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Thermophilic anaerobic treatment to industrial wastewater: number of micro-organisms and biomass

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Abstract

This paper describes the determination of the microbial population, in terms of the number, biomass and composition, of single and two phase, laboratory-scale thermophilic (55°C) anaerobic reactors, under steady-state conditions. Epifluorescence microscopy with DAPI (4',6-diamidine-2-phenylindole) as fluorochrome was used to determine the total number of microorganisms in the reactors, and autofluorescence microscopy for the number of the total methanogenic populations. The results obtained by the direct count methods were compared to the quantity of biomass contained in the system, determined by volatile suspended solids (VSS). The concentration of acidogenic bacteria was estimated by subtraction of the autofluorescence results from those of the DAPI epifluorescence microscopy.

1 Introduction

Complex kinetics, interactions and different steps of anaerobic digestion have been reported by numerous authors [1-6]. There are considered to be two main groups of bacteria involved: acidogenic and methanogenic. The stability of the system depends on the viable bacterial groups involved in the process. Understanding the functioning of anaerobic reactors requires quantitative information on microbial numbers, biomass and activities of the bacterial groups

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involved in the process. Numbers, biomass, and activities represent distinct ecological parameters. Though normally correlated to each other, these parameters should not be used in an interchangeable manner.

Direct count procedures by microscopic methods yield the highest estimates of members of microorganisms and are occasionally used for indirect calculation of biomass. Epifluorescence microscopy with fluorometric stains are widely used for direct counting of bacteria, since it does not require culturing [7-9]. In particular, DAN-specific fluorescent stains 3,6-bis [dimethylamino] acridinium chloride (acridine orange) and 4',6-diamidine-2-phenylindole (DAPI) are most often used [10].

A characteristic peculiarity of methanogens is their UV-induced blue-green autofluorescence which permits to count them by autofluorescence microscopy [11-12]. However, this method is subjected under following conditions: it only shows methanogens with a high content of F420 such as hydrogen-utilizing methanogens; acetate-utilizing methanogens belonging to the genus *Methanosaeta* can not be counted at all and genus *Methanosarcina* are found in clump made up of many individual cells. Nevertheless, it is one frequently used method to count autofluorescent methanogens in anaerobic reactors [13-18].

The main objectives of this paper are to quantify the microbial numbers and biomass content of thermophilic anaerobic reactors and to make a comparative analysis of the results obtained by different counting methods. The total count was performed by the DAPI epifluorescence microscopy method. Autofluorescent methanogens were counted by the autofluorescence microscopy method. The biomass was determined by measuring volatile suspended solids (VSS).

These techniques have been applied to the measurement of the microbial populations contained in both single and two-stage, laboratory-scale reactors. In the single-stage process the main reaction steps- acidogenesis and methanogenesis- take place in the same reactor, while in the two-stage process they take place in separate reactors.

2 Materials and Methods

2.1.Experimental plan

The experimental protocol was designed to quantify the main bacterial population contained in a laboratory-scale continuously-stirred tank reactor (**CSTR**), without recycling solids. In this reactor, the solids and liquids retention times are equal. Two types of system were used : a single-stage reactor and two-stage reactor. Single-stage reactors were operated at two hydraulic retention time (HRT): 4 and 10 days (digesters **R4** and **R10**, respectively). In the two-stage system, HRTs were 1.7 and 4 days for the acidogenic phase (reactors $\mathbf{RA_{(1.7)}}$ and $\mathbf{RA_{(4)}}$, respectively), and 4 days for the methanogenic phase (reactors $\mathbf{RM_{(RA\ 1.7)}}$ and $\mathbf{RM_{(RA\ 4)}}$, for the corresponding HRT of the acidogenic phase).

The conditions selected for the first stage were those favouring the growth acid-formers, i.e. a short HRT (1.7 days) and a low pH (5.5); these conditions

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may be inhibitory to the methane formers. The methanogenic reactor was fed with filtered acidogenic effluent to suppress acidogenic bacteria in the feed.

Quantification assays were performed when reactors reached steady-state conditions during a period of 20 days. The attainment of the steady state was verified after an initial period (3 times the HRT) by checking whether the effluent characteristic values continued at the mean of the previous measurements.

All assays were carried out in duplicate and all the results shown are the average values of all of them.

2.2. Experimental system

A schematic diagram of the laboratory-scale CSTR used in the study is shown in Figure 1.

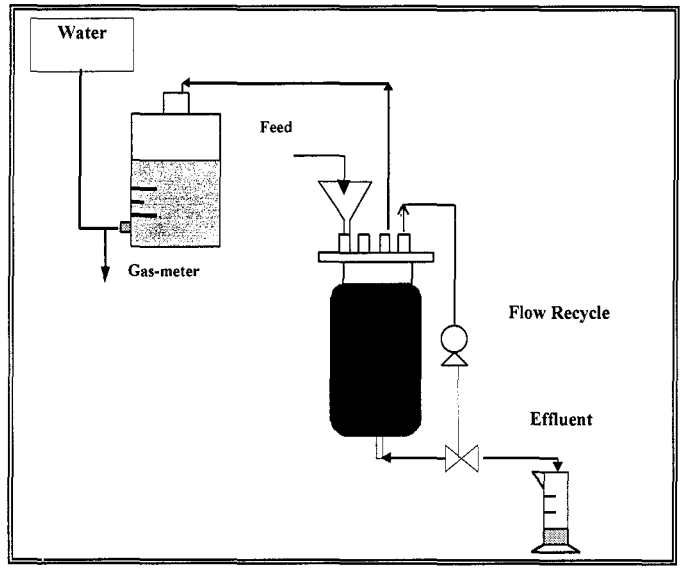

Figure 1: Schematic diagram of the experimental CSTR

The stirred tank reactor consisted of vertical cylinder tank (25 cm length and 10 cm internal diameter). The active liquid volume was 2 L. Reactor temperature was maintained at 55°C and the biogas generated was collected in a gas-meter. The feed was added in a semi-continuous mode: one dose per day. Effluent recirculation was used to mix and homogenise the liquid in the system.

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2.3. Feed solution

Distillery wastewater (vinasses) obtained from an ethanol producing wine-distillery plant located in Tomelloso (Ciudad Real, Spain) was used. In general, the vinasses showed an adequate relationship between the different macro and micro-nutrients with a favourable BOD:N:P ratio (91:4:1) suitable for microbiological treatment. A complete study of the characteristics and properties of vinasses can be found in a previous paper by the authors [19]. Before their utilisation, vinasses were transported and maintained at 4°C. The original substrate was diluted with tap water to attain the required chemical oxygen demand (COD) concentration to be used in the feed for this experiment (around 15 g $COD^{-1}L^{-1}$). This was supplemented with sodium hydroxide to maintain a neutral pH, and pH 5 in the case of the acidogenic reactor. Vinasses biodegradation batch experiments [20] indicated that this was a complex medium formed by two substrates of different nature and biodegradability: S_1, the easily biodegradable substrate fraction (80% of the total), and S_2, the recalcitrant substrate fraction. Initial COD of vinasses was 30 g COD^{-1}, and the concentration of volatile suspended solid (VSS) and bacteria was negligible.

The methanogenic reactor was fed with acidogenic effluent, filtered using a Millipore GVWP filter with a 0.22μm pore size to retain the acidogenic microorganisms, and was supplemented with sodium hydroxide to maintain pH around 8,5.

2.4 Analytical methods

The analytical determinations made in this study can be grouped in two categories: those utilised to monitor and control the anaerobic digestion process and those utilised to count the bacterial population contained in the reactors.

2.4.1. Analytical determinations utilised to monitor and control the anaerobic digestion process

All analytical determinations were carried out according to the "Standard Methods" [21]. The parameters analysed for liquid samples in both effluent and influent were pH and soluble COD, while for gaseous samples the volume of biogas produced at STP conditions and its composition (CH_4, CO_2 and H_2) were analysed [22].

2.4.2 Analytical determinations utilised to count bacterial population

Quantification of total numbers contained in the system was determined by epifluorescence microscopy with DAPI, according to Kepner et al [8].

The autofluorescent methanogens were counted by autofluorescence direct counting, using the same microscopy as that used for the epifluorescence method as described by Doddema and Vogels [11].

Biomass was determined by measuring the volatile suspended solids contained in the digester medium, according to "Standard Methods"[21].

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3 Results and Discussion

3.1. Single-stage reactors

Performance and operating parameters for the control of the anaerobic process are shown in Table 1. Microorganism concentrations, obtained by direct count and determinations of biomass, are shown in Table 2. Percentages of the autofluorescent methanogens and number of cells per gram of biomass are shown in Tables 2-3. All the results shown are the average values for the total days of the study.

Autofluorescent methanogens remain practically constant (17%) in the single-stage digesters operated under different HRTs. Nevertheless, operation under the shorter HRT assists the bacterial population to reach a greater size in the steady-state.

The correlation between direct microbial counts and biomass are shown in Figure 2.

Table 1. Performance and operating parameters for the control of the anaerobic process during period of study

Reactor	HRT	OLRo	CODr	pH_e	Biogas	CH_4	CO_2	H_2
R10	10	1.44	82.1	7.35	0.47	82	18	0
R4	4	3.75	80.1	7.60	0.80	85	15	0

HRT (days) ; organic loading rate (OLRo) as $g \cdot L^{-1} \cdot d^{-1}$ CODo ; organic removal efficiency (as percentage of initial COD) ; pH; volumetric biogas production as $L \cdot L^{-1} \cdot d^{-1}$ digester ; percentages of CH_4, CO_2, and H_2 in the biogas.

Table 2. Microorganism concentrations by direct count, biomass by VSS and number of bacteria per gram of biomass, in the single-stage reactors

Reactor	Total Population $(x10^8)^{(1)}$	Non-methanogenic Population $(x10^8)^{(1)}$	Non-methanogenic Percentage	Autofluorescent Methanogens $(x10^8)^{(1)}$	Autofluorescent Methanogenic Percentage
R10	8.06±1.92	6.77±2.07	83.18	1.29±0.50	16.82
R4	29.70±7.15	25.10±7.00	83.41	4.60±0.80	16.59

Precision of counts: 95% confidence interval
(1) Microorganisms·mL^{-1}.

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Table 3. Biomass by VSS and number of bacteria per gram of biomass, in the single-stage reactors

Reactor	VSS (g/L)	Number of bacteria ·gVSS^{-1} (x10^{12})
R10	0.47±0.09	1.86
R4	1.57±0.22	1.87

Independently of the operated HRT, the positive correlation between microorganism concentration and biomass is high and, consequently in these cases biomass determinations can be used to estimate microbial concentrations in the reactors, and vice versa.

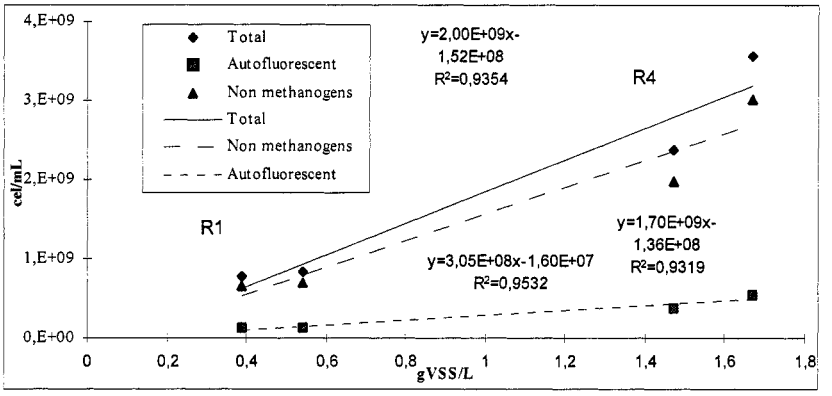

Figure 2: Correlation between microorganism concentrations by direct count and biomass as VSS, for the single-stage reactors

3.2. Two-stage reactors

Performance and operating parameters for the control of the anaerobic process are shown in Table 5. Microorganism concentrations, obtained by direct count, and determinations of biomass, are shown in Tables 6-7. Percentages of the autofluorescent methanogens and number of cells per gram of biomass are shown in Table 6. As with the single-stage-reactors, all the results shown are the average values for the total days of the study.

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Table 5. Performance and operating parameters for the control of the anaerobic process during period studied

Reactor	HRT	OLRo	CODr	pH_e	Biogas	CH_4	CO_2	H_2
$RA_{(1.7)}$	1,7	9.17	31.9	5.45	0.29	48	31	21
$RA_{(4)}$	4	3.79	30.1	5.53	0.18	66	29	7
$RM_{(RA\ 1.7)}$	4	2.43	61.5	7.70	0.32	94	6	0
$RM_{(RA4)}$	4	2.65	71.7	7.80	0.45	91	9	0

HRT (days); organic loading rate (OLRo) as $g{\cdot}L^{-1}{\cdot}\ d^{-1}$ CODo; organic removal efficiency (as percentage of initial COD); pH; volumetric biogas production as $L{\cdot}L^{-1}{\cdot}d^{-1}$ digester; percentages of CH_4, CO_2, and H_2 in the biogas.

In the initial assays, the percentages of autofluorescent methanogens remained as described above for the single-stage-reactors. However, they are, respectively, slightly lower and higher in each acidogenic and methanogenic phase of the reactor than those obtained in the single-stage system. The size of population in the acidogenic reactor is smaller when operated under a HRT of 1.7 days. This HRT is short enough to make "wash-out" of slow growing micro-organisms, possibly. Moreover, the system does not work under stable conditions, as shown by the H_2 content of the biogas (see Table 5).

Table 6. Microorganism concentrations by direct count, biomass by VSS and number of bacteria per gram of biomass, in the two-stage reactors

Reactor	Total Population $(x10^8)^{(I)}$	Non-methanogenic Population $(x10^8)^{(I)}$	Non-methanogenic Percentages	Autofluorescent Methanogens $(x10^8)^{(I)}$	Autofluorescent Methanogenic Percentages
$RA_{(1.7)}$	31.0±0.60	2.70±0.60	87.96	0.36±0.09	12.04
$RA_{(4)}$	24.40±2.40	24.20±2.40	99.29	0.17±0.05	0.71
$RM_{(RA\ 1.7)}$	5.50±2.69	4.49±2.66	79.00	1.00±0.58	21.00
$RM_{(RA4)}$	10.39±1.63	7.72±1.56	73.84	2.68±0.27	26.16

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Table 7. Biomass by VSS and number of bacteria per gram of biomass, in the two-stage reactors

Reactor	VSS (g/L)	Number of bacteria.gVSS^{-1} ($x10^{12}$)
$RA_{(1.7)}$	0.57±0.04	0.49
$RA_{(4)}$	1.05±0.09	2.35
$RM_{(RA\ 1.7)}$	0.66±0.08	0.79
$RM_{(RA4)}$	0.89±0.15	1.20

Bacterial number and biomass results are greater when the two reactors are operated under an HRT of 4 days. Autofluorescent methanogens constitute less than 1% of the total population in the first reactor phase, and 26% in the second reactor phase. Nevertheless, an effective separation of acidogenic and methanogenic phases was not obtained, thus some methanogens remained in the acidogenic reactor and vice versa.

The correlation between direct microbial counts and biomass for the two-stage system is shown in Figure 3.

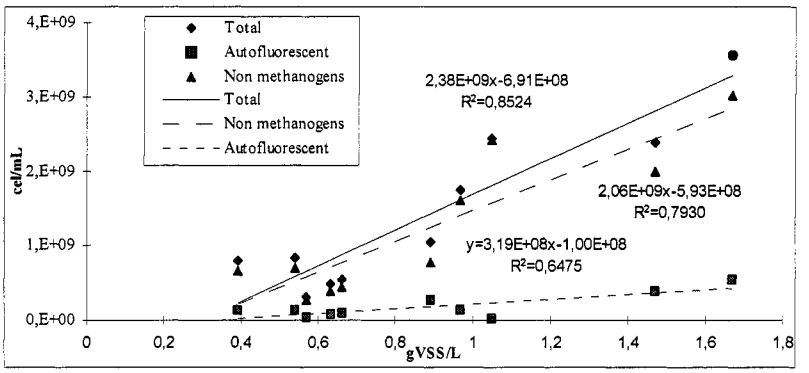

Figure 3: Correlation between microorganism concentrations by direct count and biomass as VSS, for the two-stage reactors

The correlation between direct microbial counts and biomass for the two-stage reactors are not good. In this case, biomass may increase or decrease without a corresponding change of cell numbers. Therefore, in the two-stage digesters, biomass cannot be used reliably to estimate the concentration of microorganisms, at least for the methanogenic type.

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4. Conclusions

4.1. In the single-stage reactors

There is a high correlation between direct count by DAPI epifluorescence microscopy and biomass expressed as VSS. Consequently, measurement of microbial number and biomass can be used in an interchangeable manner.

The autofluorescent methanogens constitute 17% of the total microbial community in single-stage thermophilic reactors operated under different HRTs.

4.2. In the two-phase reactors

There is no correlation between direct count and biomass parameters, so that in this case biomass changes without a corresponding change of microbial numbers, particularly of autofluorescent methanogenic numbers. In this case, measurement of microbial number and biomass should not be used in an interchangeable manner.

Autofluorescent methanogens constitute less than 1% of the total population contained in the first phase of the reactor, and more than 26% of the total in the second phase. Therefore, the effective separation of the two phases is not obtained.

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