MORPHOLOGICAL OBSERVATION AND MICROBIAL POPULATION DYNAMICS IN ANAEROBIC POLYURETHANE FOAM BIOFILM DEGRADING GELATIN

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Abstract - This work reports on a preliminary study of anaerobic degradation of gelatin with emphasis on the development of the proteolytic biofilm in polyurethane foam matrices in differential reactors. The evolution of the biofilm was observed during 22 days by optical and scanning electron microscopy (SEM) analyses. Three distinct immobilization patterns could be observed in the polyurethane foam: cell aggregates entrapped in matrix pores, thin biofilms attached to inner polyurethane foam surfaces and individual cells that have adhered to the support. Rods, cocci and vibrios were observed as the predominant morphologies of bacterial cells. Methane was produced mainly by hydrogenothrophic reactions during the operation of the reactors.
Keywords: anaerobic degradation, biofilm, microbial population dynamic, protein, gelatin.

INTRODUCTION

Immobilized cell bioreactors are the system most applicable to anaerobic wastewater treatment. Operating stability resulting from the high concentration of biomass and high cellular retention time is the main advantage that makes this technology attractive.

Studies on the dynamics of biofilm formation can be useful for achieving a better comprehension of fundamentals of biochemistry, thus resulting in important information for process design and operation. Moreover, such studies can aid in elucidating some still obscure aspects of biochemical pathways and adhesion patterns.

Protein is the main constituent of domestic sewage and it is present in a variety of industrial wastewaters. Torres (1992) reported that protein hydrolysis was the limiting step in anaerobic degradation of domestic sewage in an up-flow anaerobic sludge blanket reactor. This limitation is probably related to the complexity of the protein molecules. Therefore, studies on anaerobic protein-degrading microorganisms can be useful for verifying biochemical pathways in bioreactors.

Studying protein degradation by human intestinal bacteria, Macfarlane et al. (1986) concluded that the predominant proteolytic bacteria in fecal samples were identified as *Bacteroides* spp. and *Propionibacterium* spp.. Other proteolytic bacteria occurring in lesser numbers were identified as belonging to the genera *Streptococcus*, *Clostridium*, *Bacillus* and *Staphylococcus*. The syntrophic associations of this kind of microorganisms and methanogenic archaea were reported by Stams et al. (1994), as being of extreme importance to the process of anaerobic degradation of protein.

This paper reports on the results obtained from

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experiments carried out in differential reactors operating under anaerobic conditions in order to evaluate biofilm formation and the fate of procaryotic cell morphologies in the process. Polyurethane foam was chosen as material support for evaluating the formation and evolution of proteolytic biofilm because it has characteristics favorable to adhesion of anaerobic microorganisms.

MATERIALS AND METHODS

Four differential reactors with a volume of 15 ml were filled with cubic matrices of polyurethane foam (5 mm side). Afterwards, they were subjected to a liquid superficial velocity of 0.03 cm.s^{-1} by pumping 2 liters of a synthetic protein-based substrate from an agitated vessel. This vessel was maintained at 5°C to minimize the occurrence of biochemical reactions outside the reactor. The substrate was recycled in a closed circuit and heated to 30°C before entering the reactor.

The synthetic substrate with a chemical oxygen demand (COD) of 500 mg.l^{-1} was composed of gelatin as the sole carbon source (~219 mg of bovine albumin serum per liter) in addition to inorganic nutrient salts. The biomass used as inoculum was obtained from the effluent of an anaerobic packed-bed reactor treating a gelatin-based substrate. Biomass was added to the substrate in the agitated vessel during the first 72 hours of the experiment. Before starting, the vessel was flushed with nitrogen (100%) to remove oxygen from the liquid. Figure 1 shows the experimental apparatus used in this research.

The formation of the anaerobic biofilm in polyurethane foam particles was evaluated during 22 days. Each reactor was taken out on the 6th, 11th, 16th and 22nd days and the correspondent colonized matrices were subjected to scanning electron and optical microscopy analyses. This procedure was followed to evaluate biofilm formation and characteristics.

Samples for scanning electron microscopy were subjected to the technique developed by Nation (1983) and adapted to bacterial biofilms by Araujo (1995). For observation by optical microscopy, polyurethane foam particles were rinsed with distilled water in N$_2$ atmosphere (100%), and drops of the resulting liquid were examined under phase-contrast microscopy. Fluorescence was verified by using a UV light source attached to an Olympus BHT 2 microscope.

RESULTS AND DISCUSSION

Microbial colonization and biomass retention in polyurethane foam matrices inside the differential reactors treating gelatin occurred in different patterns. Figures 2 a, b and c show, respectively, cell aggregates entrapped in matrix pores, thin biofilms attached to inner polyurethane foam surfaces, and individual cells that had adhered to the support, probably preceding biofilm formation. These immobilization patterns were similar to those previously described by Varesche et al. (1997), who observed polyurethane foam matrices taken from a fixed-film reactor treating glucose-based substrate. Gijzen et al. (1988) reported on the adherence of methanogenic archaea to polyurethane foam matrices from a reactor treating organic acids as well. These results contradict Fynn and Whitmore's (1984) findings on the adherence of enriched methanogenic culture to polyurethane foam. According to these authors, this adhesion was weak, making removal of the biomass from the support relatively easy. Mixed anaerobic cultures, however, tend to adhere firmly to that type of support.

The morphologies observed in polyurethane foam samples are shown in Figure 3. The first samples corresponding to day 6 were yellowish or blackish, depending on their relative position in the differential reactor. The yellowish samples had a predominance of rod morphology (rod 1), resembling the genus *Clostridium*. This supposition was based on the morphology and the selective substrate (gelatin) used in the experiments. Moreover, gelatin that has not hydrolyzed is one of the keys to the presumptive identification of species in the genus *Clostridium* (Sneath, 1986).

Species of this genus metabolize multiple amino acids in a fermentative pathway (McInerney, 1988). This occurs under intensive protein degradation that involves the use of one amino acid as electron donor and another as electron acceptor, thus resulting in deamination and decarboxylation of the amino acid (Atlas, 1996). The predominant presence of *Clostridium* in anaerobic sludge samples taken from a domestic sewage treatment plant was previously reported by Siebert and Torien (1968). In fact, according to The Merck Index (1996), approximately 70% of the gelatin composition is due to the presence of only five amino acids (glycin, alanin, prolin, glutamate and arginin). Moreover, there are some well-known species of the *Clostridium* genus involved in the fermentation of these compounds (McInerney, 1988; Stams et al., 1994), such as

C. sticklandii, which degrades glycin, prolin and arginin to ornitin, acetate and aminovalerate and *C. histolyticum*, *C. purinolyticum* and *C. sporogenes*, which convert glycine to acetate and alanine to acetate and propionate (only *C. hystoliticum*).

Cocci morphologies were also found as either isolated bacteria or conglomerates in the biofilm. According to Cheng et al. (1979), *Staphylococcus* and *Streptococcus* are also proteolytic facultative anaerobes. Madigan et al. (1997) suggested that these bacteria are responsible for the yellowish color, since they have a yellow carotenoid pigment. Curved rods resembling sulphate-reducing bacteria were also observed in the yellowish matrix samples. Therefore, hydrolysis and acid fermentation were the predominant process inside the yellowish matrices.

The same morphologies associated with proteolytic and fermentation processes were found in the blackish matrices. However, the presence of rods and cocci, probably related to hydrogenothrophic methanogens, was also verified under fluorescence microscopy. Such organisms utilize acetate as the carbon source for growth but not for energy production. As amino acids and acetate stimulate the growth of some species (König & Stetter, 1988), part of the acetate removed from the liquid can probably be associated with the growth of these microorganisms and not with energy production.

Samples collected on day 11 were all blackish. The same species persisted in the samples, in addition to three other morphologies: rods (rod 2) bigger than the ones previously observed, filament bacteria and a typical Spirochaeta morphology. The rods and the filament bacteria could be related to the genera *Clostridium* or *Eubacterium* since they were hydrolytic, fermentative and strictly anaerobic microorganisms (Sneath, 1986).

Methanosaeta-like morphologies were observed in samples from the 16th day. However, the number of such microorganisms was not high. Apparently, in this process methane production by aceticlastic reactions was not effective during the first 22 days of operation, as in the typical anaerobic reactors fed with low-strength substrates and operated for long periods. The conversion of acetate to methane probably begins with the oxidation of acetate to H_2 and CO_2 in a first step and ends with the reduction of CO_2 to CH_4 using H_2. This mechanism was observed by Zinder and Koch (1984) in a thermophilic coculture of two rods fed with an acetate-containing medium, of which only one was a methanogenic organism.

Fluorescence microscopy analyses indicate significant growth of the methanogens from the 11th to the 16th day.

The samples taken on the 22nd day showed the predominance of the fluorescent methanogens. Other morphologies previously associated with the fermentative acidogenic pathway were also observed.

Although some biological characteristics of the biomass inside the matrices have changed from day 6 to day 22, the operating stability of the reactors was achieved from day 10 onward. Substrate consumption, monitored as protein contents, resulted in removal efficiencies of approximately 90%.
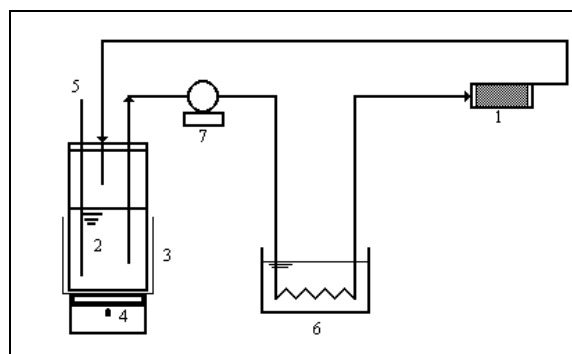
Figure 1: Scheme of the apparatus with anaerobic differential reactors. (1) Differential reactor, (2) agitated vessel, (3) ice bath, (4) magnetic stirrer, (5) sampling port, (6) heat exchanger, (7) peristaltic pump.

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(a)   (b)   (c)
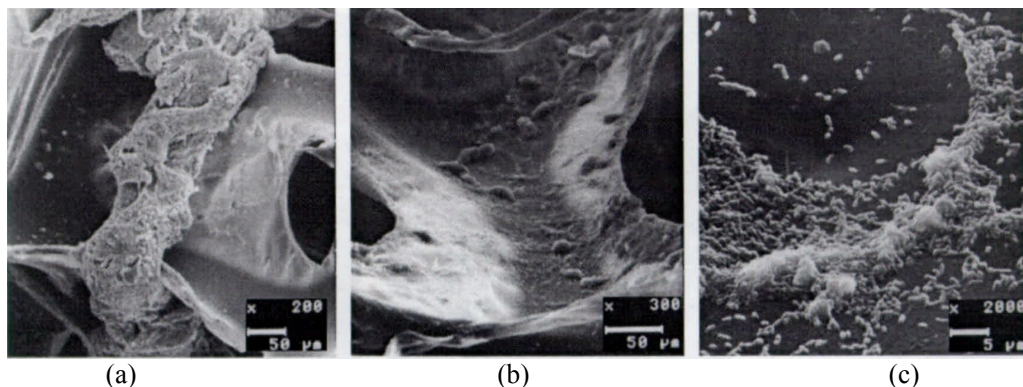
Figure 2: Microbial colonization and biomass retention in polyurethane foam matrices: (a) cell aggregates entrapped in the matrix pores, (b) thin biofilms attached to the inner polyurethane surface, and (c) individual cells that had adhered to the support.

(a)   (b)   (c)

(d)   (e)   (f)

(g)   (h)   (i)
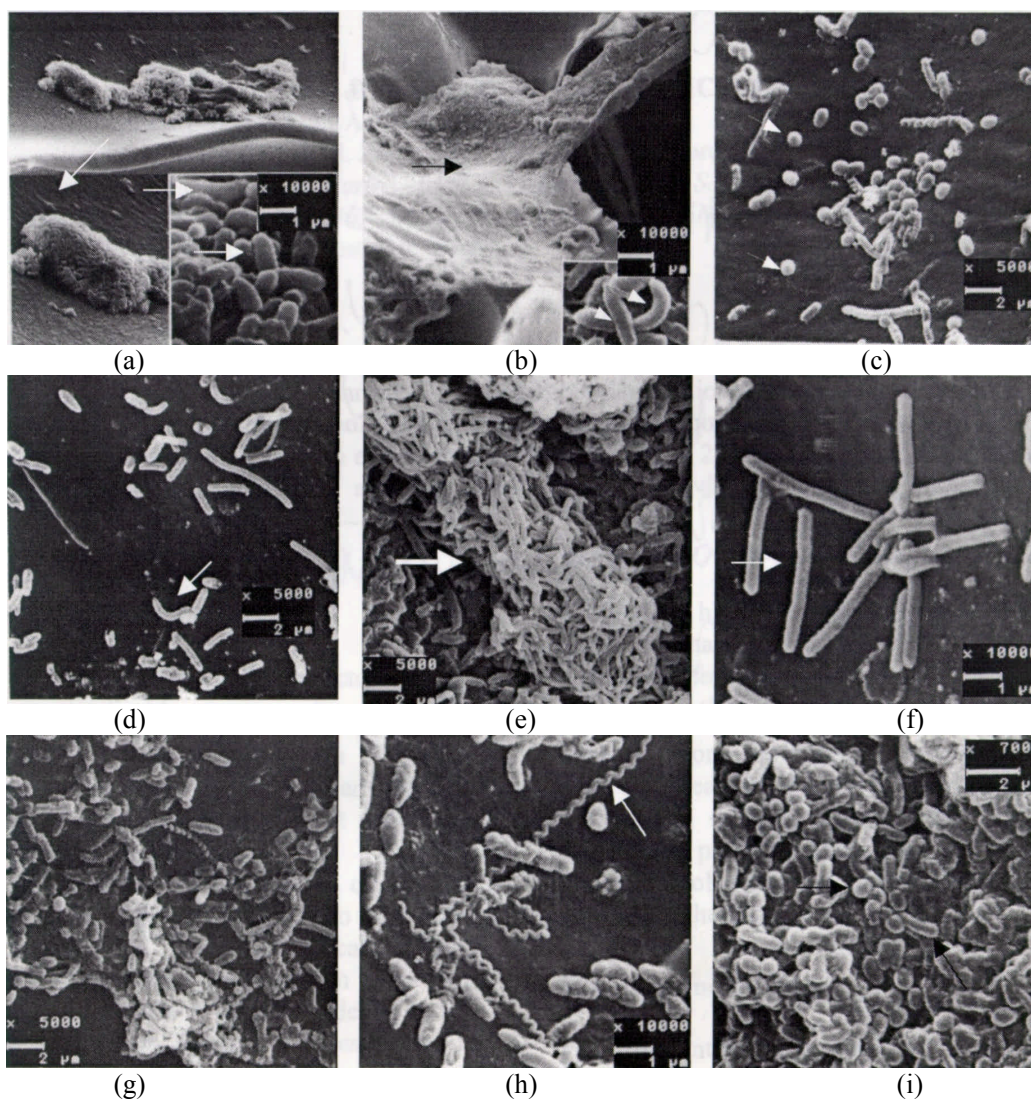
Figure 3: Morphologies observed in the polyurethane foam matrices: (a) cell aggregates with predominance of rod 1 shown in the detail; (b) thin biofilm with rod 1 and curved rods shown in the detail; (c) cocci; (d) filament bacteria, rod 1, rod 2, curved rod; (e) aggregates containing rod 2 predominantly; (f) rod 2; (g) all morphologies found; (h) Spirochaete; (i) cocci and rod 1.

CONCLUSIONS

From the results obtained in this work, the following conclusions can be drawn:

a) Three patterns of biomass immobilization could be observed on the polyurethane foam matrices: cell aggregates entrapped in matrix pores, thin biofilms attached to inner polyurethane foam surfaces and individual cells that had adhered to the support. These patterns were also observed by Varesche et al. (1997) for the same support and with glucose-based substrate.

b) Gelatin as the sole carbon source favored the growth of rods, cocci and vibrios, which were observed as the predominant morphologies attached to polyurethane foam matrices throughout the experiment.

c) The methanogenic production by aceticlastic reactions was not effective during the experiment. The conversion of acetate to methane probably begins with the oxidation of acetate to H_2 and CO_2 in a first step and ends with the reduction of CO_2 to CH_4 using H_2.

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