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MICROSTRUCTURAL ANALYSIS OF ANAEROBIC GRANULES

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SUMMARY

Photos of scanning and transmission electron microscopy showed that sludge granules treating sucrose wastewater had a 20-40 μm surface layer with diverse morphology, consisting of cocci and bacilli, and a loosely packed interior, mainly consisting of *Methanothrix*. Light microscopy using epi-fluorescent excitation showed not only the similar microstructure, but also the distribution of various genuses of methanogens.

INTRODUCTION

Since its debut in the late 1970s, the upflow anaerobic sludge blanket (UASB) process has been widely used for the treatment of various industrial wastewaters. Over 300 full-scale UASB reactors have been built worldwide (Lettinga, 1992). The success of this process relies on the formation of sludge granules with high settling ability and bio-activity. It is thus of interest to study the microstructure of the granules, which may lead to a better understanding of the mechanisms of forming granules of superior quality. In addition, this may also provide insight for a better understanding on the effect of the substrate's nature on its degradation, as well as factors that would affect the interspecies transfer of intermediates, such as hydrogen and formate. All of which could be of significance to the development of kinetic models (McCarty and Mosey, 1991).

In this study, UASB granules were examined not only by scanning electron microscopy (SEM) and transmission electron microscopy (TEM), but also by light microscopy. A histological method was used to prepare granule samples for light microscopy examination. Although SEM and TEM are able to give more detailed information on the bacterial morphology, they are unable to distinguish most of the methanogens from other bacteria of similar morphology. However, all methanogens have F_{350} and F_{420} cofactors, and thus can be readily identified by their emitted fluoresence (Edwards and McBride, 1975) under epi-fluorescent excitations at both 350 nm and 420 nm (UV and V, respectively). Therefore, using light microscope with epi-fluorescent excitation, the distribution of methanogens in the granule could also be illustrated.

MATERIALS AND METHODS

UASB granules from a bench-scale reactor treating sucrose wastewater at the

volumetric COD loading rate of 20 g/ℓ·d (Fang and Chui, 1993) were examined in this study.

To prepare for examination by light microscope, sludge granules were washed three times in a 0.1 M phosphate buffer solution at pH 7.2. They were then fixed in a phosphate buffer solution with 10% formaldehyde for 4 h (Bancroft and Stevens, 1990). Dehydration was carried out stepwise by immersing the fixed granules in a graded series of ethanol/water solutions increasing in ethanol content, from 50%, to 75%, 95%, and finally to 100%. The ethanol was then replaced with xylene in a similar fashion, i.e. immersing the granules in a graded series of xylene/ethanol solutions increasing in xylene content from 50% to 100%. The granules saturated with xylene were immersed in molten paraffin (mp. 45-55°C) at 60-65°C overnight, allowing the paraffin to penetrate into the granules. The paraffin-embedded granules were cooled down to room temperature in peel-off moulds, before being sliced in sections of 3-5 μm in thickness by a microtome (Leitz, Model 1400). The thin paraffin section was then allowed to float on a waterbath and placed on a glass slide. The slide was dried in air, put in an oven at 70°C for 10 min to melt the paraffin, and finally dewaxed with xylene. The slides were examined by a microscope (Olympus, Model BH2) under phase contrast, and epi-fluorescent (UV and V) excitations.

Prior to be examined by the scanning electron microscope (SEM, Cambridge Stereoscan 150), granules had to be fixed by soaking in a 0.1 M phosphate buffer solution (pH 7.2) with 4% glutaraldehyde for 2 hours, sliced in half after being frozen in liquid nitrogen, dehydrated with a series of water/ethanol solutions followed by another series of ethanol/carbon dioxide solutions, critical point dried with carbon dioxide, mounted on stubs with colloidal carbon, and finally coated with gold-palladium.

Prior to be examined by the transmission electron microscope (TEM, JEOL 100SX, Japan), granules were fixed overnight at 4°C in a 0.1 M cacodylate buffer solution (pH 7.4) containing 2.5% glutaraldehyde and 4% paraformaldehyde. After being washed three times in the cacodylate buffer, the granules were postfixed in cacodylate buffer with 1% osmium tetroxide for 1 hr. The granules were then washed with cacodylate buffer and dehydrated in a graded series of ethanol. Ethanol was again cleared with two exposures of propylene oxide for 15 min. The samples were then transferred to a mixture (1:1) of propylene oxide and resin (TAAB 812 Resin, U.K.) for 3 hours and in the pure resin for 12 hours for infiltration. Finally, the granules were embedded in fresh resin and polymerized at 60°C for 24 hr. Ultrathin sections were cut with an ultramicrotome (Ultracut E, Reichert-Jung, Austria) by using a diamond knife and collected on 200-mesh formvar-coated copper grids. Ultrasections were stained with uranyl acetate for 20 min. and lead citrate for 10 min. and finally examined by the TEM at an accelerating voltage of 80 kV.

RESULTS AND DISCUSSION

Over 100 granules were examined by the described methods. Figure 1 illustrates the section of a typical sucrose-grown granule under phase contrast (Figure 1a) and epi-fluorescent excitation (Figure 1b). The granule had a dense surface layer with a thickness of 20-40 μm and a loosely packed interior. The granule interior was of bright fluorescence under the UV and V excitations. Figure 2 illustrates a higher degree of magnification of the surface layer, which was consisted of, among others, a variety of hydrogen-consuming methanogenic cocci and bacilli, under epi-fluorescent excitation. The granule's interior was, however, mainly composed of bundles of acetate-consuming *Methanothrix*, which appeared as fluorescent filaments as illustrated in Figure 3.

SEM photos of a typical granule are illustrated in Figures 4 and 5, whereas TEM

photos are illustrated in Figures 6 and 7. Figures 4 and 6 clearly illustrate that the dense surface layer has a diverse population of cocci, bacilli, and filaments. Figures 5 and 7 illustrate, on the other hand, that the interior is predominantly populated with the bamboo-shaped *Methanothrix*. Unlike using light microscope with epi-fluorescent, SEM and TEM photos are unable to identify methanogens specifically. The TEM photo (Figure 7) also illustrates that the surface layer was made of microcolonies where syntrophic associations may take place (Thiele and Zeikus, 1988).

ACKNOWLEDGEMENT

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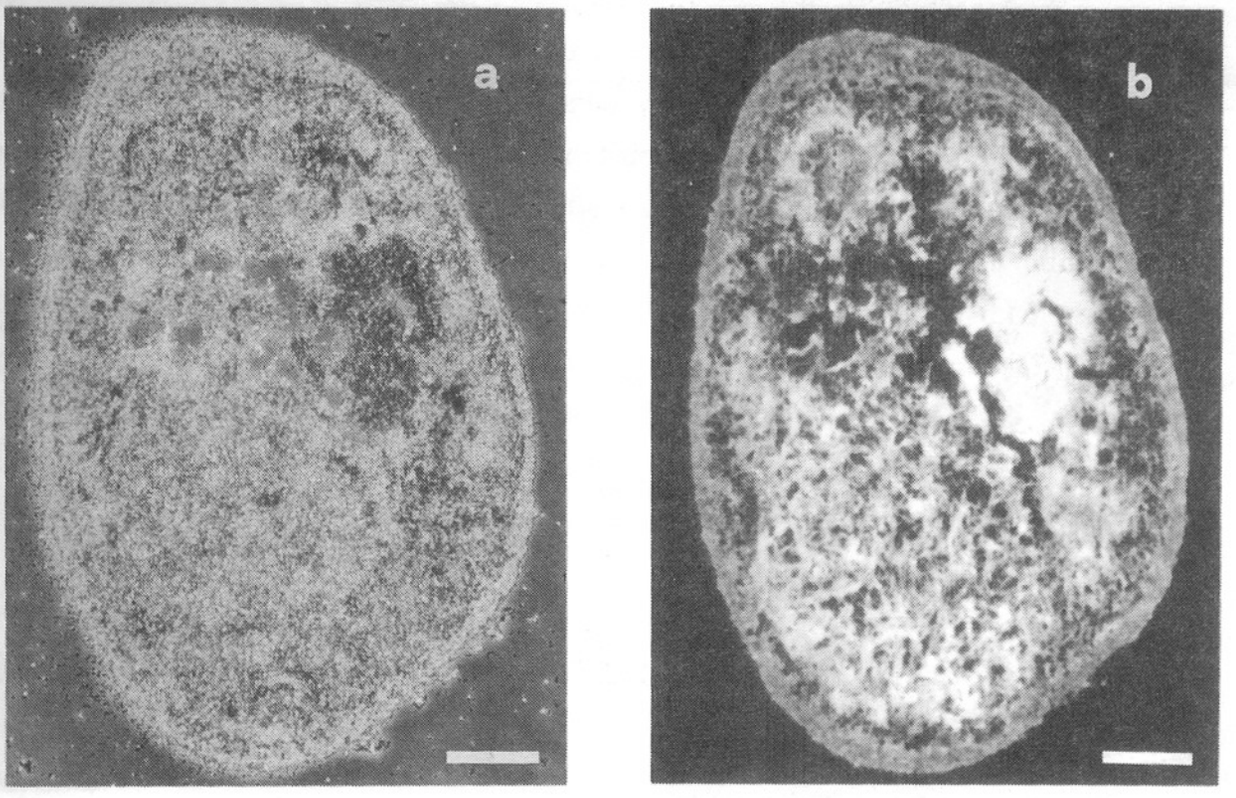

FIG. 1. Section of a typical granule under: (a) phase contrast, and (b) epi-fluorescent excitation; bar = 100 μm.

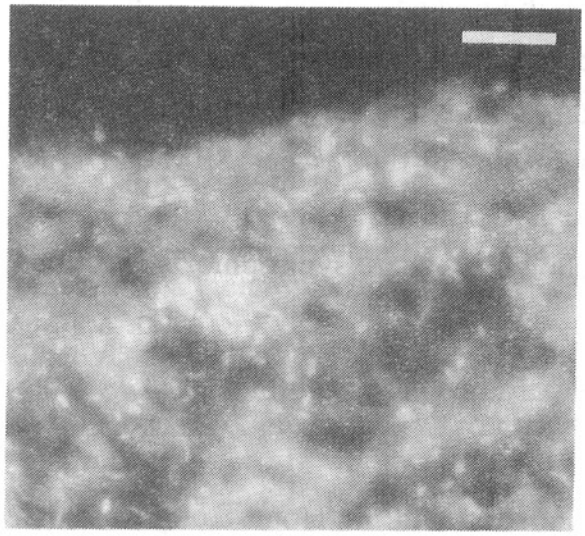

FIG. 2 Surface layer of the granule under epi-fluorescent excitation; bar = 10 μm.

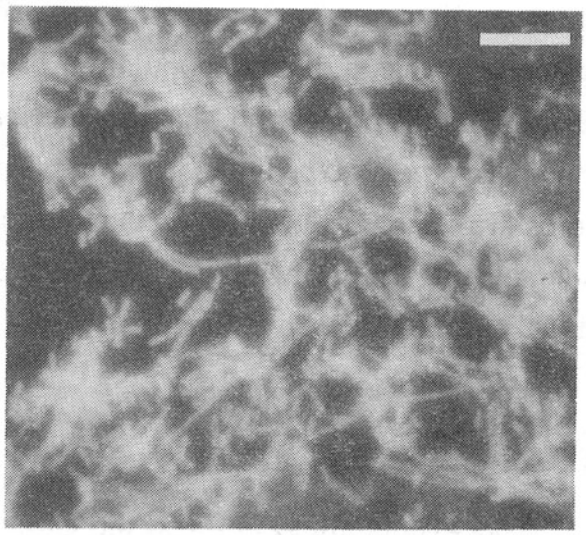

FIG. 3. *Methanothrix* in the interior of the granule under epi-fluorescent excitation; bar = 10 μm.

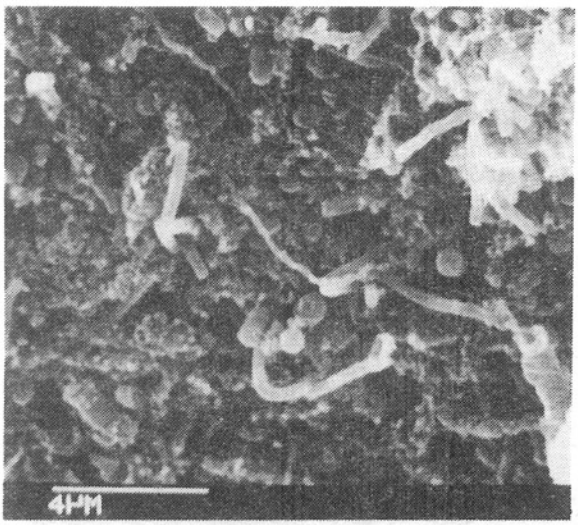

FIG. 4. SEM photo showing diverse morphology at the surface of a granule; bar = 10 μm.

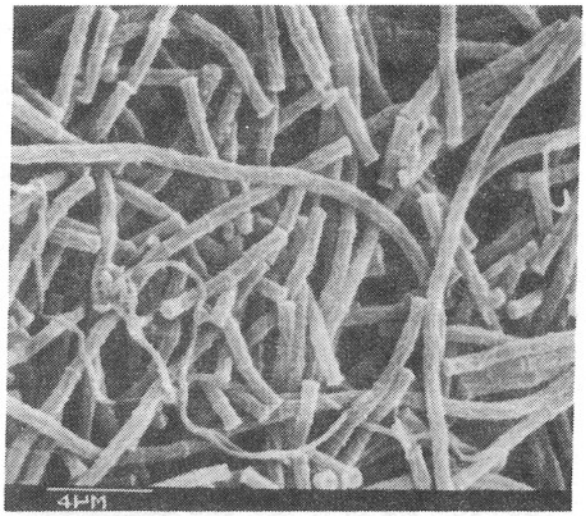

FIG. 5. SEM photo showing *Methanothrix* in the interior of a granule; bar = 10 μm.

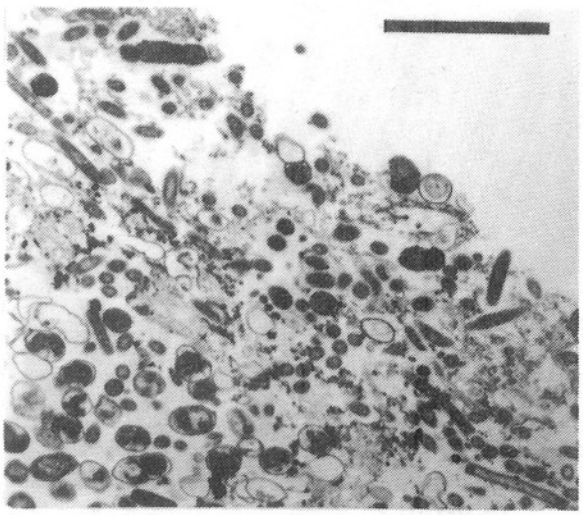

FIG. 6. TEM photo showing diverse morphology at the surface of a granule; bar = 10 μm.

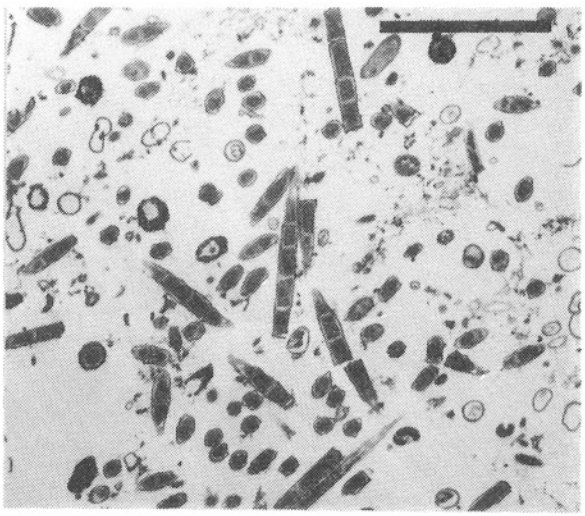

FIG. 7. TEM photo showing *Methanothrix* in the interior of a granule; bar = 5 μm.