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The use of polyurethane foam for microbial retention in methanogenic fermentation of propionate

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Summary. The use of polyurethane foam (PUF) as a microbial support carrier was evaluated with a mesophilic propionate-acclimatized sludge. The acclimatized sludge could be immobilized rapidly and stably in PUF of smaller pore size under shaking conditions. The sludge retained in PUF could maintain a high propionate metabolic activity for a long period. High conversion rates of propionate to methane of 23–65 g chemical oxygen demand (COD)·l^{-1}·day^{-1} could be achieved in reactors packed with PUF-retained sludge. A dense sludge of 0.08–0.25 g mixed-liquor volatile suspended solids (MLVSS)·cm^{-3} was retained in PUF. Microscopic analysis suggested that filamentous microorganisms, e.g., *Methanothrix* spp. could play an important role in the efficient retention of acclimatized sludge in PUF.

Introduction

In recent years, various reactors for anaerobic biological treatment of organic waste-waters have been developed. In anaerobic treatment processes, the retention of high concentrations of active cell mass is an important prerequisite for the development of high-rate treatment system. Generally, retention can be achieved by the adhesion of microorganisms on inert carriers such as sand, clay, polyvinyl chloride, and porous plastic pads in various reactor configurations.

Reticulated polyurethane foam (PUF) has been also reported to be excellent for colonization by methanogenic microflora (Fynn and Whitmore 1982, 1984; Huysman et al. 1983; Poels et al. 1984; Rozzi et al. 1989). The use of reticulated foam was first introduced for cell immobilization in aerobically activated sludge processes and has been employed successfully (Atkinson et al. 1981; Walker and Austin 1981). This process exploits the natural ability of microorganisms to form biofilms on inert materials. The porous pads added to the fermentor become naturally filled with microorganisms under fluidized conditions (Atkinson et al. 1979, 1981; Walker and Austin 1981; Fynn and Whitmore 1984).

The authors previously investigated the kinetics of inhibition of the methanogenic fermentation of propionate with propionate-acclimatized sludge as a laboratory model system (Fukuzaki et al. 1990). The rate of propionate degradation was very low and was inhibited strongly by H_2, acetate, and propionate. Although the majority of microorganisms in this acclimatized sludge were present in the form of flocs (size, 150–300 μm), when the flocs were disrupted, propionate degradation deteriorated, probably due to inefficient metabolic interaction between H_2, acetate, and propionate. This suggested that the formation of microbial aggregates was essential for the facilitation of interspecies H_2 transfer in methanogenic fermentation (Thiele et al. 1988).

In the present study, we have evaluated the use of PUF as a support carrier for the retention of sludge capable of converting propionate to methane. The acclimatized sludge could be easily retained in PUF pads where it maintained high methanogenic activity for a long period. Only little cell leakage took place under shaking conditions, suggesting that high-rate fermentation of propionate to methane might be possible using PUF-retained sludge. Hence, methanogenic fermentation of propionate was conducted in packed-bed reactors with PUF-retained sludge.

Materials and methods

Microorganism and media. Floculated propionate-acclimatized sludge (floc diameter, 150–300 μm) was used (Fukuzaki et al. 1990). A sulphate-free minimal medium (pH 6.5–6.7) contained the following (per litre of deionized water): K_2HPO_4, 0.7 g; KH_2PO_4, 0.45 g; NH_4Cl, 1.0 g; $MgCl_2 \cdot 6H_2O$, 0.82 g; $CaCl_2 \cdot 2H_2O$, 0.25 g; NaCl, 2.25 g; L-cysteine·HCl, 0.3 g; $FeCl_3 \cdot 6H_2O$, 4 mg; vitamin solution (Wolin et al. 1963) without vitamin B12, 10 ml; trace element solution (Mazumder et al. 1986)

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without Na_2EDTA and $FeSO_4$, 6 ml; titanium (III) citrate (Zehnder and Wuhrmann 1976), 0.075 mmol; propionate as sole carbon and energy source, 0.9–9.4 g. Medium preparation was as reported previously (Mazumder et al. 1986).

Immobilization of sludge in polyurethane foam. All manipulations were carried out under an O_2-free atmosphere of N_2 gas. One hundred pieces of PUF (5 mm cubes; apparent density, 0.05 $g \cdot cm^{-3}$; porosity, 97%) with pore sizes of 20, 30, 40, and 50 pores per linear inch (ppi) were added to 720-ml serum vials containing 100 ml medium with 60 mM propionate, which were then sealed with butyl rubber stoppers. The headspace of the vial was evacuated and stood for 10 min to remove internal air from the PUF, and then the headspace pressure was adjusted to 1 atm (101.3 kPa) with O_2-free N_2 gas. After repeating this manipulation twice, titanium (III) citrate was added as a reductant. For the immobilization of acclimatized sludge, 100 ml sludge [4.0 g mixed-liquor volatile suspended solids (MLVSS)$\cdot l^{-1}$] was inoculated into the vial containing PUF. The vial was incubated at 37° C with shaking (100 rpm). The concentration of free sludge outside the PUF was measured regularly to check the retention of microbial cells by the PUF.

The PUF-retained sludge obtained (10–20 pieces) was transferred piece by piece into 125-ml serum vials containing 50 ml medium with 20 mM propionate and these vial cultures were acclimatized for 1 month by transferring PUF-retained sludge to fresh medium after every batch culture.

Kinetics of propionate utilization. All cultures were conducted in 125-ml serum vials containing 50 ml medium at 37° C with shaking (100 rpm). To determine the effects of initial propionate concentration and pH on propionate utilization by PUF-retained sludge, initial propionate concentrations of 4–140 mM were used at pH levels of 6.5 and 7.0. The 15 pieces of PUF (50 ppi)-retained sludge acclimatized for 1 month was inoculated into the 125-ml vials. The propionate utilization rate was calculated from the disappearance of propionate for 24 h after incubation. The propionate utilization rate was divided by the respective MLVSS retained in PUF to express the specific rate of propionate utilization. The relationship between the specific rate of propionate utilization and initial propionate concentration was analysed by using a second-order substrate inhibition model (Fukuzaki et al. 1990):

$$q_s = \frac{q_m S}{K_s + S + S^2/K_i} \quad (1)$$

where q_s is the specific rate of propionate utilization, millimoles per gram of MLVSS per day; q_m is the maximum value of q_s; S is the initial concentration of undissociated propionic acid, millimolar; K_s is the substrate saturation constant, millimolar; and K_i is the substrate inhibition constant, millimolar.

Packed-bed reactor. Two types of reactor were used. One was a cylindrical glass column reactor (2.7 cm diameter × 17 cm length) (Fig. 1A) with a working volume of 85 ml. Medium was supplied from the bottom by a peristaltic pump (Decarf N-10, Taiyo, Japan) and effluent (gas-plus-liquid) was discharged from the top of the reactor. The other was a flat-type plastic reactor (25 cm length × 7.5 cm width × 2.7 cm height) (Fig. 1B) with a working volume of 200 ml. The depth of medium in the reactor was about 1.1 cm. Culture medium and gas were discharged from the reactor separately. These reactors were operated at 37° C without pH control.

Firstly, the acclimatized sludge was incubated with PUF (50 ppi) pads in 720-ml serum vials under shaking to gain PUF-retained sludge. After immobilization, the PUF-retained sludge was further acclimatized in repeated batch culture for 1 month. Then, the cylindrical and flat-type reactors were packed with the PUF-retained sludge, leaving 45% and 35% of apparent void fraction, respectively. Batch cultures were conducted after packing, and when gas production ceased due to propionate exhaustion,

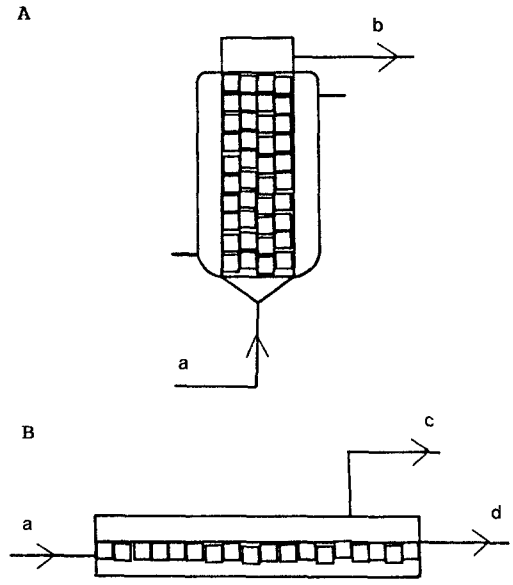

Fig. 1 A, B. Schematic diagram of packed-bed reactors. **A** Cylindrical reactor. **B** Flat-type reactor. *a*, medium inlet; *b*, gas-plus-liquid outlet; *c*, gas outlet; *d*, liquid outlet

continuous cultures were started by feeding fresh medium. The loading rate was increased after a steady state had been established, as judged from the gas production rate and propionate concentration in the effluent.

Microscopy. Electron microscopic observations were carried out as follows. PUF-retained sludge was soaked in a fixation solution consisting of 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 h. Samples were washed with deionized water three times and dehydrated by successive transfers in a series of 50, 60, 70, 80, 90, 95, and 100% ethanol solutions. Then, the sample was dried in liquid carbon dioxide to the critical point. Dry samples were mounted on scanning electron microscope (SEM) stubs with silver paint and then coated by sputtering with gold. The sections were examined and photographed with a JSM (Jeol, Tokyo, Japan).

Analyses. Fatty acids and gas composition were determined by gas chromatographs equipped with a flame ionization detector and a thermal conductivity detector, respectively (Fukuzaki et al. 1990). To determine the cell mass retained in PUF, PUF-retained sludge was dried at 105° C for 24 h, and the dry weight was determined. Mixed-liquor suspended solids (MLSS) in PUF was calculated from the difference between the weights of the dried PUF-retained sludge and PUF (apparent density, 0.05 $g \cdot cm^{-3}$). Then, dried PUF-retained sludge was ashed by burning in a crucible to constant weight, and the weight of the ash was determined (no ash originated from PUF). The MLVSS in PUF was calculated from the difference between the weights of MLSS and ash. Protein was determined by a dye-binding method described previously (Mazumder et al. 1986). Mineral composition of the ash accumulated in PUF was determined by atomic adsorption, inductively coupled plasma, or EDTA titrimetric methods (American Public Health Association, 1989) in the Kawasaki Steel Techno-research Corp., Mizushima, Japan.

Chemicals. All chemicals were of reagent grade purchased from commercial sources. Polyurethane foams (Bridgestone Co., Tokyo, Japan) were gifts from the Kanegafuchi Chemical Industry Co., Takasago, Japan. N_2 gas of >99.9999% (v/v) purity (Chugoku Teisan, Hiroshima, Japan) was used without any treatment.

Results and discussion

Immobilization of sludge in PUF

To examine the influence of the PUF pore size on microbial immobilization, four kinds of PUF with pore sizes of 20, 30, 40, and 50 ppi were used as support carriers. During incubation of the sludge with different PUFs, the concentration of free sludge outside the PUF gradually decreased as a function of the PUF pore size (Fig. 2). The efficient immobilization into PUF occurred in a culture containing PUF of smaller pore size, e.g., 50 ppi. During 10 days of shaken culture, approximately 95% of the inoculated sludge was immobilized in PUF-50 ppi, while only 20% was retained in PUF-20 ppi. Hence, PUF-50 ppi was chosen as a support carrier for subsequent experiments. From a practical viewpoint, it is of interest that immobilization of the propionate-acclimatized sludge could be performed efficiently and easily into the meshes of PUF.

Figure 3 shows SEM micrographs of microorganisms retained in PUF pads cultivated for 14 days after immobilization. Microorganisms grew entwined around the PUF matrix (Fig. 3A). A more detailed micrograph (Fig. 3B) indicates that filamentous microorganisms adhered to the matrix, suggesting that *Methanothrix*-like microorganisms were dominant and might be playing an important role in stimulating the immobilization process of the acclimatized sludge in PUF. In relation to this, it has been demonstrated that *M. soehngenii* adhered preferentially to polymeric surfaces with high hydrophobicity due to the more hydrophobic nature of their cells (Verrier et al. 1988), suggesting that adhesion of *Methanothrix* spp. to the hydrophobic surface of polyurethane foam would occur easily.

During 30 days of cultivation of PUF-retained sludge in repeated batch culture under shaking conditions (100 rpm), little cell leakage took place (10–40 mg protein $\cdot l^{-1}$ as a measure of cell concentration) in each batch culture. The degradation of propionate to methane by PUF-retained sludge occurred stoichiometrically without any accumulation of H_2 or acetate (data not shown). This suggested that a well-organized microbial ecosystem might be established by the formation of flocs within the PUF. It could be concluded that PUF was a suitable carrier for enrichment of propionate-acclimatized sludge involving obligate syntrophic bacteria.

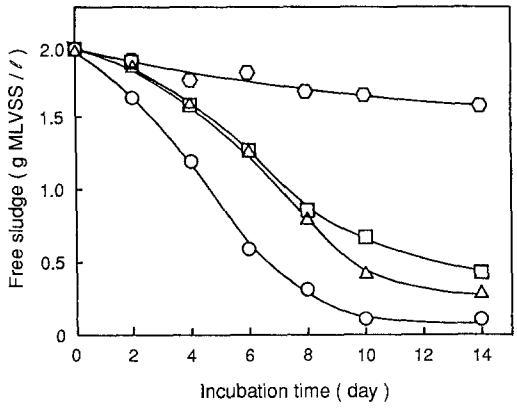

Fig. 2. Immobilization of propionate-acclimatized sludge in polyurethane foam (PUF) of different pore sizes. Each PUF was cut into 5 mm cubes before use. Immobilization of the sludge was carried out in 720-ml serum vials containing 200 ml medium and 100 pices of PUF pads. Culture was carried out at 37° C under shaking (100 rpm): ○, 20 pores per linear inch (ppi); □, 30 ppi; △, 40 ppi; ○, 50 ppi; MLVSS, mixed-liquor volatile suspended solids

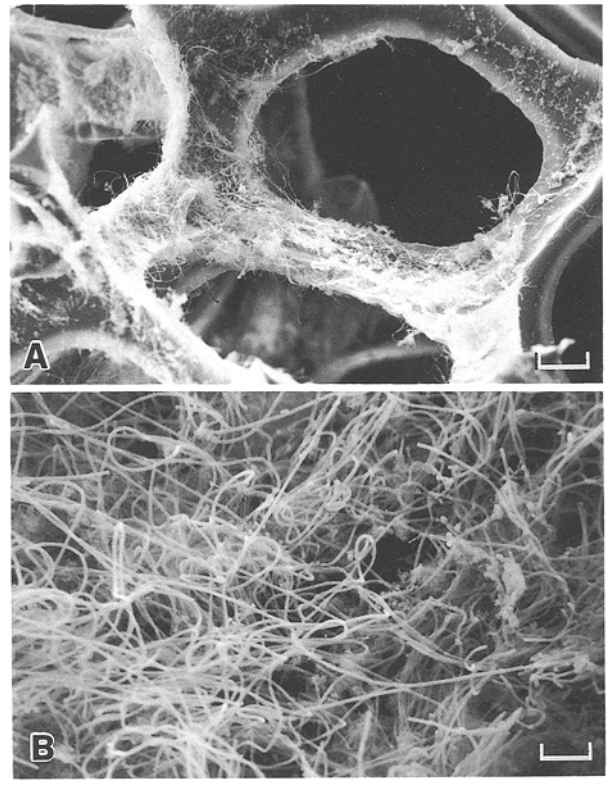

Fig. 3 A, B. Scanning electron microscope (SEM) micrographs of microorganisms retained on the PUF matrix. The PUF-retained sludge was cultivated for 14 days after immobilization. **A** Bar = 50 μm. **B** Bar = 10 μm

Kinetics of propionate consumption by PUF-retained sludge

Propionate disappearance was measured with different initial concentrations of propionate at pH values of 6.5 and 7.0. The rates of propionate utilization obtained were plotted against the concentrations of undissociated propionic acid (Fig. 4) together with previous data (broken line) obtained from flocculated sludge (150–300 μm) (Fukuzaki et al. 1990), since it has been demonstrated that inhibition by propionate was caused by undissociated propionic acid (Fukuzaki et al. 1990). The solid line in Fig. 4 was derived by data analysis based on a non-linear regression method and Eq. 1. The kinetic constants of Eq. 1 derived from data fitting were as follows: $K_s = 81.9$ μM; $K_i = 4.2$ mM; and $q_m = 2.31$ mmol $\cdot$ g MLVSS$^{-1} \cdot$ day^{-1} compared with the previous ones ($K_s = 15.9$ μM; $K_i = 0.79$ mM; and

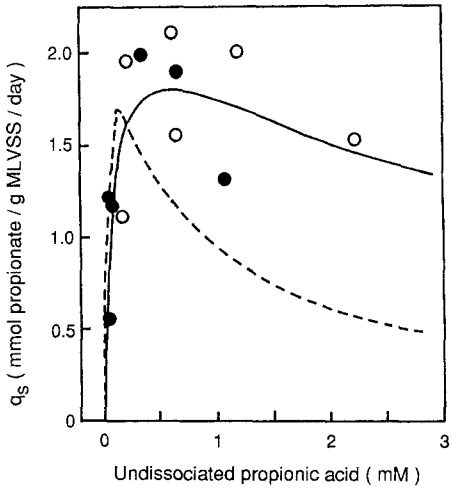

Fig. 4. Propionate inhibition against specific rate of propionate utilization (q_s) by PUF-retained sludge. Initial cell mass retained in PUF (50 ppi, 15 pieces) was 0.182 g MLVSS. Cultures were incubated in 125-ml serum vials (medium, 50 ml; headspace, initially N_2) at 37° C with shaking (100 rpm). The q_s values were calculated from the disappearance of propionate for 24 h after incubation. The concentration of undissociated propionic acid was calculated by $pK_a = 4.89$ (37° C). *Solid line,* computer simulation of data fitting by a non-linear regression method and Eq. 1 (see text). *Broken line,* computer simulation of previous data using flocculated sludge (150–300 μm) (Fukuzaki et al. 1990): ○, pH 6.5; ●, pH 7.0

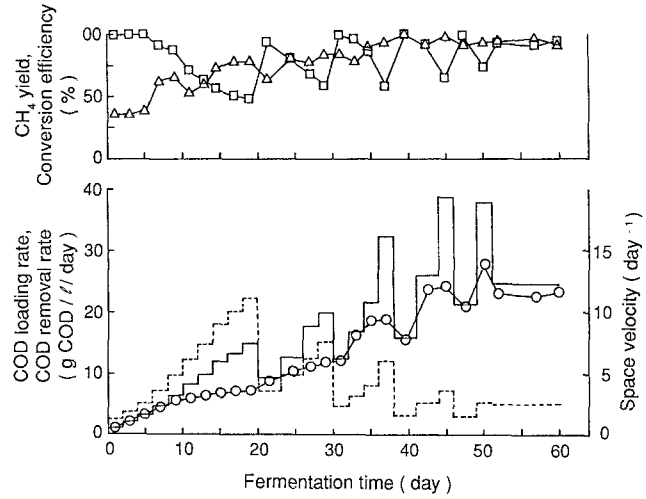

Fig. 5. Methanogenic fermentation of propionate in a cylindrical reactor packed with PUF-retained sludge. Chemical oxygen demand (COD) in the feed medium (g/l): 1.3 (0–20 days); 2.6 (20–30 days); 5.3 (30–38 days); 10.2 (38–46 days); 14.2 (46–51 days); 9.8 (51–60 days). *Solid line* (*without symbols*), COD loading rate; ○, COD removal rate; – – –, space velocity; □, conversion efficiency {[1-($COD_{effluent}$/$COD_{influent}$)] × 100}; △, CH_4 yield [(CH_4 recovered/theoretical CH_4 produced from propionate) × 100]

$q_m = 2.15$ mmol·g MLVSS^{-1}·day^{-1}). From these different phenomena between the immobilized sludge in PUF and flocculated sludge, it can be concluded that the culture of PUF-retained sludge became more stable against the inhibitory substrate of propionate.

Methanogenic fermentation of propionate in packed-bed reactors

The above results suggested the possibility of high-rate methanogenic fermentation of propionate using PUF-retained sludge. Hence, methanogenic fermentation of propionate was conducted in packed-bed reactors.

Figure 5 shows the performance of propionate degradation to methane in a cylindrical packed-bed reactor. Chemical oxygen demand (COD) loading rate (= COD concentration × space velocity) was controlled by monitoring the COD removal rate. The COD loading rate was increased stepwise until the 18th day of operation in accordance with the increase in COD removal rate. When it was judged that the COD removal rate was increased no further after 20 days of operation, the next COD loading rate was temporarily decreased. By this means, the COD removal rate could be increased up to 22.5 g COD·l^{-1}·day^{-1} after 60 days of operation, where the conversion efficiency {[1-($COD_{effluent}$/$COD_{influent}$)] × 100, %} and CH_4 yield [(CH_4 recovered/theoretical CH_4 produced from propionate) × 100, %] accounted for 93.8% and 91.2%, respectively. The COD removal rate observed was comparable to that of the propionate degradation to methane in a thermophilic digestion in an upflow anaerobic sludge blanket (UASB) reactor in which a high density of granular sludge could be retained without any supports (Wiegant et al. 1986).

During the operation, when the COD loading rate was increased sharply (e.g., 36 and 44 days of operation), the conversion efficiency decreased sharply due to the discharge of propionate by the sudden increase in COD loading rate. At the beginning of the operation until the 5th day, the CH_4 yield was remarkably low because part of gas evolved remained within the thickly packed pads in the reactor. This was due to the low

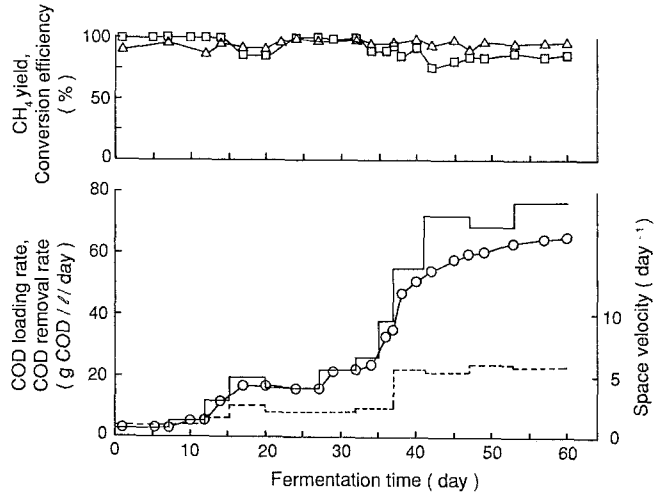

Fig. 6. Methanogenic fermentation of propionate in a flat-type reactor packed with PUF-retained sludge. COD in the feed medium (g/l): 4.3 (0–7 days); 7.8 (7–27 days); 10.6 (27–41 days); 13.4 (41–47 days); 12.0 (47–53 days); 13.8 (53–60 days). *Solid line* (*without symbols*), COD loading rate; ○, COD removal rate; – – –, space velocity; □, conversion efficiency; △, CH_4 yield

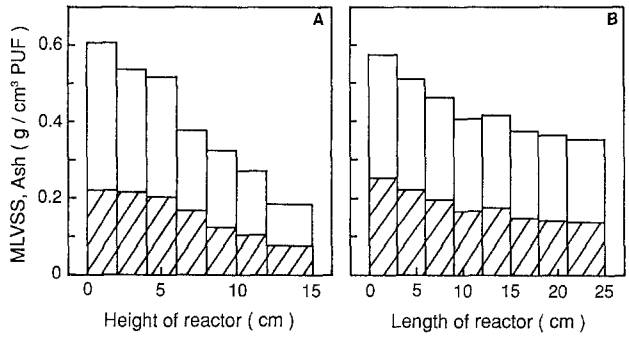

Fig. 7 A, B. Distribution of cell mass and ash in packed-bed reactors after 60 days of operation (related to Figs. 5 and 6). **A** Cylindrical reactor. **B** Flat-type reactor. The cultured PUFs in the two reactors were horizontally or vertically subdivided into seven or eight sections, and the cell mass and ash accumulated in PUF pads were analysed (see Materials and methods): ▨, MLVSS; □, ash

value of the COD loading rate, which apparently gave a low CH_4 yield. In a flat-type reactor (see later in Fig. 6) the evolved gas could not stay within the flatly packed pads, which gave a normal CH_4 yield even at the beginning of the operation. During the operation, the free cell concentration in the effluent on a protein basis was below 18 mg·l^{-1} suggesting that very little cell leakage or cell lysis was taking place.

A higher COD removal rate was also observed in a flat-type packed-bed reactor (Fig. 6) compared to the run of the cylindrical reactor. The maximum removal rate was 65.0 g COD·l^{-1}·day^{-1}, where the efficiency of propionate conversion and CH_4 yield accounted for 85.6% and 95.2%, respectively. Such a high propionate removal rate has not been reported previously (Wiegant et al. 1986). The cell concentration on a protein basis in the effluent was very low (less than 25 mg·l^{-1}).

Figure 7 shows the distribution of cell mass and ash content in PUF in the two reactors after 60 days of continuous operation. In the cylindrical reactor (Fig. 7A), cell mass accumulated densely in the PUF (0.20–0.22 g MLVSS·cm^{-3}) up to 6 cm height from the bottom of the reactor, beyond which cell mass gradually decreased up to the top of the reactor. This was probably due to an insufficient utilization of substrate in the upper part of the reactor where the evolved gas accumulated might disturb the contact between solid (PUF pads) and liquid (medium). Consequently, short channelling occurred and limited the substrate supply to the microorganisms in the PUF pads along the vertical direction of the reactor. Moreover, another growth-limiting factor seemed to be the unavoidably increasing pH value along an axis from the bottom upwards. The fresh medium pH values of 6.6±0.1 increased up to 8.0±0.1 at the top of the reactor. In fact, a high pH such as 8.0 inhibited propionate utilization by the propionate-acclimatized sludge (Fukuzaki et al. 1990).

A similar dense cell mass (0.14–0.25 g MLVSS·cm^{-3}) was accumulated in PUF in the flat-type reactor (Fig. 7B). The cell mass concentration decreased slightly along the axis from the reactor inlet.

Table 1. Mineral composition of ash accumulated in PUF after a 60-day operation in a flat-type reactor

Component	Ca	Mg	K	Na	P	Fe	Ni	Co	S
Content (g/100 g ash)	31.30	3.06	0.60	3.15	19.6	0.12	0.01	0.05	0.02

Ash was obtained by burning the whole PUF-retained sludge (380 pieces) in a crucible until obtaining a constant weight. For details of the sample used see legend to Fig. 7B

This might be caused by a pH increase along the axis mentioned above. The cell densities retained in PUF in the both reactors were much higher than those (0.2–7.5 mg dry cell·cm^{-3}) observed in continuous culture of an enriched methanogenic culture with PUF (Fynn and Whitmore 1982, 1984).

It was of significant interest to note that the ash content of MLSS corresponded to 56–63% in both reactors (see Fig. 7). It was surmised that such a high ash content was attributed to alkaline conditions caused by propionate utilization progressing in the microbial network of PUF, consequently stimulating the crystallization of inorganic salts. The mineral composition of the ash is shown in Table 1. A high amount of calcium and phosphorus and a small amount of magnesium and sodium were detected. These data suggest that the most of the ash might be composed of calcium salts, particularly calcium phosphate.

The cell mass concentrations retained in cylindrical and flat-type reactors accounted for 29.6 and 42.5 g MLVSS·l-reactor^{-1}, respectively, corresponding to 75.0 and 101 g MLSS·l-reactor^{-1}.

The results of the performance of packed-bed reactors indicated that the flat-type reactor was preferable to the cylindrical one in the methanogenic fermentation of propionate by PUF-retained sludge. In the cylindrical reactor, part of the gas evolved was retained in the reactor without being released, which might result in short channelling, whereas the flat-type reactor excelled in allowing release of the evolved gas owing to the headspace above the PUF pads. This difference might contribute to the enhancement of propionate conversion to CH_4 in the flat-type reactor. Furthermore, cell mass distribution in Fig. 7 also indicated that the configuration of the flat-type reactor allowed more available working volume than that of the cylindrical reactor.

Figure 8 shows SEM micrographs of PUF-retained sludge in the flat-type reactor after 60 days of continuous operation (see Fig. 6). Compared with Fig. 3A, considerable development of biofilm in the PUF matrix was observed (Fig. 8A). The interstices of the foam matrix were densely filled with microbial aggregates (Fig. 8B). A greater magnification of the PUF-retained sludge (Fig. 8C) showed that very dense filamentous microorganisms were dominant inside the PUF. These blunt-ended filamentous cells seemed to be *Methanothrix* spp., which were playing an important role in re-

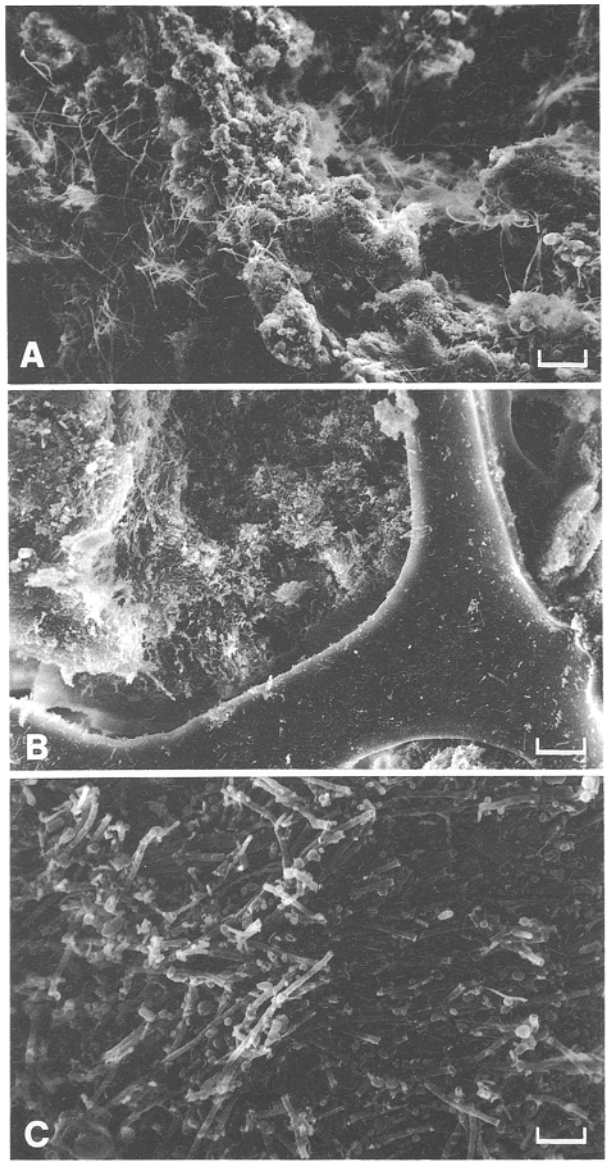

Fig. 8. SEM micrographs of the surface (**A**) and thin section (**B** and **C**) of PUF-retained sludge in a flat-type reactor after 60 days of operation (related to Figs. 6 and 7B). The *bars* represent 20 μm (**A** and **B**) or 5 μm (**C**)

taining the acclimatized microflora in the reticulated PUF.

The results obtained from the present study showed the usefulness of PUF for the retention of a methanogenic microflora by which a high rate of methanogenic fermentation might be achieved with a short start-up operation.

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