Appl Microbiol Biotechnol (1987) 27:306–314

Applied and Microbiology Biotechnology

Anaerobic digestion of whey and whey permeate with suspended and immobilized complex and defined consortia

G. Zellner, P. Vogel, H. Kneifel[1], and J. Winter*

Department of Microbiology, University of Regensburg, Universitätsstr. 31, D-8400 Regensburg and [1]Department of Biotechnology III, Kernforschungsanlage Jülich, D-5170 Jülich, Federal Republic of Germany

Summary. Whey could be anaerobically digested at space loadings up to 36 kg COD/$m^3 \cdot d$ in an upflow digester containing porous clay beads for immobilization of microorganisms. In a parallel fermenter without immobilization a space loading of only 8 kg/$m^3 \cdot d$ was reached. The start-up time was very much reduced by the support material. The COD-reduction in both reactors was 95% and volatile fatty acids in the effluent were below 10 mmol/l. During the digestion of whey a thick layer of *Methanothrix soehngenii* and occasionally *Methanobrevibacter arboriphilus* was immobilized on the clay beads. The *Methanothrix soehngenii* layer disappeared, when whey permeate was fed. *Methanosarcina* spec. became the predominant acetotrophic methanogen, probably due to the lower pH resulting from digestion of whey permeate. *Methanosarcina* spec., however, was suspended and only occasionally trapped in the pores of the clay beads. No significant adhesion of other bacteria occurred.

In a chemostat a consortium of 5 isolates from digested whey and a strain of *Methanosarcina barkeri* was able to degrade all components of whey, although at a slightly lower conversion rate than by the complex natural consortium. The total population in the whey digester was more than twice as numerous as that in the whey permeate digester. The lower number of acetotrophic methanogens seemed to be the rate-limiting step in the whey permeate digester and seemed to be responsible for the lower overall conversion rates.

Introduction

Whey is a by-product of milk processing and is abundantly obtained during cheese production. In 1984 more than 82 million tons of whey were produced all over the world and 5.8 million tons in the Federal Republic of Germany (Haefs 1985). Whey as such or whey powder may serve as a cattle feed. Whey may also be used for lactose or protein production. However, the market for whey or whey products is oversaturated. In the case of deproteination of whey for the production of a valuable human food additive, the residual whey permeate is still a waste with a high COD and must be treated before disposal.

Due to its components lactose, lactate, acetate, citrate and protein, whey is a well defined and suitable substrate for anaerobic digestion (Wildenauer and Winter 1985). Whey fermentation in completely mixed digesters (Follman and Märkl 1979) and digesters with biomass flocculation (Barford et al. 1986) is not as effective as in fixed-bed digesters, which seem to reach higher loading rates and a shorter HRT (Wildenauer and Winter 1985; Switzenbaum and Danskin 1982). In this contribution the anaerobic digestion of whey and of whey permeate was compared in digesters with and without porous clay beads for immobilization of the flora. The population in digesters operated with both substrates was determined and the suitability of defined microbial consortia of known metabolic activity as starter cultures for the fermentation of whey was tested.

Materials and methods

Substrates, organisms and culture techniques. The composition of whey and whey permeate, obtained from the Milchwerke

* Dedicated to Professor Dr. H. J. Rehm on the occasion of his 60th birthday

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Regensburg GmbH, is shown in Table 1. Initial inoculation of fermenters was with supernatant from digested sewage sludge. After a steady state was obtained with whey, microorganisms performing the digestion were isolated and characterized as described earlier (Zellner and Winter 1987), using the anaerobic technique of Balch et al. (1979) and selective media, containing 5 g/l of either lactose, glucose, galactose (fermentative bacteria), 5 g/l of ethanol, propionate, n-butyrate or lactate +30 mmol/l sodium sulfate (acidogenic, sulfate-reducing bacteria) and 5 g/l of formate, acetate or 300 kPa H_2/CO_2 (80:20%) (methanogenic population) as substrates.

Reactors and operation mode. A scheme of the reactor set-up is shown in Fig. 1. Different digesters were operated at varying hydraulic retention times (HRT) with whey or whey permeate addition via a titrator to digest at preselected pH-values, or by continuous whey addition (chemostat mode) at constant HRT. The digestion temperature was 37° C. The recirculation rate in fixed-bed and reference digesters with a suspended flora was 6 times per hour in order to avoid the formation of pH-gradients. For starter culture experiments with isolates of the whey digester New Brunswick Multigen 1000 chemostats were used and operated with sterilized whey as described earlier (Winter and Wolfe 1980).

Analyses. Analyses for the characterization of whey and the performance of the whey digesters were performed as described in detail earlier (Wildenauer and Winter 1985; Zellner and Winter 1987). Polyamines were determined after extraction, derivatization with dansyl chloride and separation by HPLC as described by Scherer and Kneifel (1983).

Table 1. Composition of cheese whey and of deproteinized whey permeate

Parameters	Cheese whey	Whey permeate
Chemical oxygen demand (COD) (g O_2/l)	75	50
Total solids content (%)	5	4.2
Volatile solids content (%)	4.4	3.8
Total nitrogen (g/l)	1.9	0.512
Ammonia nitrogen (g/l)	0.6	0.500
Protein content[a] (%)	0.81	0.075
Lactose (g/l)	40	40
Lactate (g/l)	10	10
Acetate (mmol/l)	15	18
Propionate (mmol/l)	5	4
n-Butyrate (mmol/l)	0.7	0.5
Potassium (mmol/l)	38	36
Sodium (mmol/l)	66	68
Calcium (mmol/l)	7	2
Chloride (mg/l)	38	33
pH	4.3	4.1

[a] (Total nitrogen − ammonia nitrogen) × 6.25 = protein content

Results

Whey and whey permeate fermentation in an upflow fixed bed reactor

A cylindrical digester (8 × 45 cm, Fig. 1) with clay beads as supporting material for microorganisms was inoculated with digested sewage sludge, incubated for 1 day to establish anaerobic conditions and then supplied with increasing amounts of whey by a titrator unit at a constant pH of 6.7. As shown in Fig. 2 steady state conditions were obtained 10 days after the initial start-up with a gas production rate of 11 l/l·d, equivalent to a hydraulic retention time (HRT) of 3.5 days and a space loading of approximately 24 kg/m^3·d. When whey was added continuously to give a HRT of 2.1 days and a space loading of 36 kg/m^3·d, the gas production rate increased to 14 l/l·d, but the pH decreased to 6.5 and the volatile fatty acid levels in the effluent increased slightly. A further reduction of the HRT caused a drastic pH drop, due to overloading and a breakdown of the digester.

The population established for the degradation of whey was also able to stabilize whey permeate (Fig. 2, arrow), that contained only 0.08% of protein as a nitrogen source. Unexpectedly, supplementation of ammonia or phosphate did not improve the digestion process (not shown).

The reduction of the chemical oxygen demand (COD) was 90%—95% for whey and whey permeate fermentation.

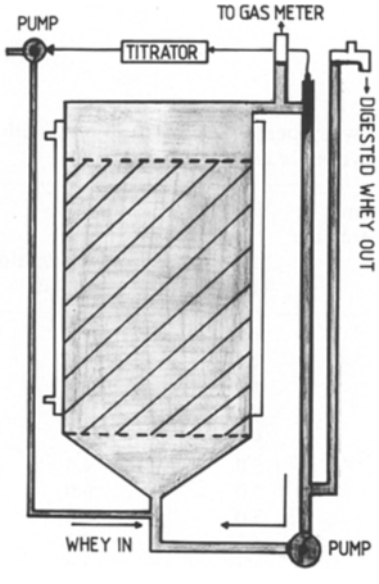

Fig. 1. Scheme of the digester set-up used for whey or whey permeate fermentation. The digesters were thermostated by a water jacket. Immobilisation of microorganisms was on clay beads which were placed within the *hatched area*

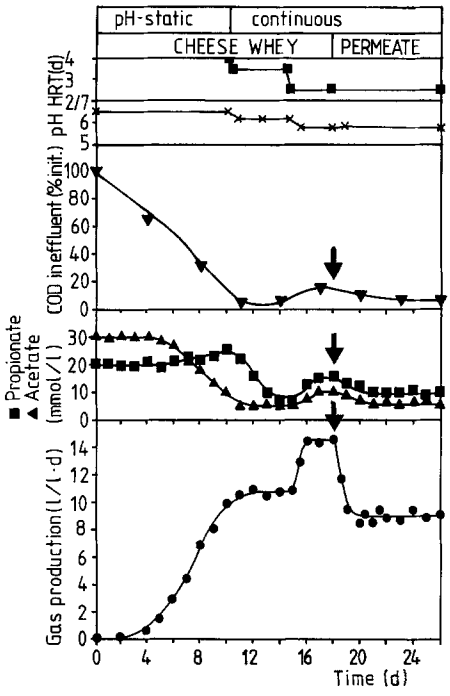

Fig. 2. Start-up of whey fermentation in a fixed-bed reactor (8 cm diameter × 45 cm height, liquid volume 1.5 l), containing clay beads (1 cm diameter, clay volume 0.8 l) and switch to whey permeate as substrate. Day 0—10: Increasing whey addition at a constant pH of 6.6, controlled by a titrator. Day 10—15: Continuous addition of 430 ml whey per day. Steady state at a pH of 6.2. Day 15—18: Continuous addition of 480 ml whey per day. Steady state at a pH of 5.7. Day 18—26: Continuous addition of 480 ml whey permeate per day

Influence of the pH on whey permeate fermentation in a fixed-bed upflow reactor

Whey permeate fermentation at constant pH-values ranging from 4.9 to 6.7 was compared, when steady state conditions for at least three HRTs were obtained (Table 2). The shortest HRTs of 3.3—3.8 days were obtained when the pH was maintained in the range between 4.9 and 5.3. However, high concentrations of acetate and of n-butyrate were found in the effluent, causing a high COD and a low gas production rate of only 4 l/l·d. A much better gas production rate (7.3 l/l·d) was observed during fermentation at pH 5.5 for a HRT of 4.3 days. No butyrate was found in the effluent, but the acetate level was still high (35 mmol/l). The lowest COD in the effluent of whey permeate digesters was obtained for a pH-range of 6.5—6.7 and a HRT of 6—7.5 days, with acetate concentrations at or below 15 mmol/l.

Comparison of whey and whey permeate fermentation in reactors with and without immobilization

Two cylindrical reactors (4 cm in diameter × 45 cm in height), of which one was filled with clay beads, were operated pH-statically. The maximum space loading in the whey digester without

Table 2. Fermentation of whey permeate in an upflow fixed-bed reactor at different pH-values, which were kept constant by titration with whey for 3 hydraulic retention times

pH-Value	Daily amount of whey permeate addition[a] (ml/d)	Hydraulic retention time (d)	Daily gas production (l/l·d)	Metabolite concentration in the effluent (in mmol/l):			Chemical oxygen demand[b] (g O_2/l)
				Acetate	Propionate	n-Butyrate	
4.9	400	3.75	4.4	64	5	35	14.0
6.3	250	6.0	3.4	20	5	0.0	4.0
6.7	200	7.5	2.5	6	0	0.0	2.0
6.5	240	6.25	2.7	15	0	0.0	2.5
6.3	260	5.80	3.7	20	2	0.0	2.6
6.1	300	5.0	4.3	27	3	0.0	3.5
6.0	300	5.0	6.0	25	10	0.0	5.0
5.7	300	5.0	6.0	25	4	0.0	5.0
5.5	350	4.3	7.3	35	5	0.0	7.0
5.25	450	3.3	4.0	60	5	12.0	8.0
4.9	400	3.75	4.0	65	5	35.0	12.0

The pH-static operation was in the sequence as stated. [a] The digester volume without the support material was 1.5 l.; [b] The COD was determined for the clarified supernatant of the effluent. Parameters for each pH-interval represent steady state conditions

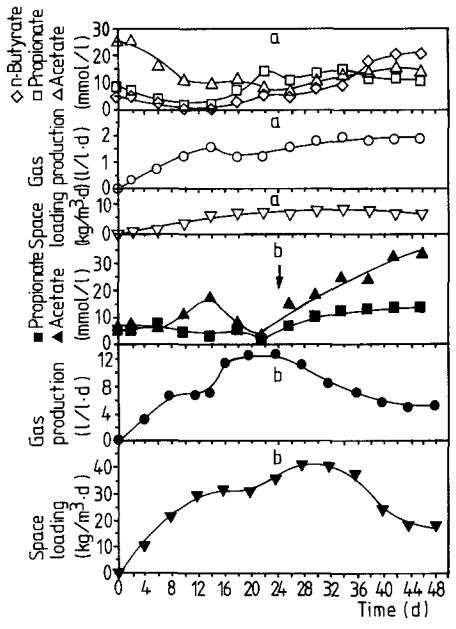

Fig. 3. Comparison of the digestion of whey in a digester (4 cm diameter × 42 cm height) with suspended biomass (**a**) and with biomass immobilization on clay beads (**b**). Whey addition in both reactors was controlled at pH 6.5 by a titrator unit. When a loading rate of 40 kg/m³·d was reached in the fixed-bed reactor, the whey substrate was replaced by whey permeate (**b**, *arrow*)

immobilization was 8 kg/m³·d at a gas production rate of 2 l/l·d (Fig. 3a). The digestion of whey in the digester containing clay beads started much faster, reached a space loading of 30—35 kg/m³·d within 24 days and a gas production rate 8—12 l/l·d (Fig. 3b). The volatile fatty acid levels were higher in the reactor without immobilization of microorganisms and butyrate was produced in addition. Similar high volatile fatty acid levels were measured, when whey in the digester with the clay beads was replaced by whey permeate as substrate (Fig. 3b, arrow). Due to the lower COD of whey permeate and the accumulation of acetate and propionate the space loading decreased (pH-static operation mode), causing a reduction of the gas production rate.

Maximum hydraulic retention time for whey permeate and stability of the fermentation in a reactor containing clay beads

When whey permeate was digested at a HRT of 3—3.8 days the pH dropped constantly within 46 days from 6.5 to 4.8, the acetate concentration in the effluent increased steadily to more than 40 mmol/l and n-butyrate accumulation began at a pH below 5.5. The COD in the clarified effluent increased to 15—20 g/l, while the gas production rate was rather constant at 10 l/l·d (Fig. 4, day 0—46). When the HRT was kept at 5 days, the pH raised, the acetate concentration in the effluent fell and n-butyrate disappeared (Fig. 4, day 46—52). The fermentation was stable for a HRT of 5 days at a pH of 5.5—6.0. The average acetate concentration in the effluent was 30 mmol/l. The acetate could be degraded within 2 days, when the whey permeate addition was reduced (day 84—86). The addition of whey permeate from another batch (starting at day 86) caused an increase of the acetate level and fluctuations in the gas production. A reduction of the HRT below 5 days led to failure of the digestion process. Acetate cleavage seemed to become rate-limiting before the

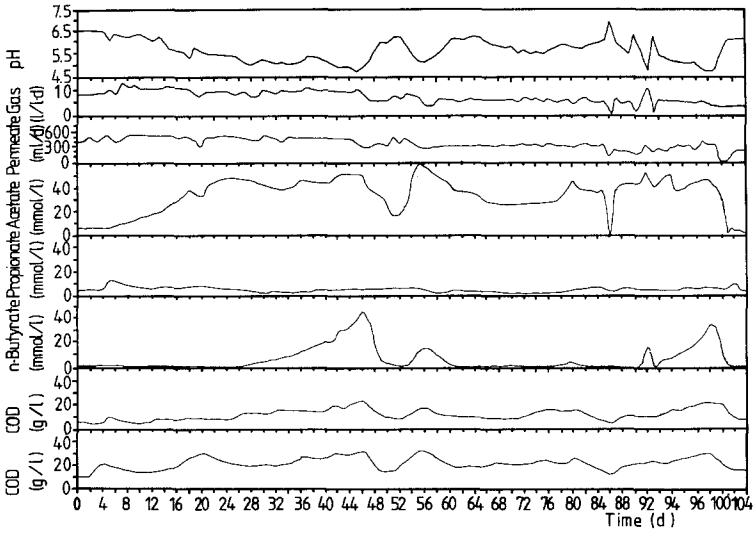

Fig. 4. Stability of whey permeate fermentation in a fixed-bed reactor with clay beads. The pH, gas production, volatile fatty acid levels and the COD in the effluent *(bottom curve)* and in the clarified effluent (second curve from bottom) are shown in dependence on the whey permeate addition per day. At day 86 another batch of whey permeate was used

Table 3. Metabolic capabilities of relevant isolates from the whey-degrading consortium

Stage	Isolate	% of culturable flora	Substrates	Fermentation products	Polyamine content (μmol/g dry weight)							
					DAP	PUT	CAD	NSD	SPD	HSD	NSM	SPM
Fermentative phase	Strain A (*Lactobacillus* spec.)	10	Lactose, Glucose, Galactose, Citrate	L-Lactate, Acetate	0.03	0.12	0.02	—	7.35	0.02	—	2.71
	Strain C	5	Lactose, Glucose, Galactose	D-Lactate, little Acetate, Ethanol, CO_2	0.02	0.27	0.04	—	17.1	—	—	0.42
	Strain F	5	Glucose, Galactose	L-Lactate, Acetate, Ethanol, Formate, CO_2	0.10	0.17	0.02	—	16.3	0.02	—	1.75
	Strains G, I, L2 *(Eubacterium limosum)*	25	Glucose, Galactose, Lactate, Protein	n-Butyrate, Acetate, Formate, CO_2, H_2, Fatty acids	—	0.07	—	—	0.06	—	—	0.27
	Others (clostridia, Fusobacterium, Bacteroides etc.)	10	n.d.	n.d.								
Acetogenic phase	Strains XVI, MP47, MP27, MP9, MP34	5	Lactate, Ethanol, Formate, Methanol	Acetate, CO_2, H_2	0.05	0.05	—	—	13.2	—	—	0.65
	MP34 (*Desulfovibrio* spec.)			Acetate, CO_2, H_2								
Methanogenic phase	Strain ZZ1 (*Methanobacterium* species)		H_2:CO_2, Formate	CH_4, CO_2	—	0.03	—	—	0.13	—	—	0.69
	Strain MZ42N *(Methanobrevibacter arboriphilus)*	5	H_2:CO_2, Formate	CH_4, CO_2	0.03	0.17	0.03	—	0.18	—	—	0.34
	Methanogenic rod *(Methanothrix soehngenii)*	20	Acetate	CH_4, CO_2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Strain XII *(Methanocorpusculum parvum)*	15	H_2:CO_2, Formate	CH_4, CO_2	51.6	14.8	0.84	—	0.28	—	—	0.39

n.d. = not determined; DAP = 1,3-Diaminopropane; PUT = Putrescine; CAD = Cadaverine; NSD = Sym-norspermidine; SPD = Spermidine; HSD = Sym-homospermidine; NSM = Sym-norspermine; SPM = Spermine

H_2/CO_2-fermenting methanogens were oversaturated, which was indicated by the occurrence of n-butyrate in the effluent.

Comparison of whey digestion by enrichment cultures and by whey-degrading isolates as starter cultures

Sterile whey was continuously fermented in chemostats, inoculated either with a mixture of whey isolates (Table 3), belonging to the genera *Lactobacillus, Eubacterium, Desulfovibrio, Methanobrevibacter arboriphilus* strain MZ42N, *Methanosarcina barkeri* and a not yet assigned genus (isolate F, Zellner and Winter 1987) (Fig. 5a) or with effluent from a whey digester (Fig. 5b). Due to a more balanced inoculum and precultivation in whey, gas production in the digester with the whey consortium started immediately at a rate of 3—3.5 l/l·d (HRT 10 days). When the HRT was reduced from 10 to 5 days at day 20, a drastic increase of the acetate level in the effluent was observed and butyrate began to accumulate. Propionate levels were high, decreased slowly and seemed not to be influenced by the increase of the loading. In the chemostat inoculated with whey isolates and *Methanosarcina barkeri* a steady state was reached after approximately 20 days at 15 days HRT (Fig. 5a). The inocula were pregrown in defined media and thus had to be adapted to whey. The consortium, however, was stable and fermentation of whey could be improved to a gas production rate of 3 l/l·d at a HRT of 10 days within a further month of operation (not shown). Thus, starter cultures may be useful, but may require adaptation when precultivated in a different medium.

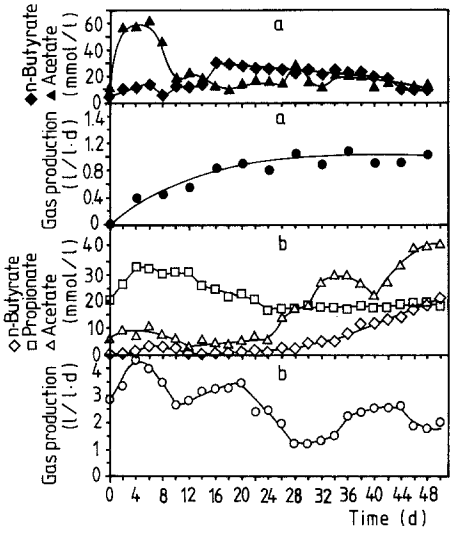

Fig. 5. Comparison of whey fermentation in chemostats by defined mixed cultures, isolated from a whey digester (**a**) and by a complex mixed enrichment culture (**b**). Whey addition was controlled by a titrator, set at pH 6.5 for the first 20 days. Then a continuous operation mode was chosen. The chemostat with the defined mixed culture reached a steady state at a HRT of 15 days, which later could be improved to a HRT of 10 days (not shown). The chemostat with the complex enrichment culture reached a steady state at a HRT of approximately 7 days. During continuous operation at 7 days HRT acetate and butyrate accumulated

Microbial population in the whey digester and in the whey permeate digester

Growth of microorganisms on the surface of pores of the clay beads with increasing incubation time is shown in Fig. 6a—c. Microscopical examination of the clay beads revealed the presence of mainly *Methanothrix soehngenii* and occasionally a *Methanobrevibacter arboriphilus,* which formed a dense layer on the surface of the clay beads after 18 days. Almost no *Methanothrix soehngenii* and rarely *Methanosarcina* spec. were found in digested whey (Fig. 6d), indicating the effective immobilization of acetoclastic methanogens. When whey was replaced by whey permeate, the pH continuously dropped below 6.5 and the biomass from the surface of the clay beads was successively detached (not shown). The *Methanothrix soehngenii* was washed out and was finally replaced by *Methanosarcina* spec., which almost exclusively represented the acetotrophic methanogens in whey permeate digesters in the pH-range of 6.5—4.9. The total population and its composition was compared in whey and whey permeate digesters (Table 4). The total population of the whey digester is twice as numerous as that of the whey permeate digester. Approximately twenty times as many acetate-cleaving methanogens, immobilized on the clay beads, were found in the whey digester than in the whey permeate digester. This may explain the higher maximum loading rates of whey digesters, compared to digesters with whey permeate as substrate. Hydrogenotrophic organisms were present in the same order of magnitude in both digesters.

The contribution of 6 fermentative, 5 acetogenic and 5 methanogenic organisms to the whey-degrading population is shown in Table 3. The most abundant fermentative bacteria belong to the genus *Eubacterium.* Acetogenic isolates were all assigned to the genus *Desulfovibrio.* The methanogenic population consisted of *Methanobac-*

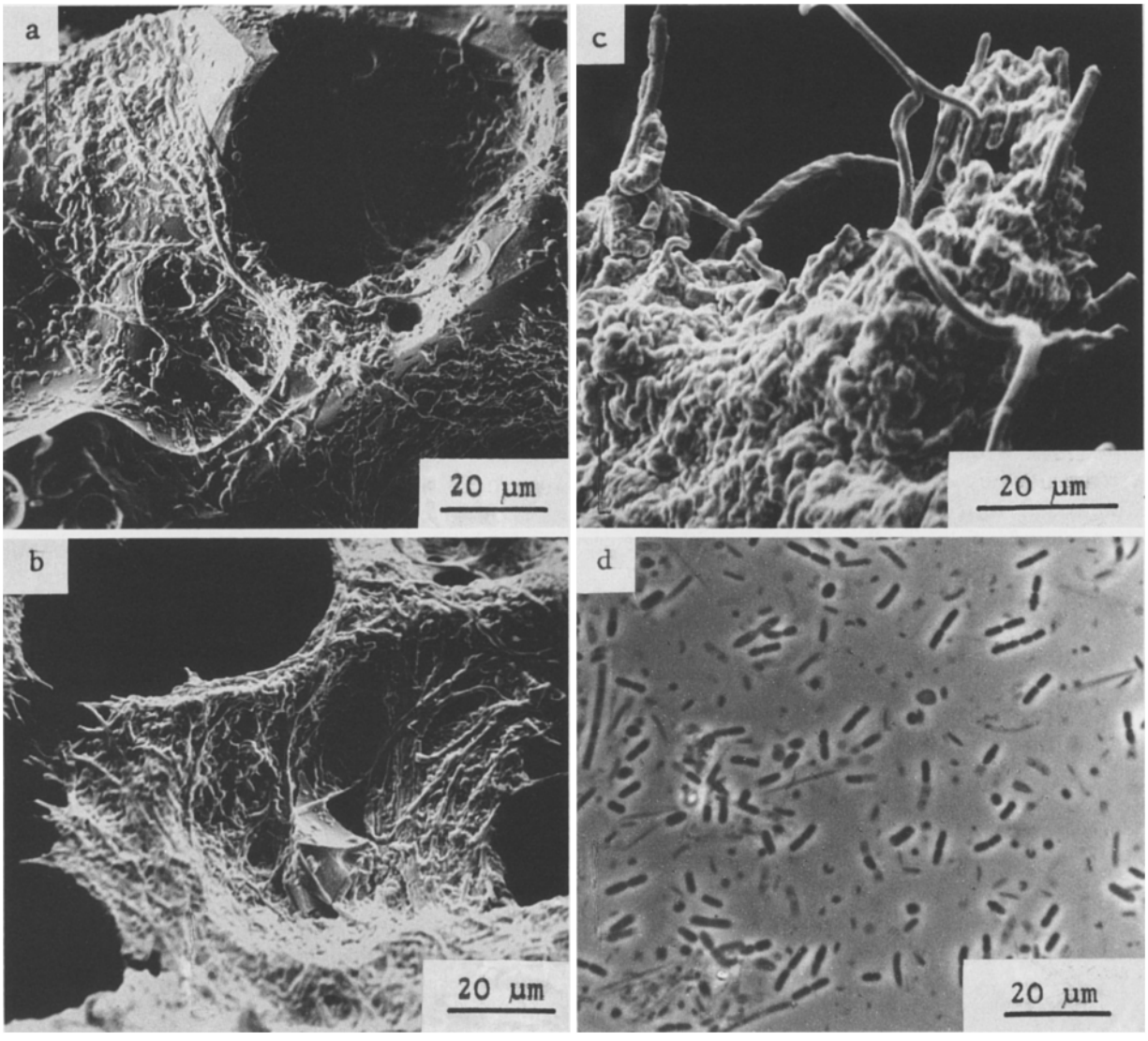

Fig. 6. Scanning electron microphotographs of the clay beads showing the immobilization of *Methanothrix soehngenii* after 5 days (**a**), 10 d (**b**) and 18 d (**c**) and phase contrast microphotograph, showing the population of the effluent (**d**)

Table 4. Proportion of fermentative, acetogenic and methanogenic bacteria in a whey digester and in a digester with whey permeate as substrate

Source of organisms[a]	Total population (microscopic count) organisms/ml	Fermentative bacteria growing on[b]			Acetogenic bacteria growing on SO_4^{--} +		Methanogenic bacteria growing on			Total culturable population[c] %
		Lactose CFU/ml	Glucose CFU/ml	Galactose CFU/ml	Lactate CFU/ml	Ethanol CFU/ml	H_2/CO_2 CFU/ml	Acetate CFU/ml	Formate CFU/ml	
Whey	1×10^{10}	4×10^9	5×10^9	5×10^9	5×10^8	n.d.	1.5×10^9	2.5×10^9	1×10^8	95
Whey permeate	4×10^9	1×10^9	1×10^9	1×10^9	1×10^9	7×10^7	1.4×10^9	1.3×10^8	1.1×10^7	81

[a] From digester effluent (whey permeate) or digester effluent into which the bacterial layers of an appropriate amount of clay beads were suspended in the anaerobic chamber

[b] Medium No. 1 (Winter 1980) + respective substrates as stated in *Materials and methods*

[c] Of the microscopically counted population; CFU = colony forming units

terium species strain ZZ1, *Methanobrevibacter arboriphilus* strain MZ42N, *Methanospirillum* spec., *Methanothrix soehngenii* and *Methanocorpusculum parvum*, which can easily be identified by his polyamine content, especially the high amount of 1,3-diaminopropane and putrescine (Table 3) and by other features (Zellner et al. 1987).

Discussion

In the digesters with clay beads as support material much higher loading rates could be obtained than in the digesters with a suspended population. While almost no fermentative, acetogenic and H_2/CO_2-consuming methanogenic bacteria were immobilized on clay beads, *Methanothrix soehngenii*, the abundant acetotrophic methanogen, and little of the *Methanobrevibacter arboriphilus* population, which tended to form aggregates as known from the literature (Zeiskus and Henning 1975), were immobilized on the clay beads in the whey digester. The time required for the start-up of the whey digester and the maximum loading rate are largely dependent on the dimensions of the reactor (Wildenauer and Winter 1985). A switch from whey to whey permeate as substrate caused no severe disturbances of the whey population, since *Eubacterium limosum* strains, the main protein degraders in whey (Zellner and Winter 1987), can also degrade sugars, and thus compete with *Lactobacillus* spec. and the not yet named isolate F (Zellner and Winter 1987). When whey permeate was the substrate the bacterial layer on the clay beads was completely dissolved and *Methanothrix soehngenii* was replaced by *Methanosarcina* sp., most probably due to a pH of below 6.4 in whey permeate digesters.

The comparison of whey digestion in completely mixed and fixed-bed digesters reveals the higher loading rate and gas production rate of the fixed-bed digester. In the fixed-bed digester n-butyrate was never found at level exceeding 0.5 mmol/l, while in the digester without microbial carrier n-butyrate was detected at higher levels. n-Butyrate production by *Eubacterium limosum* may be prevented by a well-functioning interspecies hydrogen transfer (Winter and Soutschek 1982; Zellner and Winter 1987) and low acetate levels (Ahring and Westermann 1987a, b; Pacaud et al. 1986). While Chartrain and Zeikus (1986) reported propionate as an important intermediate of whey fermentation, whey degradation may obviously proceed without propionate producing and degrading organisms. Similar as reported by Chartrain et al. (1987) for the digestion of non-sterilized raw whey in a contact digester with a designed population, the digestion of autoclaved whey by a defined microbial consortium required a lag-phase. As shown in this contribution the selection of an efficient whey-degrading consortium is not a time-consuming task and thus the use of starter cultures for whey degradation may be only of theoretical interest.

Acknowledgements. The presented work was supported by the Bundesministerium für Forschung und Technologie, D-5300 Bonn under contract PBE 0318820A. We thank Heinz Reischel and Regina Leiss for excellent technical assistance. The skillful work of D. Niesner, R. Knott, and F. Waas from the mechanical and electronical workshop is gratefully acknowledged.

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Received June 16, 1987/Accepted August 4, 1987