Anaerobic Thermophilic Colonization of Porous Support

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Original scientific paper
Received: October 10, 2005
Accepted: February 1, 2006

Biofilm development in an open-pore sintered glass material (SIRAN) was studied using a laboratory-scale anaerobic fixed-film bioreactor under thermophilic conditions. The startup and performance of that bioreactor, operating on distillery waste water feed (vinasses), were also studied.

Results obtained indicated that stepped organic loading during initial bioreactor start-up reduced the periods of adaptation in the colonization process and micro-organism attachment and biofilm formation was accelerated by the surface characteristics of the carrier. The results obtained by operating with stepped organic loading (Γ = 2.0 kg m^{-3} d^{-1} COD) over a period of 70 days suggest that a stable operation of the process (90% COD removal) and high density of biomass immobilized on the support (20 kgVSatt per m^3 SIRAN) is achieved. Epifluorescence microscopy demonstrated that, initially, attached growth was developed in crevices, where biomass was protected from shear forces and, finally, SIRAN was completely covered and biofilm developed on the entire SIRAN particles.

Key words:
Anaerobic digestion; biofilm; immobilization; open-pore sintered glass; support materials, SEM

Introduction

The anaerobic treatment of industrial wastewater has a number of potential benefits, including low energy consumption, low excess sludge production, enclosure of odours and aerosols, and rapid re-start-up after prolonged shut-down.[1] High rate anaerobic digesters which retain biomass also have high treatment capacity and low site area requirements. The major process configurations developed are upflow anaerobic sludge blanket (UASB), both upflow and downflow stationary packed beds and fluidised or expanded beds.[2–7]

One drawback associated with immobilized reactors is the length of time required for initial biofilm attachment, (time of 9 to 12 months have been reported[8]). This problem can be minimised using a stepped organic load during initial reactor start-up[9] or supplying a substrate that may be directly metabolised by microorganisms,[10] or modifying the flow velocity.[11]

The nature of the support used for biofilm attached has a significant effect on the biofilm attachment. A wide range of materials have been used as non-porous support at laboratory-and pilot-scale, including glass beads,[12] red drain clay,[13,14] sand and a number of different plastics[15] and porous materials, such as needle-punched polyester,[16] polypropylene particles,[17] granular activated carbon (GAC)[18] and sintered glass.[2,4,6,7]

Several important advances have been made in the study of biofilm microbial populations relating to their spatial structure (or architecture), their community structure, and their dependence on physicochemical parameters.[19] *Darkin* et al.[14] propose the application of the environmental scanning electron microscope (ESEM) to the direct examination of the clay interface and biofilm formation in situ within the microcosm.

This research was designed to evaluate start-up and performance of an anaerobic fixed-film bioreactor containing a porous support (SIRAN) treating distillery wastewater (vinasses), and to follow the colonisation process on SIRAN.

Materials and methods

Feed composition

The feed utilised is wine distillery water proceeding from an ethanol producing wine-distillery plant situated in Tomelloso (Ciudad Real, Spain). The vinasses were transported and maintained at 4 °C before their utilisation. This feed was diluted with tap water to attain the required feed Chemical Oxygen Demand (COD) mass concentration to be

used in this experiment (around 18 γ_{COD} = 18 g L^{-1}) and was supplemented with sodium hydroxide to maintain a neutral pH.

Fixed-film bioreactor

The colonisation process of support medium was carried out in an anaerobic digester treating vinasses of wine and using SIRAN as fixed-film support. The schematic diagram of the anaerobic fixed-film system used in the laboratory study is shown in Fig. 1. The anaerobic filter bioreactor consists of vertical cylindrical tank. The active liquid volume was 2 L, and the empty volume was 2.4 L. The temperature was maintained at 55 °C (precision of ± 1 °C) and the biogas generated was collected in a gas-meter. The operation was semi-continuous and feed was supplied by a peristaltic pump connected to a programmable timer. Effluent recirculation was used to mix and homogenise the liquid in the system.

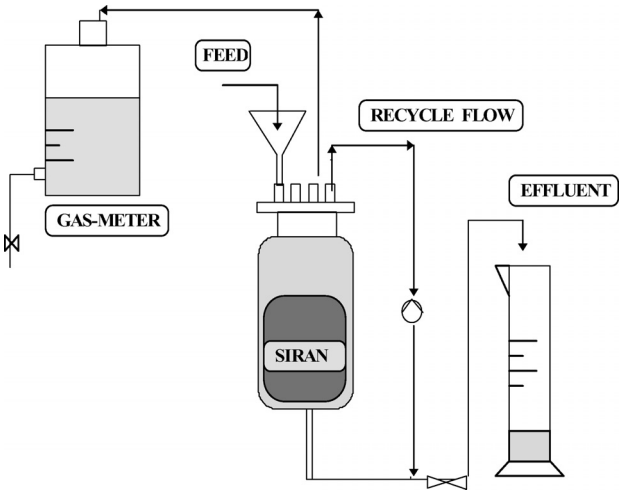

Fig. 1 – *Schematic diagram of the anaerobic fixed-film reactor used*

Characteristics of the support

The support used consisted of open-pore sintered glass beads, "SIRAN". This carrier has been developed and marketed by Schott Glaswerke, and it is produced by sintering of a mixture of glass and saltpowder followed by a washing process which elutes the non-sinterable salt. The resulting glass sponge has well-defined pore size distribution with double-pore structure.[2]

The particles were sieved for uniformity and the resulting particles had an apparent diameter of approximately 1.5 – 2 mm. This material was chosen because of its uniformity and also because it could be calcinated to measure dry organic matter concentrations. The main characteristics of SIRAN carriers are the follows: medium real density ρ = 1832 g L^{-1}; bulk density ρ_b = 570 g L^{-1}; pore volume fraction φ = 50-60 %; pore diameter d = 60-300 μm, and high volumic surface (a = 87000 m^2 m^{-3}) suitable for using as support in anaerobic fluidized-bed bioreactors.

Experimental procedure

The experimental protocol was initially carried out with 750 mL of clean support. Two litters of anaerobic thermophilic inoculum, coming from a laboratory stirred tank bioreactor, were added to the non-colonized support. The characteristics of inoculum used are: pH 7.9, P_{COD} = 710 kg m^{-3} d^{-1}, P_c = 550.2 mg L^{-1}, γ_{SS} = 1.35 kg m^{-3}, γ_{SVS} = 0.89 kg m^{-3} and Volatile Fatty Acids, (VFA) = 22.4 g m^{-3}.

The bioreactor was seeded with active biomass from a conventionally operational steady-state digester. Every day, 200 mL of medium were replaced with 200 mL of new fresh vinasses (diluted and neutralised vinasses with 15 kgm^{-3} COD, HRT: 10 days) and organic load of 1.27 kg m^{-3} d^{-1} COD. After 15 days operating in these conditions, the HTR was reduced to 8 d increasing the volume of feed to 250 mL. The organic load rate at this time was situated between 1.6 and 2.0 kg m^{-3} d^{-1}. The rate of recycle flow during this research was 8-10 L h^{-1}, in order to reduce microbial stress.

The colonization assay lasted 70 d, enough time to obtain an adequate attached biomass to the particles of support, as it will be noticed in the results obtained from the evolution of the process.

Analytical methods

All analytical determinations were performed according to "Standard Methods".[20] For liquid samples, the parameters analysed in both the effluent and the influent were pH, chemical oxygen demand (COD), both Total and Volatile Suspended Solids (TSS, VSS), and attached microbial mass (VSatt). For gaseous samples, the parameters analysed were the volume of biogas produced and its composition. COD was determined by the dichromate reflux methods. For soluble COD, the sample was first filtered as in the TSS analysis and the filtrate was used for the COD analysis. TSS and VSS were determined by the glass fibber filter method as described in "Standard Methods".[20] Gas production was measured continuously by water displacement. Measurements of methane and carbon dioxide were obtained using gas chromatography separation, accomplished by using a stainless steel column packed with Carbosieve SII (diameter 1/8", length 2 m) with thermal conductivity detector TCD.

Attached biomass concentrations were determined by removing a representative sample from the bioreactor and then ashing the dried sample to

measure the total volatile solids both attached to the particles and entrapped among them.[21]

In several stages of the colonisation process, a small fraction of colonised particles was utilised for their morphologic characterisation using optic microscopy and epifluorescence microscopy (length wave excitation at λ = 400-440 nm with barrier filter at 460 nm) and scanning electronic microscopy (SEM).

Results and discussion

The experimental protocol was designed to examine the effect of organic loading rate on the efficiency of the anaerobic thermophilic (55 °C) fixed-film bioreactor (with SIRAN support) process and evaluate the biomass attached concentration evolution in the bioreactor. The temporal evolution of organic removal rate, OLRr as kg m^{-3} COD per day and methane production P_{CH_4} as m^3 m^{-3} CH_4 per day are shown in Fig. 2.

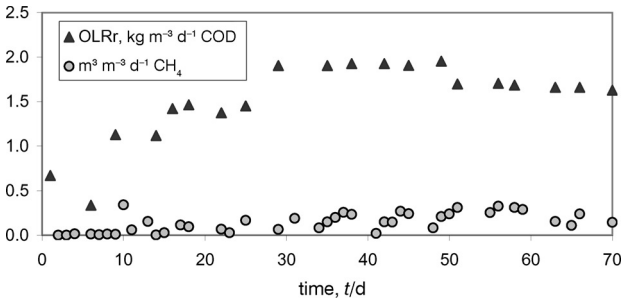

Fig. 2 – *Temporal evolution of organic removal rate, OLR_r and methane production*

During the early start-up period of the bioreactor, the organic removal efficiency (OLR = 1.27 kg m^{-3} d^{-1} COD) was 85 % soluble COD removal. This COD removal included both processes: organic matter conversion to methane and synthesis of new microbial biomass that increased suspended biomass. When the OLR increased to 1.6 kg m^{-3} d^{-1} COD (decreasing HRT until 8 days), the efficiency increased until more than 90 % of the elimination, and when the OLR was 2.0 kg m^{-3} d^{-1} COD the process achieved 95 % soluble COD removal.

The pH medium was maintained, all the time, into the range of pH values appropriate to develop the process of anaerobic digestion in the thermophilic range. The values registered are relatively high (close to 8) and provoke the gaseous CO_2 dissolution in the liquid medium and consequently the relatively low percentages of carbon dioxide analysed in the biogas.

The biogas volume and methane generated during the process was low but concordant with the literature in this field.[5,6] The volumetric concentration rate of methane generation showed a tendency to stabilise in the range approaching σ_{CH4} = 0.2–0.3 m^3 m^{-3} d^{-1} with methane yield 0.2 m^3 CH_4 per kg COD_r. Nevertheless, this value was significantly lower than the stochiometric theoretical value of 0.35 m^3 CH_4 per kg COD_r. In this sense, the synthesis of new microorganisms and the initial biomass attachment processes on the support surface involved the initial production of polysaccharide to bind the material. This phase involves a high consumption of organic material through the synthesis route (anabolism), thereby, diminishing the quantity of substrate that it transforms into methane. For this reason, the theoretical value of 0.35 m^3 CH_4 per kg COD_r is higher than the calculated experimental value.

The monitoring of the colonisation and attachment biomass processes on SIRAN was studied by evaluating the modifications, that were produced in the total content of volatile suspended solids in the liquid medium and attached to the support throughout the temporal extension of the experiment, according to the protocol described by *Shieh* et al.[21] Temporal evolution of the biomass colonisation process on SIRAN support is shown in Fig. 3. The

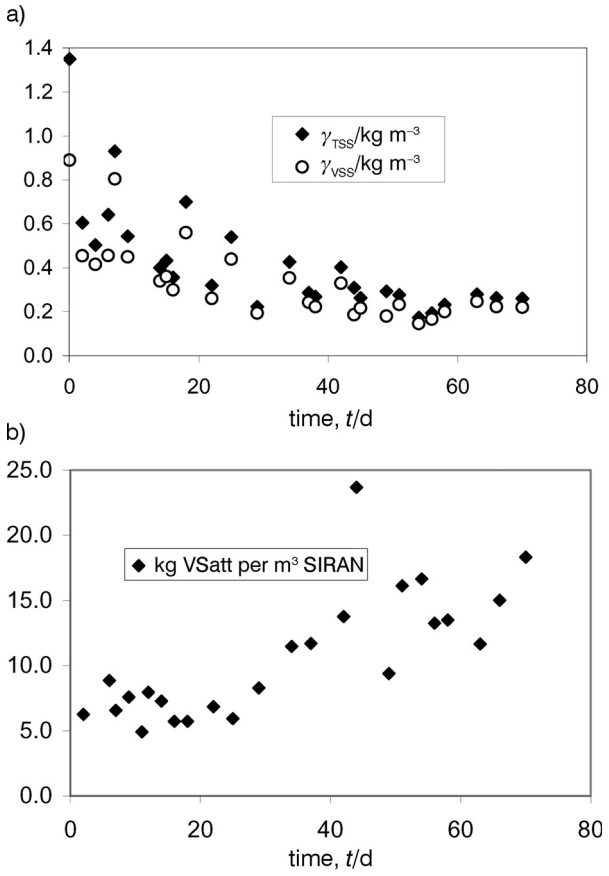

Fig. 3 – *Temporal evolution of effluent solids during the colonisation process a) total and volatile suspended solids (γ_{TSS} / kg m^{-3} and γ_{VSS} / kg m^{-3}) and b) volatile attached solids (kg VS_{att} per m^3 SIRAN)*

graphic shows total and volatile suspended solids in the medium (γ_{TSS} and γ_{VSS}) and the volatile attached solids on the support (kg VS_{att} per m^3 SIRAN) over a period of 70 d. As it is shown, the effluent VSS followed a decreasing tendency until it stabilised at 0.2 kg m^{-3}. Therefore, the whole bioreactor contained approximately 0.4 g VSS. Also, in just 2 d of contact between microorganisms and support, 6 kg VSatt per m^3 SIRAN were obtained and the attached solids follow an increasing tendency, reaching 20 kg VS_{att} per m^3SIRAN in 70 d. Hence, the physical characteristics of the SIRAN support, with double-pore structure and high surface area, favoured the adequate conditions for biomass attachment since the initial stage of the process. The oscillation of VSatt after 40 d of research can be attributed to the detachment of biofilm as consequence of the shear forces and friction phenomenon with other close particles.

The results published by *Gorris* et al.[22] indicate that the colonisation process occurs in three consecutive phases: viz. lag phase, biofilm production phase and steady-state phase, irrespective of the type of inoculum applied. This pattern reflected the overall rate of colonisation and biofilm production on beads present in the bioreactor. These stages are observed in the present study (see Fig. 4), although, the stepped COD load reduced the duration of the initial period of induction to a few hours operation. The three phases of the colonisation of material supports have also been observed and described in other studies like the one carried out by *Sich & van Rijn*.[23] These authors studied the bacterium colonisation using as support sand particles in a fluidized bed bioreactor at laboratory-and pilot-scale. The following of the colonisation process by SEM (Scanning Electronic Microscopy) led to distinguish an *initial phase* that concerns the two first days of the process, an *intermediate phase* until the day 10th and the *final phase of the colonisation*, going to the day 20th.

The final values of biomass density on the SIRAN support obtained in this research were comparable with values published by *Fox*[24] in colonisation processes on GAC (granular activated carbon) in fluidised bed systems, introducing similar profiles of evolution, although, the GAC support requires a longer period of time in order to reach the same density of attached biomass. Thus, at the end of the experiment (12 weeks), SIRAN retained over seven times the biomass retained on 0.7 mm sand and over three times the attached biomass than the anthracite during a 43 weeks period treating synthetic feed containing acetate as the only carbon source at mesophilic conditions.[24]

Bull et al.[9] indicated that stepped organic loading with co-substrate addition improved performance during the initial start-up time and appeared to aid bacterial development. Hence, the feed utilised in this study, vinasses, was a complex medium that provided all the macro-and micronutrients necessary for an adequate colonisation process of the carrier.[24]

At the end of the process, the attached biomass was more than 95 % of the total volatile solids contained in the bioreactor. Surface roughness increased the rate of accumulation of biomass on the packing support. It is most probably due to the initially colonised bacteria in the pores being pro-

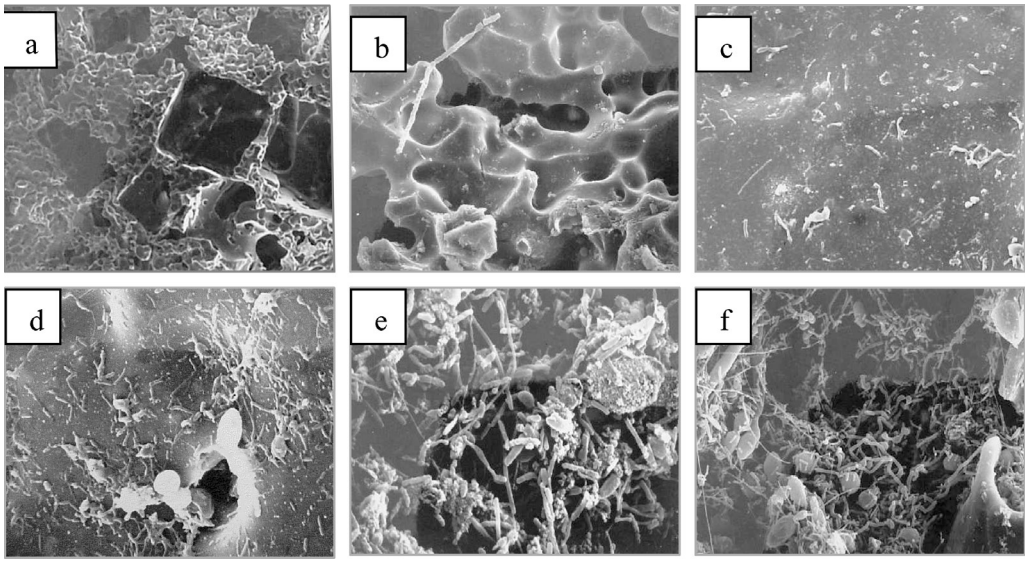

Fig. 4 – *SEM micrographs: a) non-colonized (×100); b) colonisation at 2 days (×1000); c) at 7 days (×2500); d) at 14 days from incubation (×2500); e) at 41 days (×5000) and, f) at the final steps of the process (70 days) (×2000)*

tected from shear forces and changes in environmental conditions. Under these conditions, it can be considered that the degradative activity in the system is due, mostly, to the immobilised microorganisms. These findings indicate that SIRAN is a suitable support medium for fluidised-bed, anaerobic bioreactors treating high-rate organic loadings.[6]

In several stages of the colonisation process, a small fraction of colonised particles was used for their morphologic characterisation, using optic microscopy and epifluorescence microscopy (length wave excitation at 400–440 nm with barrier filter at 460 nm) and scanning electronic microscopy (SEM).

Epifluorescence microscopic observations (λ_{exc} : 400-440 nm, λ_{barr} : 460 nm) and SEM characterizations were made on both virgin support and on colonized-beads obtained from different stages of the process. These observation corroborated the results obtained: SIRAN carrier showed a high colonisation density at a faster rate during startup. The initial adhesion occurred within the first 6 h. The surface roughness was critical to initial micro-organisms attachment and biofilm formation.[2,24,26]

Fig. 4 shows SEM micrographs of colonised SIRAN in different stages. In Fig 4.(a) can be observed non-populated particle of SIRAN™ (100 magnification), showing the high number of macro-and micropores, characteristic structure that gives it a very high surface area. The micropores, between 1–10 μm, provided the adequate area to the initial colonisation of microorganisms. The macropores protect the microorganisms from shear forces and probably friction phenomenon with other close particles.

In the micrographs shown in the Fig. 4(b)-(f), different stages of the colonisation process are shown (at 2nd, 7th, 14th, 40th, and in the final steps of the colonisation). After 2 days, biofilm growth on SIRAN media was located primarily in areas with irregular shapes and large crevices, where protection from shear forces exists. After 7 days, the bacterium colonisation increases over the surface and after 14 days, the distribution becomes more heterogeneous showing areas of higher microbial density and others with no significance amount microorganisms (biofilm production phase).

In advanced stages of colonisation, the bacteria covered the entire support surface (Fig. 4.e 4.f). The presence of *filamentous* forms morphologically similar to *Methanosaeta (Methanotrix)* and *Methanosarcina* sp.,[17,27] *bacilares forms and cocci*, mainly, were observed by Scanning Electron Microscopy (SEM). *Tessele* et al.[17] noticed the major presence of determined bacterium species attached to the support depending on the degree of colonisation.

Conclusions

Anaerobic fixed-film digesters can achieve > 95 % COD reduction at COD loading of 2.0 kg m^{-3} d^{-1}, within 70 d. Therefore, very short start-up periods are followed by a stable operation: the high biomass concentration is protected against wash-out and remains on the large inside surface area of the SIRAN carriers.

The sponge-like characteristics of the sintered glass carrier enable very fast attachment of the micro-organisms, so the start-up time was very short including high COD removal right from the beginning of the feed. In just 48 h of incubation, quantities of 6 kg VSatt per m^3 SIRAN were obtained.

The open pore structure of the carrier offers high surface areas to be colonised by active biomass and the entire carrier can be populated. This characteristic allows a high biomass colonisation capacity in short periods of time: 20 kg VSS per m^3 SIRAN in a period of 70 days.

ACKNOWLEDGEMENTS

This work was funded by a grant of the Comisión Interministerial de Ciencia y Tecnología (C.I.C.Y.T.) of the Spanish Government (No. BIO 92-0859 – Madrid, SPAIN). R. Rodríguez-Cano was supported by an Accion-MIT fellowship from the Spanish Ministry of Science and Technology.

Abbreviations and symbols

COD – Chemical Oxygen Demand
COD_r – Chemical Oxygen Demand removal
HRT – Hydraulic Retention Time
OLR_r – Organic Load Rate removed
OLR_0 – Initial Organic Load Rate
VS_{att} – Volatile Attached Solids
TSS – Total Suspended Solids
VSS – Volatile Suspended Solids
a – volumic surface, m^2 m^{-3}
d – pore diameter, μm
P – production, kg m^{-3} d^{-1}
t – time, d

Greek letters

Γ – mass concentration rate, kg m^{-3} d^{-1}
γ – mass concentration, kg m^{-3}
ρ – density, g L^{-1}
λ – length wave, nm
π – volumen concentration rate, m^3 m^{-3} d^{-1}

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