

Granule shearing enables the high resolution microbial identity

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Abstract

Granular biomass forms naturally in high-rate anaerobic treatment systems. It is very important for relevant industrial wastewater treatment systems, which generate methane as renewable chemical energy. The granules themselves are macroscopic aggregates, with a high density and a layered structure. Understanding drivers for spatial and microbial community structure can provide opportunities to enhance this technology. Until now, phylogenetic analysis has been studied either by sectioning and microscopic analysis with fluorescent *in situ* hybridization, or by whole population molecular analysis by bulk methods. This means different functional and spatial layers cannot be analysed separately by bulk molecular methods, including next generation sequencing techniques. In this work, we describe a method to successfully remove microbes selectively from successive spatial layers. The removed layers can then be analysed by whole molecular methods such as pyrosequencing, and compared to *in situ* analysis via cryosectioning and FISH. Particularly distinct and dominant (mainly bacterial) outer layer populations can be identified through both molecular analysis on sheared off layers and *in situ* analysis on the whole granules.

Key words

Anaerobic granule, shear, UASB, granule layer, cryosection-FISH

INTRODUCTION

The upflow anaerobic sludge blanket (UASB) reactor is one of the most widely-applied reactor designs for anaerobic digestion of readily degradable wastewaters. In a UASB, functional microorganisms including acidogens, acetogens and methanogens, aggregate into granules of 0.5-3mm which have a high sedimentation velocity and are therefore resistant to wash out under high hydraulic load. They are essentially a self-supporting biofilm, with observable layering (Guiot *et al.*, 1992) depending on feedstock. Microbial identity is known to be important in determining functional performance (Karakashev *et al.*, 2005).

Early studies of granule microbial communities were based on the morphology of microorganisms with scanning electron microscopy and transmission electron microscopy (Quarmby & Forster, 1995), and extensive interpretation has been based on presumptive identification. More recently, molecular techniques have been applied to identify the microbial ecology and structure of anaerobic granules (Kim *et al.*, 2011). As bulk DNA analysis methods require processing whole granules, they do not distinguish spatial information. To date, the spatial information, i.e. the position of particular organisms in the granules, can only be studied with fluorescent *in situ* hybridization (FISH). However, this is highly dependent on the choice of probes, and response of the organism to the FISH process. It is also limited to identification based on the probe specificity, and does not allow direct chemical analysis of the microbial genome. In this paper, we describe a modified version of Pereboom's abrasion technique (Pereboom, 1997) which aims to sequentially remove layers from mature granules from full-scale UASB reactors by applying shear stress and potentially allow phylogenetic and functional characterisation of each layer.

MATERIALS AND METHODS

Shear experiment

The granules collected from full scale UASB reactors at a brewery and cannery were referred as VFA (volatile fatty acid) granules and carbohydrate granules respectively. The shear experiments were performed in a standard geometry cell (1.3 L, 120 mm diameter) with a stainless steel Rushton impeller (40 mm diameter). Anaerobic granules were first gently washed over a 200 µm sieve to remove the native fines. Approximately 10 g (wet weight) sieved granules were added to 1 L 1× phosphate buffered saline (PBS) (pH 7.2) at the beginning of shear experiment and sheared at either 500 rpm (VFA granules) or 1500 rpm (carbohydrate granules). Samples were collected after 5, 20, 90, 180, 270 and 360 minutes of shearing. At each sampling point, the contents of the cell were sieved through the 200 µm sieve to separate fines and remaining granules. Fines were centrifuged to collect a pellet. Granules on the sieve were collected for further analysis. The remaining granules on the sieve were re-suspended in 1 L 1×PBS and shearing continued. Granule images were taken by high resolution camera. The images were then analysed using Quantimet image analysis software (Leica, Australia) to determine the size of granules. Total Suspended Solids (TSS) were analysed by method 2540D and R in standard methods (APHA, 1992). The sheared depth was calculated based on the volume loss obtained from TSS using the size and density of granules measured.

Pyrosequencing and Cryosection-FISH

Genomic DNA was extracted from generated fines and remaining granule (on the sieve) collected at each sampling point with the Power soil DNA isolation kit (Mo Bio, USA). Pyrosequencing analyses were performed by the Australian Centre for Ecogenomics (ACE) at The University of Queensland, using primer sets 926f (5'-AAACTYAAAKGAATTGACGG-3') and 1392r (5'-ACGGGCGGTGWGTRC-3') with Roche 454 GS FLX sequencer (Roche, Switzerland). Pyrosequencing reads were processed using QIIME (Caporaso et al., 2010) with error correction by ACACIA (Bragg *et al.*, 2012) to obtain operational taxonomic unit (OTU) tables and representative sequences in each OTU. PFA fixed granules were embedded and sectioned according to the procedure described in Batstone *et al.* (2004). FISH was performed according to the protocol described by Lee *et al.* (1999).

RESULT

Shearing was successful as demonstrated by FISH analysis of pre-sheared and post-sheared granules (Figures 1A and 1B respectively). As can be seen, the outer bacterial layer has been removed. The abundance of major bacteria obtained from outer layer samples were doubled compared to the whole granule. These bacteria were also identified by FISH with specific probes (Figure 2A and 2B).

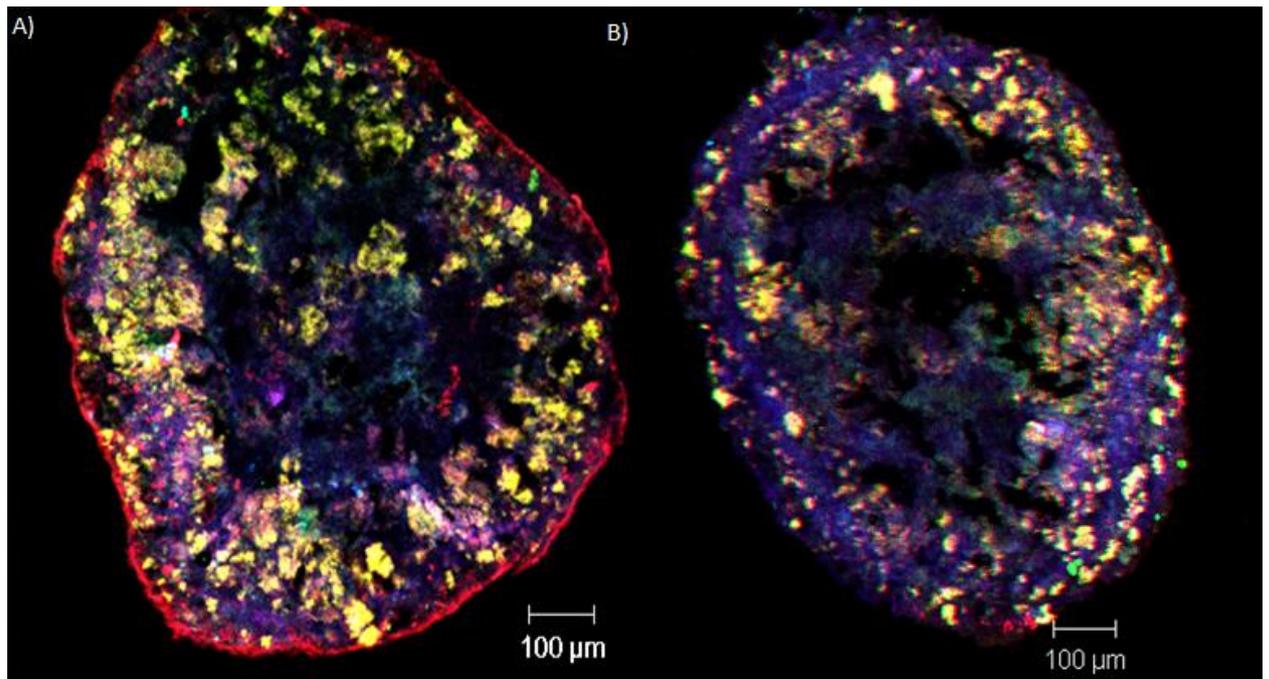


Figure 1. FISH image of the carbohydrate granules before (A) and after shearing (B). Bacteria are shown in red and form a distinct layer on the surface of carbohydrate granules. This layer is completely removed after shear experiment. Archaea are shown in yellow and cyan.

Table 1. Abundance shift of major bacteria in the outer layer obtained from pyrosequencing.

Microorganism	VFA granules		Carbohydrate granules	
	O/W*	I/W**	O/W*	I/W**
Bacteroidetes	-	-	1.47×	0.82×
Candidatus Cloacamonas	1.6×	0.4×	-	-

* Abundance in outer layer/ abundance in the whole granule

** abundance in inner layer/ abundance in the whole granule

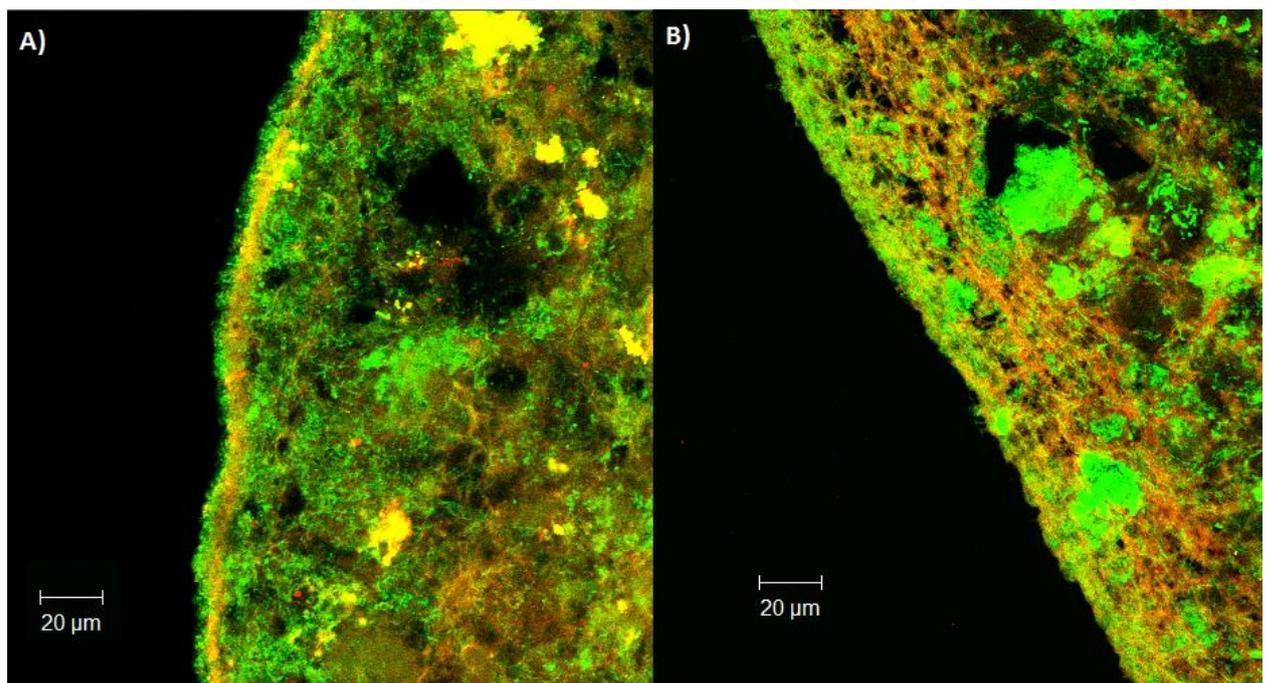


Figure 2. FISH image of major bacteria on the outer layer. Bacteria are shown in green, Bacteroidetes on the outer layer of carbohydrate granules (A) is shown in yellow, *Candidatus Cloacamonas* on the outer layer of VFA granules (B) is shown in yellow.

DISCUSSION

The relationship between spatial information and microbial community identification can be well described by this shear method. Compared to traditional methods which apply bulk DNA method to the whole granules, the shear method provided an opportunity for fast detection of major microorganism on each layer by FISH. In anaerobic granules, the bacterial community were widely spread across >20 phyla, thus the proportion of specific microorganism of the whole microbial community is usually small and not distinguishable. Without the spatial information, FISH hunting (i.e. trying all possible bacterial probes in a row) can be intensive and time-consuming. With the shear method, applying bulk DNA methods to each layer is possible. The bacterial and archaeal community can then be monitored separately and provide a higher resolution on microbial community structure of anaerobic granules. In addition, the acidogenic groups in both VFA and carbohydrate granules were effectively identified by both T-RFLP and FISH, which were rarely achieved previously (Sekiguchi *et al.*, 1999). Furthermore, the quality of DNA recovered from the shear method is far better as the shear force is relatively gentle compared to subsequent DNA extraction.

CONCLUSION

The shear method is able to provide an opportunity to selectively remove the sequential layer structure from anaerobic granules, which opened the gateway towards microbial analysis and identification of microbial aggregates under high resolution. Bacterial outer layers in both cases were presumptive acidogens, but from phylogenetically diverse groups.

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