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A Bacterial Population Analysis of Granular Sludge from an Anaerobic Digester Treating a Maize-Processing Waste

A. R. Howgrave-Graham,* F. M. Wallis

Department of Microbiology and Plant Pathology of University of Natal, PO Box 375, Pietermaritzburg 3200, Republic of South Africa

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P. L. Steyn

University of Pretoria, Lynnwood Road, Pretoria 0002, Republic of South Africa

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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.

Key words: Granular sludge, anaerobic digester, maize processing waste, bacterial population, electron microscopy.

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% 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/litre, of which protein comprised 12%.

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: (a) the acid-forming stage during which fatty acids are produced from polysaccharides, lipids and proteins, in which a wide range of microorganisms participate; (b) 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 McInerney and Bryant in 1981, has three stages. The first is the fermentative stage in which complex organic materials, e.g. carbohydrates, proteins and lipids,

*To whom correspondence should be addressed.

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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: the hydrolytic bacteria that 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; the hydrogen-producing acetogenic bacteria (obligate and facultative anaerobes) that 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; the homoacetogenic bacteria which can ferment a wide spectrum of multi- or one-carbon compounds to acetic acid; the methanogens which can ferment acetate, H_2, CO_2 and other one-carbon compounds like methanol and methylamine to methane.

All three schemes emphasize the importance of volatile fatty acids as intermediates in anaerobic digestion. However, Verstraete *et al.* (1981) state 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 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. In addition it may be determined which model best fits the Bellville clarigester, or a new one may be proposed. 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.

METHODS

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.

Microscopy

The 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% glutaraldehyde and 2% osmium tetroxide, and dehydrated in an ethanol series. For scanning electron microscopy (SEM) the specimens were critical-point dried, gold sputtercoated and examined in a Hitachi S450 SEM.

Transmission electron microscopy of the granules was carried out in a Hitachi H600 TEM after staining 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.

Isolation procedures

Lactic acid bacteria

Sludge granules were serially diluted (10^{-1} to 10^{-7}) in Ringers solution after maceration with an Ultraturrax, and each ten-fold dilution was streaked onto Rogosa agar + 1% fructose + 0·4% 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, pseudocatalase, the presence of diaminopimelic acid in the cell walls and the configuration of the lactic acid produced.

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.

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.

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

Butyric acid bacteria
Granules were pasteurized at 75°C for 10 min, streaked on *Clostridium* selective agar (Merck), and incubated in an anaerobic jar with an Anaerocult A envelope.

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 preparations. The spent medium was examined in a Hewlett-Packard 5790 gas chromatograph for the presence of propionic, acetic, isobutric, n-butyric and isovaleric acids using a 2 m glass column with Graphpac coated with carbowax plus H_3PO_4 (injection temperature = 125°, oven temperature = 70°, final temperature = 150°).

Methanogens
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). 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 by gas chromatography with the same column used for propionic acid determinations.

The effect of homogenization of the granules with an Ultraturrax 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.

RESULTS

Light microscopy
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.

Electron microscopy
SEM and TEM studies revealed that the granules contained several bacterial morphotypes in close association with each other (Figs 1–10) 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 Figs 2 and 3). Figure 3 is a cross section of a free-living spirochaete with several fibrils in the axial filament, suggestive of *Spirochaeta plicatilis*.

Fig. 1. SEM showing the diversity of bacteria within anaerobic digester granules.

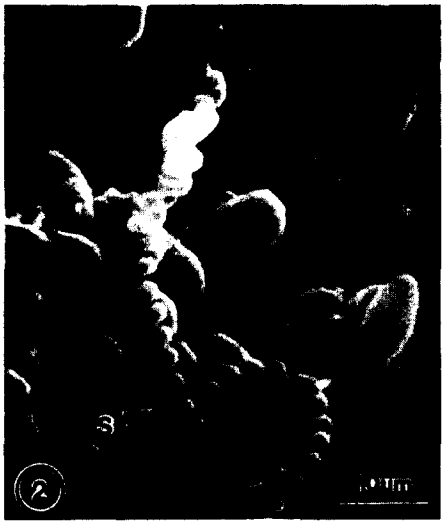

Fig. 2. SEM of Spirochaete (S) within a granule.

Fig. 4. Bacteria within granules, some with flagella (f).

Fig. 3. Cross section of *Spirochaeta plicatilis* showing fibrils (F) within the axial filament.

Fig. 5. Section through a rod showing capsular material (C).

Motile organisms varying with respect to positioning and number of flagella were abundant in the granules as shown in Figs 4, 8 and 9. Figure 4 includes a bent rod with a single polar flagellum. This organism is too small to be either a sulphate-reducer or an acetogen (Hespell & Bryant, 1979; Pfennig *et al.*, 1981).

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 (Fig. 5) while in Fig. 6 a loose network of matrix material is evident. These observations suggest that a network of polymeric strands holds the bacterial cells together. Within the granule structure dense 'capsules' were observed around groups of bacteria, isolating them from the rest of the granule (Fig. 7). The areas inside the 'capsules' appear lighter in colour than those outside them, probably due to failure of the osmium tetroxide to penetrate the dense 'capsular' barrier.

Isolation studies

Lactic acid bacteria

Twelve rod-shaped isolates varying in morphology from short to long rods, and filaments were

Fig. 6. TEM of cells and interlinking glycocalyx (G).

Fig. 8. SEM of bacteria within a granule including a vibroid laterally-flagellated cell (F) similar to *Selenomonas* and *Lachnospira*.

Fig. 7. TEM of bacteria and a dense 'capsule' (K) around a large proportion of the population.

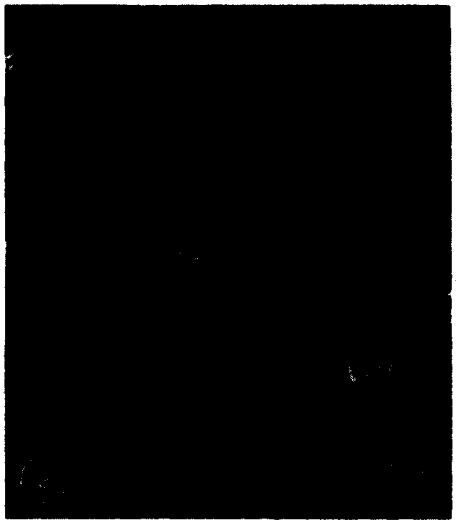

Fig. 9. Spiral bacterium, with tuft of polar flagella (f) — possibly *Desulfovibrio gigas*.

distinguished. Of special interest were two isolates of unusual spiral morphology. These isolates were all identified as members of the hetero-fermentative *Lactobacillus* group on the basis of their sugar fermentation pattern, the configuration of the lactic acid produced, the presence of di-aminopimelic acid in the cell wall and other physiological characteristics. Six of the isolates could break down starch, identifying them as hydrolytic bacteria and between the 12 isolates a wide spectrum of sugars could be metabolized.

Acetogenic bacteria

Several colonies of vibroid and coccoid bacteria were observed microscopically after incubation in roll tubes. Some vibrios showed a tumbling motility in wet preparations that was characteristic of *Selenomonas* and *Lachnospira*, 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 (Fig. 8).

The sulphate-reducing bacteria

After 5 days of incubation, the specific sulphate-reducer medium turned black, and pitch-black

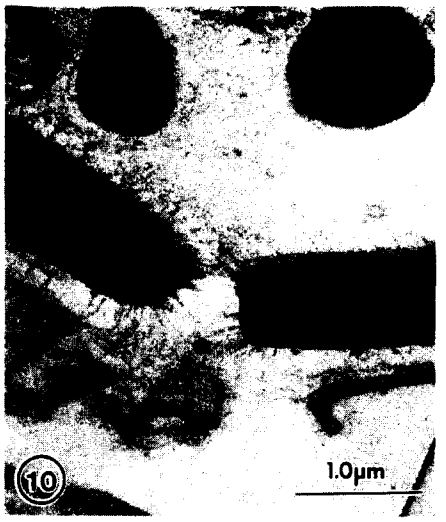

Fig. 10. Encapsulated rods resembling *Klebsiella*.

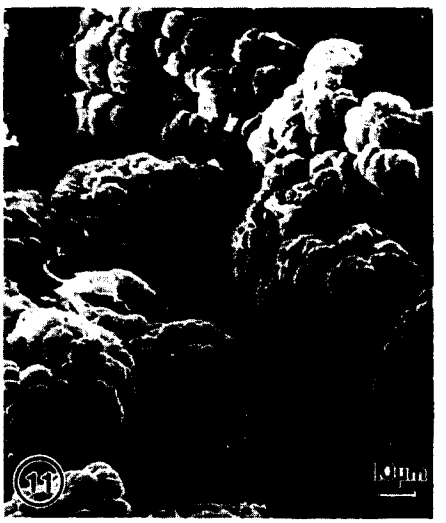

Fig. 11. *Methanosarcina*-like tetrads in acetate-containing enrichment medium.

colonies of sulphate-reducers were scattered throughout it. (The black medium was not dense enough to prevent light passing through, whereas the pitch-black colonies were.) 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* (Fig. 9). 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.

Enterobacteria

Numerous colonies developed on MacConkey and EMB agar. Of these several were identified as *Salmonella arizonae* and *Klebsiella* using API20E identification strips. Electron micrographs of granules revealed the presence of encapsulated rods resembling *Klebsiella* (Fig. 10).

Butyric acid bacteria

No *Clostridium* species were isolated but some of the bacteria present in the granules, e.g. *Selenomonas*, are capable of forming butyric acid under certain conditions.

Propionic acid bacteria

No *Propionibacterium* species were isolated but some of the granule-inhabiting bacteria, such as *Selenomonas*, can produce propionate under certain circumstances.

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 (Fig. 11). *Methanothrix*-like filaments were also obtained. Electron micrographs of granules confirmed the presence of numerous tetrads and filaments resembling these two bacteria (Fig. 12).

Physical disruption of the granules, using the Ultraturrax, resulted in death of the cells, since growth occurred at higher dilutions on facultative anaerobic plates when the granules were not homogenized than when they were. In addition, the ATP biometer, in contrast to the constant ATP reading for undisturbed granules, indicated a very high ATP reading immediately after homogenization due to release of ATP by the ruptured cells. A subsequent decrease to ATP levels far below those of undisturbed granules then occurred due to its breakdown. Sonication resulted in extremely slow breakdown 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.

Fig. 12. TEM of tetrads and filaments resembling *Methanosarcina* and *Methanothrix* respectively.

DISCUSSION

The results 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 members of the population were probably not isolated or identified. In general, the electron and light microscopic observations of the isolated and enriched bacteria supported similar observations made on entire granules, e.g. the isolation of *Klebsiella*, verifying the observation by TEM of a *Klebsiella*-like rod.

In this study, no propionic or butyric acid producers were detected, unless *Selenomonas* is considered as belonging to this 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 granules examined indicated that in this granular sludge metabolism might revolve around lactate metabolism. *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 also produces butyrate while the latter produces propionate (Hespell & Bryant, 1979).

The Enterobacteria isolated (*Salmonella arizonae* and *Klebsiella*) ferment sugars to lactate, succinate, acetate, ethanol, CO_2, H_2 and formate (Doelle, 1975).

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 fall into the first stage of this model. Since no propionibacteria and clostridia were detected, lactate is most probably further utilized by the acetogenic sulphate reducers, producing acetate, CO_2 and sulphide (Pfennig *et al.*, 1981). Sulphate-reducers would therefore comprise the third group 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 utilize acetate; some species in the former genus being obligate acetate utilizers (Zehnder *et al.*, 1980). The larger population of *Methanosarcina*-like tetrads, however, contradicts findings by Dolfing (1986) who investigated a granular sludge from a sugar factory effluent. He stated that *Methanothrix* rather than *Methanosarcina* would be the most significant methanogen in granular sludge. The present study indicates that both would be significant in granular-sludge metabolism.

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 treatment could disrupt the granules, and as these treatments proved lethal to the bacteria present 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 which could bind the organisms together to form granules. This hypothesis was extended to include other bacteria that had this property. An amino-acid analysis (Ross, W. R., pers. comm., 1984) indicated that both cysteine and cystine were absent from the feed to the digester and an unidentified

bacterium in the granules may thus be responsible for the production of this polypeptide, which could contribute 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 such a role in the bioreactor used in the present study as too few organisms with similar morphology were observed within the granules.

It is a distinct possibility that in some granular anaerobic-digester sludges the lactic acid bacteria rather than the propionic and butyric acid bacteria play a major role in the efficiency of the digestion process. If this is so, lactate and acetate would be more important intermediates than butyrate and propionate in these sludges.

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 amount of effluent being treated as well as a decrease in the amount of gluten protein entering the digester. This alteration in feed to the digester affected the nature of the sludge formed and smaller granules with reduced settleability resulted.

It can be concluded that the widely accepted four-stage model for the microbiology of anaerobic digestion comprising hydrolytic, acidogenic, acetogenic and methanogenic stages applies to 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 the selection of the specific microorganisms within the granules.

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