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Dynamics of the Immobilization of Anaerobic Meshopilic Bacteria on a Plastic Support

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ABSTRACT

In this article, a study of the dynamics of the immobilization of mesophilic anaerobic bacteria was conducted. Low-density polyethylene (LDPE) ground to fine particles was used as immobilization support in an inverse fluidized bed. After 135 d of immobilization, the biomass concentration attained was 375 μg protein/g dry plastic support. The specific rate for acetate consumption was 0.008 μmol acetate/μg protein/h. The kinetic parameters of the bioparticles (K_s = 1.067 m*M* and V_{max} = 0.00754 μmol acetate/μg protein/h) were estimated by the initial rate method at different acetate concentrations and were compared to the values obtained by a direct parameter estimation method from the dynamic assays for acetate consumption with an initial substrate concentration of 10 m*M*. The particles were prepared for scanning electron microscopy (SEM) to evaluate the biofilm qualitatively. The SEM studies showed that the surface and cavities of the support were colonized, and the bacterial structures found were bacilli similar to *Methanothrix* as the predominant microorganism and a few groups of *Methanosarcina*-like cocci. The K_s value found is between those reported for the predominant bacterial group immobilized.

Index Entries: Anaerobic bioimmobilization; fluidized bed; plastic support; specific activity; substrate affinity.

INTRODUCTION

The waste-water treatment in fluidized beds has been extensively reported as an efficient operation for high-loading organic rates, with some advantages over the Upflow Ascendent Sludge Blanket (UASB) reactor, such as a great retention of biomass in the reactor, large superficial area for biomass attachment, and the possibility to control biofilm thickness *(1–6)*. However, the cell attachment mechanism to the support materials used in these systems is not fully understood, and considerable scientific work has been done in an attempt to describe the immobilization process to different supports and by different microorganisms in anaerobic conditions *(7–11)*.

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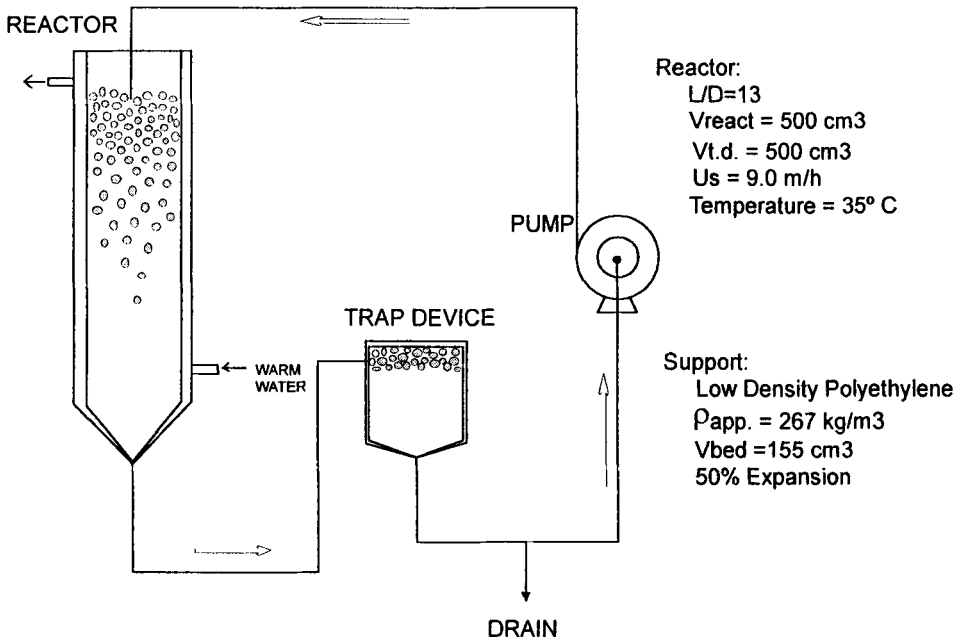

Fig. 1. Schematic diagram of the batch downflow fluidized bed used for bioimmobilization.

The objective of this work was to describe the immobilization dynamics of a mesophilic sludge on low-density polyethylene (LDPE) particles in a downflow fluidized bed similar to that of Golnzález et al. *(9)*, the specific acetoclastic activity, and the affinity for the substrate of the fully immobilized bioparticles.

MATERIALS AND METHODS

Immobilization System

The immobilization system was a batch downflow fluidized bed of 500 cm^3 (L/D 13) shown as a diagram in Fig. 1. It has a device to trap the plastic particles that flow out of the bed. The support used was LDPE pellets ground to fine particles with a size range distribution from 0.56 to 0.28 mm and with an apparent density of 267 kg/m^3. Approximately 155 cm^3 of support were fluidized at a superficial velocity of 9 m/h, and the bed expansion achieved was 50%. The reactor was operated at 35°C and pH 7.0. Every 2 d the immobilization medium *(12)* containing 1 g acetate/L and 50 mg of volatile suspended solids (VSS)/L of inoculum was replaced by fresh medium.

Inoculum

The inoculum was a mesophilic sludge with 21 g VSS/L with a specific methanogenic activity of 0.42 mmol CH_4/g VSS/h. The sludge was observed in an Olympus BH2-RFCA light and epifluorescence microscope and showed microbial structures similar to *Methanothrix* as the dominant microorganism, some grouped *Methanosarcina*-like cocci, and short bacilli, slightly curved and strongly fluorescent that could be *Methanobacterium*.

Specific Activity Determinations

The biofilm accumulation was assessed through the specific acetoclastic activity of the bioparticles at several intervals of the colonization time (15, 30, 45, 70, 100, and 135 d). All specific activity determinations were made by placing a known amount of wet particles into serologic bottles containing 20 mL of mineral medium *(12)* and 10 m*M* acetate. The acetate was analyzed with standard gas chromatography using an HP 5890 chromatograph with an AT-1000 capillary column at 120°C.

The acetate degradation rate was related to the final biomass content, which was measured by detaching the biofilm by sonication with successive washes in distilled water to quantify directly the total suspended solids (TSS) and the VSS as indicated by the standard methods *(13)*, or in phosphate buffer 0.1*M*, pH 7.0, to determine the protein content by Coomassie brilliant blue G-250 *(14)*. All quantities were related to the amount of dry support used for each determination.

Bioparticles Substrate Affinity

The affinity of the bioparticles for the substrate at concentrations of 1, 2, 3, 4, 5, 10, and 15 m*M* of acetate was estimated at the 135th d and was measured following the same methodology described. All specific activity rates were obtained by means of the initial rate method. The kinetic parameters, K_s and V_{max}, were obtained by the inverse modifications to the Michaelis-Menten model and by an integral method *(15)*. The integral method uses measurements of substrate concentration as a function of time to evaluate the kinetic parameters that fit the concentration measurements according to the linearized equation:

$$K_s \ln (S/S_0) + S_0 - S = V_{max} t \qquad (1)$$

where S and S_0 are the substrate concentrations at time t and $t = 0$, respectively.

Sample Preparation for SEM

The bioparticles were prepared following a methodology similar to that of Zellner et al. *(11)*. Prior to fixation, the bioparticles were washed with cacodylate buffer 0.1*M*, pH 7.5 (Sigma, St. Louis, MO) and placed in buffer solution with 3% of glutaraldehyde (25% aqueous solution Grade I, Sigma) for 24 h. After this time, the sample was washed again with buffer solution, dehydrated by increasing ethanol concentrations, and dried with a Labconco Lyph·Lock 4.5 lyophilizer at –50°C and 10 μ of vacuum for 3 to 5 h. The dried sample was sputtered with gold or aluminum with a Sputter-Coater Bal-Tec SCD 050 and observed at 15–25 kV in a Jeol-5200 and a Zeiss DSM 940 A scanning microscopes.

RESULTS

Immobilization Dynamics

The immobilization process was carried out for 135 d. The protein, TSS, and VSS contents attained at this time were: 0.375 mg, 106.0 mg, and 39.75 mg/g of dry support, respectively. Figure 2 shows the acetate degradation pattern at each time during the immobilization period. On the 100th d, a noticeable decrement of the specific activity was detected, owing to an unexpected malfunction of the immobilization system that led to the total leakage of the liquid phase and loss of biomass.

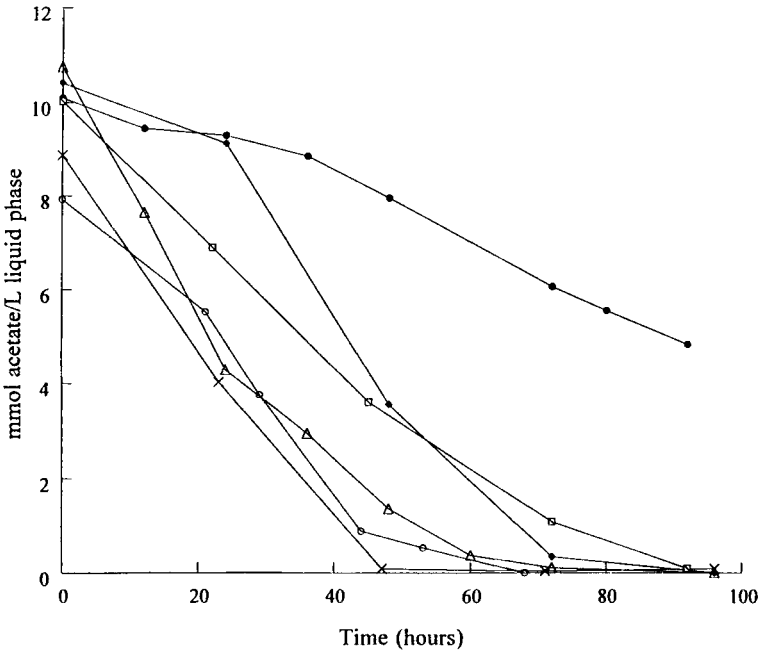

Fig. 2. Acetate degradation pattern at several times of bioimmobilization. ●, 15 d; ○, 30 d; ◆, 45 d; x, 70 d; □, 100 d; △, 135 d.

However, it can be seen that some of the activity was recovered at the 135th d, suggesting that when the biofilm is already attached to the support, the fixation of more microorganisms onto the surface is facilitated, as Meier-Schneiders et al. *(10)* have already found.

The specific acetate degradation rates at each time follow an exponential curve, and the maximum activity was found around 70th d (*see* Fig. 3). It seems that the bioimmobilization process reached a plateau after this time. This is in good agreement with previous reports that deal with batch *(9)* and continuous *(2,3,6,11)* immobilization studies, which found out that about 60 d are necessary to reach full immobilization and a steady-state organic load removal.

In this exponential curve can be distinguished two-phase bioimmobilization dynamics. The first phase corresponds to the biofilm formation, and the second one to a steady-state phase, and thereafter no activity increase is noticed, suggesting a dynamic saturation mechanism. It is not possible to say if the bioimmobilization process is limited by the rate of biomass attachment or by the biomass activity.

Affinity of the Bioparticles for the Substrate

The acetate consumption patterns at different acetate concentrations are shown in Fig. 4. It can be seen that all patterns are similar with a clear tendency to exhaust the substrate. At 15 m*M*, a slight decrement was found in the consumption rate, because there is a delay in the consumption pattern. The Michaelis-Menten-like curve constructed with the specific rates obtained is shown in Fig. 5. The maximum specific rate was found at 10 m*M*, and there seems to be a slight inhibition by the substrate at 15 m*M*.

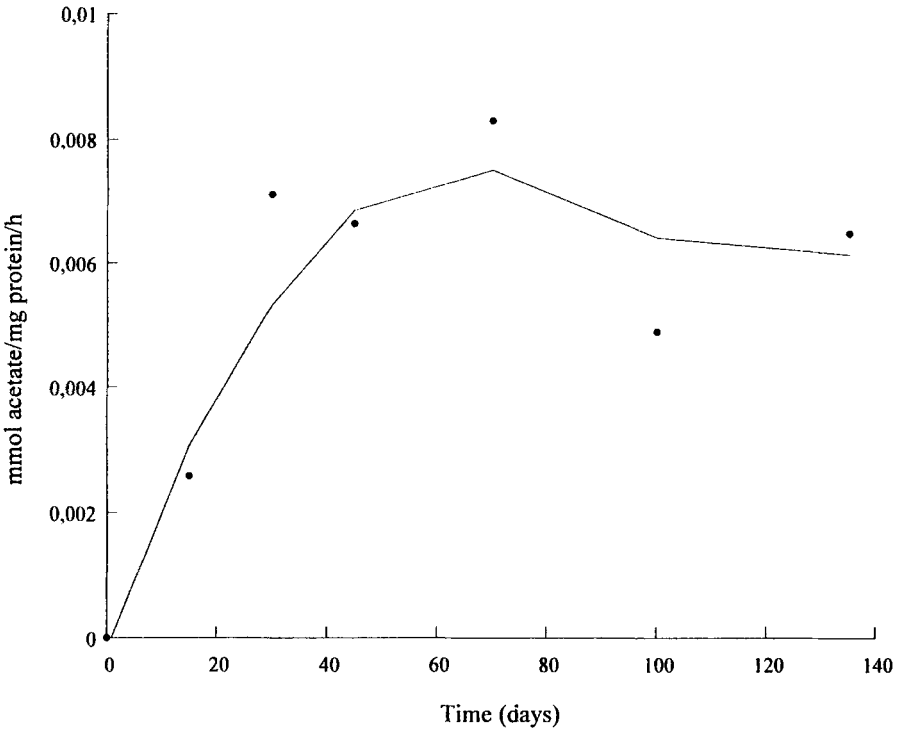

Fig. 3. Specific acetate degradation rates during the immobilization time. Note that the bioimmobilization dynamic follows an exponential behavior reaching a plateau after the 70th day of immobilization.

The kinetic parameters obtained by the Hanes-Woolf model were K_s = 1.067 m*M* and V_{max} = 0.00754 μmol acetate/μg protein/h, which are in good agreement with the experimental data (*see* Fig. 5). On the other hand, the kinetic parameters obtained by the integral method *(15)* with S_0 = 10 m*M* were K_s = 1.723 m*M* and V_{max} = 0.0098 μmol acetate/μg protein/h.

The V_{max} values obtained are similar, but there is a discrepancy between the K_s values obtained by the two methods that can be explained because the integral method considered only one substrate consumption pattern, corresponding to the highest consumption rate. Nevertheless, the K_s values obtained are in the range for those reported for *Methanothrix*. The V_{max} values are under those reported for this microorganism. It is probably because of the low biomass concentration attached to the support. The comparative values of the kinetic parameters are listed in Table 1.

SEM Studies of the Bioparticles

The SEM studies showed that the support has a furrowed surface with crevices, pleats, and cavities quite suitable for colonization (Fig. 6A,B) and apparently offered a larger surface area. The particles were colonized mainly by *Methanothrix*-like bacteria (Fig. 6C,D) forming long filaments attached to the surface of the media. The biofilm was not dense or thick; it was very superficial. The pleats and crevices were well colonized (not shown), as opposed to the smooth surfaces of the support

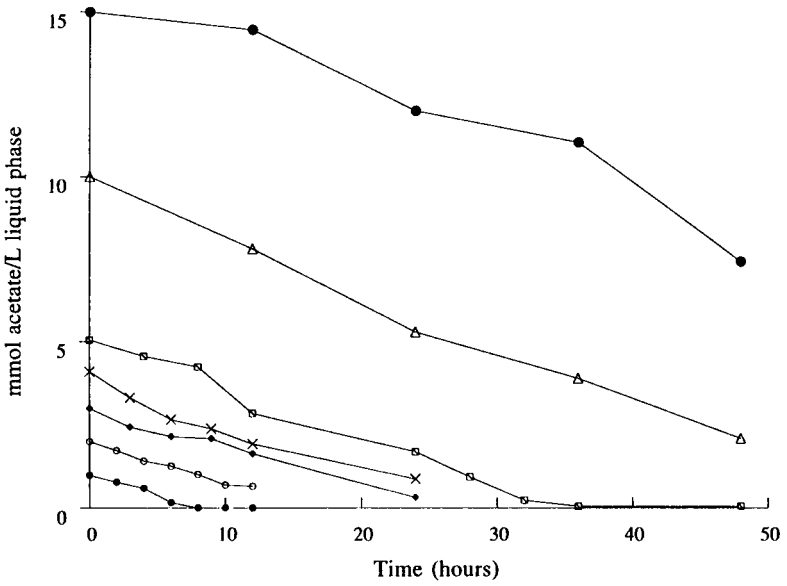

Fig. 4. Acetate consumption patterns of the bioparticles at the 135th d of immobilization. ●, acet 1; ○, acet 2; ◆, acet 3; ×, acet 4; □, acet 5; △, acet 10; ●, acet 15.

where the adhesion was poor. The *Methanosarcina*-like bacteria and the small, curved bacilli were scarcely seen on the particles.

DISCUSSION

In spite of the superficial area offered by the support, the amount of biofilm found is low, as reflected in the low acetoclastic activity. Owing to the necessary high superficial velocity used to expand the bed, the shear stress between the particles and the liquid phase produces biofilm detachment *(4)*. Low superficial velocities lead to less shear stresses, so the microorganisms settle on the surface of the support, remaining there for longer times until the irreversible adhesion occurs *(5)*. At these operation conditions, the bed does not expand enough to reach a fluidized state, and, after a time of operation, the bed fractionates and shows "channelization" or channel formation with preferential flow. This is because of the dynamic friction that the particles exert on each other and with the liquid phase because of its irregular shape and rough edge.

These factors finally determine the maximal biofilm thickness and the time to reach a steady-state phase in the bioimmobilization exponential curve. Although González et al. *(9)* found out that the system we studied is quite suitable for immobilization, this work suggests that the cell attachment mechanism and the cell reproduction rate are slow with respect to the detachment by shear stresses at which the particles are subjected in the immobilization system.

The K_s value obtained is typical to that reported earlier for *Methanothrix* bacteria, and this is supported by the SEM studies, which show that the support was colonized by *Methanothrix*-like bacteria as the most abundant acetotrophic methanogen, and the specific activities are assigned to this microorganism. The prevalence of this microorganism is owing to the low acetate concentration used to feed the reactor.

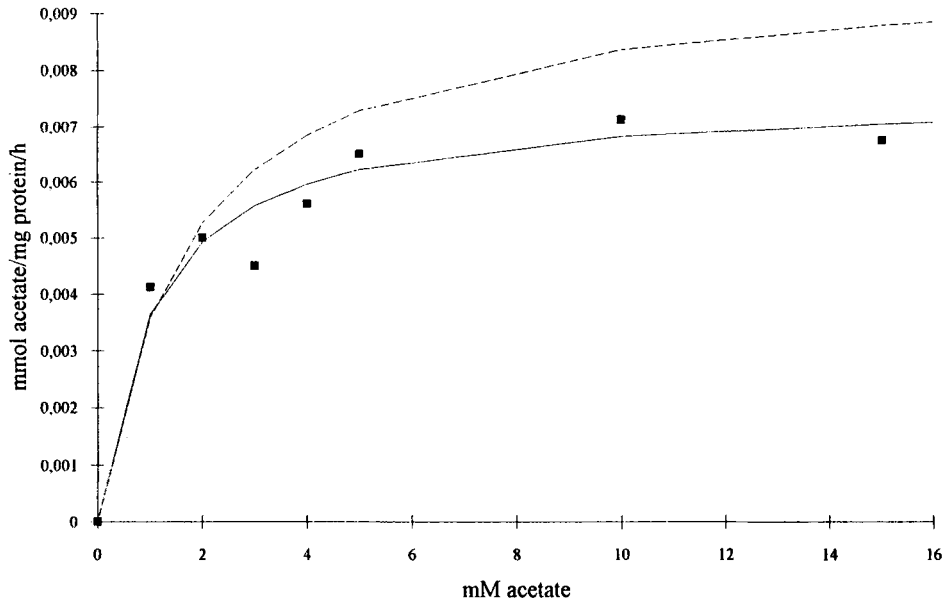

Fig. 5. Michaelis-Menten curve constructed with the kinetic parameter estimated with the Hanes-Woolf model (—) and the integral method (-----). Experimental data shown as filled squares.

Table 1
Kinetic Parameters Obtained by the Inverse Modification to the Michaelis-Menten Model and the Integral Method Compared with Those Reported in the Literature

	V_{max}, mg COD/mg VSS/d	K_s mg COD/L	References
Methanothrix	2.9–6.1	11–30	*(16)*
		45–76	*(17)*
		20	*(18)*
Methanosarcina	5.9–12	320	*(16)*
		320	*(17)*
This work	0.116	68	Hanes-Woolf
	0.15	110.24	Integral method *(15)*

With the evidence of this work and that of González et al. *(9)*, it seems that the main difference between a batch and a continuous immobilization system is that the bioimmobilization dynamics for the batch system is exponential with two stages: biofilm formation and steady-state phases.

Although for the continuous systems, the bioimmobilization pattern is sigmoidal and shows three consecutive stages: the lag, the biofilm production, and the steady-state phases, as Gorris et al. *(2)* already pointed out. The lack of a lag phase in a batch immobilization system is probably the result of the fact that there is no washout of cells from the system because it is closed. The inoculum is accumulated into the bed with subsequent reinoculations, and the microorganisms are present in great numbers since the beginning of the process. Nevertheless, the time to attain a steady-state is similar in both systems.

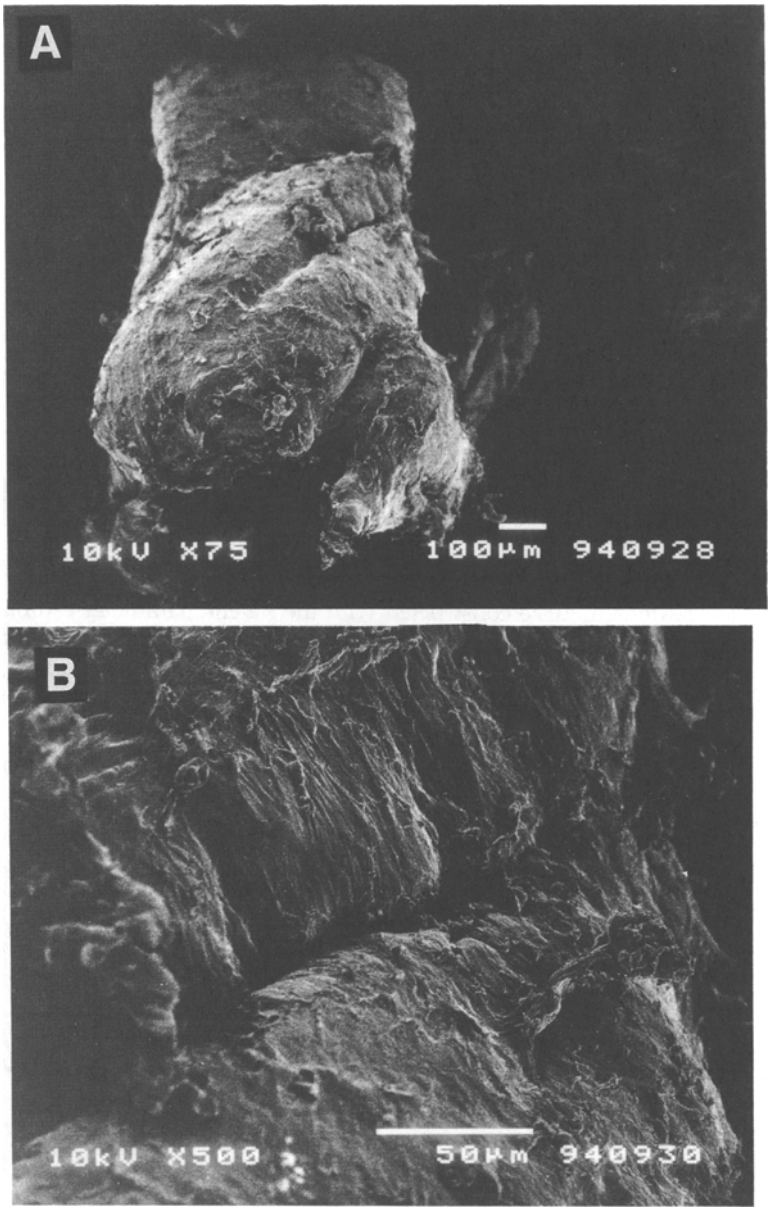

Fig. 6. **(A)** SEM photographs of the LDPE particles used as support, **(B)** a magnification that shows the furrowed surface of the support, **(C)** the *Methanothrix*-like bacteria adhered on a smooth surface of the support, and **(D)** filaments of the same microorganisms attached to a crevice of the particle.

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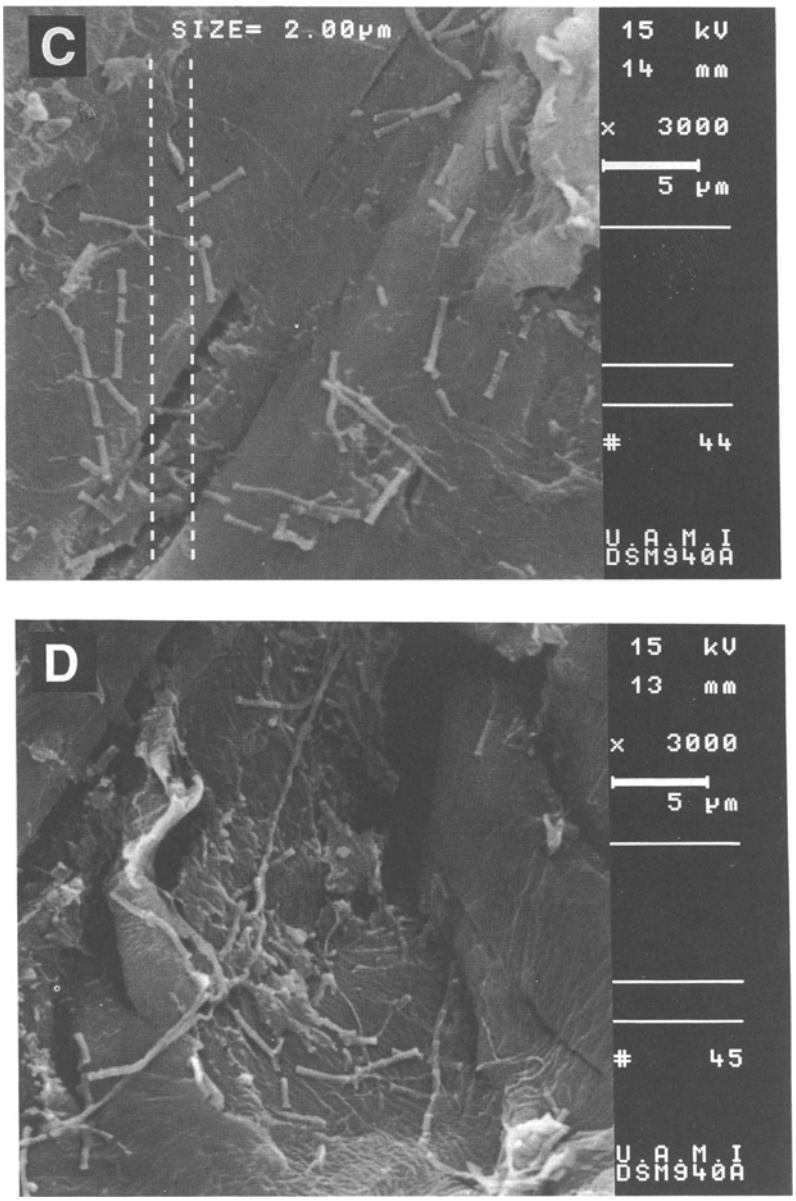

Fig. 6C and D.

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