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MICROBIAL STRUCTURE AND ACTIVITY OF UASB GRANULES TREATING DIFFERENT WASTEWATERS

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ABSTRACT

The microstructure of three types of UASB granules respectively treating sucrose, glutamate and brewery wastewaters in mesophilic conditions were analyzed by light, scanning electron and transmission electron microscopies, along with the specific methanogenic activity (SMA) of the granules. Results showed that the granule's microstructure was dependent on the nature of the substrate. Those degrading soluble carbohydrates exhibited a layered structure, while those degrading glutamate exhibited a rather uniform structure. Such a difference was explained based on the substrate's rates of acidogenesis and diffusion. A model of the typical layered structure was proposed. In addition, the acetoclastic *Methanothrix* was found as the key structural element in all the granules, suggesting that it plays an important role in granulation. Three types of syntrophic microcolonies were found to be abundant in granules degrading soluble carbohydrates: two were juxtapositioned syntrophic microcolonies, each was composed of hydrogen-producing acetogens and hydrogen-consuming methanogens, while the third was a cluster-type of syntrophic association between two microcolonies. The SMA data using individual VFA as substrate provided supporting evidence to the observations of the bacterial compositions in the granules.

KEYWORDS

Activity; brewery; glutamate; granule; microbial structure; sucrose; syntrophic microcolony, UASB.

INTRODUCTION

Since its introduction over a decade ago, the Upflow Anaerobic Sludge Blanket (UASB) process (Lettinga *et al.*, 1980) has been successfully applied to various types of wastewaters (Hulshoff Pol and Lettinga, 1986; Fang *et al.*, 1990; Fang and Chui, 1993a). Over 300 full-scale reactors have been built worldwide with most of them designed for treating wastewaters from food/beverage industries, such as brewery, potato, starch, and sugar processing (Lettinga and Hulshoff Pol, 1991).

With a population of over 1.1 billions, China has one of the largest food/beverage industries in the world. It annually produces, for instance, over 8 million tons of beer and 20 thousand tons of monosodium glutamate (MSG). Because of the high levels of organic contents in the wastewaters, brewing and MSG are the two industries among the major polluters in China. Attempts have been made in recent years in China to apply the UASB technology for the treatment of wastewaters from these two industries.

The success of the UASB process relies on the formation of active and settleable sludge granules. Researches have been carried out to study the granulation mechanism (Hulshoff Pol and Lettinga, 1983) and

the granular microbial composition (Dolfing *et al.*, 1985; Dubourguier *et al.*, 1988), as well as the microbiology of methanogens (Mah and Smith, 1981) and the syntrophic associations between the hydrogen-producing acetogens and the hydrogen-consuming methanogens (Boone and Bryant, 1980; McInerney *et al.*, 1981; Stieb and Schink, 1985; Roy *et al.*, 1986). However, only a small number of studies have been reported on the microstructure of UASB granules.

In this study, three types of UASB granules respectively treating wastewaters of sucrose, brewery and MSG, were examined for their microstructures and their methanogenic activities.

METHODS AND MATERIALS

Microstructures of the UASB granules were examined using not only light microscopy (LM, Olympus, Model BH2), but also scanning electron microscopy (SEM, Cambridge Stereoscan 150), and transmission electron microscopy (TEM, JEOL 100SX). Since all methanogens, and only methanogens, have both the F_{350} and the F_{420} cofactors, they can be readily identified under LM by their emitted fluorescence (Doddema and Vogel, 1978) under epi-fluorescent excitations at either 350 nm or 420 nm. Examinations of bisected granules under SEM could illustrate the morphology and location of each bacterium inside the granule. Examinations by TEM could illustrate, on the other hand, the ultrastructure of each bacterium. Detailed procedures for preparing granules for LM, TEM, and SEM examinations followed those reported previously (Fang and Chui, 1993b). The specific methanogenic activity (SMA) of the granules was measured in duplicate in serum vials (Hwang and Cheng, 1991). The individual substrate used for the SMA measurements included formate, acetate, propionate, butyrate, and sucrose.

The three types of granules were all obtained from UASB reactors operated at the mesophilic condition of 35-37°C. The sucrose-degrading granules were sampled from an 8.5 l reactor operating at the COD loading of 20 g l^{-1} day^{-1} (Fang and Chui, 1993a). The granules treating brewery wastewater were obtained from a full-scale wastewater treatment plant, of which the detailed operating conditions were unfortunately not available. The glutamate-degrading granules were obtained from a pilot reactor treating MSG wastewater at the COD loading of 5.0 g l^{-1} day^{-1}. Over 50 granules of each type were examined.

RESULTS

A typical sucrose-degrading UASB granule had a size of 1-2 mm. It had a 20-40 mm dense outer layer and a loosely packed interior (Fig. 1a). A section observed by LM under epi-fluorescent excitation illustrates that methanogens were distributed throughout the granule. The methanogens in the outer layer were of diverse morphologies, including cocci, bacilli, and some filaments, while those in the interior were predominantly filamentous *Methanothrix* (Fig. 1b). Figure 1c illustrates the complex bacterial composition of the granule surface. Figures 1d and 1e, which are sections of the outer layer observed respectively by SEM and TEM, also illustrate that the layer was densely packed with bacteria of various morphologies and the evidence of syntrophic microcolonies. On the other hand, Figures 1f and 1g, as observed by SEM and TEM respectively, illustrate that the granule interior was predominantly composed of *Methanothrix*. The genus of *Methanothrix* (Zehnder *et al.*, 1980) can be identified by its fluorescent, bamboo-shaped filament (Fig. 1b), rod-shaped cell (0.7-0.8 x 2.0-3.5 mm) with flat-ends (Fig. 1f and 1g), and an ultrastructure with outer and inner cell walls, and with an end-plate between adjacent cells.

A typical UASB granule treating brewery wastewater had a size of 2-4 mm and exhibited a complex layered structure (Fig. 2a). Figure 2b, which is a section observed by LM under epi-fluorescent excitation, illustrates the three-layered profile of methanogen distribution in the granule. The outer layer, about 100 mm in thickness, was composed of various types of bacteria, including cocci, bacilli, and some scattered colonies of *Methanothrix* and *Methanosarcina* (Fig. 2c). The majority of bacteria in this layer were acidogens with a small fraction of methanogens, as illustrated by its low fluorescent intensity. The middle layer (Fig. 2d), having a thickness of 100 mm, was dominated by syntrophic microcolonies (right-side of Fig. 2d) with scattered *Methanothrix* colonies (top left corner in Fig. 2d, pointed with an arrow). The central core was densely packed with short-rod-shaped *Methanothrix* (Fig. 2e and 2f).

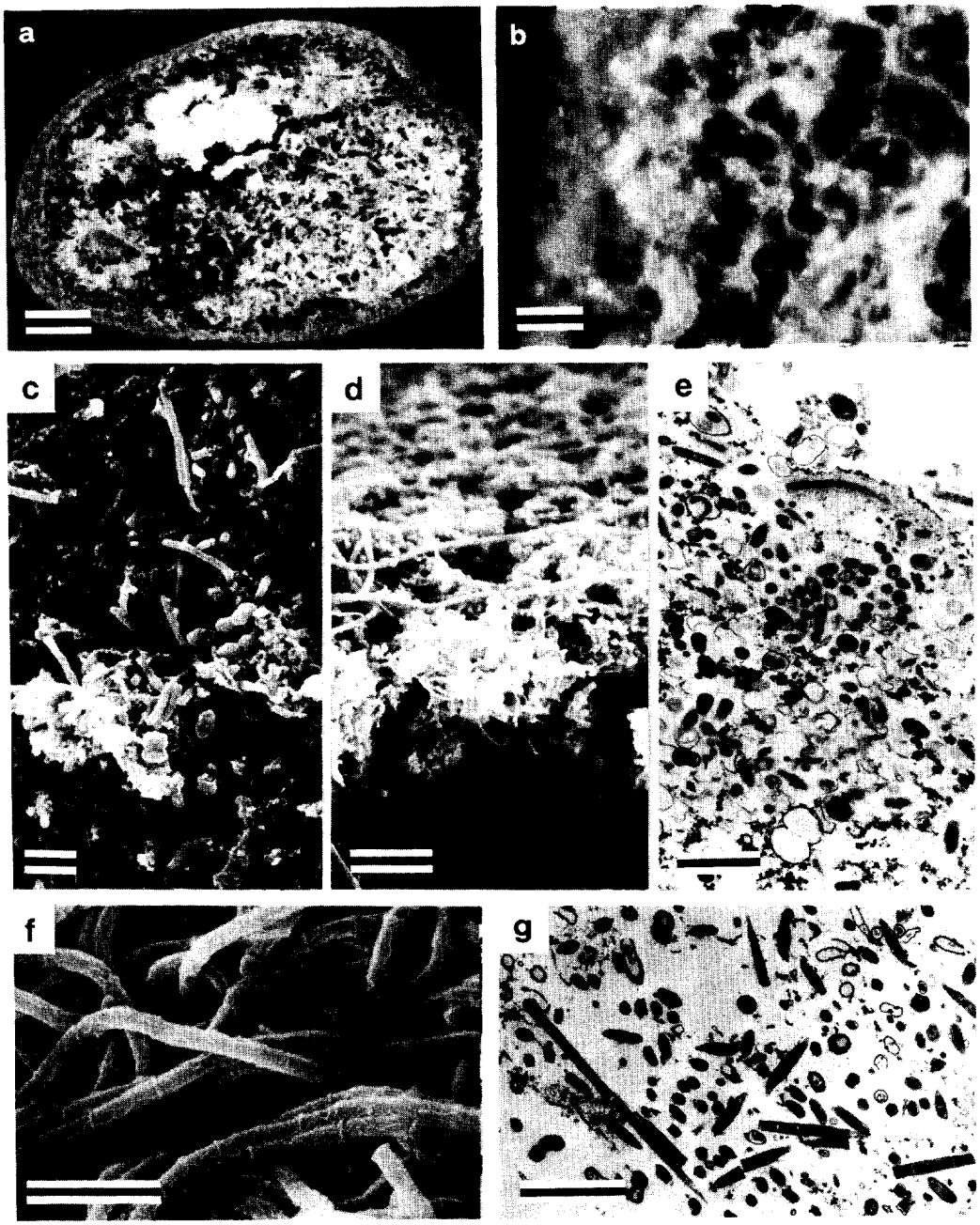

Fig. 1. Micrographs of sucrose degrading granules. (a) microtome section under epi-fluorescent excitation, bar = 100 μm; (b) microtome section at the surface under epi-fluorescent excitation, bar = 10 μm; (c) surface under SEM, bar = 2 μm; (d) section of the outer layer under SEM, bar = 8 μm; (e) ultrasection of the outer layer under TEM, bar = 2 μm; (f) *Methanothrix* at the centre core under SEM, bar = 4 μm; (g) ultrasection of *Methanothrix* at the centre core under TEM, bar = 4 μm

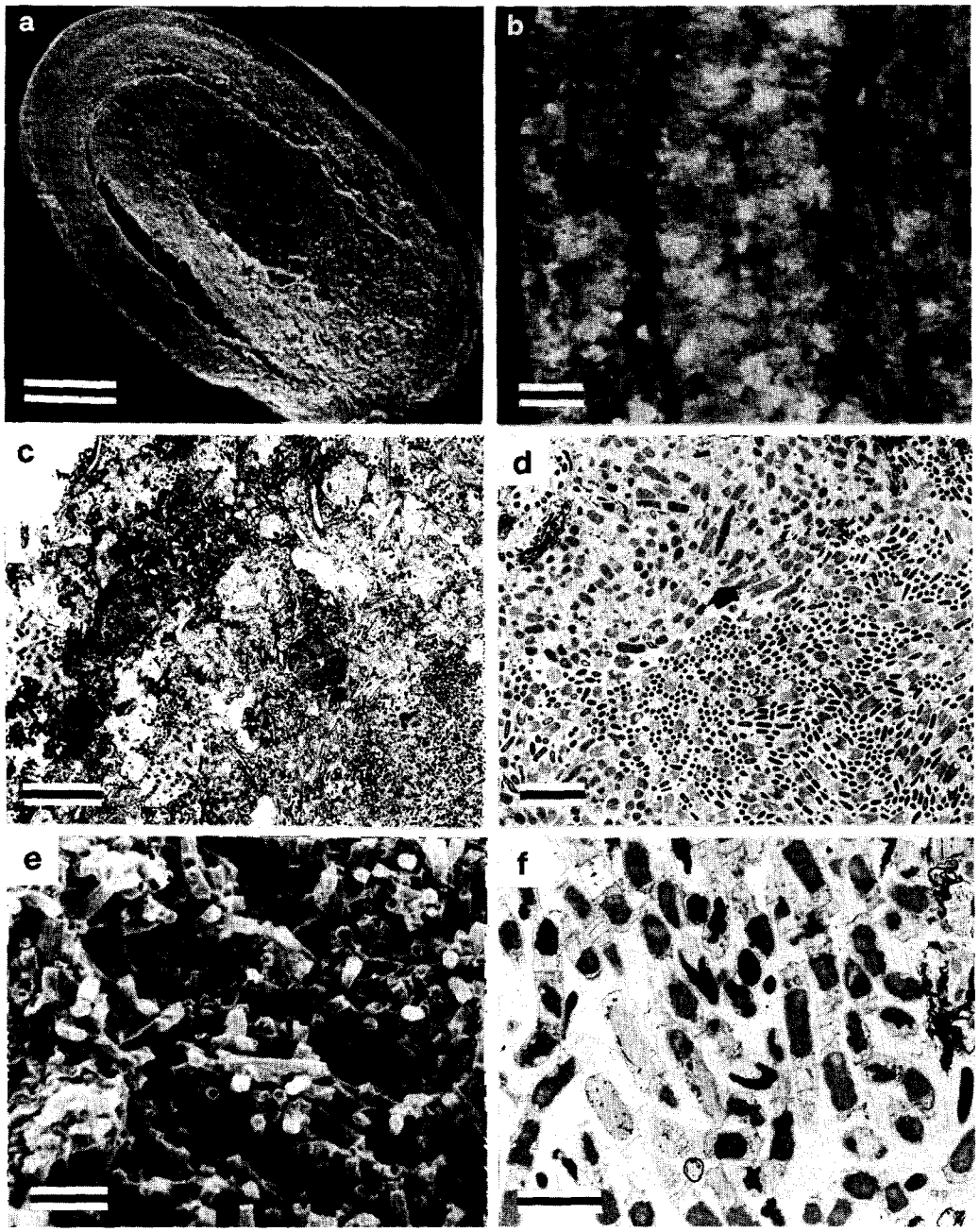

Fig. 2. Micrographs of UASB granules treating brewery wastewater. (a) bisected granule under SEM, bar = 0.5 mm; (b) microtome section under epi-fluorescent excitation, bar = 100 μm; (c) ultrasection of the outer layer under TEM, bar = 10 μm; (d) ultrasection of the mid-layer under TEM, bar = 4 μm; (e) *Methanothrix* at the centre core under SEM, bar = 4 μm; (f) ultrasection of *Methanothrix* at the centre core under TEM, bar = 2 μm

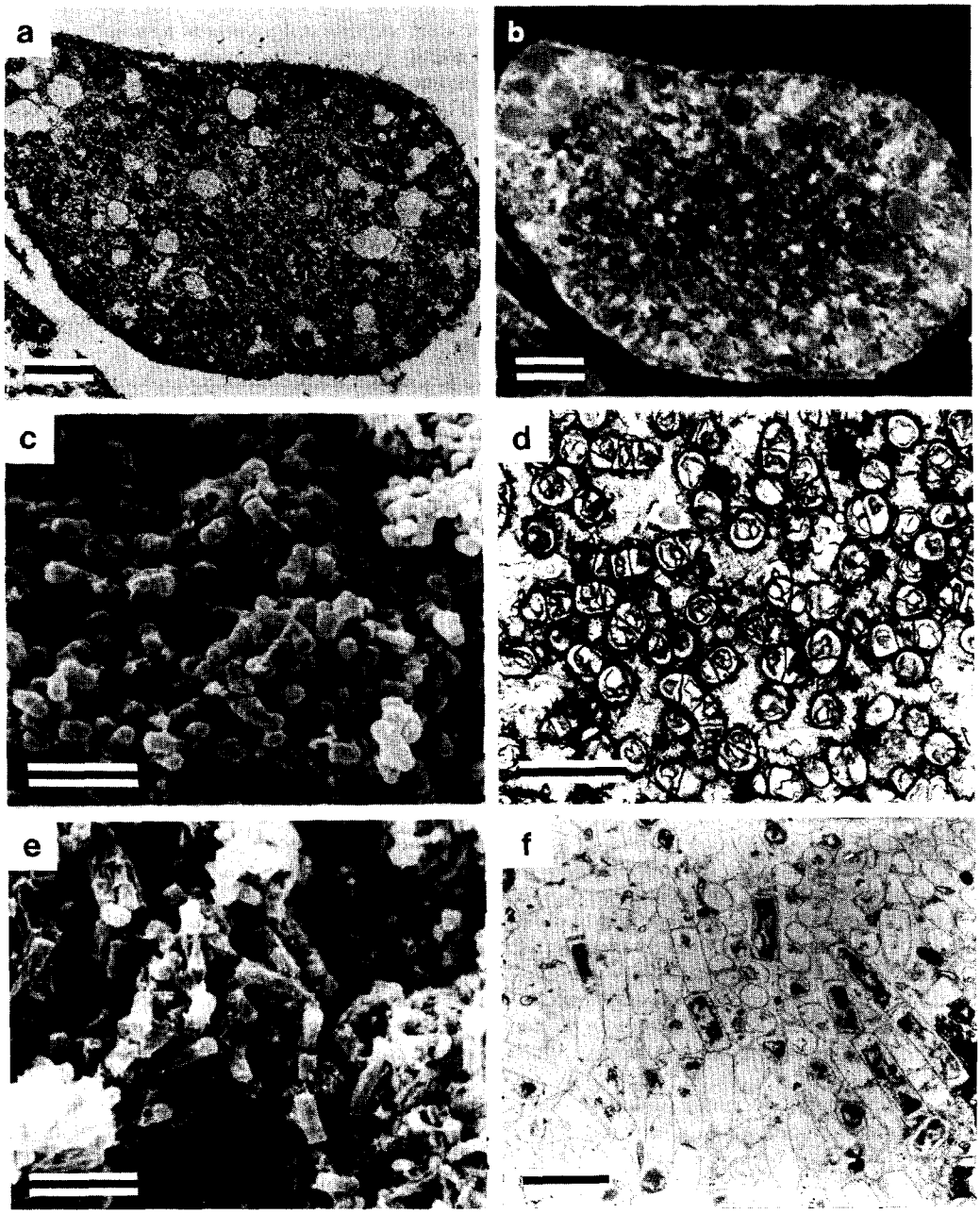

Fig. 3. Micrographs of glutamate degrading granules. (a) microtome section under bright field, bar = 10 μm; (b) microtome section under epi-fluorescent excitation, bar = 100 μm; (c) bacilli colony under SEM, bar = 4 μm; (d) ultrasection of bacilli colony under TEM, bar = 2 μm; (e) *Methanothrix* under SEM, bar = 4 μm; (f) ultrasection of *Methanothrix* under TEM, bar = 2 μm

A typical glutamate-degrading granule had a size of 0.5-1.5 mm, and did not exhibit any layered structure. Instead, it had a network of *Methanothrix* with low fluorescent intensity and packets of non-methanogenic bacilli throughout the section, as illustrated in Figures 3a and 3b. Evidence of syntrophic microcolonies was scarce. The morphologies and ultrastructures of the bacilli and the *Methanothrix* are illustrated in Figures 3c-3f. Although most cells appeared normal under SEM (Fig. 3c and 3e), micrographs of TEM (Fig. 3d and 3f) illustrate that, however, most of the bacterial cells were lysed with cell membranes separated from the cell wall.

Table 1 summarizes the SMA data of the granules using individual volatile fatty acids (VFA) and sucrose as substrates. In general, among the three types of granules, the sucrose-degrading granules exhibited the highest SMA, whereas the glutamate-degrading granules the lowest.

TABLE 1. Specific Methanogenic Activities (SMA) of Granules Using Volatile Fatty Acids and Sucrose as Substrates

Sludge treating wastewater of	SMA (g CH_4-COD g VSS^{-1} day^{-1})				
	formate	acetate	propionate	butyrate	sucrose
sucrose	1.22	1.20	0.52	0.61	0.75
brewery	1.26	0.49	0.13	0.12	0.28
MSG	0.02	0.17	<0.01	0.20	0.06

DISCUSSION

Overall Granule Structure

So far, only a limited number of studies had been reported on the microstructure of UASB granules. MacLeod *et al.* (1990) examined the sucrose-degrading granules and proposed a three-layered structure of UASB granules. The outer and middle layers were composed of mainly acidogens and syntrophic microcolonies, respectively, while the centre core was composed of *Methanothrix*-like methanogens. Such a layered structure has later been confirmed by Guiot *et al.* (1992) for glucose-degrading granules. On the other hand, Grotenhuis *et al.* (1991) reported that there was no layered structure for UASB granules treating propionate, ethanol, and sugar-refinery wastewaters. In propionate-degrading granules, *Methanothrix* and *Methanobrevibacter arboriphilus* clustered together with the presumably propionate-oxidizing acetogens being found throughout the granule. These conflicting observations, therefore, seem to suggest that the bacteriological composition, and thus the microstructure, of a sludge granule is dependent on the substrate.

In this study, both UASB granules treating sucrose and brewery wastewaters exhibited a layered structure. Such a structure was prominent, in particular, for the granules treating brewery wastewater, as illustrated in Figure 2. The centre core of a granule treating brewery wastewater was predominantly composed of *Methanothrix*. The middle layer consisted of two types of microcolonies: one was composed solely of *Methanothrix*, whereas the other was the syntrophic microcolony of hydrogen-producing acetogens and hydrogen-consuming methanogens. The outer layer had a very complex bacterial composition, including not only acidogens of various morphologies, but also *Methanothrix*. Unlike in MacLeod's model where it was found only in the centre core, *Methanothrix* was present throughout the granule. Figure 4 illustrates a typical layered-structure model found in this study.

However, the layered structure seems to be limited to granules treating soluble carbohydrates as substrate, such as those in the sucrose, glucose and brewery wastewaters. There was no layered structure observed for

granules degrading non-carbohydrate substrates, as reported by Grotenhuis *et al.* (1991) and as found in this study for glutamate-degrading granules.

The granule's bacterial profile, and thus its microstructure, conceivably depends on the concentration profiles of the substrates and metabolites, such as VFA, acetate, hydrogen, etc., inside the granule. These profiles, in turn, are dependent on the rates of degradation and diffusion of the substrates and metabolites. The overall process of anaerobic degradation involves at least three steps, i.e. acidogenesis, acetogenesis and methanogenesis. When degrading soluble carbohydrates, acidogens are concentrated in the outer layer of the granule, not only because of the availability of carbohydrates in the bulk solution but also because of the higher rate of acidogenesis as compared to those of acetogenesis and methanogenesis (Noike *et al.*, 1985). The VFA, which are produced by the acidogens and concentrated in the outer layer, diffuse inward, due to concentration gradients, and become substrates for syntrophic acetogens or acetate-degrading methanogens in the middle layer. Furthermore, since it is the metabolite of the acetogens in the middle layer, acetate is the key substrate for the bacteria in the centre core. This seems to explain the three-layered structure as illustrated in Figure 4.

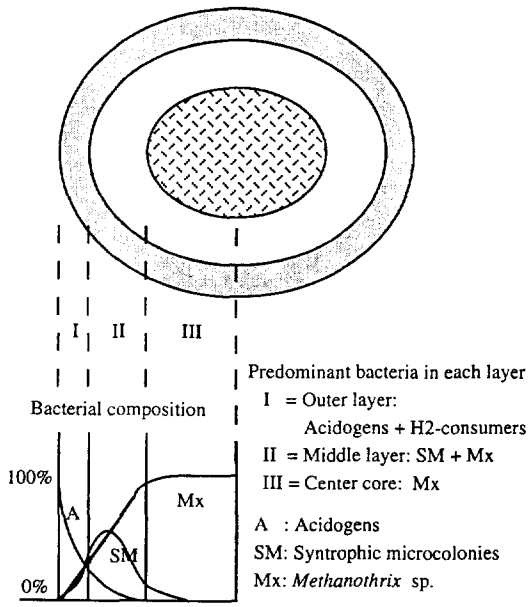

Fig. 4. Proposed layered structure and bacterial composition for the granules treating soluble carbohydrates.

On the other hand, in the case of anaerobic degradation of proteins or amino acids, such as glutamate, the acidogenesis becomes the rate-limiting step. It is therefore conceivable that the glutamate, because of its slow degradation and molecular diffusion, was rather evenly distributed throughout the granule, and, accordingly, so were the other metabolites, such as VFA, acetate, hydrogen, etc. Consequently, bacteria were rather evenly distributed inside the granule and there was no layered structure in the glutamate-degrading granules.

The Role of *Methanothrix* and *Syntrophic* Microcolonies in Granules

SEM and TEM micrographs illustrate that *Methanothrix* was a key structural element in all the granules observed. In the glutamate-degrading granule which had no layered structure, a *Methanothrix* network was found throughout the granules. In granules treating brewery wastewater, *Methanothrix* not only was the predominant bacterium in the centre core of the granule and one of two types of microcolonies found in the

middle layer, but also was found in smaller amount in the outer layer of granules. This seems to suggest that *Methanothrix* filaments likely play an important role in sludge granulation.

Propionate and butyrate are among the major intermediates in the anaerobic degradation of soluble carbohydrates. The ultimate degradation of propionate and butyrate involves three different groups of bacteria, i.e. syntrophic acetogens, hydrogen-consuming methanogens and acetate-consuming methanogens. However, the degradation of propionate and butyrate are thermodynamically unfavorable, unless the concentrations of the two metabolites, hydrogen and acetate, are being kept at very low levels. The syntrophic acetogens, thus, have to grow in the vicinity of the hydrogen-consuming and acetate-consuming methanogens (McInerney *et al.*, 1981; Boone and Bryant, 1980; Stieb and Schink, 1985; Roy *et al.* 1986).

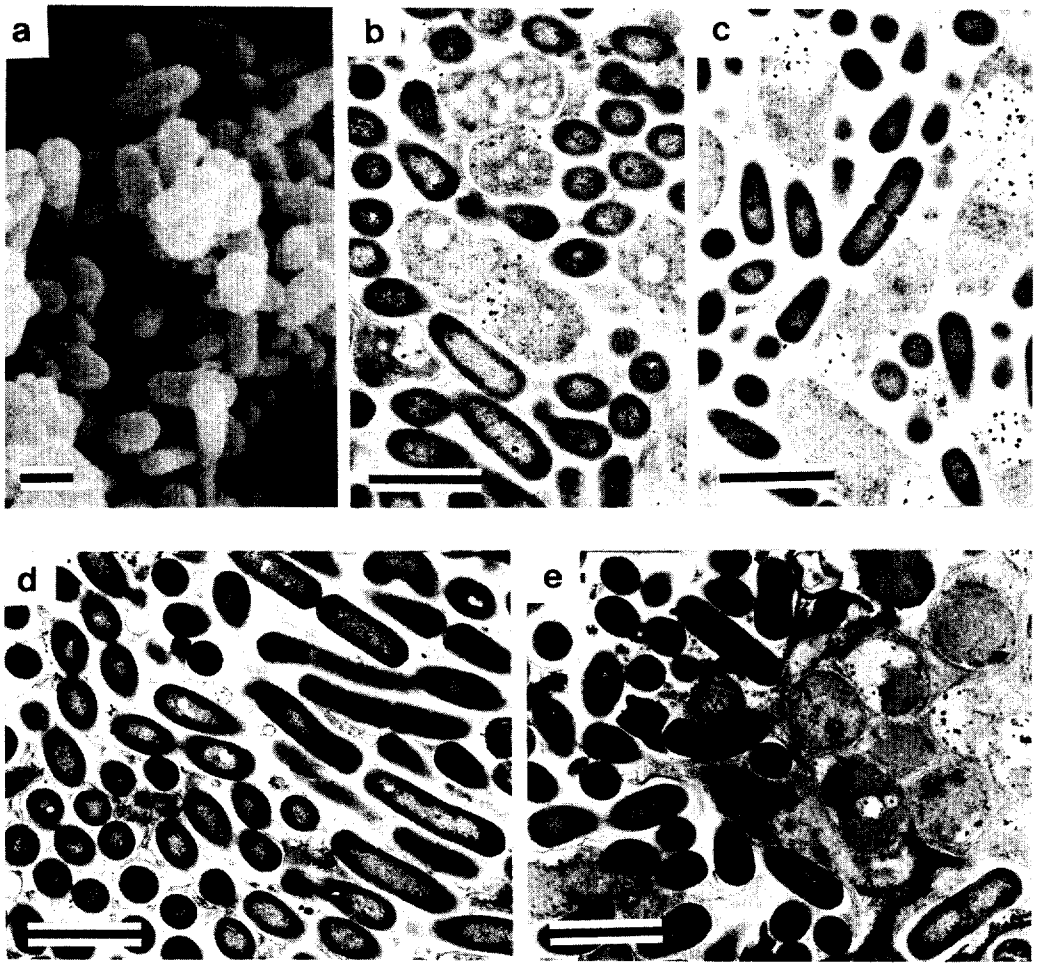

Fig. 5. Micrographs of different syntrophic microcolonies in UASB granules treating brewery wastewater (bar = 1 μm). (a) juxtapositioned syntrophic microcolonies under SEM; (b) one type of juxtapositioned syntrophic microcolonies with close interspecies distance under TEM; (c) another type of juxtapositioned syntrophic microcolonies with wider interspecies distance under TEM; (d) *Methanobrevibacter*-like colony under TEM; (e) cluster-type syntrophic microcolonies under TEM.

Such a syntrophic relationship could be enhanced if the syntrophic bacteria are in juxtaposition (Fig. 5a) so that the diffusion distance for the metabolites is minimized (Boone *et al*, 1988; Thiele and Zeikus, 1988). Two types of juxtapositioned syntrophic microcolonies, each being composed of hydrogen-producing

acetogens and hydrogen-consuming *Methanobrevibacter*-like methanogens, were observed in this study for the granules treating brewery wastewater (Fig. 5b and 5c). The two microcolonies could be distinguished from their differences in the ultrastructure of the cells of the hydrogen-producing bacteria and the interspecies distance. Observations of similar syntrophic microcolonies had also been reported by a number of investigators (Dubourguier *et al.*, 1988, Stams *et al.*, 1989; MacLeod *et al.*, 1990; Grotenhuis *et al.*, 1991). However, it was also observed in the granules treating brewery wastewater that a microcolony of hydrogen-consuming *Methanobrevibacter*-like bacteria (Fig. 5d) appeared in syntrophic association with another microcolony of presumably hydrogen-producing bacteria (pointed by an arrow in Fig. 5e). This cluster type of syntrophic association had not been reported in any previous studies.

Specific Methanogenic Activity

The SMA data in Table 1 show that the glutamate-degrading granules had bio-activities nearly one order of magnitude lower than those of the other two types of granules. This is reflected by the observations of its low fluorescent intensity under LM and the abundance of lysed cells under TEM. The low activity could be attributed to the high chloride content (over 6,000 mg l^{-1}) in the treated MSG wastewater. The SMA data also show that in general the sucrose-degrading granules had higher activities than the granules treating brewery wastewater.

All three types of granules exhibited relatively high SMA in degrading acetate. This is reflected by the abundance of acetoclastic *Methanothrix* in all granules as observed by microscopes. All methanogen require either acetate, or formate, or H_2/CO_2 as energy and carbon source. All the formate-degrading methanogens can also convert H_2/CO_2 (Boone and Whitman, 1988). Comparison of SMA using formate and acetate as substrate in Table 1 shows that majority of methane produced by granules treating brewery wastewater was via formate (or its hydrogen equivalent), instead of acetate. This is also reflected by the abundance of formate-and-H_2/CO_2-degrading *Methanobrevibacter* in this type of granule. Granules treating sucrose and brewery wastewater exhibited appreciable SMA degrading propionate and butyrate. This is also reflected by the presence of juxtapositioned syntrophic microcolonies observed in these granules.

CONCLUSION

1. The microstructure of the UASB granules was dependent on the nature of the substrate. Granules treating sucrose and brewery wastewaters exhibited a three-layered structure. On the other hand, granules degrading glutamate exhibited a rather uniform structure. This could be explained from the difference in the substrate's rate of acidogenesis, which would affect the concentration profiles of the substrate and metabolites in the granule, and consequently its microstructure.

2. The acetoclastic *Methanothrix* was found as the key structural element in all the granules. This seems to suggest that *Methanothrix* likely plays an important role in sludge granulation.

3. Syntrophic microcolonies were found in granules degrading soluble carbohydrates, for which propionate and butyrate were the two key intermediates. There were two types of juxtapositioned syntrophic microcolonies, each was composed of hydrogen-producing acetogens and hydrogen-consuming methanogens. In addition, a cluster type of syntrophic association between two microcolonies was also observed.

4. The SMA data using individual VFA as substrate provided supporting evidence to the observations of the bacterial compositions in the granules.

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