Bioresource Technology 100 (2009) 492–496

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Short Communication

Application of flowcell technology for monitoring biofilm development and cellulose degradation in leachate and rumen systems

C. O'Sullivan [a,*], P.C. Burrell [b], M. Pasmore [d], W.P. Clarke [a], L.L. Blackall [c]

[a] Division of Environmental Engineering, The University of Queensland, St. Lucia, Brisbane, Queensland 4072, Australia
[b] Water Quality and Research, Water Distribution, Brisbane City Council, 315 Brunswick Street, Fortitude Valley, Queensland 4006, Australia
[c] Advanced Wastewater Management Centre, The University of Queensland, St. Lucia, Queensland 4072, Australia
[d] Sr. Principal Engineer, Baxter Healthcare Corporation, 25212 W IL Route 120, Round Lake, IL 60073, USA

ARTICLE INFO

Article history:
Received 7 March 2008
Received in revised form 13 June 2008
Accepted 16 June 2008
Available online 8 August 2008

Keywords:
Cellulose
Anaerobic digestion
Flowcell
Leachate
Rumen

ABSTRACT

In this study, a flat plate flowcell was modified to provide a reactor system that could maintain anaerobic, cellulolytic biofilms while providing the data needed to carry out a chemical oxygen demand mass balance to determine the cellulose digestion rates. The results showed that biofilms could be observed to grow and develop on cellulose particle surfaces from both anaerobic digester leachate and rumen fluid inocula. The observations suggest that the architecture of rumen and leachate derived biofilms may be significantly different with rumen derived organisms forming stable, dense biofilms while the leachate derived organisms formed less tenacious surface attachments. This experiment has indicated the utility of flowcells in the study of anaerobic biofilms.

1. Introduction

Since their development flowcells have become the tool of choice for the *in situ* investigation of aerobic biofilms (Caldwell and Lawrence, 1988; Palmer, 1999). A flowcell is a chamber that facilitates the growth of microbial biofilms under controlled conditions on a surface which can be mounted directly onto a microscope stage for visualisation. This offers the advantage that direct observations of the biofilm can be made in a non-destructive manner. Therefore, sequential images of the same biofilm can be made over time, allowing dynamic biofilm behaviour to be assessed.

In past studies of cellulose degradation, observations of the cellulolytic biofilms were made by collecting slurry grab samples from the reactor and subjecting these samples to light microscopy, fluorescence *in situ* hybridisation (FISH) and/or electron microscopy (Barnes, 2003; O'Sullivan et al., 2005a; Song et al., 2005). This has meant that when observations were made, a different set of biofilms on different cellulose particles were viewed at each sampling event. This means that events such as maturation, thickening, sloughing and loss of biofilms could only be inferred by comparing biofilms viewed at one time step to others at another time step. While inferences about the bulk properties of the biofilms can be made from observations of the grab samples, the destructive nature of sampling meant that any conclusions made from observations are dependant on the comparability of the samples. Flowcells provide a means of observing changes in biofilm composition and structure in real time (Palmer, 1999).

Studies of anaerobic biofilms using flowcells have been carried out but they are relatively recent and they are much less common than aerobic studies. Hansen et al. (2000, 2001) used an anaerobic flowcell system, similar to that employed in this study, to investigate biofilm formation by the oral bacteria *Porphyromonas gingivalis* under anaerobic and micro-aerophilic conditions. In this paper they noted that growth of anaerobic biofilms in flowcells had only been attempted a few times and the data from several previous studies had not been published. They also noted that anaerobic chemostats were commonly used but that this technology had not been extended to the *in situ* study of anaerobic biofilms.

A study of methanogenic biofilms using flowcell technology was conducted by Jones et al. (1997). In this study, a flowcell chamber capable of maintaining anaerobic conditions was developed using materials commonly found in microbiology labs (e.g., microscope slides and cover slips). They used the flowcell to examine cell attachment, colonisation and biofilm development by butyrate or hexanoate degrading methanogenic cultures enriched from an anaerobic landfill. They found that while materials such as cellophane, glass and plastic supported biofilm growth, cellulosic materials such as wood and cotton remained poorly colonised

* Corresponding author. Tel.: +61 7 3346 9820; fax: +61 7 3365 4599.
E-mail address: c.osullivan1@uq.edu.au (C. O'Sullivan).

0960-8524/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2008.06.028

even after three weeks. They concluded that non-degradable materials such as glass and plastic play an important role in landfills by providing a substrate on which biofilms can develop. This finding is in contrast to the accepted theory that cellulolytic bacteria are plentiful in landfills and require intimate contact with cellulose fibres in order for solubilisation to proceed effectively (Beguin and Aubert, 1994; Boone et al., 1993; Chynoweth and Pullammanappallil, 1996; Song et al., 2005). However, Jones et al. (1997) noted that the lack of colonisation of cellulosic materials in their study may be due to the exclusion of cellulolytic organisms from the enrichment culture.

The aim of this experiment was to demonstrate the utility of flowcell technology in studies of anaerobic, cellulolytic biofilms. The flowcell was used to compare the architecture and time-resolved behaviour of cellulolytic biofilms from rumen and leachate inocula growing on the surfaces of cellulose fibres. These observations were then related to process data gained from chemical oxygen demand (COD) balances.

2. Methods

2.1. Flowcell reactor setup

The flat plate flowcell consisted of a Perspex (polymethyl methacrylate) base into which a 52 mm × 12 mm chamber with a depth of 2 mm had been machined. This chamber was connected to inlet and outlet ports and was open at the top surface. A recess in the Perspex base plate was sized to fit a 60 mm × 40 mm coverslip which formed the upper surface of the chamber and provided the means of viewing the biofilms inside. The coverslip was held in place by a silicone gasket, which ensured that a liquid and gas-tight seal was maintained, and a metal face plate. Coverslips with marked grids were used to assist in locating particular cellulose fibres for observation at each sampling time.

The flowcell reactor configuration was completed by connecting a flat plate flowcell to a fully anaerobic media vessel at the inlet and outlet ports by means of Norprene tubing (Saint-Gobain Performance Plastics, Ohio, USA) to create a fully recycled system. The media vessel was a 2 L Schott bottle fitted with gas and slurry sampling ports as described for previous digestions (O'Sullivan et al., 2005a), however the bottle was also fitted with inlet and outlet ports to allow for media to flow through the flowcell. This created a fully recycled flowcell system connected to an anaerobic digester with provision for all sampling necessary to perform COD balances.

Prior to use the flowcell, media bottle and all tubing were autoclaved to ensure sterility. The coverslip, which formed the upper surface of the flowcell, was coated with silicone glue on its underside. Cellulose fibres were dusted onto the wet glue and it was allowed to dry overnight. This treatment meant that, when the flowcell was assembled, the cellulose fibres were suspended from the inside upper surface of the chamber. This meant that the fibres were fixed in a position which allowed for viewing using objective lenses with shorter working distance (up to 40× objective) while being in constant contact with the bulk media within the flowcell. Suspending the cellulose fibres from the upper surface of the flowcell also ensured that cells that appeared on the cellulose surfaces were definitely attached (either temporarily or permanently) to the fibres and not just resting on the surface as may be the case with cells on the lower surface of the chamber. Once the silicone glue was set, the flowcell chamber was assembled and connected to the media vessel.

Two reactor systems were prepared. One was inoculated with rumen fluid and the other with leachate from a leach bed anaerobic digester treating mixed organic fraction of municipal solid waste (MSW). The reactor vessels were filled with 1 L of basal medium (artificial saliva (Coleman, 1987)) supplemented with microcrystalline cellulose (Sigmacell, 50 μm nominal particle diameter), ammonium chloride and either sterile rumen fluid or sterile leachate. The composition of this mixture, before addition of the inoculum was (in g/L unless otherwise stated) $NaHCO_3$ 5.9; K_2HPO_4 5.0; KH_2PO_4 4.0; NaCl 0.52; CaCl (anhydrous) 0.035; $MgSO_4 \cdot 7H_2O$ 0.07; NH_4Cl 1.5; and clarified, sterile rumen fluid or sterile digester leachate at a concentration of 200 ml/L.

The leachate inoculum was collected from a 220 L leach bed anaerobic digester that was treating a simulated waste mix designed to mimic the organic fraction of MSW. The waste in the reactor was made up of defined amounts of food waste, yard waste and paper waste as described by Kayhanian and Hardy (1994) in a medium of sodium bicarbonate solution at 11.2 g/L.

Rumen fluid was collected from a fistulated steer that was feeding on a forage diet at the time of sampling. The collected material was filtered through four layers of nylon mesh (1 mm × 1 mm) to remove course solids prior to being placed into a nitrogen purged, insulated flask which provided an anaerobic, temperature controlled environment for the duration of transportation.

Once assembled and filled with media, the systems were purged with 80%:20% N_2/CO_2 gas mixture for 15 min to adjust the pH of the media and ensure an anaerobic headspace within the reactor vessels and the flowcells. One reactor was inoculated with 10% (v/v) leachate. In keeping with the findings of previous work, which indicated that initial cell density affects the rate of cellulose degradation during anaerobic digestion (O'Sullivan et al., 2008), the other reactor was inoculated with 1% (v/v) rumen fluid so as to provide comparable cell densities in both the leachate and rumen inoculated reactors at the outset of the experiment.

A flow rate of 1 mL/min was chosen for the media flow through the flowcell as the dimensionless Reynolds number calculated for this flowrate was less than 2000 indicating laminar flow conditions. The contents of the media vessels were stirred only during sampling of the slurry with the contents remaining fully settled at all other times. In the rumen inoculated system the flow was continuous except at sampling and viewing times. In the leachate inoculated system flow was continuous for the first four days after which flow was ceased for 12 h. The flow remained continuous for the rest of the digestion except for sampling and viewing times.

2.2. Microscopy and image collection

The flowcell systems were held in an incubator at 37 °C at all times except during sampling. This meant that the flowcell chambers were not permanently mounted on a microscope stage. At sampling times flow of media through the flowcells was stopped, the reactors were removed from the incubator and gently agitated to mix the contents before samples of gas and slurry were collected. The reactor vessel was then returned to the incubator but the flowcell was retained on the outside by passing the connecting tubes through a small notch in the incubator door. The flowcell was mounted onto the stage of an Olympus CX40 light microscope. Three cellulose fibres in each reactor were tracked over time. The fibres were located using the gridded coverslip. The fibres were viewed with a 40× objective lens under phase contrast microscopy. Images were collected using an Olympus C5060 digital camera mounted to the trinocular fitting of the microscope via a c-mount fitted with a C5060 digital camera adapter.

2.3. Analytical procedures

As in previous experiments the VFA production, the quality and quantity of biogas (methane, carbon dioxide and hydrogen) produced and ammonia and ammonium–nitrogen (NH_3–N) consumption was measured at each sampling time. A nitrogen balance was used to determine the biomass generation rate. Based on this data,