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Study on the development of methanogenic microflora during anaerobic digestion of sugar beet pulp

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Summary. The pattern of increase in cell number in 12 different groups of bacteria was studied during anaerobic digestion of enzymatically prehydrolysed sugar beet pulp in a 70-l fermentor with sequential feeding over a period of 130 days. Glucose-fermenting bacteria accounted for 90% of the total microflora as estimated by direct epifluorescence. Strictly anaerobic bacteria were largely dominant; only 10% were methanogens. Sulphate-reducing bacteria accounted for 0.1% of the total microflora. The yield of biogas was compared with the numbers of bacteria.

Introduction

Methanogenic fermentation of solid, agro-industrial, cellulosic by-products is limited mainly by the rate of hydrolysis of the polymeric compounds. These substrates are usually devoid of appropriate microflora and, to be efficiently fermented, require suitable inoculation for biogas production. Two decades ago several authors showed that strictly anaerobic bacteria formed the dominant population in digestors and that methanogens accounted for about 10% of the total microflora (Mah and Sussman 1967; Siebert et al. 1967; Siebert et al. 1968; Toerien and Siebert 1967).

The total bacterial count was generally estimated to be 10^{10} bacteria/ml in stabilized digestors. These results were obtained after addition of rumen fluid or digestor juice to the enumeration media (Spoelstra 1968; Hobson and Shaw 1973; Iannotti et al. 1978; Ueki et al. 1978; Touzel et al. 1981).

In the present study the development of the methanogenic microflora during the anaerobic digestion of sugar beet pulp was investigated.

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Materials and methods

Anaerobic digestion. The anaerobic digestion of sugar beet pulp was performed after pretreatment by enzymatic hydrolysis with fungal cellulases, in a 70-l fermentor with a sequential feeding as described previously (Labat et al. 1984). The inoculum was a mixture of swine and cow slurries. Bacterial counts were performed on the inoculum and during the 130 days of the digestion process.

Sample preparation. The samples were taken anaerobically from the digestor in serum bottles filled with O_2-free N_2, and placed in an anaerobic flexible PVC glove box (La Calhene, Bezons, France). After homogenization in a Potter apparatus, the samples were diluted (1:10) by adding anaerobically a 1 ml sample to Hungate tubes (Bellco Glass Inc., Vineland, NJ, USA) containing 9 ml anaerobic reduced solution. The solution had the following composition: K_2HPO_4, 0.3 g; mineral solution (Balch et al. 1979), 50 ml; $NaHCO_3$, 5 g; resazurin, 0.5 mg; cysteine-HCl·H_2O, 0.5 g; distilled water, 900 ml; atmosphere N_2—CO_2 (80%—20%); $Na_2S \cdot 9H_2O$ (0.5 g/l) was added after autoclaving using the Hungate technique (Hungate 1969); final pH was 7.2.

Different media for specific bacterial enumerations were inoculated with syringes filled with O_2-free N_2 using a gas manifold (Balch et al. 1979) outside the glove box.

Bacterial quantifications. Two types of enumerations were performed:

1. Bacterial MPN determination according to metabolites produced during growth on appropriate media for methanogenic, fermentative and dissimilatory nitrate-reducing bacteria. Sulphur- and sulphate-reducing bacteria were estimated using a filter method.
2. Bacterial MPN determination according to the sensitivity to oxygen.

Results were related to total bacterial counts (TB) obtained by direct microscopic examination with the epifluorescence technique (Hobbie et al. 1977): 5 ml of the diluted samples ($1:10^4$) were filtered on 0.2 µm black stained Nuclepore filter (Nuclepore Corp., Pleasanton, CA, USA) and stained (5 min) with acridine orange solution (Hobbie et al. 1977). Fluorescent bacteria were easily counted with a 100×100 µm ocular micrometer under incidental UV light with special filters (Zeiss BP 450—490, FT 510, LP 520).

Methanogenic bacteria. Modified (1 g/l yeast extract and Biotrypcase instead of 2 g/l) medium 1 of Balch et al. (1979) was used and adjusted to a final pH 7.2. The medium was dispensed in 5 ml aliquots into Hungate tubes, then reduced with Na_2S, using the Hungate technique. The tubes were gassed under 80% N_2—20% CO_2 gas phase (1 atm) or 80% H_2—20% CO_2 gas phase (2.5 atm) and sterilized (110°C, 35 min).

Three enumerations were performed:
1. H_2-Oxidizing methanogens (HOMB) for which a H_2–CO_2 gas phase was used;
2. autotrophic H_2-oxidizing methanogens (AUMB) using the same gas phase but with a medium lacking yeast extract, Biotrypcase and rumen fluid;
3. aceticlastic methanogens (AMB) with acetate (5 g/l) as growth substrate; N_2–CO_2 was the gas phase.

After 3 weeks of incubation at 35°C, CH_4 was analysed by FID gas chromatography (Varian Aerograph 2700) using a $2\,m \times 1/8''$ stainless steel column with Porapak Q 80—100 mesh at 200°C. The temperatures of the injector and detector were 150°C and 240°C respectively. Gas flows were as follow: N_2, 25 ml/min; H_2, 30 ml/min; air, 300 ml/min. Under these conditions, elution time of CH_4 was 45 s.

Acidogenic bacteria. The Modified medium of Zeikus et al. (1980) was used. Sodium selenite (17.3 mg/l) and 12 ml of an alkaline bromothymol blue solution (2 g/l) were added to the medium. N_2–CO_2 was the gas phase with 5 g/l glucose (GFB) or sodium lactate (LFB) as carbon and energy sources. Growth was indicated by the yellow coloration appearing during medium acidification.

Dissimilatory nitrate-reducing bacteria (NRB). These bacteria were enumerated in the basal medium for methanogens of Balch et al. (1979) by addition of 2 g/l of acetate and succinate but without sulphate and Na_2S; 0.5 g/l $NaNO_3$ was added to Hungate tubes containing O_2-free N_2 and 10% acetylene as gas phase. Final pH was 7.2. N_2O was analysed by TCD gas chromatography (Girdel 30) operating with dual $1.8\,m \times 1/8''$ stainless steel columns with Carbosphere 60—80 mesh at 80°C. The temperatures of injector and detector were 105°C and 150°C, respectively, with helium as carrier gas (45 ml/min) and 250 mA power filaments.

Sulphate-reducing bacteria (SRB). An amount of 50 ml of a 10^{-2} diluted sample was filtered on 0.22 µm HA-type Millipore filter (Millipore Corp., Bedford, MA, USA). The filters were then placed in screw-cap tubes (125×16 mm) filled to the top with the following medium:

1. Solution A (lactate users SRBlac): KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7\,H_2O$, 2 g; $Na_2SO_4 \cdot 10\,H_2O$, 2 g; $CaCl_2 \cdot 2\,H_2O$, 0.1 g; NH_4Cl, 1 g; sodium lactate 5 g; distilled water, 800 ml.
2. Solution A′ (acetate users SRBac): same as solution A except that lactate was replaced by sodium acetate 5 g/l.
3. Solution B: K_2HPO_4, 5 g; distilled water, 100 ml.
4. Solution C: Mohr salt, 0.5 g; distilled water, 100 ml.

Solutions A (or A′) and B were autoclaved and solution C was sterilized by filtration before mixing together and dispensing aseptically into screw-cap tubes containing 1 ml sterile water with sedimented FeS (5% w/v) as reductant. After incubation at 35°C, black coloration by FeS formation from Mohr salt was compared with a reference value obtained from calibration curves set up with suitable dilutions of *Desulfovibrio vulgaris* cells (SRBlac) or enrichment mixture on acetate (SRBac) which were estimated by direct microscopic counts.

Sulphur-reducing bacteria (S°RB). These bacteria were counted like sulphate-reducing bacteria, but sulphate was omitted and sodium acetate was the only carbon and energy source. Elemental sulphur was deposited on the filters (Millipore 0.45 µm) before inoculation. A standard curve was obtained with a pure strain of *Desulfuromonas acetoxidans*.

Facultative anaerobic bacteria (FAB). FAB were estimated in Petri dishes containing Nutrient Agar (Difco Lab., Detroit, MI, USA) after aerobic incubation.

Non oxygen-sensitive anaerobic bacteria (Non-OSAB). These bacteria were estimated by MPN determination in the same medium as acidogenic bacteria (GFB medium) with 8 g/l Bacto-Agar (Difco). Samples of 0.2 ml were inoculated aerobically in liquified agar medium, anaerobically prepared and dispensed, then reliquified just before use and stored during inoculation in a 45°C water-bath. After addition of $Na_2S \cdot 9\,H_2O$ (0.5 g/l), the medium was then aerobically dispensed in long tubes (400×8 mm). Incubation was at 35°C. Colonies were counted in the last dilution tubes; MPN counts were also followed by color change in the acidified medium. The number of non-oxygen-sensitive, strictly anaerobic bacteria (Non-OS SAB) was obtained by subtraction of the number of FAB.

Oxygen-sensitive strictly anaerobic bacteria (OS SAB). Petri dishes with 2% agar medium (GFB) were prepared and inoculated inside the anaerobic glove box and incubated in anaerobic containers as described by Balch et al. (1979). The gas phase was N_2–CO_2 (1.5 atm). The number of OS. SAB was obtained by subtraction of Non-OSAB from the number obtained in Petri dishes.

To all media, except for SRB, S°RB, FAB and AUMB, clarified sterilized rumen fluid (50% v/v) was added to improve bacterial growth. Nine bacterial counts were performed during the anaerobic digestion of hydrolysed sugar beet pulp. Each count evaluated 12 different bacterial groups. Bacterial counts were done weekly during the first 3 weeks, then biweekly over one month and every three weeks over 9 weeks. For each MPN determination, 4 successive dilutions (0.2 ml) were inoculated in 5 tubes per dilution. Results were calculated according to classical McCrady's tables (McCrady 1918).

Results

Total microflora (TB)

The bacterial number increased one hundred times from the inoculum from about 10^8 to 10^{10} cells/ml during the first 4 weeks of incubation (Fig. 1). After 5 weeks of digestor course, air was accidentally introduced into the fermenter at the

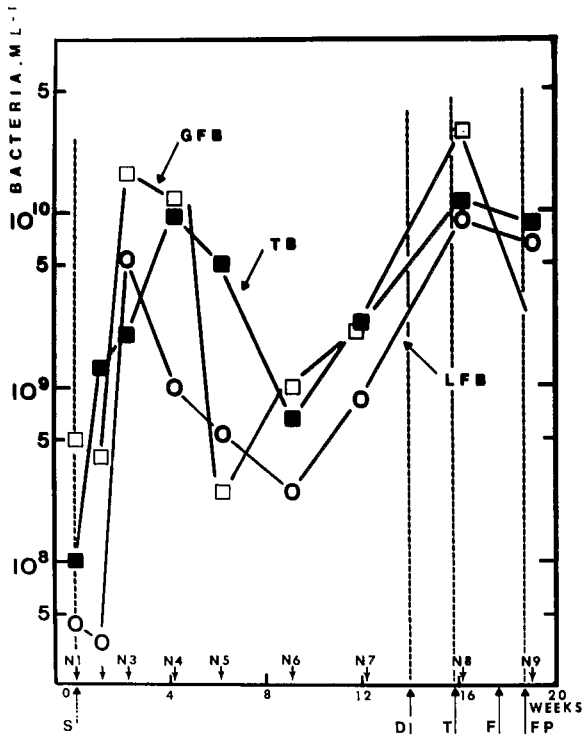

Fig. 1. Counts of total microflora (TB) and fermentative bacteria on glucose (GFB) and lactate (LFB). N1 to N9: successive enumerations; S: simple loading; D: double loading; T: three-fold loading; F: four-fold loading; FP: fermented pulps added. Simple loading means: 2 l solubilized pulps (20 g vs/l) per day was added (vs = volatile solids)

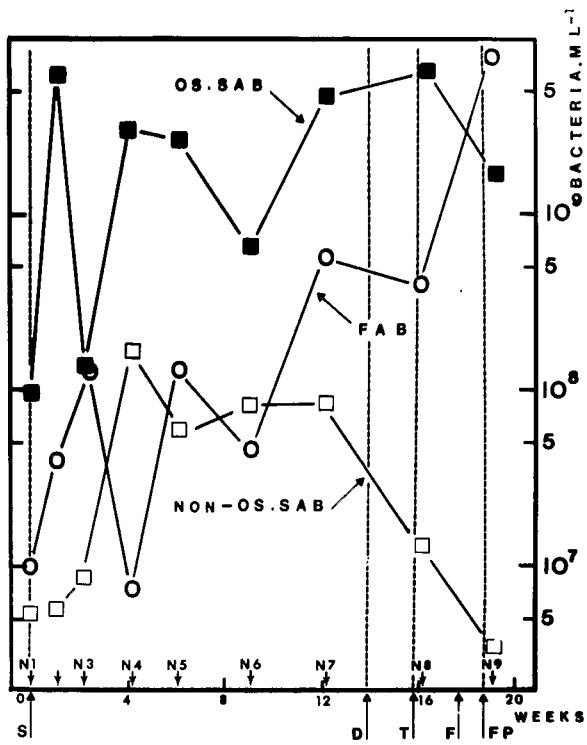

Fig. 2. Counts of bacteria depending of their sensitivity to oxygen. FAB: facultative anaerobic bacteria; NON-OS. SAB: non oxygen-sensitive strictly anaerobic bacteria; OS. SAB: oxygen-sensitive strictly anaerobic bacteria

level of the recycling pump and resulted in a decrease of the total bacterial number to 10^9 cells/ml, before it again slowly reached the level of 10^{10} bacteria/ml. Increased feeding by doubling then tripling and quadrupling the initial daily load did not affect bacterial cell numbers. Figures 1—4 show the dynamics of different bacterial groups during methanogenic fermentation of solubilized sugar beet pulp as compared with succession of total microflora shown in Fig. 1.

Sensitivity of bacteria against oxygen

The oxygen-sensitive, strictly anaerobic bacteria (OS. SAB) represented more than 80% of the total microflora during stabilized digestion (Fig. 2). Non-oxygen-sensitive strictly anaerobic bacteria (Non-OS. SAB) were less numerous and accounted for less than 5% of total microflora. Facultative anaerobic bacteria (FAB) accounted for about 10% of the microflora. They increased up to 90% of total microflora when loading was performed with fermented solubilized pulps 2 weeks before the end of the experiment. In contrast, the number of strictly anaerobic bacteria fell.

Fermentative bacteria (Fig. 1). They represented the highest number of the total microflora and were affected by air contamination. Glucose fermentative bacteria (GFB) were more numerous than lactate fermentative bacteria (LFB), which were predominant when the fermenter was loaded with fermented pulps.

Methanogenic bacteria (Fig. 3). They accounted for less than 10% of total microflora with prevalence of aceticlastic bacteria (AMB) on hydrogen oxidizers (HOMB). After accidental introduction of air, the restoration of the stabilized level took longer to establish, probably due to the slow doubling time of methanogenic cells. Their number fell drastically when fermented pulps were introduced. Autotrophic methanogens (AUMB) were less numerous (10^6 bacteria/ml), showing the relative dependence of most methanogens to growth factors.

Dissimilatory nitrate-reducing bacteria (Fig. 3). They were 10^6 per ml in the inoculum and their density decreased rapidly as strict anaerobiosis took place and residual nitrate disappeared.

Sulphate-reducing bacteria (Fig. 4). Not numerous in the inoculum (10^2 per ml), their number increased during digestion to reach 0.1% of total microflora. Their density, however, fell quickly after air contamination and was never totally restored.

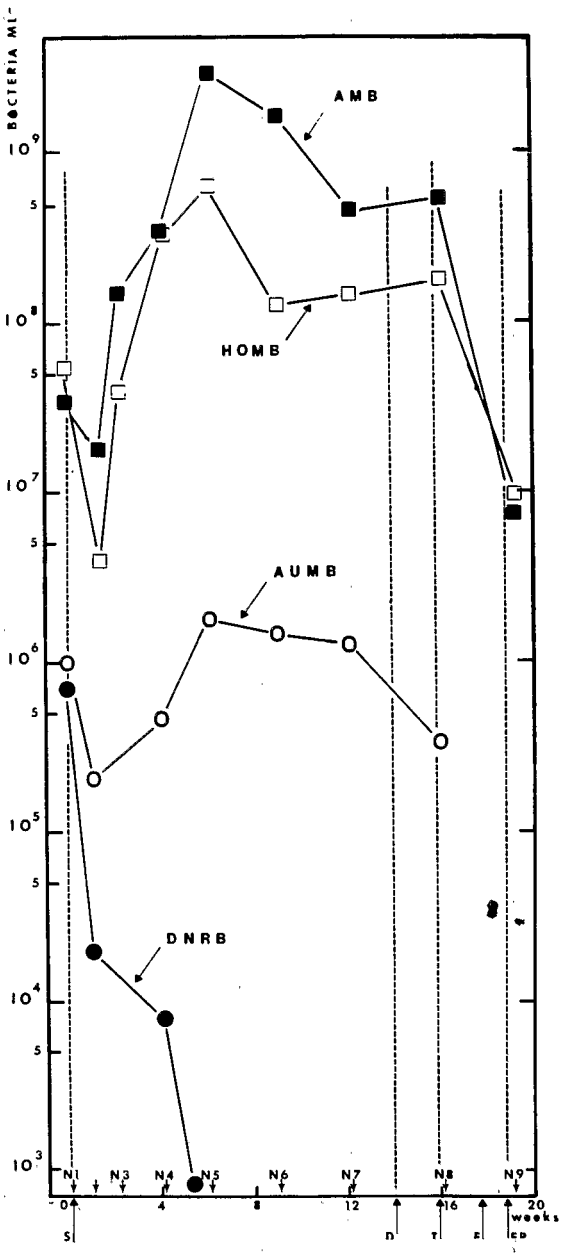

Fig. 3. Counts of methanogenic and nitrate-reducing bacteria. AMB: aceticlastic methanogenic bacteria; HOMB: hydrogen oxidizing methanogenic bacteria; AUMB: autotrophic methanogenic bacteria; DNRB: dissimilatory nitrate-reducing bacteria

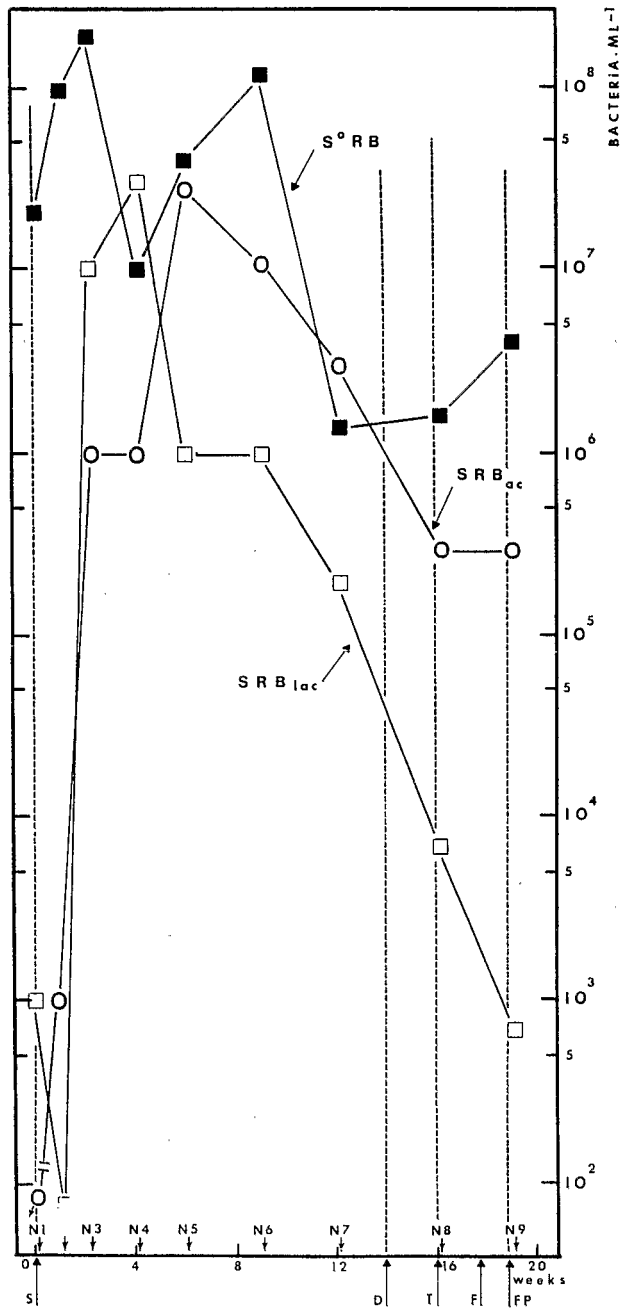

Fig. 4. Counts of sulphate- and sulphur-reducing bacteria. SRBlac: sulphate-reducing bacteria on lactate; SRBac: sulphate-reducing bacteria on acetate; S°RB: sulphur-reducing bacteria

Sulphur-reducing bacteria (Fig. 4). Their number was higher than that of SRB but it is known that some sulphate reducers (Biebl and Pfennig 1977) as well as some methanogens (Stetter and Gaag

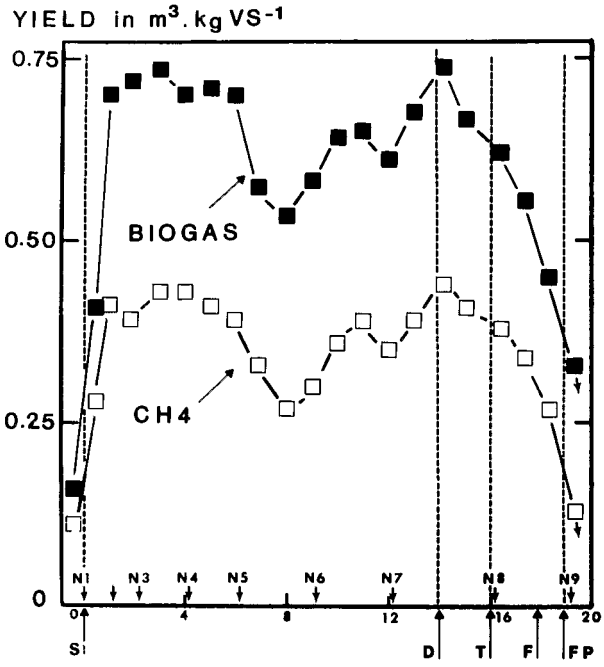

Fig. 5. Yields of biogas and CH_4 during anaerobic digestion of solubilized sugar beet pulp

1983) can use elemental sulphur as the final electron acceptor for sulphidogenesis. Their number increased after loading with fermented solubilized pulps.

Comparison with yield of biogas. Biogas production during anaerobic digestion of hydrolysed sugar beet pulps has been reported elsewhere (Labat et al. 1984). As compared with the yield curve (Fig. 5), air contamination influenced biogas yield which decreased as the number of anaerobic bacteria decreased. This decrease was also obtained when loading increased: the density of fermentative bacteria increased (Fig. 1) while the number of methanogens remained at an intermediary level (Fig. 3). Finally, when fermented pulps were introduced, the facultative anaerobic bacteria became the dominant microflora and the density of methanogens fell drastically as did the yield of biogas (Fig. 5) and the pH of the digestor (see Labat et al. 1984).

Discussion

The inclusion of bacteria inside clumps or aggregates in the digestor could influence the result of the total count. We reduced this problem by using a Potter apparatus and a polycarbonate membrane filter in order to obtain a single focal plane on the filter surface, but the Potter method surely does not fully disintegrate clumps of *Methanosarcina* spp. Here the purpose was to study the dynamics of the microflora rather than to determine the absolute accurate bacterial concentration. The results showed that total bacterial number could not be used as an indication of the good "health" of a digestor, since this count did not correlate with biogas yield.

The density of sulphur reducers was relatively high (10^8 per ml). This could be due to the possibility of a black coloration of the filter after growth by S°RB plus some facultative sulphate reducers and methanogens growing on acetate and able to use sulphur as final electron acceptor. The dominant morphological type among the counted bacteria was, however, a short, straight and non-sporulated rod.

After stabilization of the digestion, the number of aceticlastic methanogens was about 10 times higher than the number of the H_2–CO_2 users. We isolate two aceticlastic strains from extreme dilution MPN tubes, a *Methanosarcina mazei*-like species and a *Methanothrix* species. In the beginning, the *Methanosarcina* species was the dominant aceticlastic methanogen. However, after about 16 weeks of loading, the *Methanothrix* species became the dominant one.

This study is one of the first reports on a correlation between the biogas yield of a digestor and the succession of its microflora. It describes the development of the main bacterial groups during anaerobic digestion of a cellulosic substrate from initial inoculum (mixture of wastes) to stabilized digestion. We also report on some accidental events during the fermentation process and their effect on the development of the microflora.

The possibility of increasing the performance of a digestor by controlling its microflora appears theoretically possible or envisageable. The factors for optimizing such a complex system are numerous; analysis of various bacterial groups during the digestion process could indicate bad running of the digestor. Two or three specific counts could possibly explain failure of digesters, but could not contribute to optimization of the yield of biogas.

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