Direct Observation of Nitrifying Biofilms on Particles by Electron Microscopy

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Abstract

Structural examinations of a biofilm immobilized on small cement ball (CB) particles in a three-phase fluidized bed reactor for a highly concentrated nitrification process were carried out. With transmission and scanning electron microscopes, a diverse microbial arrangement was determined. The scanning electron micrographs showed that a large number of clustering bacteria was covered by extracellular polymers. Between the clusters, many open spaces were found. The transmission electron micrographs showed a regular arrangement of bacteria inside the biofilm. Most clusters of *Nitrosomonas* sp.-like bacteria were found near the surface of the biofilm and the majority of *Nitrobacter* sp.-like bacteria were observed inside the biofilm. At least 14 types of bacteria were found in the mixed culture nitrifying biofilms.

Key words : biofilm, electron microscopy, microbial ecology, nitrification, *Nitrosomonas* sp., *Nitrobacter* sp., oxygen effects, three-phase fluidized bed

INTRODUCTION

A fluidized bed reactor with biofilm attached particles was highly effective for the biological treatment of wastewater¹⁻⁴⁾. Although there are many reports on the kinetics and dynamics of the nitrification process of a biofilm in a fluidized bed reactor, little information is available about the distribution of immobilized nitrifying bacteria and the structure of a biofilm. The structure of the biofilm could play an important role in the biological treatment of wastewater, because substrates transport from the bulk liquid to the microbial clusters depends on the biofilm structure.

Most of the studies concerning nitrifying biofilms have described the population dynamics and distribution of microorganisms in the biofilm by an indirect method such as examining the results of the reaction process by microsensors⁵⁾, or counting bacteria by the MPN method⁶⁾ or by the molecular probes⁷⁾. Although the former method is effective for

evaluating the biological reaction process in the system, it does not directly show the condition of the microorganisms. The latter methods only show the dominant bacteria which grow in a specific substrate, and other bacteria which only occur in small populations may be overlooked.

Microscopic methods are an important tool to examine *in situ* bacterial adhesion, growth, succession and biofilm formation at solid surfaces⁸⁻⁹⁾. With a fluidized bed reactor, the biofilm structure in an anaerobic system such as that with a methanogenic biofilm¹⁰⁾ and a denitrifying biofilm¹¹⁾ has been frequently studied. However, most of the studies on the biofilm in an aerobic process were carried out in a rotating biological contactor¹²⁾ or with attached biofilm in a laboratory device¹³⁾. Very little *in situ* observation of nitrifying biofilms in a fluidized bed reactor has been done except for the study by scanning electron microscopy (SEM)⁶⁾. Since SEM only shows the surface of the biofilm, the structure and distribution

inside the biofilm cannot be elucidated.

Our study examined the microbial ecology in a mixed-culture nitrifying biofilm immobilized on particles based on direct observation by both SEM and transmission electron microscopy (TEM). This observation was conducted simultaneously with a nitrification process, leading to an explanation of the nitrification process in the biofilm where natural competition between ammonia-oxidizing bacteria and nitrite-oxidizing bacteria can be observed.

MATERIAL AND METHODS

Reactor A nitrifying biofilm was obtained in a laboratory-scale three-phase fluidized bed bioreactor in which cement ball (CB) particles were used as media. The reactor was made of acrylic tube (5 cm in diameter and 320 cm in height). The influent ammonia-nitrogen concentration was varied in the range 70-105 g/m³, the temperature was maintained at 30 °C, and pH of the influent was kept at 8. The reactor was aerated with pure air, being operated at a superficial liquid velocity and superficial gas velocity of 69.1 and 432 m/d.

Seeding sludge and substrate The reactor was inoculated with mixed-culture microorganisms originally obtained from our laboratory inoculum tank, these microorganisms having been acclimated by ammonia-nitrogen feeding for several years. The substrate was continuously fed into the reactor, the proportions of the main substrate being $\text{NH}_4\text{HCO}_3 : \text{NaHCO}_3 : \text{K}_2\text{HPO}_4 = 10 : 3.5 : 1.4$.

Electron microscopy observation

Biofilm attaching particles were collected from the steady-state-operation reactors several times 3 months after start-up. The sample locations were chosen according to characteristics such as the diameter of the biofilm. For each sampling, 2 ml of particles were immediately fixed with Karnovsky liquid consisting of 8 % glutaraldehyde, paraformaldehyde, and a 0.2 M cacodylate buffer (pH 7.4) at 5 °C for 2 to 4 hours. The samples were then washed and stored in a buffer until required for SEM or TEM observation.

The Karnovsky-fixed samples were de-

hydrated in an ethanol series (25, 50, 75, 90 and 100 %) and then freeze-dried. Each sample was then sputter-coated with platinum by an ion-sputterer (Hitachi E 101) and examined with a scanning electron microscope (Hitachi S-570 or Hitachi S-2400).

The Karnovsky-fixed samples were postfixed with 1% OsO_4 for 40 min and then washed twice in the buffer. All samples were dehydrated in an ethanol series (25, 50, 75, 90 and 100 %) and then infiltrated with *n*-butyl glycidyl ether. Each sample was then embedded in epoxy resin (Quetol 651), and the embedded biofilms were cut into thin sections of about 60-140 nm with a glass knife and collected on 150-mesh copper grids. The thin sections from the outer and inner part of the biofilms were poststained for 15 min with uranyl acetate and then for 5 min with lead citrate, before being observed in a transmission electron microscope (JEOL JEM-100CX) operated at 80 kV. TEM photographs which clearly showed the location of the outer and inner part of the biofilms were used to examine the bacteria in terms of structure, composition, size, and distribution. To obtain information about the bacterial distribution, many electron micrographs of the thin section of the biofilms were taken, and then these were put together so that a map of the biofilms could be clearly seen.

Bacterial identification The bacterial suspension taken from the biofilms was tested for nitrification activity as described in Bergey's Manual⁽¹⁾. These results and the morphology of the nitrifying bacteria appearing in the TEM micrographs were compared to the description and the TEM micrographs in the manual⁽¹⁾. Small-population bacteria were identified from the TEM micrographs by measuring their size, position, shape and cytomembrane characteristics such as number of layers, thickness and spatial distribution. In addition, colony counts for the heterotrophic bacteria and a denitrifying activity test were also conducted.

RESULTS AND DISCUSSION

A SEM image of a nitrifying biofilm shows that thin extracellular polymers covered almost all of the bacterial clusters of the

biofilm's surface (Fig. 1). Short plump rod bacterial cells were found most frequently as part of the biofilm surface being loosely packed by the extracellular polymers to form an irregular shape (Fig. 1, A). Spaces can be clearly seen between the clusters of cells (Fig. 1, B), which act as channels for the substrate.

Figure 2 shows the TEM image of a cross-section of a nitrifying biofilm. Many kinds of microbial communities existed in the outer part. The shapes of the microorganisms included spheres (cocci), helices and rods, the dominant shape being that of short plump rods (Fig. 2, A and B). These dominant cells were frequently observed in clusters from 2 to 10 μm in size. There were clearance and smooth boundaries between the microorganism clumps and extracellular polymers. Viable bacteria and dead bacteria could be distinguished clearly by the cell contents, where dead cells without contents (Fig. 2, E) remained as cell walls because of the lysis process. A large amount of extracellular polymer (Fig. 2, F) can be clearly observed as dark-light areas existing between cells and between clusters.

All of the sections of the biofilm showed a similar arrangement, population, and distribution of bacterial cells. This indicates that the structure of these nitrifying biofilms had the same pattern. Figure 2, A and Fig. 3 (a) show that the short plump rod clusters ($1.0 \times 0.6 \mu\text{m}$) were similar in morphological appearance to *Nitrosomonas* sp.¹⁴⁾ These clusters were always located near the surface of the biofilm where the oxygen concentration was high. To differentiate, this bacterium is called *Nitrosomonas*-like type 1. Irregular cells of $1.0 \times 0.5 \mu\text{m}$ with similar size to that of *Nitrosomonas* sp., as shown in Fig. 2, B and Fig. 3 (b), could be observed inside those of type 1. These are called *Nitrosomonas*-like type 2. On the basis of these characteristics and the positive reaction in the nitrification test, these microorganisms were tentatively identified as belonging to the *Nitrosomonas* genus whose cells were uniformly covered with multilayered cytomembranes.

The inner layer of the biofilm, which was close to the CB particle surface and might have had a low concentration of substrate,

Fig. 1 Scanning electron micrograph of a nitrifying biofilm attaching particle. Thin extracellular polymers covered the irregular form of the bacterial clusters (A). The space between the clusters has the function to channel liquid (B). Bar, 6 μm .

displayed a network of dead cells (Fig. 2, E). In this layer, various cell types were found in clusters covered closely and tightly with firm extracellular polymers (Fig. 2, C and Fig. 3 (c)). The majority of tight clusters, containing only one morphological type of short rods or pear shapes, were $0.9 \times 0.5 \mu\text{m}$ long. These clusters are believed to be *Nitrobacter* sp. Figure 4 shows a TEM image of the inner part of the nitrifying biofilm. The *Nitrobacter*-like bacteria seem to be pressed into an irregular form (Fig. 4, A) because of gathering in tight clusters. In the same section, other bacteria which were not found in tight clusters had a normal form (Fig. 4, B). The *Nitrobacter*-like and *Nitrosomonas*-like cells were morphologically distinguished from each other because the *Nitrobacter*-like cell has cytomembranes composed of 4 - 6 layers of paired membrane existed in partial peripheral region of the cell (Fig. 3 (c)) while the *Nitrosomonas*-like one is uniformly covered with multilayered cytomembranes¹⁴⁾ (Figs. 3 (a) and (b)). An

(a)

(b)

(c)

Fig. 3 TEM image of nitrifying bacteria observed in the biofilm. (a) *Nitrosomonas*-like type 1 bacteria with regular shaped peripheral cytomembranes (arrow). Bar, 1 μm ; (b) *Nitrosomonas*-like type 2 bacteria with irregular shaped peripheral cytomembranes (arrow). Bar, 1 μm ; (c) *Nitrobacter*-like bacteria which only exist in a part of the peripheral region of the cell (arrow). Bar, 2 μm .

Fig. 2 Transmission electron micrograph of a nitrifying biofilm (cross-section). A. Clusters of *Nitrosomonas*-like type 1 bacteria; B. Clusters of *Nitrosomonas*-like type 2; C. Clusters of *Nitrobacter*-like bacteria; D. Other bacteria; E. Dead bacteria; F. Extracellular polymer; G. Spaces. Edge of the biofilm is shown by the arrow. Bar, 5 μm .

Fig. 4 TEM image of the inner part of the nitrifying biofilm. A. Clusters of *Nitrobacter*-like bacteria; B. Other bacteria; C. *Nitrosomonas*-like type 2 bacteria; D. Spaces between clusters; E. Dead bacteria. Bar, 5 μm .

abundant network of dead cells was also found in this layer because of the limited substrate and oxygen concentration.

Identification from the TEM images by the apparent shape, size and position of the cells indicated that at least 14 types of bacterial cells were present in these nitrifying biofilms. At least three of these were nitrifying bacteria, two being *Nitrosomonas*-like bacteria and one type being a *Nitrobacter*-like bacterium. The other 11 types were found in small populations spread among the nitrifying bacteria. Nine types were found both near the biofilm surface (outer part of the biofilm) and near the CB particle surface (inner part of the biofilm), while two types were always in the outer part and three types were always in the inner part of the biofilm. Based on the morphology, one type was similar to *Hyphomicrobium* sp., a denitrifying bacteria (Fig. 5, C). The existence of denitrifying bacteria was confirmed because the biofilm suspension gave a positive reaction in the denitrification test. The others were identified as heterotrophic microorganisms, evidence for this being that the biofilm suspension was capable of producing heterotrophic colonies on an agar plate. From the form, color, and bacterial shape observation of the colonies, 8 types of heterotrophic colonies appeared to exist in the biofilm suspension.

Fig. 5 TEM image of the outer part of the nitrifying biofilm. A. *Nitrosomonas*-like type 1 bacteria; B. *Nitrosomonas*-like type 2 bacteria; C. *Hyphomicrobium*-like bacteria. Edge of the biofilm is shown by an arrow. Bar, 5 μm .

CONCLUSION

In the nitrification process with a high ammonia concentration, the same pattern in the biofilm structure and composition of the microorganisms were observed by SEM and TEM as follows. *Nitrosomonas*-like bacteria were located near the surface of the biofilm and *Nitrobacter*-like bacteria were located in the inner part. The bacterial cells were abundant near the surface of the biofilm, while dead bacterial cells were found in large quantities in the inner part of the biofilm. At least 14 types of bacteria were found in the mixed-culture nitrifying biofilm. Three types were nitrifying-like bacteria, while other 10 types were heterotrophic-like and one was denitrifying-like.

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