# The Evolution of Biomass in a Two-phase Anaerobic Treatment Process During Start-up

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This paper reports the experimentation carried out in a semi-continuous, laboratory-scale, two-reactor system fed with diluted wine vinasses. The principal objective of this research was to observe the variations in auto-fluorescent methanogens and non-methanogenic bacteria at differing rates of HRT and OLR. The experiments were conducted with two different HRTs in the acidogenic phase: 1.7 and 4 days; whereas in the methanogenic phase, HRT was maintained at 4 days.

In the acidogenic phase, the numbers of methanogens and non-methanogens decreased considerably when the HRT was 1.7 days. This HRT is short enough to make "wash-out" of slow-growing microorganisms possible and it may also be the minimum retention time bordering on process failure conditions. Phase separation (acidogenesis and methanogenesis) was not achieved in either of the two reactors. A moderate level of methanogenic activity was permitted in the first reactor.

*Key words:*
Epifluorescence, methanogenesis, thermophilic anaerobic digestion.

## Introduction

Anaerobic treatment of industrial wastewater has become a viable technology in recent years due to the rapid development of high-rate reactors, low excess sludge production, and enclosure of odours and aerosols.[1] The treatment capacity of an anaerobic digestion system is primarily determined by the amount of active population retained within the system, which in turn is influenced by wastewater composition, system configuration and operation of anaerobic digestion.

Anaerobic digestion can be considered a three-step process, even though it is really a coupled sequence of microbiological interactions. In the initial stage, complex organic materials are depolymerized and converted to $CO_2$, $H_2$ and fatty acids, mainly acetic, propionic and butyric. In the next stage, all the higher acids are converted to acetic acid. In the final stage, a biogas containing methane and $CO_2$ is produced along two different pathways: from acetic acid (acetoclastic methanogens) and from $CO_2$ and $H_2$ ($H_2$-utilizer methanogens).

In anaerobic reactors, acidogenic bacteria convert soluble organic material mainly to acetate, propionate, butyrate, $H_2$, and $CO_2$, presumably via the known pathway of metabolism. The optimum pH of acidogenic bacteria is 5.2 to 6.5, and the specific growth rate is over 2 days. Some of the products of acidogenic bacteria, namely acetate, and $H_2$, can be metabolized by methanogenic bacteria, but others, such as propionate and butyrate, cannot. Propionate and butyrate must be further degraded to acetate and $H_2$ by acetogenic bacteria. These bacteria grow very slowly, with a minimum doubling time of 3.6 days. The optimum pH of these microorganisms is 6 to 7. Methanogens are among the most fastidious of anaerobes; they require or are stimulated by growth factors such as vitamins, unusual trace minerals (Co, Ni), fatty acids (acetate) or specific cofactors (coenzyme M) unique to methanogenic microorganisms. In a typical anaerobic digester, the two major methanogenic precursors are acetate and $H_2$-$CO_2$. Approximately 70 % of the digester methane comes from acetate and the remainder from $CO_2$ reduction to $CH_4$. Methanogenic bacteria grow more slowly than acidogenic bacteria, at a rate similar to acetogens. The optimum pH environment for methanogens is within the range 7.5 to 8.5.[2,3]

The two-stage anaerobic treatment process has several advantages over conventional processes. Firstly, it permits the selection and enrichment of different bacteria in each digester; in the first phase, complex pollutants are degraded by acidogenic bacteria into volatile fatty acids (VFA), which are subsequently converted to methane and carbon dioxide by acetogenic and methanogenic bacteria in the second phase. Secondly, it increases the stability of the

process by controlling the acidification-phase in order to prevent overloading and the build-up of toxic material. Thirdly, the first stage may act as a metabolic buffer, preventing pH shock to the methanogenic population; in addition, low pH, a high organic loading rate and a short hydraulic retention time are all factors which favour the establishment of the acidogenic phase, and preclude the establishment of methanogens.

Acidogenic and methanogenic microorganisms differ, not only in terms of their nutritional and pH requirements, but also with respect to their physiology, growth and nutrient uptake kinetics, and in their particular ability to withstand environmental changes. Consequently, conditions that are favourable to the growth of acid-forming bacteria (short HRT, low pH) may be inhibitory to methane-forming bacteria[4]. An advantage of two-phase digesters is that their operating conditions may be selectively determined in order to maximise not only acid- but also methane-forming bacterial growth. In some cases, nonetheless, (wastewater with a high content of biorecalcitrant substance) a certain level of methanogenic activity is permitted in the acidogenic reactor, since these bacteria consume $H_2$, produced in the acidogenic phase.[5] When $H_2$ removal by methanogens is less efficient, $H_2$ blocks electron disposal by proton reduction, and acidogenic bacteria must channel electrons to other disposal sites. This results in increased production of reduced fermentation products such as propionate and butyrate. However, the resulting low $H_2$ concentration accompanies the formation of acetate as the major soluble product. Furthermore, the continuous removal of $H_2$ stimulates its own formation, and $H_2$, together with $CO_2$, then become important products.

## Materials and methods

All the experiments were carried out in thermophilic conditions and employed vinasses as substrate, obtained from an ethanol-producing wine-distillery plant situated in Tomelloso (Ciudad Real, Spain). In general, the vinasses showed an adequate relationship between the different macro- and micro-nutrients, with a favourable ζCOD:N:P ratio for microbiological treatment, and an acid pH (approximately 3.7). The concentration of volatile suspended solids and bacteria in the vinasses was negligible. A complete study of the characteristics and properties of the vinasses may be found in previous papers published by the authors.[6,7] The results obtained indicated that this was a complex substrate, with an overall 20 % biorecalcitrant fraction. Some of the characteristics of the vinasses are detailed in Table 1.

Table 1 – *Characteristics of wastewater used*

| Quantity | Value |
|---|---|
| pH | 3.35-5.00 |
| COD, $\gamma_{O2}$ / g L$^{-1}$ | 30.0 |
| BOD$_5$, $\gamma_{O2}$ / g L$^{-1}$ | 12.0 |
| Suspended solids $\gamma$ / mg L$^{-1}$ | 120 |
| Polyphenols $\gamma_{galic\ acid}$ / mg L$^{-1}$ | 500 |

A study was undertaken of the changes in the microbial populations of a two-phase, anaerobic digestion system operating under different conditions. The HRT of the acidogenic reactor varied between 1.7 and 4 days, whilst the HRT in the methanogenic reactor was maintained at 4.0 days.

Quantification of the total microbial population was determined by epifluorescence microscopy with DAPI, in accordance with the procedure employed by *Kepner*.[8] The autofluorescent methanogen count was performed by F420 autofluorescence micoscopy, as described by *Doddema* and *Vogels*.[9] By this method, the number of methanogens with a high content of F420, such as hydrogen-utilizing methanogens, may be evaluated. The concentration of the non-methanogenic population was estimated by subtracting the results of the autofluorescence microscopy from those obtained by epifluorescence microscopy with DAPI. Quantification assays were performed over a period of 6 months.

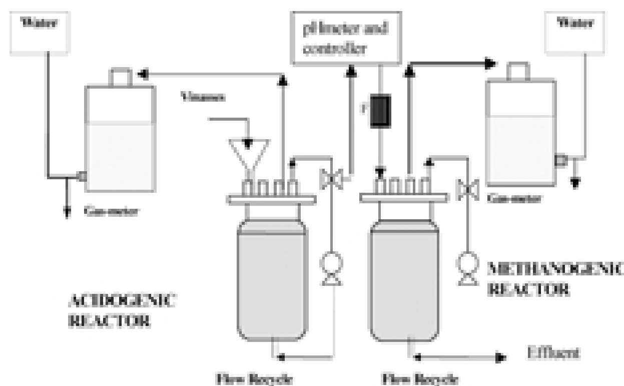
In order to establish an active anaerobic bacterial population, the reactors were seeded with effluent from a CSTR single-stage reactor, namely, an inoculation reactor. The operating characteristics used in this reactor, are presented in Table 2.

Table 2 – *Operating characteristics of inoculation reactor*

| *Quantity* | *Value* |
|---|---|
| Temperature | 55 °C |
| HRT | 4 days |
| Organic loading rate (OLRo) | 3.79 g·L$^{-1}$·d$^{-1}$ CODo |
| Organic removal efficiency (as fraction of initial COD) | 78.9 % |
| pH (units) | 7.80 |
| Volatile suspended solids | 1.57 g L$^{-1}$ |

The ratios of non-methanogenic and autofluorescent methanogens contained in the inoculation reactor were 84:16, respectively.[10]

Figure 1 shows a schematic diagram of the laboratory-scale, two-phase system, composed of two




F i g . 1 – *Schematic diagram of the two-stage anaerobic digestion system*

CSTR in series: the first phase is a 1-litre fermentative stage, and the second is a methanogenic phase, with a 5-litre working volume.

The reactor temperature was maintained at 55 °C and the biogas generated was collected in a gas-meter. Effluent recirculation was used to mix and homogenise the liquid in the system, and the solid and liquid retention times were equal.

The acidogenic reactor was fed with vinasses (15 g $L^{-1}$ COD). Prior to their use, they were supplemented with sodium hydroxide to maintain pH 5. The methanogenic reactor was fed with filtered acidogenic effluent (to suppress the acidogenic bacteria in the feed), and pH was controlled within the range 7.5–7.9 by the addition of NaOH. A GVWP Millipore membrane filter was used, with a pore diameter of 0.22 $\mu$m, and this was changed weekly. The lowest HRT of the acidogenic reactor (1.7 days) produced sufficient acidogenic effluent to permit feeding of the methanogenic reactor in accordance with the HRT corresponding to this phase.

The analytical procedures employed to monitor and control the anaerobic digestion process were conducted in accordance with "Standard Methods"[11]. The quantities employed in the analysis of the liquid samples of, both, the effluent and influent were pH, volatile fatty acids and soluble COD; in the case of gaseous samples, the parameters used were the volume of biogas produced in STP conditions and the composition thereof ($CH_4$, $CO_2$ and $H_2$).

Soluble COD was determined by the dichromate reflux method: the sample was first filtered using a Millipore AP4004705 filter with a 0.45 $\mu$m pore diameter; the filtrate was then used for the COD analysis. Volatile fatty acids were measured by gas chromatography, according to the method described by *Fang.*[12] Gas production was measured continuously by water displacement. Gas composition ($CH_4$, $CO_2$ and $H_2$) was determined using a modified gas chromatography method previously described by *Nebot* et al.[13]

## Results and discussion

The performance and operating quantities for the control of the anaerobic process are shown in Table 3. Microorganism concentrations are shown in Table 4. All the results shown are the average values for the total days of the study.

T a b l e 3 – *Performance and operating quantities for the control of the anaerobic process during the period studied*

| Reactor | HRT | OLRo | CODr | $pH_e$ | Biogas | $CH_4$ | $CO_2$ | $H_2$ |
|---|---|---|---|---|---|---|---|---|
| | $t$/d | $g \cdot L^{-1} \cdot d^{-1}$ CODo | $w$/% | | $L\ L^{-1} \cdot d^{-1}$ digester | $\varphi$/% | $\varphi$/% | $\varphi$/% |
| Acidogenic | 1.7 | 9.17 | 31.9 | 5.45 | 0.29 | 48 | 31 | 21 |
| Methanogenic | 4 | 2.43 | 61.5 | 7.70 | 0.32 | 94 | 6 | 0 |
| Acidogenic | 4 | 3.79 | 30.1 | 5.53 | 0.18 | 66 | 29 | 7 |
| Methanogenic | 4 | 2.65 | 71.7 | 7.80 | 0.45 | 91 | 9 | 0 |

Organic loading rate (OLRo) ; organic removal efficiency (as mass fraction of initial COD) ; volumetric biogas production.

T a b l e 4 – *Microorganism number concentrations in the two-stage reactors*

| Reactor | HRT | Total Population $C/10^{8(1)}$ | Non-methanogenic Population $C/10^{8(1)}$ | Autofluorescent Methanogenic Population $C/10^{8(1)}$ |
|---|---|---|---|---|
| Acidogenic | 1.7 | 3.10±0.60 | 2.70±0.60 | 0.36±0.09 |
| Methanogenic | 4 | 5.50±2.69 | 4.49±2.66 | 1.00±0.58 |
| Acidogenic | 4 | 24.40±2.40 | 24.20±2.40 | 0.17±0.05 |
| Methanogenic | 4 | 10.39±1.63 | 7.72±1.56 | 2.68±0.27 |

Precision of counts: 95% confidence interval

(1) Microorganisms · $mL^{-1}$.

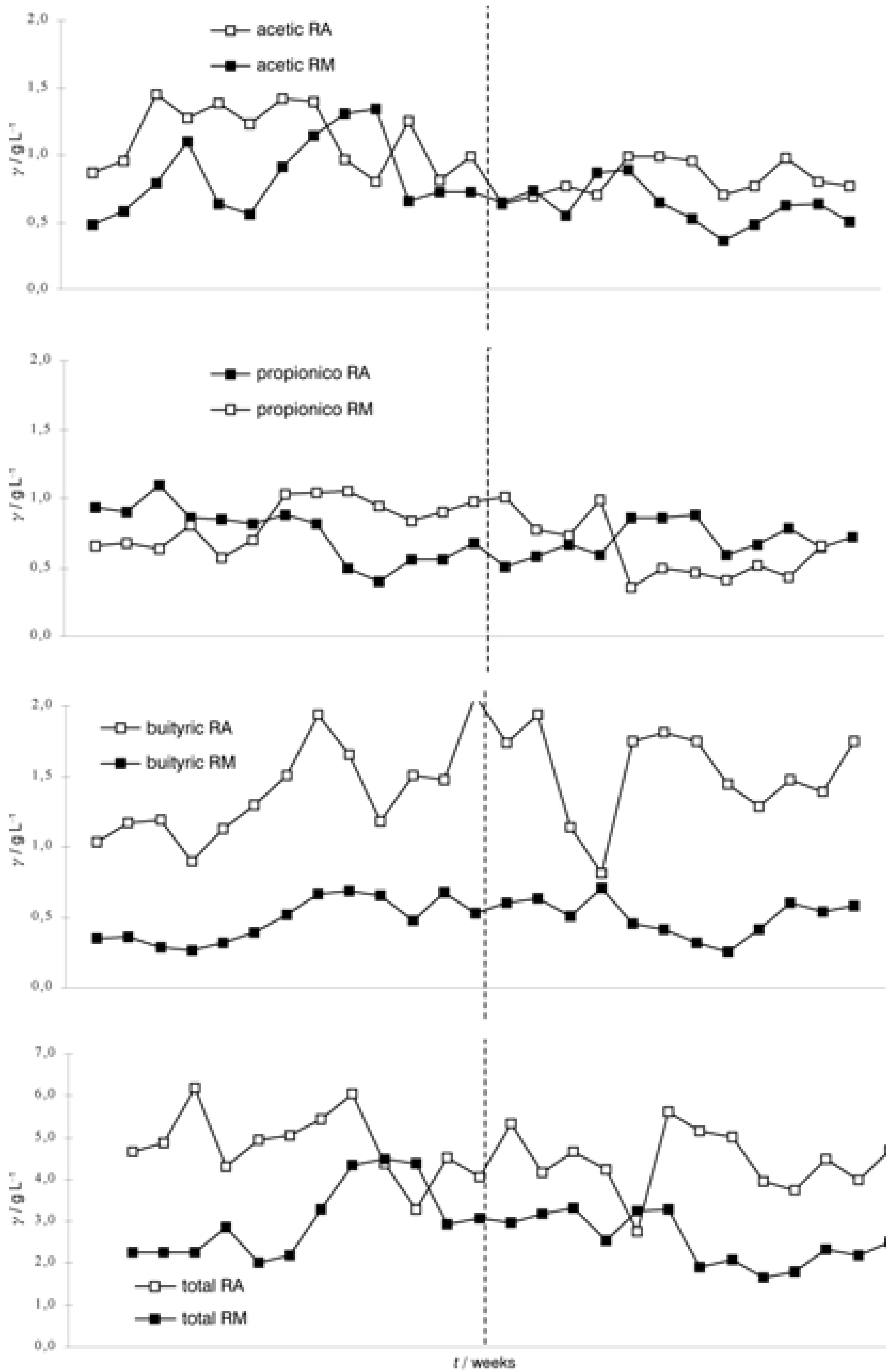



Fig. 2 – *Volatile Fatty Acid of Two-Stage System*

During start-up, the total numbers of non-methanogenic bacteria and fluorescent methanogenic bacteria were smaller when the acidogenic phase was operated at an HRT of 1.7 days and with an $\gamma_{OLRo}$ of 9.17 $g_{COD}$ $L^{-1}$ $d^{-1}$. The total autofluorescent methanogenic population was 12 % in the acidogenic reactor, as opposed to 21 % in the methanogenic reactor. This HRT was short enough to make "wash-out" of slow-growing microorganisms possible. In addition, the system was operating under unstable conditions, as shown by the $H_2$ content of the biogas (see Table 1).

The amount of autofluorescent methanogens and non-methanogenic bacteria increased significantly during start-up, when the acidogenic reactor was operated at an HRT of 4 days (OLRo 3.79 $g_{COD}$ $L^{-1}$ $d^{-1}$). In this case, not only the size of the aforementioned populations increased, but their percentages as well. As a result, autofluorescent methanogens constituted 0.71 % and 26 % of the total population in the acidogenic and methanogenic reactors, respectively. These percentages are significantly lower and higher in each acidogenic and methanogenic phase of the two-stage system than those obtained in the single-stage inoculation reactor.

Phase separation (acidogenesis and methanogenesis) was not achieved in the two reactors. The fact, that $H_2$-utilizer methanogenic bacteria require less strict growth conditions than the acetogenic type, and hence can resist the operating conditions imposed (low pH and short HRT), may explain why they remain in the acidogenic reactor.

Figure 2 shows the evolution of volatile fatty acids in the acidogenic and methanogenic reactors. It may be observed that the evolution is similar in both phases. The level of VFA decreased in both reactors when the HRT was 4 days in both phases. When the HRT was 1.7 days in the acidogenic phase, the higher concentrations of propionate and acetate contained in the digester indicate that the reactor was not operating under stable conditions. The butyrate resultant of acidogenic metabolism was transformed to methane by acetogens and methanogens in the second reactor.

## Conclusions

The principal objective of this research was to observe the effect of changes in HRT on auto-fluorescent methanogens and on the non-methanogenic bacterial population in a two-stage anaerobic digestion system treating wastewater from a wine-distillery factory during start-up. The acidogenic reactor was operated at different rates of HRT and OLR, and, consequently, the number of auto-fluorescent methanogens and non-methanogenic bacteria and their relative percentages, varied. When the acidogenic reactor was operated at an HRT rate of 4 days, auto-fluorescent methanogens constituted 0.71 % and 26 % of the total microbial population in the acidogenic and methanogenic reactors, respectively.

### Abreviations and symbols

$COD_o$ – Initial Chemical Oxygen Demand
$COD_r$ – Chemical Oxygen Demand
*CSTR* – Continuously-Stirred Tank Reactor
*DAPI* – 4',6-diamidine-2-phenylindole
*HRT* – hydraulic Retention Time
*OLRo* – Initial Organic Loading Rate
*RA* – Acidogenic Reactor
*RM* – Methanogenic Reactor
*t* – time
*VSS* – Volatile Suspended Solids
*C* – microorganism concentration, m $L^{-1}$
*w* – mass fraction, %
$\varphi$ – volume fraction, %
$\gamma$ – mass concentration, d
$\zeta$ – mass ratio

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