MICROBIOLOGICAL INVESTIGATIONS INTO GRANULAR SLUDGE FROM TWO ANAEROBIC DIGESTERS DIFFERING IN DESIGN AND INDUSTRIAL EFFLUENT PURIFIED

BY

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ABSTRACT

Due to a combination of selection criteria, sludges from upflow anaerobic digesters treating industrial waste waters consist primarily of well-settling, dense agglomerates called granules. Quantification of the component mixed microbial populations of these granules has been severely restricted by the inability of researchers to disrupt them without concomitantly destroying numerous cells. *In situ* quantification using light and electron microscopy is complicated by the high cell numbers and bacterial diversity; the small cell size; and the destructive nature of electron microscopy preparative techniques preventing the viewing of more than a small percentage of the population at a time. For these reasons, in this investigation, standardization of qualitative electron microscopic techniques was performed prior to their application to granules. Isolation and electron and light microscopic techniques were applied to granules from a full-scale clarigester treating effluent from a maize-processing factory. In addition, a method using montaged transmission electron micrographs (TEMs) taken along a granule radius, and image analysis, was developed for bacterial quantification within granules. This method, together with antibody probe quantification, was applied to granules from an upflow anaerobic sludge blanket (UASB) digester treating a brewery effluent. The clarigester granules contained a metabolically and morphologically diverse population of which many members were not isolated or identified. By contrast, the UASB digester granules consisted primarily of morphotypes resembling *Methanothrix, Methanobacterium* and *Desulfobulbus*, in order of predominance. However, only about

one-third of the population reacted with antibody probes specific to strains of bacterial species expected to occur within these granules. According to the antibody probe library used, the *Methanobacterium*-like cells observed in TEMs were probably *Methanobrevibacter arboriphilus*. From this study it is apparent that different anaerobic digester designs, operational parameters, and the chemical composition of the waste water purified, are factors which influence the formation and maintenance of granules differing with respect to their microbial populations. Until the difficulties associated with quantification are overcome, the processes governing granule formation and/or population selection will remain obscure.

DECLARATION

I hereby declare that the contents of this thesis, except where the contrary is indicated, is the result of my own investigation, under the supervision of Prof. F.M. Wallis, Department of Microbiology and Plant Pathology, University of Natal, Pietermaritzburg, and has not been submitted to any other university for degree purposes.

ALAN R. HOWGRAVE-GRAHAM

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CHAPTER 1

INTRODUCTION

Qualitative and quantitative population studies on microbial associations are often impeded by bacterial attachment to each other. To surmount this obstacle researchers have had either to disrupt such associations using physical means (most, harsh enough to result in simultaneous cell lysis), or perform *in situ* observations using light or electron microscopy. Quantification subsequent to disruption would be impaired while qualitative results might still be valid. Microscopic quantification of large bacterial aggregates is hampered by the three dimensional structure of closely knit populations while qualitative studies are relatively simple, especially if the research tool is an electron microscope.

Anaerobic digester granular sludges consist of numerous such aggregates which accumulate in upflow reactors due to the imposition of a variety of selection pressures. These aggregates (known as granules or pellets) are discreet particles of attached biomass wherein the component subpopulations are closely associated to improve nutrient transfer between the bacteria which, by interaction, convert a variety of waste waters primarily to methane and other gases. Granules vary in bacterial species composition with digester operational parameters and the effluent being purified; but as a consequence of the difficulties indicated above, bacterial quantification has been applied primarily on disrupted granules. In addition, there is

a lack of consistency regarding the electron microscopy preparative steps and the diversity of granule species composition is too often overlooked, with authors assigning generalized characteristics to granules based on the results of limited experiments.

In this thesis, the populations within granules from a clarigester purifying waste water from a factory that produces starch, glucose and other products from maize are examined and, for reasons beyond the control of the author, only partially compared to those of granules within an upflow anaerobic sludge bed (UASB) reactor treating brewery effluent. For reasons discussed later in the thesis, the clarigester granule study is only qualitative whereas the results from the UASB granule investigation are both qualitative and quantitative. For the quantitative aspects of the study, the transmission electron microscopy techniques had first to be standardized and optimised and a reliable method for cell quantification from transmission electron micrographs had to be developed.

As most of the chapters in this thesis have already been published or submitted for publication, there is some repetition and the method of referencing differs between chapters depending on the journal in which they appear. For this reason each chapter has its own list of references.

The main aim and objectives of this study were:

a) to establish a standard electron microscopy protocol best suited to the study of granules from different types of anaerobic digester;

b) to develop and apply a reliable method for cell quantification within anaerobic digester granules using transmission electron micrographs of granule cross-sections;

c) to confirm or refute previous studies which indicate that different digester designs, feed composition and operating conditions result in the formation of macroscopically similar but ultrastructurally and microecologically very different sludges.

CHAPTER 2

LITERATURE REVIEW

Anaerobic processes for biological stabilization of soluble and insoluble waste organic matter occur in both natural and controlled ecosystems (Pohland and Ghosh, 1971). As early as 1895 anaerobic digestion was applied by man for the removal of domestic sewage suspended solids from waste waters using septic tanks (Callander and Barford, 1983). About 1950, anaerobic digestion was applied to the purification of food processing waste waters e.g. from meat packing and winery operations. Since those early days, many advances have been made in digester design and performance which were necessitated by increased energy costs and environmental awareness (Callander and Barford, 1983).

In South Africa, the motivation for successful anaerobic digestion of industrial or domestic waste waters is an urgent requirement for preservation of our limited water resources. In 1982 Bekker predicted that development of this country would result in water demand exceeding supply before 1992. Fortunately, this prediction has not materialized. Water has been identified as South Africa's most limiting natural resource and every effort must be made to conserve it. This can be achieved by applying three strategies: a) reducing water consumption, b) increasing water reuse and c) developing new water sources (Toerien and Maree, 1987). Before reuse, however, water must be treated to an acceptable standard for either direct reuse,

or discharge into the aquatic environment without serious and/or unacceptable water quality deterioration.

The biological treatment of waste water may be performed using either aerobic or anaerobic processes. Both may be construed as complex mixed culture/mixed substrate systems which rely upon interacting biological and physical mechanisms which will affect process performance (Hamer *et al.*, 1985). Aerobic and anaerobic biotreatment processes result from the growth and activity of a wide range of microbial species and strains with concomitant production of microbial biomass, carbon dioxide and other products from biodegradable pollutants. In biotreatment processes, the objectives are to minimize the biomass yield and to maximize gas production with maximum pollutant conversion or removal from the effluent (Hamer *et al.*, 1985). Aerobic effluent treatment processes may be variants of the activated sludge or trickling filter type processes. Initially both were designed for the biotreatment of municipal and domestic sewage (Hamer *et al.*, 1985) but have more recently been adapted for the treatment of some weak (i.e. low in pollutants) industrial effluents. In the activated sludge process dispersed microbial flocs bio-oxidize pollutants, while in trickling filters, attached, stationary microbial films are responsible for the bio-oxidation processes. Essentially the microbes in both types of process oxidize soluble and insoluble biodegradable pollutants after first entrapping the particulate matter in either the flocs (Gujer, 1980) or the films. These flocs and films are also capable of removing much non-biodegradable soluble matter

from solution while non-biodegradable insoluble matter may be removed from suspension by entrapment.

Many of the principles of biotreatment apply to both aerobic and anaerobic waste water treatment processes. The major differences between them are that: a) aerobic treatment processes require oxygen while anaerobic processes require an absence of oxygen (digesters must thus be designed accordingly); and b) aerobic processes can be used only for the treatment of weak effluents. Ross (1989) indicated that organic industrial wastes with chemical oxygen demand (COD) of 2 to 200 g l^{-1} are too concentrated to be treated satisfactorily by aerobic methods. For this reason the feasibility of treating industrial waste waters by anaerobic digestion in South Africa was recognised and researched by Toerien and Hattingh (1969), Kotze *et al.* (1969) and Pretorius (1969). Since these pioneering studies many high-strength industrial or domestic waste waters have been successfully treated by anaerobic digestion.

2.1 ADVANTAGES AND DISADVANTAGES OF ANAEROBIC DIGESTION

The anaerobic treatment of domestic waste waters has a long history in South Africa, mainly involving the stabilization of sewage sludges in separate heated digesters (Toerien and Maree, 1987). The anaerobic digestion of industrial waste waters, however, has a much wider application internationally (Ross, 1989) and its

success can be directly ascribed to its many advantages over aerobic systems. Among these are:

- the anaerobic conversion of pollutants via a series of metabolic reactions results in the formation of gaseous end products, specifically carbon dioxide and methane, the latter being a combustible by-product (Ditchfield, 1986) which may be used as a surplus energy source. When treating industrial waste water streams containing more than 3,000 mg l^{-1} COD (Olthof and Oleszkiewicz, 1982) anaerobic treatment uses less energy and generates less sludge than does aerobic treatment (Toerien and Maree, 1987).

- it has been applied to waste water streams with COD as low as 1,000 mg l^{-1} and as high as 200,000 mg l^{-1} (Olthof and Oleszkiewicz, 1982; Ross, 1989). By contrast, aerobic processes are incapable of treating the higher loads without prior dilution (Ross, 1989).

- anaerobic processes may be acclimatized to degrade some recalcitrant organic compounds that aerobic treatment cannot degrade (Olthof and Oleszkiewicz, 1982).

- at high organic loading, anaerobic processes degrade soluble wastes better than do aerobic processes (Olthof and Oleszkiewicz, 1982).

- hydraulic retention times in anaerobic digesters are comparable with (Olthof and Oleszkiewicz, 1982), or better than (Ross, 1989), those for aerobic treatment.

- anaerobic treatment requires only 10-20% of the nutrients required for aerobic treatment and, where needed, alkalinity can be controlled by recycle (Ross, 1989).

- the anaerobic process is totally enclosed, eliminating odour problems and bacterial aerosols (Vriens *et al.*, 1990).

- anaerobic digesters occupy less land space than activated sludge or trickling-filter systems (Vriens *et al.,* 1990).

In spite of the above advantages anaerobic digestion alone is incapable of purifying waste water to a standard suitable for discharge into rivers in South Africa. For this reason it should only be considered for the stabilization of sewage sludge or as a pre-treatment for the removal of a large percentage of the COD prior to aerobic purification. A disadvantage of anaerobic digestion for the stabilization of sewage sludge is that the separated heated digesters represent one of the largest capital cost elements in the construction of local sewage plants (McGlashan, 1986 cited by Toerien and Maree, 1987).

2.2 DESIGN OF DIGESTERS EMPLOYED IN ANAEROBIC WASTE WATER TREATMENT

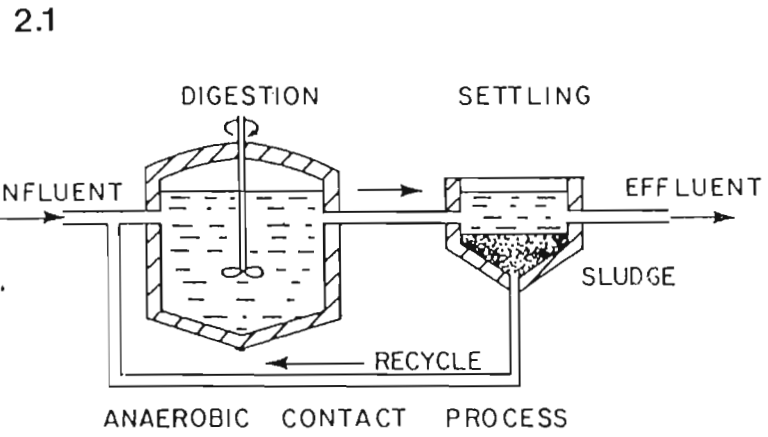
The choice of waste water treatment plant should be based upon the following factors (Vriens *et al.*, 1990):

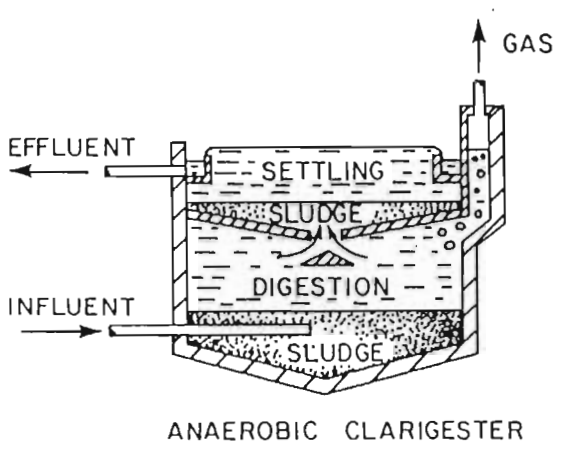
- the required quality of the final effluent
- as low as possible capital and working costs
- it must be simple to operate and run
- it must not give rise to problems of infestation and odour
- it must be compact

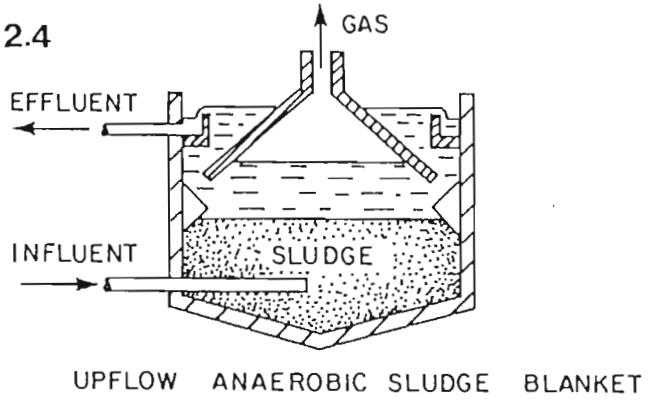
In the middle of the nineteenth century, the involvement of bacteria in the anaerobic decomposition of organic deposits was known (McCarty, 1982) but it was only in 1882 that this knowledge was first applied to the creation of a suitable environment for these bacteria to carry out anaerobic digestion. Since then, numerous digester modifications have been made to improve performance. Among the later designs were the septic tank, the Travis tank, and the Imhoff tanks which combined digestion and sedimentation in a single unit (McCarty, 1982). Since these digesters did not employ mechanical mixing, phase separation between solid and liquid resulted, with a sludge layer at the bottom and a scum layer at the top of the tank (McCarty, 1982). This led to the design of: a) conventional or low-rate digesters with intermittent mixing, sludge feeding and withdrawal (Kasan, 1988); and b) high rate

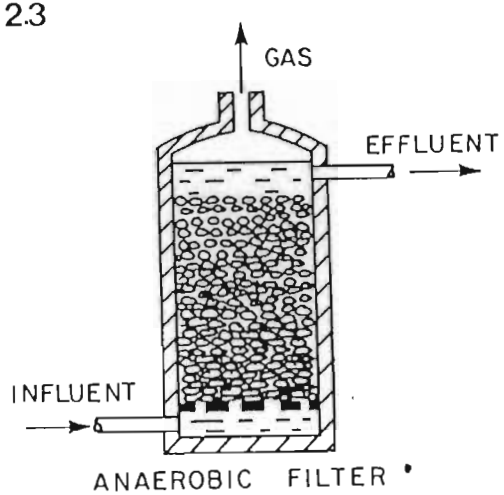
digesters with continuous mixing and continuous or intermittent sludge feed and withdrawal (Kasan, 1988). Both of these types of digester are commonly used for sewage sludge digestion (McCarty, 1982).

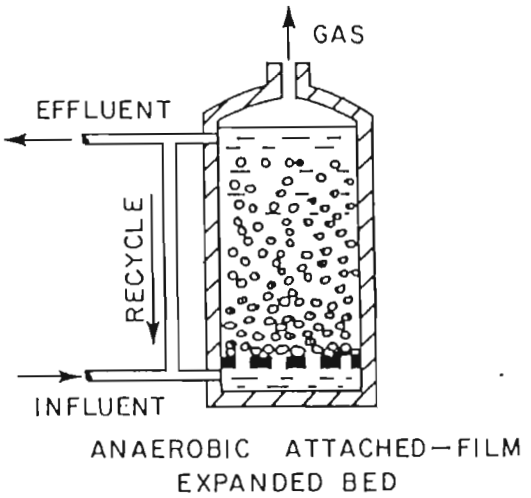
The continued interest in the anaerobic treatment of industrial waste waters has resulted in different reactor designs aimed at maintaining and retaining large bacterial populations (Toerien and Maree, 1987). Schroepfer *et al.*, (1955) (cited by McCarty, 1982) developed the "anaerobic contact process" (Figure 2.1) to treat packing house wastes while the so-called "clarigester" (Figure 2.2) was successful in full-scale treatment of winery wastes (Stander,1966) and incorporates a digestion and a settling compartment. Young and McCarty (1969) developed the "anaerobic filter" (Figure 2.3), a fixed-film reactor process which was later used to treat wheat starch waste water. The "upflow anaerobic sludge blanket" (UASB) (Figure 2.4) which employs granular particles, containing bacteria, was developed by Lettinga *et al.* (1979). The "anaerobic filter" was extended by Switzenbaum and Jewel (1980) in developing the "anaerobic attached-film expanded bed" reactor (Figure 2.5) in which waste water passes upwards through a bed of suspended material to which bacteria are attached. Most of the digesters depicted in Figures 2.1 to 2.5 are capable of treating waste waters in an upflow mode allowing early retention of active biomass in the reactor, with or without the use of a simple gas-solid-liquid separator. This is especially desirable for waste waters with low levels of suspended matter (Verstraete, 1989). In addition to these more conventional reactors the "anaerobic











FIGURES 2.1-2.5 Various designs of anaerobic digesters (McCarty, 1982)

baffled reactor" and the "anaerobic fluidized bed reactor" (Maragno and Campos, 1991) and, more recently, membrane assisted processes (Ahmadun, 1994; Ross *et al.*, 1994) and multiplate anaerobic reactors (Guiot *et al.*, 1994) have been applied to the anaerobic treatment of waste waters. Two-stage (Anderson *et al.*, 1993; Kunst, 1994) and three-stage (Kubler and Schertler, 1994) anaerobic digesters are designed to enhance effluent purification by separating the trophic groups to reduce toxicity effects, while hybrid reactors (Malaspina *et al.*, 1994; Versprille *et al.*, 1994) incorporate two or more of the above designs.

The upflow-mode digesters discussed above have several advantages over mixed-reactors. These include their low energy consumption as no stirring is required, their excellent biomass retention due to gravity settling of the sludge, and their good COD removal capacity.

For these reasons upflow digesters, especially UASB digesters, are being utilized more and more for the treatment of industrial waste waters. Most of these digesters operate in the mesophilic range (Kasan, 1988); several operate thermophilically (Visser *et al*, 1991; Soto *et al*, 1991; Macario *et al*, 1991); while some can be operated under psychrophilic conditions (Koster and Lettinga, 1985). To maintain the former two conditions heating is often required, especially in colder climates, and this is usually achieved using the methane generated by the digesters themselves.

2.3 INDUSTRIES UTILIZING ANAEROBIC DIGESTION FOR WASTE WATER TREATMENT

The wastes purified by anaerobic digestion must be largely biodegradable as the process is microbial and all the nutrients required for metabolism must be present in the waste water or added prior to digestion. A wide range of industrial waste waters, typically with a predominantly soluble, or colloidal content, and a high COD (2-200 g l^{-1}) which is mainly organic (Ross, 1989) has been treated by anaerobic digestion (Table 2.1).

TABLE 2.1 Some industrial wastes processed successfully by anaerobic treatment

Wastes treated successfully	
Abattoir (Ross, 1989) Apple processing (Ross, 1989) Baker's yeast (van der Merwe & Britz, 1994) Barley stillage (Toerien & Maree, 1987) Beer brewery (Ross, 1989) Beet sugar wastes (Toerien & Maree, 1987) Cheese whey (Toerien & Maree, 1987) Chemical industry waste (Toerien & Maree, 1987) Citric acid production (Toerien & Maree, 1987) Dairy waste (Toerien & Maree, 1987) Guar gum (Toerien & Maree, 1987) Industrial effluents (Britz *et al.*, 1994) Landfill leachate (Britz *et al.*, 1994) Maize processing (Ross, 1989)	Malting (Ross *et al.*, 1992) Meat packaging waste (Toerien & Maree, 1987) Methanol from cotton seeds (Toerien & Maree, 1987) Palm oil waste (Toerien & Maree, 1987) Petrochemical (Augoustinos *et al.*, 1986) and (Marx and Vernin, 1992) Pharmaceutical waste (Toerien & Maree, 1987) Piggery waste (Toerien & Maree, 1987) Pulp and paper (Vlok & Bohmer, 1989) Sewage (Britz *et al.*, 1994) Wine distillery (Toerien & Maree, 1987) Yeast production (Toerien & Maree, 1987)

2.4 MICROBIOLOGY OF THE ANAEROBIC DIGESTION PROCESS

The anaerobic digester designs discussed above have one common purpose, i.e. to ensure that the right "climate" is created for the microorganisms occurring within them. These microorganisms are responsible for the conversion of a mixture of complex compounds to a narrow range of simple compounds, viz. methane and carbon dioxide. This requires a mixed microflora (Zeikus, 1979). However, the microflora of digesting sludge do not simply comprise a range of species which ferment different substrates, such as sugars or proteins, to methane and carbon dioxide. The ecology of the system is much more complicated and involves an interacting succession of microbes which influence each others growth and metabolism (Holland *et al.*, 1987). According to Kasan (1988) the organic matter within the waste water is converted into oxidized materials, new cells, energy for their life processes, and characteristic gaseous end products. This conversion occurs in three distinguishable phases:

(a) liquefaction of solids

(b) digestion of the solubilized solids

(c) gas production

McCarty (1982) Kasan (1988), and Vriens *et al.*, (1990) described three stages in anaerobic digestion, viz. the non-methanogenic or acidogenic stages (incorporating

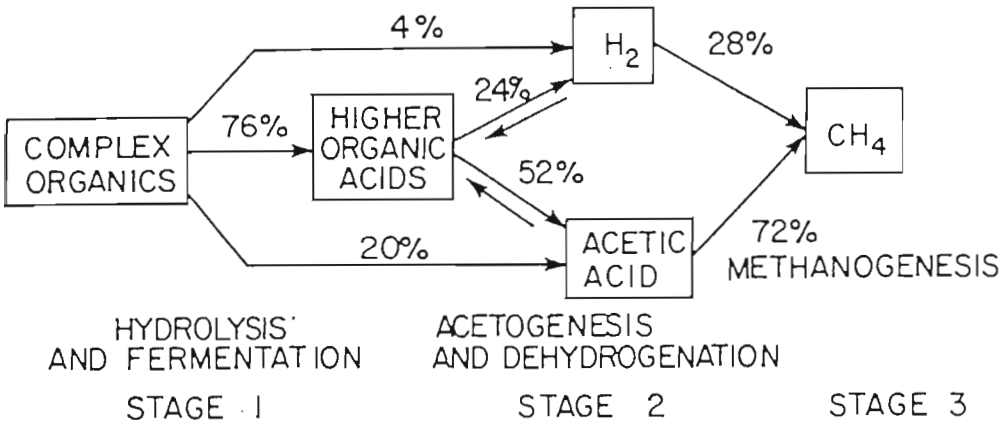


FIGURE 2.6 Biochemical stages in biomethanization (McCarty, 1982).

"hydrolysis and fermentation" and "acetogenesis and dehydrogenation") and the methanogenic stage (Figure 2.6).

According to van Andel and Breure (1984) these stages entail four processes: Firstly, the organic polymers must be hydrolysed into smaller subunits to be transported into the bacterial cells. In this way, proteins give rise to amino acids, polysaccharides to sugar monomers and fats or oils to polyols and long chain fatty acids. Next, the smaller subunits are fermented in a series of reactions. These processes provide the energy for the non-methanogenic population. The fermentation products comprise a variety of small organic compounds, mainly so-

called volatile fatty acids, the gases carbon dioxide and hydrogen, and some lactic acid and ethanol. This stage of the process is designated as "acidogenesis". The third process entails oxidation of the more reduced products to acetic acid, carbon dioxide and hydrogen as these are the sole substrates that can be used by the methanogenic bacteria. The bacteria performing this oxidation are called acetogens. For this conversion to occur, the partial pressure of hydrogen must be kept low by hydrogen uptake by, e.g. the methanogens. Finally, the methanogens carry out methane fermentation by one or both of two reactions: in the first, carbon dioxide and hydrogen are converted to methane and water; e.g. by *Methanobacterium*, while in the second (acetoclastic methanogenesis) acetate is converted into methane and carbon dioxide; e.g. by *Methanosarcina*. The whole sequence of reactions can be considered as a microbial food chain where a product excreted by one species is assimilated by another group of microbes (van Andel and Breure, 1984).

Understandably, the characteristics and rate of addition of the waste water being treated and the prevailing digestion conditions will influence the nature of the microbial population within anaerobic digesters. Ultimately, interactions of these variables will select that microbial population most suited to the specific anaerobic digestion process underway. However, to prevent the inhibition of specific microbial groups within a digester it may be necessary to alter the digestion process by adding specific nutrients to the waste water. The vast numbers, and great diversity of microbes isolated from, and identified within, anaerobic digesters (Tables 2.2 and

2.3), as well as the many species not yet identified, make it extremely difficult to characterize (qualitatively or quantitatively) their individual roles. Although the importance of syntrophic microbial associations within anaerobic digesters had been long recognised, details of the interactions occurring between species had not been fully elucidated by 1987 (Toerien and Maree, 1987). This situation still exists today. Tables 2.2 and 2.3 list bacterial genera and species that have been isolated from anaerobic digesters over the past four decades.

TABLE 2.2 Non-methanogenic bacteria identified in anaerobic digesters

Genus	Bacterial species	Reference
Aerobacter	*A.aerogenes*	Toerien & Hattingh (1969)
Aeromonas	*A. hydrophila* *Aeromonas* sp.	Esterhuyse *et al.* (1992) Kotzé *et al.* (1968)
Acetobacter	*Acetobacter* sp.	Zeikus (1979)
Acinetobacter	*A. calcoaceticus* *A. lwoffii*	Britz & Joubert (1986) Britz *et al.* (1994)
Actinomyces	*Actinomyces* sp.	Britz *et al.* (1994)
Alcaligenes	*A. bookerii* *A. faecalis* *A. viscolactis* *Alcaligenes sp.*	Toerien & Hattingh (1969) McCarty *et al.* (1962) Toerien & Siebert (1967) McCarty *et al.* (1962) Kotzé *et al.* (1968); Britz *et al.* (1994)
Agrobacterium	*Agrobacterium* sp.	Dolfing *et al.* (1985)
Anaeroplasma (mycoplasma)	*Anaeroplasma* sp.	Rose & Pirt (1981)

TABLE 2.2 (Cont.)

Genus	Bacterial species	Reference
Bacillus	*B. cereus*	Toerien *et al.* (1967), Toerien & Hattingh (1969)
	B. cereus var. mycoides	Toerien *et al.* (1967), Toerien & Hattingh (1969)
	B. circulans	Toerien *et al.* (1967), Toerien & Hattingh (1969)
	B. endorhythmos	Buck *et al.* (1954)
	B. firmus	Toerien & Siebert (1967)
	B. knefelkampi	Cookson & Burbank (1965), Burbank *et al.* (1966)
	B. megaterium	Toerien *et al.* (1967), Toerien & Hattingh (1969)
	B. pantothenticus	Toerien & Hattingh (1969)
	B. pumilis	Toerien & Siebert (1967)
	B. sphaericus	Toerien & Siebert (1967)
	B. subtilis	Toerien & Hattingh (1969)
	Bacillus sp.	Toerien & Hattingh (1969), Britz *et al.* (1994)
Bacteroides	*Bacteroides sp.*	Post *et al.* (1967) Britz & Joubert (1986)
Bordetella	*Bordetella* sp.	Britz *et al.* (1994)
Butyrivibrio	*Butyrivibrio* sp.	Novaes *et al.* (1988)
Citrobacter	*C. freundii*	Tracy *et al.* (1989) Britz *et al.* (1994)
Clostridium	*C. aminovalericum*	Hardman & Stadman (1960)
	C. bifermentans	Tracy *et al.* (1989), Britz *et al.* (1994)
	C. bryantii	Dolfing (1986)
	C. carnofoetidum	Dolfing (1986)
	C. ramosum	Britz *et al.* (1994)
	C. thermocellum	Dolfing (1986)
	C. thermohydrosulfuricum	Novaes (1986)
	Clostridium sp.	Dubourguier *et al.* (1988)
Desulfobulbus	*D. elongantus*	Dubourguier *et al.* (1988)
Desulfotomaculum	*Desulfotomaculum* sp.	Zeikus (1979)
Desulfovibrio	*D. wolfeii*	Novaes (1986)
	D. hungateii	Schink (1986)
	Desulfovibrio sp.	Schink (1986)

TABLE 2.2 (Cont.)

Genus	Bacterial species	Reference
Enterobacter	*E. aerogenes* *E. agglomerans* *E. cloacae*	Esterhuyse *et al.* (1992) Tracy *et al.* (1989) Britz & Joubert (1986)
Enterococcus	*E. faecalis*	Britz *et al.* (1994)
Escherichia	*E. coli* *E. intermedia* *Escherichia sp.*	McCarty *et al.* (1962), Cookson & Burbank (1965) Burbank *et al.* (1966), Toerien & Siebert (1967) Toerien & Hattingh (1969) Kotzé *et al.* (1968)
Fusobacterium	*Fusobacterium* sp.	Britz & Joubert (1986), Britz *et al.* (1994)
Klebsiella	*K. faecalis* *K. lactis* *K. oxytoca* *K. pneumoniae* *Klebsiella sp.*	Rose & Pirt (1981) Tracy *et al.* (1989) Tracy *et al.* (1989) Tracy *et al.* (1989) Burbank *et al.* (1966)
Leptospira	*L. biflexa* *Leptospira sp.*	Toerien & Siebert (1967) Maki (1954)
Micrococcus	*M. candidus* *M. luteus* *M. varians* *M. ureae* *Micrococcus* sp.	Toerien & Hattingh (1969), Toerien & Siebert (1967) Toerien & Siebert (1967) McCarty *et al.* (1962), Toerien *et al.* (1967) Toerien & Hattingh (1969) Kotzé *et al.* (1968)
Moraxella	*Moraxella* sp.	Britz *et al.* (1994)
Neisseria	*N. catarrhalis*	McCarty *et al.* (1962)
Paracolobacterium	*P. intermedium* *P. coliforme*	Toerien & Siebert (1967) Toerien & Siebert (1967)
Pasteurella	*Pasteurella* sp.	Britz *et al.* (1994)
Pelobacter	*P. carbinolicus*	Dubourguier *et al.* (1986)
Peptostreptococcus	*Peptostreptococcus* sp.	de Haast & Britz (1987)

TABLE 2.2 (Cont.)

Genus	Bacterial species	Reference
Propionibacterium	*P. acidipropionici*	Riedel & Britz (1992)
	P. frendenreichii	Riedel & Britz (1992)
	P. jensenii	Riedel & Britz (1992)
	P. rubrum	Mostert *et al.* (1992)
	P. thoenii	Riedel & Britz (1992)
Proteus	*P. vulgaris*	Toerien & Siebert (1967)
Pseudomonas	*P. aeruginosa*	Toerien & Hattingh (1969)
	P. ambigua	Toerien & Hattingh (1969)
	P. denitrificans	Burbank *et al.* (1966)
	P. maltophila	Tracy *et al.* (1989), Britz *et al.* (1994)
	P. oleovorans	Toerien & Hattingh (1969)
	P. perolens	Toerien & Siebert (1967)
	P. pseudomallei	Toerien & Hattingh (1969)
	P. reptilivora	McCarty *et al.* (1962), Toerien & Siebert (1967)
	P. riboflavina	Toerien & Siebert (1967)
		Burbank *et al.* (1966), Toerien & Hattingh (1969)
	Pseudomonas sp.	Kotzé *et al.* (1968) Toerien & Hattingh (1969)
Sarcina	*S. cooksonii*	Cookson & Burbank (1965), Burbank *et al.* (1966)
	S. lutea	McCarty *et al.* (1962)
Selenomonas	*Selenomonas* sp.	Zoutberg *et al.* (1988)
Serratia	*S. indicans*	Burbank *et al.* (1966)
Sporolactobacillus	*Sporolactobacillus* sp.	Sharma & Hobson (1986)
Staphylococcus	*S. aureus*	Tracy *et al.* (1989), Britz *et al.* (1994)
	S. hominis	Tracy *et al.* (1989), Britz *et al.* (1994)
Streptococcus	*S. diploidus*	Buck *et al.* (1954)
	S. faecalis	Rose & Pirt (1981)
	S. lactis	Rose & Pirt (1981)
Streptomyces	*S. bikiniensis*	Toerien & Siebert (1967)
Syntrophobacter	*S. wolinii*	Boone & Bryant (1980)
Syntrophomonas	*S. sapovorans*	Dubourguier *et al.* (1988)
	S. wolfei	Schink (1986)

TABLE 2.3: Methanogens identified in anaerobic digesters

Genus	Species	Reference
Methanobacterium	*M. bryantii* *M. formicicum* *M. thermoautotrophicum*	Macario & Conway de Macario (1988)
Methanobrevibacter	*M. arboriphilus* *M. smithii*	Macario & Conway de Macario (1988)
Methanococcus	*Methanococcus* sp.	Zeikus (1979)
Methanogenium	*M. marsinigri*	Ahring & Schmidt (1992)
Methanoplasma *Methanosaeta*	*M. concilii* *M. thermoacetophila*	Patel and Sprott (1990) Patel and Sprott (1990)
Methanoplasma (mycoplasma)	*M. elizabethii*	Dubourguier *et al.* (1988) Rose and Pirt (1981)
Methanosarcina	*M. barkeri* *M. mazei* *M. thermophila*	Mah and Smith (1981) Ahring *et al.* (1993) Macario & Conway de Macario (1988)
Methanospirillum	*M. hungateii*	Zeikus (1979) Dolfing (1986) Macario & Conway de Macario (1988)
Methanothrix (Methanosaeta - Patel & Sprott, 1990)	*M. soehngenii*	Dolfing *et al.* (1985) Fannin *et al.* (1983) Ohtsubo *et al.* (1991)

The gas produced during anaerobic digestion is about 55% to 75% methane (which is produced via the mechanisms shown in the flow chart illustrated in Figure 2.6), 25% to 45% CO_2, and trace amounts of such gases as H_2S, H_2 and N_2. Normally, the organic acids produced are utilized as soon as they are formed; failing which, the methane producers will be inhibited due to a pH decrease (Kasan, 1988).

The bacteria indicated in Tables 2.2 and 2.3 probably represent only a small percentage of those present and active in anaerobic digesters, the remainder not yet having been isolated or characterised. Table 2.2 lists the non-methanogenic bacteria. These have not been grouped according to function as there is often overlap: e.g. *Clostridium*, which may be considered an acidogen, is also capable of hydrolytic activity. Figure 2.6 is a simplification of the biochemical processes occurring in anaerobic digestion. A better understanding of how complicated the process is can be obtained by considering Figure 2.7 which presents a generalized scheme of the metabolic processes performed by bacteria listed in Tables 2.2 and 2.3.

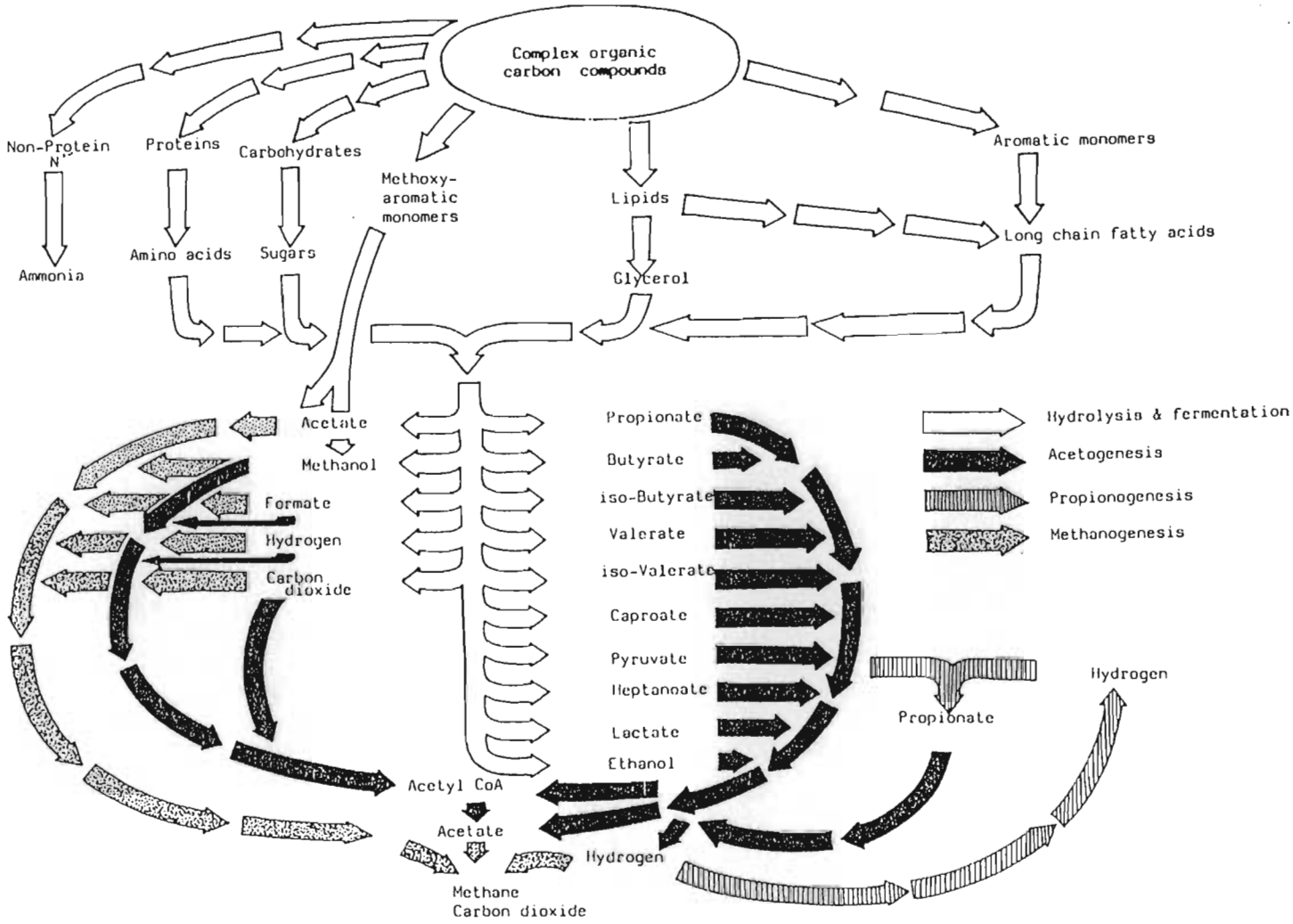


FIGURE 2.7 Methanogenic fermentation carbon and electron flow in anaerobic digestion (Senior, 1986)

Thus to obtain a comprehensive understanding of the mechanisms applicable to all anaerobic digestion systems would be a monumental if not impossible task. The situation is further complicated by the occurrence of other processes within digesters besides the direct conversion of waste water nutrients to the end products shown in Figures 2.6 and 2.7. These include:

(a) biomass increase within one or more sub-populations due to cell growth or because of extracellular polymer production (Dolfing *et al.*, 1985).

(b) predation by protozoa (Dubourguier *et al.*, 1988).

(c) bacteriophage action (Prensier *et al.*, 1988).

(d) "cryptic" growth i.e. parasitism by one bacterial species on another, requiring lysis of the latter (Hamer *et al.*, 1985) e.g. predation by *Bdellovibrio*.

(e) autolysis due to, for example, toxicity (Dubourguier *et al.,* 1988).

2.4.1 Microbial populations within granular anaerobic sludges

The formation of dense aggregates comprising a mixed microflora significantly complicates population studies as many of the bacteria present may not be isolateable and quantification becomes impossible when such a variety of microorganisms is present. This problem may be partly overcome if the aggregate can be disrupted without killing the cells. As a result, research on microbial

populations of digester granules has been undertaken largely on disrupted granules (Dolfing, 1986) or has relied upon bacterial morphology studies or random isolations (Britz and Joubert, 1986; Dolfing, 1986; Dubourguier *et al.*, 1988; Tilche and Yang, 1988).

The metabolic processes within granular anaerobic digester sludges must be essentially the same as within other anaerobic digester sludges. This indicates that many of the bacteria listed in Tables 2.2 and 2.3, or bacteria with similar catabolic processes, must occur within anaerobic digester granules. In addition, at least some of these bacteria must be capable of contributing to granule formation and all must be able to live within granules.

The resulting proximity of the bacteria to each other should result in more efficient substrate conversion since the catabolic end products of the members of one trophic group would be released into the granule to be utilized by members of the next trophic group in their vicinity (Schink and Thauer, 1988). As a result, less metabolites will be lost from the digester.

Dolfing (1986) observed that even the harshest chemical treatments were incapable of disrupting granules while sonication and homogenization were more successful. However, the latter two treatments are probably detrimental to the granule population and will thus adversely affect quantification. Electron micrographs of

granules (Dolfing *et al.*, 1985; Grotenhuis *et al.*, 1986; Guiot *et al.*, 1991; Prensier *et al.*, 1988) reveal a diverse population including rods, filaments, cocci and spirochaetes, with rods resembling *Methanothrix* being the most commonly reported morphotype.

2.5 GRANULATION IN UPFLOW DIGESTERS

As previously mentioned, the upflow mode of anaerobic digestion is increasing in popularity both in South Africa and internationally (see Proceedings of First and Second South African Anaerobic Digestion Symposia, 1986 and 1989; Sixth International Symposium on Anaerobic Digestion, 1991). The reactor design currently receiving most attention is the UASB digester (Figure 2.4), the advantages of which were discussed in Section 2.2.

Since upflow digesters lack complete mechanical mixing and UASB digesters lack a settling compartment it would seem that these designs should be incapable of proper functioning as: (a) there would be minimal microorganism-substrate contact; and (b) the biomass should float out of the digester, severely compromising COD removal. Contradictively, under suitable operating conditions upflow reactors are extremely efficient at conversion of COD to gas as the contained biomass forms aggregates called "granules" (Dubourguier *et al.*, 1988; Dolfing, 1986) or "pellets" (Ross, 1984; Sam-Soon *et al.*, 1990). The excellent settling properties of these

"granules" or "pellets" (Dolfing, 1986) eliminates the need for a settling compartment or support material, without compromising substrate conversion efficiencies.

Typically these granules have diameters of 0.5-4.0 mm and settling velocities of 0.012 m second^{-1} (Alibhai and Forster, 1986). The mixed microbial populations within granules/pellets also produce gas which, when trapped within them, allows the granules to float upwards toward the digester gas/liquid interface until the gas is released, whereupon the granules settle back to the sludge bed. This, together with the upflow velocity of the incoming waste water, creates sufficient mixing and selection pressure favouring only those species which can avoid being washed out of the system. One selection pressure regarded as extremely significant by Hulshoff Pol *et al.* (1988) is the minor differences that exist in the settling properties (density) of individual cell types within planktonic populations and between bacterial agglomerates. Campos and Anderson (1991) indicated that the liquid upflow velocity is a significant selective process while Thaveesri *et al.* (1994) found that high liquid surface tension of the waste water enhanced granule production when hydrophobic cells were the predominant population. Conversely, hydrophilic cells formed granules under low surface tension conditions.

No clear distinction has been made between the terms "granule", "pellet", "floc" and "aggregate". Dolfing (1986), according to Sam-Soon *et al.* (1990) did, however,

tentatively propose the following distinction between bacterial conglomerates within anaerobic digesters:

- flocs conglomerates with a loose structure
- pellets conglomerates with a denser structure than flocs and existing as separate entities
- granules dense pellets having a granular appearance

The need for standardizated terminology was reiterated at the Sixth International Symposium on Anaerobic Digestion (1991) in São Paulo, Brazil. In this thesis the term "granules" (synonymous with "pellets") will refer to all compact, well settling, microbial aggregates essential for efficient operation of upflow digesters. Granules contain microbial populations comprising many of the bacteria listed in Tables 2.2 and 2.3 as well as many unidentified species including, possibly, spirochaetes (Prensier *et al.*, 1988), capable of performing anaerobic digestion as depicted in Figure 2.8.

Bochem *et al.* (1982) described sarcina-type granules containing an acetate splitting methanogen of the genus *Methanosarcina* which predominated only when high acetate concentrations were maintained in the effluent, possibly due to its low affinity ($K_m \approx 60$ mM) for this substrate. Wiegant and Lettinga (1985) subsequently reported that UASB digesters containing sarcina-type granules tend to give operational

problems because of the inherent instability of these granules which, nonetheless, show good settleability and specific activity while intact. Wiegant (1988) identified two types of granule in anaerobic digesters, i.e. sarcina and filamentous. Granules comprising filamentous bacteria consist primarily of *Methanothrix* (also known as *Methanosaeta*) and predominate in effluents with low acetate concentrations notwithstanding the high affinity ($K_m \approx 2$ mM) this bacterium has for acetic acid (Hulshoff Pol *et al.*, 1988). de Zeeuw (1988) used the same criteria as Wiegant (1988) to group sludge granules in UASB reactors but included additional characteristics such as predominance, or otherwise, of rod-shaped bacteria, granule shape, and bacterial attachment to inert particles (Table 2.4), referred to in Figure 2.8 as "attachment nuclei". Figure 2.8 is a compilation by Colleran (1988) of theories proposed by de Zeeuw (1988), Hulshoff Pol *et al.* (1988) and Wiegant (1988), concerning the formation of stable well-settling granules. Any or all of these factors may play a role in this process and will depend on operating conditions including: pH, pH_2 and nitrogen status (Sam-Soon *et al.*, 1990); sludge retention time (Colleran, 1988); sugar to volatile fatty acid ratio (Grotenhuis *et al.*, 1991); Ca^{2+} concentration (Vanderhaegen *et al.*, 1991); and surface tension (Thaveesri *et al.*, 1994).

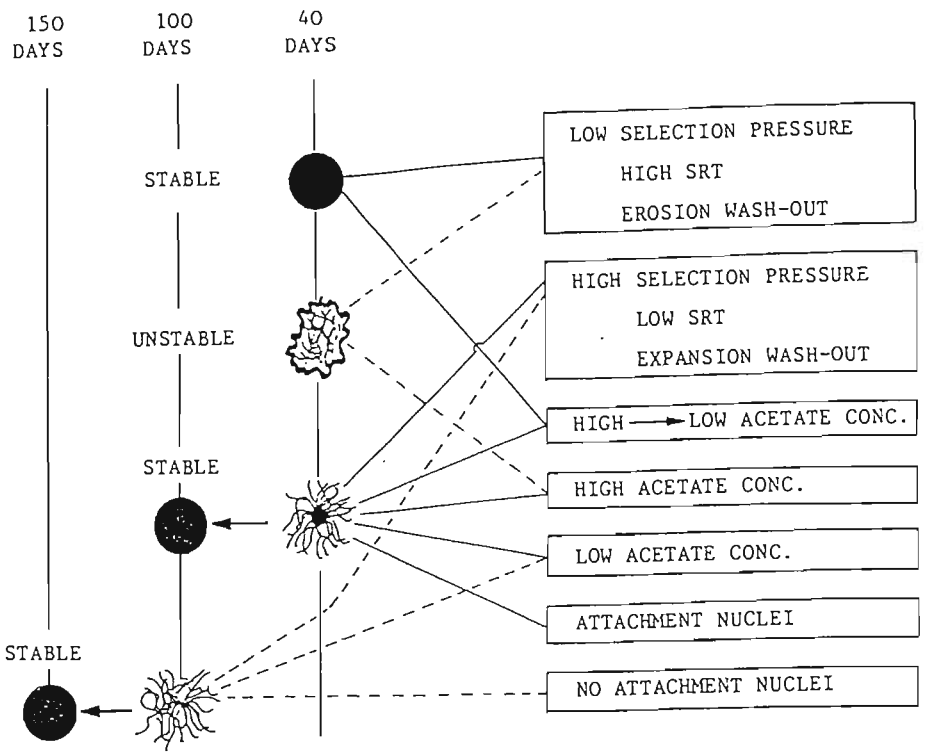


FIGURE 2.8 Suggested factors governing the granulation of anaerobic sludge (Colleran, 1988).

Sam-Soon *et al.* (1987) found that within a granular sludge bed, the granules differed with respect to function from bottom to top. They identified three digester zones as follows:

(i) a lower active zone in which: (a) acidogens generate short chain fatty acids (principally acetic and propionic), carbon dioxide and hydrogen. The hydrogen is generated at such a rate that a high hydrogen partial pressure (pH_2) is created; (b) hydrogenotrophic methanogens generate methane from hydrogen

and carbon dioxide; and (c) acetoclastic methanogens convert some acetate to methane and carbon dioxide.

(ii) an upper (active) zone which becomes active when, due to the action of hydrogenotrophs, the pH_2 has been reduced to such low values that: a) acetogens can convert propionate to acetate, H_2 and CO_2; and b) acetoclastic methanogens convert all the acetate to CH_4 and CO_2.

(iii) an upper (top of reactor) inactive zone in which no further significant biochemical reactions take place.

TABLE 2.4 Different types of macroscopic sludge pellets formed in UASB reactor start-up experiments (de Zeeuw, 1988)

Granule type	Description
A.	Compact spherical granules mainly composed of rod-shaped bacteria resembling *Methanothrix soehngenii*. Also called rod-granules.
B.	More or less spherical pellets mainly consisting of loosely intertwined filamentous bacteria attached to an inert particle. Also called filamentous granules. The prevailing bacteria resemble *Methanothrix soehngenii*.
C.	Compact spherical granules composed predominantly of *Methanosarcina*-type bacteria.

According to MacLeod *et al.* (1990) Guiot *et al.* (1991) and Vanderhaegen *et al.* (1991), type B anaerobic digester granules (Table 2.4) contain an acetoclastic core of *Methanothrix* to which a layer of H_2-consuming methanogens intermixed with H_2-producing acetogens was presumed to adhere. An outer layer of adhering acidogens and H_2-consuming organisms would subsequently be generated. This theory was supported by Fang *et al.* (1994) for granules degrading soluble carbohydrates, but not for those fed glutamate since the structure of the latter was too uniform.

As indicated previously, granules are compact structures containing mixed microbial populations which, to maintain their structural integrity require some adhesion mechanism. Adhesion may be by the incorporation of extracellular glycocalyx into granules as observed by Ross (1984) and Dolfing *et al.* (1985), and/or the interlinking of filaments as described by Wiegant (1988). In addition the use of pili to interconnect the bacteria might also play a role. It is probably not necessary for more than a few of the species present to facilitate adhesion thereby creating a microniche for entrapment of the remainder of the bacteria.

Various theories regarding the primary factors governing the formation of stable, well-settling granules have been advanced by de Zeeuw (1988) and Hulshoff Pol *et al.* (1988). These were summarized schematically (Figure 2.8) by Colleran (1988). The requirement for extracellular polymers (glycocalyx) in granulation within upflow digesters had previously been speculated on by Ross (1984) and Sam-Soon *et al.*

(1987). The occurrence of such glycocalyx in granules has been widely reported, Ross (1984), Dolfing *et al.* (1985), Sam-Soon *et al.* (1987) and Vanderhaegen *et al.* (1991). Vanderhaegen *et al.* (1991) proposed that in field reactors the sugar fermenting acidogens form sufficient biomass and polymers to act as "nucleation centres", in which the rest of the methanogenic associations develop. This theory of "nucleation centres" supports the earlier hypothesis of Colleran (1988) that "attachment nuclei" played a pivotal role in the formation some stable granules (see Figure 2.8).

The use of low selection pressure (defined by Colleran, 1988, as "the sum of the hydraulic loading rate and the gas loading rate"); a high sludge retention time (SRT) and the low biomass loss due to "erosion type" sludge washout (whereby friction caused by turbulence results in sloughing off of protrusions, thereby streamlining the granules, improving their settleability, and concomitantly causing washout of the removed surface materials), together with maintenance of low acetate concentrations (following reduction from the higher levels pertaining immediately after start-up) permits the most rapid development of stable, *Methanothrix*-like dominated type A granules (Table 2.4), because these organisms have a high affinity for acetate. This is achievable using a variety of crude starting sludges but both sludge concentration and the COD of the feed are critical factors (Colleran, 1988; Hulshoff Pol *et al.*, 1988).

de Zeeuw (1988) proposed that type A granules (Table 2.4) arise through colonisation by *Methanothrix* of the central cavities of *Methanosarcina* clumps which are selected for in the initial digestion stages by the high acetate concentrations present. This was supported by Colleran (1988) who observed that small, young granules had centres composed exclusively of *Methanothrix*, with *Methanosarcina* on the outside. She observed that subsequent growth yielded denser *Methanothrix*-type granules and also that loss of the outer sarcinal layers occurred. A similar theory proposing that *Methanothrix* formed "nucleation centres" was advanced by MacLeod *et al.* (1990) while, as previously mentioned, Vanderhaegen *et al.* (1991) indicated that sugar-fermenting acidogens form sufficient biomass and polymers to act as nucleation centres for the development of the remainder of the granule population.

Sam-Soon *et al.* (1987), studying anaerobic digestion of an apple juice concentrate containing high sugar and negligible nitrogen, postulated that granulation is mediated by *Methanobacterium* strain AZ (*Methanobrevibacter arboriphilus*). This organism synthesizes all amino acids except cysteine and, under high pH_2 conditions, if ammonia is added, a high intracellular ATP/ADP ratio is generated but the organism's growth is restricted by cysteine limitation. The high ATP/ADP ratio induces an over-production of amino acids which are secreted as extracellular polypeptide which enmeshes the bacterial population to form granules. These authors determined that cysteine augmentation of the feed decreased polymer

production and granulation. They observed that nitrogen limitation had an inhibitory effect on granulation as it inhibited production of excess amino acids and thus extracellular polymer. Conversely, Dolfing *et al.* (1985) showed that granules from a sugar refinery waste contained high percentages of carbohydrate rather than polypeptide. Riedel and Britz (1992) also detected extracellular polysaccharides. The postulate of Sam-Soon *et al.* (1987) that granulation may be due largely to *Methanobrevibacter arboriphilus* is contrary to the findings of Vanderhaegen *et al.* (1991) and Riedel and Britz (1992) who suggested that *Methanothrix* and *Propionibacterium*, respectively were the major contributors to granulation. Later in this thesis evidence based upon the results of ruthenium red and osmium tetroxide stained granule sections suggest the presence of polysaccharides rather than polypeptides. These conflicting results indicate that the agents responsible for granulation may be dependent upon the system within which it occurs and the chemical composition of the waste water treated.

Thus, the factors may that contribute to the formation of stable granules can be summarized as follows:

(a) a slightly acid to neutral pH (Ten Brummeler *et al.*, 1985, Sam-Soon *et al.* 1990).

(b) a high sludge retention time (Colleran, 1988)

(c) "erosion type "of sludge washout (Colleran, 1988)

(d) a selection force must be present whereby the free living cells are washed out of the reactor system (Hulshoff Pol *et al.*, 1988)

(e) an environment with a high pH_2 (Sam-Soon *et al.*, 1990)

(f) a limited supply of cysteine either from the feed or becoming available through the activity or lysis of other organisms present (Sam-Soon *et al.*, 1990)

(g) a nitrogen supply, in the free or saline ammonium form, well in excess of the metabolic requirements of the organisms (Sam-Soon *et al.*, 1990)

(h) a considerable supply of sugars relative to volatile fatty acids is required as non-acidified waters give rise to more abundant granular growth than waters in which all the carbohydrates are first fermented to lower volatile fatty acids (Grotenhuis *et al.*, 1991)

(i) the concentration of essential nutrients, specifically Ca^{2+}, should attain levels of 30 mg l^{-1} or more (Vanderhaegen *et al.*, 1991)

(j) low liquid surface tension of the waste water being treated (Thaveesri *et al.*, 1994).

Vanderhaegen *et al.* (1991) also identified the following factors as strongly detrimental to the formation and growth of granular sludge pellets: high ammonium and protein levels (which contradicts the findings of Sam-Soon *et al.*, 1990), and high levels of free suspended solids. It is also well known that granulation cannot occur in completely mixed reactors (Lettinga *et al.*, 1979, Sam-Soon *et al.*, 1990) due to excessive shear forces, but is readily induced in UASB digesters. Granulation will also be inhibited by a variety of toxic compounds (Cohen, 1991; Sam-Soon *et al.*, 1991).

In conclusion, all the factors inducing or contributing to granulation are not yet known and contradictory theories indicate that they may vary from system to system. It is probable that variations in any of these parameters will result in the formation of different granule types (see Table 2.4). The glycocalyx may, or may not be, significant for granulation but probably plays a role in protecting the bacteria from toxicity effects (Costerton and Irvin, 1981).

2.6 CONCLUSION

This review of the literature gives a clear indication of the complexity of anaerobic digestion processes and the multiplicity of bacteria involved; therefore, a complete understanding of the processes within each system would be very difficult to achieve. The study of granules within upflow digesters is even more complicated as

they comprise a large, diverse bacterial population adhering so closely to each other that determination of interspecies interactions and quantification studies are impossible. For this reason most granulation studies comprise case histories incorporating the monitoring of operational parameters and/or population studies using random species isolation or light and electron microscopy. Digestion studies have led to some contradictions in defining optimum granulation conditions. These differences may be explained by the fact that no two systems are alike. Thus, to achieve successful granulation, each system must be assessed individually. However, some requirements essential for granulation within anaerobic digesters have been defined and these must be applied to ensure efficient digester operation. Unfortunately qualitative and quantitative studies of processes and microbial populations within anaerobic digesters can remain only tentative until techniques are developed for the disruption of granules without adversely affecting the internal population, and until such time as better techniques are developed for the investigation of intact granules.

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CHAPTER 3

A COMPARISON BETWEEN THE TWO ANAEROBIC DIGESTER DESIGNS, THEIR PERFORMANCE, EFFLUENTS PURIFIED AND BACTERIAL MORPHOTYPES IN THE GRANULAR SLUDGES

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EVALUATION OF TWO UPFLOW ANAEROBIC DIGESTERS PURIFYING INDUSTRIAL WASTE WATERS HIGH IN ORGANIC MATTER

by

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ABSTRACT

Two full-scale anaerobic digesters, one a clarigester purifying a maize processing waste water and the other with an upflow anaerobic sludge blanket (UASB) configuration treating brewery effluent, contained well-settling, granular sludges efficient in pollutant removal. Due to differences in both digester design and feed composition, the sludges differed in activity and microbial population. The clarigester granules contained a diverse population with a multiformity of hydrolytic, acidogenic and acetogenic bacteria while the numerically predominant methanogens, in order of significance, were *Methanosarcina* and *Methanothrix*. These granules did not reconstitute on re-start up following digester shutdown and possible reasons for this are discussed. The UASB granules contained a more uniform population with three major microbial morphotypes, the predominant methanogens being *Methanothrix* and, possibly, *Methanobacterium* based on enumeration from electron micrographs. In this paper the differences in digester design, feed composition, sludge microbiology and process performance are discussed.

3.1 INTRODUCTION

Successful pretreatment of industrial waste waters by upflow anaerobic digesters relies upon the capacity of their sludges to settle by gravity. For this to occur the biomass must aggregate either by the microbes attaching to some support material or to each other. For the latter to occur a specific digester design must be combined

with certain selection criteria for bacterial aggregation (Colleran, 1988). Granules (also referred to as pellets) so formed are capable of degrading effluents rich in organic matter due to the presence of a diverse bacterial population including hydrolytic, acidogenic, acetogenic and methanogenic species (Zeikus, 1982). de Zeeuw (1988) described three granule types distinguished on the basis of their microbial composition. Two of these i.e. type A (compact spherical granules mainly composed of rod-shaped bacteria resembling *Methanothrix soehngenii*) and type C (compact spherical granules composed predominantly of *Methanosarcina*-type bacteria) are discussed in this paper. The influence of digester design and effluent characteristics on the nature of the sludges produced are also discussed. In addition, possible solutions to some of the problems experienced during operation of these digesters are offered.

3.2 DIGESTER DESIGN

The type A granules were produced in a Biothane designed anaerobic sludge blanket (UASB) system (Figure 3.1); the only dedicated, full-scale UASB currently operating in South Africa (Isherwood, 1991). It was commissioned at Prospecton, Natal, in 1985 and incorporates a preconditioning tank and 20% recycle for the treatment of brewery waste water. Without the conditioning tank the operating capacity is 1480 cubic metres (Ross, 1989); inclusion of the preconditioning tank increases the total capacity to 1700 cubic metres (Isherwood, 1991).

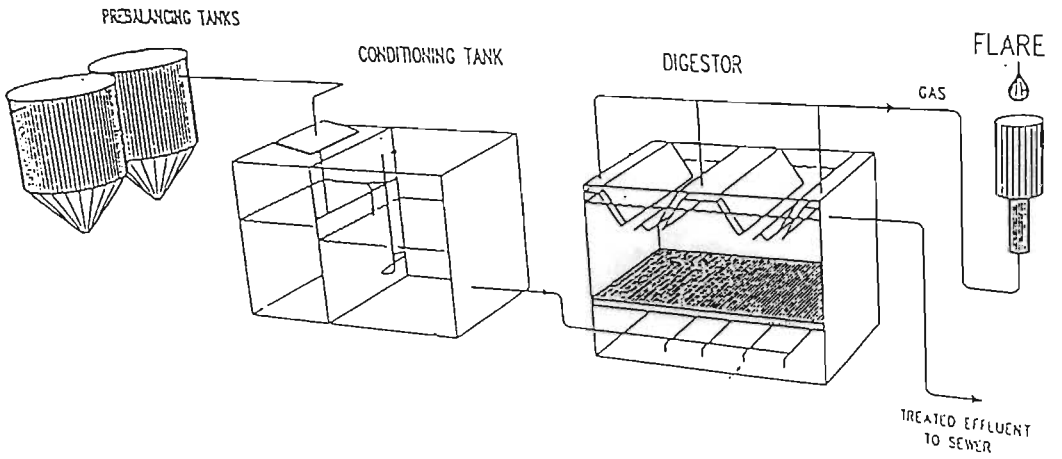


FIGURE 3.1 Biothane UASB at Prospecton treating brewery waste water (Isherwood, 1991)

The type C granules were established in three underground, unheated Dorr-Oliver clarigesters, each with a digester capacity of 623 cubic metres and a clarifier capacity of 347 cubic metres (Ross, 1984). Although originally designed for treating raw sewage, these digesters were commissioned in 1962 in Bellville, Cape, for the treatment of maize processing waste water, following replacement of the original inlet with a bottom one, thereby reversing the flow (Ross, 1989). These clarigesters then became upflow digesters incorporating a settling stage and a scraper system to transfer the settled sludge back to the digester compartment (Figure 3.2).

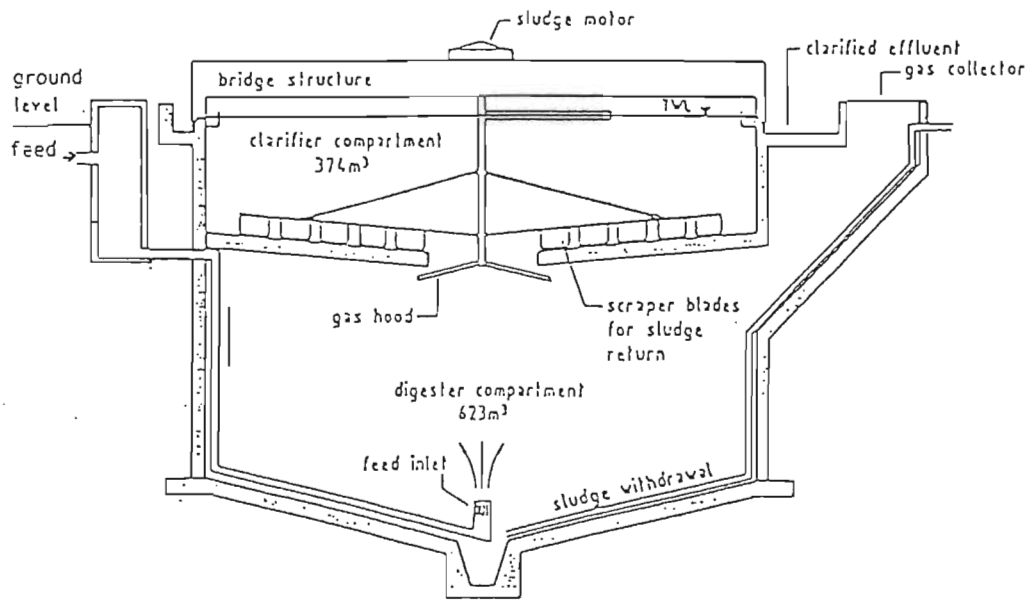


FIGURE 3.2 Reverse flow clarigester in Bellville for maize processing waste water treatment (Ross, 1989)

3.3 INFLUENT SUBSTRATE CHARACTERISTICS

These varied considerably in both systems depending on the processing conditions. Some general values are listed in Table 3.1. Lactic acid bacteria are used for steeping during maize processing and this results in a higher lactic acid concentration to the clarigesters. Initially gluten (20 to 60% total protein) comprised 7% (m/v) of the COD in the maize processing waste water. Subsequent recovery of

this component for animal feed coincided with the clarigesters' inability to produce granules upon start up (P. Harrigan, personal communication).

TABLE 3.1 Brewery and maize processing waste water characteristics

	Brewery	**Maize processing waste**
COD (mg l^{-1})	2000-6000	7200
Sugars [mg l^{-1}(wet mass)]	500	400
Ethanol [mg l^{-1}(wet mass)]	820	40
Protein [mg l^{-1}(wet mass)]	100	500
pH	7.0 (after neutralization)	4.3

The buffering capacity within the clarigesters was sufficient to obviate the necessity for pH adjustment (Ross, 1989) while the pH of the effluent to the UASB has to be neutralized (as indicated in Table 3.1) and urea nitrogen supplemented (Isherwood, 1991; Dudley *et al.*, 1993). Both the clarigester (Ross, 1984) and UASB substrates contained negligible amounts of volatile fatty acids and acetate was not detected in either feed. The amino acids of the feed were analyzed and are recorded in Table 3.2.

TABLE 3.2 Amino acid analysis of brewery and glucose/starch waste waters as determined by the Department of Animal Science (University of Natal) and the CSIR, respectively

Amino acid	Concentration in waste water (mg l^{-1})	
	Brewery	Glucose/Starch
Alanine	9.4	39.8
Arginine	5.8	24.5
Aspartic acid	12.0	22.5
Cysteine	4.9	0
Glutamic acid	24.4	26.9
Glycine	7.0	9.0
Histidine	2.7	56.5
Iso-leucine	6.6	15.9
Leucine	11.1	85.7
Lysine	6.7	24.5
Methionine	2.1	18.4
Phenylalanine	6.7	49.3
Proline	10.5	42.6
Serine	6.5	18.5
Threonine	6.5	25.5
Tryptophan	0	6.5
Tyrosine	4.4	32.2
Valine	7.6	26.9

All chemical analyses on the maize processing waste water are courtesy of the (then) National Institute for Water Technology of the CSIR while the Department of Animal Science of the University of Natal provided amino acid analysis results and South African Breweries other analyses on the brewery effluent. Table 3.2 indicates the full amino acid profiles of both waste waters since Sam-Soon *et al.* (1987) had postulated that, for granule formation, amino acids other than cysteine must be present in excess whereas cysteine must be absent. The data indicates that only the glucose/starch waste water meets these requirements.

3.4 PROCESS PERFORMANCE OF THE DIGESTERS

The operating conditions of the two digesters are listed in Table 3.3. Both systems were subject to wide day-to-day fluctuations in volume and organic strength of the influent.

The clarigesters are underground and, being unheated, their temperatures varied according to that of the incoming flow (Ross, 1984). These clarigesters are no longer used for the purification of maize processing waste water as: (a) they have exceeded their life expectancy and were prone to breakdown; (b) extensions to the Bellville sewage works has eliminated the necessity of pretreatment; (c) they did not produce granular sludge during start-up once gluten-free substrate was used. The

latter problem may have been exacerbated by concomitant deviations from previous start up procedures e.g. reduction in the volume of settled sewage inoculum used (W.R. Ross, personal communication).

TABLE 3.3 Clarigester and UASB operational conditions

	Clarigester	**UASB**
Feed load rate (tons COD d^{-1})	2-3	15
Sludge load rate (kg COD kg TSS^{-1} d^{-1})	0.06	0.63
Soluble COD removal (%)	96	93-96
Operating temperature (^0C)	15-25	32-38
Retention time (h)	89	10
Methane production rate (m^3 kg^{-1} sCOD removed)	ND	0.6

ND = not determined

The UASB is currently maintained at 32-38^{0}C using the methane evolved as energy source. As can be seen from Table 3.3, the system is operating effectively in the bioconversion of organic matter. Performance remains consistently good and municipal tariffs for further treatment of the effluent have been significantly reduced through on-going optimisation of the digester operating conditions. The previously experienced washout of floccular material has been overcome by the addition of ferric chloride and by minimising the amount of suspended solids entering the

digester. The ferric chloride is ionized in solution and the metal ion adsorbs readily to cell surfaces. In addition, iron sulphides produced by the sulphate-reducing bacteria (SRB) are deposited within granules thereby increasing their density and thus settleability. Installation of a second in-line filter has eliminated the possibility of distribution pipe blockages which could cause uneven loading and disruption of the sludge blanket, making the digester sensitive to contamination in the feed - viz. normal quantities of disinfectants and oil.

3.5 SLUDGE CHARACTERISTICS

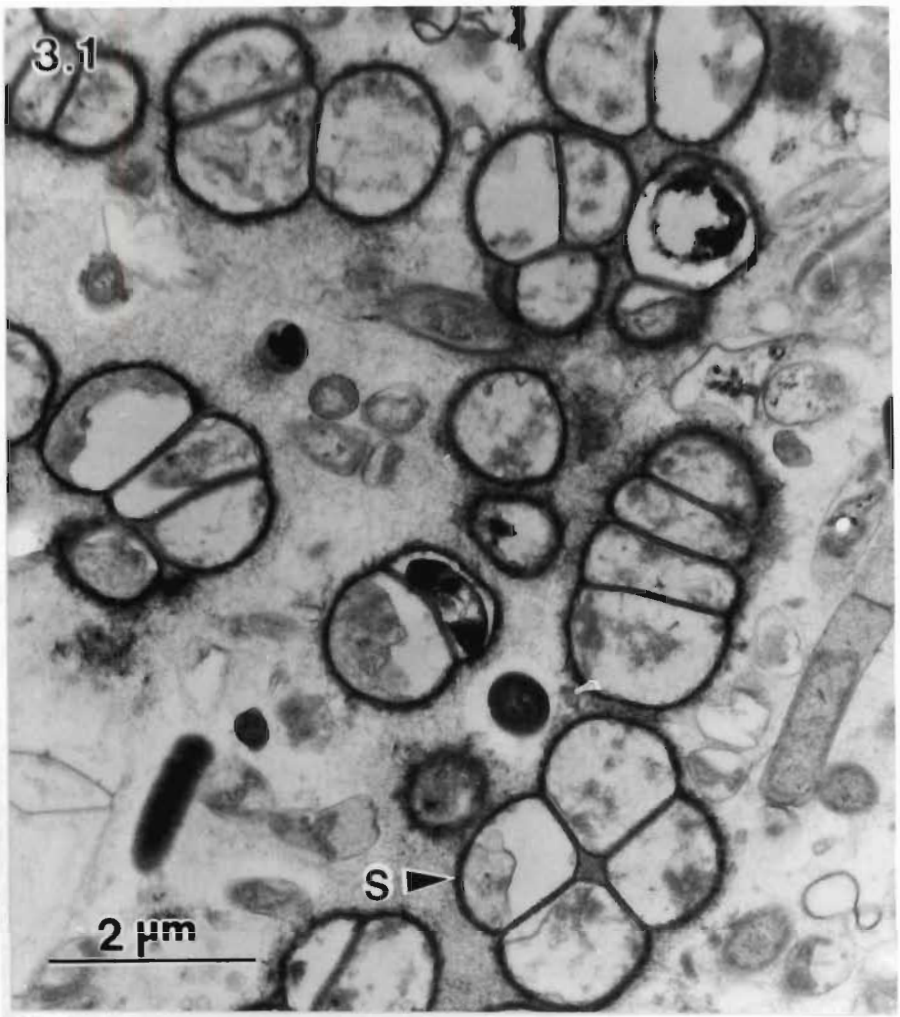
Both the clarigesters and the UASB digester, when operating optimally, contained well-settling sludges consisting of macroscopically similar granules. Those purifying the maize processing waste water were 1-2.5 mm in diameter while the brewery granules were initially 0.5-2 mm in diameter increasing to 5 mm after clearing of the feed pipes.

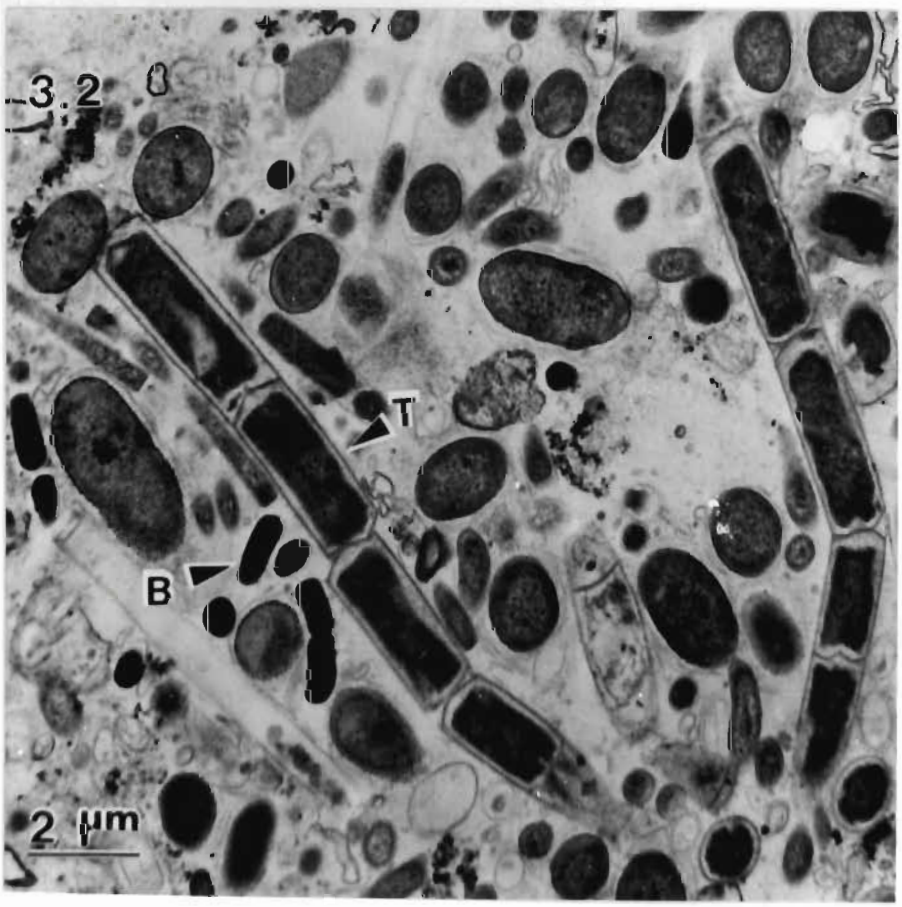
Electron microscopy using the methods discussed in Chapters 4 and 5 revealed that the clarigester granules were type C (Plate 3.1) while the UASB digester contained type A granules (Plate 3.2) according to de Zeeuw's (1988) description. Bacterial isolations from the type C granules revealed the occurrence of a diverse population of hydrolytic, acidogenic, acetogenic and methanogenic bacteria including, in order of significance, *Methanosarcina* and *Methanothrix*, (Howgrave-Graham *et al.*, 1991).

These results are numerically and volumetrically based upon visual observation of numerous electron micrographs of many individual granules. Electron microscopic quantification indicated that on a biomass basis the UASB granules contained 55% (v/v) *Methanothrix*-like cells and 11% (v/v) *Methanobacterium*-like cells (Howgrave-Graham and Wallis, 1993) (Chapter 6). A large proportion of the remaining population were *Desulfobulbus*-like. Cells showing morphological characteristics different from the groups mentioned were rare. No *Methanosarcina*-like cells were detected.

PLATE 3.1 *Methanosarcina*-like cells (S) in the type C granules

PLATE 3.2 *Methanothrix*- (T) and *Methanobacterium*-like cells (B) in the type A granules


3.1
S
2 µm


3.2
T
B
2 µm

3.6 DISCUSSION

Both the clarigesters and the UASB were capable of producing granular sludges which were efficient in COD removal. The granules were similar macroscopically but electron microscopic and isolation studies revealed that the bacterial populations therein differed considerably. This, together with substrate composition, suggests a concurrent variation in metabolic processes, especially during the hydrolytic and acidogenic stages which are known to be influenced by the composition of the feed (Zeikus, 1982).

Discrepancies between the two sludge types would be due to: the different initial sludge inocula (the UASB was inoculated with imported granular sludge while the clarigester sludge originated from settled sewage); differences between the substrates (e.g. more ethanol was present in the brewery feed while the COD and lactate were higher in the maize processing waste water); the digester configurations; and differences in operational parameters e.g. temperature and hydraulic load. From Table 3.2 it may be concluded that the abundance of all amino acids but cysteine may have led to the evolution of type C granules in the clarigester according to the theory of Sam-Soon *et al.* (1987), assuming that *Methanobacterium arboriphilus* strain AZ was present in this sludge (see Chapter 2 for more details). The present author, however, feels that this explanation is too simplistic and that

other factors such as: pH, pH_2 and nitrogen status; sludge retention time; sugar to volatile fatty acid ratio; Ca^{2+} concentration; and surface tension are significant.

In this study it was found that type C granules (de Zeeuw, 1988), contrary to popular belief (Colleran, 1988), were established on an acetate-free medium. The presence of lactate (which is easily converted to acetate by acetogens), and the high gluten content of the maize processing waste effectively replaced acetate in selecting for *Methanosarcina* containing granules, although the specific role of gluten in this process is unknown.

The large diversity in bacterial morphotypes (many of which were isolated and identified - see Chapter 5) indicated a high hydrolytic, acidogenic and acetogenic capacity within the clarigester granules while the UASB granules consisted primarily of methanogens. This raises the question of how the non-methanogenic metabolism is performed. Standard electron microscopy preparative techniques result in the erosion of the outermost layer of granules (unpublished data) which may contain the non-methanogenic populations as indicated by Guiot *et al.* (1991). Alternatively, acidogens and acetogens may adhere to the inside surfaces of the UASB digester or the conditioning tank, which was found, upon emptying, to be lined with a biofilm. The incorporation of a conditioning tank would then have converted the UASB into a two-stage digester with much of the hydrolytic and acidogenic activity occurring in the attached biofilm, while most acetogenesis and methanogenesis would occur

in the main digester compartment where the methanogens and obligate sytrophs are closely associated.

From this study it can be concluded that the creation and maintenance of resilient anaerobic digester granular sludges in upflow digesters can be achieved with diverse substrates and under various operating conditions, the nature of which determines the composition of the microbial population.

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CHAPTER 4

STANDARDIZATION OF ELECTRON MICROSCOPY METHODS

A condensed version of this chapter was published in 1991 in "Letters in Applied Microbiology" (vol.13, pp.87-89) as:

PREPARATION TECHNIQUES FOR THE ELECTRON MICROSCOPY OF GRANULAR SLUDGE FROM AN ANAEROBIC DIGESTER

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ABSTRACT

Several fixation and dehydration techniques for scanning and transmission electron microscopy of glycocalyx and microbial populations within granules from an upflow anaerobic sludge blanket digester purifying a brewery waste water were compared. Sputter-cryo and freeze-drying techniques prior to scanning electron microscopy (SEM) allowed viewing of the glycocalyx whereas, in contrast, standard fixation and dehydration techniques were suitable for examination of underlying microbial populations by both SEM and transmission electron microscopy. None of the techniques resulted in granule disruption and evidence gained from the use of different fixatives indicated that the glycocalyx was comprised mostly of carbohydrate.

4.1 INTRODUCTION

Upflow anaerobic sludge blanket (UASB) digesters have been widely used for the purification of industrial effluents (Dolfing, 1986). Success using this system relies upon the formation of dense granules consisting of anaerobic bacteria interspersed with glycocalyx which may or may not play a role in maintaining the structural integrity of the granules. Electron microscopic investigation of these granules is difficult because little or no glycocalyx remains following standard preparative electron microscopy techniques (see electron micrographs in:

Dubourguier *et al.*, 1987; Tilche & Yang, 1987; Yoda *et al.*, 1989; Howgrave-Graham *et al.*, 1991).

With respect to biofilms, Richards & Turner (1984) suggested that partial disappearance of glycocalyx was due to the unsuitability of glutaraldehyde as a fixative since subsequent acetone dehydration resulted in glycocalyx removal. However, these authors, and others (Costerton & Irvin, 1981), did observe some interlinking strands of glycocalyx within bacterial biofilms following standard preparative techniques for electron microscopy. In contrast, sputter-cryo techniques revealed that the biofilm was coated with a dense layer of glycocalyx which obscured the underlying microbial population (Richards & Turner, 1984).

Attempts to disrupt granules for population studies using all but the harshest treatments have failed (Dolfing *et al.*, 1985), possibly because the chemical composition of the glycocalyx remains unresolved. Most reports speculate that carbohydrates (Dolfing *et al.*, 1985) and polypeptides (Sam-Soon *et al.*, 1987) constitute the main components of the glycocalyx.

In this study, various fixation and dehydration techniques used prior to transmission and scanning electron microscopy of granules were evaluated. Our results have enabled us to suggest the composition of the glycocalyx and establish its role in the structural integrity of these granules.

4.2 MATERIALS AND METHODS

4.2.1 Anaerobic digester granular sludge

This was obtained from a full scale UASB digester treating a brewery effluent at Prospecton, Natal, South Africa. Some granules were processed while fresh, others were stored for 3 months at 4°C before processing.

4.2.2 Electron microscopy

A Jeol 100CX transmission electron microscope was used to view stained and cryo-sections, while a Hitachi S-570 scanning electron microscope was used for examination of sputter-cryo, air-dried, freeze-dried, and stained granules.

4.2.3 Fixation techniques

Granules were fixed in 3% (v/v) glutaraldehyde and/or 2% (m/v) osmium tetroxide in 0.05 M cacodylate buffer prior to dehydration. Alternatively, 4% (v/v) formaldehyde in distilled water was used as fixative. Either glutaraldehyde fixation, or no chemical treatment preceded sputter-cryo (specimens frozen to -180°C, gold-palladium coated and examined in the SEM at this temperature); air-dried (overnight at 22°C), or freeze-dried (ampoules containing granules prefrozen to -60°C in an acetone bath and then lyophylized at 10^{-2} Torr) samples. Glutaraldehyde fixation was used for cryo-ultramicrotomy.

4.2.4 Dehydration

Increasing concentrations of ethanol, acetone or 2,2 dimethoxypropane were used as dehydrating agents following fixation. Critical point drying (Hitachi HCP-2 CPD) was applied to SEM samples prior to coating with gold/palladium. Sputter-cryo, air-dried and freeze-dried samples were coated but not critical point dried.

4.2.5 Staining and embedding

Specimens for TEM examination were embedded in Spurr's resin (Spurr, 1969). Uranyl acetate and lead citrate were used to stain some sections while others were stained using ruthenium red (Springer & Roth, 1973).

4.3 RESULTS AND DISCUSSION

Neither glutaraldehyde, osmium tetroxide nor formaldehyde adequately fixed the glycocalyx with the result that subsequent acetone, ethanol or 2,2 dimethoxypropane dehydration caused its dissolution. SEM studies revealed mainly square-ended filaments resembling *Methanothrix* interspersed with short rods (Plate 4.1) and occasional spirochaetes, while TEM studies showed in addition smaller round-ended rods and large cocci (Plate 4.2). In contrast sputter-cryo (Plate 4.3), freeze-dried and air- dried granules showed a dense mat

of glycocalyx which obscured the underlying microbial population. Air-drying of samples caused most distortion of the glycocalyx. In stored granules a number of cells had autolysed resulting in "ghost-forms" within the granules (Plate 4.4).

TEM showed that cryo-ultramicrotome and resin-embedded sections were similar but the former contained more extracellular material (compare Plates 4.5 and 4.6 with Plates 4.2 and 4.4). The only effect of ruthenium red was to stain the extracellular material more darkly.

Costerton and Irvin (1981) indicated that in bacterial biofilms, the glycocalyx consists of 99% water with the result that it condenses during standard TEM and SEM dehydration procedures. According to these authors, this condensed glycocalyx is, however, still visible as strands interconnecting the cells. In this study on granules such glycocalyx strands were only observed if acetone, ethanol or 2,2 dimethoxypropane dehydration was performed on refrigerated material following protracted storage (see Plate 4.7).

Our results showed that sputter-cryo or freeze-drying can be successfully used as fixation for the scanning electron microscopy of anaerobic digester granule glycocalyx. These methods can also be used to determine the efficacy of various glycocalyx extraction methods. Alternatively, standard SEM or TEM fixation and dehydration procedures are useful if the microbial population rather than the

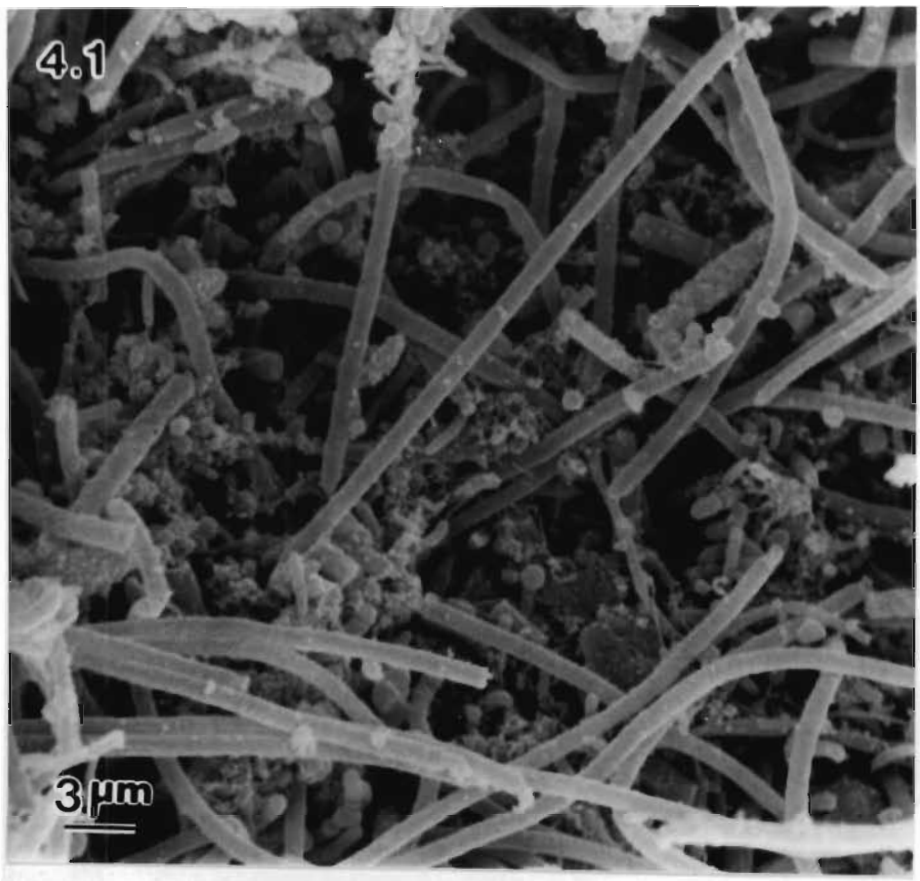
glycocalyx is to be examined. It is immaterial, in this case, whether glutaraldehyde, osmium tetroxide or formaldehyde is used for fixation as none of these fixatives prevented glycocalyx loss during subsequent dehydration steps. Likewise the dehydrating agent used was also immaterial as the glycocalyx was completely washed out of fresh granules whether ethanol, acetone or 2,2 dimethoxypropane were used.Since glutaraldehyde fixes proteins strongly but has limited lipid or carbohydrate fixing capacity (Hayat, 1981), if the glycocalyx was proteinaceous it would be preserved despite lipid and carbohydrate wash-out during dehydration. Conversely, osmium tetroxide has high lipid fixing capacity but is a poor fixative of proteins and carbohydrates (Hayat, 1981). Hence, in the fresh granules, the glycocalyx probably consisted primarily of carbohydrates, since neither osmium tetroxide, glutaraldehyde, nor a combination of these preserved the glycocalyx from dissolution during dehydration. Much of the glycocalyx of older granules, which is more resistant to dissolution, might consist of complexes of the above compounds as all the fixatives preserved strands of it.

The role of the glycocalyx in adhesion of the bacteria to each other to maintain the granule's structural integrity was shown to be minimal as none of the above SEM and TEM preparative techniques resulted in disruption of the granules. Thus some other feature e.g. pili or filamentous forms, must be responsible for maintaining granule integrity. The assumption that the presence of glycocalyx is

not necessarily to maintain granule integrity, is based on observations that despite treatments which are known to remove glycocalyx (Richards and Turner, 1984), the treated brewery waste granules retained their structural integrity (Plate 4.8), and also that treated granules contained remarkably little glycocalyx (Plates 4.1, 4.2, 4.4, 4.7).

PLATE 4.1 SEM of glutaraldehyde fixed, ethanol dehydrated granules showing the microbial population

PLATE 4.2 TEM of glutaraldehyde/osmium tetroxide fixed, ethanol dehydrated granule microorganisms


4.1
3 µm

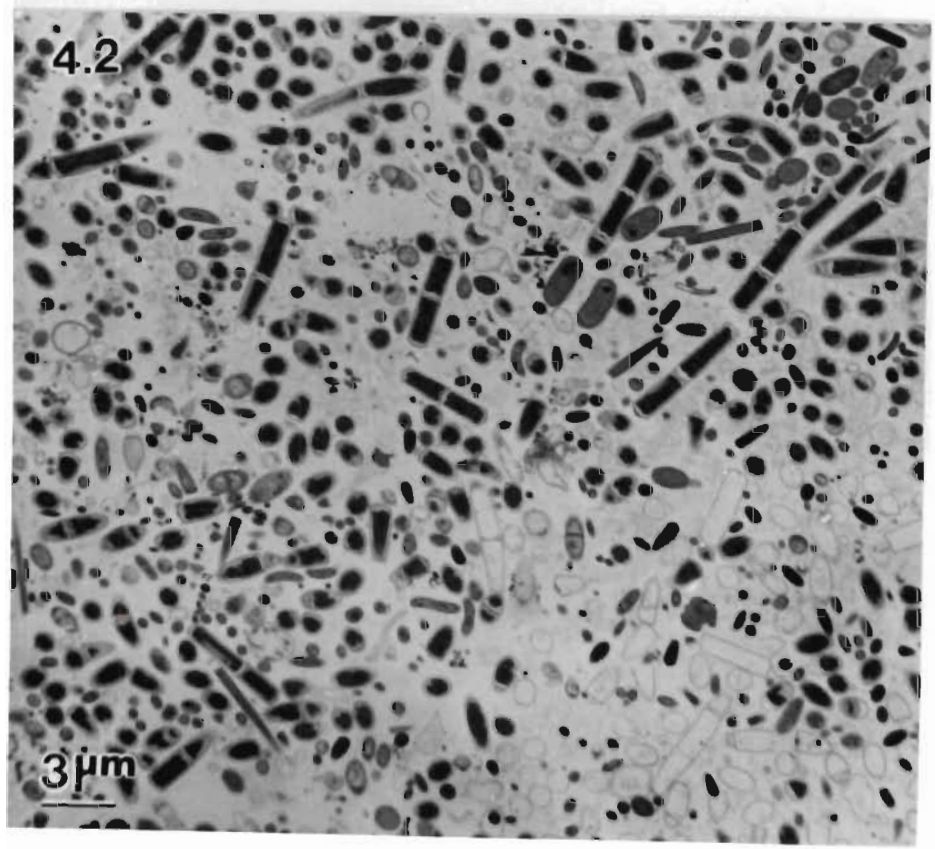
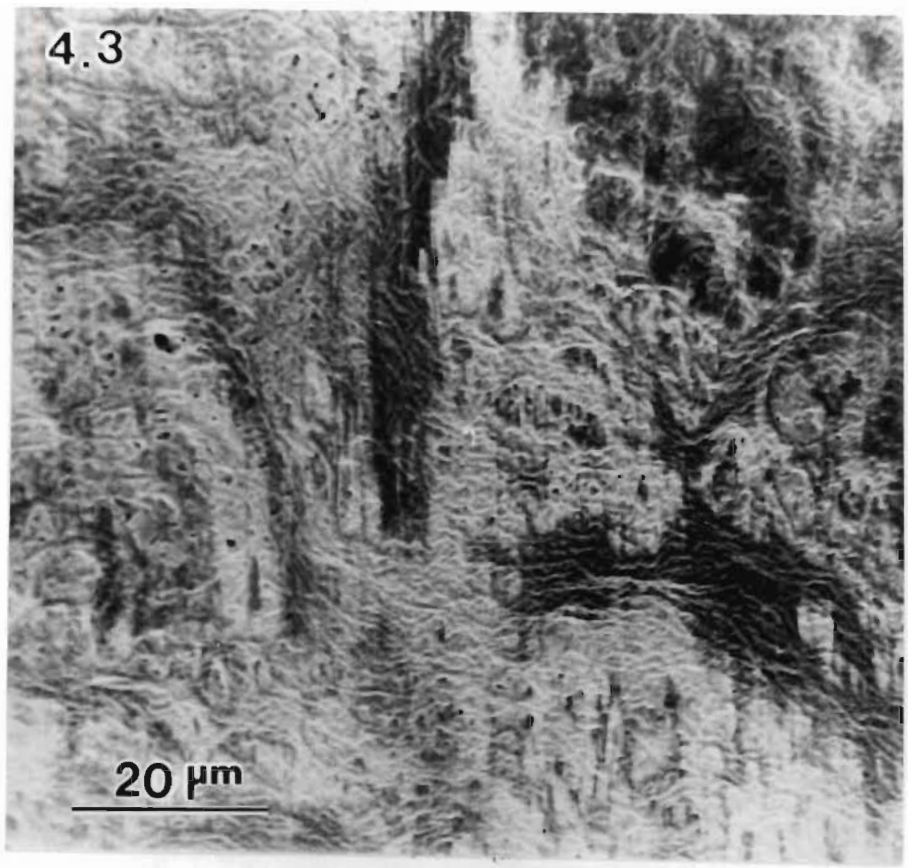
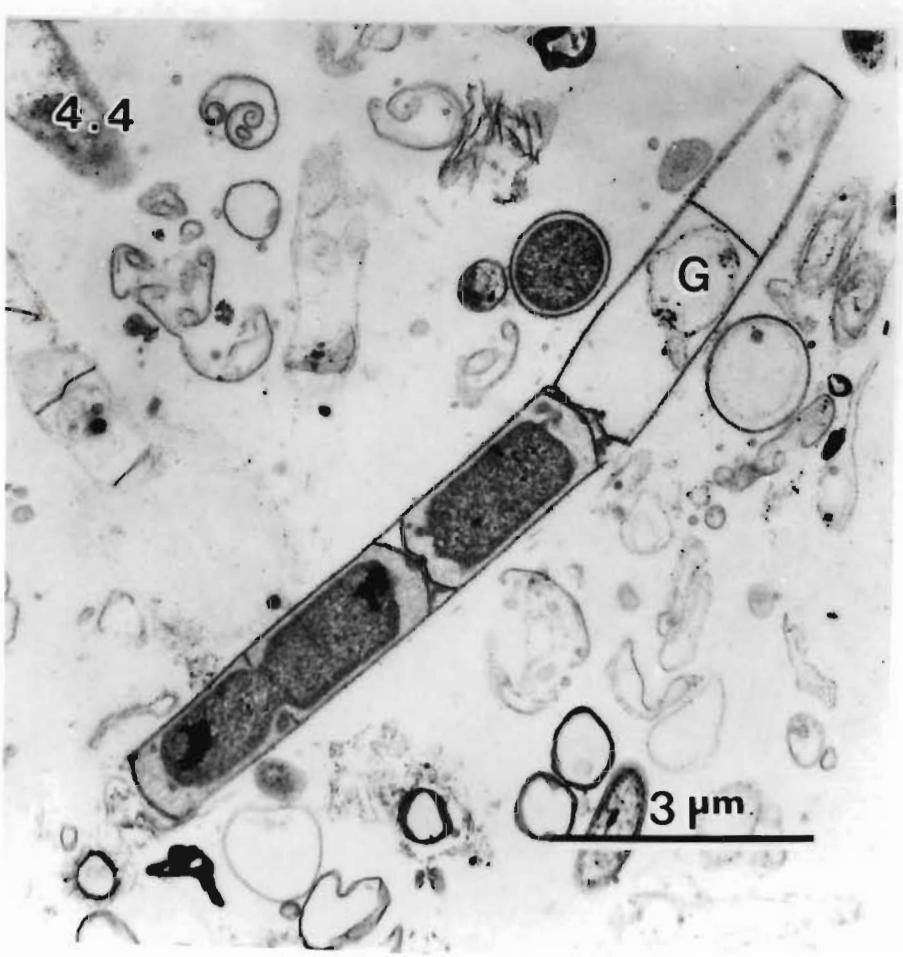

4.2
3 µm

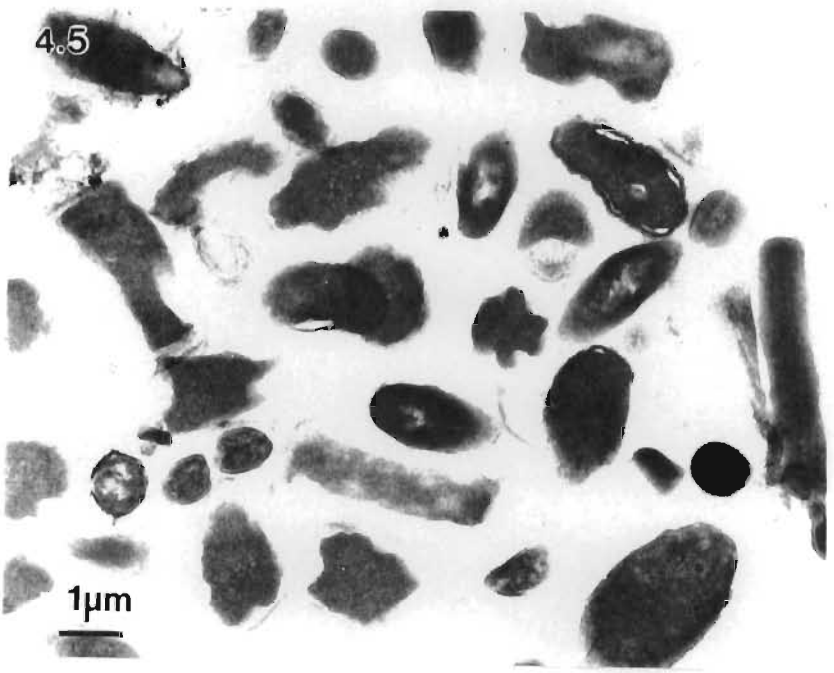
PLATE 4.3 Sputter-cryo of a granule surface showing the enveloping glycocalyx

PLATE 4.4 TEM of microbial filament showing "ghost-forms" (G)


4.3
20 µm


4.4
G
3 µm

PLATES 4.5 and 4.6 TEMs of a cryo-ultramicrotome section through a granule from a digester treating brewery waste water


4.5
1µm

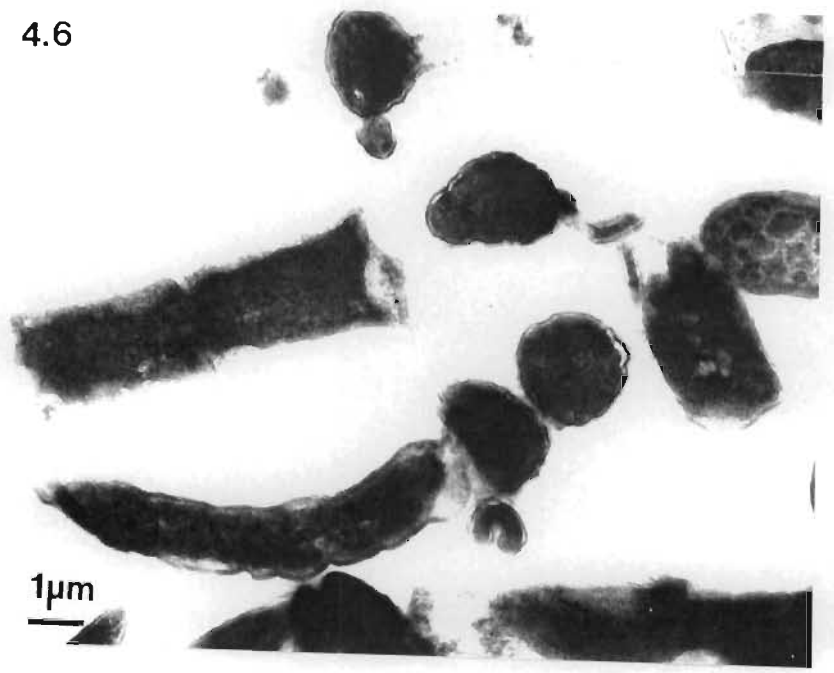
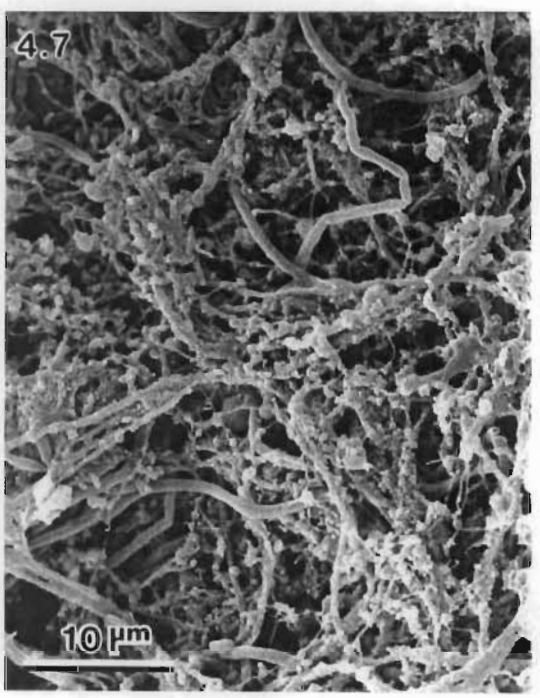
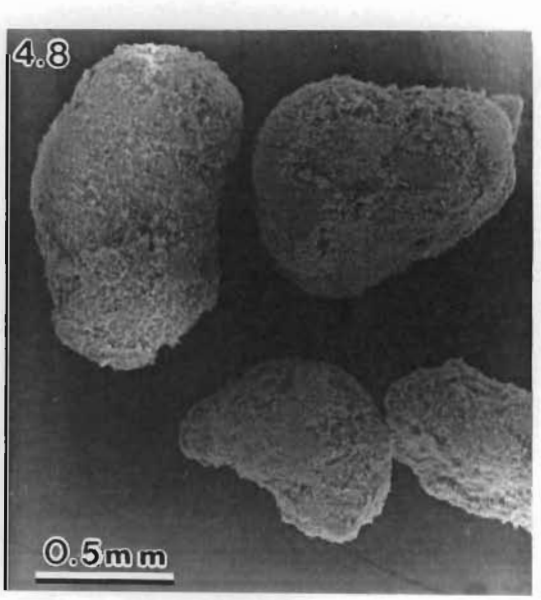

4.6
1µm

PLATE 4.7 SEM of a portion of the surface of a refrigerated granule after glutaraldehyde fixation and ethanol dehydration

PLATE 4.8 SEM of entire granules after glutaraldehyde fixation and ethanol dehydration


4.7
10 μm


4.8
0.5mm

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CHAPTER 5

BACTERIA DETECTED WITHIN CLARIGESTER GRANULES TREATING A MAIZE-PROCESSING WASTE WATER

A condensed version of this chapter was published in 1991 in "Bioresource Technology" (vol. 37, pp.149-156) as:

A BACTERIAL POPULATION ANALYSIS OF GRANULAR SLUDGE
FROM AN ANAEROBIC DIGESTER
TREATING A MAIZE PROCESSING WASTE

by:

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ABSTRACT

Microbial population studies were conducted on a dense granular sludge with excellent settling, thickening and nutrient removal properties from a South African clarigester treating effluent from a factory producing glucose and other carbohydrates from maize. The bacterial population comprised a heterogeneous group including acetogens, enterobacteria, sulphate reducers, spirochaetes, heterofermentative lactobacilli and methanogens. The presence of these bacteria, and lack of propionic acid and butyric acid bacteria, suggests that the microbial activity of this anaerobic digester involved acetate and lactate metabolism rather than propionate or butyrate catabolism as a source of precursors for methane production.

5.1 INTRODUCTION

A dense granular sludge with excellent settling and thickening properties was developed in a full scale upflow clarigester in Bellville (Republic of South Africa) treating an effluent rich in carbohydrates and containing the minerals and growth factors necessary to maintain the bacteria within the granules in a viable state. The products of the factory (also present in the feed to the clarigester) included starch, maize dextrins, glucose and dextrose syrups, maize gluten (20 - 60% total protein) and germ oil in varying quantities (see Ross, 1984, for further

details). The total solids and nutrients occurred in far higher concentrations in the clarigester feed than in the effluent from the clarigester (Ross, 1984). The bacteria present in the granules were found to be capable of removing 93% of the chemical oxygen demand (COD) from the feed based on an initial substrate concentration of 7200 mg l^{-1} of which protein comprised 12% (m/v).

Three major models have been postulated to describe the metabolism of anaerobic digestion. The oldest model proposed is a two-stage process involving two major bacterial groups, namely: (i) the acid forming stage during which fatty acids are produced from polysaccharides, lipids and proteins, in which a wide range of micro-organisms participate; (ii) the methane forming stage involving the methanogens which convert these acids to CO_2 and CH_4, but which also use CO_2 and H_2 to form methane.

A later model described by McInery and Bryant in 1981 has three stages. The first is the fermentative stage in which complex organic materials, e.g. carbohydrates, proteins and lipids are converted to fatty acids, alcohols, CO_2, ammonia and some H_2. In the second stage, hydrogen-producing acetogenic bacteria break down the products of the first group to hydrogen, carbon dioxide, acetate and sometimes other acids. The final stage involves the methanogens which utilize H_2, CO_2 and acetate to produce biomass and methane.

A four stage model has been described by Zeikus (1982), Zoetemeyer (1982) and Sam-Soon *et al.* (1987) and is now widely accepted. In this model four metabolic groups of microorganisms are recognized: (i) the hydrolytic bacteria ferment a variety of complex organic molecules like polysaccharides, lipids and proteins to acetic acid, H_2 and CO_2, other one carbon compounds, organic acids larger than acetic acid and neutral compounds larger than methanol; (ii) the hydrogen-producing acetogenic bacteria (obligate and facultative anaerobes) can ferment organic acids larger than acetic acid (e.g. butyrate or propionate) as well as neutral compounds larger than methanol (e.g. ethanol and propanol) to H_2 and acetate; (iii) the homoacetogenic bacteria which can ferment a wide spectrum of multi- or one-carbon compounds to acetic acid; and (iv) the methanogens which can ferment acetate, H_2 ,CO_2 and other one-carbon compounds like methanol and methylamine to methane.

All three schemes emphasise the importance of volatile fatty acids as intermediates in anaerobic digestion. However, Verstraete *et al.* (1981) stated that the fermentation pattern of anaerobic digestion can be manipulated by inoculating a substrate with a particular type of organism, e.g. *Lactobacillus* spp., which will produce mainly lactate, or *Propionibacterium* spp., which will convert the bulk of the same substrate to propionic acid.

The purpose of this study was to isolate and identify the bacteria, for ultimate determination of the nature of the biochemical processes occurring within anaerobic digester sludge. Such information could lead to optimization of anaerobic digester sludge settleability and COD-removal capacity by manipulation of the conditions in the digester to select for a population with good granulation capability. Light and electron microscopy was used to elucidate the relationships of the various bacteria within the granules in an attempt to explain the good settling properties of the sludge.

5.2 MATERIALS AND METHODS

5.2.1 Sampling

Granules were obtained from the Bellville clarigester and kept refrigerated under anaerobic conditions. All tests and isolations were carried out before the granules were two weeks old.

5.2.2 Microscopy

For light microscopy granules were crushed between a microscope slide and cover slip and viewed under white and UV light with a Zeiss phase contrast microscope.

For electron microscopy the granules were fixed in 3% (v/v) glutaraldehyde and 2% (m/v) osmium tetroxide, and dehydrated in an ethanol series. For scanning electron microscopy the specimens were critical point dried, gold sputter-coated and examined using a Hitachi S450 SEM.

Transmission electron microscopy of the granules was carried out in a Hitachi H600 TEM after having been stained with ruthenium red, osmium tetroxide, uranyl acetate and lead citrate, as described by Springer and Roth (1973), embedded in Spurr's (1969) Resin and sectioned with a glass knife.

5.2.3 Isolation procedures

5.2.3.1 Lactic acid bacteria

Sludge granules were serially diluted (10^{-1} to 10^{-7}) in Ringer's solution after maceration with an ultraturrax, and each ten-fold dilution was streaked onto Rogosa agar + 1% (m/v) fructose + 0.4% (m/v) potassium sorbate (to suppress yeasts and catalase positive organisms), and incubated at 32°C in an anaerobic jar with an anaerocult A envelope.

The colonies that developed were streaked out repeatedly until pure cultures were obtained. Pure cultures were subsequently examined microscopically for morphological characteristics and motility. These bacteria were tested for the

spectrum of sugars they could ferment, the formation of catalase, the presence of diaminopimelic acid in the cell walls and the configuration of lactic acid isomer(s) produced.

5.2.3.2 Acetogenic bacteria

Roll tubes were made using the technique described by Hespell and Bryant (1979) for the isolation of *Selenomonas, Succinovibrio, Butyrivibrio, Succinomonas* and *Lachnospira*. The tubes were inoculated with granule dilutions as described for the lactic acid bacteria and incubated at 32°C, taking great care to ensure anaerobic conditions at all times. Colonies were subinoculated until pure cultures were obtained and then examined microscopically.

5.2.3.3 Sulphate-reducing bacteria

Roll tubes were prepared using the media and methods described by Pfennig *et al.* (1981) for the isolation of *Desulfovibrio* and other sulphate reducers. After inoculation with granule dilutions the tubes were incubated at 32°C. Characteristic black colonies of sulphate reducers were transferred to sterile roll tubes and examined by phase contrast microscopy.

5.2.3.4 Enterobacteria

Granular sludge dilutions were streaked on Eosin Methylene Blue and MacConkey agar and incubated anaerobically. Following purification, cultures were identified using API 20E identification strips. In addition Gram stain, catalase and oxidase tests were carried out.

5.2.3.5 Butyric acid bacteria

Granules were pasteurised at 75°C for 10 minutes, streaked on *Clostridium* selective agar (Merck), and incubated in an anaerobic jar with an anaerocult A envelope.

5.2.3.6 Propionic acid bacteria

Granular sludge dilutions were streaked onto modified yeast extract and lactate medium (Britz, 1975) and incubated at 32°C in anaerobic jars. Pure cultures were examined using both Gram stain and liquid mount preparation. The spent medium was examined using a Hewlett-Packard 5790 gas chromatograph for the presence of propionic, acetic, *iso*-butyric, n-butyric and *iso*-valeric acids using a 2 m glass column with Graphpac coated with carbowax plus H_3PO_4 (injection temperature = 125°C, oven temperature = 175°C, final temperature = 150°C) and an FID detector. The standards used were 100 mg l^{-1} solutions of acetic,

propionic, n-butyric, *iso*-butyric and *iso*-valeric acids in 0.03 M aqueous oxalic acid. The injection volume was 5 μl and the carrier gas was N_2 with a flow rate of 20 ml min^{-1}.

5.2.3.7 *Staphylococcus*

Detection of many large clumps of cocci using light- and electron-microscopy motivated attempts to isolate this genus. For enrichment, trypticase soy broth containing 10% (m/v) NaCl was used prior to inoculation onto Baird-Parker agar (Merck). Isolates were Gram stained, tested for acid production from glucose (anaerobically and aerobically), mannitol utilization and catalase activity.

5.2.3.8 *Bacillus*

Isolation of this genus was performed subsequent to the detection (using TEM) of spores resembling those of *Bacillus* as depicted by Gould and Hurst (1969). Granules were pasteurized, as for the isolation of butyric acid bacteria, prior to inoculation into tubes containing the medium described by Norris *et al.* (1981) for the isolation of *Bacillus* from soil, water and other natural environments. Isolates were then tested for aerobic and anaerobic growth on glucose, Gram reaction and spore production.

5.2.3.9 Methanogens

Since SEM, TEM and fluorescence microscopy revealed the presence of three methanogenic morphotypes, namely, *Methanothrix* and *Methanosarcina*, (both acetate utilizers), and an unknown rod (which also fluoresced at 420 nm) these were enriched for. The first two genera were enriched using the methods of Zehnder *et al.* (1980) and the third methanogen, using the methods of Bryant *et al.* (1967). Methane production was tested using a Hewlett-Packard 5790 gas chromatograph with a Petrocol DH 150 column at a temperature of 35°C, using helium as the carrier gas with a flow rate of 20 ml sec^{-1}. An FID detector was used at a temperature of 200°C; the injection volumes were 25 μl and the standards were methane, ethane, propane, *iso*-butane, n-butane and n-pentane.

The effect of homogenization of the granules with an ultra-turrax homogenizer (necessary for inoculation of the anaerobic roll tubes) was gauged by plating out whole and homogenized granules onto Enterobacteria media (see above) as well as by determining the change in ATP content after homogenization using a Lumac ATP biometer.

5.3 RESULTS

5.3.1 Light microscopy

Whole granules were black and varied in shape and size as viewed using bright field microscopy (Plate 5.1). Weakly fluorescent long filaments and strongly fluorescent sarcinae and single rods were present within the granules. Nonfluorescent motile vibrios, rods, cocci, spirilla and sarcinae were observed, the sarcinae appearing black under phase contrast.

5.3.2 Electron microscopy

SEM and TEM studies revealed that the granules contained several bacterial morphotypes in close association with each other (Plates 5.2 to 5.14) confirming work carried out by Dolfing (1986). As confirmed by light microscopy, sarcinae, vibrios, cocci, filaments and a variety of rods were present.

Spirochaetes were observed in both SEM and TEM preparations (See Plates 5.3 and 5.4). Plate 5.4 is a cross section of a free-living spirochaete with several fibrils in the axial filament, suggestive of *Spirochaeta plicatilis*.

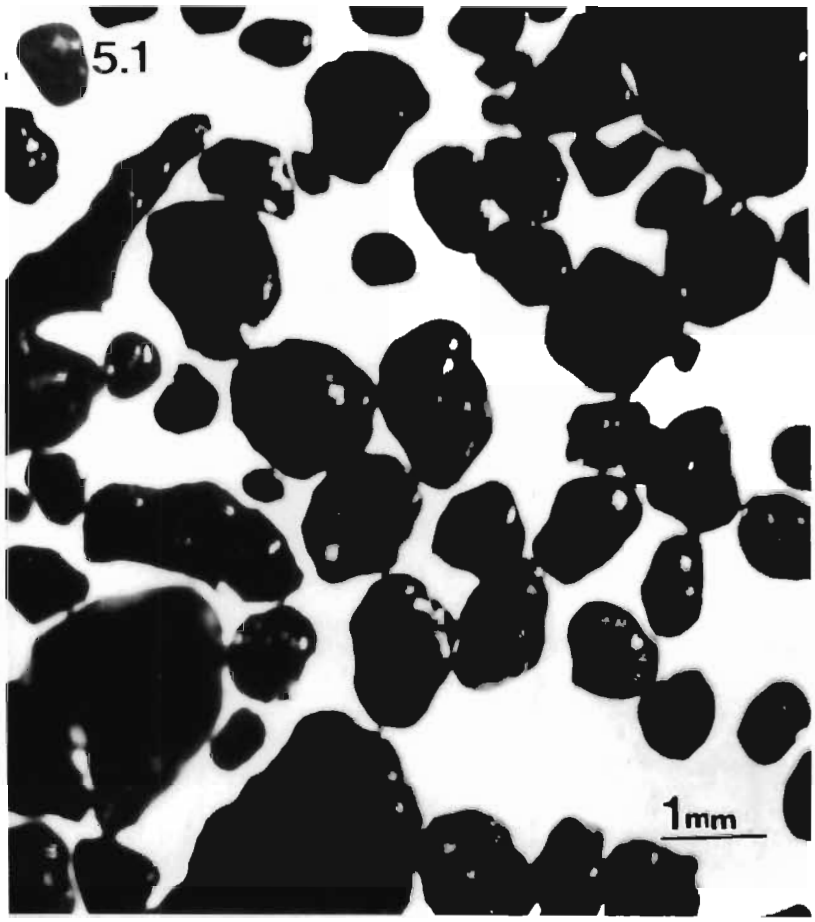
Motile organisms varying with respect to positioning and number of flagella were abundant in the granules as shown in Plates 5.5 and 5.10. Plate 5.5 includes a bent rod with a single polar flagellum. This organism is too small to be either a sulphate reducer or an acetogen (Hespell and Bryant, 1979; Pfennig *et al.*, 1981).

5.3.2.1 Use of the electron microscope to determine the adhesive properties of the bacteria within the granules

Within the granules interlinking strands between the bacterial cells appear to be extensions of bacterial capsules (Plate 5.6) while in Plate 5.7 a loose network of matrix material is evident. Contradictory to the study on brewery granules, these observations suggest that a network of polymeric strands may hold the bacterial cells together in these *Methanosarcina* containing granules. It is also possible that the strands are actually condensed glycocalyx as discussed in Chapter 6. Within the granule structure dense membranous compartmentalization was observed around groups of bacteria, isolating them from the rest of the granule (Plate 5.8). The areas inside the "compartments" appear lighter in colour than those outside them, possibly due to failure of the osmium tetroxide to penetrate the electron dense barrier. Alternatively these barriers may be interfaces within granules separating areas of different hydrophobicities.

PLATE 5.1 Light micrograph of granules from a clarigester treating maize-processing waste water

PLATE 5.2 SEM showing the diversity of bacteria within anaerobic digester granules


5.1
1mm

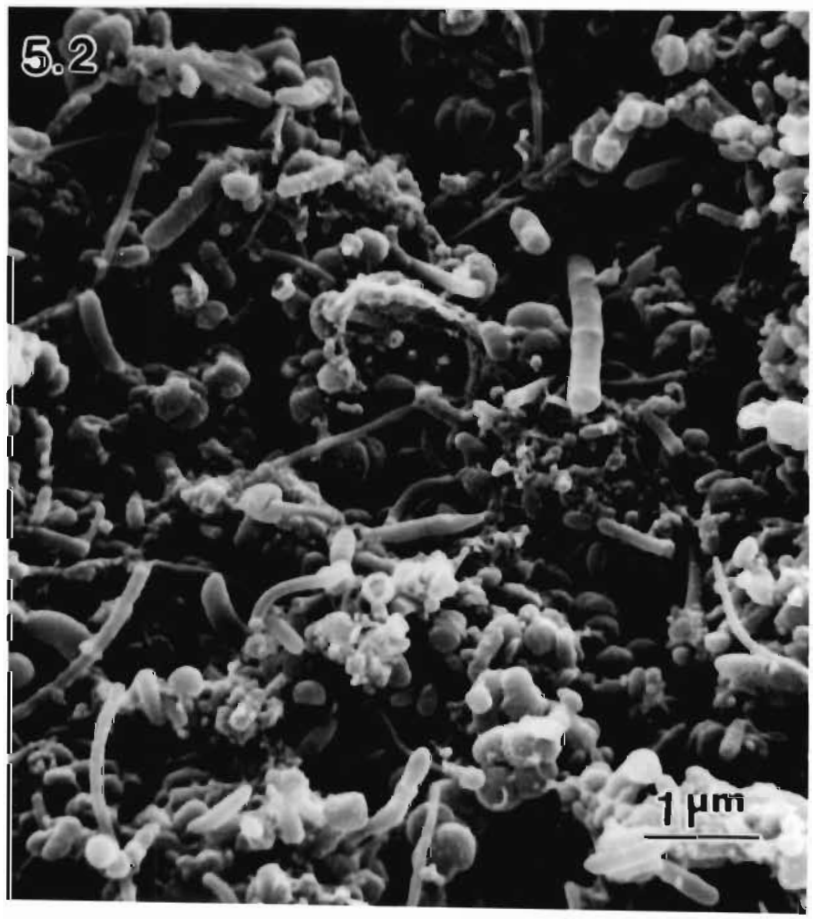
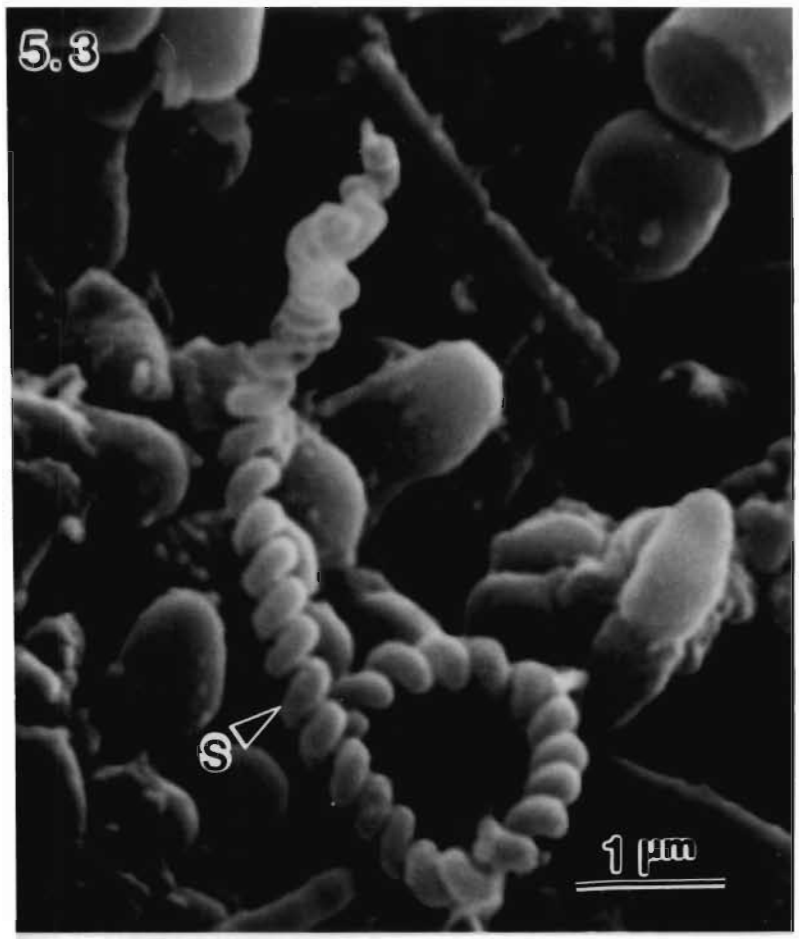
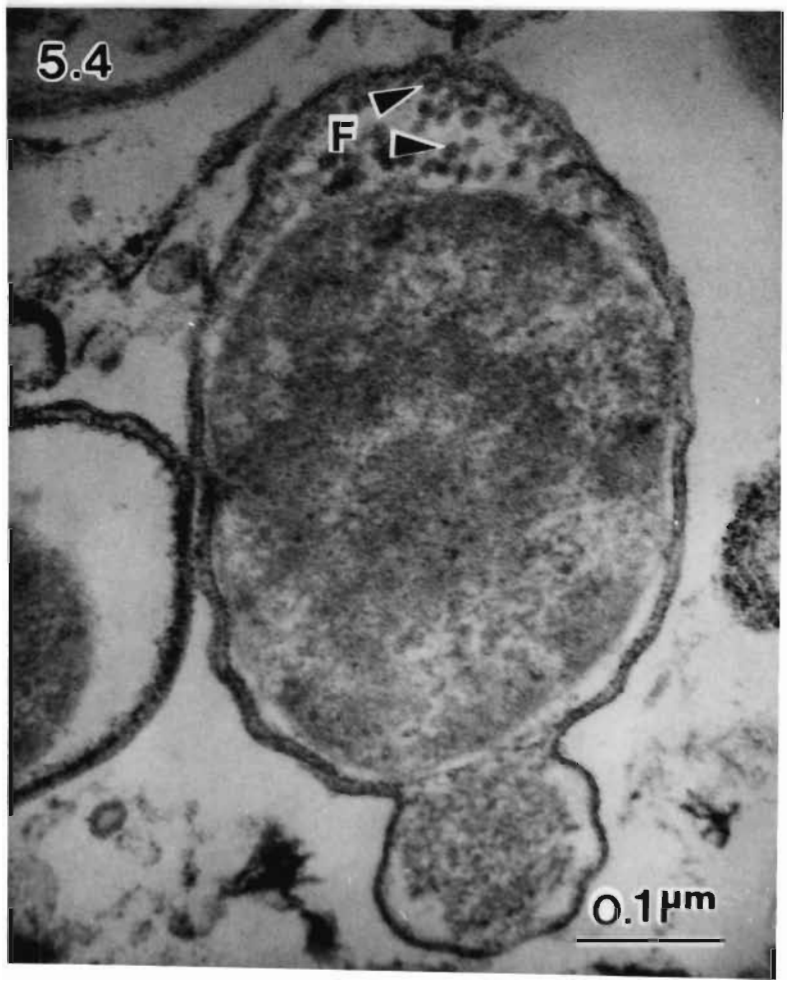

5.2
1 µm

PLATE 5.3 SEM of spirochaete (S) within a granule

PLATE 5.4 Cross section of *Spirochaeta plicatilis* (probable identity) showing fibrils (F) within the axial filament


5.3
S
1 µm


5.4
F
0.1µm

5.3.3 Isolation studies

The organisms identified, and their characteristics, are listed in Table 5.1 and include the following:

5.3.3.1 Lactic acid bacteria

Eleven rod-shaped isolates varying in morphology from short to long rods, and filaments were distinguished. Of special interest were two isolates of unusual spiral morphology. These isolates were all identified as members of the heterofermentative *Lactobacillus* group on the basis of their sugar fermentation pattern, the configuration of the lactic acid isomer(s) produced, the presence of diaminopimelic acid in the cell wall and other physiological characteristics (see Appendix 1). Six of the isolates could break down starch, identifying them as hydrolytic bacteria and between the eleven isolates a wide spectrum of sugars could be metabolized (see Appendix 1).

TABLE 5.1 Summary of characteristics used for bacterial identification

	Staphylococcus	*Bacillus*	*Lactobacillus*	*Methanosarcina*
Cell morphology	Cocci, singly or in clumps, non-motile	Rods	Non-motile rods (short, long or coiled)	Cocci in tetrads and clumps
Gram	+	+	+	Variable
Acid from glucose	+	+	+	ND
Catalase	+	+	-	ND
Anaerobic growth	+	+	+	+
Aerobic growth	+	+	ND	-
Methane production	ND	ND	ND	+
Fluorescence at 420 nm	-	-	-	+
Critical sugar utilization tests	Mannitol-vl	Mannitol-vl	Different isolates varied	ND
Spore production	-	+	-	-
Other	Grows in 10% (m/v) NaCl but no colony pigmentation		Grows at pH 3.9 and forms lactic acid	Only grows in the presence of acetate

ND = not determined

Salmonella arizonae and *Klebsiella* were identified using API 20E identification systems and sulphate reducers by their ability to reduce sulphate anaerobically.

5.3.3.2 Acetogenic bacteria

Several colonies developed after incubation of the roll tubes. Microscopic examination showed that these consisted of vibroid and coccoid bacteria. Some of the vibrios showed a tumbling motility in wet preparations characteristic of *Selenomonas* and *Lachnospira* when grown on this selective medium (Hespell and Bryant, 1979), while others showed translational motility. This motility was short lived due to the lack of anaerobiosis during microscopic examination. SEM studies verified the presence of vibroid, laterally flagellated cells with dimensions and morphology characteristic of these two bacteria (Plate 5.9).

5.3.3.3 Sulphate-reducing bacteria

After five days incubation, the specific sulphate-reducer isolation medium turned black, and scattered, pitch black colonies of sulphate reducers were observed. Microscopic examination of these colonies revealed spiral-shaped bacteria with a corkscrew motility. These organisms resembled *Desulfovibrio gigas* as described by Pfennig *et al.* (1981). Electron microscopy of the granules confirmed the presence of spiral bacteria with polar tufts of flagella resembling *Desulfovibrio gigas* (Plate 5.10). Other vibroid, motile sulphate reducers were also present. In the roll tubes white colonies surrounded by clear halos were observed within the blackened medium. The white colonies consisted of coccobacilli, often diplococci, but also single cells and clumps of cells. All attempts to subculture the organisms

from the white colonies were unsuccessful, preventing further study of these bacteria.

5.3.3.4 Enterobacteria

Numerous colonies containing Gram negative rods developed on MacConkey and EMB agar. Of these, several were identified as *Salmonella arizonae* and *Klebsiella* using API20E identification strips. All isolates were oxidase negative and catalase positive, while electron micrographs of the granules revealed the presence of encapsulated rods resembling *Klebsiella* (Plate 5.11).

5.3.3.5 Butyric acid bacteria

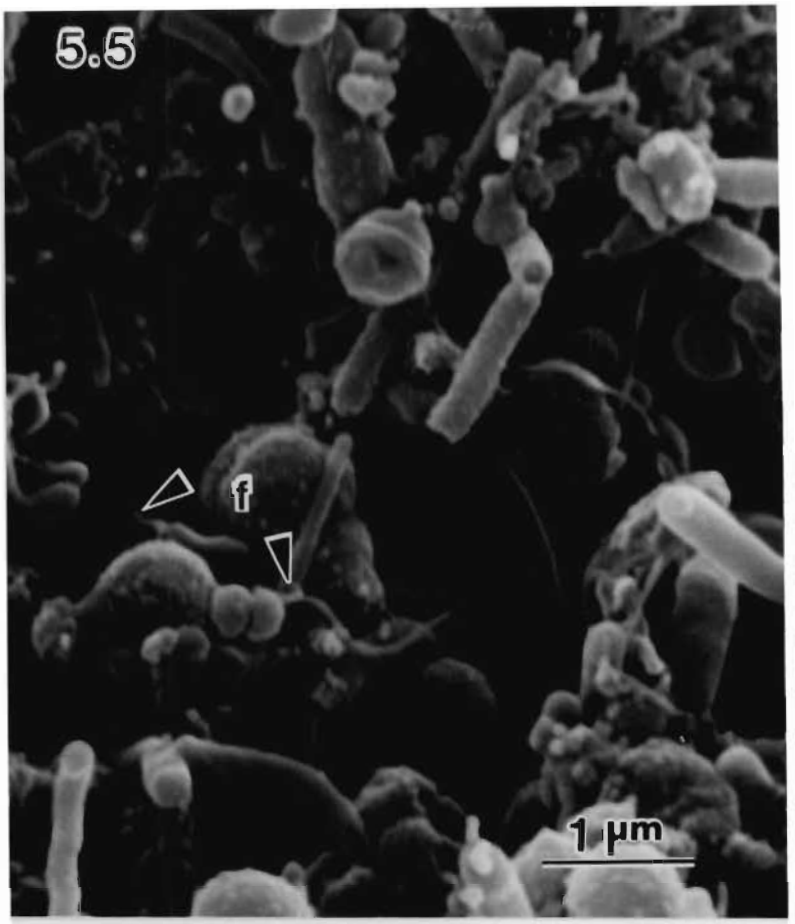
No *Clostridium* species were isolated but some of the other bacteria isolated from the granules, e.g. possibly *Selenomonas*, are capable of forming butyric acid under certain conditions (Hespell and Bryant, 1979).

5.3.3.6 Propionic acid bacteria

No *Propionibacterium* species were isolated but some of the granule-inhabiting bacteria such as *Selenomonas*, if present, can produce propionate under certain circumstances (Hespell and Bryant, 1979).

PLATE 5.5 Bacteria within granules, some with flagella (f)

PLATE 5.6 Section through a cell showing capsular material (C)


5.5
f
1 µm

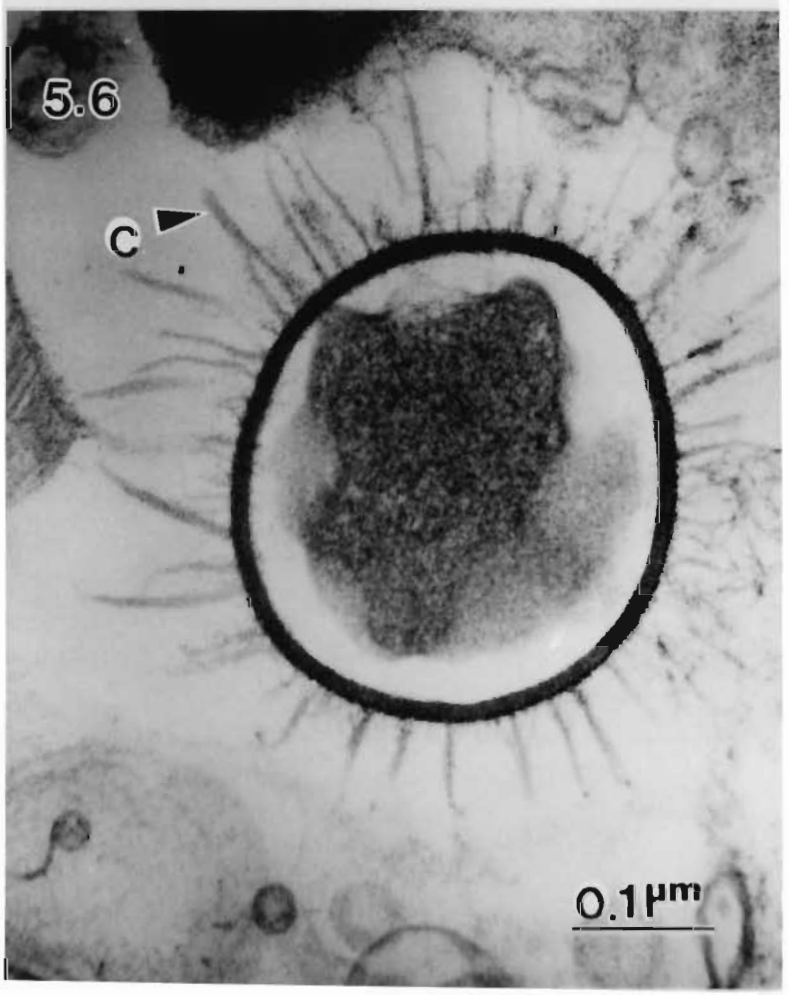
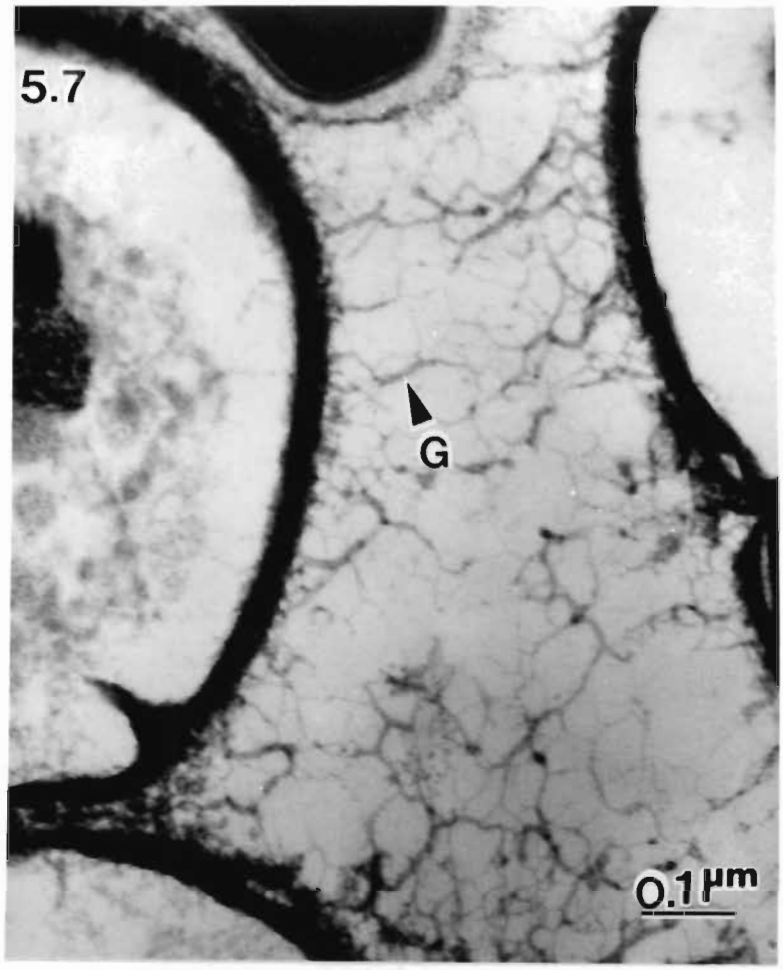

5.6
C
0.1µm

PLATE 5.7 TEM of cells and interlinking glycocalyx (G)

PLATE 5.8 TEM showing compartmentalization within a granule. Note electron-dense bounding structure (K)


5.7
G
0.1 µm

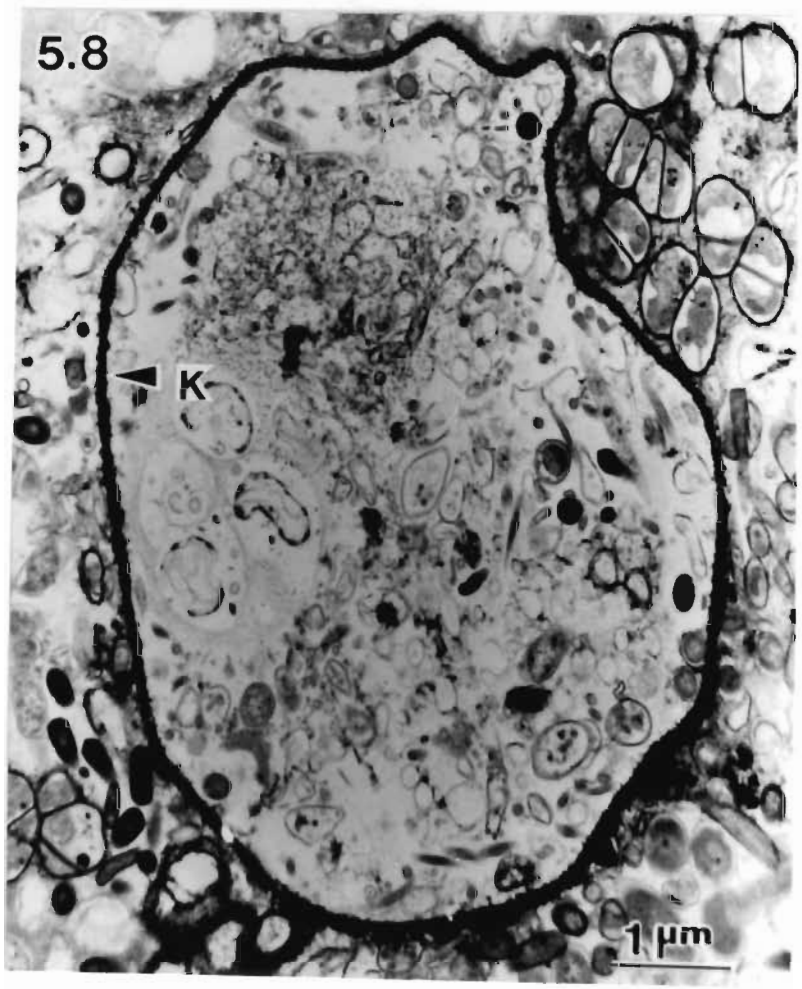
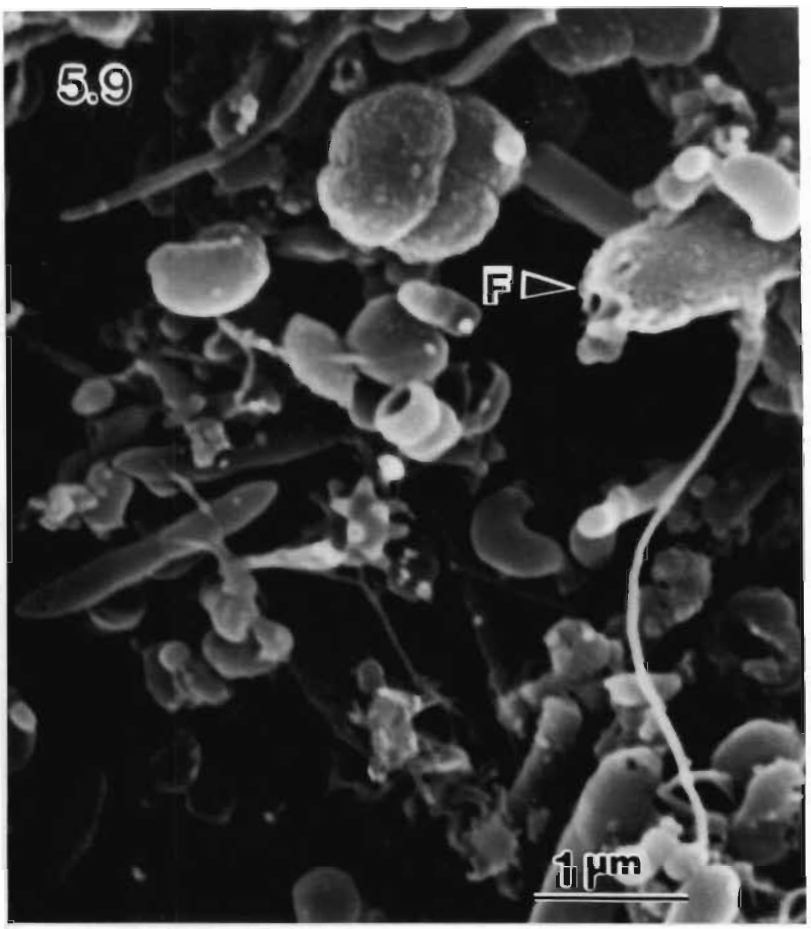

5.8
K
1 µm

PLATE 5.9 SEM of bacteria within a granule including a vibroid, laterally flagellated cell (F) similar to *Selenomonas* or *Lachnospira*

PLATE 5.10 Spiral bacterium, with tuft of polar flagella (f). Possibly *Desulfovibrio gigas*


5.9
F
1 µm


5.10
f
1 µm

5.3.3.7 *Staphylococcus*

Cultures of non-motile Gram positive cocci (single or in irregular clusters) were obtained using the enrichment and isolation methods described. They were catalase positive and capable of anaerobic and aerobic growth on glucose with simultaneous acid production. Their ability to grow in 10% (m/v) NaCl, together with the above characteristics indicates that they were possibly *Staphylococcus*. The colonies were not pigmented and mannitol could be utilized as described for some strains of *Staphylococcus* (Schleifer, 1986).

5.3.3.8 *Bacillus*

Colonies of Gram positive spore forming, catalase positive and mannitol negative rods were isolated that were capable of both aerobic and anaerobic growth. They were capable of producing acid from glucose and their spores (Plate 5.14) resembled those of *Bacillus*.

5.3.3.9 Methanogens

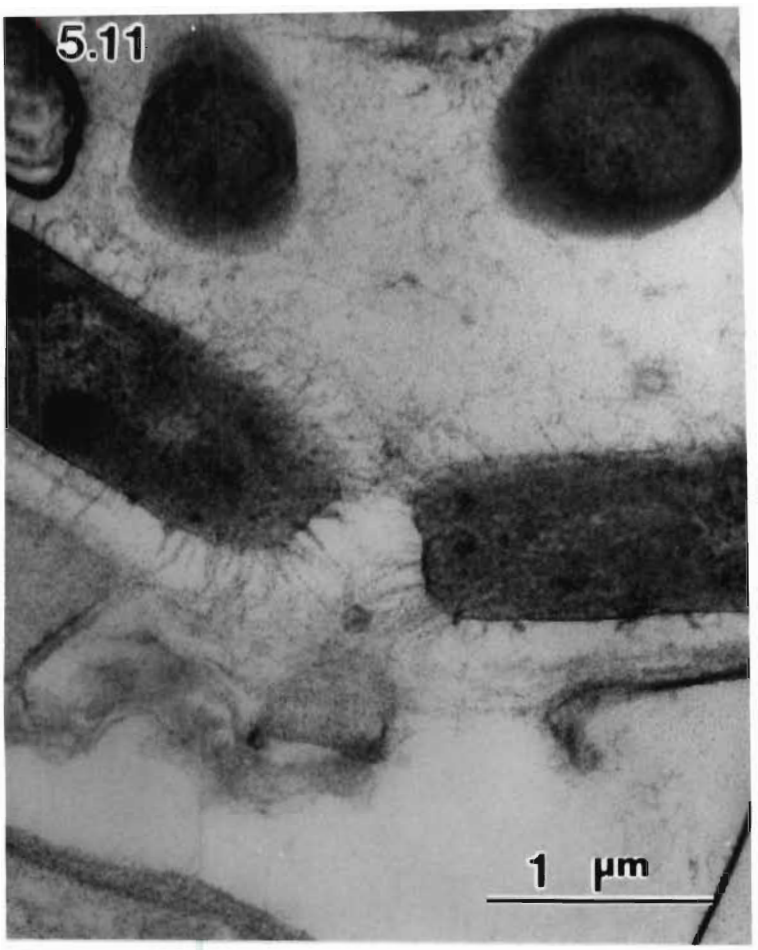
Use of the acetate containing medium for *Methanothrix* and *Methanosarcina* enrichment resulted in the concentration of *Methanosarcina*-like tetrads (similar to those described by Zhilina, 1971) which clumped together and were covered by an extracellular polymeric matrix (Plate 5.12). *Methanothrix*-like filaments were also

obtained. Electron micrographs of granules confirmed the presence of numerous tetrads and filaments resembling these two bacteria (Plate 5.13).

Physical disruption of the granules, using the ultraturrax, resulted in death of many of the cells, since growth was observed at higher dilutions on facultative anaerobic plates when the granules were not homogenized than when they were. In addition, ATP biometry indicated a very high ATP reading immediately after homogenization of the granules due to release of ATP by the ruptured cells. A subsequent decrease in ATP content to levels far below those of undisturbed granules then occurred due to its denaturation. Sonication resulted in extremely slow disruption of the granules. The harsh treatment required for granule disruption in this experiment proved to be destructive to the microbial population and this would seriously complicate any attempt at quantification.

PLATE 5.11 Encapsulated rods resembling *Klebsiella*

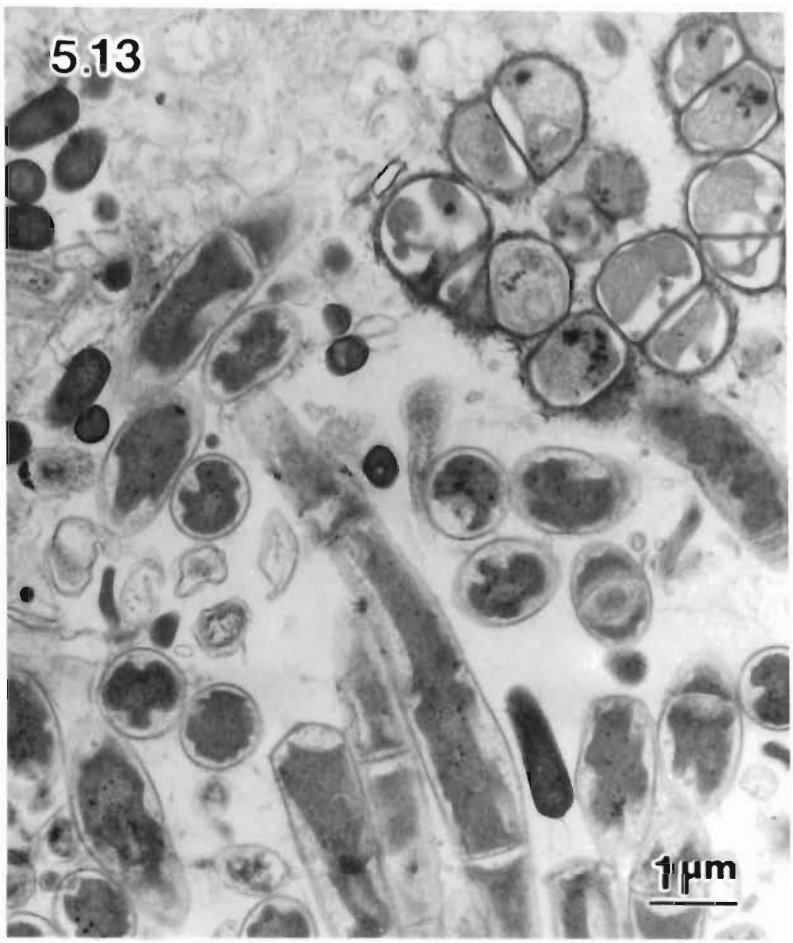
PLATE 5.12 *Methanosarcina*-like tetrads in acetate-containing enrichment medium

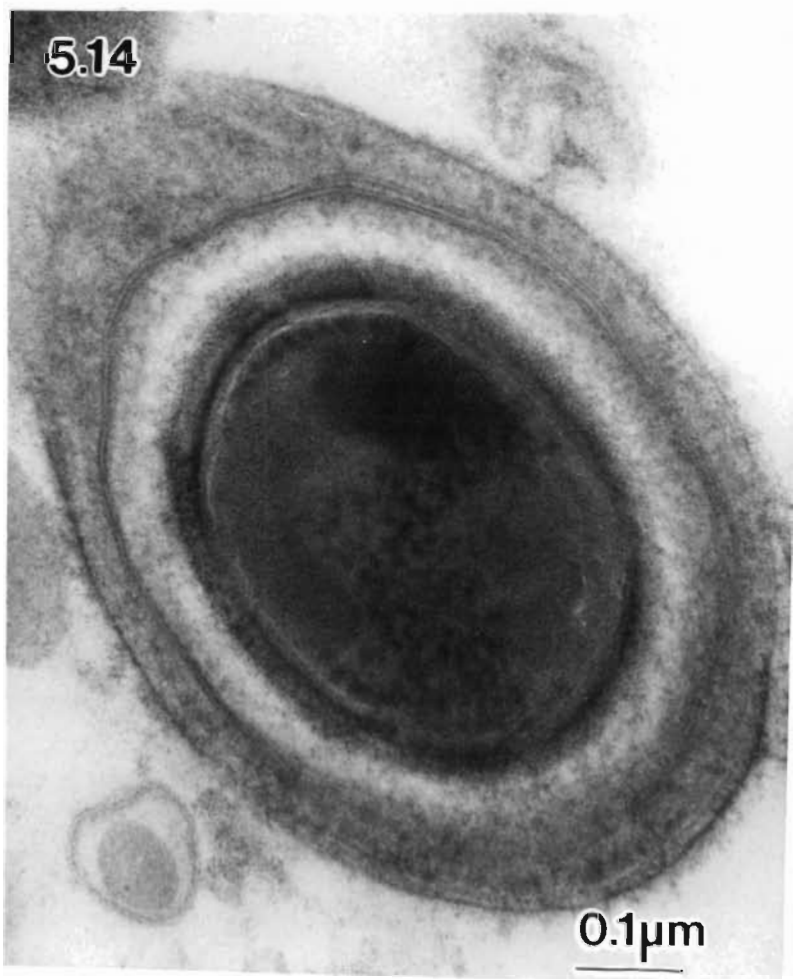

5.11
1 µm


5.12
1 µm

PLATE 5.13 TEM of tetrads and filaments resembling *Methanosarcina* and *Methanothrix*, respectively

PLATE 5.14 Endospore resembling that of *Bacillus*


5.13
1µm


5.14
0.1µm

5.4 DISCUSSION

The results obtained give a good indication of which bacterial groups occur in the clarigester granules. However, although a wide diversity of bacteria was identified in the granules, some non-culturable members of the population were undoubtedly not isolated in pure culture and identified.

In general, the electron- and light-microscopic observations of the isolated and enriched bacteria supported observations made on entire granules. For example, the isolation of *Klebsiella* verified the observation by TEM of a *Klebsiella*-like rod, and the presence of *Bacillus* was deduced after spores resembling those of this genus were detected on electron micrographs, and confirmed when a number of isolates of this genus were obtained.

The Enterobacteria isolated (*Salmonella arizonae* and *Klebsiella*) ferment sugars to lactate, succinate, acetate, ethanol, CO_2, H_2 and formate (Doelle, 1975) while *Staphylococcus* and *Bacillus* can both utilize a wide variety of carbohydrates and amino acids (which vary according to species and strain) with concomitant acid and gas production (Schleifer, 1986; Sneath, 1986).

Lachnospira can break down dextrins and hexoses to ethanol, lactate, acetate, formate, CO_2 and H_2. *Butyrivibrio* and *Selenomonas* can break down dextrins and starch, respectively, to lactate, formate, acetate and CO_2 and H_2. The former

organism produces butyrate while the latter can, under certain circumstances, produce propionate (Hespell and Bryant, 1979). In this study, no propionic or butyric acid producers were detected, unless *Selenomonas* is present and considered as belonging to the former group. This suggests that these bacteria play an insignificant role in this type of anaerobic digestion.

The presence of lactic acid bacteria was to be expected since the steeps liquor discharged into the clarigester had high *Lactobacillus* counts. These organisms normally produce lactate, ethanol and/or acetate, CO_2, glycerol and mannitol from glucose, lactose and other sugars (Doelle, 1975). Some of these bacteria were able to hydrolyse starch. The ease with which these bacteria were isolated in large numbers from all the clarigester granules examined indicated that in this granular sludge, metabolism might revolve around lactate rather than butyrate. It was for this reason that emphasis was placed on the isolation and identification of the lactic acid group of bacteria (see Appendix 1).

All the above organisms comprise the second stage of the proposed four-stage model. However, as many of them are capable of hydrolytic activity they could also be part of the first stage of this model. Since no propionibacteria and clostridia were detected, lactate is most probably further utilised by the acetogenic sulphate reducers and other syntrophic organisms not isolated, producing acetate, CO_2 and sulphide (Pfennig *et al.*, 1981). Sulphate reducers and other syntrophs would, therefore, comprise the third stage of this model.

The methanogenic group is able to transform most of the end products produced by organisms comprising the third stage to methane and CO_2. *Methanosarcina* and *Methanothrix* can both utilise acetate; some species of the former genus in fact being obligate acetate utilisers (Zehnder *et al.*, 1980). In the present study, the population of *Methanosarcina*-like tetrads was apparently larger in number and volume than the *Methanothrix* population, as observed in numerous electron micrographs of many granules. This contradicts the findings of Dolfing (1986) who investigated a granular sludge from a sugar factory effluent and found that *Methanothrix* rather than *Methanosarcina* was the most significant methanogen present. The present study indicates that members of both genera would be significant in granular sludge populations.

Spirochaeta plicatilis has unfortunately never been isolated in pure culture and its metabolic processes are thus poorly understood.

Due to their highly stable structure only extremely harsh treatments could disrupt these clarigester granules. Unfortunately, these treatments proved lethal to the bacteria present, so quantitative studies could not be carried out.

The methanogens isolated tended to form stable aggregates, thus possibly contributing to the stability of the granules. This confirms postulates by Yoda *et al.* (1989) who indicated that a syntrophic relationship between acetogens and methanogens may play a key role in forming a dense biofilm. Work by Sam-Soon

et al. (1987) indicated that *Methanobacterium* strain AZ produces polypeptide in the absence of cysteine and that this polypeptide could bind the organisms together thereby aiding in the formation of granules. This hypothesis was extended to include other bacteria with this property. An amino acid analysis of the feed to the clarigester (see Table 3.2) indicated that both cysteine and cystine were absent. Thus, if the "cysteine-hypothesis" (Sam-Soon *et al.*, 1987) is valid, an unidentified bacterium in the granules may be responsible for the production of the polypeptide and this could be contributing to granulation. Zoutberg *et al.* (1988) found that *Selenomonas ruminantium* was responsible for the formation of aggregates within an anaerobic gas-lift reactor; this organism, however, is unlikely to play a pivotal role in our bioreactor as too few *Selenomonas*-like cells were observed within the granules.

Successful anaerobic treatment of waste water from industries where lactic acid bacteria are inoculated (for e.g. steeping) is probably more reliant upon these bacteria, than propionic and/or butyric acid bacteria. If this is so, lactate and acetate would be more important intermediates than butyrate and propionate in sludges involved in digestion.

It is noteworthy that subsequent to this study, the maize processing factory started to recover the gluten previously discharged to the clarigester. This resulted in a decrease in the volume of effluent being treated as well as a decrease in the amount of gluten protein entering the digester. This alteration in

feed composition affected the nature of the sludge formed and resulted in smaller granules with reduced settleability being produced. It can be concluded that the widely accepted four stage model for the microbiology of anaerobic digestion comprising hydrolytic, acidogenic, acetogenic and methanogenic stages can be applied to this granular sludge. It should, however, be noted that the nature of the digester feed, and/or the bacteria in the feed, would probably play a significant role in determining the micro-organisms that establish within the granules.

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CHAPTER 6

DEVELOPMENT OF A METHOD TO QUANTIFY BACTERIAL MORPHOTYPES WITHIN ANAEROBIC DIGESTER GRANULES BY IMAGE ANALYSIS OF TRANSMISSION ELECTRON MICROGRAPHS

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QUANTIFICATION OF BACTERIAL MORPHOTYPES WITHIN ANAEROBIC DIGESTER GRANULES FROM TRANSMISSION ELECTRON MICROGRAPHS USING IMAGE ANALYSIS

by

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ABSTRACT

Image analysis was applied to sequential transmission electron micrographs of an ultrathin section from the central region of an anaerobic digester granule to quantify the constituent bacterial morphotypes present. Our experience indicates that this procedure is suitable for the determination of populations of small spherical granules only and that it would be a useful technique for monitoring granule development. The cell area data determined in this study should permit rapid future quantification of *Methanothrix*- and *Methanobacterium*-like cells from cell counts derived from transmission electron micrographs.

6.1 INTRODUCTION

In Upflow Anaerobic Sludge Blanket (UASB) digesters active biomass retention independent of flow rate, and thus efficient digester performance, relies upon the formation of compact, rapidly settling sludge aggregates, called granules. These have been described as spherical (de Zeeuw, 1988) or spheroid (Wu *et al.*, 1991) and comprise a bacterial association of diverse morphotypes. Quantification of the microorganisms is complicated by the structural integrity of the granule which cannot be disrupted without destroying a large proportion of the population (Dolfing *et al.*, 1985; Howgrave-Graham *et al.*, 1991). Recent quantitative studies have relied upon standard viable number and total cell number techniques on disrupted granules, often combined with light microscopy using methanogen-

specific fluorescent antibodies to count cells within paraffin wax embedded sections (Grotenhuis *et al.* , 1991; Schmidt *et al.*, 1992). Wu *et al.* (1991) counted more than 6700 cells on transmission electron (TE) micrographs of granules and extrapolated morphotype concentrations from these data. Cell concentration calculations from cross section counts are likely to be erroneous unless the morphotypes are the same size, as smaller cells are less likely to be detected in random sections than larger ones. The random orientation of cells observed in all published TE micrographs of granules should also be considered during quantification as, irrespective of cell type, very few would have the same area in the observed plane of the section. Total cell area determinations within carefully selected cross sections would allow a far more accurate quantification of morphotypes within granules. For this, image analysis, which has until now only been applied to sizing granules (Dubourguier *et al.*, 1988; Dudley *et al.*, 1991) but has been successfully used in quantifying bacteria within other mixed ecosystems (Jones *et al.*, 1992; Meijer *et al.*, 1990), could be applied.

In this study the feasibility of applying image analysis to TE micrographs of UASB granules for determination of morphotype concentration and distribution therein is evaluated. As this chapter is aimed at exploring new combinations of techniques it cannot be construed as representative of granules in general, e.g. only one granule was used and only a very small percentage of this granule's cross-sectional area was analyzed.

6.2 MATERIALS AND METHODS

6.2.1 Anaerobic digester granule

The granule originated from a full-scale digester purifying brewery waste water at Prospecton, Natal (South Africa) and prepared immediately for TE microscopy as described below.

6.2.2 Transmission electron microscopy

One spherical granule of 0.35 mm diameter was fixed, dehydrated and embedded according to the method of Howgrave-Graham and Wallis (1991). The resin block was trimmed on the sides until the granule was clearly visible, allowing the position opposite its centre to be etched on the block with a glass ultramicrotome knife under a dissecting microscope. The front of the block was then trimmed away using glass knife ultramicrotomomy until half the granule had been pared to the centrally located etch mark. Ultrathin sections were then cut through the granule centre with tungsten-coated glass knives and picked up on formvar coated copper grids of 3 mm diameter with slots of 2 x 0.5 mm (the cross-bars on normal copper grids obscure much of each section, obstructing accurate cell quantification). To confirm that the sections used were central, the granule cross section diameter was monitored throughout trimming and sectioning. Sections were viewed with a Jeol 100 CX TE microscope and overlapping electron

micrographs, taken at 2000x magnification from the centre of the granule outwards along a radius (with minimum artifacts) to the granule perimeter were assembled to form a montage. Plate 6.1 shows such a montage.

6.2.3 Image capture

A Seescan Solitaire image analyzer fitted with a CCD video camera and macro zoom lens was used to capture images of 335 μm^2 from TE micrograph negatives placed on a light box. This approach alleviated the problem of light reflection from prints. Nine sequential images were captured along a radial line drawn on the print montage to aid accurate image orientation. A point equidistant from three points on the granule perimeter was taken as the granule centre.

6.2.4 Image analysis and processing

A program, written by Seescan, stored information on the position (relative to the granule centre), area and number of all cells in three morphological categories distinguished by the image analyzer. These were: (i) *Methanothrix*-like cells (bamboo-shaped rods); (ii) *Methanobacterium*-like cells (small electron-dense rods), as observed by Wu *et al.* (1991); and (iii) all bacterial morphotypes present (Plate 6.1). Attempts to analyze for *Desulfobulbus*-like cells (large coccoid to diptheroid cells) were abandoned as this morphotype was not successfully distinguished by thresholding. In Chapter 7, however, these organisms are

quantified by multiplying the average area of several cells manually "coloured in" by cell numbers as suggested later in this chapter.

The captured images were recalled and processed separately for each of the three cell morphotype categories. *Methanobacterium*-like cells, being the darkest and smallest were usually easily thresholded out and quantified, but interactive steps were required to ensure that all cells were accurately represented according to the print montage. The *Methanothrix*-like cells occurred in localized colonies allowing large areas of each image to be deleted by the "exclude" function before these cells were thresholded; this procedure followed deletion of the already quantified *Methanobacterium*-like cells. Interactive steps in conjunction with the reference print montage were again required to ensure that an accurate representation was obtained. Quantification of the total cell concentration was also achieved by applying thresholding and interactive steps. Before each image was processed, its coordinates in relation to the granule centre were recorded and the area of the measuring frame determined. The threshold levels varied according to the contrast and light intensity in the images.

6.3 RESULTS AND DISCUSSION

Figure 6.1 details area and number distributions for each of the three morphotypes within an area of 2950 μm^2 which comprised 0.031% of the total

cross sectional area of the analyzed granule. A total of 1281 cells were counted and size determinations indicated that they occupied 18% of the total montage area. The 685 (53% of the total cell population) *Methanothrix*-like cells constituted 55% of the total cell area while the 326 (25% of the total cell population) *Methanobacterium*-like cells constituted only 11% of the total cell area. From these data, and assuming that the ratios of the three cell-morphotype categories determined from the cross section examined are representative of those throughout the granule volume, it can be concluded that *Methanothrix*-like cells constitute the largest component of the bacterial population in the granule examined. *Methanobacterium*-like cells occupied a far smaller fraction of the total granule than their numbers would suggest.

The accuracy of our results depends also on the validity of the assumptions that the granule was perfectly spherical, that the area examined was truly representative of all other areas that could have been captured, and that the centre of the granule was accurately chosen. Errors resulting from these assumptions could be minimized by processing more images from several radii. This is especially applicable towards the granule periphery where statistical errors would tend to be exacerbated. This would, however, be a monumental task particularly with larger granules, as it takes at least ninety minutes to process each image.

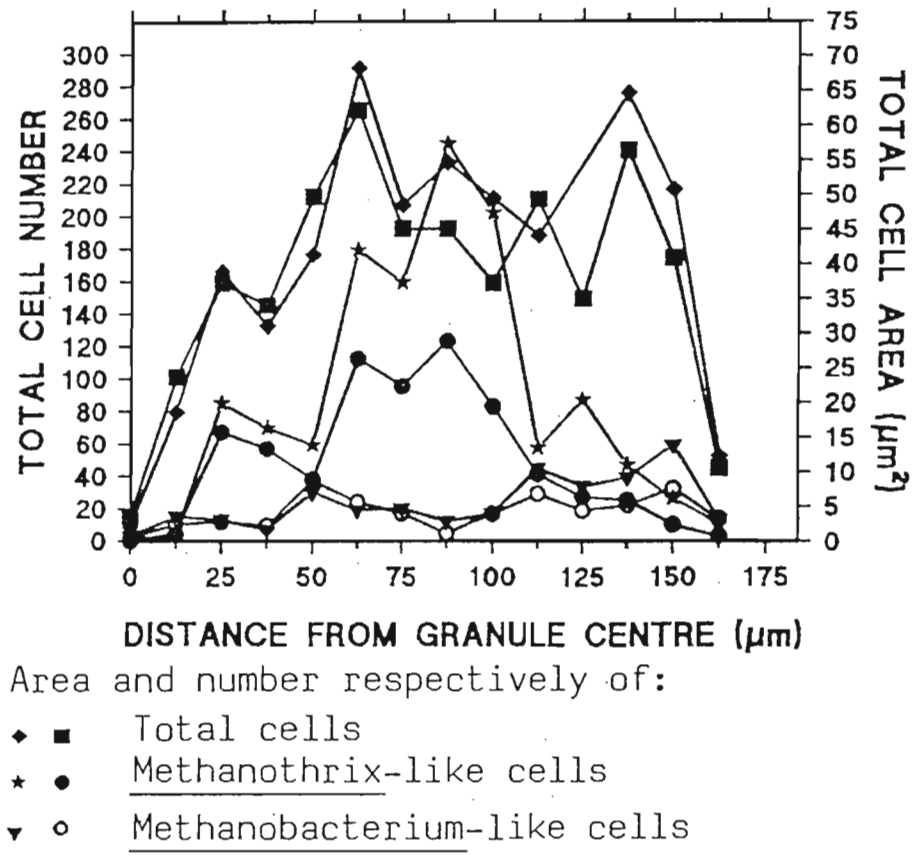


FIGURE 6.1 Cell morphotype distribution derived from captured electron micrograph images. "Total cell number" and "total cell area" represent all cells quantified between radii as indicated on the x axis.

A possible alternative would be to combine the data extrapolated from image analysis with the counting technique of Wu *et al.* (1991). This would entail multiplying the average cross-sectional area of each morphotype by cell numbers obtained from counts made at each radius (or set of radii) on TE microscopic montages.

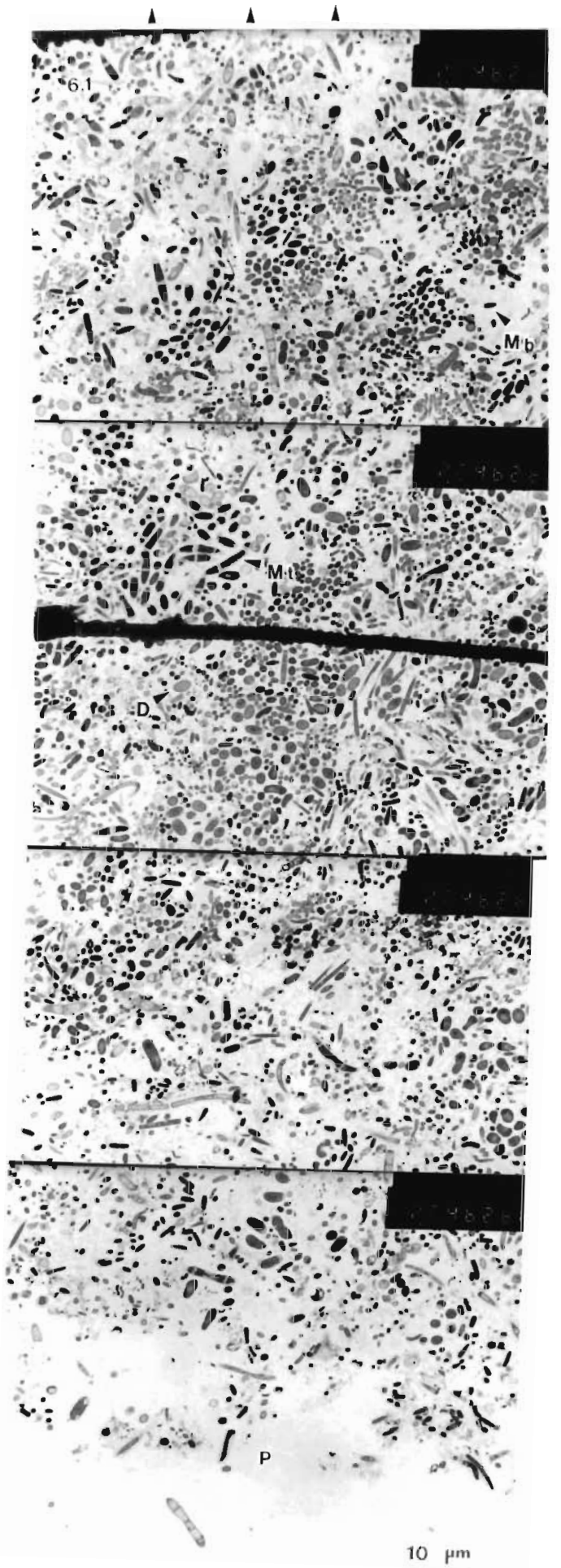
This approach would eliminate calculation errors associated with quantifications based on cell counts alone, which assume that all cells are orientated identically in the plane of the section and that all cells of the same type are of the same size. *Methanothrix*- and *Methanobacterium*-like cells were calculated to have average cross sectional areas of 0.421μm^2 and 0.178 μm^2 , respectively. In the present study, as in any future studies based on similar data, it must be recognized that cell orientation will always be random.

The data in Figure 6.1 indicate that the centre of the granule was almost devoid of cells while *Methanothrix*-like cells were localized in colonies between 60 and 137 μm from the granule centre. *Methanobacterium*-like cells although more abundant at 50 to 85μm and 100 to 162μm from the granule centre, were mostly found in association with *Desulfobulbus*-like cells (see Plate 6.1). The locality of each cell type within the granule is probably determined by the physicochemical environment and competition for substrate. The validity of the above results could be enhanced by capturing and analyzing more images or, preferably, by applying the modified counting technique suggested above.

It can be concluded from this work that quantification of cell morphotypes within spherical granules containing mixed populations is possible using image analysis on TE micrographs provided the granules are small and: (a) the morphotypes quantified can be distinguished, using thresholding, from the other cells; or (b) are localized in sub-colonies allowing exclusion of the surrounding

morphologically different cells. This quantification procedure would be especially useful in monitoring granule development. A time saving alternative is to count cells on TE micrographs and multiply this figure by an average cross sectional area of the cells quantified using image analysis.

PLATE 6.1 Montage of transmission electron micrographs of an anaerobic digester granule revealing its perimeter (P) and the direction (along a radius) of its centre (arrowheads). *Methanothrix*-like (Mt), *Methanobacterium*-like (Mb) and *Desulfobulbus*-like (D) cells are also indicated


6.1
M b
M t
D
P
10 µm

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CHAPTER 7

QUANTITATIVE ANALYSIS AND MAPPING OF BACTERIA IN BREWERY GRANULES USING TRANSMISSION ELECTRON MICROSCOPY AND STRAIN SPECIFIC ANTIBODY PROBES

A condensed version of this chapter has been submitted for publication to Applied and Environmental Microbiology as:

QUANTITATIVE ANALYSIS AND MAPPING OF BACTERIA IN A COMPLEX CONSORTIUM USING A COMBINATION OF TRANSMISSION ELECTRON MICROSCOPY WITH IMMUNOTECHNOLOGY

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ABSTRACT

Bacterial morphotypes within granules from an upflow anaerobic sludge blanket digester (UASB) purifying a brewery waste water were quantified and localized *in situ* from transmission electron micrographs (TEMs) along granule radii. Cell numbers were translated to biomass by incorporating relative size factors determined by image analysis. Cells within homogenized granules were counted using a haemocytometer while methanogen and acidogen strains were enumerated using antibody probes. Granule cross sections showed two distinct strata: a 500 μm thick outer cortex consisting primarily of cells, and an inner medulla virtually devoid of cells. *Methanothrix*-like cells (26.2% of the population or 46.3% (v/v) of the biomass) and *Methanobacterium*-like cells (37% of the cell number or 27.1% (v/v) of the biomass) concentrated in two bands at 100-150 and 250-450 μm below the granule surface whereas *Desulfobulbus*-like cells (9.6% of the cell number or 19.0% (v/v) of the cell mass) were evenly distributed throughout the cortex. Other morphotypes (27.2% of the population or 7.6% (v/v) of the biomass) were concentrated near the granule surface. A methanogen antigenically close to *Methanothrix soehngenii* OPFIKON formed 0.22% of the haemocytometer-determined population of 6.95×10^6. Both of these figures were lower than those obtained from TEM quantification, probably due to most *Methanothrix*-like cells in TEMs being immunologically unrelated to this strain, and bacterial disruption during granule homogenization, respectively. As the

bacteria identifiable with available probes (for reference organisms in culture collections) are a minority, the majority being not antigenically identical to the reference organisms, many numerically significant bacteria in granular sludges remain to be isolated and specifically identified.

7.1 INTRODUCTION

In the purification by upflow anaerobic digesters of organic matter-rich industrial waste waters the application of a specific set of selection criteria (7) results in the formation of "granules": spherical, or near spherical, dense bacterial consortia which are retained in the digesters by sedimentation, ensuring continuous nutrient removal.

The microbial composition of these granules has been the subject of numerous studies with a variety of methods. Some studies involved physical disruption of the granules followed by incubation and quantification using standard plate or MPN counts (11, 16, 37). Due to the stability of the granule structure (15, 20) and the regular occurrence of bacterial clumps or filaments (36), these techniques provide only an estimation of the number of viable survivors, and clumps of survivors, that can grow under the new culture conditions. Metabolic activity studies (10) may be used to estimate potential biomass activity but substrate

utilization and metabolite (e.g., methane) production rates, are only useful in determining prevailing biomass activity.

For direct light microscopic quantification, the coenzyme F420 levels may be related to the methanogen biomass content (18), but its rapid oxidation precludes it from being used after exposure to air. The smallness of bacteria within relatively large granules and their population density complicate light microscopic quantification of individual species. The latter quantification can only be achieved following granule disruption and subsequent dilution of the suspension prior to counting with a haemocytometer (23, 35). With this method, damage to the cells may be visually assessed. Quantification of methanogenic strains using antibody probes on disrupted granules has increased in popularity (1, 12, 16, 23, 31, 35) but is limited to the strains for which probes are available. An added advantage of the use of antibody probes is that they may also be used to determine the location of positively reacting microbes and colonies within histological sections of granules (28).

Electron microscopy has long been used for qualitative examination of anaerobic digester granules (11, 12, 15, 20, 29, 33, 34, 37), whereas cell quantification from transmission electron micrographs has only recently been applied (22, 37). This procedure allows only distinctive morphotypes within mixed populations to be

quantified, and if bacterial counting is applied alone, errors due to different cell sizes may be incurred; this problem may be overcome by incorporating image analysis (22). As it is impossible, using this procedure, to count all the cells within one or more granules, montages of spherical granule cross-sectional radii may be used for quantification, providing the data are submitted to statistical evaluation. This method may also be applied to determine the microbial distribution within granules (22).

Thus, no single technique is sufficient for quantifying all the bacteria present within complex consortia. In this study, a combination of transmission electron microscopy and fluorescence microscopy with antibody probes was used for the identification, and quantitative determination and mapping of methanogenic and non-methanogenic strains within anaerobic digester granules treating brewery waste water.

7.2 MATERIALS AND METHODS

7.2.1 Anaerobic digester granules

These were obtained from a full-scale mesophilic upflow anaerobic sludge blanket (UASB) digester treating a brewery effluent at Prospecton, Natal, South Africa. Details on the digester, operating conditions and effluent characteristics

have been described previously (19). Fresh spherical, or nearly spherical, granules were placed in 3% (v/v) glutaraldehyde in cacodylate buffer for electron microscopy; or Telly' solution for histochemistry and immunohistochemistry (28); or formalin for immunological testing (23).

7.2.2 Granule sizing

Four entire granules, sectioned for TEM, were individually sized from cross-sectional diameters (22, and see Chapter 6). Thirty-four granules used for immunological bacterial quantification were photographed on a graph paper background with a polaroid camera, prior to disruption, for image analyzer sizing with a Kontron Vidas 2.0 system as previously described (13). From areas so determined, granule volumes were extrapolated using the formulae for determining the area within a circle and the volume of a sphere. The procedures are outlined in Figure 1 as a flow-chart. After disruption by 30 depressions of the plunger of a Teflon piston tissue grinder (Tri-R Instruments, Rockville Center, N.Y.) and centrifugation for 10 minutes at maximum r.p.m. (12,535 RCF) using 1.5 ml polypropylene centrifuge tubes in a Beckman microcentrifuge, the granule biomass volume was again determined by subtraction of the supernatant volume withdrawn.

7.2.3 Transmission electron microscopy

Four typical granules were ethanol dehydrated and embedded in Spurr's resin (32) for sectioning with a diamond knife after fixation with glutaraldehyde and osmium tetroxide in cacodylate buffer as previously described (21, and see Chapter 4). Only sections through the granule centre were picked up on 2 x 1 mm formvar-coated slot grids (22), and stained with uranyl acetate and lead citrate for viewing with a Jeol 100CX transmission electron microscope at 80 kv. In contrast with the work reported in Chapter 6 (22), transmission electron micrographs (TEMs; 1000× magnification) were taken sequentially from the periphery to the centre of each granule as indicated in Figure 7.1. This was necessary because the granules used in this study were older and hence larger than those used in our previous study (1.5-2.0 mm vs 0.35 mm diameter). The larger granules had larger "hollow" cores which made estimation of the granule centre difficult. The TEMs were assembled into montages for bacterial counting (Figure 7.1).

7.2.4 Panel of reference microorganisms

Reference methanogens of known physiological and taxonomic (4, 5), and antigenic characteristics (24, 26), together with syntrophs and acetogens (similarly characterized) were used in the slide immunoenzymatic assay constellation as positive, negative and morphotype controls as described (25, 27).

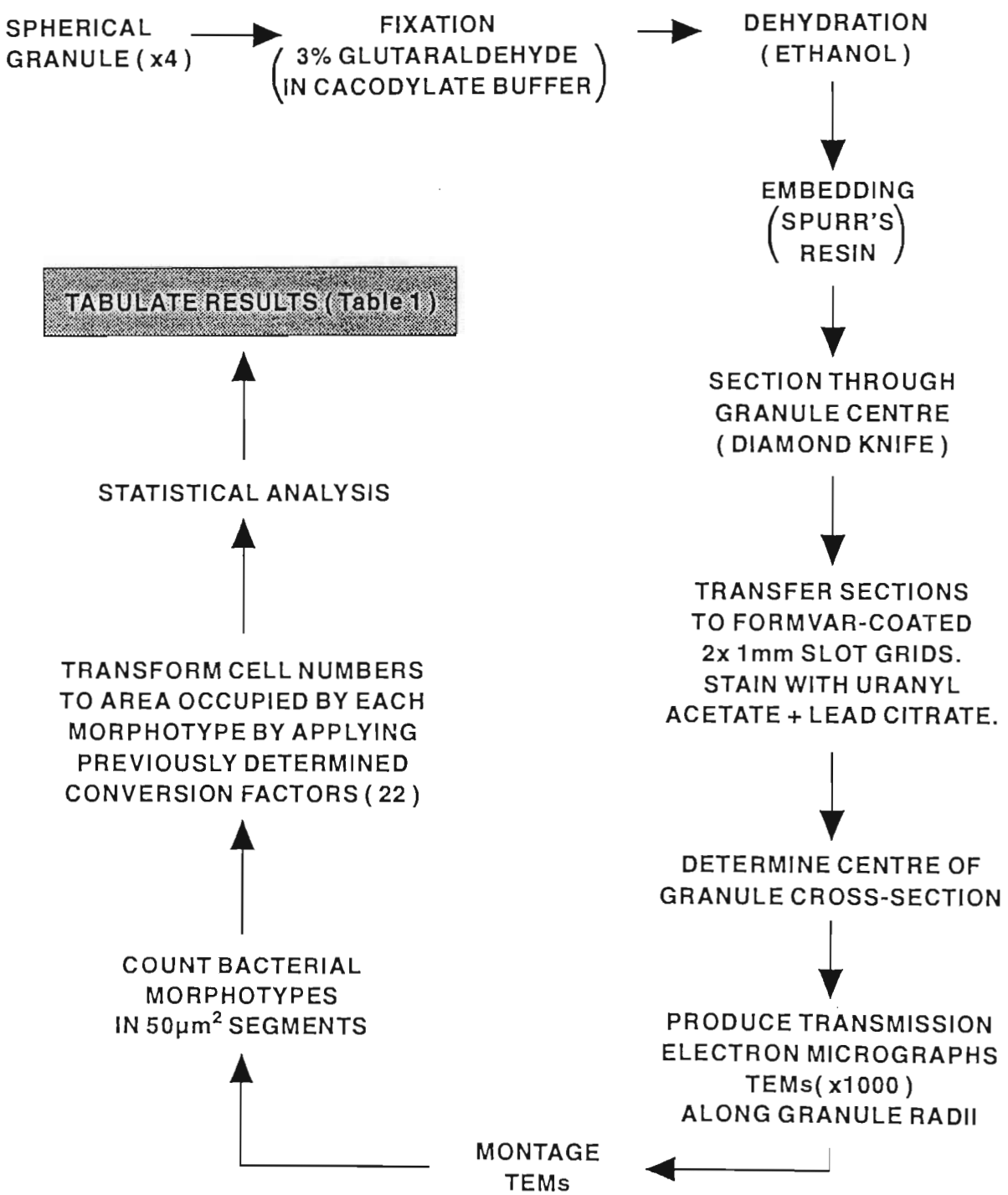


FIGURE 7.1 Flow chart of procedures used for quantitative analysis and mapping of bacterial morphotypes in spherical granules.

The reference organisms for our study were mesophilic and morphologically similar to bacteria observed in the brewery granules. These organisms are listed below in the order prescribed by the antigenic fingerprinting method (the number preceding each bacterium defines its position in the fingerprint (24)): 1, *Methanobrevibacter smithii* PS; 2, *Methanobacterium formicicum* MF; 4, *Methanobacterium bryantii* MoH; 5, *Methanobacterium bryantii* MoHG; 6, *Methanosarcina barkeri* R1M3; 7, *Methanospirillum hungatei* JF1; 8, *Methanobrevibacter ruminantium* M1; 9, *Methanobrevibacter arboriphilus* DH1; 10, *Methanobrevibacter smithii* ALI; 14, *Methanococcus voltae* PS; 18, *Methanosarcina mazei* S6; 19, *Methanosarcina barkeri* W; 21, *Methanobrevibacter arboriphilus* AZ; 22, *Methanobrevibacter arboriphilus* DC; 23, *Methanomicrobium mobile* BP; 30, *Methanothrix soehngenii* OPFIKON; and 33, *Methanocorpusulum parvum* XII. The non-methanogens used as reference were the syntrophs: 1, *Syntrophomonas wolfei;* 2, *Syntrophospora bryantii;* 3, *Syntrophococcus sucromutans;* and 4, *Desulfobulbus propionicus;* and the acetogens: 1, *Clostridium populeti;* 3, *Acetogenium kivui;* 5, *Clostridium formicoaceticum;* and 6, *Acetobacterium woodii.*

7.2.5 Antibody probes

Calibrated antibody probes were derived from antisera against the reference bacterial strains listed above and were used for antigenic fingerprinting (24, 26).

7.2.6 Histochemistry and immunohistochemistry

Granule fixation, dehydration and paraffin embedding using an automated Ultratechnicon system was carried out as by Macario *et al.* (28), as was the sectioning with an 820 Spencer microtome (American Optical Instrument Company, Buffalo, N.Y.). Thin sections (5μm) collected on 14 mm circles of slide-immunoenzymatic-assay (SIA) slides (8) were cleared of paraffin and rehydrated (9) prior to Gram or indirect immunofluorescence staining as described (28, 35). A Zeiss Axiophot (Carl Zeiss, Germany) was used for microscopic observations and photography.

7.2.7 Bacterial enumeration and identification

Four classes of bacteria were counted in 50 $\times$ 50 μm^2 of granule cross-sectional areas using the montaged-electron micrographs described above (Figure 7.1). Classes A, B and C consisted of *Desulfobulbus-*, *Methanobacterium-* ,and *Methanothrix*-like morphotypes, respectively; while class D included "all other" bacterial morphotypes. Counting, which progressed from the granule periphery inwards along marked radii, was performed using a Suntex 560 colony counter. Data for all four granules were analyzed statistically using the F-test to determine whether the granules differed significantly with respect to morphotype numbers at corresponding locations within the granules. To correct for errors resulting from

size differences between the cell morphotypes, the number of *Methanothrix*-like cells was multiplied by 0.421 and the *Methanobacterium*-like cells by 0.178; values which represent average cross-sectional areas of these bacteria as previously determined by image analysis (22, Chapter 6). Likewise, the *Desulfobulbus*-like cell numbers were multiplied by 0.458; a figure similarly obtained by image analysis (unpublished data). The conversion factor for total cell number, viz., 0.237 was determined previously (22, Chapter 6). Included in "all other" morphotypes (type D cells) were those bacteria that could not be distinguished by image analysis as type A (*Desulfobulbus*-like), B (*Methanobacterium*-like), or C (*Methanothrix*-like) cells. Morphological diversity among this group prohibited derivation of a conversion factor for determining average cell area. However, the overall conversion factor of 0.273 for all categories of cell types (22, Chapter 6) could be used to derive a conversion factor (X) for type D cells by extrapolation through weighting the conversion factors of types A,B and C cells by the proportion of the total number of bacteria in these classes (see Table 7.2 and below):

$$\frac{(26.2\times0.421)+(37.0\times0.178)+(9.6\times0.458)+(27.2\times X)}{} = 0.237\times100$$

$$X = \frac{23.7000-(11.0302+6.5860+4.3968)}{27.2}$$

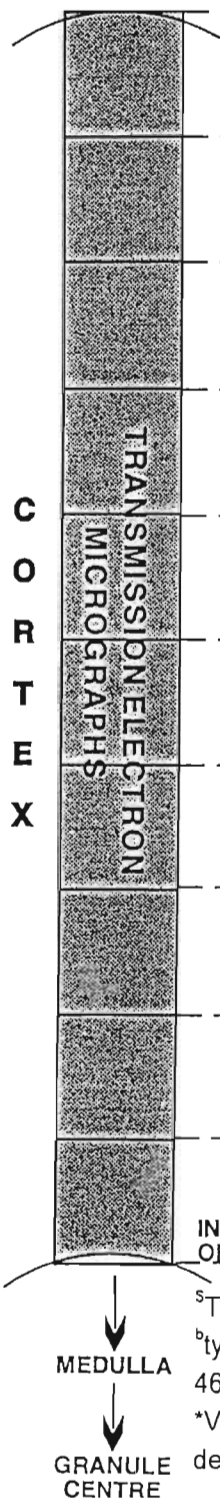
$$=0.062$$

These data were expressed as the relative total area occupied by each cell type (Table 7.2) and their distribution within the granules (Table 7.1).

Cell identification and enumeration based on immunological techniques involving the SIA constellation method (25) was also undertaken. The numbers of cells within positively reacting filaments were estimated visually.

Total cell numbers in resin-embedded granules were extrapolated from the cell counts from TEM montages, while those in granules disrupted for immunological tests were counted with a haemocytometer (improved Neubauer Haemocytometer; Max levy, Philadelphia, P.) using standard procedures (23, 30). Bacterial numbers within filaments had to be estimated as in immunological studies, but since the initial granule disruption procedure was inadequate to separate individual cells for quantification, a 1 ml 26 3/8 tuberculin syringe and needle were employed to further disrupt the aggregates for antibody probe and haemocytometer quantification.

TABLE 7.1 Schematic representation of bacterial morphotype quantification from transmission electron micrograph montages along a granule radius (cell numbers and areas quoted are averaged between four granules)



DISTANCE FROM GRANULE PERIPHERY (µm)	Morphotype distribution in 50µm² segments expressed as: CELL No.	CELL AREA[$]
	A = 102	47[a]
	B = 458	82[b]
	C = 43	18[c]
50	D = 711	44[d]
	A = 141	65[a]
	B = 483	86[b]
	C = 247	104[c]
100	D = 682	42[d]
	A = 157	72[a]
	B = 685	122[b]
	C = 406	171[c]
150	D = 486	30[d]
	A = 121	55[a]
	B = 551	98[b]
	C = 306	129[c]
200	D = 359	22[d]
	A = 173	79[a]
	B = 399	71[b]
	C = 381	160[c]
250	D = 313	19[d]
	A = 142	65[a]
	B = 564	100[b]
	C = 331	139[c]
300	D = 287	18[d]
	A = 127	58[a]
	B = 672	120[b]
	C = 487	205[c]
350	D = 314	20[d]
	A = 184	84[a]
	B = 417	74[b]
	C = 630	265[c]
400	D = 217	14[d]
	A = 81	37[a]
	B = 324	58[b]
	C = 677	285[c]
450	D = 266	17[d]
	A = 102	47[a]
	B = 315	56[b]
	C = 214	90[c]
500	D = 189	12[d]

[$]Total area occupied by: [a]type A (*Desulfobulbus*-like cells = 19.0% [b]type B (*Methanothrix*-like) cells = 27.1%; [c]type C (*Methanobacterium*-like) cells = 46.3%; and [d]type D ("other morphotypes") cells = 7.6%*.

*Value calculated using a conversion factor of 0.062 (see Materials and Methods for details).

TABLE 7.2 Percentage of total cell number and of total cell-occupied area represented by each morphotype category

Morphotype Category	Cell no.	% of total cell no.	Conversion factor[@]	% of total cell-occupied area
Total cell number	57990	-	0.237	100
Type A[•]	15199	26.2	0.421	46.6
Type B[•]	21470	37.0	0.178	27.8
Type C[•]	5547	9.6	0.458	18.5
Type D[•]	15774	27.2	0.062	7.1

[•] See Table 7.1 for footnote identity

[@] See text for details

7.3 RESULTS

7.3.1 Granule size

The four granules prepared for TEM varied in diameter from 1.5 to 2.0 mm with an average of 1.8 mm which is translated to a volume of 3.05 mm^3, assuming that the granules were perfectly spherical. TEMs revealed that there were very few cells in the core region of each granule, thus confirming previous work (33).

Irrespective of size, the granules appeared hollow with nearly all of the cells occurring in an outer shell 0.45 to 0.5 mm thick.

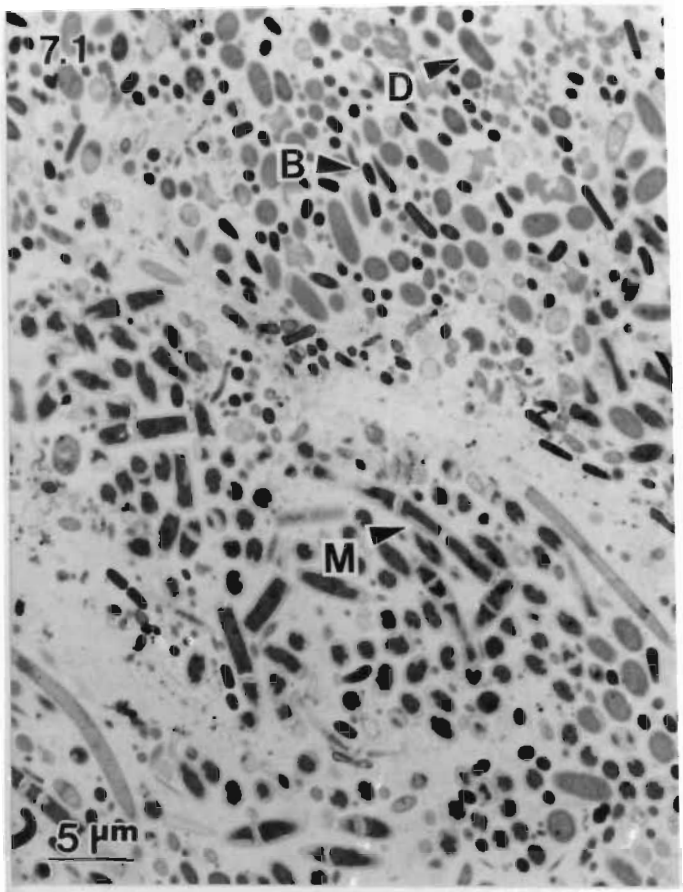
The 34 non-dehydrated granules used for immunological cell quantification had a combined volume of 202.6 mm^3 with an average volume of 6 mm^3 as determined by image analysis prior to disruption. This volume decreased to 90 mm^3 after homogenization.

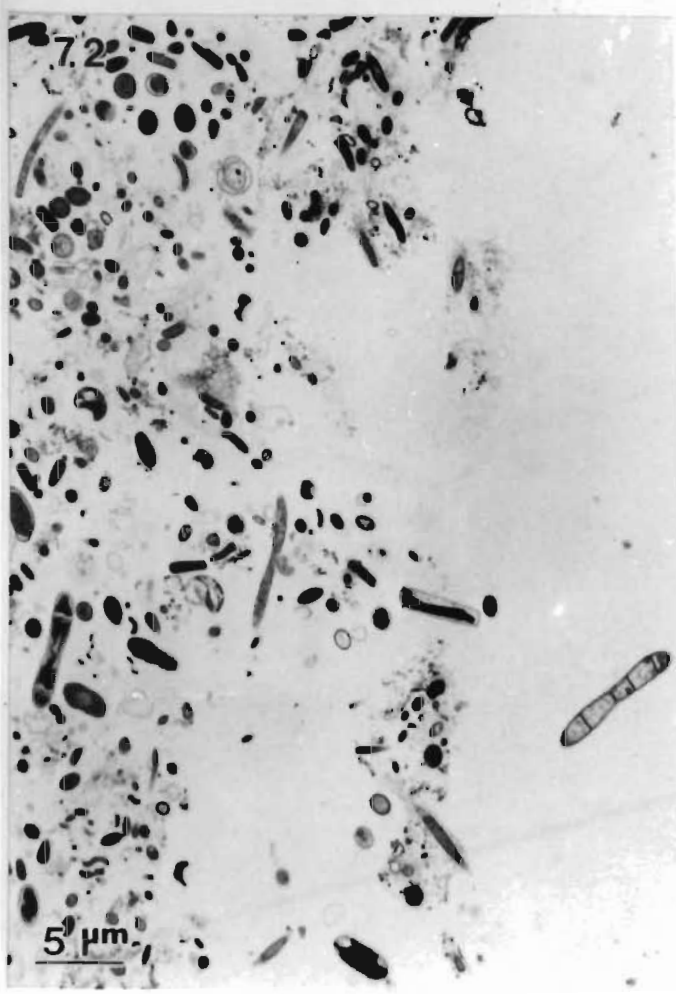
7.3.2 Transmission electron microscopy

Cell packing was consistently dense throughout the cortex of the granules (Plate 7.1) except at the periphery (Plate 7.2) and adjacent to the medulla where cells were less numerous. The three most abundant bacterial morphotypes, i.e., *Methanothrix*-, *Methanobacterium*- and *Desulfobulbus*-like cells are indicated in Plate 7.1.

PLATE 7.1 Transmission electron micrograph of a central region of a granule cortex. M = *Methanothrix*-like, B = *Methanobacterium*-like, and D = *Desulfobulbus*-like, cells

PLATE 7.2 TEM of a peripheral region of a granule cortex. Note smaller numbers of cells compared to the inner cortical region shown in Plate 7.1 above


7.1
D
B
M
5 µm


7.2
5 µm

7.3.3 Population localization using TEMs

A total of 57,990 cells were counted along the four montages and categorized according to morphology (*Desulfobulbus*-, *Methanobacterium*-, *Methanothrix*-like cells, and "all other" bacteria), and location, i.e., depth below the granule surface. Analysis of variance showed that the only significant differences in cell numbers between the four granules occurred within the group referred to as "all other" cells. There were significant differences between *Methanothrix*- and *Methanobacterium*-like cell numbers at different locations within the granules while *Desulfobulbus*-like bacteria were evenly distributed throughout the cortex. Table 7.1 and Figure 7.2 indicate the average cell number distribution throughout the cortices of the four granules examined. Figure 7.3 is a plot of mean total area occupied by bacteria belonging to each of the four morphological categories in 2,500 μm^2 areas at increasing depths beneath the granules' surfaces. There were no significant differences between granules with respect to total cell numbers at different depths, but the cell density was highest at 150 μm from the periphery while least cells occurred in the 450-500 μm strata bordering on the "hollow" medullae (see Table 7.1).

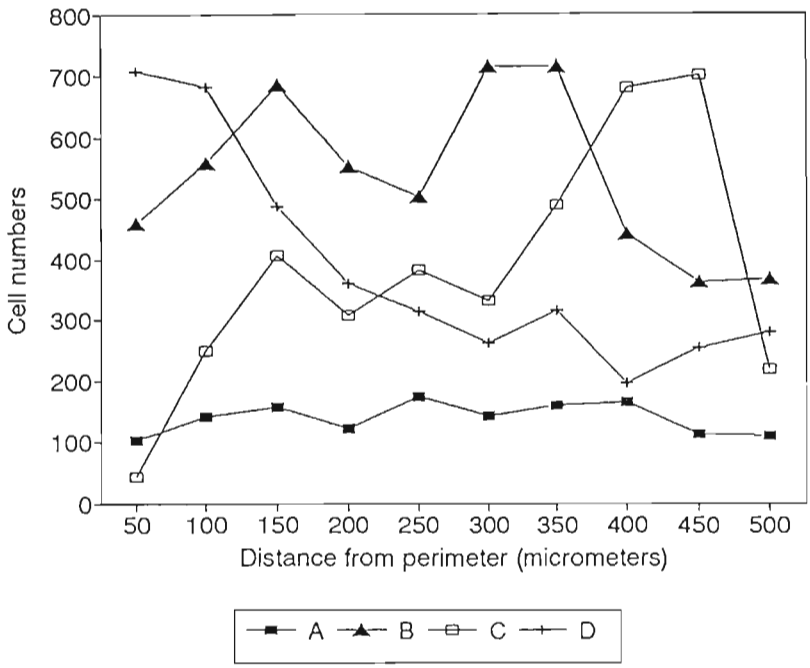


FIGURE 7.2 The distribution of cell numbers along a granule radius. A = *Desulfobulbus*-like, B = *Methanobacterium*-like, and C = *Methanothrix*-like cells. D = "all other" cell morphotypes.

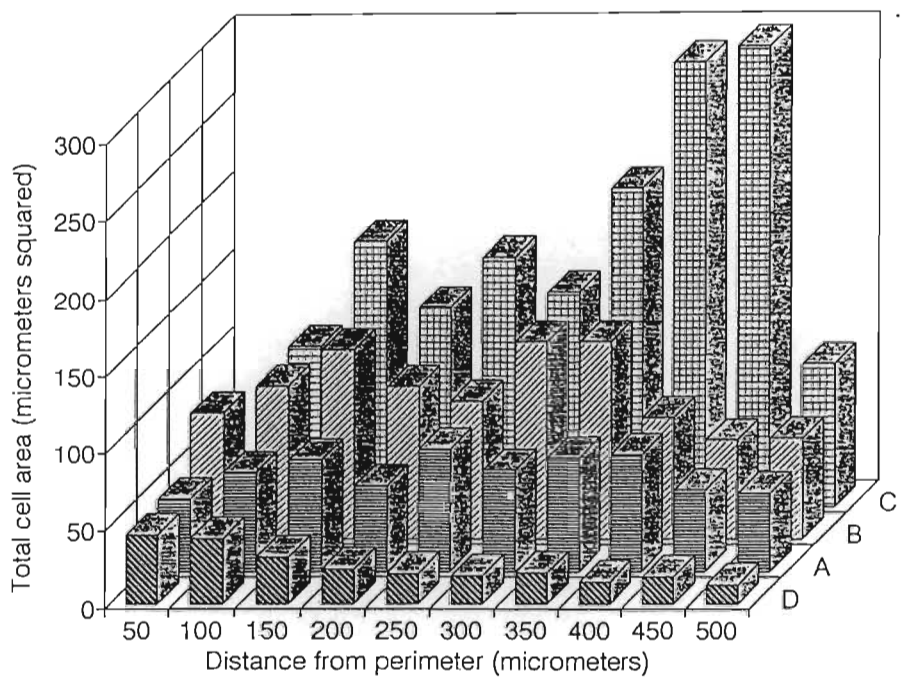


FIGURE 7.3 The distribution of type A, B, C and D cells within a granule cortex expressed as total cell areas (derived from Figure 7.2).

7.3.4 Bacterial quantification from TEMs

Of the total (for all four granules) cross sectional area of 10 mm^2 only 1.25% i.e., 0.1 mm^2 (total montaged area) out of 8 mm^2 cortex was captured on TEMs for analysis as the medullae were virtually devoid of cells. Combined results for the four montages revealed the presence of 57,990 cells occupying an area of 13,744 μm^2. Of these, there were 15,199 *Methanothrix*-like cells with a total area of 6,399 μm^2 comprising 26.2% of the population or 46.6% of the total cell area, and thus biomass (Figure 7.4) (based upon the assumptions made in Chapter 6). The 21,470 (37% of the population) smaller *Methanobacterium*-like cells occupied 3,822 μm^2 or 27.8% of the total cell area; the 5,547 (9.6% of the population) *Desulfobulbus*-like cells occupied 2,541 μm^2, constituting 18.5% of the total area containing bacterial cells; while 982 μm^2 (7.1% of the total area) was occupied by "all other" cell morphotypes. Thus, all cell types occupied 55% of the total cortical area examined per granule.

As cortex (consistently approximately 500 μm thick, irrespective of granule size) comprised 80% of the four granules' cross sectional area, of which cells occupied 55%, it may be calculated that 44% (v/v) of our granular sludge is cellular biomass, assuming that our specimens are representative of all granules in the digester and that bacterial cells are similarly distributed along all radii.

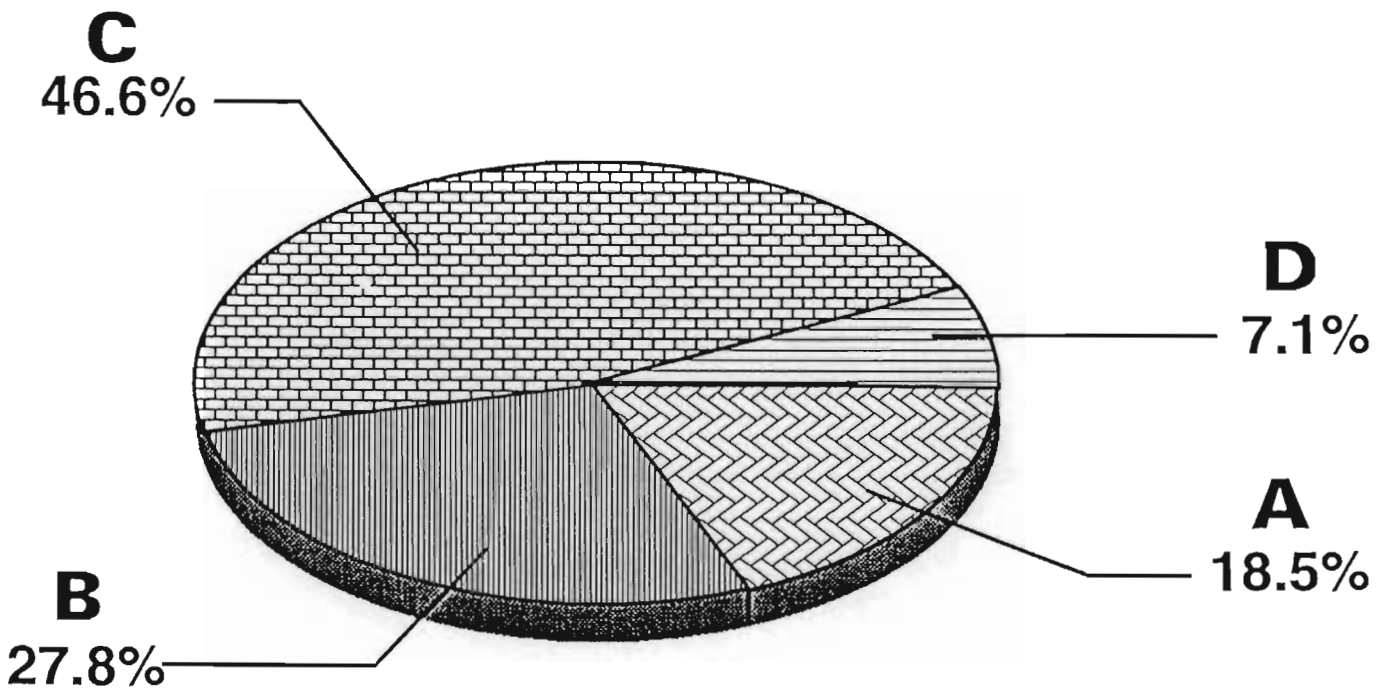


FIGURE 7.4 Respective areas occupied by *Desulfobulbus*-like (A), *Methanobacterium*-like (B) and *Methanothrix*-like (C) cells, and "all other" morphotypes (D).

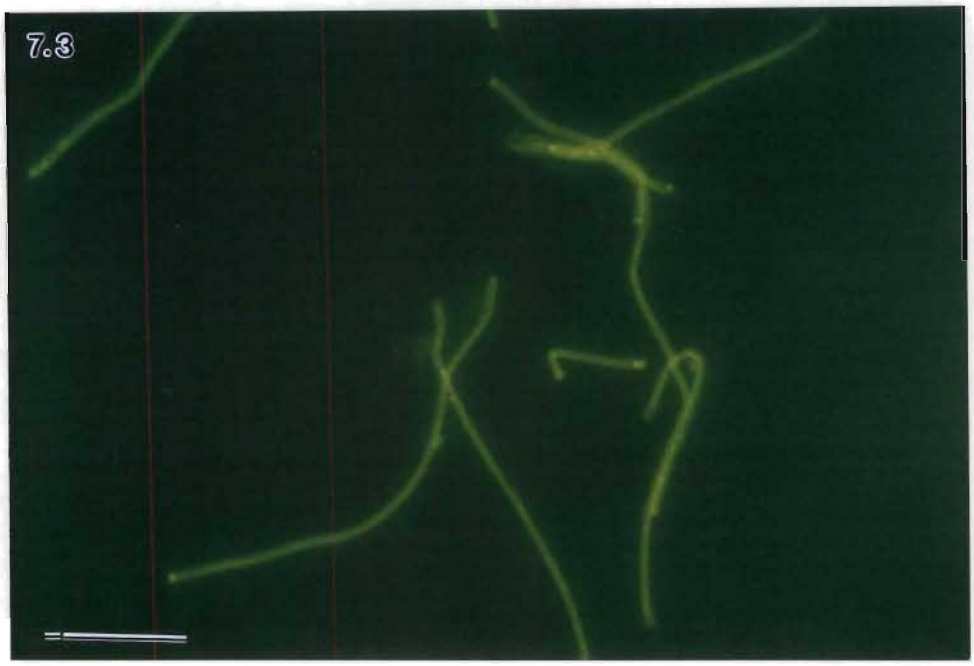
It can be extrapolated from the above data that *Methanothrix*-like, *Methanobacterium*-like and *Desulfobulbus*-like cells occupy 0.205, 0.122 and 0.081 ml per ml of granule biomass, respectively. *Methanothrix* varies in size from 0.7-1.2 $\times$ 2-6 μm (6) and is typically cylindrical. The volume of *Methanothrix* is thus between 0.77 and 6.8 μm^3 indicating that it occurs at a concentration of 3.01×10^7-2.66×10^8 per ml of whole granule.

7.3.5 Immunological bacterial enumeration

The 34 granules used for this study contained a total of 1.4×10^6 cells in the 90 μl of disrupted biomass, i.e., 1.56×10^7 cells per ml. This may be translated to 6.95×10^6 cells per ml in whole granules. Of these, 39.1% are accounted for (Table 7.3) while all other strains tested for were not detected. Plate 7.3 is a fluorescence light micrograph of *Methanothrix soehngenii* OPFIKON in pure culture stained with its specific antibody probe, while Plate 7.4 is of filaments within disrupted granules cross-reacting with a *Syntrophococcus sucromutans* probe. In Plate 7.5, a probe prepared against *Methanobrevibacter arboriphilus* DH1 is seen to react with a pure culture of this organism while in Plate 7.6 rods within disrupted granules are antigenically related and morphologically similar (but not identical) to this bacterium.

PLATE 7.3 Fluorescent light micrograph of a *Methanothrix soehngenii* OPFIKON pure culture stained with a specific antibody probe prepared against this organism. Bar = 20 μm

PLATE 7.4 Filaments within disrupted granules cross-reacting with an antibody probe prepared against *Syntrophococcus sucromutans*. Bar = 20 μm


7.3

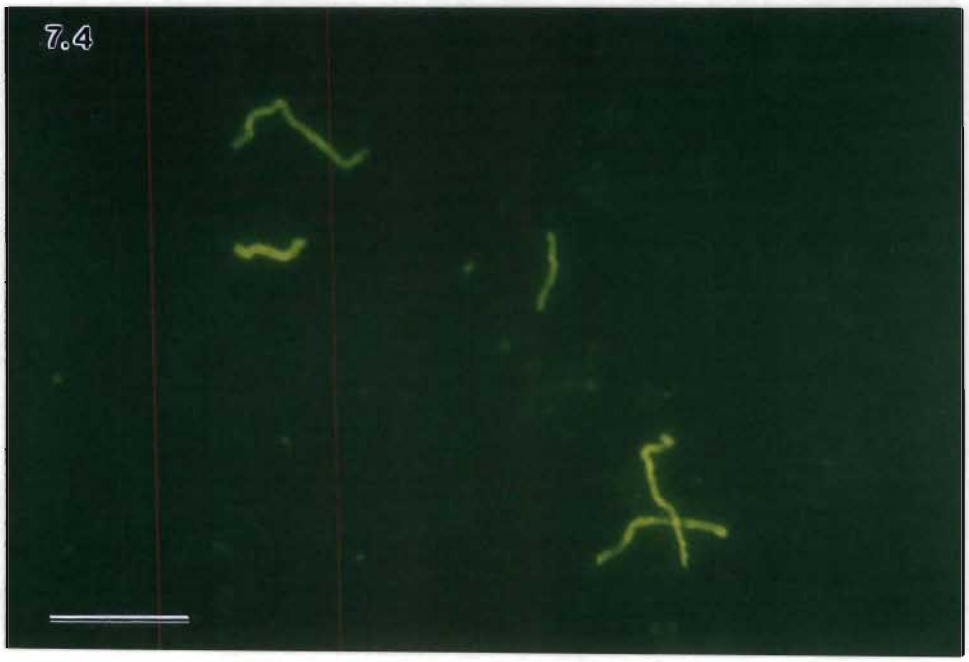
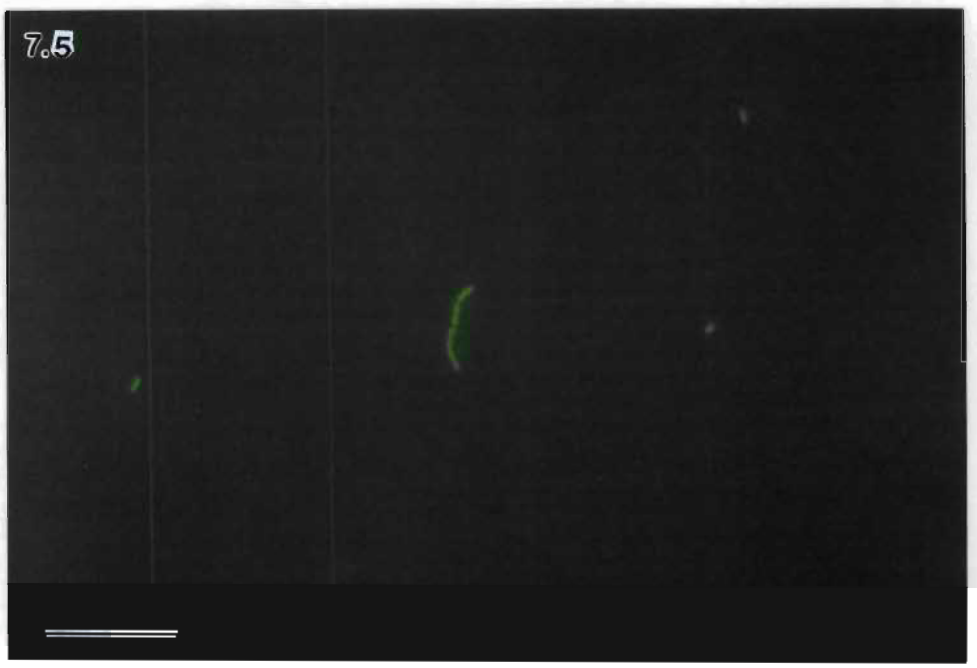

7.4

PLATE 7.5 Fluorescent light micrograph of antibody probe for *Methanobrevibacter arboriphilus* DH1 reacted against a pure culture of this bacterium (positive control) and;

PLATE 7.6 reacted with cells in disrupted granules. Bars = 20 μm


7.5

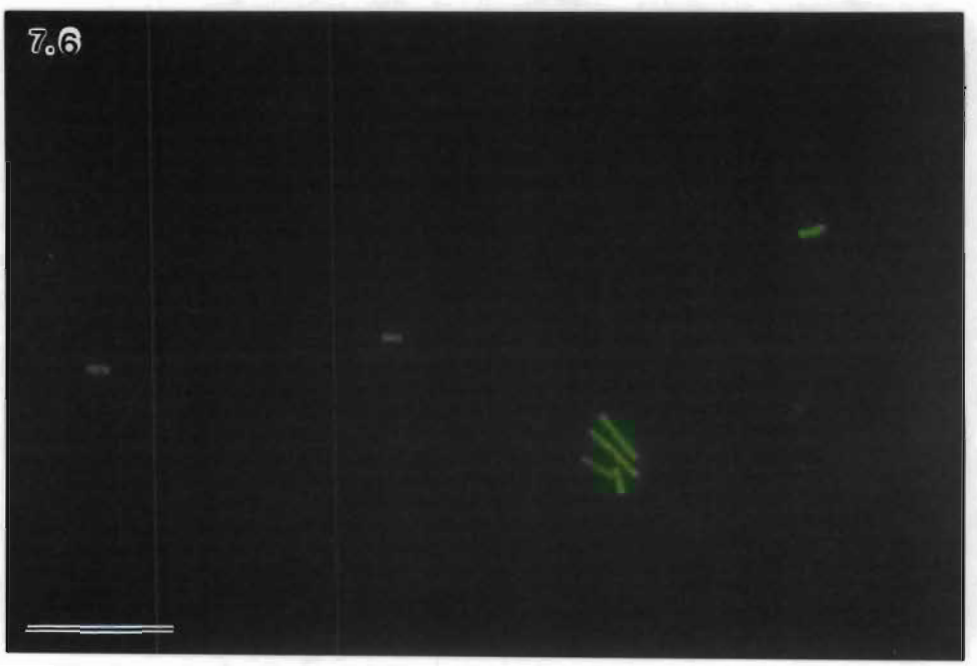

7.6

TABLE 7.3 Bacterial numbers (in order of predominance) within brewery granules as quantified using antibody probes

Bacterial strain	Cell number per ml	% of total population
Methanobrevibacter arboriphilus DH1	5.0×10^6	32.05
Filament cells cross-reacting with *Syntrophococcus sucromutans* probe	4.31×10^5	2.76
Methanobacterium bryantii MoHG	2.51×10^5	1.61
Methanobrevibacter arboriphilus AZ	1.89×10^5	1.21
Methanobrevibacter smithii ALI	9.4×10^4	0.6
Syntrophococcus sucromutans	5.25×10^4	0.34
Methanothrix soehngenii OPFIKON	3.37×10^4	0.22
Methanospirillum hungatei JFI	1.58×10^4	0.1
Filament cells cross-reacting with *Methanosarcina barkeri* W probe	1.28×10^4	0.08
Desulfobulbus propionicus	9.33×10^3	0.06
Syntrophomonas wolfei	5.86×10^3	0.04
Methanobrevibacter ruminantium M1	4.73×10^3	0.03
Methanomicrobium mobile BP	3.8×10^3	0.024
Clostridium populeti	3.47×10^3	0.05

Populations of less than 2×10^3 cells per ml: *Methanocorpusulum parvum* XII; *Acetobacterium woodii*.

7.3.6 Gram stain and histological observations

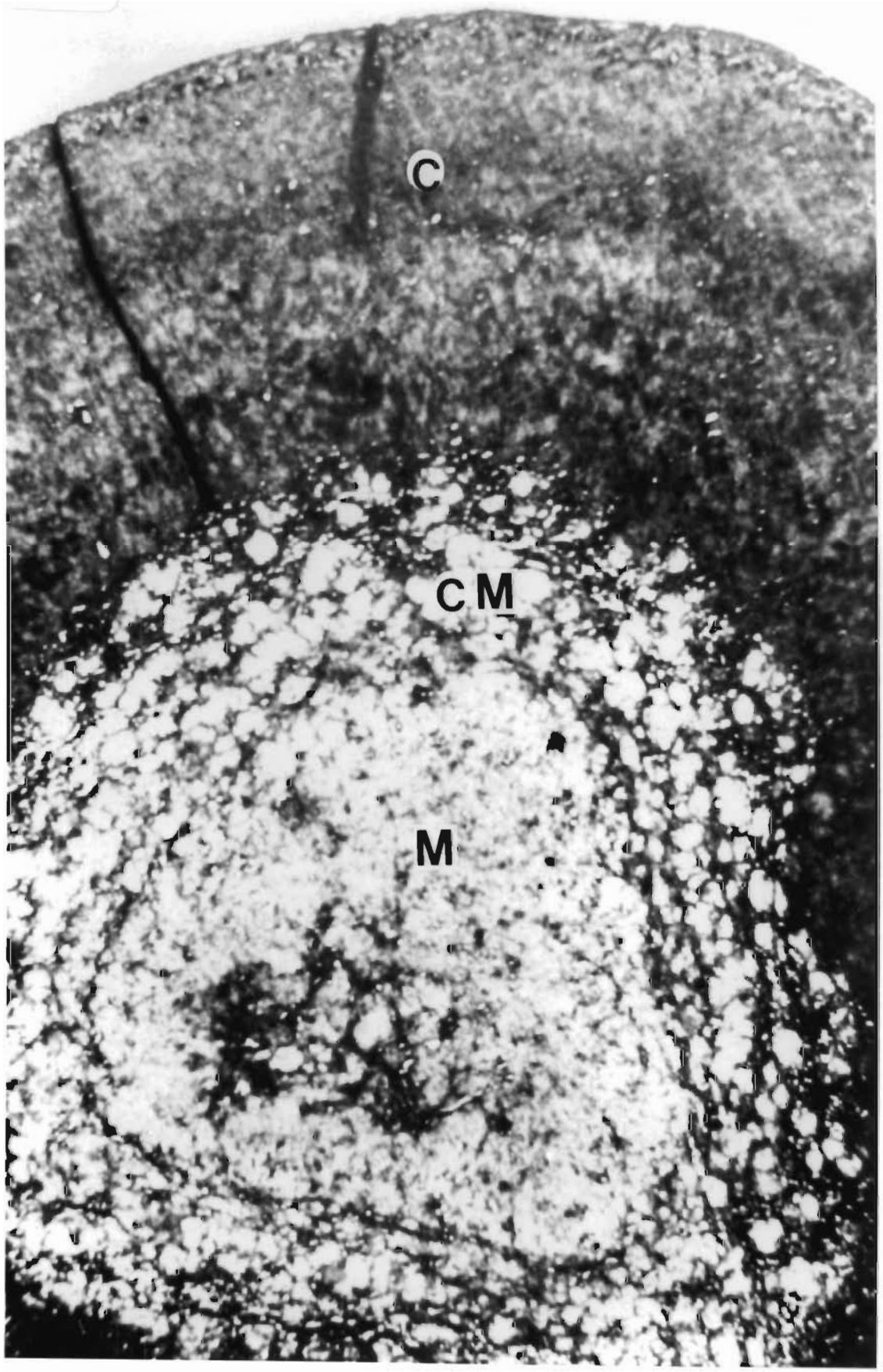
Most cells stained Gram negative as did the histological sections (Plate 7.7) which clearly revealed the cortex and medulla, as did interference phase microscopy (Plates 7.8 and 7.9) (by contrast the medulla was washed out during TEM preparation giving granules a hollow appearance). Small, darkly-staining colonies were observed in the peripheral regions of the cortex which were enveloped in a loose web of glycocalyx. Sections stained with antibody probes revealed that bacteria antigenically close to *Methanobrevibacter arboriphilus* DH1 were dispersed throughout the granules (Plate 7.10) while bacteria antigenically related to *Methanobacterium bryantii* MoHG often occurred in discreet colonies (Plate 7.11).

7.4 DISCUSSION

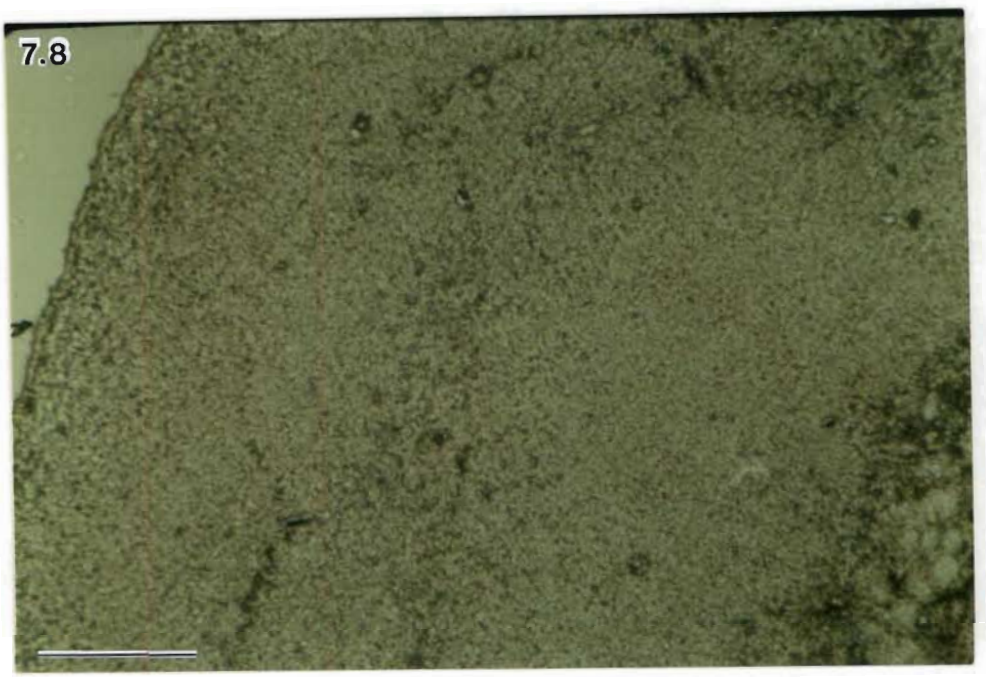
This study represents the only *in situ* quantitative localization of different bacterial types within anaerobic digester granules wherein errors due to differences in cell sizes are accommodated and where results are compared to those of immunological quantification on homogenized granules. There were discrepancies between the results obtained with the two techniques. However, both techniques have merits and the results can be reliably compared to observations by other authors.

PLATE 7.7 Thin, Gram-stained histological section of a granule. The cortex (C) and medulla (M) are visible, as is an intercortico-medullary region (CM)

7.7



PLATES 7.8 and 7.9 Interference phase light micrographs of a thin histological section through a granule. Bars = 100 μm


7.8

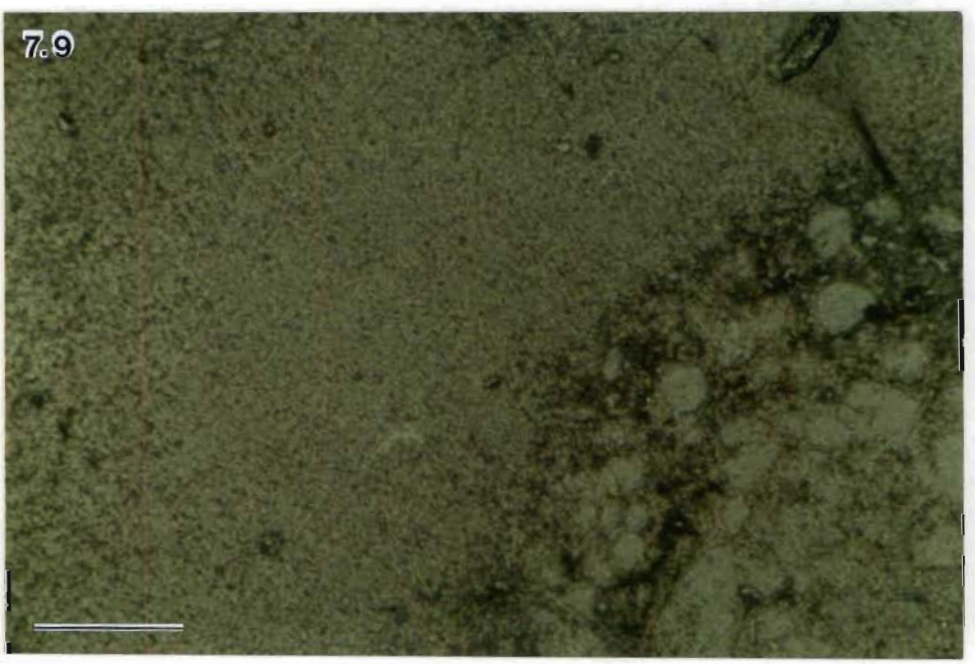
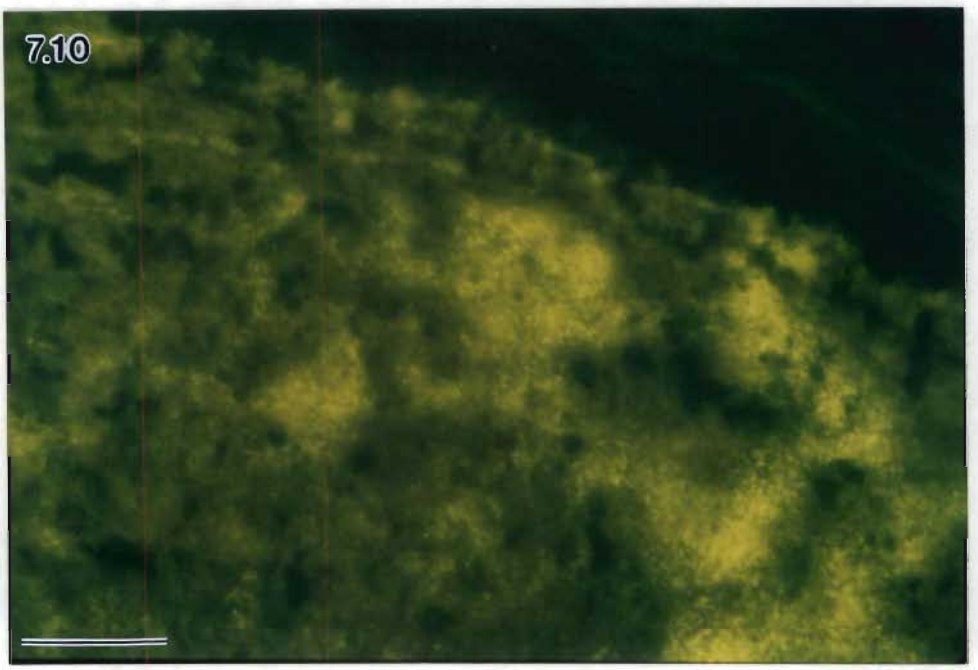
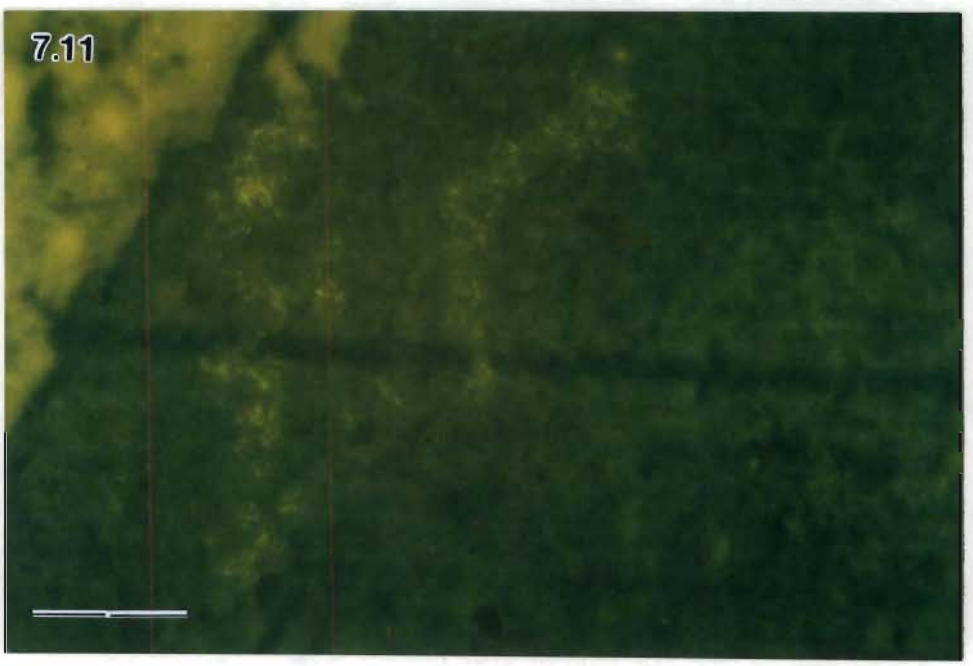

7.9

PLATE 7.10 A thin histological section of a granule reacted with an antibody probe for *Methanobrevibacter arboriphilus* DH1. Bar = 20 μm

PLATE 7.11 A thin histological granule section reacted with an antibody probe for *Methanobacterium bryantii* MoHG. Bar = 20 μm


7.10


7.11

The granules treating brewery waste water showed the stratification described by other authors for granules grown on hexose feed or brewery effluent (2, 3, 14, 16, 17, 28, 29, 35). Microcolonies of *Methanothrix*-like cells and mixed colonies of juxtaposed syntrophic bacterial species resembling *Methanobacterium*- and *Desulfobulbus*-like cells (14, 15, 29, 37) were also observed on the TEMs. Qualitative studies of the TEMs, however, revealed no organised bacterial layering within the cortex as described by others (14, 17, 29), while the medulla was virtually devoid of cells. A statistical analysis of bacterial numbers and area throughout the cortex of four granules did, however, confirm that stratification does occur within granules. *Methanothrix*- and *Methanobacterium*-like cells are concentrated in a band 100 to 150 μm from the granule surface. *Methanobacterium*-like cells form a second band at 250 to 350 μm while *Methanothrix*-like cells form a second band at 400 to 450 μm within the granule cortex (Figure 7.5). *Desulfobulbus*-like cells are evenly distributed throughout the cortical region, while cell types with morphologies different from those mentioned above, although also present throughout the cortex, were more prevalent at the granule periphery. Noteworthy is that, although the number of *Methanothrix*-like cells are lower than that of *Methanobacterium*-like cells, they comprise the highest granule area, and thus occupy the most volume (see Figures 7.2 & 7.3).

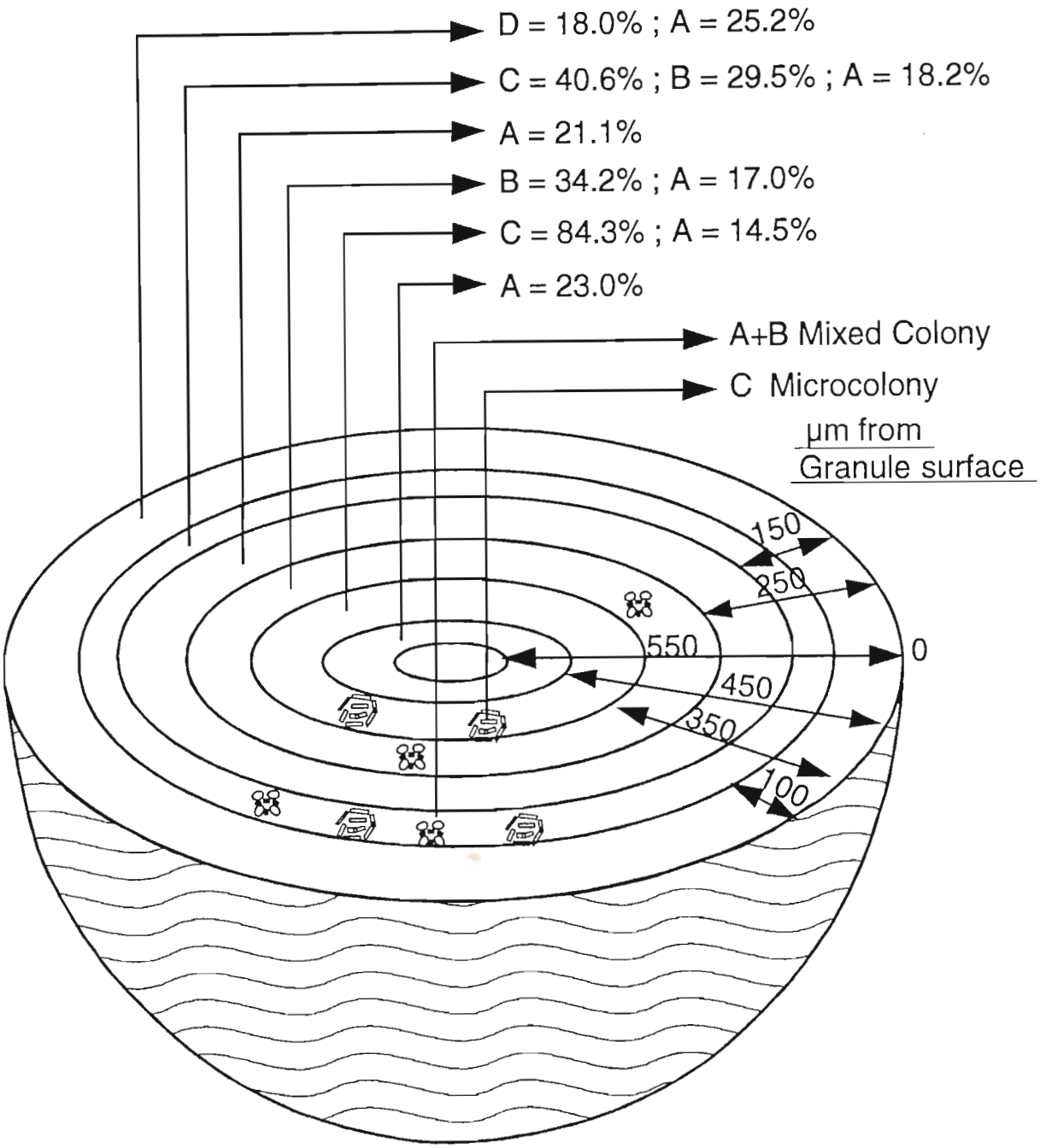


FIGURE 7.5 Schematic representation of the area distribution of *Desulfobulbus*-like (A), *Methanobacterium*-like (B), *Methanothrix*-like (C), and "all other" cells (D) throughout a granule

In their study on granules purifying brewery waste water Wu *et al.* (37), obtained bacterial counts of over 6,700 cells from TEMs and determined that there were 1.31×10^7 *Methanothrix*-like cells per ml. This value is approximately half of the minimum figure of 3.01×10^7 cells obtained in the present study, based on a combination of TEM area determinations and the volume of *Methanothrix* obtained from the literature (6). Approximately 0.11% of this population reacted positively with the antibody probe produced against *Methanothrix soehngenii* OPFIKON. There were also filaments which resembled *Methanothrix*, but did not react with the probe for OPFIKON; these may represent other *Methanothrix* immunotypes.

There is also discrepancy between the present haemocytometer quantification results and TEM quantification by Wu *et al.* (37). Their total granule cell density was 5.78×10^7 cells per ml, which is higher than the present figure for disrupted granules of 6.95×10^6 cells per ml. However, the present TEM studies revealed that *Methanothrix*-like cells, at a minimum of 3.01×10^7 cells per ml, represented 26.2% of the total population, therefore there must have been at least 1.15×10^8 cells per ml in the granules. All the above values, however, fall within the range 4.6×10^6 to 2.1×10^{12} (depending upon substrate), determined by Grotenhuis *et al.* (16) using the MPN method on disrupted granules. Discrepancies in the above results were probably due to: the granule disruption procedures used for

haemocytometer counts; the shrinkage of granules during ethanol dehydration for TEM (this was subsequently determined by image analysis to be up to 50%; unpublished data); the present quantification study from TEMs being more extensive than that of Wu *et al.* (37), with cell distribution throughout granule cortices being considered. As only 39% of the cells counted with the haemocytometer were accounted for in the immunological quantifications, a large proportion of the population did not react with the antibody probes used, probably including at least one strain of *Methanothrix*.

This study quantitatively confirms qualitative observations by other authors of stratification within anaerobic digester granules (3, 14, 29) (Table 7.1). However, the theory that granules fed a brewery waste water or hexose based medium have inner cores of *Methanothrix* (3, 14, 29) does not always hold true since our granule centres were virtually devoid of cells. A previous study conducted in this laboratory (33) which showed that a fluorescent band associated with methanogens migrated from the cortical region to the granule centre upon increase of the brewery waste water flow rate to the anaerobic digester, may explain this discrepancy. It is unlikely that an even nutrient gradient occurs between the granule periphery and its centre as similar cells often aggregate in microcolonies, more abundant in some strata and separated by other cell types. The cells are arranged in such a way that they can interact closely and the lack

of cells in the medulla indicates that conditions for growth are unfavourable at more than 500μm from the granule surface. In smaller granules (less than 1 mm in diameter) a honeycomb-matrix-like glycocalyx occurs in the medullary region (33) suggesting that nutrient diffusion was sufficient to allow microbial growth throughout the granules. Upon increase in granule size, this central population would starve and lyse, to form a medulla virtually devoid of cells. The high density of *Methanothrix*- and *Methanobacterium*-like cells observed at 250 to 450 μm below the granule surface in electron micrographs suggests that most methanogenesis possibly occurs here; while the even distribution of *Desulfobulbus*-like cells indicates that sulphate reduction might occur throughout the granule cortex. The concentration of "all other" morphotypes near the granule surface suggests that hydrolysis and acidogenesis is probably most active in this region. Large cell numbers in the cortex indicates that most metabolic activity occurs here while the medulla may act as a gas trap to promote flotation in larger granules thereby assisting in digester mixing.

The high proportion of *Methanobrevibacter arboriphilus* cells identified using antibody probes (34% of the haemocytometer cell count) and the low proportion of *Methanobacterium bryantii* (1.61%) cells may indicate that bacteria until now described as *Methanobacterium*-like, possibly did not belong in this genus.

From this study it is concluded that the various techniques commonly applied to quantify cells within anaerobic digester granules have to be used in a complementary fashion. For example, cell enumeration from TEM montages of spherical granule radii gives statistically accurate data but does not allow positive bacterial identification and is subjected to error resulting from ethanol dehydration shrinkage. Antibody probe quantification requires prior granule disruption which may result in cell damage. Future studies should be directed towards the preparation of gold-labelled antibodies specific for granule isolates to stain granule cross sections for *in situ* TEM quantification.

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CHAPTER 8

CONCLUSIONS

One of the major findings of this research was that the granules from the two digesters studied differed in several respects. Granular sludge produced in the upflow anaerobic digester purifying waste water from a maize processing factory differed in microbial composition from granules within another upflow anaerobic digester purifying brewery effluent. The former granules, although macroscopically similar to those from the brewery contained an extremely diverse population, some members of which were isolated and identified. Hydrolytic bacteria, acidogens and acetogens were detected and the methanogens present included *Methanosarcina* and possibly *Methanothrix* (*Methanosaeta*), both being numerically significant members of the population. The brewery granules, which were initially imported for digester inoculation and allowed to acclimatize for seven years prior to this study, contained a more uniform population consisting primarily of methanogens, a few of which were identified, as were some non-methanogens, using antibody probes.

Based on biomass extrapolations from cell cross-sectional area determinations, *Methanothrix* morphotypes were predominant in brewery granules, while cells resembling *Methanobacterium* and *Desulfobulbus* constituted most of the

remaining cell biomass. The low numbers of possibly hydrolytic or acidogenic bacteria (as determined using transmission electron microscopy (TEM) and antibody probes) in these granules indicate that either: the few cells of these types detected were exceptionally active; the electron microscopic preparative methods, in this instance, did not preserve enveloping glycocalyx, and therefore these cells were washed out of the granules; or, possibly, hydrolytic, acidogenic and acetogenic activity occurs in the pre-conditioning tank where biofilm formation was detected. No *Methanosarcina*-like tetrads were detected in these granules.

Differences between the two granule types may be attributed to differences in digester design, operational parameters and the nature of the effluent purified. In both cases the upflow digester configuration provided conditions which selected for well settling granular sludge with good retention properties.

For the maize waste water processing plant it became apparent that formation of a sludge with desirable properties was dependent on the chemical composition of the feed and the operating conditions in the digester, since after recovery of the gluten and application of different start-up procedures no granules were produced. Fresh granules were thus not available for quantification using antibody probes or TEM and image analysis. The latter analysis would, however,

be far more complicated on these granules due to the large diversity of morphotypes.

The brewery digester granules were coated with a layer of glycocalyx which, however, was washed out when standard electron microscopic preparative techniques were employed. This material could be conserved, however, by modifying the fixation and dehydration procedures used. These findings emphasise the importance of precisely describing the procedures and reagents used for electron microscopy of granules. As maize-processing digester granules were no longer available during this stage of the study, a comparison could unfortunately not be made.

TEM montages were extremely useful for the extrapolation of relative bacterial populations within spherical brewery digester granules after image analysis was used to quantify the areas of each morphotype. Unfortunately, these procedures preclude positive identification of the bacteria. The use of antibody probes overcame this problem but at the cost of not being able to determine the locality of identified cells within the granules. In addition, the significance of such results was dependent upon how many of the species for which antibody probes were available occurred within the granules under investigation; only one-third of the bacteria detected reacted with the specific antibody-probe library available. This

serological approach suggested that cells which resemble *Methanobacterium* on TEMs were probably *Methanobrevibacter arboriphilus*. Granule disruption was also too harsh to allow the structural integrity of all cells to be maintained. These problems could possibly be overcome by using pure cultures isolated from granule populations to produce specific antibody probes conjugated to gold or some other metal which would allow both positive bacterial identification and localization on TEM montages.

This investigation confirmed that different types of granules occur within different anaerobic upflow digesters depending upon operational conditions, waste water purified and/or digester design. The study is unique in that it incorporates the use of TEM, image analysis and antibody probes to obtain the most accurate quantitative assessment possible of microbial populations within such granules; an undertaking generally acknowledged as extremely difficult due to the close proximity of cells to each other, and the resistance of granules to all but the harshest disruption methods which destroy many of the cells. Application of the techniques described in this thesis are not limited to granules as mixed populations within any aggregate of known dimensions may be determined qualitatively and/or quantitatively as described.

This thesis has shown that granules treating different waste waters differ in both structure and microbial composition. Each granule type will thus have specific nutritional and physicochemical requirements which must be determined and fulfilled for successful operation. Specific additives, e.g. ferric chloride, may also enhance biomass retention by improving settleability, thereby allowing shorter retention times and thus increasing digester productivity.

APPENDIX I

CHARACTERISTICS USED IN *Lactobacillus* IDENTIFICATION

MORPHOLOGY OF ROD-SHAPED *Lactobacillus* spp.

R_1 - Long immotile rods occurring singly, in pairs or chains with a corkscrew morphology.

R_3 - Short immotile rods occurring singly or in pairs and sometimes, short chains.

R_4 - Short immotile rods occurring singly or sometimes in pairs. Few elongated rods.

R_7 - Short immotile rods forming long chains.

R_8 - Short to long immotile rods occurring singly or in pairs.

R_9 - Very long immotile rods usually forming long chains but sometimes occurring singly or in pairs.

M_2 - Short to very long immotile rods occurring singly or in pairs.

M_3 - Short to long immotile, usually single rods but sometimes occurring in pairs or short chains.

M_4 - Short to long single immotile rods, the latter being curved into corkscrew shapes.

M_7 - Short to long, immotile, bent rods.

M_8 - Short to very long immotile straight rods occurring singly or sometimes in pairs.

SUGAR UTILIZATION TEST RESULTS FOR THE *Lactobacillus* ISOLATES

(R_x AND M_x)

	R_1	R_3	R_4	R_7	R_8	R_9	M_2	M_3	M_4	M_7	M_8
Amygdalin	-	-	-	+	-	-	-	-	-	-	-
Arabinose	-	+	+	-	-	-	-	+	-	-	+
Cellobiose	-	-	-	+	-	-	-	-	-	-	-
Cellobiose to gas	-	-	-	+	-	-	-	-	-	-	-
Esculin	+	-	-	+	-	+	+	-	+	+	-
Fructose	+	-	-	+	ND	-	+	+	+	+	+
Galactose	-	-	-	+	-	+	-	+	-	-	+
Gluconate to Gas	-	-	+	+	-	-	-	-	-	-	-
Glucose + Citrate to Gas	-	+	+	-	+	-	-	+	-	-	+
Glucose-citrate to Gas	-	+	+	-	+	-	-	ND	-	-	+
Inositol	-	-	-	-	-	-	-	-	-	-	-
Inulin	-	-	-	+	-	-	-	-	-	-	-
Lactose	-	-	+	-	-	-	-	-	-	-	-
Malate to gas	-	-	+	+	-	-	-	-	-	-	-
Maltose	-	-	+	-	+	-	+	-	+	+	-
Mannitol	-	-	-	-	-	-	-	-	-	-	-
Mannose	+	-	-	+	-	+	+	-	+	+	-
Melezitose	-	+	-	+	+	-	-	+	-	-	+
Melibiose	-	+	+	-	+	-	-	+	-	-	+
Raffinose	-	-	+	-	+	-	-	-	-	-	+
Rhamnose	-	-	-	-	-	-	-	-	-	-	-
Ribose	-	-	+	+	-	-	-	-	-	-	-
Salicylic acid	+	-	-	+	-	+	-	-	+	+	-
Citrate to gas	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	+	-	+	-	-	-	-	-
Starch	+	-	-	+	-	-	+	-	+	+	-
Sucrose	+	+	+	+	+	-	+	+	+	+	+
Trehalose	+	-	-	+	-	-	+	-	+	+	-
Xylose	-	+	-	-	-	-	-	-	-	-	-

ND = Not determined

METABOLIC ANALYSIS RESULTS OF *Lactobacillus* ISOLATES

	R_1	R_3	R_4	R_7	R_8	R_9	M_2	M_3	M_4	M_7	M_8
Catalase	+	+	+	+	+	+	+	+	+	+	+
DAP in cell wall	-	-	-	-	-	-	-	-	-	-	-
Gas from glucose	-	+	+	-	-	-	-	-	-	-	-
Lactic acid configuration	D	DL	DL	L	DL	L	DL	DL	D	DL	DL
Growth at 30°C	-	+	-	+	+	+	-	+	-	-	+
Growth at 4°C	+	-	-	-	-	-	-	-	+	+	-
NH_3 production	+	+	+	-	+	+	+	+	-	+	+
Growth at pH 3.9	+	+	+	+	+	+	-	+	-	-	+
Growth with 10% (m/v) NaCl	+	+	-	+	+	-	-	-	-	-	-

DAP = diaminopimelic acid