of *Mb. formicicum* strain T1N in the reactor, liquid medium (16 liters) was made in a 20 liter carboy (medium reservoir) sealed with a butyl rubber stopper. The medium composition was (per liter of distilled water): $MgCl_2 \cdot 6H_2O$, 0.32 g; $CaCl_2 \cdot 2H_2O$, 0.32 g; NaCl, 0.8 g; NH_4Cl, 0.8 g; KH_2PO_4, 0.32 g; sodium acetate, 0.08 g; sodium formate, 6.81 g; formic acid (88%), 6.44 mL; $Na_2S \cdot 9H_2O$, 0.12 g; resazurin, 0.002 g; trace element solution (Kenealy and Zeikus, 1981), 5 mL; vitamin solution (Wolin *et al.*, 1963), 5 mL. After autoclaving for 90 min at 121 °C, an O_2-free nitrogen gas was used to pressurize the headspace of the carboy up to 40 kPa in order to maintain anaerobic conditions. Vitamin solution, formic acid-sodium formate solution, and sodium sulfide solution (15%, v/v) were added into the carboy anaerobically after medium was cooled to room temperature. The pH of the medium was 4.0, and total concentration of sodium formate plus formic acid was 220 mM.

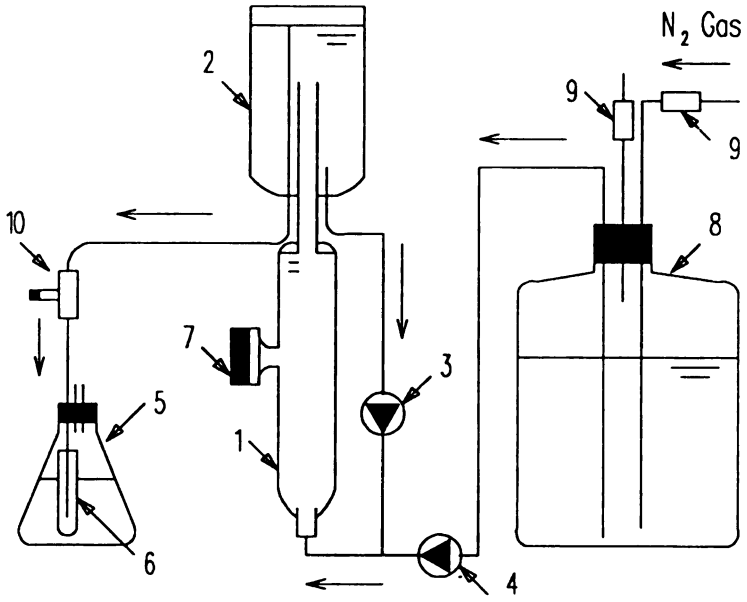


Figure 7.1 Schematic diagram of upflow reactor system. 1: column reactor; 2: settler; 3: recycle pump; 4: feed pump; 5: effluent collecting flask; 6: water sealer; 7: sampling port with a butyl rubber stopper; 8: medium reservoir; 9: steriled air filter; 10: glass "T" with a butyl rubber stopper.

For development of granules using defined fatty acid degrading cultures as the inoculum, liquid medium (16 liters) was prepared in a 20 liter carboy, using the procedure described above. The medium composition was (per liter of distilled water): $MgCl_2 \cdot 6H_2O$, 0.32 g; $CaCl_2 \cdot 2H_2O$, 0.32 g; NaCl, 0.8 g; NH_4Cl, 0.8 g; KH_2PO_4, 0.32 g; $Na_2S \cdot 9H_2O$, 0.08 g; resazurin, 0.002 g; and trace element solution, 5 mL. The concentrations of acetate, propionate, and butyrate were adjusted by adding the mixtures of the sodium salt and acid forms of the volatile acids. pH of the medium was 4.7.

7.2.8 Reactor start-up and operation

i) Anaerobic technique prior to inoculation

To ensure successful start-up, anaerobic conditions were established in the reactor systems prior to inoculation. The reactors, including feed pump tubing and recycle pump tubing, were autoclaved at 121 °C for 30 min before inoculation. Initially, an HCl solution (1N) was added into the water sealer installed in the effluent flask, and the reactors were flushed with oxygen-free N_2 gas for 30 min. Subsequently, the basal medium (pH7.0 to 7.1) pre-reduced with 0.5 mM Na_2S and buffered with sodium bicarbonate (30 mM) and potassium phosphate (30 mM) was added to fill the reactor. Recycle of medium was started, and N_2 flushing was stopped. Anaerobic conditions in the reactor were monitored using the redox potential indicator resazurin. Finally, the carboy containing the feed medium was connected to the reactor under aseptic conditions. The reactor was inoculated through the sampling port, and feeding was initiated.

ii) Inoculation with *Methanobacterium formicicum* strain T1N

Mb. formicicum strain T1N was used as inoculum in order to examine if this

organism could develop granule-like aggregates by itself using formate as substrate. Inoculum of concentrated strain T1N clumps (30 mL in volume and about 0.12 g dry weight) formed about 5 mL compact cell bed in the reactor. After inoculation, sodium formate stock solution (1.1 mL of 4 M) was added to the reactor to achieve a starting concentration of ca. 20 mM. The reactor was placed on recycle without any addition of feed for the initial 12 hours. When significant gas production was observed, the feed pump was turned on and the reactor fed continuously. During the operational period, flow rate, effluent pH, methane and hydrogen content in biogas, and formate concentration in the effluent were monitored daily.

iii) Inoculation with defined cultures

Well-grown monocultures and syntrophic cultures were used as inoculum. The inoculation sequence of different species was as follows: initially, actively growing cells of *Mb. formicicum* strain T1N (40 mL, 0.22 g SS) were inoculated and about 10 mM sodium formate was added into the reactor; after 8 hours, aggregates of *Ms. mazei* strain T18 (0.05 mL) and cells of *Methanothrix* strain M7 culture (30 mL, 0.05 gSS) were inoculated and then the reactor was fed continuously at 7 mL/hr. Finally, a coculture of strain BH and *Mb. formicicum* strain T1N (30 mL, 0.025 gSS) and a triculture of propionate degrader strain PT, *Mb. formicicum* strain T1N and *Methanothrix* M7 (30 mL, 0.06 gSS) were added into the reactor one after another at intervals of 12 hours. After inoculation, pH and fatty acid concentrations in the reactor were determined initially on alternate days and then daily. Methane and hydrogen contents in biogas were determined every other day.

iv) Maximum VFA degradation rates and apparent K_m

To determine the maximum VFA degradation rates of the granules developed from the defined cultures, another reactor with the same size was prepared and filled

with pre-reduced basal medium (pH 7.0) as described above. The basal medium in the reactor contained acetate (5mM), propionate (5mM) and butyrate (5mM). The granules (free of flocs) in the original reactor were transferred anaerobically using a 10 mL glass syringe with a 18 gauge needle into the new reactor. This inoculum contained 5 mL of compact granule. After inoculation, the new reactor was continuously fed with a medium containing propionate (30 mM) and butyrate (30 mM). The maximum degradation rates for propionate and butyrate were determined on the basis of the following day's operational data (VFA concentrations in the reactor and feed rate) using following equations:

$$dS/dt = V_R \cdot (S_{t1}-S_{t2})/(1000 \cdot (t_2-t_1)+Q \cdot (S_0-0.5 \cdot (S_{t1}+S_{t2})) \qquad (7\text{-}1)$$

where, dS/dt is degradation rate (mmol/hr); V_R is the liquid bulk volume of reactor system including recycle tubing, 220 mL in this experiment; S_{t1} and S_{t2} are propionate or butyrate concentration (mM) at time t_1 and t_2, respectively; Q is feed rate (mL/hr). S_0 is propionate or butyrate concentration in feed (propionate, 30 mM; and butyrate, 30 mM). Acetate degradation rate was calculated as:

$$dS_a/dt = V_R \cdot (S_{at1}-S_{at2})/(1000 \cdot (t_2-t_1)+Q \cdot (S_{a0}-0.5 \cdot (S_{at1}+S_{at2}))+dS_p/dt+ 2 \bullet dS_b/dt \qquad (7\text{-}2)$$

where, dS_a/dt, dS_p/dt and dS_b/dt are degradation rates (mmol/hr) for acetate, propionate and butyrate, respectively. In equation (7-2), acetate produced from syntrophic propionate and butyrate degradation is included but valerate formed in the reactor was ignored for this purpose since valerate concentrations were low (<0.1 mM).

The determination of apparent K_m values of propionate and butyrate using the granules developed with the defined species is described in Appendix C.

7.3 THE ROLE OF *Methanobacterium formicicum* STRAIN T1N IN GRANULE FORMATION

Mb. formicicum strain T1N, isolated from the R-granules (a predominant species), exhibited strong aggregation. The role of *Mb. formicicum* strain T1N in granule formation, was evaluated using SEM observation, its population in granules, adhesion behavior, and aggregate formation (by a pure culture) in a continuous fed upflow reactor.

7.3.1 Electron microscopic observation of aggregates formed by *M. formicicum* strain T1N

As described in Chapter 5, *Mb. formicicum* strain T1N was very adhesive and formed aggregates. Under SEM observation (Figure 7.2A), the aggregates had a complicated three-dimensional structure. Cationized ferritin (CF) staining is a method that can be used reveal the surface topology of bacterial cells, presence of extracellular polymers and other extracellular structures via. SEM observation (Lamed *et al.*, 1987). CF stained strain T1N cells showed abundant massive fibers forming a network among the cells and protuberant-like structures (formed by CF particles) on the cell surface (Figure 7.2B). These fibers formed by the polymers, connected the cells to each other to form aggregates. The presence of CF particles on the cell surface and the fibers reveals the existence of anionic groups on the polymers and the surface of cells.

The polymers on the surface of *Mb. formicicum* strain T1N appear to contribute to the formation of aggregates and may also contribute to granular structure.

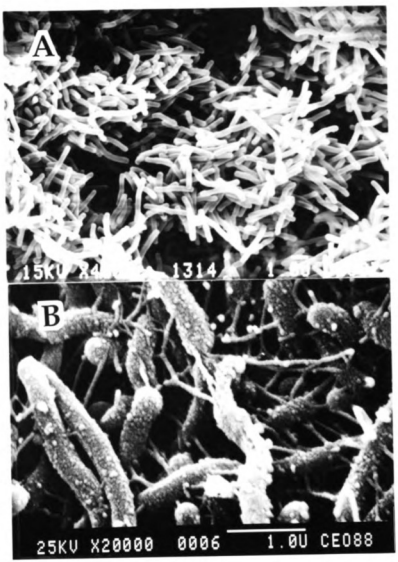


Figure 7.2 SEM photographs of *Methanobacterium formicicum* strain T1N. (A) Sample treated using normal procedure showed the complicated structure of aggregates. (B) Fibers formed by extracellular polymers which connected cells to each other to form aggregates and CF particles on the surface of CF treated cells.

7.3.2 Effect of tetracycline on methanogenesis of disrupted R-granules

In the R-granules, the prevalent H_2-CO_2 and formate-utilizing methanogens were *Mb. formicicum* strain T1N and *Mb. formicicum* strain RF plus considerably lower populations of a *Msp. hungatei* strain BD (Chapter 5). The former was tetracycline sensitive and the latter two strains were resistant to tetracycline. Therefore, tetracycline was added to the disrupted granules under a H_2-CO_2 atmosphere to estimate the population of *Mb. formicicum* strain T1N in the R-granules.

After incubation, methane concentration in both test bottles (plus and minus tetracycline) increased logarithmically at an identical rate (doubling time of about 8 hours). After the initial 12 hours, however, the specific methane production rate in the bottles receiving 1 mg/mL of tetracycline was 40 to 60% of that in the bottles without tetracycline. This indicated that approximately half of the H_2-CO_2 utilizing methanogens in the R- granules were inhibited by addition of tetracycline. Since only *Mb. formicicum* strain T1N was tetracycline-sensitive, strain T1N was present in great numbers in the R-type granules.

7.3.3 Re-aggregation of dispersed cells

Re-aggregation results of *Mb. formicicum* strain T1N and the R-granules are presented in Figure 7.3. Aggregates of *Mb. formicicum* strain T1N and the granules were disrupted completely after washing with DD-water and sonication. No aggregation occurred after the suspensions were vortexed for 2.0 min in DD-water. Significant aggregation, however, was observed in the suspensions of strain T1N and the disrupted granules to which NaCl solution was added (4 mM) before vortexing the sample for 2.0 min. In the suspension of strain T1N, almost all cells aggregated

together as clumps. Clumps also formed quickly in suspensions of disrupted granules, but many free cells still remained in the suspensions. Most free cells from the granules were identified as *Methanothrix*-like and *Methanospirillum*-like bacteria. Other salts, such as potassium phosphate, sodium acetate, sodium bicarbonate, and potassium chloride also induced re-aggregation of the dispersed cells when provided at the same concentration (4 mM).

The effect of pH on re-aggregation of *Mb. formicicum* strain T1N dispersed cells and disrupted granules was identical. The suspended cells of strain T1N and disrupted granules had the same tendency to re-aggregate at a pH range of 3.6 to 8.5 in the various buffers, but then did not re-aggregate at pH 11 or higher.

Mb. formicicum strains MF and RF were also examined for re-aggregation performance. The cells of these strains were dispersed by washing with DD-water and sonication, and were homogeneously suspended in DD-water. Neither of the strains formed any aggregates after vortexing the suspensions for 2 min. After sonication, sodium chloride (2 to 30 mM) or potassium phosphate (10 to 30 mM) was added, re-aggregation of cells of strains MF and RF did not occur.

These results indicate that *Mb. formicicum* strain T1N has re-aggregation characteristics which other *Mb. formicicum* strains tested did not. Therefore, the re-aggregation of disrupted R-granules may be mainly attributed to strain T1N. This fact suggested that this strain might play a more important role in the structure of the R-granules than do other methanogenic strains.

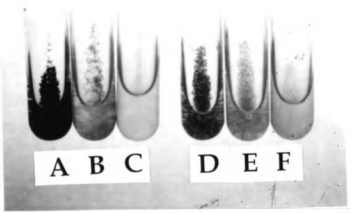


Figure 7.3 Comparison of aggregation of dispersed cells between the R-granules and *Methanobacterium formicicum* strain T1N aggregates. (a) Intact R-granules. (b) Clumps formed by dispersed cells of disrupted granules after addition of NaCl (2 mM). (c) Cell suspension of granules, dispersed by washing with DD-water and sonicating. (d) Intact aggregates formed by *Mb. formicicum* strain T1N. (e) Aggregates re-formed by dispersed cells of strain T1N after addition of NaCl (2mM). (f) Cell suspension of strain T1N, dispersed by washing with DD-water and sonicating.

7.3.4 Aggregate formation in laboratory-scale reactor by *Methanobacterium formicicum* strain T1N

Granule-like aggregates were developed using a monoculture of *Mb. formicicum* strain T1N grown on formate in the laboratory upflow reactor system described in the Materials and Methods section. Operational results of growth of strain T1N in the upflow reactor is presented in Figure 7.4. The formate conversion rate in the reactor increased gradually as feed flow rates were increased. During the initial three weeks, relatively high concentrations of formate remained in the reactor. Gradually the formate concentration dropped from 20 mM to 2.2 mM. Within three week's continuous operation, formate levels decreased to lower than the detection limits of HPLC (<2 mM). Hydrogen was produced from formate during methane production. The hydrogen partial pressure ranged between 5 and 26 Pa staying generally between 10 and 20 Pa. The initial effluent pH was about 6.9 to 7.0. As formate levels decreased, the pH rose to 7.2 to 7.3. Methane production increased from about 6.7 mL (STP) CH_4/hr to 28 mL (STP)/hr after 1 month of operation. Methane content in the biogas increased from 35% at the beginning to 46% in three weeks, and remained at that level thereafter.

After inoculation, initial feed flow rate of about 5.5 mL/hr was gradually increased to 24 mL/h by day 38 of continuous operation. The bacteria soon formed aggregates, some of them accumulated in the bottom of the reactor, some were captured in the top settler, and others washed out from the reactor with the effluent. During the first three weeks, flocs accumulated in the settler were recycled back to the reactor every day by increasing the recycle rate and shaking the settler gently to allow the clumps and flocs to become entrained in the recycle flow. Initially, the volume of

flocs was about 5 mL. The effluent OD_{660} increased from 0.10 at the beginning to 0.23 after 20 days but then dropped to 0.12 to 0.14, indicating wash-out of light flocs. Microscopic examination revealed that small flocs and free cells were in the effluent. Dense aggregates were retained in the reactor. These eventually formed granule-like aggregates after 20 days that were easily identified via naked-eye-observations.

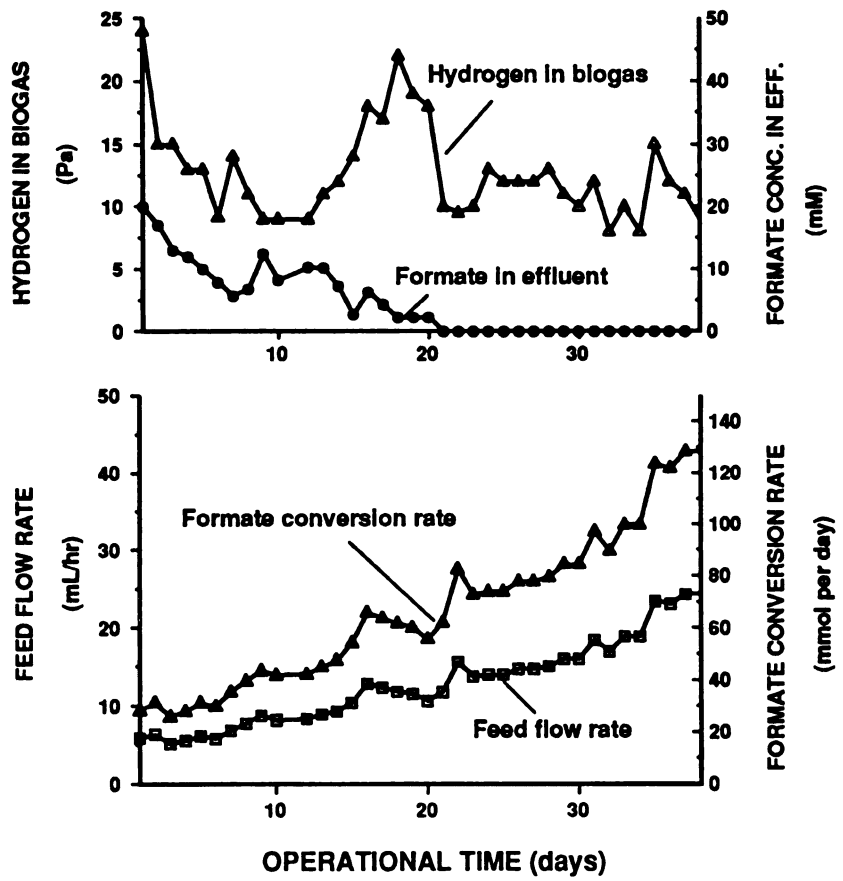


Figure 7.4 Operational results of upflow reactor inoculated with *Methanobacterium formicicum* strain T1N and operated at 35 °C. (A) Formate concentration in reactor and H_2 partial pressure in biogas. (B) Feed flow rate and formate conversion rate.

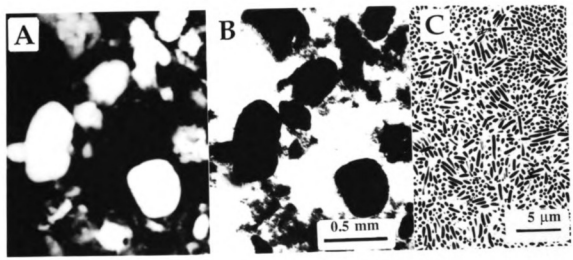


Figure 7.5 Granule-like aggregates formed by *Methanobacterium formicicum* strain T1N in the upflow reactor. (a) Aggregates with flocs under UV-light. (b) under visible light. (c) TEM photograph of a thin section of a aggregate showing dense microbial structures.

After 40 days of operation, the reactor was opened to examine the aggregates. About 4 mL granule-like aggregates were harvested. The aggregates formed by strain T1N appeared yellow in color and spherical in shape (Figure 7.5B). These aggregates fluoresced with a blue-green color under UV-light (Figure 7.5A). Some flocs or clumps were found among the granule-like aggregates. In the settler about 15 mL compact clumps were observed. Also, a thin layer of biofilm of strain T1N was found

inside recycle tubing and on the glass wall of both the reactor and settler. TEM examination of cross sections of the granule-like aggregates formed by strain T1N had high cell densities (2.6 x 10^8 cells/cm^2) which was approximately 4.2 x 10^{12} cells/cm^3. The cells were packed together side to side in regular pattern (Figure 7.5C).

The size distribution of the granule-like aggregates is illustrated in Figure 7-6. All the aggregates were less than 0.7 mm in diameter with 60% of total number of measuring between 0.1 to 0.2 mm. About 70% of total aggregate volume, however, is contributed by the aggregates with a diameter ranging from 0.4 to 0.6 mm.

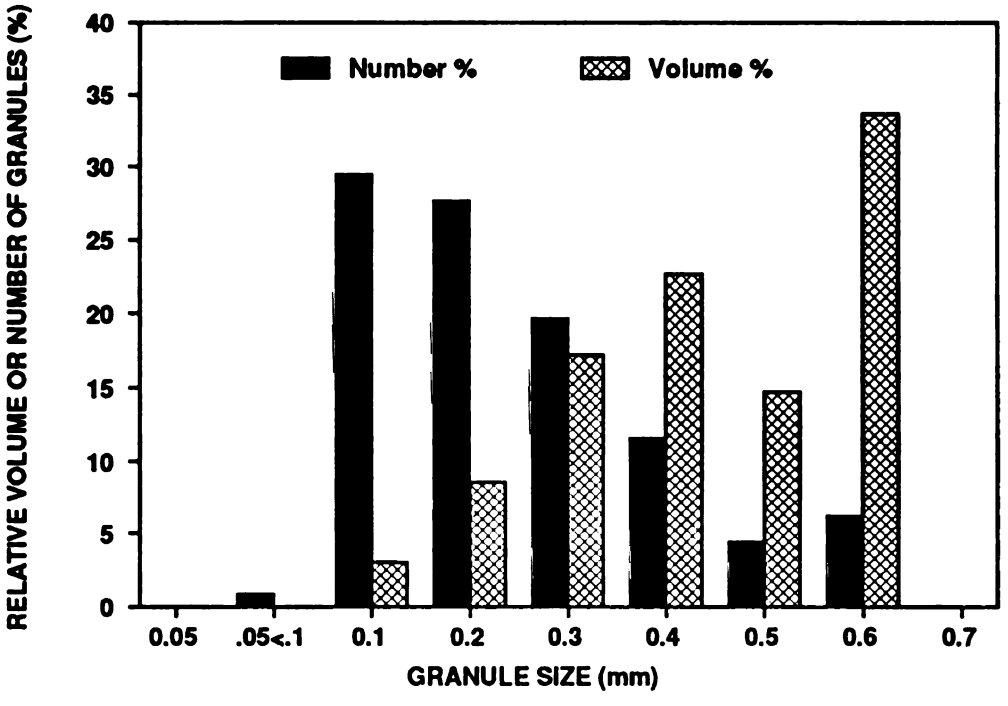


Figure 7.6 Size distribution of granule-like aggregates formed by *Methanobacterium formicicum* strain T1N in the relation to total numbers and aggregate volume.

7.4 GRANULE FORMATION BY DEFINED SYNTROPHIC FATTY ACID DEGRADING CULTURES

7.4.1 The extracellular polymers of *Methanothrix* strain M7

Methanothrix strain M7 was a predominant species in the R- granules (Chapter 5). This organism was observed to form rosette clumps in the stationary phase. The three dimensional structure of the clumps of *Methanothrix* strain M7 showed that the cells formed a complicated network (Figure 7.7A), which may entrap other bacterial species. CF-stained cells exhibited a fiber-like structure which appeared to connect cells to each other (Figure 7.7B). This indicates that strain M7 produces extracellular polymers. This result supported the hypothesis that *Methanothrix* M7 may be one of key species required for formation of the R-granules.

7.4.2 Hydrophobicity of cell surfaces of various methanogens

Hydrophobicity is an important parameter for describing the characteristics of the cell surface. It can significantly influence the adherence of bacterial cells to support media as well cells to cells. Hydrophobicity can be estimated by measuring the reduction of cell concentration in aqueous phase after addition of hydrocarbon solvent (Rosenberg *et al.*, 1980).

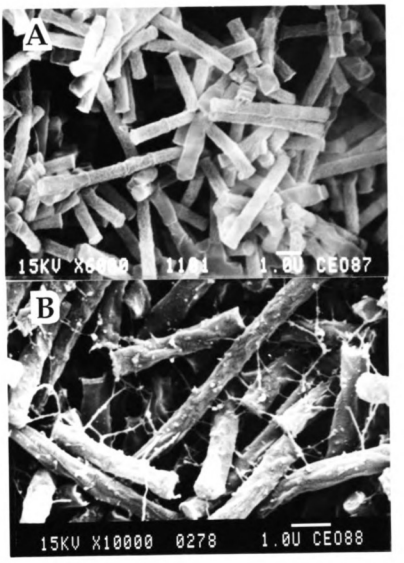


Figure 7.7 Scanning electron microphotographs of *Methanothrix* strain M7. (A) Sample treated by normal procedure shows microstructure of clumps. (B) Cationized ferritin (CF) stained cells show polymer fibers connecting the cells each other and the presence of CF particles on cell surface.

Five strains of methanogens were tested for hydrophobicity, including *Mb. formicicum* strains MF, T1N and RF, *Msp. hungatei* strain BD, and *Methanothrix* strain M7. The three hydrocarbons used (hexadecane, xylene and hexane) exhibited similar results for hydrophobicity tests. The results using hexane as the apolar phase are summarized in Table 7.1. The cells of strain T1N formed aggregates in the aqueous phase containing potassium phosphate very quickly and could not be adsorbed by hexadecane, hexane or xylene. All cells remained in the aqueous phase as aggregates. Thus, it was not possible to accurately determine hydrophobicity of strain T1N cells. Less than 6% of strain MF cells remained in the aqueous phase indicating that strain MF cells were significantly hydrophobic. Strain RF cells were also hydrophobic. On the other hand, cells of *Msp. hungatei* strain BD remained in aqueous phase, indicating these cells were more hydrophilic. After the suspension of *Methanothrix* strain M7 (OD_{400} 0.21) was vortexed with hexane, the OD_{400} in the aqueous phase was reduced by 85%. Most cells were partitioned to the apolar phase, indicating that the surface of these cells was hydrophobic in nature.

It is known that two hydrophobic surfaces will attract each other when they come together, while two hydrophilic surfaces will repel each other (Rapporteur *et al.*, 1984). The hydrophobicity test explained why it is possible for *Mb. formicicum* strains MF and RF and *Methanothrix* strain M7 to form aggregates or clumps but it is impossible for *Msp. hungatei* strain BD. Therefore, based on these results, *Mb. formicicum* species and *Methanothrix* M7 are likely to be more important in granule formation than *Msp. hungatei* strain BD.

Table 7.1 Hydrophobicity and cell adhesion properties of methanogenic species obtained from the R-granules

Organism	OD_{400} in liquid phase		Hydrophobic	Aggregation	Re-aggregation
	before	after			
Methanobacterium formicicum					
strain T1N	0.45	0.02	unknown	yes	yes
strain MF	0.40	0.02	+	yes	no
strain RF	0.45	0.05	+	yes	no
Methanospirillum hungatei					
strain BD	0.20	0.18	-	yes	no
Methanothrix					
strain M7	0.21	0.03	+	yes	no

1) Non-polar phase was hexane (0.8 mL), and aqueous phase was phosphate buffer (pH 7.0, 3.6 mL).
2) The change in optical density of cells at 400 nm (OD_{400}) before and after vortexed for 2 min was used to estimate hydrophobicity. The reduction of OD_{400} was due to the adsorption of cells to apolar phase except the case for *Mb. formicicum* strain T1N.

7.4.3 Evaluation of aggregation behavior of methanogens and syntrophic cultures

The aggregation behavior of various syntrophic fatty acid degrading associations and pure methanogen cultures are summarized in Table 7.2. The relative aggregation percentages (R) were estimated on the basis of optical density at 660 nm (OD_{660}) of liquid and the microscopic examination. The OD_{660} of pure cultures of *Mb. formicicum* strain T1N and *Ms. mazei* strain T18 was very low (< 0.02) because cells formed aggregates; thus R for strain T1N and strain T18 was taken as 100%. Most cells of *Methanothrix* strain M7 formed rosette clumps but some remained as dispersed cells (R = 70%). *Msp. hungatei* strain BD grew as dispersed cells only (R=0%).

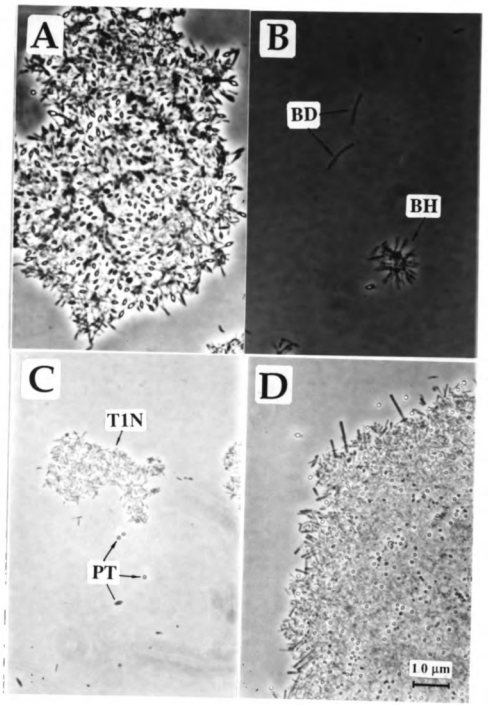
Cells of *Mb. formicicum* strain T1N and strain BH in coculture formed dense aggregates. Some of the aggregates were as large as 0.2 to 0.4 mm in diameter after two transfers. Microscopic examination indicated that the cells of the two strains grew homogeneously (Figure 7.8A). When the aggregates formed, the medium appeared clear (OD_{660} <0.04) and only very few cells were found free in the liquid (R = 0.95). No aggregates were observed in cocultures of strains BH and *Msp. hungatei* strain BD. Microscopic examination revealed that *Msp. hungatei* strain BD grew as single filaments while strain BH formed small clumps consisting of only a few cells. It was also observed during microscopic observation that the cells of the two strains were always separated from each other (Figure 7.8B). This coculture was assigned an R value of zero. The clump-forming characteristic of strain BH indicates the presence of adhesive cell surface polymers on the surface of strain BH cells.

Table 7.2 Aggregation behavior of pure methanogen and defined syntrophic methanogenic cultures

Culture	Observation	Relative aggregation (%)
strain T1N	Aggregates	100
strain T18	Aggregates	100
strain M7	Aggregates and free cells	70
strain BD	Free cells	0
strains T1N+BH	Dense aggregates formed with diameter 0.2-0.4 mm, few free cells	95
strains BD+BH	No aggregates, but very small clumps of strain BH. BD grew as single cells	25
strains T1N+PT	Strain T1N formed clump, but strain PT grew as free cells outside T1N clumps.	20
strains T1N+PT+M7	Small aggregates contained the three strains. Free cells also presented.	70

Note: After 1-2 months incubation at 37 °C.

Figure 7.8 Aggregate formation by syntrophic methanogenic cocultures and triculture. (A). Aggregate formed by *Mb. formicicum* T1N and butyrate-degrading strain BH. (B) Cells of *Msp. hungatei* BD did not form any clumps or aggregates with strain BH. (C) Cells of propionate degrader strain PT did not grow within the aggregate of *Mb. formicicum* T1N. (D) The cells of strain PT grew together with the cells of *Methanothrix* M7 and *Mb. formicicum* T1N to form syntrophic aggregate.


A
B
BD
BH
C
T1N
PT
D
10 µm

Cells of propionate degrader strain PT and *Mb. formicicum* strain T1N did not grow homogeneously in coculture. Microscopic observation revealed that cells of strain PT were always found outside the clumps of strain T1N (Figure 7.8C). However, in the triculture consisting of strain PT, strain T1N and *Methanothrix* strain M7, a larger number of aggregates formed. These aggregates were homogeneously comprised of cells from the three strains (Figure 7.8D). Some free cells were also observed. The R values for coculture and triculture were 20% and 70%, respectively.

7.4.4 Granule formation by defined species

The anaerobic granules degrading VFA should contain at minimum an acetate-utilizing methanogen, an H_2-CO_2-utilizing methanogen, and syntrophic fatty acid degraders. Development of syntrophic anaerobic granules was attempted using the following organisms: *Mb. formicicum* strain T1N, *Methanothrix* strain M7, *Ms. mazei* strain T18, syntrophic propionate degrader strain PT and butyrate degrading strain BH. This microbial composition design was based on these criteria: i) these species possessed the capability to completely mineralize acetate, propionate and butyrate into CH_4 and CO_2; ii) they had been demonstrated to form aggregates in their pure culture or their syntrophic associations.

Granules were formed using the defined species. Results, including the concentrations of fatty acids in the influent and effluent, VFA degradation rates per reactor, COD volumetric loading rates and COD volumetric removal rates, are presented in Figure 7.9. The operational conditions of the reactor and the results obtained are summarized in Table 7.3. For the initial 31 days of operation, the feed contained 5 mM sodium acetate, 10 mM sodium propionate and 10 mM butyric acid. Subsequent to day 30, propionate and butyrate were gradually increased to 15 and 16.6

mM. After 57 days of operation, the reactor was with fed fresh medium containing 30 mM propionate and 30 mM butyrate (no acetate). Based on the stoichiometry of methane production form propionate and butyrate (Table 1.4 in Chapter 1), about 70 to 75% of the methane should be produced through acetate as the terminal intermediate.

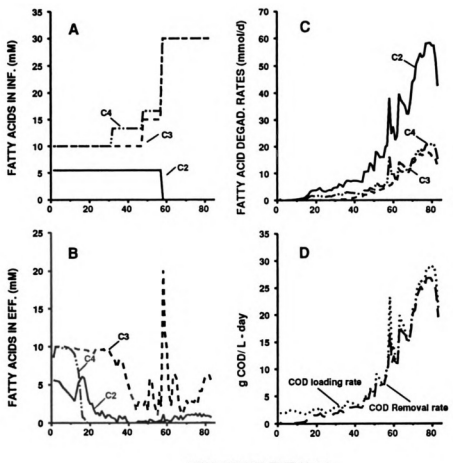


Figure 7.9 Results of reactor system used to study formation of syntrophic anaerobic granules by defined species. (a) VFA concentrations in the influent. (b) VFA concentrations in the effluent. (c) VFA degradation rates of the reactor vs. operational period. (D) COD volumetric loading rates and volumetric COD removal rates.
C2: acetate; C3: propionate; and C4: butyrate.

Table 7.3 Operational conditions and performance of a reactor inoculated with defined syntrophic methanogenic cultures

Operation date	Feed VFA (mM) C_2	C_3	n-C_4	Feed rate ml/hr	pH	COD load g/L/day	COD removal (%)	CH_4 (%)	H_2 (Pa)
day 1 to 31	5	10	10	5.3-8.0	6.2-7.0	2.0-3.0	57-59	70-75	5-9
day 32 to 47	5	10	13.3	7.5-11.3	7.0-7.2	3.2-5.0	64-80	74-75	3-5
day 48 to 57	5	15	16.6	11.3-16.8	7.0-7.1	6.5-9.7	78-80	74-75	3-4
day 58 to 82	0	30	30	13.2-29.0	7.3-7.4	13-29	61-83	79-80	3-4

(1) C_2: acetate; C_3: propionate; n-C_4: butyrate.
(2) The date sequence begins on the first day when all starter cultures added into reactor.
(3) COD load was calculated on the basis of stoichiometry of complete oxidation of substrates to CO_2 (g COD/mol): acetate, 64; propionate, 112; butyrate, 160.

After start-up, acetate was degraded immediately. Butyrate concentration remained at 10 mM for about 10 days before significant butyrate degradation occurred. The growth rate of propionate degraders was very slow. Significant propionate degradation occurred only after 37 days. Fatty acid degradation rates in the reactor increased almost logarithmically during the operational period. The initial pH in the reactor was 6.23. This gradually increased up to about 7.0 in 16 days as the fatty acid concentrations decreased. After the feed was switched to a propionate-butyrate mixture, the pH increased from 7.0 to 7.3. The hydrogen partial pressure which was about 17 Pa at the beginning, dropped to 3 to 5 Pa on day 16 and remained at this level during the rest of the operational period. The methane content in biogas was 70 to 75% when reactor was fed with acetate-propionate-butyrate mixture. This increased slightly to 75 to 76% when fed with propionate-butyrate mixture, as would be anticipated. The increase in the COD volumetric removal rates was proportional to the increase in the COD volumetric loading. At the beginning, the COD removal rate (total reactor system) was less than 0.1 g COD/L-d with a COD removal of less than 3%. At the end of the operation, the volumetric COD removal rate had increased to 22.5 g COD/L-day with COD removal efficiencies of 80 to 83%. An attempt to monitor the specific volumetric loading rate and specific COD removal rate on the basis of biomass of granules was impossible since both flocs and biofilms were presented in the reactor system.

At the beginning, the reactor contained about 5 mL bacterial bed consisting of inoculated cultures of apparently low density aggregates. After 17 days of continuous operation, most of the light fraction of the aggregates were washed out of column reactor into the settler. A few dense small aggregates with a brownish-yellow color, remained at the bottom of the reactor. The granule-like aggregates were observed at

the bottom of the reactor after 20 days of operation. These gradually developed to form a granule bed in the reactor. By day 82, about 7 mL of granules had developed in the reactor. During this period (after about 50 days), attachment of bacteria to the reactor wall became visible. This process of attachment and subsequent growth of biofilms continued until the termination of the experiment at day 82, roughly 50% of the total biomass was attached to the reactor wall and the rest was in the granule bed. The attached biofilms were yellowish in color while the color of granules was brownish yellow in color.

7.4.5 Microbial structure of granules

The granules developed by the defined species were of irregular shape and about 1 mm in diameter (Figure 7.10). Microscopic examination revealed that both granules and biofilms had the same microbial composition and both had all five of the species inoculated into the reactor at readily observable numbers. Under SEM examination, the cells of *Methanothrix* strain M7, *Mb. formicicum* strain T1N, propionate degrading strain PT and butyrate degrading strain BH were frequently observed on the surface of granules (Figure 7.10). The cells of *Ms. mazei* strain T18 were observed only occasionally. TEM cross thin section revealed that strain T1N, strain M7, strain PT and strain BH (except strain T18) grew homogeneously within the granules (Figure 7.10). *Ms. mazei* strain T18 grew as individual aggregates inside the granules and did not appear to mix with other bacteria. On the outer surface of the aggregates strains T18 and T1N grew in what appeared to be an attached layer (Figure 7.10). This layer of strain T1N appeared to serve as a bridge between T18 aggregates and the other syntrophic bacteria growing together. The overall cell density of strain T18 in these granules was hard to determine by direct microscopic count due to aggregation and

because the size of *Ms. mazei* T18 cells was irregular. The aggregates of strain T18 occupied about 30-35% of total surface of cross section of the granules. The density of the four strains in the granules was determined (Table 7.4). The cell density of the granules was 8.7 x 10^7 cells/cm^2. Direct counting of the individual morphotype-cells on the electron microphotographs led to the approximate ratio among the strains. The ratio of T1N:M7:BH:PT was 100:59:24:13.

7.4.6 Characterization of granules

The maximum specific conversion rates for acetate, propionate and butyrate were determined using batch test in the reactor described in the Materials and Methods section. The results of acetate, propionate and butyrate conversion rates of the syntrophic anaerobic granules are presented in Table 7.4. The granules had high fatty acid degradation rates, specific gravity of 1.023, and density of 58 gSS/L. Based on the stoichiometry of complete oxidation of acetate, propionate and butyrate to methane and CO_2, the maximum COD removal rate of the granules was 3.22 g COD/gSS-d at 35 °C. These granules had a normal distribution with 0.8 mm as the mean diameter based on the total number of granules (Figure 7.11). The volume of granule bed was principally due to the granules with a size not larger than 1.0 mm in diameter. This indicates that the granules had a relatively uniform size distribution.

The apparent half velocity coefficients, K_m of the granules were determined to be 0.038 mM for propionate and 0.189 mM for butyrate, respectively. These values can be considered as the respective kinetic parameters of propionate-degrading strain PT and butyrate-degrading strain BH. These values are basically the same as those of the R-granules in Table 3.6 as expected since strains PT and BH are prevalent VFA-degrading species in the R-granules.

Figure 7.10 Granules formed by defined species. Left: Overview of the granules. Below: The surface of a granule under SEM. BH: butyrate degrading strain BH; M7: *Methanothrix* strain M7; PT: propionate degrader strain PT; T1N: *Mb. formicicum* strain T1N. Right: Homogeneous growth of strains T1N, M7, BH and PT formed the bulk of granules on TEM tin section. Individual aggregate of *Ms. mazei* T18 was associated to the granule bulk through *Mb. formicicum* strain T1N cells.

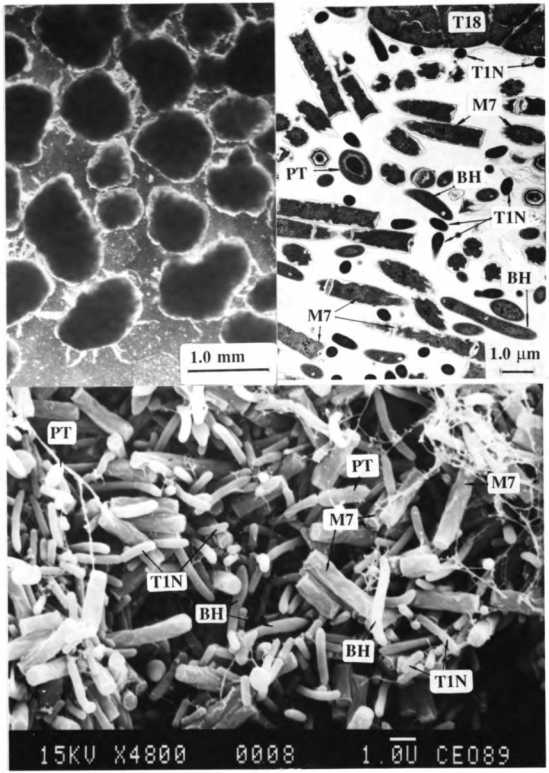

1.0 mm
T18
T1N
M7
PT
BH
T1N
BH
M7
1.0 µm
PT
PT
M7
M7
T1N
BH
BH
T1N
15KV X4800 0008 1.0U CE089

Table 7.4 Physical-chemical, metabolic and microbial characteristics of the granules developed by the defined species

Physical-chemical	
Density (gSS/L)	58
VSS/SS	0.88
specific gravity	1.023
Specific fatty acid conversion rate[a] (mmol/gSS-d)	
Acetate	43.1
Propionate	8.96
Butyrate	17.1
Apparent K_m (mM)[b]	
Propionate	0.038
butyrate	0.189
Specific COD removal rate (g COD/gSS-d)	3.22
Ratio among various defined species[c] (%)	
Mb. formicicum strain T1N	50.8
Methanothrix strain M7	30.0
Butyrate degrading strain BH	12.4
Propionate degrading strain PT	6.8

a) Determined in reactor fed with VFA mixture continuously at 35 °C. Acetate, propionate and butyrate concentrations in reactor were 5 mM, 6 mM and 1 mM, respectively. Feed contained 0.3 mM sodium sulfide as reductant.

b) The determination of K_m is described in Appendix C.

c) Determined by sequently counting the number of identified individual morphotype-cells on the cross thin section under TEM. The total cells number was 954. The number of *Methanosarcina mazei* T18 was not count into the total cell number.

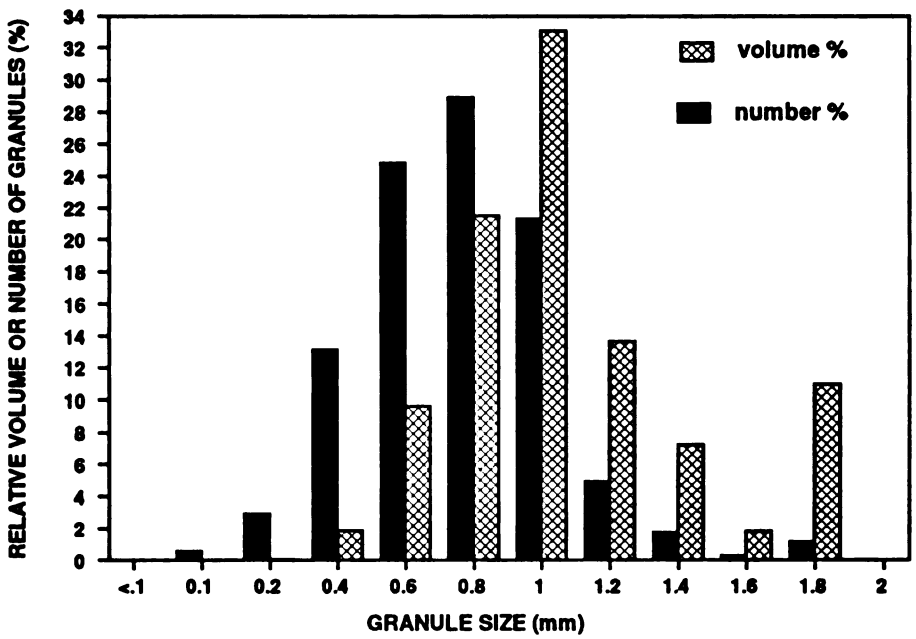


Figure 7.11 Size distribution of the granules formed by defined species.

7.5 DISCUSSION

7.5.1 The role of *Methanobacterium formicicum* species in granule formation

Mb. formicicum species (strains T1N, RF and MF) possessed aggregate-forming characteristics. They may, therefore, form a network by bridging between cells to form a granule structure when they are predominant species in granules. Recently, Koornneef *et al.* (1990) reported that methanogens antigenically related to *Mb. formicicum* strain MF were observed in all granules from five different sources. This indicates that *Mb. formicicum* is likely to be a prevalent species in anaerobic granules. *Mb. formicicum* strains T1N and RF were identified as the predominant species in the

R-granules developed on a VFA mixture (Chapter 5). Based on the TEM observation, these strains formed granule structure together with *Methanothrix* strain M7 and other bacteria.

Among the three strains, *Mb. formicicum* strain T1N was investigated more in detail. This strain was able to re-aggregate after disaggregation and formed granules in monoculture. The property of aggregation and granule-like aggregate formation of strain T1N may be linked to polymers on the cell surface, which appear to promote cells adhering to one another. The cells of strain T1N can re-aggregate under a wide pH range (3.6 to 8.5). SEM observation demonstrated that large numbers of CF particles apparently interacted with anionic groups of polymers and the surface of strain T1N. This indicates that the polymers may be strongly polarized with anionic groups. The presence of anionic groups is also supported by disaggregation at low ionic strength and subsequent re-aggregation observed as results of adding small amount of different salts. Identification and characterization of the polymers needs further study. Polymers may also produced by other strains of *Mb. formicicum* species and may be somewhat different from those produced by strain T1N since clump or aggregate-forming is a common property of this species. This property appears to make *Mb. formicicum* species important in formation of granules treating wastewater when hydrogen and formate metabolism is necessary for substrate metabolism.

In addition to *Mb. formicicum*, other H_2-CO_2 utilizing methanogens e.g. *Methanobrevibacter* sp. were also prevalent methanogens in granules (Dubourguier *at al.*, 1988a, 1988c). Their role in anaerobic granule formation needs further study.

7.5.2 Ecophysiological role of different species in granular structure

The R-granules were found to be composed primarily of H_2-CO_2 and formate-

utilizing and acetate-utilizing methanogens plus syntrophic fatty acid degraders (Chapter 3 and 5). The key species in granule formation, therefore, are likely to belong to these groups. All the species tested for compatibility and aggregation i.e. *Mb. formicicum* strain T1N, *Methanothrix* strain M7, *Ms. mazei* strain T18, *Msp. hungatei* strain BD, butyrate degrading strain BH and propionate degrader strain PT, are prevalent organisms in the R-granules. The role of each species in the formation of aggregates and resultant of the granules formed by these species is discussed below.

Mb. formicicum strain T1N is believed to be one of the essential organisms responsible for granule formation of the R-granules. This organism formed aggregates (which grew as big as 0.3 mm or more in diameter) together with strain BH. It also formed homogeneous aggregates with strains PT and *Methanothrix* strain M7 in triculture. Strain T1N grew as the major species in granular matrix together with all other species. Strain T1N, therefore, appears to play an important role in bridging the gap between the aggregates of *Methanosarcina* and the aggregates formed by other species to form granules.

That *Methanothrix* species play an essential role in granular structure has been proposed by several investigators (Wiegant and de Man, 1986; Dubourguier *et al.*, 1988a; Hulshoff Pol *et al.*, 1988; Hulshoff Pol, 1989). The results from this work support this as well. In the granules formed using the defined species, *Methanothrix* strain M7 formed a network together with *Mb. formicicum* strain T1N that appeared to support the granular structure. It was observed that this strain possesses binding properties that make aggregates with strain T1N and propionate degrading strain PT. These latter two species did not aggregate with other partners. This characteristic of strain M7 appears to be important in the formation of syntrophic anaerobic granules.

Ms. mazei strain T18 has a strong aggregation character similar to other strains

of *Methanosarcina* species. However, the role of *Methanosarcina* in granulation has been questioned (Lettinga *et al.*, 1985; Hulshoff Pol *et al.*, 1988). Except for granules developed in the presence of high acetate concentrations, *Methanosarcina* species are not normally found in high numbers. In the development of the defined species granules, *Ms. mazei* strain T18 contributed ca. 30% of cross section or 16.4% in total volume of the granules. TEM observation revealed that cells of strain T18 were always present as individual aggregates in granules and were bound to bulk matrix of granules via. *Mb. formicicum* strain T1N cells. The addition of *Methanosarcina* appeared to enhance the granulation progress. It is possible that aggregates of *Methanosarcina* provide nuclei for granule formation. Whether this role is essential for granule formation and if this role can be adequately filled by inert materials cannot be determined from these experiments.

Msp. hungatei strain BD did not grow together with strain BH in syntrophic association as aggregates, although the latter could form aggregates by itself. Strain BD was observed occasionally inside the brewery granules (Chapter 4) and only on the surface of the R-granules (Chapter 5). This indicates that *Msp. hungatei* strain BD is not essential for structures of these two granules. Therefore, we did not use it as starter culture for development of granules. However, it did grow in the clumps formed by a non-spore-forming propionate degrader strain PW (one of the prevalent propionate degraders in R-type granules) (Chapter 5).

Strain BH readily formed aggregates when it grew with *Mb. formicicum* strain T1N. Results from SEM microphotograph indicated that this strain produced polymer and, therefore, also contributed to maintenance of the granular structure. The role, if any, of spore-forming propionate degrader, strain PT, in granulation was not determined. This organism was essential, however, for propionate metabolism.

Formation of syntrophic anaerobic granules appears to be the result of contributions by a number of the species used as inoculum. Aggregate formation is likely to be the essential step that leads to granule formation. The aggregate formation by the cells of one species or multi-species is likely related to the characteristics of cell surface (extracellular polymer formation, hydrophobicity, etc.). Extracellular polymer formation by the species (*Mb. formicicum* strain T1N, *Methanothrix* strain M7, and strain BH) which induce aggregation is probably the key in helping form and stabilize granules. *Msp. hungatei* strain BD did not produce polymer and is, therefore, probably less important in granule formation.

7.5.3 Hydrophobicity and adherence

Hydrophobicity is an important characteristic of the cell surface. It probably affects the formation of aggregates and granules. The hydrophobic bacteria may play a greater role in granule formation than hydrophilic ones.

The cell surface of *Methanothrix* strain M7 appeared quite hydrophobic. This is consistent with the results from testing of *Methanothrix soehngenii* strain FE (Verrier *et al.*, 1988). *Msp. hungatei* strain BD was hydrophilic. So was *Msp. hungatei* strain JF1 (Verrier *et al.*, 1988). *Mb. formicicum* strains MF and RF were identified as being hydrophobic. The hydrophobicity of *Mb. formicicum* strain T1N could not be determined using the Rosenberg' method (1980) because this strain formed aggregates very quickly in the aqueous phase. It may, however, have the same hydrophobicity as other strains (MF and RF). *Ms. mazei* T18 was not judged using Rosenberg's method. The extracellular polymers of *Ms. mazei*, however, have been reported to be highly hydrophilic (Kreisl and Kandler, 1986). The hydrophobicity of strain BH and propionate degrading strain PT could not be determined because they did grow as

monocultures. Based on the hydrophobicity analysis, the aggregate formation by cocultures of strain T1N and strain BH is due to their hydrophobicity. This is consistent with the observation that hydrophobic bacteria tend to adhere to a higher extent to solid surfaces than do hydrophilic bacteria (Kjelleberg, 1984), and hydrophobic methanogens such as *Mtrx. soehngenii* strain FE, *Methanobrevibacter arboriphilus* strain AZ adhere well to hydrophobic inert support materials (Verier *et al.*, 1988). Based on TEM and SEM observation, the majority of cells in the R-granules were *Methanothrix* strain M7 and *Mb. formicicum* species (Chapter 3 and 5). The role of these two species in granule formation may be, in part, related to their hydrophobicity.

7.6 SUMMARY

1) *Methanobacterium formicicum* strain T1N was one of predominant species in the R-granules. This strain grew as aggregates in liquid medium and produced extracellular polymers that were identified by a cationized ferritin staining method. The cells, dispersed by washing with distilled water and sonicating could re-aggregate when a low level of salts (4 mM) was added to the medium. The R-granules had similar re-aggregation capability presumably due to the presence of a large number of cells of *Mb. formicicum* strain T1N in the granules.

2) Granule-like aggregates were formed in a laboratory upflow reactor (35 °C) within 40 days using *Mb. formicicum* strain T1N as inoculum and formic acid as the sole substrate. The size of the granules was between 0.1 to 0.7 mm with 2.6×10^8 cells/cm^2 on cross thin section.

3) *Methanothrix* strain M7 also had clump-forming characteristics. The presence of extracellular polymers, which apparently connected other cells to

complicated network, was identified using the cationized ferritin staining method.

4) The cell surface of *Mb. formicicum* species and *Methanothrix* strain M7 were more hydrophobic while *Methanospirillum hungatei* strain BD had a hydrophilic surface. In syntrophic association, clumps or aggregates were formed in a coculture consisting of strain BH plus *Mb. formicicum* strain T1N and a triculture consisting of propionate degrading strain PT, *Mb. formicicum* strain T1N and *Methanothrix* strain M7.

5) Anaerobic granules were developed in a lab-scale upflow reactor fed with a VFA mixture using defined culture consisting of *Mb. formicicum* strain T1N, *Methanothrix* strain M7, *Methanosarcina mazei* strain T18, strain BH and propionate degrader strain PT as inoculum. The maximum fatty acid degradation rates of the granules were 43.1, 8.96 and 17.1 mmol/gSS-d for acetate, propionate and butyrate, respectively. The maximum specific COD removal rate was 3.22 gCOD/gSS-d. The average size of the granules was about 0.8 mm.

6) Transmission electron microscopic observations revealed that *Mb. formicicum* strain T1N, *Methanothrix* strain M7, butyrate degrading strain BH and propionate degrading strain PT formed the bulk of the granule as homogeneous syntrophic association with a ratio of 100:59:24:13, respectively. Cells of *Ms. mazei* strain T18 existed inside the granules as its own aggregates which were apparently linked to rest of the granule via *Mb. formicicum* strain T1N. The interaction of different species in the formation of the aggregates and granules is likely related to both polymer formation and the hydrophobicity of the surface of some of the species.

CHAPTER 8

SUMMARY AND CONCLUSIONS

1. Considerable amounts of methanogenic bacteria (i.e. approximately 10^8 cells/g VSS) were enumerated in four activated sludges from different sources, indicating that activated sludge could be used as inoculum material for anaerobic reactors.

2. Anaerobic granules were developed from either anaerobic digested sewage sludge, aerobic activated sludge or defined microbial species isolated from granules as inoculum.

3. Specific organic loading rate was an important parameter affecting granulation process. The granulation progress using carbohydrate-containing wastewaters was basically similar to that using volatile fatty acid (VFA) mixtures as substrate and digested sludge as inoculum reported in literatures.

4. Anaerobic granules capable of high COD removal rates (≥7 gCOD/gVSS) were developed using an VFA mixture with low sulfate level (0.15 mM) at 35 °C. One granule type had a rod-type *Methanothrix* sp. as predominant species (the R-granules) and displayed higher mass and cell density than the other (the F-granules) which had filament-type *Methanothrix soehngenii*-like cells as predominant species. These two types displayed significant difference in operational performance. This was

due to the predominant acetate utilizing methanogen populations.

5. Both R- and F-granules had similar properties in substrate kinetics and microbial population levels for methanogens and syntrophic acetogens.

6. Sulfate reducing bacteria did not play a significant role in metabolism of H_2, formate and acetate in the R-granules in the absence or presence of sulfate. Sulfate reducing bacteria did not play a significant role in butyrate degradation but they were involved, to a limited extent, in syntrophic propionate degradation.

7. In granules developed on a sulfate-containing brewery wastewater (0.6 to 1.3 mM as SO_4^{2-}). sulfate reducing bacteria played a significant role in degradation of propionate and ethanol either via sulfate reduction or syntrophic acetogenesis but did not metabolize H_2, formate, and acetate to any significant extent.

8. At least three types of microcolonies were observed inside the brewery granules. One of these microcolonies consisted of *Methanothrix*-like rods, while the remaining types were mainly composed of non-methanogenic rods together with *Methanobacterium*-like rods. One of the non-methanogenic rods that had an irregular morphotype was, tentatively, identified as a *Desulfovibrio* sp. The remaining rods were observed to be propionate degrading, sulfate reducing bacteria. These SRB, based upon TEM and SEM observation, may play an important role in granular structure for these granules.

9. Five strains of methanogens were isolated from the R-granules and characterized. The prevalent methanogens in the R-granules were identified as *Methanobacterium formicicum* strains T1N and RF, and *Methanothrix* strain M7. A spore-forming, syntrophic propionate degrader strain PT and sulfate-reducing, propionate degrader, strain PW (which can also degrade propionate syntrophically) were isolated as the prevalent propionate degraders in the granules. Both strains are

different from other propionate degraders isolated to date. Butyrate degrading strain BH was isolated and primarily identified to be *Syntrophospora bryantii* sp., as prevalent syntrophic butyrate degrader. A rod-shaped, syntrophic isobutyrate-butyrate degrader, strain IB, was also isolated as another prevalent butyrate degrader in the granules.

10. The combination of all isolated strains can completely mineralize all VFAs from reactor feed (acetate, propionate and butyrate) and metabolic intermediates (isobutyrate, 2-methylbutyrate and valerate) formed in the reactors.

11. Syntrophic propionate degradation in the R-granules was performed by strains PT and PW together with H_2 utilizing methanogens. Syntrophic butyrate degradation was carried out by strains BH and IB.

12. Reversible isomerization between butyrate and isobutyrate occurred during syntrophic butyrate and isobutyrate degradation by the R-, F- and brewery granules. In R-granules, this reaction was performed by syntrophic isobutyrate-butyrate degrading strain IB. The reversible isomerization was coupled with syntrophic butyrate oxidation. The most thermodynamically favorable condition for the syntrophic degradation of butyrate or isobutyrate in conjunction with the isomerization was at an equal concentrations of isobutyrate and butyrate.

13. Valerate and 2-methylbutyrate were produced during syntrophic butyrate degradation by R-, F-, and brewery granules when propionate was present. In the R-granules, strain BH synthesized valerate from propionate only during syntrophic butyrate degradation. Isobutyrate-degrading strain IB was responsible for the synthesis of 2-methylbutyrate. The metabolic pathway for syntrophic valerate degradation by strains IB and BH appeared to follow β-oxidation while the pathway involved in the degradation of 2-methylbutyrate appeared to be more complicated.

14. Hydrogen and formate accumulated at an equilibrium level during syntrophic ethanol degradation by the brewery granules. Energetic analysis suggests both are intermediates of ethanol degradation.

15. Syntrophic degradation of isobutyrate and butyrate by the triculture consisting of strain IB, *Mb. formicicum* strain T1N and *Methanosarcina mazei* T18 was completely inhibited in the presence of a high hydrogen partial pressure (>2000 Pa) or a measurable level of formate (>10 to 200 μM). Significant level of formate (more than 1 mM) was detected during the perturbation with hydrogen (17 to 40 kPa). Both hydrogen and formate were likely electron carriers in the syntrophic degradation.

16. A reversible reaction between hydrogen production from formate and formate synthesis from H_2 plus HCO_3^- was observed to occur during methanogenesis by the R-granules and brewery granules. This reaction was observed during the growth of H_2-CO_2 and formate utilizing methanogens including *Mb. formicicum* species (strains MF, T1N and RF) and *Methanospirillum hungatei* strain BD. Formate synthesis was also performed by *Ms. mazei* strain T18. Unlike other strains, this strain was not able to catabolize formate to methane.

17. *Mb. formicicum* species actively formed cell aggregates and extracellular polymers, that appeared to play an important role in granule formation. Granule-like aggregates were formed in a continuously-feeding, laboratory upflow reactor using *Mb. formicicum* strain T1N as inoculum and formic acid as substrate.

18. *Methanothrix* strain M7 also had clump-forming characteristics. The presence of extracellular polymers, which apparently connected other cells to for complicated network, was identified using a cationized ferritin staining method. The cell surface of *Mb. formicicum* species and *Methanothrix* strain M7 were more hydrophobic while *Msp. hungatei* strain BD had a hydrophilic surface. In syntrophic

association, clumps or aggregates were formed in a coculture consisting of strain BH plus *Mb. formicicum* strain T1N and a triculture consisting of propionate degrading strain PT, *Mb. formicicum* strain T1N and *Methanothrix* strain M7.

19. Anaerobic granules actively degrading fatty acids were developed when *Mb. formicicum* strain T1N, *Methanothrix* strain M7, *Ms. mazei* strain T18, butyrate-degrading strain BH and propionate-degrading strain PT were used as inoculum.

CHAPTER 9

RECOMMENDATION FOR FUTURE RESEARCH

1. Further research is needed to evaluate the factors influencing stability of granules, including the composition of polymers of granules, factors effecting stability of polymers, and factors enhancing and inhibiting polymer production.

2. The factors enhancing granulation are to be further characterized. They can be either addition of microbial aggregates as bio-nuclei; or addition of inorganic and organic particles as nuclei. It is advisable to develop a fast-screening techniques to assay suitability of non-granular inoculum.

3. Development of granules with specific biodegradation capability is needed to investigate. These granules can degrade toxic compounds such as aromatic compounds, chlorinated compounds.

4. In this study, *Methanothrix*, and *Methanobacterium formicicum* have been demonstrated as important species for granulation. The role of other methanogens such as *Methanobrevobacter*, which was suggested to be a important species for granulation, is needed to examine. Hydrolytic bacteria, which are prevalent species in the granules developed on complex wastewaters, and sulfate-reducing bacteria, which formed syntrophic microcolonies with methanogens (see Chapter 4), are also needed to be evaluate their role in granule formation.

5. Some isolates in this study need to be characterized further. *Methanothrix* strain M7 (has a somewhat different morphology from *Methanothrix soehngenii.* Recent DNA-DNA hybridization data showed less-relatedness between these two bacteria. If strain M7 is a new species, it needs to be classified using other methods (such as comparison of sequence 16SrRNA). The taxonomy of syntrophic acetogen strains BH, IB and PT, and sulfate-reducing bacteria strain needs to be determined.

APPENDICES

APPENDIX A

MATERIALS, GENERAL ANALYTIC METHODS AND MEDIA

1. Chemicals, gases and materials

All chemicals used in this study except for Chapter 2 were of analytical grade and were obtained from Sigma Chemical Co. (St. Louis, MO) or Mallinckrodt., Inc.(Paris, KY) except butyric acid (Eastman Kodak Co., Rochester, NY), and yeast extract and purified agar (Difco Co., Detroit, MI). Nitrogen , hydrogen, CO_2 gases and mixtures of N_2-CO_2 (95:5), N_2-CO_2 (70:30) and H_2-CO_2 (80:20) were obtained from Union Carbide Corp., Linde Division (Warren, MI). Before use, all gases were passed over copper filings heated to 370 °C to remove traces of O_2.

Pressure tubes (27 mL in volume, Bellco Glass, Inc., Vineland, NJ) and serum bottles (58 mL or 158 mL in volume, Wheaton Scientific, Millville, NJ) with butyl rubber stoppers (American Scientific Products, McGaw Park, IL) were used for growth of anaerobic bacteria or batch experiments.

2. Analytical methods

i) Methane and CO_2

Methane was analyzed with a Hewlett-Packard model 5890A gas chromatograph

(GC) equipped with a flame ionization detector (FID) using a chromosorb 101 column and N_2 as a carrier gas (10 mL/min). The column temperature used was 60 °C. The temperatures of injector and detector were 100 and 250 °C, respectively. Hydrogen was analyzed with the same model GC equipped with a thermal conductivity detector (TCD) and a carbosphere column (Analabs-Foxboro, North Haven, CT) with N_2 (10 mL/min) as a carrier gas. The temperatures of column, injector and detector were 60, 100, and 100 °C, respectively.

CO_2 was measured using a Series 580 TCD GC (GOW-MAC Instrument Co., Bridgewater, NJ) with a TCD and Cabosphere SS column with He as a carrier gas (50 mL/min) and a column temperature of 100 °C. The temperatures of injector and detector were 100 and 150 °C, respectively. The detector current was 150 mA. Gas samples were withdrawn from the gas sampling ports on upflow anaerobic reactors or the headspace of pressure tubes or serum bottles with 1 mL glass syringes (Container Corp., Sioux City, IW) equipped with a gas tight MININERT syringe valves (Alltech Associates, Inc., Deerfield, IL) and 25 gauge needles. An injection volume of 0.4 mL was used for all cases.

ii) Volatile fatty acids

Fatty acids (acetate, propionate, butyrate, isobutyrate, 2-methyl butyrate, valerate) were determined with a Hewlett-Packard model 5890A GC equipped with a FID and Chromosorb 101 column (180 °C) and N_2 as a carrier gas (20 mL/min). The temperatures of injector and detector were 200 and 250 °C, respectively. Methanol and ethanol were also analyzed by this GC with a FID using a column temperature of 150 and 170 °C, respectively. Liquid samples were withdrawn from sampling ports on the reactor or the pressure tubes with 1 mL sterilized syringe with 23 gauge needle.

Samples were centrifuged at 2,500 x g for 5 min with an Eppendorf 5415

centrifuge (Brinkmann Instruments Inc., Westury, NY), and the supernatant was acidified using 10 N H_3PO_4 (10:1, vol. sample/vol. acid). The injection amount of samples was 3 µL for most experiments but up to 6 µL was injucted for the K_m determination in Appendix C. The detect limits of different VFAs ranged from 0.002 to 0.02 mM, depending upon the injection amount and the sensitivity of GC used.

iii) Formate, glucose and protein

Formate was determined by an enzymatic method with formate dehydrogenase by the method of Hopner and Knapper (1974). The sensitivity of this analysis was 5 µM. Samples were withdrawn from anaerobic serum bottles or pressure tubes and centrifuged at 2,500 x g for 3 min in an Eppendorf 5414 centrifuge. The supernatant was immediately transferred into a -20 °C freezer. Prior to determination, the samples were thawed at room temperature and centrifuged again. The supernatant was stored in an ice box prior to use. The samples were diluted to obtain a formate concentration of approximately 0.5 to 1.5 mM if necessary.

Glucose was determined using a YSI glucose analyzer equipped with an enzyme electrode sensor (YSI model 27, Yellow Springs Instruments Co., Yellow Springs, OH). The pre-treatment of samples were the same as that for formate determination.

To determine the protein content of granules, samples were transferred into a HCl solution (0.05N) and subsequently dispersed by repeatedly passing the granules through a glass syringe (10 mL) and a 23 gauge needle. Protein in the samples was determined by the Lowry method (Hartee, 1972) with bovine serum albumin as standard.

iv) Suspended solids, volatile suspended solids, and specific gravity

Suspended solids (SS) were determined by centrifugation (Vesilind, 1974) or filtration (APHA *et al.*, 1985). Volatile suspended solids (VSS) and specific gravity

of granules were determined according to Standard Methods (APHA *et al.*, 1985).

v) Anion concentration determination

Concentration of anions (soluble sulfate, phosphate, chloride and nitrate) were determined using a Dionex 4001i Ion-Chromatography Module/SP (Dionex Corp., Itasca, IL) with a Dionex conductivity detector and Ionpac A54A column. The determination required that the concentration of the anions was lower than 15 to 20 mg/L in order to obtain a linear response. The suspended solids in samples were removed using centrifugation prior to the determination. The samples were diluted with double distilled water as necessary, depending on the concentration of anion to be measured.

vi) Insoluble carbonate content

The insoluble carbonate content in granules was determined using a 1 mL sample centrifuged for 5 min in an Eppendorf 5415 centrifuge at 2,500 x g. The pellet was re-suspended in distilled water and the centrifugation repeated. The pellet was then weighed and divided into two portions. One half was used for dry weight (or SS) determination and the remainder was transferred into a 158 mL serum bottle. The bottle was sealed with a butyl stopper and the CO_2 background concentration was determined by GC. HCl (6.5 N, 5 mL) was then injected into the bottle, and the sample was vigorously agitated for 30 min. The carbonate content in the sample was calculated using the increase in CO_2 partial pressure, and the calculated dissolved CO_2 in the liquid.

vii) Optical density

The optical density or light absorbance of bacterial cultures and reactor effluent were determined using a spectrophotometer model SIMADZU UV-160 (Simadzu Corp., Kyoto, Japan) at 400 or 660 nm.

3. Preparation of media and stock solutions

i) Basal medium (BS)

Basal (BS) medium was used for growing methanogens and syntrophic acetogens. It was modified from the phosphate buffered basal (PBB) medium (Kenealy and Zeikus, 1981). The following components were added into one liter double distilled water: NaCl, 0.9 g; $MgCl \cdot 6H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; NH_4Cl, 1.0 g; trace mineral solution (for BS), 10 mL; and resazurin solution (0.2%), 1.0 mL. pH was adjusted to 7.2 to 7.4 using NaOH solution (1N). This medium was boiled under an oxygen-free nitrogen atmosphere for 20 min and then, when it became cool, was dispensed into pressure tubes or serum bottles under the nitrogen atmosphere. The tubes or bottles were sealed with butyl rubber stoppers and aluminum cramps and then autoclaved under 121 °C for 30 min.

ii) Basal medium for sulfate-reducing bacteria (BSS)

The medium for growth of sulfate-reducing bacteria was modified from the medium used for the growth of *Desulfobulbus propionicus* (Widdel and Pfennig, 1984). The following chemicals were added to one liter double distilled water: Na_2SO_4, 3.0 g; KH_2PO_4, 0.2 g; NH_4Cl, 0.3 g; NaCl, 1.0 g; $MgCl_2 \cdot 6H_2O$, 0.4 g; KCl, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.15g; $NaHCO_3$, 5 g; and resazurin solution (0.2%), 1.0 mL. The medium was prepared by adding all above chemicals except for $NaHCO_3$, boiled under the oxygen-free nitrogen atmosphere for 20 min and then was allowed to cool under an N_2-CO_2 gas mixture (70:30) atmosphere. Subsequently, $NaHCO_3$ (5 g) was added into the cooled medium and completely dissolved. The medium was dispensed to serum bottles or pressure tubes under the N_2-CO_2 atmosphere and then autoclaved at 121 °C for 30 min.

Sulfate-free BSS medium was also prepared for the growth of methanogens and syntrophic acetogens and for other special tests. In this case, Na_2SO_4 was not added.

iii) Trace mineral solutions

(1) Trace mineral solution for the BS medium

This mineral solution was used in the BS medium for the growth of methanogen and syntrophic acetogens, prepared as described by Kenlealy and Zeikus (1981). It contains (per one liter double distilled water): Nitrilotriacetic acid (NTA), 12.8 g; $FeSO_4 \cdot 7H_2O$, 0.10g; $MnCl_2 \cdot 4H_2O$, 0.10 g; $CoCl_2 \cdot 6H_2O$, 0.17 g; $CaCl_2 \cdot 2H_2O$, 0.10 g; $ZnCl_2$, 0.10 g; $CuCl_2 \cdot 2H_2O$, 0.02 g; H_3BO_3, 0.01 g; Na_2MoO_4, 0.01 g; NaCl, 1.00 g; Na_2SeO_3, 0.017 g; and $NiSO_4 \cdot 6H_2O$, 0.26 g. Initially, NTA was added to 200 mL of double distilled water, and the pH was then adjusted to 6.5 using an KOH solution (5N) until all crystals of NTA were completely dissolved. Subsequently, about 600 mL of the water was added to above solution. Each compound was then dissolved in the solution in the sequence listed. Finally, the water was added to the solution to attain an one liter volume.

The trace mineral solution was added to the basal medium prior to autoclaving.

(2) Trace mineral solution for BSS medium

This mineral solution was used in the BSS medium and sulfate-free BSS medium. It was modified from the trace mineral solution used for *Desulfobulbus* sp. (Widdel and Pfennig, 1984). It contains (per 1000 mL double distilled water): HCl (12N), 6.4 mL; $FeCl_2 \cdot 4H_2O$, 1.5 g; $ZnCl_2$, 0.07 g; $MnCl_2 \cdot 4H_2O$, 0.1 g; H_3BO_3, 0.06 g; $CoCl_2 \cdot 6H_2O$, 0.19 g; $CuCl_2 \cdot 2H_2O$, 0.002 g; $NiCl_2 \cdot 6H_2O$, 0.024 g; and $Na_2MoO_4 \cdot 2H_2O$, 0.36 g. First, $FeCl_2 \cdot 4H_2O$ was dissolved in the HCl, and then this solution was mixed with 990 mL of the water. Subsequently, the other salts were dissolved to the solution. pH was adjusted to 6.0 with NaOH solution (1N). Finally, the solution

volume was made up to 1000 mL.

The trace mineral solution was dispensed to several serum bottles (158 mL), and these bottles were then sealed with butyl rubber stoppers and aluminum cramps. Each bottle contained 50 mL of the solution. These bottles were repeatedly flushed with oxygen-free nitrogen and subsequently drawn with vacuum for 30 min, and then autoclaved at 121 °C for 25 min. The trace mineral solution (for BSS) was added to the BSS medium before inoculation.

iv) Vitamin solution

The vitamin solution used was that of Wolin *et al.* (1963). It contains (per 1000 mL double distilled water): biotin, 2 mg; folic acid 2 mg; pyridoxine-HCl (B_6), 10 mg; thiamine-HCl (B_1), 5 mg; riboflavin (B_2), 5 mg; nicotinic acid, 5 mg; pantothenic acid, 5 mg; cyanocobalamin (B_{12}), 0.1 mg; and P-aminobenzoic acid (PABA), 5 mg. The above compounds were added in the sequence listed to ensure complete dissolution. The solution was then filter sterilized (via 0.25 µM filter) and stored in serum bottles, which had been vacuum flushed with oxygen-free nitrogen gas and then autoclaved.

v) Stock solutions

(1) 2.5% $Na_2S{\cdot}9H_2O$ stock solution was prepared under an oxygen-free N_2 atmosphere. Double distilled water was boiled for 15 min in a flask under an N_2 gas atmosphere and then cooled for 5 min. A portion of this water was removed and set aside under an N_2 gas atmosphere. Crystals of $Na_2S{\cdot}9H_2O$ (25 g) were transferred into the flask (containing near 1000 mL water) and dissolved by stirring. When the solution was cooled to room temperature (25 °C), concentrated HCl (12 N) was added to adjust pH to 9.5 under the N_2 atmosphere. The solution was then brought to a volume of 1000 mL with the water set aside, dispensed into serum bottles, and autoclaved at 121 °C for 25 min.

(2) The following stock solutions were prepared by directly dissolving solid chemicals in double distilled water, repeatedly vacuum-flushing with the oxygen-free nitrogen gas for 30 min, and autoclaving under 121 °C for 25 min, which including:

-- 10% sodium bicarbonate (10 g $NaHCO_3$ in 100 mL water);

-- 2.76 M phosphate solution (15 g KH_2PO_4 and 29 g K_2HPO_4 in 100 mL water);

-- 1 M Na_2SO_4 solution;

-- 4 M sodium formate, 1 M sodium acetate, 4 M sodium acetate, 1 M sodium propionate, and 1 M sodium butyrate solutions.

(3) The following stock solutions were prepared by adding the respective quantity of solvents in 50 mL of double distilled water (neutralizing with 10 N NaOH solution, if it was a fatty acid except for formic acid), and then bringing to the volume of 100 mL. The solutions were then dispensed into serum bottles, repeatedly vacuum-flushed with N_2, and then autoclaved, which including:

-- 1 M isobutyrate, 1 M valerate, 1 M 2-methylbutyrate, 0.5 M caproate, 0.5 M 4-methylvalerate, 0.25 M heptanoate, 0.25 M 2-methylhexanoate, 0.1 M caprylate, and 0.1 M pelargoate solutions;

-- 50% formic acid, 20% methanol, 1 M ethanol, 1 M propanol, 1 M isopropanol, 1 M butanol, and 1 M isobutanol solutions.

(4) The following stock solutions were prepared using filter sterilization as the procedures for preparation of vitamin solution:

-- 0.5 M $FeSO_4$ solution;

-- 1 M sodium pyruvate solution;

-- 100 g/L glucose and 100 g/L lactose solutions;

-- 1 M urea solution;

-- 1 M cysteine-HCl solution.

4. Media for growth and maintenance of different microbial species

i) Methanogens

(1) *Methanobacterium formicicum* strains T1N, RF and MF

The BS medium (50 mL) in 158 mL serum bottles was supplemented with following solutions: vitamin, 0.5 mL; 10% bicarbonate, 0.5 mL; 2.76 M phosphate, 0.25 mL; 1 M sodium acetate; 0.2 mL; and 2.5% $Na_2S \cdot 9H_2O$, 0.5 mL. The bottles were then pressurized with an H_2-CO_2 (80:20) gas mixture to 1.5 atm. Inoculum of 1 to 2% (vol/vol) was then added using sterile syringes.

(2) *Methanospirillum hungatei* strain BD

The BS medium (50 mL) in 158 mL serum bottles was supplemented with following solutions: vitamin, 0.5 mL; 10% bicarbonate, 2.0 mL; 2.76 M phosphate, 0.10 mL; 1 M sodium acetate; 0.2 mL; and 2.5% $Na_2S \cdot 9H_2O$, 0.5 mL. The bottles were then pressurized with an H_2-CO_2 (80:20) gas mixture to 1.5 atm. Inoculum at 2 to 4% (vol/vol) was then added.

(3) *Methanosarcina mazei* strain T18

The BS medium (50 mL) in 158 mL serum bottles was supplemented with following solutions: vitamin, 0.5 mL; 10% bicarbonate, 1.0 mL; 2.76 M phosphate, 0.1 mL; 4 M sodium acetate; 0.25 mL; and 2.5% $Na_2S \cdot 9H_2O$, 0.4 mL. The bottles were then pressurized with H_2-CO_2 (80:20) gas mixture to 1.0 atm. The settled aggregates (0.1 to 0.2 mL) were used as inoculum.

(4) *Methanothrix* strain M7

The BS medium (50 mL) in 158 mL serum bottles was supplemented with following solutions: vitamin, 0.5 mL; 10% bicarbonate, 2.5 mL; 2.76 M phosphate, 0.2

mL; 4 M sodium acetate; 0.2 mL; and 2.5% $Na_2S \cdot 9H_2O$, 0.2 mL. The bottles were then pressurized with N_2-CO_2 (70:30) gas mixture to 1.0 atm. Inoculum of 5 to 10% (vol/vol) was then added.

ii) Syntrophic acetogens

(1) Coculture of strain PT plus *Methanobacterium formicicum* strain T1N

The BS medium (50 mL) in 158 mL serum bottles was supplemented with following solutions: vitamin, 0.5 mL; 10% bicarbonate, 2.0 mL; 2.76 M phosphate, 0.25 mL; 1 M sodium propionate; 0.75 mL; and 2.5% $Na_2S \cdot 9H_2O$, 0.5 mL. The bottles were then pressurized with N_2-CO_2 (70:30) gas mixture to 1.0 atm. Inoculum of 10-20% (vol/vol) was the added.

(2) Coculture of strain PW plus *Methanospirillum hungatei* strain BD (or *Methanobacterium formicicum* RF)

The sulfate-free BSS medium (50 mL) in 158 mL serum bottles was supplemented with following solutions: vitamin, 0.5 mL; 1 M sodium propionate; 0.75 mL; 0.5 M Na_2SO_4, 0.02 mL; trace mineral solution (for BSS), 0.2 mL; and 2.5% $Na_2S \cdot 9H_2O$, 0.5 mL. The bottles were then pressurized with N_2 gas mixture to 1.0 atm. Inoculum of 5 to 10% (vol/vol) was then added.

(3) Monoculture of strain PW

The BSS medium (50 mL) in 158 mL serum bottles was supplemented with following solutions: vitamin, 0.5 mL; 1 M sodium propionate; 0.75 mL; trace mineral solution (for BSS), 0.2 mL; and 2.5% $Na_2S \cdot 9H_2O$, 0.5 mL. The bottles were then pressurized with N_2 gas mixture to 1.0 atm. Inoculum of 5 to 10% (vol/vol) was then added.

(4) Coculture of strain BH and *Methanobacterium formicicum* strain T1N

The BS medium (50 mL) in 158 mL serum bottles was supplemented with

following solutions: vitamin, 0.5 mL; 10% bicarbonate, 2.0 mL; 2.76 M phosphate, 0.25 mL; 1 M sodium butyrate; 0.5 mL; and 2.5% $Na_2S \cdot 9H_2O$, 0.5 mL. The bottles were then pressurized with N_2-CO_2 (70:30) gas mixture to 1.0 atm. Inoculum of 5 to 10% (vol/vol) was then added.

(5) Triculture strain IB, *Methanobacterium formicicum* strain T1N and *Methanosarcina mazei* strain T18

The BS medium (50 mL) in 158 mL serum bottles was supplemented with following solutions: vitamin, 0.5 mL; 10% bicarbonate, 2.0 mL; 2.76 M phosphate, 0.2 mL; 1 M sodium isobutyrate; 0.5 mL; and 2.5% $Na_2S \cdot 9H_2O$, 0.4 mL. The bottles were then pressurized with an N_2-CO_2 (70:30) gas mixture to 1.0 atm. Inoculum of 5 to 10% (vol/vol) was added.

5. Media for substrate conversion tests

These media were used in substrate conversion or degradation tests by anaerobic granules performed in Chapter 3, 4, 5 and 6.

In VFA (acetate, propionate, and butyrate) and formate conversion tests, the BS medium (50 mL) in 158 mL serum bottles was supplemented with following stock solutions: 10% bicarbonate, 2.5mL; 2.76 M phosphate, 0.2 mL; 2.5% $Na_2S \cdot 9H_2O$, 0.2 mL; and respective substrate stock solution. The bottles were then pressurized with 1 atm N_2-CO_2 (70:30) gas mixture.

When H_2-CO_2 (80:20) gas mixture was used as substrate (1.0 atm), addition of bicarbonate (10%) was 3.0 mL.

Sulfate was supplied, when required for test sulfate reduction, by addition of $FeSO_4$ stock solution (0.5 M).

APPENDIX B

DETERMINATION OF MAXIMUM SUBSTRATE CONVERSION RATES AND SPECIFIC GROWTH RATES IN REACTORS

1. The maximum substrate conversion rates

The maximum substrate conversion rates by the R- and F-granules were determined in laboratory-scale upflow reactors which are described in Chapter 3 (Figure 3.1), based on substrate consumption with the exception of formate. Prior to addition of the substrate pulse, all bacterial flocs and fine particles accumulated in the top settlers were completely removed using syringes. The feed was stopped for half an hour, but not the recycle, in order to allow the bacteria to consume most of the substrates. The maximum substrate conversion rates were determined using pulse feed of respective stock solutions. The following stock solutions were used: sodium formate, 4 M; formic acid, 50% (vol/vol); sodium acetate, 4 M; sodium propionate, 1 M; sodium butyrate, 1 M; sodium isobutyrate, 1 M; sodium valerate, 1 M; methanol, 20% (vol/vol); ethanol, 1 M; and glucose, 100 g/L. They were prepared anaerobically as described in Appendix 1.

The stock solutions (except for formate and formic acid) were slowly injected into the top settler through a syringe and were mixed by shaking the settler gently and

increasing the recycle rate from about 30 mL/min to 60 mL/min. The injection volume of the respective substrates was calculated to attain concentrations of 40 mM methanol, 20 mM ethanol, 15 mM acetate, 10 mM propionate, 10 mM butyrate, 10 mM isobutyrate, 10 mM valerate, and 2.0 g/L glucose within the reactors, respectively. Approximately 10 minutes after the injection, liquid samples were withdrawn through a sampling port on the reactor every 10 to 15 minutes for analysis to obtain the time-dependent substrate concentration change and the maximum substrate conversion rates per reactor (mmol/hr).

The maximum volumetric conversion rates (mol/L granules-day) and the maximum specific conversion rates (mmol/gVSS-d) were then calculated based on the granule volume (liter) in the reactors and the granule bed density (gVSS/L granules).

i) Acetate, propionate, isobutyrate, valerate, methanol, ethanol and glucose

The equation for the calculation of substrate conversion rates of acetate, propionate, valerate, methanol, ethanol, and glucose is described as follows:

$$dS/dt = (S_{t1} - S_{t2}) \cdot V_R / (1000 \cdot (t_2 - t_1)) \qquad \text{(B-1)}$$

where, dS/dt is substrate conversion rate per reactor (mmol/hr); S_{t1} and S_{t2} are substrate concentration (mM) inside reactor at time t_1 and t_2, respectively; V_R is the liquid bulk volume of the total reactor system (mL).

Initially, the value for V_R was calibrated by injecting a certain amount (V_p, 1 mL was used) of sodium propionate solution (1 M) to the reactors using equation (B-1) and following equation:

$$V_R = (1000 \cdot V_p/1000 - T \cdot dS_p/dt)/S_T \qquad \text{(B-2)}$$

where, V_p is the volume of propionate solution (mL); S_T is the propionate concentration after T hr (1/6 hr i.e. 10 min was used) of injection; dS_p/dt is the maximum propionate conversion rate (mmol/hr). The value for V_R was calculated by assuming that an initial

value of V_R equals the total reactor volume (265 mL) minus the granule bed volume. By iteratively estimating the initial dS_p/dt from equation (B-1), and then calculating the value of V_R from equation (B-2) until the deviation between two V_R values was less than 0.5%, the calculated values for V_R were approximately 225 and 235 mL for reactors R and F in Chapter 3, respectively.

As soon as the value for V_R was obtained, it was used for the calculation of the other substrate conversion rates.

ii) Butyrate

For butyrate conversion, the total conversion rate, the conversion rate of butyrate to acetate, and conversion rate of butyrate to isobutyrate were determined simultaneously, because isomerization between butyrate and isobutyrate occurred during the pulse feed tests (see Chapter 6):

$$\text{Isobutyrate}^- \rightleftharpoons \text{Butyrate}^- \rightarrow 2\ \text{Acetate}^- + 0.5\ CH_4$$

The total maximum butyrate conversion rates were calculated using equation (B-1), the conversion rate of butyrate to isobutyrate was

$$(dS/dt)_{ib} = (S_{ibt2} - S_{ibt1}) \cdot V_R / (1000 \cdot (t_2 - t_1)) \qquad \text{(B-3)}$$

where, $(dS/dt)_{ib}$ is conversion rate for butyrate to isobutyrate (mmol/hr); S_{ibt1} and S_{ibt2} are isobutyrate concentration (mM) inside reactor at time t_1 and t_2, respectively. The rate of conversion of butyrate to acetate was calculated as follows,

$$(dS/dt)_b = (S_{bt1} + S_{ibt1} - S_{bt2} - S_{ibt2}) \cdot V_R / (1000 \cdot (t_2 - t_1)) \qquad \text{(B-4)}$$

where, $(dS/dt)_b$ is conversion rate for butyrate to acetate (mmol/hr); S_{bt1} and S_{bt2} are butyrate concentration (mM) inside reactor at time t_1 and t_2, respectively.

iii) Formate

The conversion rates of formate were determined based on formation of products (methane and hydrogen). Initially, a formate stock solution (4M, pH 7) was

injected into the settler of the reactor to attain a concentration of ca. 30 mM formate within the reactor system. After 10 minutes, a certain amount of 50% anaerobic formic acid solution (pH 2) was slowly injected into the reactor every two min to keep the pH inside the reactor between 6.9 and 7.1. Methane and hydrogen content in the biogas were analyzed every 3 min and gas production was measured by a liquid displacement method (35 °C). During the pulse feed test of formate, methane and hydrogen were produced since the granules performed the following reactions (see Chapter 6):

$$4HCOOH + H_2O + H^+ \rightarrow CH_4 + 3\ HCO_3^-$$

and $$HCOOH + H_2O \rightarrow H_2 + HCO_3^- + H^+$$

In the short-term test, the formate used for growth was ignored. The total conversion rate, the rate of formate to methane, and the rate of formate to hydrogen were calculated by using the following equations,

$$dS_f/dt = dV/dt\{(4 \cdot f_{CH4} + f_{H2}) \cdot 273/[(273+35) \cdot 22.4]\} \quad \text{(B-5)}$$

$$(dS/dt)_{CH4} = dV/dt\ \{4 \cdot f_{CH4} \cdot 273/[(273+35) \cdot 22.4]\} \quad \text{(B-6)}$$

and $$(dS/dt)_{H2} = dV/dt \cdot f_{H2} \cdot 273/[(273+35) \cdot 22.4] \quad \text{(B-7)}$$

where, dS_f/dt, $(dS/dt)_{CH4}$, and $(dS/dt)_{H2}$ are the total conversion rate (mmol/hr), the rate of formate to methane, and the rate of formate to hydrogen, respectively; dV/dt is measured biogas production (mL/hr), f_{CH4} and f_{H2} are average methane and hydrogen fraction in biogas.

2. Specific growth rates of different trophic groups

The specific growth rates for acetate, propionate and butyrate degraders were determined in reactors operated at 35 °C. During the determination period (6 to 7 days were used), concentrations of acetate, propionate and butyrate in the reactors were controlled at relatively steady levels (see Table 3.5 in Chapter 3) by adjusting feed rate

on a daily basis. All the biomass (including granules and flocs) were kept inside reactors. The specific growth rates of the different trophic bacteria in the reactor, including granules and flocs, were determined based on the increase in substrate conversion (to methane or acetate plus methane) rates (mmol/day per reactor) which were assumed as proportional to the respective biomass. The substrate conversion rates of propionate, butyrate and acetate were calculated using equations (B-8), (B-9) and (B-10), respectively

$$dS_p/dt = (S_{pin}-S_{pef})\cdot Q \qquad \text{(B-8)}$$

$$dS_b/dt = (S_{bin}-S_{bef}-S_{ibef})\cdot Q \qquad \text{(B-9)}$$

and $$dS_a/dt = (S_{ain}+S_{cin}-S_{aef}-S_{cef})\cdot Q + dS_p/dt + 2\cdot dS_b/dt \qquad \text{(B-10)}$$

where dS_p/dt, dS_b/dt, and dS_p/dt are conversion rates (mmol/d per reactor) of propionate, butyrate and acetate, respectively; S_{pin}, S_{bin}, S_{cin} and S_{ain} were the influent concentrations (mM) of propionate, butyrate, ethanol, and acetate, respectively; S_{pef}, S_{bef}, S_{cef} and S_{aef} were the effluent concentrations (mM) of propionate, butyrate, ethanol and acetate, respectively; Q is feed rate (L/d). In above equations, the effect of isomerization between butyrate and isobutyrate on the conversion rate of butyrate is considered while the effect of formation of valerate and 2-methylbutyrate on the conversion rates for propionate and butyrate was ignored because of their low concentrations (<0.05 mM). For the acetate conversion rate, the amounts of acetate produced from the degradation of propionate, butyrate and ethanol were taken in account. The small fraction of fatty acid conversion contributed by glucose (2mM) in medium B was ignored. The specific growth rates were calculated using linear semi-logarithmic regressions of the conversion rates vs time (see Figure 3.3).

APPENDIX C

DETERMINATION OF APPARENT HALF VELOCITY COEFFICIENTS (K_m) USING TIME-COURSE TESTS

1. Equations

In anaerobic digestion, Monod kinetics are widely used to model the process of substrate degradation, especially for fatty acids (acetate, propionate and butyrate). Under normal concentration ranges observed in digesters, these equations fit experimental observations quite well (Lawerence and McCarty, 1970; O'Rourke, 1968). The Monod equations are expressed as

$$dX/dt=-Y\cdot dS/dt-bX \quad \text{(C-1)}$$

$$\mu=\mu_{max}\cdot S/(K_s+S)-b \quad \text{(C-2)}$$

$$dS/dt=-V_{max}\cdot X\cdot S/(K_s+S) \quad \text{(C-3)}$$

where, X = microorganism concentration, g/L

S = substrate concentration, mM (or g/L)

Y = yield coefficient, g organism/mmol (or g) substrate converted

μ = microorganism growth rate, 1/d

μ_{max} = microorganism maximum growth rate, 1/d

V_{max} = maximum specific substrate conversion rate,

mmol (or g) substrate/g organism-d

K_s = half-velocity coefficient, mmol substrate/L (or g/L)

b = microorganism decay rate, 1/d

When reaction occurs over a short period (compared with the doubling time of microorganisms), the growth of the organisms can be ignored. In this case, equation (C-3) is equivalent to the Michaelis-Menten equation:

$$-dS/dt=k\cdot S/(K_m + S) \qquad \text{(C-4)}$$

where, k = maximum substrate conversion rate, mmol/L-d (or g/L-d)

K_m = Michaelis-Menten coefficient, mmol/L (or g/L)

The value of K_s in equation (C-3) can, therefore, be estimated using the value of K_m observed during short term substrate conversion. For the microorganisms growing as flocs (or granules), the value of K_m determined is influenced by substrate diffusion within the flocs (Ngian *et al.*, 1977; Dolfing, 1985). Usually, the value determined is considered to be an apparent K_m value.

The apparent K_m value can be estimated from an integrated solution to the Michaelis-Menten equation (Halwachs, 1978):

$$t/U = K_m/k\cdot(1/U\cdot\ln(1/(1-U))-1) + (S_0 + K_m)/k \qquad \text{(C-5)}$$

where, $U= (S_0-S_t)/S_0$, S_0 is the initial substrate concentration, S_t is the substrate concentration at time t. The advantage for this method is that only one time-course experiment is required to estimate the kinetic parameters.

The apparent K_m can also be determined using a Lineweaver-Burk plot:

$$1/v = K_m/k\cdot(1/S) + (1/k) \qquad \text{(C-6)}$$

or

$$S/v = K_m/k + (1/k)\cdot S \qquad \text{(C-7)}$$

where, v = dS/dt. This technique requires a series of experiments, consisting of

several initial substrate concentrations, to obtain the degradation rate at different substrate concentrations. Simply, the average rate and average substrate concentration obtained from a single time-course experiment can introduce a relatively large error.

2. Determination of K_m values of R- and F-granules

i) Procedures

The apparent K_m values of the R- and F-granules (see Chapter 3) were determined for acetate, propionate, isobutyrate, butyrate and valerate. Determination was performed using serum bottles (158 mL) in a 35 °C shaking water bath (125 strikes/min). The BS medium (50 mL) used was buffered with 90 mM $NaHCO_3$ and pressurized with 0.4 atm CO_2 gas, resulting in a pH of 6.8. Sodium sulfide (0.35 mM) was used as a reductant. Prior to these tests, granules (approximately 1 mL) were withdrawn anaerobically from operational reactors using a glass syringe (1 mL) equipped with an 18 gauge needle and transferred into a serum bottle containing the reduced medium which had been warmed in the 35 °C water bath. To reduce the mass transfer effects, the granules were further dispersed by repeatedly passing the granules through a glass syringe (1 mL) with a 25 gauge needle and re-injecting into the bottle. After adding the respective substrates (2 mM), the bottles were incubated at 35 °C for 2 hours for activation of the respective trophic group. Subsequently, liquid samples were withdrawn from every 1 to 0.1 hours for determination of substrate conversion till the substrate was consumed. Finally, the disrupted granules retained in the vials were centrifuged at 4000 rpm using a Sorvall RC5C centrifuge with SS34 rotor (Du Pont Co., Newtown, CT) for 5 min. The pellet was used for suspended solids (SS) determination.

In this assay, the plot for the integrated solution to Michaelis-Menten equation

(C-5) was used for the determination of K_m for acetate, propionate, isobutyrate and valerate. During syntrophic butyrate degradation, the concentration of isobutyrate formed was significant since the reversible isomerization between isobutyrate and butyrate occurred (see Chapter 3 and Chapter 6). It, therefore, was impossible to use the plot for the integrated solution. The K_m for butyrate was determined using the Lineweaver-Burk plot (C-7). The conversion rate (dS_b/dt) was calculated follows:

$$dS_b/dt=(S_{b1}-S_{b2}+S_{ib1}-S_{ib2})/(t_2-t_1) \qquad \text{(C-8)}$$

where, S_{b1},S_{b2},S_{ib1} and S_{ib2} are butyrate and isobutyrate concentration at time t_1 and t_2, respectively.

ii) Results and discussion

The results of the K_m determination for the both R- and F-granules are presented in Table C.1. Figure C.1 to C.10 describe the time-course of respective substrate degradation and the plots using the integrated solution (equation C-5) or modified Lineweaver-Burk expression. The lower standard deviation (<4%) among the determined K_m values were observed for acetate degradation while higher standard deviation (22 to 37%) was observed for isobutyrate degradation. In general, the standard deviation for these assays was ca. 2.3 to 37% using the integrated solution (C-5), and ca. 3.1 to 9.3% using the Lineweaver-Burk plot.

Assays were completed within 6 hrs for acetate degradation (Figures C.1 and C.2), 13 hours for propionate degradation (Figure C.3 and C.4), and 5 hours for butyrate (Figures C.5 and C.6), isobutyrate (Figures C.7 and C.8) and valerate degradation (Figures C.9 and C.10). Compared with the doubling time of 30 hours to 7 days for acetate utilizing methanogens, 5 to 7 days for syntrophic propionate degraders, and 50 to 70 hours for syntrophic butyrate degraders, growth of the microorganisms can be considered insignificant. In all the assays (pH 6.8 to 7.0), the

concentrations of fatty acids were low, and potential inhibition due to non-ionized acids was eliminated. During the degradation of propionate, isobutyrate, butyrate and valerate, the acetate concentration and hydrogen partial pressure were low, resulting in favorable energetics for degradation.

During the assay for acetate degradation, the initial acetate concentrations were approximately 6.0 to 7.0 mM, the data were well fit to the integrated solution (Figures C.1 and C.2) with good reproducibility (Table C.1). This indicates that acetate degradation in the granules can be expressed via Monod or Michaelis-Menten equation within this concentration range.

The data for the propionate assay fit equation (C-5) as shown in Figures C.3 and C.4. However, a slightly higher standard deviation was observed in the K_m values calculated for the R-granules.

During the assay for butyrate, significant formation of isobutyrate was observed (Figures C.5 and C.6). The integrated solution (C-5) could not be used, therefore, to describe the degradation of butyrate to methane (or β-oxidation). Considering that isobutyrate was degraded via butyrate (see Chapter 6), equation (C-8) was used to calculate the rate of butyrate degradation. The average concentration between two sequenced samples was used for plot. The data obtained were fit to equation C-7 and good regression coefficients were obtained.

When isobutyrate was degraded, a low level of butyrate (<0.06 mM) was observed (Figures C.7 and C.8). Considering butyrate was an intermediate of isobutyrate degradation (see Chapter 6), the K_m was determined using the integrated equation. The data was fit by equation (C-5), as shown in Figures C.7 and C.8. A relative high standard deviation was observed from the calculated K_m values. This may be due to neglecting the isomerization reaction.

The degradation of valerate appeared to fit equation C-5 although during the assay low levels of acetate and propionate accumulated as intermediates (Figures C.9 and C.10).

3. Determination of K_m value of *Methanothrix* strain M7 for acetate

i) Procedures

Cells of *Methanothrix* strain M7, isolated from the R-granules (see Chapter 5), were grown on acetate at 35 ℃ in serum bottles containing 60 mL medium. After supplementing the bottles with acetate 4 times (15 mM per time), the absorbency density (OD) of the cell suspension in the bottles reached 0.14 at 660 nm. The cultures were than used for the K_m determination. The assay was performed in the 35 ℃ shaking water bath (125 strikes/min). Liquid samples were withdrawn from the bottles periodically for acetate analysis. The initial concentration of acetate was approximately 4.0 mM. The pH change (from 7.03 to 7.05) during this assay was insignificant.

ii) Results and discussion

The assay lasted 38 to 45 hours. The doubling time of tested cultures were observed to be 7 days, being much longer than the doubling time under optimum conditions (see Chapter 5). The effect of cell growth was, therefore, ignored. A typical acetate degradation curve and the plot for the integrated solution is presented in Figure C.11. The K_m for acetate degradation was ca. 0.41±0.03 mM. This is consistent to the K_m values obtained from the R-granules, in which *Methanothrix* strain M7 was predominant acetate-utilizing species.

4. Determination of K_m values of the granules developed using defined species

i) Procedures

The apparent K_m values for the degradation of propionate and butyrate were determined for the granules developed using defined species which were isolated from the R-granules (see Chapter 7). The procedures were the same as those for the R- and F-granules. The K_m values were calculated using the plot for the integrated solution.

ii) Results and discussion

The assay for propionate degradation was completed within 7 hours. The acetate produced during propionate degradation was low (<0.05 mM). The data were well fit to the integrated solution, as shown in Figure C.12. The K_m value determined was ca. 0.038 mM, being close to the values obtained from the R-granules.

The isomerization of butyrate to isobutyrate was not observed in this assay since the defined species did not include isobutyrate-butyrate degrading species. The K_m value for butyrate degradation determined was ca. 0.189 mM (Figure C.13).

5. Conclusion

1) The data of degradation of acetate, propionate, isobutyrate, butyrate and valerate at low concentrations in a short term can be expressed using Michaelis-Menten relation.

2) The apparent half velocity coefficient (K_m) for acetate, propionate, isobutyrate and valerate can be estimated the plotting data from time-course experiments using the integrated solution to Michaelis-Menten equation. The K_m value for butyrate degradation can also estimated using this method if isomerization between butyrate and isobutyrate is not present.

3) When isomerization occurs, the K_m value for butyrate degradation can be estimated using Lineweaver-Burk plot.

Table C.1 Results of apparent K_m values (mM) for R- and F-granules

Substrate	R-granules			F- granules		
	K_m	R	Average K_m	K_m	R	Average K_m
Acetate	0.430	0.9963	0.43±0.01	0.414	0.9893	0.42±0.016
	0.427	0.9846		0.402	0.9946	
	0.420	0.9799		0.433	0.9920	
Propionate	0.069	0.9956	0.048±0.018	0.0369	0.9781	0.037±0.005
	0.036	0.9916		0.0376	0.9875	
	0.0378	0.9913				
Butyrate	0.143	0.9913	0.15±0.014	0.186	0.9888	0.19±0.006
	0.148	0.9964		0.194	0.9768	
	0.169	0.9996				
Isobutyrate	0.156	0.9882	0.15±0.034	0.190	0.9941	0.15±0.056
	0.190	0.9941		0.110	0.9854	
	0.121	0.9950				
Valerate	0.121	0.9975	0.134±0.013	0.0925	0.9867	0.100±0.009
	0.146	0.9975		0.110	0.9848	
	0.136	0.9992		0.0976	0.9923	

(1) Apparent K_m values for degradation of acetate, propionate, isobutyrate, and valerate were calculated using equation (C-5).
(2) The K_m values for degradation of butyrate were calculated using equation (C-7).

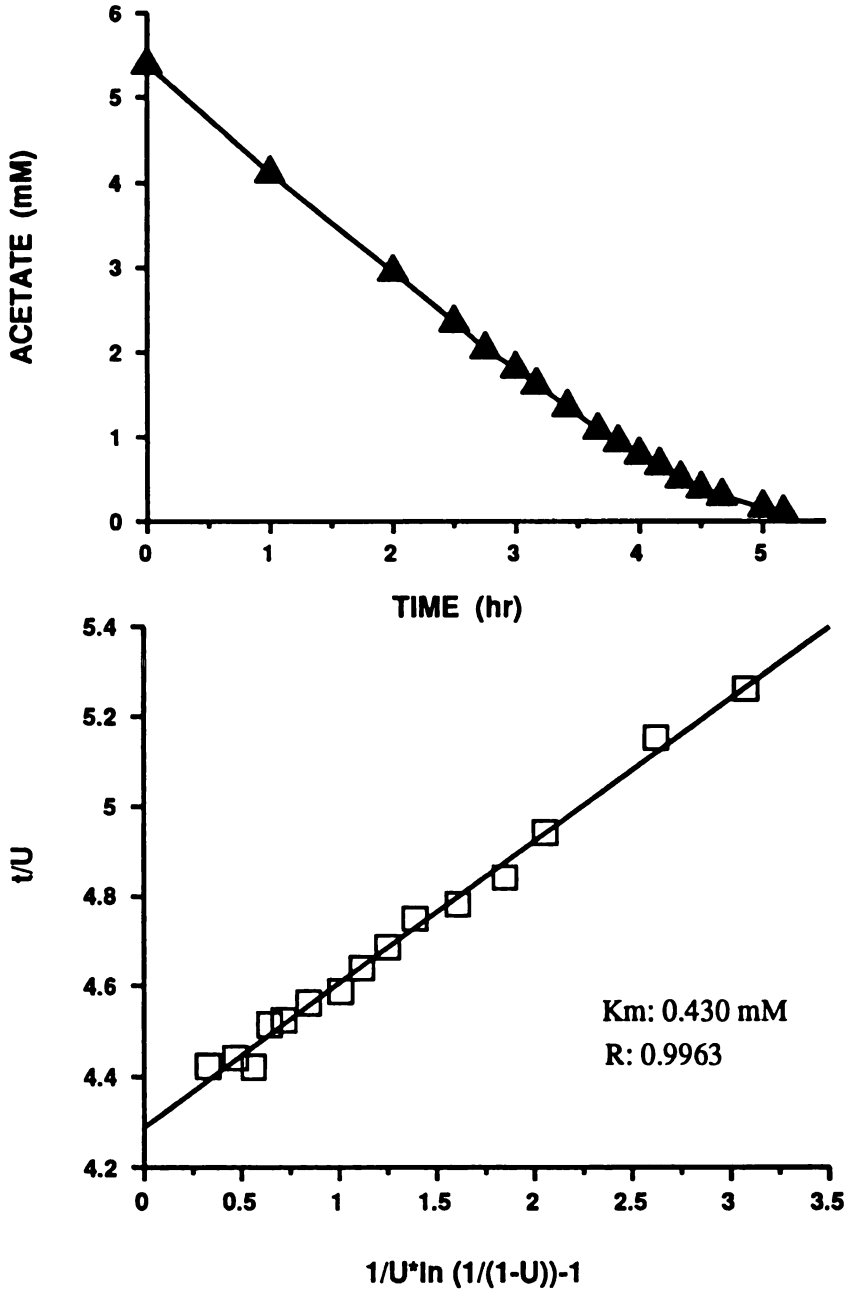


Figure C.1 Time-course of acetate degradation by the R-granules and the plot for determination of K_m using equation C-5.

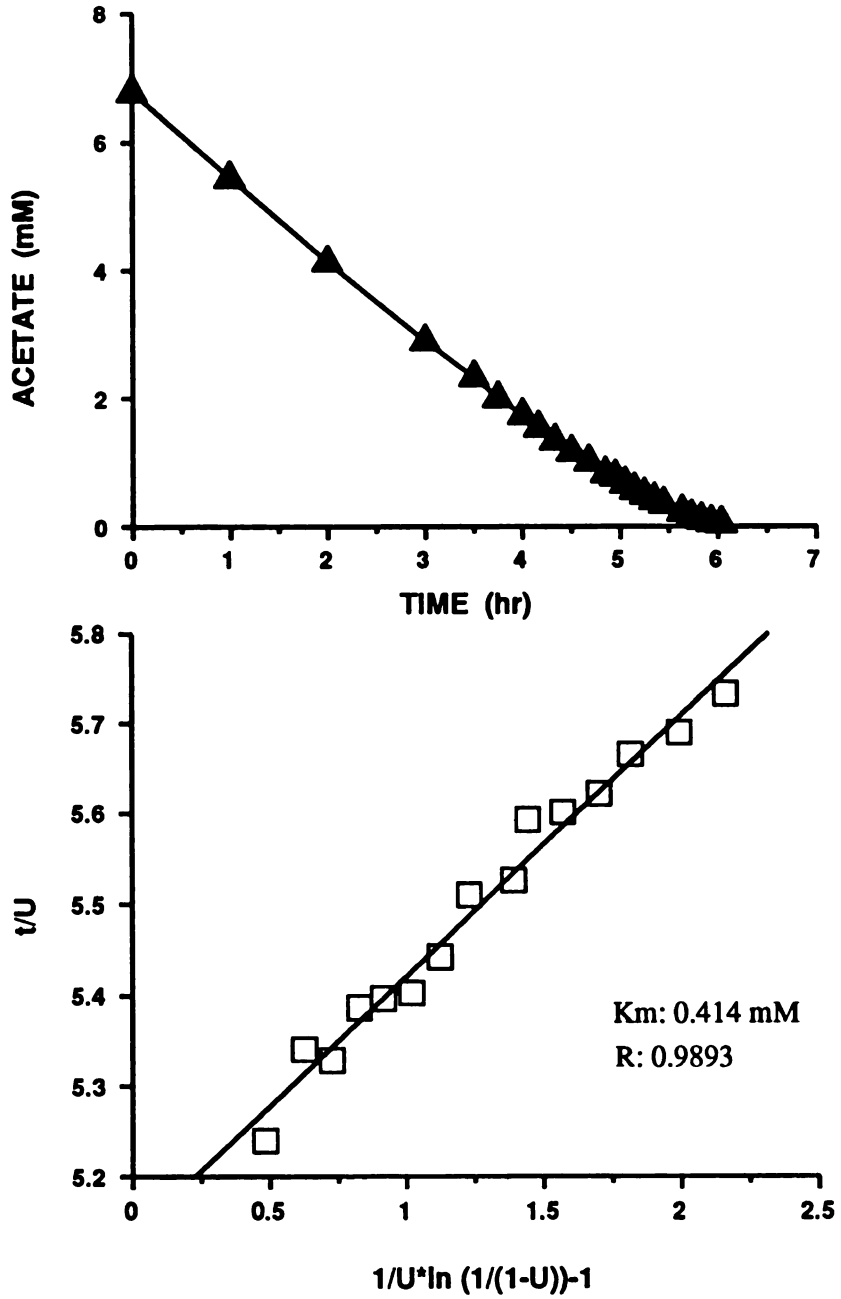


Figure C.2 Time-course of acetate degradation by the F-granules and the plot for determination of K_m using equation C-5.

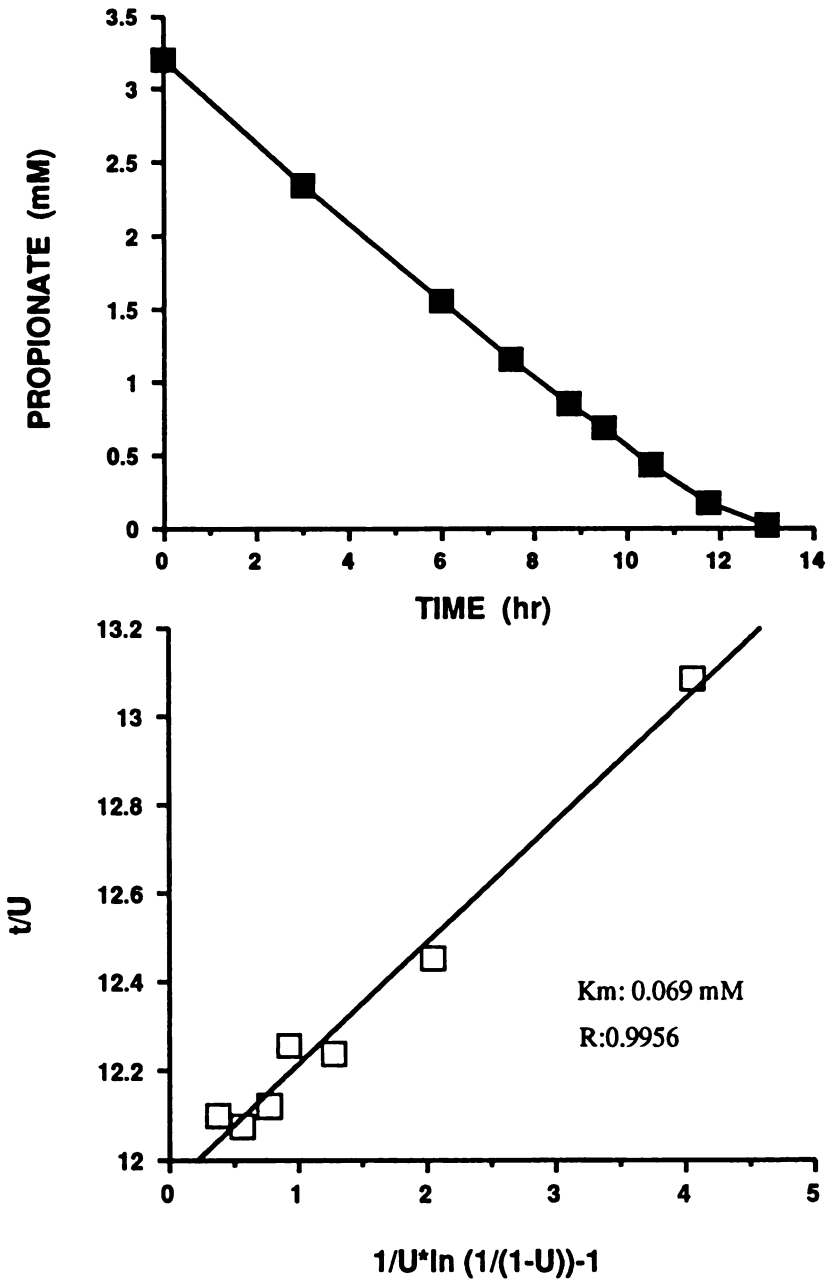


Figure C.3 Time-course of propionate degradation by the R-granules and the plot for determination of K_m using equation C-5.

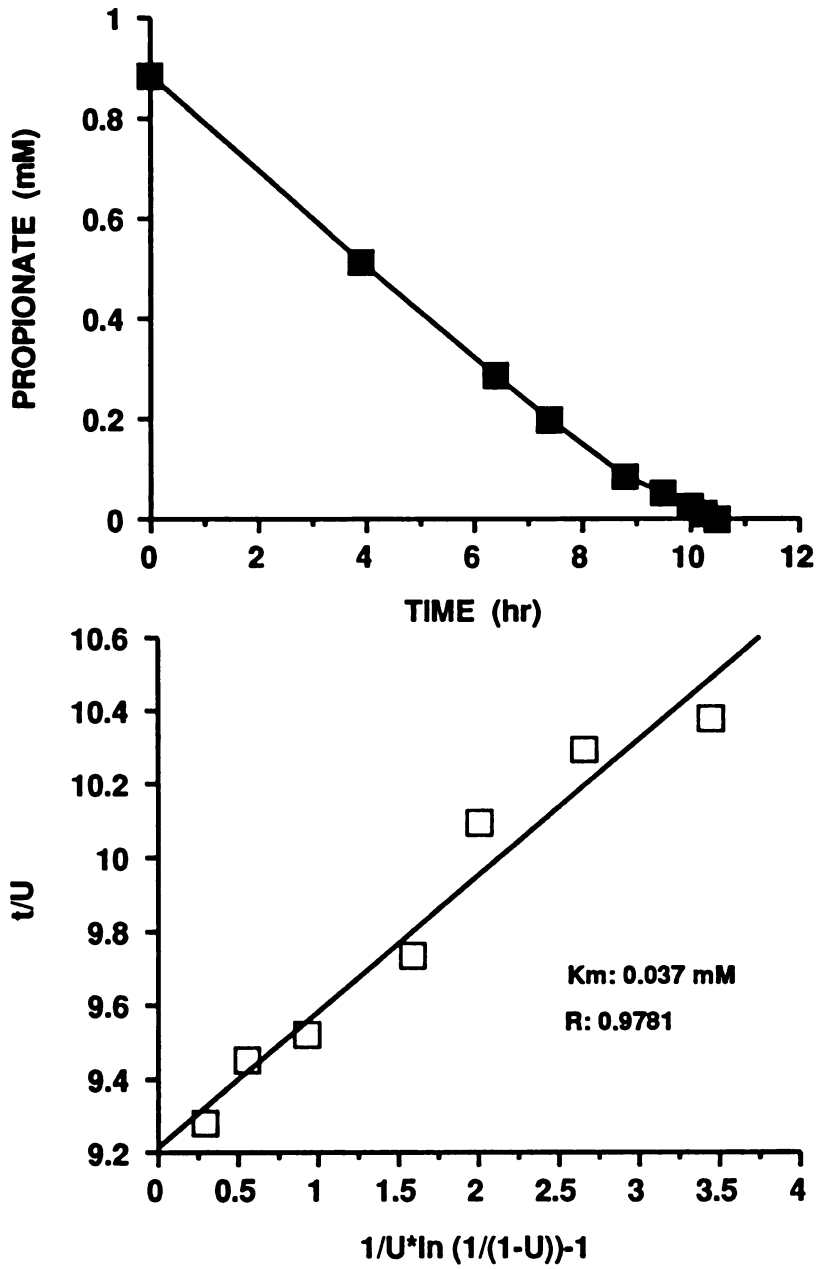


Figure C.4 Time-course of propionate degradation by the F-granules and the plot for determination of K_m using equation C-5.

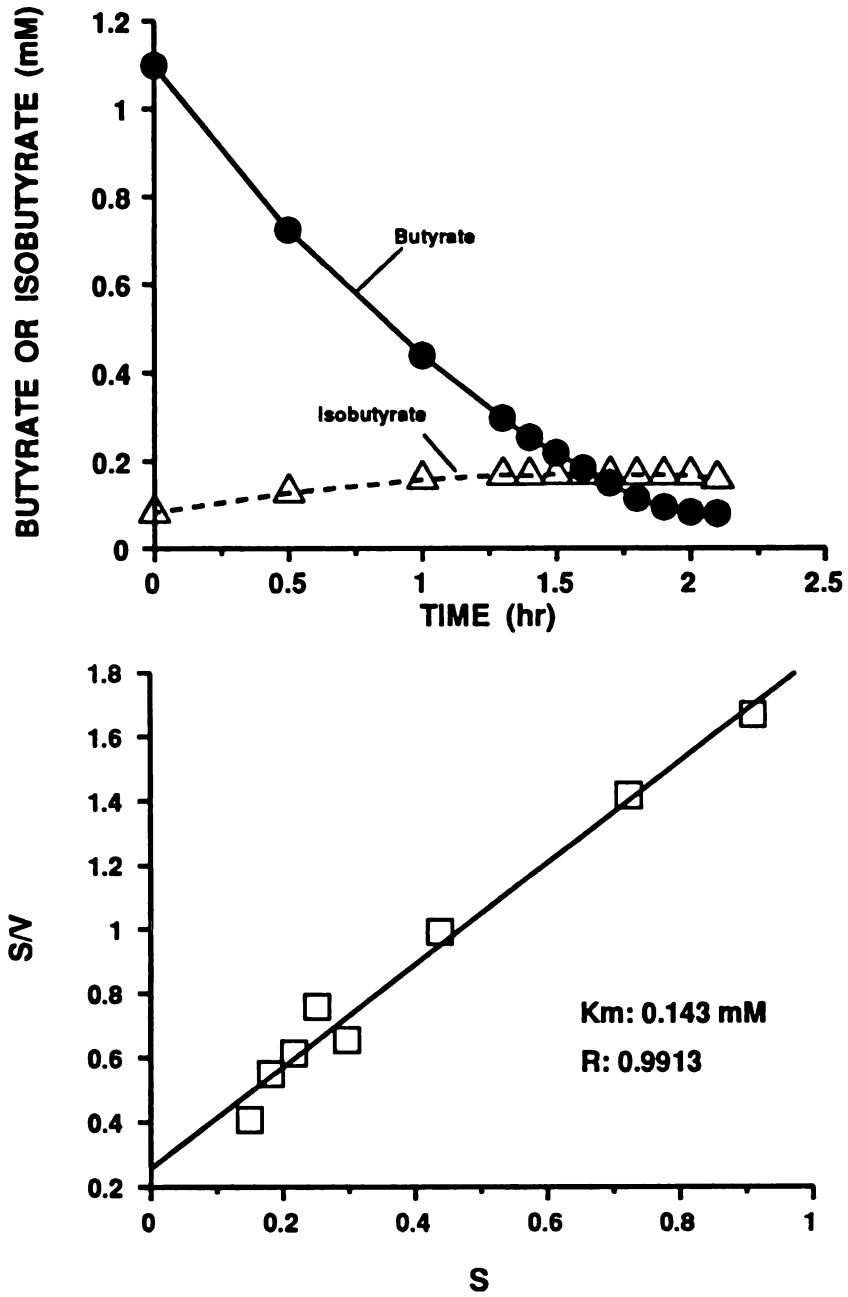


Figure C.5 Time-course of butyrate degradation by the R-granules and the plot for determination of K_m using equations C-7 and C-8. Significant level of isobutyrate accumulated during butyrate degradation.

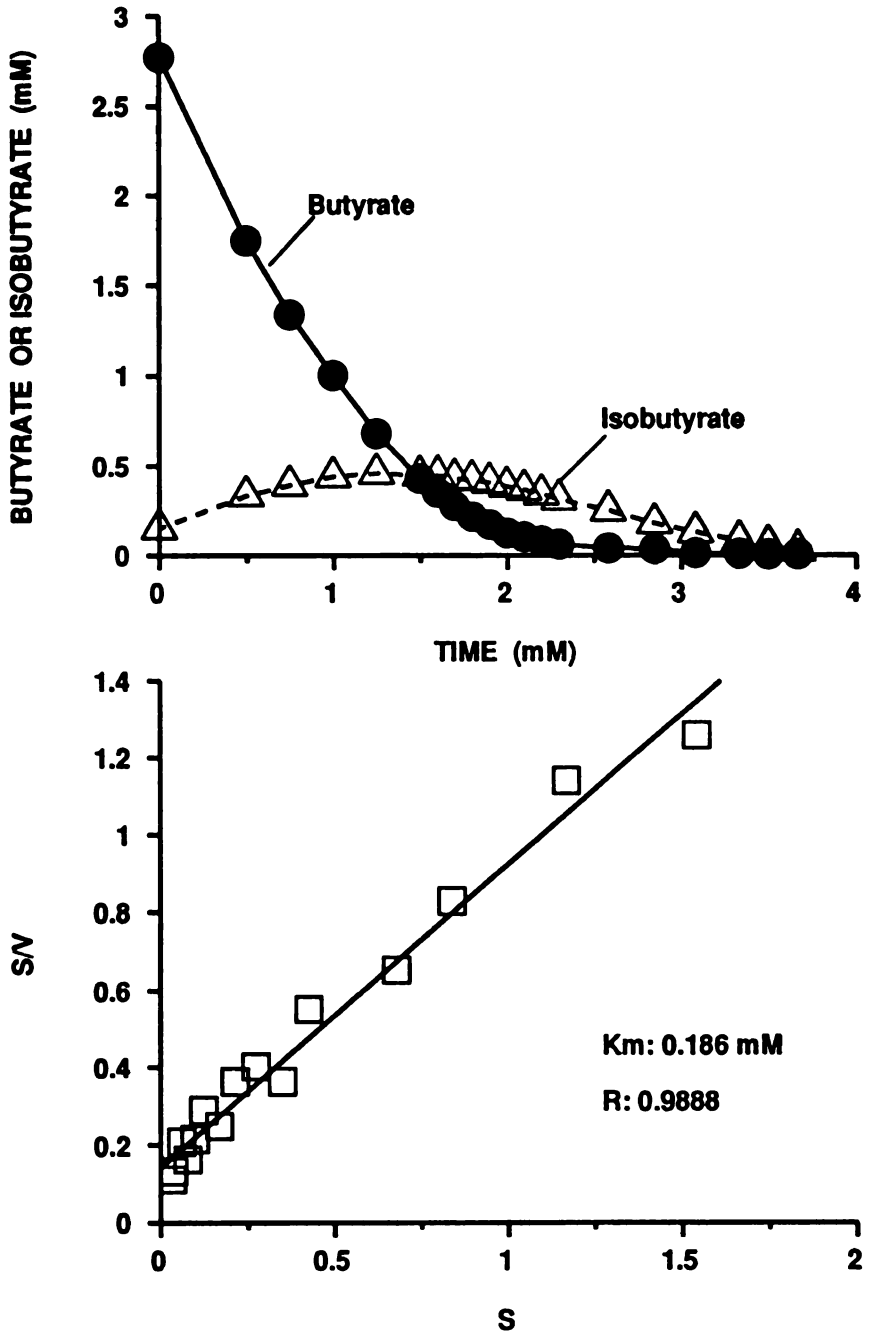


Figure C.6 Time-course of butyrate degradation by the F-granules and the plot for determination of K_m using equations C-7 and C-8. Significant level of isobutyrate accumulated during butyrate degradation.

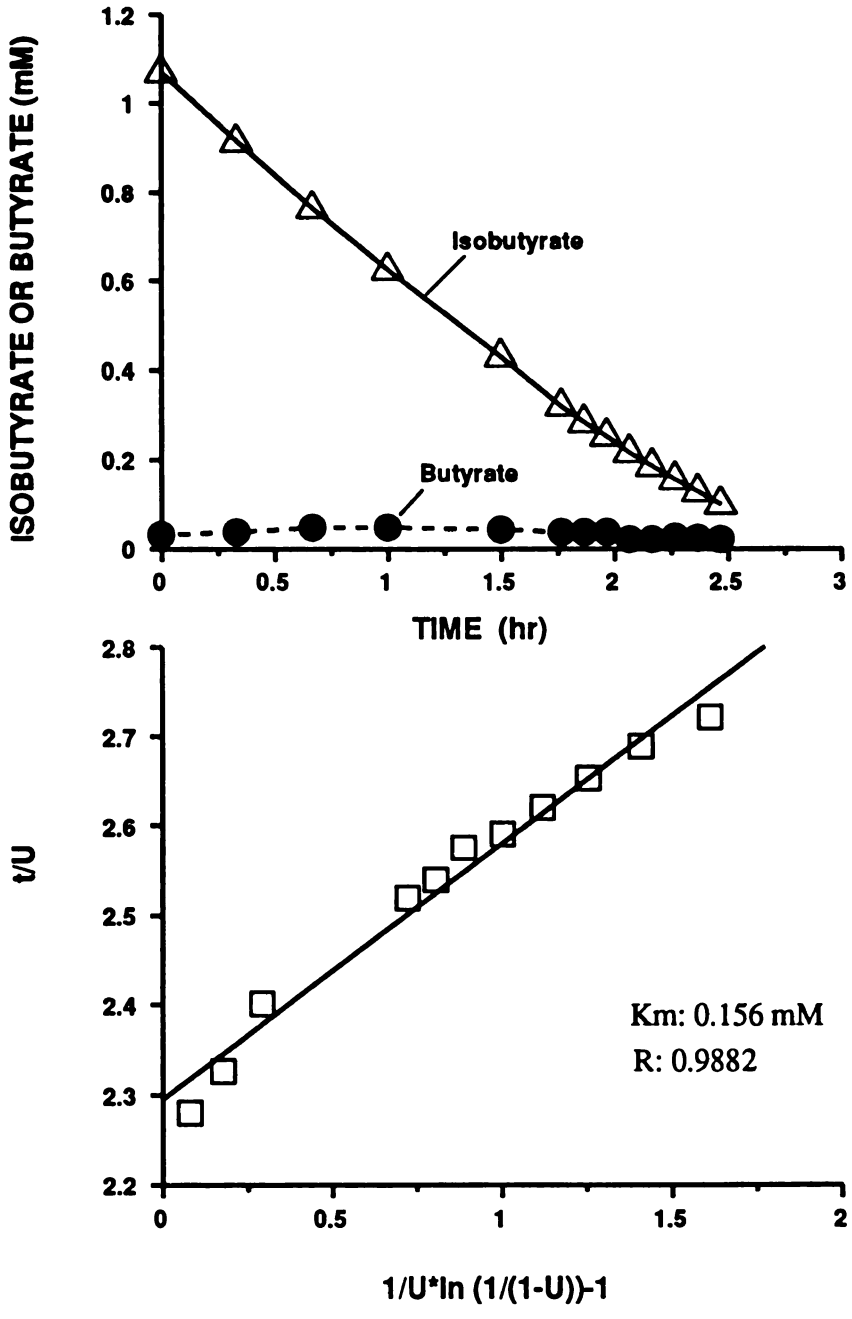


Figure C.7 Time-course of isobutyrate degradation by the R-granules and the plot for determination of K_m using equation C-5. Trace amount of butyrate accumulated as an intermediate during isobutyrate degradation.

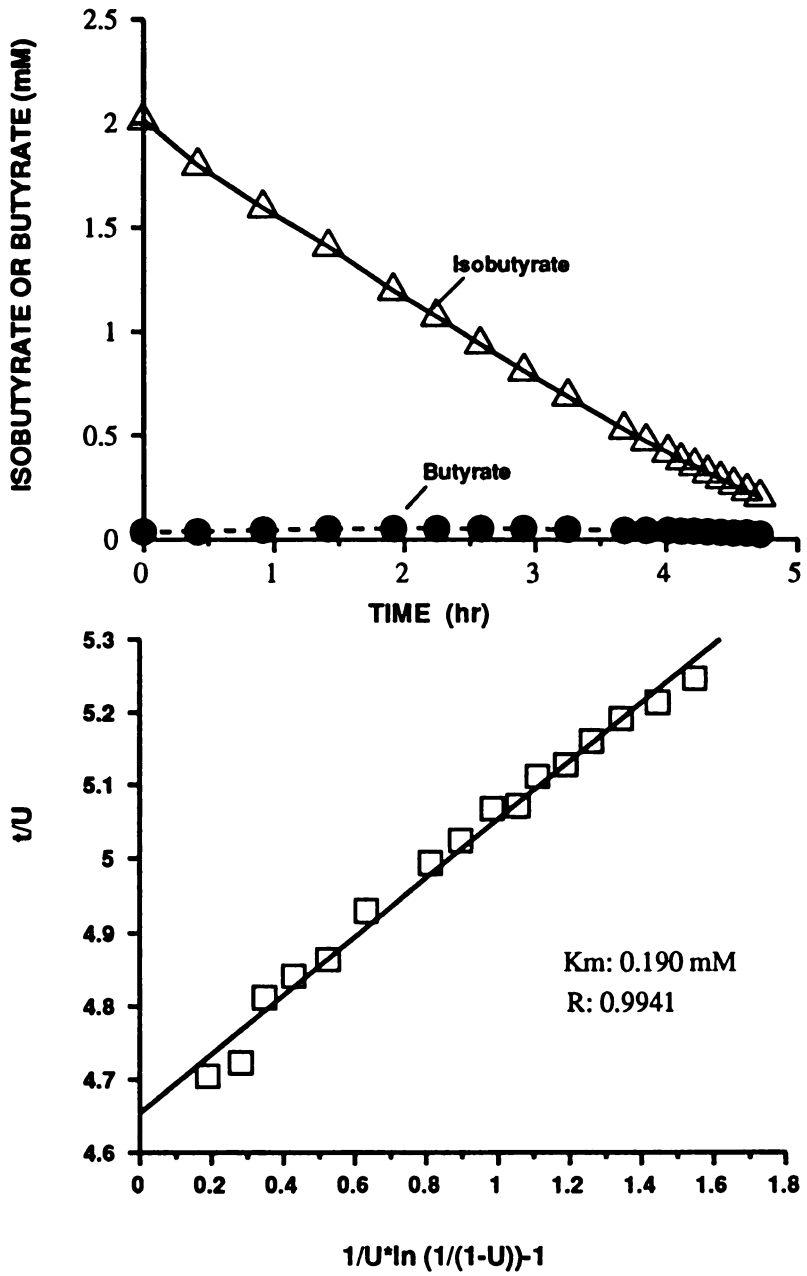


Figure C.8 Time-course of isobutyrate degradation by the F-granules and the plot for determination of K_m using equation C-5. Trace amount of butyrate accumulated as an intermediate during isobutyrate degradation.

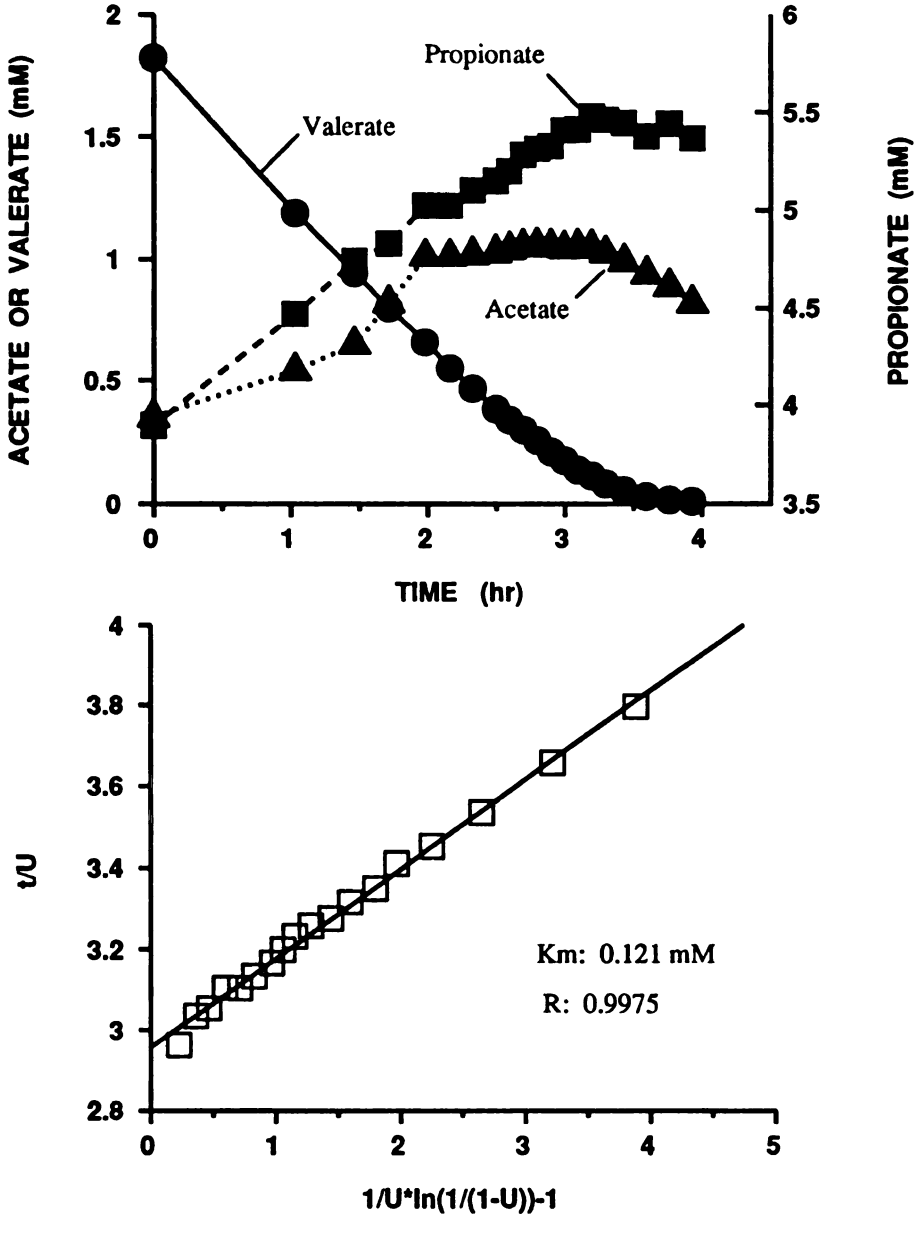


Figure C.9 Time-course of valerate degradation by the R-granules and the plot for determination of K_m using equation C-5. Acetate and propionate accumulated during valerate degradation.

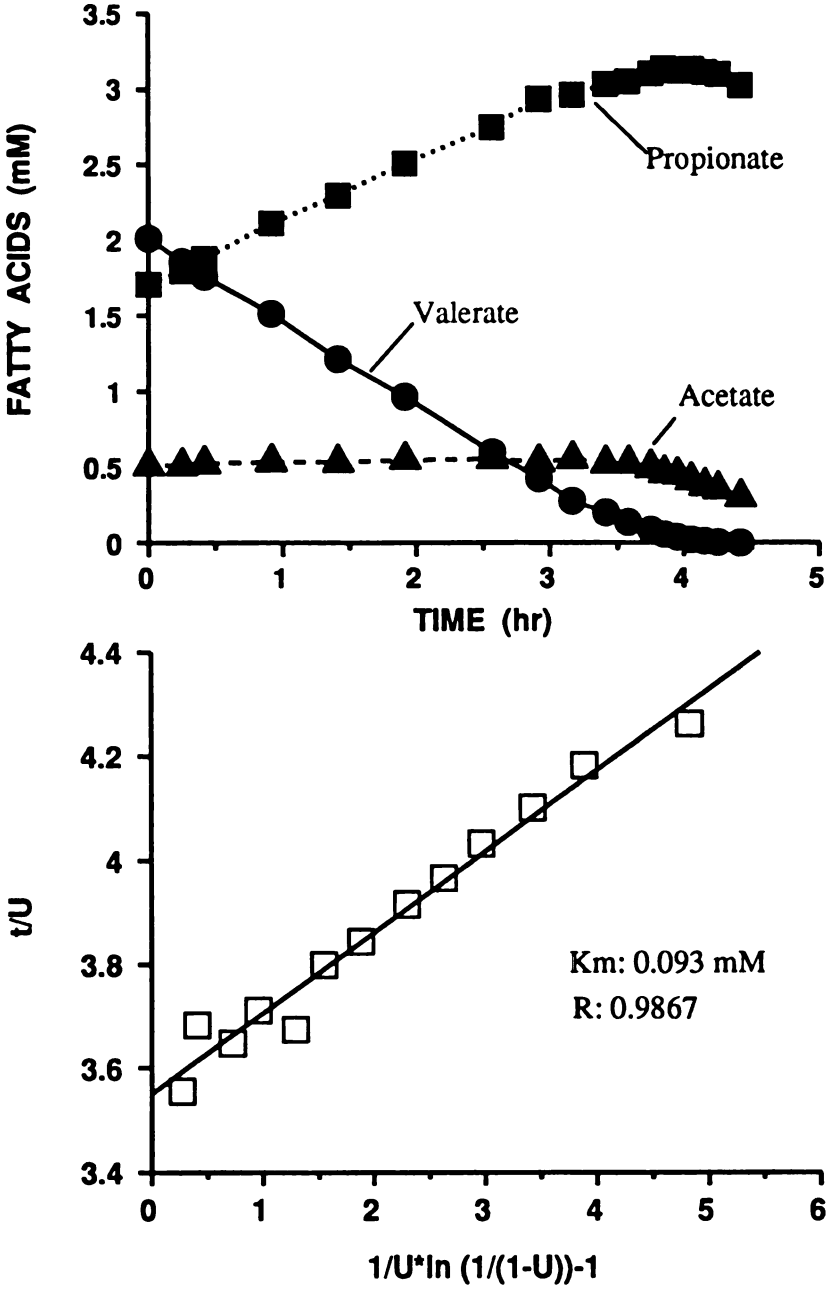


Figure C.10 Time-course of valerate degradation by the F-granules and the plot for determination of K_m using equation C-5. Acetate and propionate accumulated during valerate degradation.

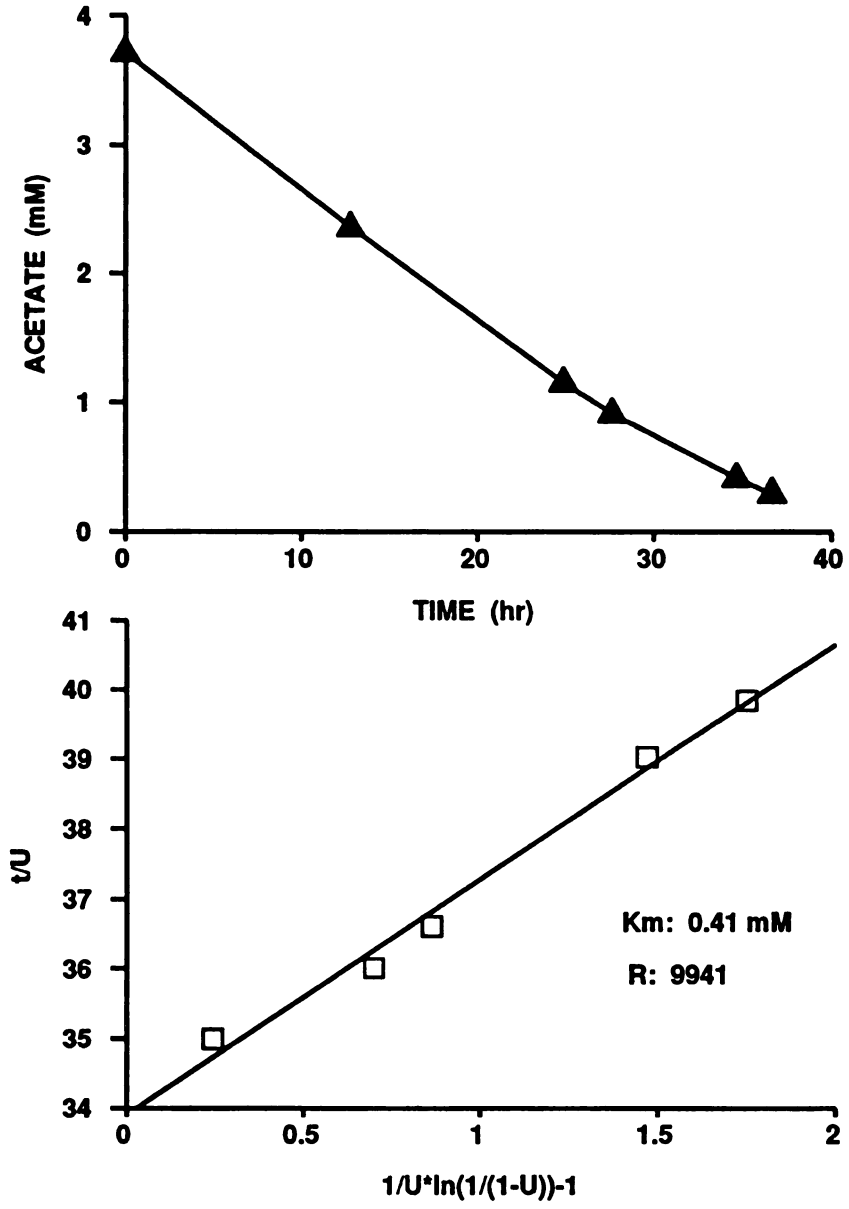


Figure C.11 Time-course of acetate degradation by *Methanothrix* strain M7 and the plot for determination of K_m using equation C-5.

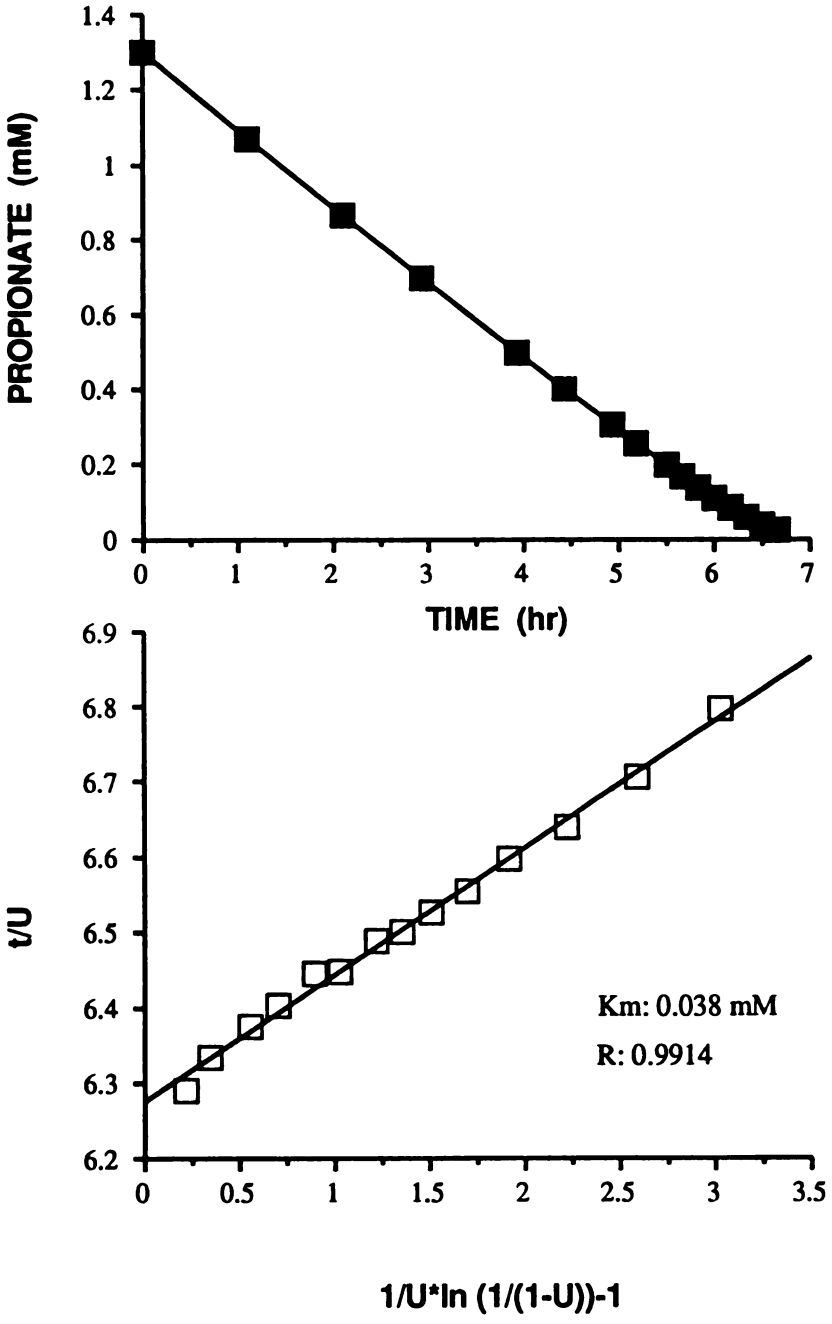


Figure C.12 Time-course of propionate degradation by the granules developed using defined species and the plot for determination of K_m using equation C-5.

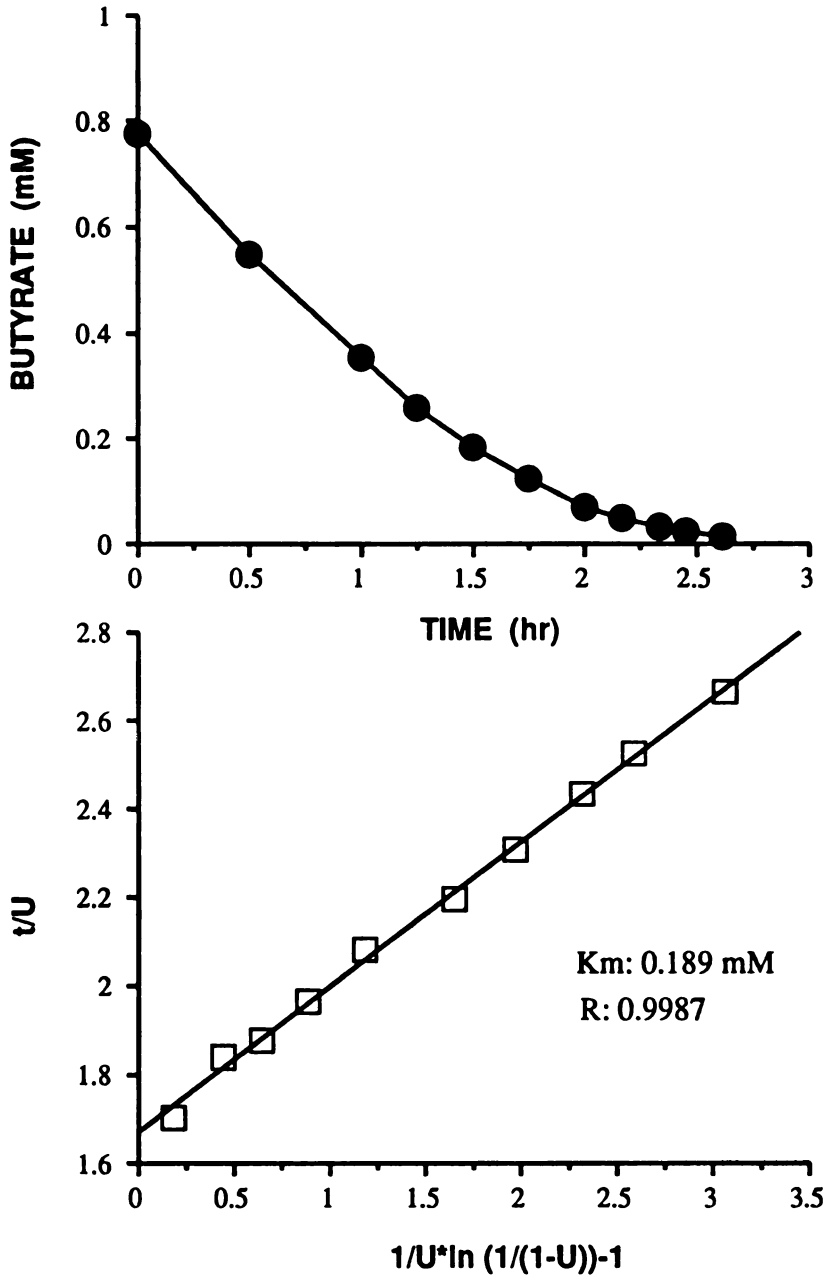


Figure C.13 Time-course of butyrate degradation by the granules developed using defined species and the plot for determination of K_m using equation C-5. Isobutyrate formation was not observed.

APPENDIX D

LIGHT MICROSCOPIC, SCANNING ELECTRON MICROSCOPIC AND TRANSMISSION ELECTRON MICROSCOPIC EXAMINATION

1. Light microscopy

Phase-contrast and epifluorescence observations of microorganisms were made with an Olympus microscope model BH2 equipped with a mercury lamp and an automatic-exposure camera. The autofluorescence of methanogens was observed with a B(IF-490) excitation filter. Kodak plus-X pan films were used for photographs.

2. Scanning electron microscopy (SEM)

The samples of bacterial cultures were withdrawn from anaerobic serum bottles using 1 mL syringe with a 18 gauge needle. Aggregated cells were immediately washed with 0.9% NaCl five times. Dispersed cells were centrifuged (at 10,000 rpm) to obtain a cell pellet which was, then, washed in a 0.9% NaCl solution. This centrifugation-washing procedure was repeated for 4 to 5 additional times. The samples of granules were taken from the reactors and washed with 0.9% NaCl. The washed samples (aggregates, centrifuged pellets, or granules) were then fixed in 0.1 M phosphate buffer (pH 7.0) containing 4% glutaraldehyde for more than 30 min, washed with 0.1 M phosphate buffer for 10 min, and then dehydrated using a graded series of

ethanol solutions (25, 50, 75, 85, 95, and 100% of ethanol). The samples were dried by the critical-point method and sputter coated with gold.

Samples for X-ray analysis were prepared essentially the same as those for SEM except they were sputter coated with carbon instead of gold.

For the preparation of cationized-ferritin (CF) stained samples, cell suspension (1 mL), washed with 0.9% NaCl solution, was transferred to the surface of a FE membrane (4 μM, 13 mm in diameter, Nuclepore Co., Pleasanton, CA). Subsequently, liquid was removed from the membrane by vacuum. The cells were stained by overlaying with diluted CF (10%) for 10 min, then washed with 0.9% NaCl, and fixed in 5% glutaraldehyde-phosphate buffer (0.1 M, pH 7.0) initially at room temperature for one hour and then over night at 4 °C. The cells and membrane were dehydrated through the graded series of ethanol solutions, dried and sputter coated with gold.

SEM microphotographs were taken with an JSM 35C scanning electron microscope (JEOL, Tokyo, Japan) equipped with a tracor Northern energy-dispersive X-ray spectrometer Model 5500 (EDX Instruments, Middletown, WI).

3. Transmission electron microscopy (TEM)

Samples of bacterial cultures were washed using 0.1 mM phosphate buffer (pH 7.2) by centrifugation four times. Granule samples were washed with the phosphate buffer directly. The samples (centrifuged pellets or granules) were fixed in 0.1 M phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde for 12 to 16 hrs at 4 °C. The fixed samples were rinsed 3 times at ambient temperature in 0.1 mM phosphate buffer (pH 7.2), post-fixed with 1% OsO_4 in the same buffer, dehydrated through a graded series of ethanol solutions followed by propylene oxide. Samples were embedded in polybed 812 (Polyscience Inc., Warrington, PA). Thin sections were cut

with a LKB Ultratome and then post-stained with uranyl acetate and lead citrate. TEM examination was performed using a Philips CM-10 electron microscope.

4. Cell density

The cell density in the granules was determined by counting the number of cells on TEM microphotographs of thin cross sections based on the number of different morphotypes per cm^2.

5. Granule size analysis.

Size distribution of granules was determined using photographs. The granules in an operating reactor were completely mixed by inverting the reactor several times. The reactor was opened, and a portion of the mixed granules was poured into petri dishes. The liquid was evenly spread to obtain a single granule layer. The petri dishes were placed under a dissecting photomicroscope (Olympus SZH). Along with an internal size standard, photographs of different areas of the preparation were then taken. To estimation of the size distribution (percentage of certain sized granules in total granule number and the percentage of certain sized granules in total granule volume), the shape of the granules was assumed to be spherical. The mean diameter of each granule measured by averaging the maximum diameter and minimum diameter of the granule on the photographs was used for the calculation. The total number of each type of granules for size distribution estimation was not less than 150. The size distribution was calculated using the following computer program.

```
5 REM: MAIN PROGRAM FOR NUMBER OF GRANULES VS. THE AVERAGE SIZE AND
GRANULE BED VOLUME.
10 REM: DATA FOR THE INTERVAL OF DIAMETERS
12 DATA (********)
14 REM: DATA FOR AVERAGE DIAMETER MEASURED FROM MICROSCOPIC PHOTOGRAPHS;
NUMBER 100000 IS USED FOR END OF THE DATA.
16 DATA (********), 100000
18 INPUT "NUMBER OF THE INTERVAL OF DIAMETER"; N
20 REM: INPUT MULTIPLY FACTOR WHICH WAS CALCULATED BASED ON INTERNAL SIZE
STANDARD ON THE PHOTOGRAPHS
24 INPUT "MULTIPLY FACTOR", FACTOR
30 GOSUB 100
40 END


100 REM: SUBPROGRAM FOR NUMBER OF GRANULES VS. THE AVERAGE SIZE AND
GRANULE BED VOLUME.
110 DIM FRACTIONVOLUME (N), FRACTIONNUMBER (N), SIZE(N)
120 NUMBER=0
130 FOR I=1 TO N
135 READ SIZE(I)
138 NEXT I
140 READ DIAMETER
145 IF DIAMETER >=10000 GOTO 1400
150 NUMBER=NUMBER+1
```

```
155 DIAMETER=DIAMETER/FACTOR
160 VOLUME=3.1416*DIAMETER^3/6
165 TOTALVOLUME=TOTALVOLUME+VOLUME
170 FOR I=1 TO N-1
180 IF DIAMETER>SIZE(I) GOTO 1140
1100 FRACTIONNUMBER(I)=FRACTITONNUMBER(I)+1
1120 FRACTIONVOLUME(I)=FRACTIONVOLUME(I)+VOLUME
1140 IF DIAMETER>SIZE (N-1) GOTO 1200
1160 NEXT I
1180 GOTO 140
1200 FRACTIONNUMBER(N)=FRACTITONNUMBER(N)+1
1220 FRACTIONVOLUME(N)=FRACTIONVOLUME(N)+VOLUME
1240 GOTO 140
1400 PRINT "NUMBER OF GRANULES"; NUMBER
1420 PRINT "MULTIPLY FACTOR"; FACTOR
1440 PRINT "SIZE <"; DIAMETER (1); "mm"; "NUMBER (%)";
FRACTIONNUMBER(1)*100/NUMBER, "VOLUME (%)";
FRACTIONVOLUME(1)*100/TOTALVOLUME
1460 FOR I=2 TO N-1
1480 PRINT DIAMETER (I-1);"=<SIZE<"; DIAMETER (I); "mm"; "NUMBER (%)";
FRACTIONNUMBER(I)*100/NUMBER, "VOLUME (%)";
FRACTIONVOLUME(I)*100/TOTALVOLUME
1500 NEXT I
1520 PRINT "SIZE >="; DIAMETER (N); "mm"; "NUMBER (%)";
FRACTIONNUMBER(N)*100/NUMBER, "VOLUME (%)";
```

```
FRACTIONVOLUME(N)*100/TOTALVOLUME

1540 RETURN
```

APPENDIX E

FREE ENERGY CALCULATIONS FOR BIOCHEMICAL REACTIONS

1. General equation

In this study, Gibbs free energies (ΔGf°) of formation from the elements for compounds were obtained from Thauer *et al.* (1977) except for the value of isobutyrate (354.61 kJ/mol), which was estimated by using van Krevelen and Chermin equation and assuming that per CH_3, per CH_2 and per CH group, Gf^0 decreases by -18.16, 8.57 and 33.32 kJ/mol, respectively, from butyrate (Perry's Chemical Eng. Handbook, 16th ed., 1984).

Free energy changes were calculated under physiological conditions (pH 7) using the following equations:

$$\Delta G' = \Delta G^{\circ\prime} + RT \sum V_i \ln A_i \qquad \text{(E-1)}$$

where $\Delta G'$ = free energy change of reaction under physiological conditions

$\Delta G^{\circ\prime}$= the increment of free energy under standard physiological conditions (25 °C, 1 atm and pH 7)

R = gas constant 8.3143 J/K° mol

T = Temperature (°K)

V_i = the stoichiometric coefficient for component A_i in a biological reaction (i=1,2,3...,n) and is negative for reactants and positive for products

A_i = the physiological concentrations of the component i in the reaction (mol/L)

2. Formate synthesis from hydrogen plus bicarbonate

Formate synthesis from hydrogen plus bicarbonate:

$H_2 + HCO_3^- \longrightarrow formate^- + H_2O$

$\Delta G^{o'}$=-1.3 kJ/reaction (E-2)

At 35 °C and pH 7, the free energy available for formate synthesis was calculated as following:

$\Delta G' = -1.3 + 5.9 \log \{[formate^-]/[H_2][HCO_3^-]\}$ (E-3)

3. Isomerization between butyrate and isobutyrate

$butyrate^- \longrightarrow isobutyrate^-$ (E-4)

$\Delta G^{o'}$=-1.98 kJ/reaction

At 37 °C and pH 7, the free energy available for the formation of isobutyrate from butyrate and the formation of butyrate from isobutyrate were calculated as followings, respectively:

$\Delta G' = -1.98 + 5.94 \log \{[isobutyrate^-]/[butyrate^-]\}$ (E-5)

and

$\Delta G' = 1.98 + 5.94 \log \{[butyrate^-]/[isobutyrate^-]\}$ (E-6)

4. Interspercies electron transfer

i) Ethanol degradation:

Interspecies hydrogen transfer during syntrophic ethanol degradation:

$$\text{ethanol} + H_2O \longrightarrow \text{acetate}^- + H^+ + 2H_2$$

$$\Delta G^{o\prime} = +9.6 \text{ kJ/reaction} \qquad \text{(E-7)}$$

Interspecies formate transfer:

$$\text{ethanol} + 2HCO_3^- \longrightarrow \text{acetate}^- + H^+ + 2\ \text{formate}^- + H_2O$$

$$\Delta G^{o\prime} = +6.9 \text{ kJ/reaction} \qquad \text{(E-8)}$$

The $\Delta G^{o\prime}$ is the increment of free energy for the reactions under standard conditions (273 °K, 1 atm), pH 7.0 and 1 M concentration. The free energy at pH7 under non-standard conditions (35 °C or 308 °K, pH 7.0) was calculated as the following equations:

Interspecies hydrogen transfer:

$$\Delta G' = 9.6 + 5.9 \log \{[\text{acetate}^-][H_2]^2/[\text{ethanol}]\} \qquad \text{(E-9)}$$

Interspecies formate transfer:

$$\Delta G' = 6.9 + 5.9 \log \{[\text{acetate}^-][\text{formate}^-]^2/[\text{ethanol}][HCO_3^-]^2\} \qquad \text{(E-10)}$$

The initial pH of syntrophic ethanol degradation was proximately 7.0. The change during ethanol degradation was not significant because the medium had high HCO_3^--CO_2 buffer capacity (HCO_3^- 59 mM). The influence of pH change on free energy change was ignored.

ii) Butyrate and isobutyrate degradation:

Interspecies H_2 transfer

$$\text{butyrate}^- + 2\ H_2O \longrightarrow 2\ \text{acetate}^- + 2\ H_2 + H^+ \qquad \text{(E-11)}$$

$$\Delta G^{o\prime} = +48.1 \text{ kJ/reaction}$$

$$\text{isobutyrate}^- + 2\ H_2O \longrightarrow 2\ \text{acetate}^- + 2\ H_2 + H^+ \quad \text{(E-12)}$$

$\Delta G^{o\prime}$=+50.08 kJ/reaction

$$1/2\ \text{butyrate}^- + 1/2\ \text{isobutyrate}^- + 2\ H_2O \longrightarrow 2\ \text{acetate}^- + 2\ H_2 + H^+$$

$\Delta G^{o\prime}$=+49.09 kJ/reaction (E-13)

Interspecies formate transfer

$$\text{butyrate}^- + 2\ HCO_3^- \longrightarrow 2\ \text{acetate}^- + H^+ + 2\ \text{formate}^- \quad \text{(E-14)}$$

$\Delta G^{o\prime}$=+45.5 kJ/reaction

$$\text{isobutyrate}^- + 2\ HCO_3^- \longrightarrow 2\ \text{acetate}^- + H^+ + 2\ \text{formate}^- \quad \text{(E-15)}$$

$\Delta G^{o\prime}$=+47.48 kJ/reaction

$$1/2\ \text{butyrate}^- + 1/2\ \text{isobutyrate}^- + 2\ HCO_3^- \longrightarrow 2\ \text{acetate}^- + 2\ \text{formate}^- + H^+$$

$\Delta G^{o\prime}$=+46.49 kJ/reaction (E-16)

The values of $\Delta G'$ under pH 7 and 37 °C for above respective reactions were calculated based on eqaution (E-1) and $\Delta G^{o\prime}$ for respective reactions.

iii) Butyrate degradation coupled with isomerization between butyrate and isobutyrate:

(1) When butyrate concentration is much higher than isobutyrate, isobutyrate will accumulate during butyrate degradation by isobutyrate-butyrate degraders. Suppose that *p* mole of isobutyrate is formed from butyrate when one mole of butyrate is degraded with formate as electron carriers:

$$(1+p)\ \text{butyrate}^- + 2HCO_3^- \longrightarrow 2\ \text{acetate}^- + H^+ + 2\ \text{formate}^- + p\ \text{isobutyrate}^-$$

$\Delta G^{o\prime}$=+45.5-1.98 *p* kJ/reaction (E-17)

where, *p* is a variable which ≥0. Under pH 7.0 and 37°C, the free energy was calculated as

$$\Delta G' = 45.5 - 1.98\ p + 5.94 \log \{[\text{acetate}^-]^2[\text{formate}^-]^2[\text{isobutyrate}^-]^p / [\text{butyrate}]^{(1+p)}[HCO_3^-]^2\} \quad \text{(E-18)}$$

(2) When isobutyrate concentration is much higher than butyrate, butyrate will accumulate during isobutyrate degradation by isobutyrate-butyrate degraders. Suppose that q mole of butyrate is formed from isobutyrate when one mole of butyrate is degraded with formate as electron carriers:

$$q \text{ isobutyrate}^- + 2HCO_3^- \longrightarrow 2 \text{ acetate}^- + H^+ + 2 \text{ formate}^- + (q\text{-}1) \text{ butyrate}^-$$

$$\Delta G^{o\prime} = +47.48 + 1.98\, q \text{ kJ/reaction} \qquad \text{(E-19)}$$

where, q is a variable which ≥ 0. Under pH 7.0 and 37°C, the free energy was calculated as

$$\Delta G' = 47.48 + 1.98\, q + 5.94 \log \{[\text{acetate}^-]^2[\text{formate}^-]^2[\text{butyrate}^-]^{(q-1)} / [\text{isobutyrate}]^q[HCO_3^-]^2\} \qquad \text{(E-20)}$$

(3) When butyrate and isobutyrate are at nearly equal concentrations, both are degraded at the same rates:

$$1/2 \text{ butyrate}^- + 1/2 \text{ isobutyrate}^- + 2HCO_3^- \longrightarrow 2 \text{ acetate}^- + H^+ + 2 \text{ formate}^-$$

$$\Delta G^{o\prime} = +47.48 + 1.98\, q \text{ kJ/reaction} \qquad \text{(E-21)}$$

Under pH 7.0 and 37°C, the free energy was calculated as

$$\Delta G' = 46.49 + 5.94 \log \{[\text{acetate}^-]^2[\text{formate}^-]^2 / [\text{butyrate}^-]^{1/2}[\text{isobutyrate}]^{1/2}[HCO_3^-]^2\} \qquad \text{(E-22)}$$

APPENDIX F

CALCULATION FOR SUBSTRATE CONVERSION VIA SULFATE REDUCTION

1. Reactions

During anaerobic digestion in the presence of sulfate, the following substrate can be converted via sulfate reduction or/and methanogenesis:

$$4\ H_2 + SO_4^{2-} + H^+ \longrightarrow 4\ H_2O + HS^- \quad \text{(F-1)}$$

$$4\ H_2 + HCO_3^- + H^+ \longrightarrow 3\ H_2O + CH_4 \quad \text{(F-2)}$$

$$4\ \text{formate}^- + SO_4^{2-} + H^+ \longrightarrow 4\ HCO_3^- + HS^- \quad \text{(F-3)}$$

$$4\ \text{formate}^- + H_2O + H^+ \longrightarrow 3\ HCO_3^- + CH_4 \quad \text{(F-4)}$$

$$\text{acetate}^- + SO_4^{2-} \longrightarrow 2\ HCO_3^- + HS^- \quad \text{(F-5)}$$

$$\text{acetate}^- + H_2O \longrightarrow HCO_3^- + CH_4 \quad \text{(F-6)}$$

$$2\ \text{ethanol} + SO_4^{2-} \longrightarrow 2\ \text{acetate}^- + 2\ H_2O + HS^- + H^+ \quad \text{(F-7)}$$

$$2\ \text{ethanol} + HCO_3^- \longrightarrow 2\ \text{acetate}^- + H_2O + CH_4 + H^+ \quad \text{(F-8)}$$

$$4\ \text{propionate}^- + 3\ SO_4^{2-} \longrightarrow 4\ \text{acetate}^- + 4\ HCO_3^- + 3\ HS^- + H^+ \quad \text{(F-9)}$$

$$4\ \text{propionate}^- + 3\ H_2O \longrightarrow 4\ \text{acetate}^- + HCO_3^- + 3\ CH_4 + H^+ \quad \text{(F-10)}$$

$$2\ \text{butyrate}^- + SO_4^{2-} \longrightarrow 4\ \text{acetate}^- + HS^- + H^+ \quad \text{(F-11)}$$

$$2\ \text{butyrate}^- + HCO_3^- + H_2O \longrightarrow 4\ \text{acetate}^- + CH_4 + H^+ \quad \text{(F-12)}$$

2. Calculation of results in Table 4.5

i) Conversion of H_2, formate and acetate

The substrate converted via sulfate reduction was calculated directly from the ratio of molar mass of methane produced in the presence versus the absence of sulfate. For example, the ratio for methane produced from hydrogen was 0.94 and 0.98 for intact and disrupted granules, respectively. Hydrogen converted via sulfate reduction was, therefore, ca. 6% and 2%. In this calculation, the percentagies could also be calculated using substrate consumed and sulfate converted and equation (F-1), (F-3) and (F-4). In Table 4.5, the results was calculated based on CH_4 produced rather than sulfate removed because sulfate consumption was insignificant during conversion of hydrogen, formate and acetate and, therefore the determination of CH_4 was more accurate than sulfate.

ii) Conversion of propionate

Propionate conversion via sulfate reduction can be calculated using two methods:

(1) In the basis of methane produced in the presence versus the absence of sulfate.

When propionate is degraded via methanogenesis, 1 mol of propionate produces 0.75 mol of CH_4 and 1 mol acetate (equation F-10) and 1 mol acetate produces 1 mol of methane (equation F-6). Experimental results showed that the ratio of CH_4 produced in the presence versus the absence of sulfate for acetate conversion and propionate conversion were 0.96 and 0.72, respectively (Table 4.5). The following equation can be obtained based on mass balance

$$(0.75\ F + 0.96)/(1.0+0.75)=0.72$$

where, F is the fraction of methane produced via syntrophic propionate conversion and F = 0.4. The propionate converted via sulfate reduction, therefore, is

(1-0.4) * 100% = 60%.

(2) In the basis of consumption of propionate and sulfate

When substrate consumption for cell synthesis is ignored, propionate conversion via sulfate reduction can be calculated on the basis of sulfate consumption. According to equation (F-9), conversion of 1 mmol propionate to 1 mmol acetate requires 0.75 mmol of sulfate. In Table 4.5, sulfate consumed (mmol per mmol substrate) was 0.007 and 0.41 during conversion of acetate and propionate, respectively. The fraction of propionate converted to acetate via sulfate reduction is

((0.41-0.007)/1.0)/0.75=0.54 (or 54%)

The difference between these two calculations may be due to the analytic error, especially in the determination of sulfate consumption during acetate conversion, and assimilatory uptake of propionate and acetate by microorganisms. In Table 4.5, the value of 60% rather than 54% was used as the result since the calculation on the basis of methane production was more accurate and free of the influence of assimilatory substrate uptake.

iii) ethanol conversion

The ratio of CH_4 produced from acetate and syntrophic acetogenesis is 1.0:0.5 per mole ethanol, based on equations (F-6) and (F-8). In Table 4.5, the ratio of CH_4 produced in the presence versus the absence of sulfate for acetate conversion and ethanol conversion were 0.96 and 0.88, respectively. On the basis of CH_4 production, the fraction of ethanol to acetate via sulfate reduction was calculated as

1-(0.88 * 1.5-0.96)/0.5=0.28 (or 28%)

On the basis of ethanol consumed and sulfate consumed, the fraction of ethanol

to acetate via sulfate reduction is

((0.18-0.007)/1.0)/0.5=0.34 (or 34%).

In Table 4.5, the value of 28% was used as result according the same reasons discussed for propionate conversion.

3. Calculation of results in Table 5.2

The calculation of percentages of substrates converted via sulfate reduction for H_2, formate, acetate, and propionate was the same as described above, on the basis of methane production. Butyrate converted via sulfate reduction was also estimated on the basis of methane production.

The ratio of CH_4 produced from acetate and syntrophic butyrate conversion is 2:0.5 per mole of butyrate, based on equations (F-6) and (F-12). In Table 5.2, the ratio of methane produced in the presence versus the absence of sulfate was 0.997 and 0.986, respectively. On the basis of methane production, the fraction (F) of methane produced via sulfate reduction was estimated from the following mass balance

(0.5 F + 0.997 * 2)/2.50=0.986

Thus, F was 0.942 or 94.2% and butyrate degraded via sulfate reduction was 5.8%.

LIST OF REFERENCES

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Aguilar, A., C. Casas, J. Lafuente, and J. M. Lema. 1990. Kinetic modelling of isomerization of n- and i-butyrate. J. Ferment. and Bioeng. 69:261-264.

Ahring, B.K., and P. Westermann. 1984. Isolation and characterization of a thermophilic, acetate-utilizing methanogenic bacterium. FEMS Microbiol. Lett. 25:47-52.

Ahring, B.K., and P. Westermann. 1985. Methanogenesis from acetate: physiology of a thermophilic, acetate-utilizing methanogenic bacterium. FEMS Microbiol. Lett. 28:15-19.

Ahring, B.K., and P. Westermann. 1987a. Thermophilic anaerobic degradation of butyrate by a butyrate-utilizing bacterium in coculture and triculture with methanogenic bacteria. Appl. Envron. Microbiol. 53: 429-433.

Ahring, B. K., and P. Westermann. 1987b. Kinetics of butyrate, acetate, and hydrogen metabolism in a thermophilic, anaerobic butyrate-degrading triculture. Appl. Environ. Microbiol. 53: 434-439.

Alibhai , K. R. K., and C. F. Forster. 1986. An examination of the granulation process in UASB reactors. Environ. Tech. Lett. 7:193-200.

Andrews, J. F. 1968. A dynamic model of the anaerobic digestion process. In 23rd Puredue industrial waste conference proceedings. pp 285-310.

APHA-AWWA-WPCF. (ed.). 1980. Standard methods for the examination of water and wastewater, 15th edition. American Public Health Association, Washington, D.C.

APHA-AWWA-WPCF (ed.).1985. Standard methods for the examination of water and wastewater, 16th edition. American Public Health Association, Washington, D.C.

Archer, D. B., 1988. Report on the microbiological aspects of granulation. In Lettinga, G., A. J. B. Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol(ed.), Granular anaerobic sludge; microbiology and technology. Pudoc, Wageningen, Netherlands, 1988. pp107-111.

Balch, W. E., S. Schoberth, R. S. Tanner, and R. S. Wolfe. 1977. *Acetobacterium*, a new genus of hydrogen-oxidizing, carbon dioxide-reducing, anaerobic bacteria. Int. J. Syst. Bacteriol. 27:355-361.

Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe. 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. 43: 260-296.

Banat, I. M., E. B. Lindstrom, D. B. Nedwell, and M. T. Balba. 1981. Evidence for coexistence of two distinct functional groups of sulfate-reducing bacteria in salt marsh sediment. Appl. Environ. Microbiol. 42: 985-992.

Barbrosa, R. A., and G. L. Sant' Anna Jr. 1989. Treatment of raw domestic sewage in an UASB reactor. Wat. Res. 23:1483-1490.

Baron, S. F. and J. G. Ferry. 1989. Reconstitution and properties of a coenzyme F_{420}-mediated formate hydrogenlyase system in *Methanobacterium formicicum*. J. Bacteriol. 171:3854-3859.

Beaty, P. S., M. J. McInerney, and N. Q. Worfford. 1986. Energetics of H_2- producing syntrophic bacteria. Antonopoulos, A. A. (ed.). Biotechnological advances in processing municipal wastes for fuels and chemicals. Argonne Natural Laboratory, Argonne, IL. pp67-83.

Beaty, P. S., and M. J. McInerney. 1987. Growth of *Syntrophomonas wolfei* in pure culture on crotonate. Appl. Environ. Microbiol. 147: 389-393.

Beaty, P. S., and M. J. McInerney. 1990. Nutritional features of *Syntrophomonas wolfei*. Appl. Environ. Microbiol. 56:3223-3224.

Bochem, H. P., S. M. Schoberth, B. Sprey, and P. Wergler. 1982. Thermophilic biomethanation of acetic acid: morphology and ultrastructure of a granular consortium. Can. J. Microbiol. 28:500-510.

Bolle, W. L., J. van Breugel, G. C. Van Eybergen, N. W. F. Kossen, and W. van Gils. 1986. Kinetics of anaerobic purification of industrial wastewater. Biotech. Bioeng. 28: 542-548.

Boone, D. R., and M. P. Bryant.1980. Propionate-degrading bacterium, *Syntrophobacter wolinii* sp. nov. gen. nov., from methanogenic ecosystems. Appl. Environ. Microbiol. 40: 626-632.

Boone, D. R., J. A. G. F. Menaia, J. E. Boone, and R. A. Mah. 1987. Effects of hydrogen pressure during growth and effects of pregrowth with hydrogen on acetate degradation by *Methanosarcina* species. Appl. Environ. Microbiol. 53:83-87.

Boone, D. R., and W. B. Whitman. 1988. Proposal of minimal standards for describing new taxa of methanogenic bacteria. Int. J. Syst. Bacteriol. 38: pp212-219.

Boone, D. R., R. L. Johnson., and Y. Liu. 1989. Diffusion of the interspecies electron carrier H_2 and formate in methanogenic ecosystems and its implications in the measurement of K_m for H_2 or formate uptake. Appl. Environ. Microbiol. 55: 1735-1741.

Breznak, J. A., J. M. Switzer, and H. -J. Seitz. 1988. *Sporamura termitida* sp. nov., a H_2/CO_2-utilizing acetogen isolated from termites. Arch. Microbiol. 150:282-288.

Brummeler, E., L. W. Hulshoff Pol, J. Dolfing, G. Lettinga, and A.J.B. Zehnder. 1985. Methanogenesis in an anaerobic sludge blanket reactor at pH 6 on an acetate-propionate mixture. Apll. Environ. Microbiol. 49: 1472-1477.

Bryant, M. P., E. A. Wolin, M. J. Wolin, and R. S. Wolfe. 1967. *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. Arch. Microbiol. 59:20-31.

Bryant, M. P. 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. Am. J. Clin. Nutr. 25: 1342-1328.

Bryant, M. P. 1977. Microbiology of the rumen. In Stevenson, M. J. (ed.), Duke's physiology of domestic animals, 9th ed. Cornell University Press, Ithaca, NY. pp287-304.

Bryant, M. P., L. L. Campbell, C. A. Reddy, and M. R. Crabill. 1977. Growth of *Desulfovirio* in lactate or ethanol media low in sulfate in association with H_2-utilizing methanogenic bacteria. Appl. Environ. Microbiol. 33:1162-1169.

Bryant, M. P. 1979. Microbial methane production-theoretical aspects. J. Anim. Sci. 48:193-201.

Bryant, M. P., and D. R. Boone. 1987. Isolation and characterization of *Methanobacterium formicicum* MF. Int. J. System. Bacteriol. 37:171.

Chartrain, M., and J. G. Zeikus. 1986a. Microbial Ecophysiology of whey biomethanation: intermediary metabolism of lactose degradation in continuous culture. Appl. Environ. Microbiol. 51: 180-187.

Chartrain, M., and J. G. Zeikus. 1986b. Microbial Ecophysiology of whey biomethanation: Characterization of bacterial trophic populations and prevalent species in continuous culture. Appl. Environ. Microbiol. 51: 188- 196.

Clark, J. N. 1988. Anaerobic digestion of whey in a pilot-scale upflow anaerobic sludge blanket digester. In Tilche, A., and A. Rozzi. (eds.) Poster-papers, fifth international symposium on anaerobic digestion. Bologna, Italy, 22-26 May 1988. pp489-493.

Cohen, A. 1982. Optimization of anaerobic digestion of soluble carbohydrate containing wastewaters by phase separation. Ph.D thesis, Universiteit van Amsterdam, Amsterdam, The Netherlands.

Cohen, A., J. M. van Gemert, R. J. Zoetemeyer, and A. M. Breure. 1984. Main characteristics and stoichiometric aspects of acetogenesis of soluble carbohydrate containing wastewaters. Process Biochem. Dec:228-232.

Colleran, E. 1988. Report on technological aspects of granulation. In Lettinga, G., A. J. B. Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol(ed.), Granular anaerobic sludge; microbiology and technology. Pudoc, Wageningen, Netherlands, 1988. pp237-240.

Conrad, R., F. L. Lupton, and J. G. Zeikus. 1987. Hydrogen metabolism and sulfate-dependent inhibition of methanogenesis in an eutrophic lake sediments. FEMS Microbiol. Ecol. 45:107-115.

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

Crawford, G. V., and G. H. Teletzke. 1986. Performance of a hybrid anaerobic process. In Proceedings of the 41st industrial waste conference, Purdue University, West Lafayette, Indiana. Lewis Publisher, Inc., MI. pp196-208.

Dolfing J., 1985. Kinetics of methane formation by granular sludge at low substrate concentrations, the influence of mass transfer limitation. Appl. Microbiol. Biotechnol. 22: 77-81

Dolfing, J., and W. G. B. M. Blomen. 1985. Activity measurements as a tool to characterize the microbial composition of methanogenic environments. J. Microbiol. Methods. 4: 1-12

Dolfing, J., A. Griffioen, A. R. W. van Neerven, and L. P. T. M. Zevenhuizen. 1985. Chemical and bacteriological composition of granular methanogenic sludge. Can. J. Microbiol. 31:744-750.

Dolfing, J. 1987. Microbiological aspects of granular methanogenic sludge, Ph.D Thesis, Agricultural University, Wageningen, the Netherlands.

Dolfing, J., and J. M. Tiedje. 1988. Acetate Inhibition of methanogenic, syntrophic benzoate degradation. Appl. Environ. Microbiol. 54: 1871-1873.

Dubourguier, H. C., G. Prensier and G. Albagnac. 1988a. Structure and microbial activities of granular anaerobic sludges. In Lettinga, G., A. J. B. Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol(ed.), Granular anaerobic sludge; microbiology and technology. Pudoc, Wageningen, Netherlands, 1988. pp18-33.

Dubourguier, H. C., M. N. Buisson, J. P. Tissier, G. Prensier and G. Albagnac. 1988b. Structural characteristics and metabolic activities of granular methanogenic sludge on a mixed defined substrate. In Lettinga, G., A. J. B. Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol (ed.), Granular anaerobic sludge; microbiology and technology. Pudoc, Wageningen, Netherlands, 1988. pp78-86.

Dubourguier, H. C., D. B. Archer, G. Albagnac, and G. Prensierc. 1988c. Structure and metabolism of methanogenic microbial conglomerates. In Hall, E. R.,and P. N. Hobson (ed.), Anaerobic Digestion 1988. Pergramon Press, Oxford, U.K. pp13-23.

Dwyer, D. F., E. Weeg-Aerssens, D. R. Shelton, and J. M. Tiedje. 1988. Bioenergetic conditions of butyrate metabolism by a syntrophic, anaerobic bacterium in coculture with hydrogen-oxidizing methanogenic and sulfidogenic bacteria. Appl. Environ. Microbial. 54:1354-1359.

Endo, G., and Y. Tohay. 1985. High rate treatment of night soil by anaerobic upflow sludge blanket (UASB) process. In China State Biogas Association (ed.), Anaerobic digestion 1985. Guangzhou, China. pp441-443.

Endo, G., and Y. Tohay. 1988. Ecological study on anaerobic sludge bulking caused by filamentous bacteria growth in an anaerobic contact process. In Olson, B. H., and D. Jenkins. (ed.), Proceedings of international conference on water and wastewater microbiology. Volume 2, 34 (1). Newport Beach, California.

Fathepure, B. Z. 1983. Isolation and characterization of an acetoclastic methanogen from a biogas digester. FEMS Microbiol. Lett. 19:151-156.

Ferguson, T. J., and R. A. Mah. 1983. Effect of H_2-CO_2 on methanogenesis from acetate or methanol in *Methanosarcina* sp. Appl. Environ. Microbiol. 46:348-355.

Gorris, L. G. M., and J. M. A. van Deursen. 1988. Biofilm development in lab-scale methanogenic fluidized bed reactors. Lettinga,G., A.J.B. Zehnder, J.T.C. Grotenhuis, and L.W. Hulshoff Pol(eds). Granular anaerobic sludge; microbiology and technology. Pudoc, Wageningen, Netherlands, 1988. pp87-95

Gorris, L. G. M., T. M. de Kok, B. M. Kroon, C. van der Drift, and G. D. Vogels. 1988. Relationship between methanogenic cofactor content and maximum specific methanogenic activity of anaerobic granular sludges. Appl. Environ. Microbiol. 54:1126-1130.

Grotenhuis, J. T. C., J. van Lier, C. M. Plugge. 1988b. Effect of calcium removal on size and strength of methanogenic granules. In Tilche, A., and A. Rozzi. (eds.) Poster-papers, fifth international symposium on anaerobic digestion. Bologna, Italy, 22-26 May 1988. pp117-120.

Guiot, S. R., and L. van den Berg. 1985. Performance of an upflow anaerobic reactor combining a sludge blanket and a filter treating sugar waste. Biotech. Bioeng.27: 800-806.

Guiot, S. R., K.L. Kennedy, and L. van den Berg. 1985. Comparison of UBF and UASB reactor concepts. Poster presented at 4th International symposium on anaerobic digestion, Guangzhou, China, Nov. 11-15, 1985.

Guiot, S.R., S.S. Gorur, and K.J. Kennedy. 1988. Nutritional and environmental factors contributing to microbial aggregation during upflow anaerobic sludge bed-filter (UBF) reactor start-up. In Hall, E. R.,and P. N. Hobson (ed.), Anaerobic Digestion 1988. Pergramon Press, Oxford, U.K. pp.47-53.

Gujer, W., and A. J. B. Zehnder. 1983. Conversion processes in anaerobic digestion. Wat. Sci. Tech. 15: 127-167.

Guyot, J. P., and A. Brauman. 1986. Methane production from formate by syntrophic association of *Methanobacterium bryantii* and *Desulfovibrio vulgaris*. Appl. Environ. Microbiol. 52: 1436-1437.

Guyot, J. P., A. Noyola, and O. Monroy. 1990. Evolution of microbial activities and population in granular sludge from an UASB reactor. Biotechnol. Lett. 12:155-160.

Habets, L.H.A., and J.H. Knelissen. 1985. Application of the UASB-reactor for anaerobic treatment of paper and board mill effluent. Wat. Sci. Tech. 17:61- 75.

Hall, E. R., and M. Jovanovic. 1982. Anaerobic treatment of thermal sludge conditioning liquor with fixed-film and suspended growth processes. In Proceedings of the 37th industrial waste conference, Purdue University, West Lafayette, Indiana. pp 719.

Hawlwachs, W. 1978. K_m and V_{max} from only one experiment. Biotech. Bioeng. 20:281-285.

Harada, H., G. Endo, Y. Tohya, and K. Momonoi. 1988. High rate performance and its related characteristics of granulated sludges in UASB reactors treating various wastewaters. In Tilche, A., and A. Rozzi. (eds.) Poster-papers, fifth international symposium on anaerobic digestion. Bologna, Italy, 22-26 May 1988. pp1011-1020.

Hartee E. F. 1972. A modification of the Lowry Method, that gives a linear photometric response. Anal. Biochem. 48:422-426.

Heertjes, P. M., and R. R. van der Meer. 1978. Dynamics of liquid flow in an upflow reactor used for anaerobic treatment of wastewater. Biotech. Bioeng. 20: 1577-1594.

Hemens, J., P. G. Meiring, and G. J. Stander. 1962. Full scale anaerobic digestion of effluent from the production of maize starch. Water and Waste Treatment. 9:16-18.

Henson, J. M., and P. H. Smith. 1985. Isolation of a butyrate-utilizing bacterium in coculture with *Methanobacterium thermoautotrophicum* from a thermophilic digester. Appl. Environ. Microbiol. 49: 1461-1466.

Heyes, R. H., and R. J. Hall. 1983. Kinetics of two subgroups of propionate-using organisms in anaerobic digestion. Appl. Environ. Microbiol. 46: 710-715.

Hickey, R. F. and R. F. Owens. 1981. Methane generation from high strength industrial wastes with the anaerobic fluidized bed. Biotech. Bioeng. Symp. Ser. No.11, 399.

Hilpert, R., J. Winter, W. Hammes and O. Kandler. 1981. The sensitivity of archaebacteria to antibiotics. Zbl. Bakt. Hyg., I. Abt. Orig. C2:11-20.

Hilton, M. G., and D. B. Archer. 1988. Anaerobic digestion of a sulfate-rich molasses wastewater: inhibition of hydrogen sulfide production. Biotech. Bioeng. 31: 885-888.

Hirsch, R. 1984. Microcolony formation and consortia. Marshall, K.C. (eds.). Microbial adhesion and aggregation. Springer Verlag, Berlin. pp 373-393.

Hopner, T., and J. Knappe. 1974. Determination with formate dehydrogenase. In Bergmeryer, H. U. (eds.), Methods of enzymatic analysis. Academic Press Inc., New York and London, pp1551-1555.

Hu, J. -C., W. -M. Wu, and X. -S. Gu. 1985. Experimental studies on the treatment of beer yeast and saccharification wastewater by UASB reactor. Energy Sources Research Committee and International Cooperation Bureau of Academia Sinica(eds). Sino-Japanese Symposium on Renewable Energy Resources. Chengdu, China. pp35-43.

Hulshoff Pol, L. W., J. Dolfing, W. de Zeeuw, and G. Lettinga. 1982. Cultivation of well adapted pelletized methanogenic sludge. Biotech. Letters. 4: 329-332.

Hulshoff Pol, L. W., W. J. de Zeeuw, C. T. M. Velzeboer and G. Lettinga. 1983. Granulation in UASB reactor. Wat. Sci. Tech. 15: 291-304.

Hulshoff Pol, L. W., K. Heijnekamp and G. Lettinga. 1988. The selection pressure as a driving force behind the granulation of anaerobic sludge. In Lettinga, G., A. J. B. Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol (ed.), Granular anaerobic sludge; microbiology and technology. Pudoc, Wageningen, Netherlands, 1988. pp153-161

Hulshoff Pol, L. W. 1989. The phenomenon of granulation of anaerobic sludge. Ph.D Thesis, Department of Environmental Engineering, Agricultural University, Wageningen, The Netherlands.

Hungate, R. E. 1966. The rumen and its microbes. Academic Press, New York.

Hungate, R. E. 1967. Hydrogen as an intermediate in the rumen fermentation. Arch. Microbiol. 59:158-164.

Huser, B. A., K. Wuhrmann, and A. J. B. Zehnder. 1982. *Methanothrix soehngenii* gen. nov. sp. nov., a new acetotrophic non-hydrogen-oxidizing methane bacterium. Arch. Microbiol. 132: 1-9.

Isa, Z., S. Grusenmeyer, and W. Verstraete. 1986. Sulfate reduction relative to methane production in high-rate anaerobic digestion: microbiological aspects. Appl. Environ. Microbiol. 51: 580-587.

Iza, J., P. A. Garcia, I. Sanz, and F. Fdz-Polanco. 1988. Granulation results in anaerobic fluidized bed reactors. In Lettinga, G., A. J. B. Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol. (ed.). Granular anaerobic sludge; microbiology and technology. Pudoc, Wageningen, Netherlands, 1988. pp195-202.

Jain, M. K., T. E. Thompson, E. Conway de Macario, and J. G. Zeikus. 1987. Speciation of *Methanobacterium* strain Ivanov as *Methanobacterium ivanovii* sp. nov. System. Appl. Microbiol. 9:77-82.

Jain, M. K., and J. G. Zeikus. 1987. Methods for isolation of auxotrophic mutants of *Methanobacterium ivanovii* and initial characterization of acetate auxotrophs. Appl. Environ. Microbiol. 53:1387-1390.

Jain, M. K., L. Bhatnagar, and J. G. Zeikus. 1988. A taxonomic overview of methanogens. Indian J. Microbiolol. 28:143-177.

Jeris, J. S., and P. L. McCarty. 1965. The biochemistry of methane fermentation using ^{14}C traces. J. WPCF. 37:178-192.

Jewell, W. J., M. S. Switzenbaum, and J. W. Morris. 1981. Municipal wastewater treatment with the anaerobic attached microbial film expanded process. J. WPCF. 53:482-490.

Jiang, Q. 1989. University of Arizona, personal communication.

Jones, W. J., D. P. Nagle, and W. B. Whitman. 1987. Methanogens and diversity of archaebacteria. Microbiol. Rev. 51: 135-177.

Kamagata, Y., and E. Mikami. 1990. Some Characteristics of two morphotypes of *Methanothrix soehngenii* from mesophilic anaerobic digesters. J. Ferment. Bioeng. 70:272-274.

Kaspar, H. F., and K. Wuhrmann. 1978. Kinetic parameters and relative turnover of some important catabolic reactions in digesting sludge. Appl. Environ. Microbiol. 36:1-7.

Kenealy, W., and J. G. Zeikus. 1981. Influence of corrinoid antagonists on methanogen metabolism. J. Bacteriol. 146: 133-140.

Killilia, E. and E. Colleran. 1988. Comparison of fixed-bed and hybrid fixed bed/sludge bed reactors for anaerobic digestion of cheese whey. In Tilche, A., and A. Rozzi. (eds.) Poster-papers, fifth international symposium on anaerobic digestion. Bologna, Italy, 22-26 May 1988. pp541-546.

Kimata, T., T. Kawai, M. Tada, K. Tanaka, K. Shinabe, and K. Shimizu. 1990. Anaerobic treatment of thermal conditioning liquor. Paper presented at the 63rd annual conference of the water pollution control federation, October 7-11, 1990, Washington D.C.

Kjelleberg. S. 1984. Adhesion to inanimate surfaces. In Marshall, K. C. (ed.) Microbial adhesion and aggregation. Springer-Verlag, Berlin. pp51-70.

Klapwijk, A., H. Smit, and A. Moore. 1981. Denitrification of domestic wastewater in upflow sludge-blanket reactor without carrier material for the biomass. Cooper, R. F., and B. Atkinson. (eds.) Biological fluidized bed treatment of water and wastewater. Ellis Horwood Limited, Chichester. pp205- 216.

Kobayashi, H. A., E. Conway de Macario, R. S. Williams, and A. J. L. Macario. 1988. Direct characterization of methanogens in two high-rate anaerobic biological reactors. Appl. Environ. Microbiol. 54:693-698.

Koch, M., J. Dolfing, K. Wuhrmann, and A.J.B. Zehnder. 1983. Pathways of propionate degradation by enriched methanogenic cultures. 45: 1411-1414.

Koornneef, E., A. J. L. Macario, J. T. C. Grotenhuis, and E. C. de Macario. 1990. Methanogens revealed immunologically in granules from five upflow anaerobic sludge blanket (UASB) bioreactors grown on different substrates. FEMS Microbiol. Ecology. 73:225-230.

Koster, I. W., and G. Lettinga. 1984. The influence of ammonium-nitrogen on the specific activity of pelletized methanogenic sludge. Agricultural Wastes. 9:205-216.

Kreisl, P., and Kandler. 1986. Chemical structure of the cell wall polymer of *Methanosarcina*. System. Appl. Microbiol. 7: 293-299.

Kristjansson, J. K., 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.

Laanbroek, H. J., T. Abee, and J. L. Voogd. 1982. Alcohol conversions by *Desulfobulbus propionicus* Lindhorst in the presence and absence of sulfate and hydrogen. Arch. Microbiol. 133:178-184.

Lamed, R., J. Naimark, E. Morgenstern and E. A. Bayer. 1987. Scanning electron microscopic delineation of bacterial surface topology using cationized ferritin. J. Microbiol. Methods. 7:233-240.

Lanting, J., J. A. Jordan, M. T. Schone, A. Kull, W. W. Carey, and B. L. Kitney. 1988. Thermophilic anaerobic digestion of coffee wastewater. In Proceedings of the 47th industrial waste conference, Purdue University, West Lafayette. Lewis Publisher Inc., MI. pp513-524.

Lawrence, A. W., and P. L. McCarty. 1969. Kinetics of methane fermentation in anaerobic treatment. J. WPCF. 41: R1-R17.

Lauerer, G., J. K. Kristjansson, T. A. Langworthy, H. König, and K. O. Stetter. 1986. *Methanothermus sociabilis* sp. nov., a second species within the *Methanothermaceae* growing at 97 °C. Syst. Appl. Microbiol. 8:100-105.

Lee, M. J., and S. H. Zinder. 1988. Isolation and characterization of a thermophilic bacterium which oxidizes acetate in syntrophic association with a methanogen and which grows acetogenically on H_2-CO_2. Appl. Environ. Microbiol. 54:124-129.

Leigh, J. A., F. Mayer, and R. S. Wolfe. 1981. *Acetogenium kivui*, a thermophilic hydrogen-oxidizing, acetogen bacterium. Arch. Microbiol. 129: 275-280.

Lema, J.M., C. Casas, A. Aguilar, and J. Lafuente. 1988. On the isomerization between n-butyrate and i-butyrate. In Tilche, A., and A. Rozzi. (ed.), Poster-papers, fifth international symposium on anaerobic digestion. Bologna, Italy, 22-26 May 1988. pp49-51.

Lettinga G., van Velsen A. F. M., Hobma S. W., W. de Zeeuw., A. Klapwijk. 1980 Use of the upflow sludge blanket (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. Biotechnol. Bioeng. 22:699-734.

Lettinga, G., R. Roersma, P. Grin, W. de Zeeuw, L. Hulshoff Pol, L. van Velsen, S. Hobma and G. Zeeman. 1981. Anaerobic treatment of sewage and low strength waste waters. In Hughes, D.E., D. A. Stafford, B. I. Wheatley, W. Baader, G. Lettinga, E. J. Nyns, W. Versraete, and R. L. Wentworth (eds.), Anaerobic digestion 1981. Elsevier biomedical Press, Amsterdam, 1982. pp271-291.

Lettinga, G., S. W. Homa, L. W. Hulshoff Pol, W. de Zeeuw, P. de Jong, D. Grin, and R. Roersma. 1983a. Design, operation and economy of anaerobic treatment. Wat. Sci. Tech. 15:177-196.

Lettinga, G., R. Roersma, and D. Grin. 1983b. Anaerobic treatment of raw domestic sewage at ambient temperatures using a granular bed UASB reactor. Biotechnol. Bioeng.25: 1701-1723.

Lettinga, G., L. W. Hulshoff Pol, I. W. Koster, W. M. Wiegant, W. J. de Zeeuw, A. Rinzema, D.C. Grin, R.E. Roersma, and S.W. Hobma. 1984. High-rate anaerobic wastewater treatment using the UASB reactor under a wide range of temperature conditions. Biotechnol. Genet. Energ. Rev. 2: 253-284.

Lettinga, G., W. J. de Zeeuw, L. Hulshoff Pol, W. M. Wiegant, and A. Rinzema. 1985. Anaerobic wastewater treatment based on biomass retention with emphasis on the UASB process. In China State Biogas Association (ed), Anaerobic digestion 1985. Guangzhou, China. pp279-301.

Lettinga, G., and L. Hulshoff Pol. 1990. Agricultural University, Wagenegen, the Netherlands, personal communication.

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

Lorowitz, W. H., H. X. Zhao, and M. P. Bryant. 1989. *Syntrophomonas wolfei* subsp. nov., a long-chain fatty-acid-degrading, anaerobic, syntrophic bacterium; *Syntrophomonas wolfei* subsp. wolfei subsp. nov.; and emended descriptions of the genus and species. Int. J. Syst. Bacteriol. 39:122-126.

Lovley, D. R., and M. Klug. 1982. Intermediary metabolism of organic matter in the sediments of a eutrophic like. Appl. Environ. Microbiol. 43: 552-560.

Lui, Y., D. R. Boone, R. Sleat, and R. A. Mah. 1985. *Methanosarcina mazei* LYC, a new methanogenic isolate which produces a disaggregating enzyme. Appl. Environ. Microbiol. 49:608-613.

Lui, K., X. Lui, H. Xu. 1985. The research of treating the starch wastes by improving the UASB reactors. Poster presented at the 4th International Symposium on Anaerobic Digestion. Guangzhou, China, Nov. 11-15, 1985.

Macario, A. J. L., and E. Conway de Macario. 1983. Antigenic fingerprinting of methanogenic bacteria with polyclonal antibody probes. Syst. Appl. Microbiol. 4:451-458.

Macario, A. J. L., and E. Conway de Macario. 1985. Monoclonal antibodies of defined molecular specifity for identification and for probing their ecologic niches. In: Macario, A. J. L., and E. Conway de Macario (ed.), Monoclonal antibodies against bacteria, Vol. 2. Academic Press, Inc., Orlando, FL. pp213-247.

Macario, A. J. L., and E. Conway de Macario. 1988. Quantitative immunologic analysis of the methanogenic flora of digestors reveals a considerable diversity. Appl. Environ., Microbiol. 54:79-86.

MacDonell, M.T., E. Russek, and R. R. Colwell. 1984. An interactive microcomputer program for the computation of most probable number. J. Microbiol.Meth. 2: 1-7.

Mackie, R. I., and M. P. Bryant. 1981. Metabolic activity of fatty acid-oxidizing bacteria and the contribution of acetate, propionate, butyrate, and CO_2 to methanogenesis in cattle waste at 40 and 60° C. Appl. Environ. Microbiol. 41: 1363-1373.

Mah, R. A. 1980. Isolation and characterization of *Methanococcus mazei.* Curr. Microbiol. 3:321-326.

Mah, R. A., and D. A. Kuhn. 1984. Transfer of the type species of the genus *Methanococcus mazei* to the genus *Methanosarcina*, naming it *Methanosarcina mazei* (Barker 1936) comb. nov. etemend. and conservation of the genus *Methanococcus* (Approved Lists 1980) with *Methanococcus vannielii* (Approved Lists 1980) as the type species. Int. J. Syst. Bacteriol. 34:263-265.

Mahoney, E. M., L. K. Varangu, W. L. Cairns, and N. Kosaric. 1984. Cell surface and aggregation studies of microbes from anaerobic systems. Paper presented at the 57th WPCF conference, New Orleans, Louisiana, October 1-4, 1984.

Manjunath, D. L., I. Mehrotra, and R. P. Mathur. 1989. Treatment of cane sugar mill wastewater in upflow anaerobic sludge blanket (UASB) reactors. In 44th Purdue industrial waste conference proceedings. Lewis Publishers, Chelsea, MI. pp215-223.

Maupin, P., and T. D. Pollard. 1983. Improved preservation and staining of hela cell action filaments, clathrb coated membranes, and other cytoplasmic structures by tannic acid glutaraldehyde-saponin fixation. J. Cell. Biol. 96:51-62.

McCarty, P. L. 1974. Anaerobic process. Presented at the Birmingham course on design aspects of biological treatment, International Association of Water Pollution research, U.K.

McCarty, P. L. 1982. One hundred years of anaerobic treatment. In Hughes, D. E., D. A. Stafford, B. I. Wheatley, W. Baader, G. Lettinga, E. J. Nyns, W. Versraete, and R. L. Wentworth (eds.), Anaerobic digestion 1981. Elsevier biomedical Press, Amsterdam. pp3-22.

McInerney, M. J., M. P. Bryant, and D. A. Stafford. 1979a. Metabolic stages and energetics of microbial anaerobic digestion. In Stafford, D. A., B. I. Wheatley and D. E. Hughes (ed.), Anaerobic digestion. Applied Science Publishers Ltd., Cardiff, U.K. pp91-98.

McInerney, M. J., M. P. Bryant, and N. Pfenning. 1979b. Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. Arch. Microbiol. 122:129-135.

McInerney, M. J., and M. P. Bryant. 1980. Syntrophic Association of H_2-producing alcohol and fatty acid-degrading bacteria in anaerobic degradation of organic matter. In Gottscbalk et al.(eds), Anaerobes and anaerobic in fections. Gustav Fiscber Verlag, Stuttgart, New York . pp117-126.

McInerney, M. J., and M. P. Bryant. 1981. Anaerobic degradation of lactate by sytrophic associations of *Methanosarcina barkeri* and *Desulfovibrio* species and effect of H_2 on acetate degradation. Appl. Environ. Microbiol. 41:346-354.

McInerney, M. J., M. P. Bryant, R. B. Hespell, and J. W. Costerton. 1981. *Syntrophomonas wolfei* gen. nov., an anaerobic, syntrophic, fatty acid-oxidizing bacterium. Appl. Environ. Microbiol. 41: 1029-1039.

McInerney, M. J. 1988. Anaerobic hydrolysis and fermentation of fats and proteins. In Zehnder, A. J. B. (ed.), Biology of anaerobic microorganisms, John Wiley & Sons, New York. pp373-416.

Min, H., and S. H. Zinder. 1989. Kinetics of acetate utilization by two thermophilic acetotrophic methanogens: *Methanosarcina* sp. strain CALS-1 and *Methanothrix* sp. strain CALS-1. Appl. Environ. Microbiol. 55:488-491.

Mink, R. W., and P. R. Dugan. 1977. Tentative identification of methanogenic bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33:713-717.

Moller, B., R. Ossmer, B. H. Howard, G. Gottschalk, and H. Hippe. 1983. *Sporomusa*, a new genus of gram-negative anaerobic bacteria including *Sporomusa sphaeroides* spec. nov. and *Sporomusa ovata* spec. nov. Arch. Microbiol. 139: 388-396.

Morvai, L., P. Mihàltz, L. Czakò, M. Pèterfy, and J. Hollò. 1990. The influence of organic load on granular sludge development in an acetate-fed system. Appl. Microbiol. Biotechnol. 33:463-468.

Mountfort, D.O., and M. P. Bryant. 1982. Isolation and characterization of an anaerobic syntrophic benzoate-degrading bacterium from sewage sludge. Arch. Microbiol. 133: 249-256.

Mountfort, D. O., W. J. Brulla, L. R. Krumholtz, and M. P. Bryant. 1984. *Syntrophus buswellii* gen. nov., sp. nov.: a benzoate -degrading bacterium from sewage sludge. Arch. Microbiol. 133:249-256.

Mountfort, D. O. 1987. The rumen anaerobic fungi. FEMS Microbiol. Rev. 46:401-408.

Mucha, H., F. Lingens, and W. Trosch. 1988. Conversion of propionate to acetate and methane by syntrophic constortia. Appl. Microbiol. Biotechnol. 27:581-586.

Murray, W. D., and L. van den Berg. 1981. effects of nickel. cobalt, and molybdenum on performance of methanogenic fixed-film reactors. Appl. Environ. Microbiol. 42: 502-505.

Nagai, S., and N. Nishio. 1990. Application of *Methanosarcina barkeri* cells for the production of useful chemicals. In T. K. Ghose (ed.), Bioprocess engineering: the first generation. Ellis Horwood Limited Publishers, Chichester, U.K. pp291-302.

Ngian, R. F., S. H. Lin, and W. R. B. Martin. 1977. Effect of mass transfer resistance on the Lineweaver-Burk plots for flocculating microorganisms. Biotech. Bioeng. 19: 1773-1784.

Nishio, N., S. Y. Eguchi, K. Ryuno, H. Kawashima, and S. Nagai. 1983a. Isolation of a formate-utilizing methanogenic bacterium by a chemostat culture technique. J. Ferment. Technol. 61: 437-441.

Nishio, N., S. Y. Eguchi, H. Kawashima, and S. Nagai. 1983b. Mutual conversion between H_2 plus CO_2 and formate by a formate-utilizing methanogen. J. Ferment. Technol. 61:557-561.

Novaes, R. F. V., C. M. Rech, M. G. Figueiredo and L. A. Giaj-Levra. 1988. Bacterial identification of granular sludge from domestic sewage UASB-reactor. In Tilche, A., and A. Rozzi. (ed.), Poster-papers, fifth international symposium on anaerobic digestion. Bologna, Italy, 22-26 May 1988. pp61-64.

Oi, S., G. Dhavises, P. Sriprasetsak, T. Tanaka, and T. Makoto. 1985. Methane fermentation of agro-waste and grasses. China State Biogas Association (eds). Anaerobic Digestion 1985. Guangzhou, China. pp.145-157.

Oleszkiewitz, J. A., E. R. Hall, and J. Z. Oziemblo. 1986. Performance of laboratory anaerobic hybrid reactors with varying depths of media. Environ. Technol. Lett. 7:445-452.

O'Rourke J. T. 1969. Kinetics of anaerobic waste treatment at reduced temperatures. Ph.D. Thesis. Stanford University, CA, U.S.A.

Orozco. A. 1988. Anaerobic wastewater treatment using an open plug flow baffled reactor at low temperature. In Tilche, A., and A. Rozzi. (eds.) Poster-papers, fifth international symposium on anaerobic digestion. Bologna, Italy, 22-26 May 1988. pp759-762.

Ozturk, S. S., B. O. Palsson, and J. H. Thiele. 1989. Control of interspecies electron transfer flow during anaerobic digestion: dynamic diffusion reaction models for hydrogen gas transfer in microbial flocs. Biotech. Bioeng. 33:745- 757.

Patel, G. B., L. A. Roth, L. van den Berg, and D. S. Clark. 1976. Characterization of a strain of *Methanospirillum hungatei.* Can. J. Microbiol. 22: 1404-1410.

Patel, G. B., A. W. Khan , and L. A. Roth. 1978. Optimum levels of sulfate and iron for the cultivation of pure culture of methanogens in synthetic media. J. Appl. Bacteriol. 45: 347-356.

Patel, G. B., L. A. Roth, and G. D. Sprott. 1979. Factors influencing filament length of *Methanosprillum hungatii.* J. Gerneral Microbiol. 112:411-415.

Patel, G. B. 1984. Characterization and nutritional properties of *Methanothrix concilii* sp. nov., a mesophilic acetoclastic methanogen. Can. J. Microbiol. 30: 1383-1396.

Patel, G. B., and G. D. Sprott. 1990. *Methanosaeta concilii* gen. nov., sp. nov. ("*Methanothrix concilii*") and *Methanosaeta thermoacetophila.*, comb. nov. Int. J. Syst. Bacterial. 40:79-82.

Patte, K. C., and A. I. Versprille. 1982. Application of the U.A.S.B.- concept for wastewater treatment. In Hughes, D. E., D. A. Stafford, B. I. Wheatley, W. Baader, G. Lettinga, E. J. Nyns, W. Versraete, and R. L. Wentworth (ed.), Anaerobic digestion 1981. Elsevier Biomedical Press, Amsterdam, pp 121-133.

Perry, R. B., D. W. Green, and J. O. Maloney (ed.). 1984. Peery's Chemical Engineers' Handbook, 16th edition. McGraw-Hill, Inc., New York. pp3-275 to 3-335.

Phelps, T. J., R. Conrad. and J. G. Zeikus. 1985. Sulfate-dependent interspecies H_2 transfer between *Methanosarcina barkeri* and *Desulfovibrio vulgaris* during coculture metabolism of acetate or methanol. Appl. Environ. Microbiol. 50: 589-594.

Porter, K.G., and Y. S. Feig. 1980. The use of DAPI for identifying and counting aquatic microflora. Limn. Ocean. 25:943-948.

Prensier, G., H. C. Dubourguier, I. Thomas, G. Albagnac, and M. N. Buisson. 1988. Specific immunological probes for studying the bacterial associations in granules and biofilms. In Lettinga, G., A. J. B. Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol (ed.), Granular anaerobic sludge; microbiology and Technology. Pudoc, Wageningen, Netherlands, 1988. pp55-61.

Rinzema, A., J. van Lier, and G. Lettinga. 1988. Sodium inhibition of acetoclastic methanogens in granular sludge from a UASB reactor. In Lettinga,G., A. J. B. Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol(ed.), Granular anaerobic sludge; microbiology and technology. Pudoc, Wageningen, Netherlands, 1988. pp 216-222.

Robinson, R. W., D. E. Akin, R. A. Nordstedt, M. V. Thomas, and H. C. Aldrich. 1984. Light and electron microscopic examinations of methane-producing biofilms from Anaerobic fixed-bed reactors. Appl. Environ. Microbiol. 48:127-136.

Robinson, R. W. 1986. Life cycles in the methanogenic archaebacterium *Methanosarcina mazei*. Appl. Environ. Microbiol. 52:17-27.

Rosenberg, M., D. Gutnick, and E. Rosenberg. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. 9:29-33.

Roy, F., G. Albagnac, and E. Samain. 1985. Influence of calcium addition on growth of highly purified syntrophic cultures degrading long-chain fatty acids. Appl. Environ. Microbiol. 49: 702-705.

Roy, F., E. Samain, H. C. Dubourguier, and G. Albagnac. 1986. *Syntrophomonas sapovorans* sp. nov., a new obligately proton reducing anaerobe oxidizing saturated and unsaturated long chain fatty acids. Arch. Microbiol. 145: 142-147.

Samain, E., H. C. Dubourguier, and G. Albagnac. 1984. Isolation and characterization of *Desulfobulbus elongatus* sp. nov. from a mesophilic industrial digester. Syst. Appl. Microbiol. 5:391-401.

Samain, E., R. Moletta, H. C. Dubourguier, and G. Albagnac. 1987. Propionate conversion to butyrate in an anaerobic digester. In Antonopoulos, A. A. (eds). Biotechnological advance in processing municipal wastes for fuels and chemicals. Noyes Data Corp., Park Ridge, N.J., U. S. A., 1987. pp223-233.

Sam-Soon, P. A. L. N. S., R. E. Looewenthal, P. L. Dold, and G. V .R. Marais. 1988. Pelletization in upflow anaerobic sludge bed reactors. In Hall, E. R., and P. N. Hobson (ed.), Anaerobic Digestion 1988. Pergramon Press, Oxford, U.K. pp55-60.

Sax, R. I., 1985. Anaerobic pretreatment of brewery wastewater on the industrial scale. In Proceedings of the 40th industrial waste conference, Purdue University, West Lafayette, Indiana. pp59-68.

Sayed, S. W. de Zeeuw, and G. Lettinga. 1984. Anaerobic treatment of slaughterhouse waste using a flocculent sludge UASB reactor. Agricultural Wastes, 11:197-226.

Schauer, N. L., and J. G. Ferry. 1980. Metabolism of formate in *Methanobacterium formicicum*. J. Bacteriol. 42:800-807.

Schauer, N. L., D. P. Brown, and J. G. Ferry. 1982. Kinetics of formate metabolism in *Methanobacterium formicicum* and *Methanospirillum hungatei*. Appl. Environ. Microbiol. 44:549-554.

Schink, B., and R. K. Thauer. 1988. Energetics of syntrophic methane formation and the influence of aggregation. In Lettinga, G., A. J. B. Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol. (ed.), Granular anaerobic sludge; microbiology and technology. Pudoc, Wageningen, Netherlands, 1988. pp5-17.

Schönheit, P., J. K. Kristjansson, R. K. Thauer. 1982. Kinetic methannism for the ability of sulfate reducers to out-compete methanogens for acetate. Arch. Microbiol. 132:285-288.

Schroepfer, G. J., W. J. Fuller, A. S. Johnson N. R. Ziemke, and J. J. Andrerson. 1955. The anaerobic contact process as applied to packinghouse wastes. Sewage Ind. Wastes. 27:460-486.

Shelton, D. R., and J. M. Tiedje. 1984. Isolation and partial characterization of bacteria in an anaerobic consortium that mineralizes 3-chlorobenzoic acid. Appl. Environ. Microbiol. 48: 840-848.

Shen, L. 1985. A pilot scale experiment on alcohol distillery wastewater treatment with upflow anaerobic sludge blanket reactor. In China State Biogas Association. (ed.), Anaerobic digestion 1985. Guangzhou, China. pp416-417.

Sierra-Alvarez, R., L. W. Hulshoff Pol, G. Lettinga. 1988. Start-up of a UASB reactor on a carbohydrate substrate. In Lettinga, G., A. J. B. Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol (ed.), Granular anaerobic sludge; microbiology and technology. Pudoc, Wageningen, Netherlands. pp223-228.

Smith, R. L., and M. J. Klug. 1981. Electron Donors utilized by sulfate-reducing bacteria in eutrophic lake sediments. Appl. Environ. Microbiol. 42: 116-121.

Smith, M. R., and R. A. Mah. 1966. Kinetics of acetate metabolism during sludge digestion. Appl. Environ. Microbiol. 14:368-371.

Snoeyink, V. L., and D. Jenkins. 1980. Water chemistry. John Wiley & Sons, New York.

Stander, G. J., G. C. Cillie, W. R. Ross, and R. D. Baillie. 1967. Treatment of wine distillery wastes by anaerobic digestion . In Proceedings of the 22nd industrial waste conference, Purdue University, West Lafayette, Indiana. pp892-907.

Stetter, K. O., M. Thomm, J. Winter, G. Wildgraber, H. Huber, W. Zilling, D. Janécovic, H. König, P. Palm, and S. Wunderl. 1981. *Methanothermus fervidus* sp. nov., a novel extremely thermophilic methanogen isolated from an Icelandic hot spring. Zent. Bakteriol. Hyg., I. Abt. Orig. C2, 166-178.

Stieb, M., and B. Schink. 1985. Anaerobic oxidation of fatty acids by *Clostridium bryantii* sp. nov., a sporeforming, obligately syntrophic bacterium. Arch. Microbiol. 140: 387-390.

Stieb, M., and B. Schink. 1986. Anaerobic degradation of isovalerate by a defined methanogenic coculture. Arch. Microbiol. 144: 291-295.

Stieb, M., and B. Schink. 1989. Anaerobic degradation of isobutyrate by methanogenic enrichment cultures and by a *Desulfococcus multivorans* strain. Arch. Microbiol. 151:126-132.

Switzenbaum, M. S., and R. B. Eimstad. 1987. Analysis of anaerobic biofilms. Environ. Technol. Lett. 8:21-32.

Takashima, M., and R. E. Speece. 1988. Mineral nutrient requirements for high rate methane fermentation of acetate at low SRT. Presented at the 61st Annual Conference of Water Pollution Control federation, Dallas, Texas, Oct. 5, 1988.

Tatton, M. J., D. B. Archer, G. E. Powell, and M. L. Parker. 1989. Methnogenesis from ethanol by defined mixed continuous cultures. Appl. Environ. Microbiol. 55:440-445.

Taylor, B. F., and R. S. Oremland. 1979. Depletion of adenosine triphosphate in *Desulfovibrio* by oxyanions of group VI elements. Curr. Microbiol. 3:101-103.

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

Thiele, J. H., M. Chartrain and J. G. Zeikus. 1988a. Control of interspecies electron flow during anaerobic digestion: the role of floc formation in syntrophic methanogenesis. Appl. Environ. Microbiol. 54: 10-19.

Thiele, J. H., W.-M. Wu, S. Pankratz, and M. K. Jain. 1988b. High performance granular biocatalysts for syntrophic biomethanation systems. Poster presented at 1988 ASM Annual Meeting, Miami Beach, FL., 8-13 May, 1988.

Thiele, J. H., and J.G. Zeikus. 1988a. Control of interspecies electron flow during anaerobic digestion: the role of formate versus hydrogen transfers during syntrophic methanogenesis in flocs. Appl. Environ. Microbiol. 54: 20-29.

Thiele, J. H., and J. G. Zeikus. 1988b. Interactions between hydrogen-and formate-producing bacteria and methanogens during anaerobic digestion. In Erickson, L. E., and D. Y.C. Fung (eds.), Handbook on anaerobic fermentations. Marcel Dekker, Inc., New York. pp537-595.

Thiele, J. H., W. -M. Wu, M. K. Jain and J. G. Zeikus. 1990. Ecoengineering high rate anaerobic digestion systems: analysis of improved syntrophic biomethanation catalysts. Biotech. and Bioeng. 35:990-999.

Tholozan, J. L., E. Samain, and J. P. Grivet. 1988a. Isomerization between n-butyrate and isobutyrate in enrichment cultures. FEMS Microbiol. Ecology. 53: 187-191.

Tholozan,J. L., E. Samain, J. P. Grivet, and G. Albaganc. 1988b. Acetogenesis pathways from propionate in enrichment culture. In Tilche, A., and A. Rozzi. (eds.), Poster-papers, fifth international symposium on anaerobic digestion. Bologna, Italy, 22-26 May 1988. pp85-90.

Tholozan, J. L., E. Samain, J. P. Grivet, and G. Albagnac. 1990. Propionate metabolism in a methanogenic enrichment culture. Direct reductive carboxylation and acetogenesis pathways. FEM Microbiol. Ecology. 73:291-298.

Tilche, A., and X. Yang. 1988. Light and scanning electron microscope observations of the granular biomass of experimental SBAF and HABR reactors. In Lettinga, G., A. J. B. Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol(ed.), Granular anaerobic sludge; microbiology and technology. Pudoc, Wageningen, Netherlands, 1988. pp170-178.

Tilche, A. 1990. Discussion report on upflow and dowflow anaerobic filters. Paper presented at International workshop, anaerobic treatment technology for municipal and industrial wastewater, September 23-26, 1990, Valladolid, Spain.

Tomei, F. A., J. S. Maki, and P. Mitchell. 1985. Interaction in syntrophic associations of endospore-forming, butyrate-degrading bacteria and H_2-consuming bacteria. Appl. Environ. Microbiol. 50: 1244-1250.

Touzel, J. P., and G. Albagnac. 1983. Isolation and characterization of *Methanococcus mazei* strain MC3. FEMS Microbiol. Lett. 16:241-245.

Touzel, J. P., G. Prensier, J. L. Roustan, I. Thomas, H. C. Dubourguier, and G. Albagnac. 1988. Description of a new strain of *Methanothrix soehngenii* and rejection of *Methanothrix concilii* as a synonym of *Methanothrix soehngenii.* Int. J. Syst. Bacteriol. 38:30-36.

Touzel, J. P. 1989. personal communication.

Tramper, J., J. W. van Groenestijn, K. Ch. A. M. Luyben, and L. W. Hulshoff Pol. 1984. Some physical properties of granular anaerobic sludge. In Houwink, E. H., and R. R. van der Meer (eds.), Innovations in biotechnology. Elservier Science Publisher B. V., Amsterdam. pp145-155.

Traore, A. S., M. -L. Fardeau, C. E. Hatchikian, J. Le Gall, and J. -P. Belaich. 1983. Energetics of growth of a defined mixed culture of *Desulfovibrio vulgaris* and *Methanosarcina barkeri*: interspecies hydrogen transfer in batch and continuous cultures. Appl. Environ. Microbiol. 46:1152-1156.

van den Berg, L., and C. P. Lentz. 1979. Comparison between up- and down flow anaerobic fixed film reactors of varying surface-to-volume ratios for the treatment of bean blanching waste. Proceedings of the 34th Purdue Industrial Waste Conference, May, 1979. Ann Abor Science Publishers Inc., Ann Arbor, MI. pp319-325.

van der Hoek, J. P. 1988. Granulation of denitrifying sludge. In Lettinga,G., A. J. B. Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol(ed.). Granular anaerobic sludge; microbiology and technology. Pudoc, Wageningen, Netherlands, 1988. pp203-210.

van der Meer, R. R. 1979. Anaerobic treatment of wastewater containing fatty acids in upflow reactors. Ph.D Thesis, Technical University of Delft, the Netherlands.

Verrier, D., B. Mortier, H. C. Dubourguier and G. Albagnac. 1988. Adhesion of anaerobic bacteria to inert supports and development of methanogenic biofilms. In Hall, E. R.,and P. N. Hobson (ed.), Anaerobic Digestion 1988. Pergramon Press, Oxford, U.K. pp61-69.

Vesilind, P. A. 1974. Treatment and disposal of wastewater sludges. Ann Arbor Science Publisher, Ann Arbor, Michigan.

Vuoriranta, P. J., J. A. Rintala, and H. Kirjavainen. 1985. Sludge characteristics in UASB-reactor treating thermomechanical pulping effluents. Wat. Sci. Tech. 17: 313-316.

Wang, Z. X., Z. P. Chen, and Z. S. Qian. 1985. Status quo and prospects on the study of anaerobic disposal for industrial wastewater. In China State Biogas Association (ed.), Anaerobic Digestion 1985. Guangzhou, China, 1985. pp259-277.

Westermann, P., B. K. Ahring, and R. A. Mah. 1989a. Threshold acetate concentrations for acetate catabolism by aceticlastic methanogenic bacteria. Appl. Environ. Microbiol. 55:514-515.

Westermann, P., B. K. Ahring, and R. A. Mah. 1989b. Acetate production by methanogenic bacteria. Appl. Environ. Microbiol. 55:2257-2261.

Widdel, F., and N. Pfennig. 1981. Sporulation and further nutritional characteristics of *Desulfotomaculum acetoxidans*. Arch. Microbiol. 129: 401- 402.

Widdel, F., and N. Pfennig. 1982. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids, II. incomplete oxidation of propionate by *Desulfobulbus propionicus* gen. nov., sp. nov. Arch. Microbiol. 131: 360-365.

Widdel, F., and N. Pfennig. 1984. Dissimilatory sulfate- or sulfur-oxidizing bacteria. In Krieg, N. R., and J. G. Holt. (ed.), Bergey's manual of systematic bacteriology, Vol.1. Williams and Wilkins, Baltimore London. pp663- 679.

Widdel, F. 1986. Growth of methanogenic bacteria in pure culture with 2- propanol and other alcohols as hydrogen donors. Appl. Environ. Microbiol. 51: 1056-1062.

Widdel, F. 1988. Microbiology and ecology of sulfate- and sulfur-reducing bacteria. In Zehnder, A. J. B. (ed.), Biology of anaerobic microorganisms. John Wiley & Sons, New York. pp469-585.

Wiegant, W. M., J. A. Claassen, A. J. M. L. Borghans, and G. Lettinga. 1983. High rate thermophilic anaerobic digestion for the generation of methane from organic wastes. in van den Brink, W. J. (ed), Proceedings of anaerobic wastewater treatment conference, Nov. 23-25, 1983, Noordwykerhow, the Netherlands. pp392-411.

Wiegant, W, M., J. A. Claassen, and G. Lettinga. 1985. Thermophilic anaerobic digestion of high strength wastewaters. Biotech. Bioeng. 27: 1374-1381.

Wiegant, W. M., and G. Lettinga. 1985. Thermophilic anaerobic digestion of sugars in upflow anaerobic sludge blanket reactors. Biotech. Bioeng. 27:1603-1607.

Wiegant, W. M., and A. W. A. de Man. 1986. Granulation of biomass in thermophilic upflow anaerobic sludge blanket reactors treating acidified wastewaters. Biotechnol. Bioeng. 28: 718-727.

Wiegant, W. M. 1988. The 'spaghetti theory' on anaerobic sludge formation, or the inevitability of granulation. In Lettinga,G., A. J. B. Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol (ed.). Granular anaerobic sludge; microbiology and technology. Pudoc, Wageningen, Netherlands, 1988. pp146-152.

Wijbenga, D. J., and H. Th. P. Bos. 1988. Physical and biological factors influencing the growth of anaerobic granular sludge. In Lettinga,G., A. J. B. Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol. (ed.), Granular anaerobic sludge; microbiology and technology. Pudoc, Wageningen, Netherlands, 1988. pp229-234.

Williams, A. G. 1986. Rumen holotrich ciliate protozoa. Microbiol. Rev. 50:25-49.

Winslow, C. -E. A., and E. B. Phelps. 1911. Investigations on the purification of Boston sewage. J. Infectious Diseases. 8:259-288.

Woese, C. R., and R. S. Wolfe.(ed.). 1985. The bacteria, a treatise on structure and function, Volume VIII: archaebacteria. Academic Press, Inc., Orlando, Florida.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. 51:221-271.

Wolfe, R. S., and I. J. Higgins. 1979. Microbial Biochemistry of methane - a study in contrasts. In Quayle, J. R. (eds), International review of biochemistry, Microbial biochemistry, Volume 21. Univ. Park Press, Baltimore. pp267-353.

Wolin, E. A., M. R. J. Wolin, and R. S. Wolfe. 1963. Formation of methane by bacterial extracts. J. Bio. Chem. 238:2882-2886.

Wu, W.-G. 1989. Kansas University, personal communication.

Wu, W.-M. 1984. Granular sludge in upflow anaerobic sludge blanket (UASB) reactor and its Properties. M.S. Thesis, Tsinghua university, Beijing, China(in Chinese).

Wu, W.-M., J. -C. Hu, and X. -S. Gu. 1985. Properties of granular sludge in upflow anaerobic sludge blanket (UASB) reactor and its formation. In China State Biogas Association (ed.), Anaerobic Digestion 1985. Guangzhou, China. pp.339-354.

Wu, W.-M., J.-C., Hu, and X. -S. Gu. 1986. Effect of granulation of sludge in upflow anaerobic sludge blanket (UASB) reactor on solid-liquid separation. Acta. Scient. Circumstant. (in Chinese). 6: 86-95.

Wu, W. -M., J. -C. Hu, X. -S. Gu, X. Q. Zhu, Y. -Z. Zhao, H. Zhang, and Y. -S. Xu. 1987a. Methanogenic bacteria in aerobic activated sludge. Acta. Scient. Circumstant (in Chinese).7: 100-106.

Wu, W.-M, J. -C. Hu, X. -S. Gu, Y. -Z. Zhao, H. Zhang, and G. -G. Gu. 1987b. Cultivation of anaerobic granular sludge in UASB reactors with aerobic activated sludge as seed. Water Res. 21: 789-799.

Wu, W.-M, R. F. Hickey, L. Bhatnagar, M. K. Jain, and J. G. Zeikus. 1989. Fatty acid degradation as a tool to monitor anaerobic sludge activity and toxicity. In 44th Purdue industrial waste conference proceedings. Lewis Publishers, Chelsea, MI. pp225-233.

Wu, M.-M., and M. K. Jain. 1990. Mechanism of isomerization and interspecies electron transfer during isobutyrate-butyrate degradation by a syntrophic biomethanation triculture. Poster presented at 1990 ASM Annual Meeting, Anaheim, CA, 13-17 May, 1990.

Wu, W.-M., R. F. Hickey, and J. G. Zeikus. 1991a. Formate synthesis from hydrogen by hydrogen utilizing methanogens. Poster presented at 1991 ASM Annual Meeting, Dallas, TX, 5-9 May, 1991.

Wu, W.-M., R. F. Hickey, and J. G. Zeikus. 1991b. Characterization of metabolic performance of methanogenic granules treating brewery wastewater: the role of sulfate-reducing bacteria. App. Environ. Microbiol. 57: (accepted for publication).

Wu, W.-M, and R. F. Hickey. 1991. The contribution of sulfate reducing bacteria to anaerobic degradation and COD removal by anaerobic granular sludge. Paper presented at the 46th industrial waste conference, Purdue University, West Lafayette, IN, May 14-16, 1991.

Yadav, V. K., and D. B. Archer. 1988. Specific inhibition of sulphate-reducing bacteria in methanogenic co-culture. Lett. Appl. Microbiol. 7: 165-168.

Yoda, M., M. Kitagawa, and Y. Miyaji. 1989. Granular sludge formation in the anaerobic expanded macro-carrier be process. Wat. Sci. Tech. 21:109-120.

Young, J. C., and P. L. McCarty. 1967. The anaerobic filter for waste treatment. In Proceedings of the 22nd Purdue industrial waste conference. Ann Abor Science Publisher Inc., Ann Abor, MI. 1967. pp559-574.

Young, J. C., and M. F. Dahab. 1983. Effect of media design on the performance of fixed-bed anaerobic reactors. Wat. Scie. Tech. 15: 369-383.

Young, J. C. 1990. Factors affecting the design and performance of upflow anaerobic filters. Paper presented at International workshop, anaerobic treatment technology for municipal and industrial wastewater, September 23-26, 1990, Valladolid, Spain.

Zeeuw, W. J. de. 1984. Acclimatization of anaerobic sludge for UASB-reactor start-up. ph.D Thesis, Agricultural University, Wagenegen, the Netherlands.

Zeeuw, W. J. de. 1988. Granular sludge in UASB-reactors. In Lettinga, G., A. J. B.

Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol (ed.), Granular anaerobic sludge; microbiology and Technology. Pudoc, Wageningen, Netherlands, 1988. pp132-145.

Zehnder, A. J. B., and K. Wuhrmann. 1977. Physiology of a *Methanobacterium* strain AZ. Arch. Microbiol. 111:199-205.

Zehnder, A. J. B., B. A. Huser, T. D. Brock, and K. Wuhrmann. 1980. Characterization of an acetate decarboxylating non hydrogen-oxidizing methane bacterium. Arch. Microbiol. 124: 1-11.

Zehnder, A. J. B., K.. Ingvorsen and T. Marti. 1981. Microbiology of methane bacteria. In Hughes, D. E., D. A. Stafford, B.I. Wheatley, W. Baader, G. Lettinga, E. J. Nyns, W. Versraete, and R. L. Wentworth (ed.). Anaerobic digestion 1981. Elsevier Biomedical Press, Amsterdam. pp45-68.

Zeng, Y. J., and W.-G. Wu. 1985. A study of meat packing plant wastewater treatment with upflow anaerobic sludge blanket process. In China State Biogas Association (ed.), Anaerobic Digestion 1985. Guangzhou, China, 1985. pp327-337.

Zeikus, J. G., and R. S. Wolfe. 1973. Fine structure of *Methanobacterium thermoautotrophicum*: effect of growth temperature on morphology and ultrastructure. J. Bacteriol. 113: 461-467.

Zeikus, J. G., and M. R. Winfrey. 1976. Temperature limitation of methanogenesis in aquatic sediments. Appl. Environ. Microbiol. 31:99-107.

Zeikus, J. G. 1977. The biology of methanogenic bacteria. Bacteriol. Rev. 41: 514-541.

Zeikus, J. G. 1979. Microbial population in digesters. In Stafford, D. A., B.I. Wheatley and D. E. Hughes (ed.), Anaerobic digestion. Applied Science Publishers Ltd., Cardiff, U.K., 1979. pp75-103.

Zeikus, J. G., A. Ben-Bassat, and P. W. Hegge. 1980. Microbiology of methanogenesis in thermal, volcanic environments. J. Bacteriol. 143: 432-464.

Zeikus, J. G. 1983. Metabolic communication between biodegradative populations in nature. In Slater, J.H. (eds). Microbes in their natural environment. Wiley Interscience., New York. pp423-462.

Zeikus, J. G., R. Kerby, and J. A. Krzycki. 1983. Single carbon chemistry of acetogenic and methanogenic bacteria. Science. 227: 1167-1173.

Zellner , G., and J. Winter. 1987. Secondary alcohol as hydrogen donors for CO_2-reduction by methanogens. FEMS Microbiol. 44:323-328.

Zhao, Y., H. Zhang, G. Liu, L. Lian, Y. Yiao, and W. Zhou. 1985. Microbial population in rural biogas digesters of China. In China State Biogas Association (ed.). Anaerobic Digestion 1985. Guangzhou, China. pp.477-495.

Zhao, H., D. Yang, C. R. Woese, and M. P. Bryant. 1990. Assignment of *Clostridium bryantii* to *Sytrophospora bryantii* gen. nov., comb. nov. on the basis of a 16S rRNA sequence analysis of its crotonate-grown pure culture. Int. J. Syst. Bacteriol. 40:40-44.

Zinder, S. H., S. C. Cardwell, T. Anguish, M. Lee and M. Koch. 1984a. Methanogenesis in a thermophilic (58°C) anaerobic digestor: *Methanothrix* sp. as an important aceticlastic methanogen. Appl. Environ. Microbiol. 47: 796-807.

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

Zinder, S. H., and M. Koch. 1984. Non-acetoclastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic culture. Arch. Microbiol. 138: 263-272.

Zinder, S. H., T. Anguish, and A. L. Lobo. 1987. Isolation and characterization of a thermophilic acetotrophic strain of *Methanothrix*. Arch. Microbiol. 146:315-322.

Zoetemeijer, R. J., A. J. C. M. Matthijsen, J. C. van der Heuvel, A. cohen, and C. Boelhouwer. 1981. Acetogenesis of soluble carbohydrate-containing wastewaters. Tri. Cebedeu. 34:444-465.

Zoutberg, G. R., R. Mulder, M. J. Teixeira, de Mattos and O. M. Neijssel. 1988. Aggregate formation in anaerobic gaslift reactor. In Lettinga, G., A. J. B. Zehnder, J. T. C. Grotenhuis, and L. W. Hulshoff Pol (ed.), Granular anaerobic sludge; microbiology and technology. Pudoc, Wageningen, Netherlands, 1988. pp34-42.