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Microbial Ecology of Fermentative Microbes in Anaerobic Granules

Yang Lu

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The University of Queensland, Australia

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Abstract

Granules are large, self-supporting biofilms that form naturally in high-rate anaerobic treatment systems and are extremely important to reactor functionality. The biofilm structure has functional and phylogenetic layering, important both scientifically and to function. Fermenters, found on the surface of anaerobic granules, are associated with granule strength and uptake of the primary substrate. Compared to acetogens and methanogens, very limited work has been done on fermenters, particularly in granules. Due to their high diversity and difficulty in selective isolation, fermenters are best studied through culture-independent techniques. Until now, the microbial distribution could only be analysed through sectioning and microscopic analysis with fluorescent *in situ* hybridization. The whole granule could be analysed by DNA extraction and microbial community profiling methods but this did not provide spatial information. This thesis develops a method to remove microbes selectively from successive spatial layers through hydraulic shearing and demonstrates its application on anaerobic granules of three different types (collected from brewery, cannery and dairy wastewater treatment plants). Outer layers, in particular, could be selectively sheared as confirmed by FISH and TRFLP. Further analysis with 454 pyrosequencing showed that a shift in dominant population from presumptive fermenters (such as *Bacteroidales* and *Anaerolinea*) in outer layers to syntrophs (such as *Syntrophomonas* and *Geobacter*) in inner layers, with progressive changes through the depth. The method was further leveraged through covariance based deep metagenomic sequencing, with metagenomic analysis used so far to align phylogenetic information. This leveraged the shear based method to provide covariance information for the metagenomic analysis. Shear based phylogenetic and metagenomic aligned well internally and with cryosection-FISH analysis. Information provided could be used with more specific probes (particularly *Bacteroidales* and *Anaerolinea*) to confirm that these organisms were key fermenters and highly abundant in outer layers. This study indicated that fermenters were a relatively diverse but discrete population within the granule. Further analysis of the metagenomic information is required to identify roles of specific microbes. The phylogenetic approach was also used in a reactor study with different feeds (gelatine, glucose, VFA) to identify how the microbial population shifted during growth of fermenters. While physical strength was not influenced, the

dominant fermentative groups were strongly impacted by the feed matrix, and inner layer community less changed, but still substantially affected.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

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Contributor	Statement of contribution
Yang Lu	Performed experiments (100%) Wrote the paper (60%)
Frances Slater	Supervised experiments (40%) Wrote the paper (20%) Edited the paper (20%)
Ricardo Bello-Mendoza	Designed experiments (40%) Edited the paper (20%)
Damien Batstone	Designed experiments (40%) Supervised experiments (60%) Wrote the paper (20%) Edited the paper (60%)

Contributions by others to the thesis

I was principally response for all the research leading to the outcomes as described, conducted the literature review, drafted the written papers, and generated follow up versions, including final submission based on feedback from my supervisors. This thesis includes the reporting of some important contributions made by other researchers that I have collaborated with through the duration of my PhD.

Associate Professor Damien Batstone from AWMC, as my principal supervisor, contributed with experimental design and critical revising my writings.

Doctor Frances Slater from AWMC, as my co-supervisor for the first and half year of my PhD, assisted with analysing TRFLP data (Chapter 3).

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Keywords

Anaerobic, Granule, Shear, UASB, Microbial ecology, Biofilm, Granule architecture, Pyrosequencing, Illumina.

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List of Abbreviations

ACE	Australian Centre for Ecogenomics
AD	Anaerobic Digestion
BCA	Bicinchoninic acid assay
BVF	Bulk Volume Fermenter
COD	Chemical Oxygen Demand
DNA	Deoxyribo-Nucleic Acid
EGSB	Extended Granular Sludge Bed
EPS	Extracellular Polymeric Substances
FISH	Fluorescent in situ Hybridization
HMMs	Hidden Markov Models
HPLC-UV	High Performance Liquid Chromatography-UV
HRT	Hydraulic Retention Time
IC	Internal Circulation
MDS	Multiple Dimensional Scaling
NGS	Next Generation Sequencing
PBS	Phosphate Buffered Saline
PCA	Principle Component Analysis
PCoA	Principle coordination analysis
PCR	Polymeric Chain Reaction
rRNA	Ribosomal Ribo-Nucleic Acid
SEM	Scanning Electron Microscope
SRT	Sludge Retention Time
TEM	Transmission Electron Microscopy
TRFLP	Terminal Restriction Fragment Length Polymorphism
UASB	Upflow Anaerobic Sludge Bed
VFAs	Volatile Fatty Acids

Chapter 1. Introduction

This chapter develops the motivation for research and reviews current literature relevant to the project. This thesis aims to investigate the microbial community (especially the fermentative community) on anaerobic granules in terms of architecture and distribution, diversity and identity, and function. The literature review introduces background of anaerobic granules, reviews current and potential techniques for microbial study of anaerobic granules and identifies the knowledge gaps in the understanding of fermentative communities in anaerobic granules. Fundamental knowledge on microbial community gained in this thesis should expand understanding of anaerobic granules and provide opportunity for further enhancement.

1.1 Research Motivation

Anaerobic granules are the major functional elements in the widely applied wastewater treatment anaerobic digester unit operation - the Upflow Anaerobic Sludge Blanket reactor (UASB) and related variants such as Extended Granular Sludge Bed (EGSB) and Internal Circulation (IC) reactors. Functionality of anaerobic granules relies on physical properties including size, density and strength, as well as the microbial properties including microbial capability and spatial distribution. However, this technology has failed in cases where biomass is lost due to destruction of low strength granules. Due to its high efficiency, UASB technology is currently applied in brewery, cannery and dairy industries to treat wastewater consisting of soluble organic compounds. Attempts to apply this technology to other wastes treatment processes such as fatty wastewater, solid wastes treatments or manufacturers generally specify solids concentrations in the influent of $<1000 mg \cdot L^{-1}$ have generally not succeeded.

Anaerobic digestion (AD), in general, is applicable for the digestion of substrates with high solids concentration. Fermenters, grown on the surface of anaerobic granules, are responsible for the physical strength and initial steps of substrate digestion. Thus manipulating fermenters possibly provides an opportunity to enhance granule strength, boost the efficiency and/or

broaden the range of substrates anaerobic granules can process. As such, this thesis is focusing on investigate the microbial ecology of fermentative microbes with a view for enhancement in existing processes and future application of anaerobic granular processes.

1.2 Anaerobic Digestion Process

AD is a biological decomposition process that converts organic matter to methane and carbon dioxide in the absence of oxygen. AD can be implemented in a wide variety of technologies with low cost infrastructure and is relatively easy to operate and control. The final product of AD, biogas, typically contains 50-70percent methane (but may be higher depends on substrate and operational conditions) and 30-40percent carbon dioxide. Methane gas has an energy content of 55MJ·kg^{-1} (Lide, 1995). With one-third conversion efficiency of heat into electrical energy, the electricity generation is 5.1kWh·kg^{-1} CH_4. Biogas is less harmful to the environment as it is derived from short cycle organics instead of fossil carbon, and hence does not contribute to global warming. It has the potential to be used for heat and energy generation instead of fossil fuel. AD relies on the cooperative and sequential action of a number of different microorganisms (Gujer and Zehnder, 1983). There are four major steps in AD as shown in Figure 1.1.

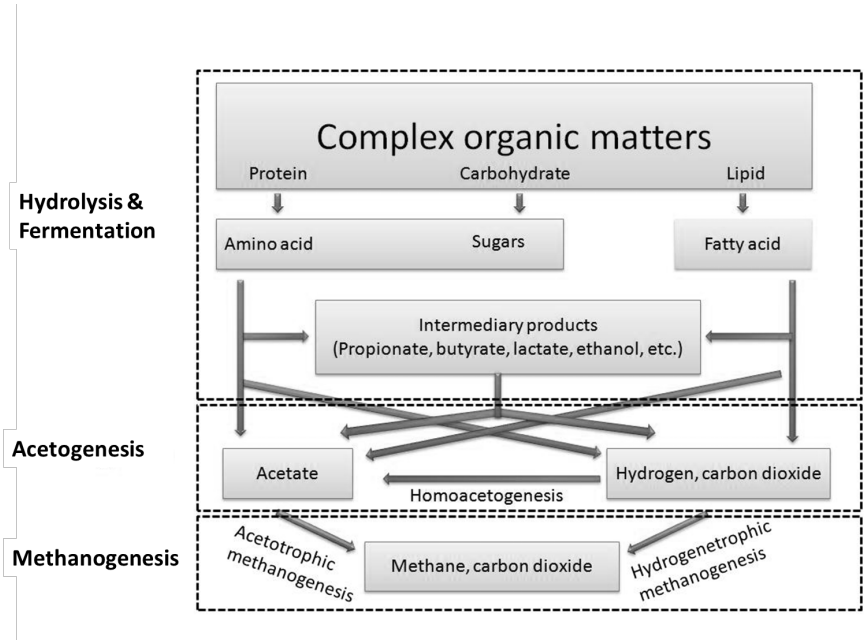


Figure 1.1 Major conversion steps in anaerobic digestion of complex organic matter.

1.2.1 Hydrolysis

Carbohydrates, proteins and lipids are the major organic components in solid wastes and high-strength wastewater. Hydrolysis is generally considered as a rate-limiting step for conversion of complex compounds and breaks down complex insoluble substrates to soluble form. As the first degradation step of complex substrates, hydrolysis is important but is generally not present in anaerobic granules. Anaerobic granules are normally applied to solubilised wastewater (such as brewery) where hydrolysis is not required. Applying UASB to complex organics requires hydrolytic capability to be introduced into anaerobic granules. In addition, as the hydrolysis of these molecules is facilitated by extra-cellular enzymes secreted by microorganisms, direct or close contact to substrate is favoured for hydrolysis. Fermenters, which are mainly identified on the surface of anaerobic granules, are the only potential candidates for hydrolysis (note they also utilise the hydrolysis products directly).

1.2.1.1 Hydrolysis of cellulose

The conversion of cellulose $(C_6H_{10}O_5)n$, to glucose can be presented as Equation [1]:

$$C_6H_{10}O_5 + H_2O \rightarrow C_6H_{12}O_6 \qquad [1]$$

Anaerobic microorganisms which are able to actively hydrolyse crystalline cellulose generally belong to family *Syntrophomonodaceae, Lachnospiraceae, Eubacteriaceae* or *Clostridiaceae* (Garrity, 2001). They are able to produce a multi-enzyme complex. The complex combines a multiple catalytic domain with an optimized carbohydrate binding module with low specificity (Schwarz, 2001). Thus the complex is able to attack and cleave a large range of carbohydrate substrates. As the efficiency is boosted by the enzyme structure, excess production of individual enzyme can be avoided. Furthermore, as a strong bridging structure between microorganism and insoluble substrate, it also transports soluble hydrolysis products. In particular, *Clostridia* is able to produce cellulosome, in which the components of cellulosome strongly bond to each other by a dockerin domain (Tokatlidis et al., 1991). Most *Clostridia* which possess cellulosome system belong to *Clostridium thermocellum* (Sakka et al., 1994) or *Clostridium aldrichii* (Yang et al., 1990). Other than *Clostridiales*, there are only two strains reported to possess cellulosome, which are *Thermobifida fusca* (Irwin et al., 1998) and *Fibrobacter succinogenes* (Fields et al., 2000). Although none of aforementioned bacterium has ever been identified in anaerobic granules, the higher classification phylum

Firmicutes has been commonly found in anaerobic granules (Diaz et al., 2006; Tsushima et al., 2010, Nakasaki et al., 2013).

1.2.1.2 Hydrolysis of Protein

Proteins are natural polymers comprised of different amino acids joined by peptide bonds. The backbone of a protein is a repeating sequence of one nitrogen and two carbon atoms. The structure of protein can be fibrous or globular. Insoluble fibrous proteins are normally used as structural material such as collagens. Globular proteins are normally dissolved in water and mainly used for biological functions. Protein hydrolysis in anaerobic digestion is mainly carried by proteolytic bacteria mainly from the class *Clostridia* (McInerney, 1988). Proteases are the extracellular enzymes secreted to breakdown proteins. Most proteases found in C*lostridium spp.* are metallo proteases which typically contain an essential metal atom (e.g. zinc) and with pH optima in the neutral pH range of 6 to 8. Unlike the hydrolysis of carbohydrate, the rate of protein hydrolysis is slower and generally unaffected by the availability of hydrogen-utilising bacteria (Nagase and Matsuo, 1982). Proteases have been found in *Clostridium histolyticum* (Hu et al., 2002)*, Clostridium sporogenes* (Allison and Macfarlane, 1990)*, Clostridium perfringens* (Shimamoto et al., 2001) *and Clostridium botulinum* (Suzuki et al., 2009). In particular, *Clostridium histolyticum* is well known for the production of proteolytic enzymes such as collagenase and clostripain. Phylum *Firmicutes* were commonly found in anaerobic granules (Diaz et al., 2006; Tsushima et al., 2010, Nakasaki et al., 2013), thus the hydrolysis of either cellulose or protein can be potentially developed on anaerobic granules.

1.2.2 Fermentation

The products of hydrolysis are converted to volatile fatty acids (VFAs) (e.g. acetate, butyrate, lactate and propionate etc.) and alcohols (e.g. ethanol, methanol etc.) by fermentation. Examples of products from glucose fermentation are listed in Equations [2] to [7] in Table 1.1.

Table 1.1 Possible products from glucose fermentation.

Products	Reaction	
Acetate, Propionate	$3C_6H_{12}O_6 \rightarrow 4CH_3COO^- + 2CH_3COO^- + 2HCO_3^- + 8H^+$	[2]
Lactate	$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOO^- + 2H^+$	[3]
Ethanol	$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3CH_2OH + 2HCO_3^- + 2H^+$	[4]
Ethanol, Acetate	$C_6H_{12}O_6 + 3H_2O \rightarrow CH_3CH_2OH + CH_3COO + 2HCO_3^+ + 2H_2 + 3H^+$	[5]
Butyrate	$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COO^- + 3H^+ + 2HCO_3^- + 2H_2$	[6]
Acetate	$C_6H_{12}O_6 + 4H2O \rightarrow 2CH_3COO^- + 4H^+ + 2HCO_3^- + 4H_2$	[7]

Fermenters are widely spread across more than 20 phyla but are commonly from families of *Streptococcaceae* or *Enterobacteriaceae* and the genera of *Bacteroides, Clostridium, Butyrivibrio, Eubacterium, Bfidobacterium*, or *Lactobacillus* (Novaes, 1986). Fermentation rate is directly related to the substrate uptake rate. Recently, Kim et al. (2011) has reported that genus *Aeromonas* (capable for carbohydrate fermentation) and specie *Clostridium sticklandii* (utilizing specific amino acids) are common and abundant during the start-up of anaerobic mixed tank reactors, irrespective of difference in wastewater composition and suggested these two groups are numerically and functionally important.

1.2.2.1 Fermentation of Glucose

Fermentation has been extensively studied in pure culture and mixed culture systems (Kalia et al., 1994; Fabiano and Perego, 2002; Kleerebezem et al., 2008). Glucose is converted to pyruvate by glycolysis through *Embden-Meyerhof-Parnas* pathway or *Entner-Doudoroff* pathway (by archaea) (Rose et al., 1976) with key enzymes listed in Figure B1 of Appendix B. Under the action of different dehydrogenases, nicotinamide adenine dinucleotide (NADH) produced during glycolysis is re-oxidized to NAD^+ and produces formate and hydrogen with the carbon being oxidised or reduced to butyrate, acetate, ethanol, propionate and lactate. Most pure culture fermentation studies have been conducted on genera *Enterobacter* (Fabiano and Perego, 2002), *Bacillus* (Kalia et al., 1994) and *Clostridium*. In mixed culture systems, *Clostridium spp.* has been reported as the dominant microorganism (Mizuno et al., 2000; Lu et al., 2011). A recent study of glucose fermentation identified two distinct classes: *Gammaproteobacteria* and *Bacteroidia*, and three different genera of class *Clostridia* (Kleerebezem et al., 2008).

1.2.2.2 Fermentation of Amino Acids

There are 20 amino acids commonly found in nature with varying molecular weight and structure. Amino acids are digested through two mechanisms: Stickland reactions and uncoupled oxidation. Stickland reactions are degradation of paired amino acids. One amino acid serves as electron donor and is oxidised to an organic acid and carbon dioxide. It also releases and produces hydrogen for reduction of the other amino acid. The other amino acid serves as electron acceptor and is reduced to an organic acid with the same chain length (Andreesen et al., 1989). Amino acids can be electron donors, acceptors or both (e.g., Leucine). Under a shortage of amino acid which can act as electron acceptor, uncoupled degradation may occur (Orlygsson et al., 1993). There is a shortfall of 10% acceptors in most amino acids (Nagase and Matsuo, 1982). Amino acid reactions are shown in Table B1 of Appendix B. Most Stickland reactions are facilitated by group I of genus *Clostridium* (e.g. *Clostridium stiklandii*) (Ramsay and Pullammanappallil, 2001; Fonknechten et al., 2010). For example, proline is the common amino acid utilised by all members of this group (Kenklies et al., 1999; Jackson et al., 2006). Some *Clostridium spp.* can only utilise specific amino acids, for example group IV *Clostridium putrefaciens* can only metabolise serine and threonine and group V *Clostridium propionicum* can only metabolise alanine, serine, threonine, cysteine and methionine. The classification of known anaerobic bacteria capable of degrading amino acid is shown in Table B2 of Appendix B.

1.2.3 Acetogenesis

Obligate hydrogen-producing acetogens convert ethanol and VFAs to acetate, hydrogen and carbon dioxide. Electrons from the anaerobic oxidation of these compounds are transferred to form hydrogen. Hydrogen formation is not favourable thermodynamically at 25°C and 1atm with positive Gibb's free energy changes (Dolfing, 1988). Thus hydrogen partial pressure needs to be maintained at low level by hydrogen-utilising methanogens with electrons effectively transferred through the hydrogen as a mediator. For example, propionate oxidation to acetate is shown in Equation [8]:

$$CH_3CH_2COO^- + 3H_2O \leftrightarrow CH_3COO^- + H^+ + HCO_3^- + 3H_2 \quad [8]$$

Propionate oxidation is thermodynamically favourable at hydrogen partial pressures below 10^{-4}atm. Butyrate and ethanol oxidation is shown in Equation [9] and [10]:

$$CH_3CH_2CH_2COO^- + 2H_2O \leftrightarrow 2CH_3COO^- + 2H_2 + H^+ \quad [9]$$

$$CH_3CH_2OH + 2H_2O \leftrightarrow CH_3COO^- + H^+ + 2H_2 \quad [10]$$

Equation [9] and [10] are favourable at hydrogen partial pressures of 10^{-3}atm and 1atm for butyrate and ethanol respectively (Pohland, 1992). Anaerobic biofilms provide an environment for hydrogen-producing acetogens to grow in close proximity to hydrogen-consuming methanogens and form a syntrophic relationship (Hungate, 1967). Methanogens consume hydrogen and keep the hydrogen partial pressure extremely low. This provides thermodynamically favourable conditions for acetogens to degrade the aforementioned organic compounds into acetate, H_2 and CO_2 (Dolfing, 1988).

Homoacetogens can produce acetate from a mixture of H_2/CO_2 and from excess electrons derived from organics. Due to a high affinity of hydrogen utilisers, accumulation of H_2 and CO_2 is generally avoided in anaerobic digestion systems. Homoacetogens outcompete methanogens for H_2 only at low temperatures (Kotsyurbenko et al., 1993) due to thermodynamic enhancements at low temperature (Kotsyurbenko et al., 1996). Model homoacetogens are *Clostridium aceticum* and *Acetobacterium woodii* isolated from sewage sludge (Novaes, 1986; Lay et al., 1998).

1.2.4 Methanogenesis

Methanogenesis occurs through three major pathways: acetotrophic/acetoclastic pathway, hydrogentrophic pathway, and methylotrophic pathway. Methanogens are obligate anaerobes and methanogenesis is considered to be the rate-limiting process in anaerobic treatment of soluble wastewater.

The acetoclastic pathway is a major catabolic process contributing up to 72 percent of the total methane generation from acetate in anaerobic digestion (Gujer and Zehnder, 1983). It is favoured under mesophilic conditions and high concentration of acetate (Zinder & Koch, 1984). Cleavage of acetate occurs via acetyl coenzyme pathway to produce methane as shown in Equation [11] followed by [12]:

$$2\ CH_3CH_2OH + CO_2 \leftrightarrow 2CH_3COOH + CH_4 \quad [11]$$

$$CH_3COOH \leftrightarrow CH_4 + CO_2 \quad [12]$$

The two most important genera of acetotrophic methanogens are *Methanosarcina* and *Methanosaeta* (Ferry, 1993). Based on microbial community analysis of 22 full-scale anaerobic digesters (Raskin et al., 1995), *Methanosaeta* was the dominant methanogen in these digesters.

The hydrogentrophic pathway contributes up to 28 percent of the methane production in anaerobic treatment systems (Khanal, 2008). Hydrogen and carbon dioxide are utilised as a source of electrons and carbon by methanogens as described in Equation [13]:

$$CO_2 + 4H_2 \leftrightarrow CH_4 + 2H_2O \quad [13]$$

Hydrogen-producing fermentative bacteria and hydrogen-producing acetogens are the main suppliers of hydrogen in anaerobic digestion systems. At high temperatures (50-65°C), high acetate concentrations and/or absence of *Methanosaeta* (Karakashev et al., 2006), H_2 and CO_2 are mainly produced by acetate oxidation. Hydrogen-utilising methanogens are mainly affiliated to the genera *Methanospirillum*, *Methanobacterium* and *Methanogenium* (Imachi et al., 2009).

Methylotrophic pathways catabolise compounds that contain methyl groups, such as methanol, mono- , di- and trimethylamine, and dimethyl sulphide (Deppenmeier et al., 1996). An example with methanol is shown in Equation [14]:

$$CH_3OH + H_2 \leftrightarrow CH_4 + H_2O \quad [14]$$

The ability to use formate for electron transfer was discovered in syntrophic butyrate-degrading microorganisms and propionate-degrading microorganisms (Schink, 1997). Further studies identified that *Syntrophobacter fumaroxidans* can grow well on propionate when cultivated with methanogens which can use both hydrogen and formate as an electron donor while not grow with methanogens that use hydrogen only (Harmsen et al., 1998). Formate is chemically and thermodynamically similar to hydrogen with the reaction between the two mediated by an extracellular enzyme formate dehydrogenase, which catalyses both forward and reverse reactions, and maintains the two in equilibrium. Formate dehydrogenase-encoding genes were also found in butyrate degrading bacteria *Syntrophomonas wolfei* (McInerney et al., 1981) and *Syntrophus aciditrophicus* (McInerney et al., 2007).

1.3 Anaerobic Digestion Technology

Anaerobic digestion relies on a complex consortium of micro-organisms and therefore anaerobic processes are designed to retain this functional biomass as sludge while promoting growth and metabolism in the reactor. Thus the applicability, performance and economy of the system are closely related to Sludge Retention Time (SRT). For solid waste, hydrolysis becomes the rate-limiting step and a long SRT (>10d) is typically required for substrate degradation. The longer treatment time is sufficient for growth of anaerobic microbes and therefore dedicated biomass retention systems are often not required. Continuous mixed-liquor reactors (such as a Continuous Stirred-Tank Reactor, CSTR) are most commonly applied for treatment of complex organic solids. In this reactor configuration, biomass is distributed evenly in the reactor by the mixing system and leaves the process as part of the treated effluent. SRT (which is equal to Hydraulic Retention Time, HRT, in CSTR) is typically in the range of 12 to 30d depending on substrate type and process conditions.

A much shorter HRT (<1d) may be used to treat wastewater with highly soluble feeds depending on concentration, pH and other process conditions. A HRT of <1d is not sufficient for growth of slower growing anaerobic microorganisms, particularly acetoclastic methanogens. Therefore at such a short HRT, separated biomass retention is required. There are several high-rate anaerobic reactor configurations which allow this including the anaerobic filter (Young and Mccarty, 1969), the downflow stationary fixed-film reactor (Lomas et al., 1999), the UASB reactor (Lettinga and Pol, 1991), the fluidized-bed reactor (Tavoulareas, 1991), and the expanded-bed reactor (Verstraete et al., 2005). As it is relatively simple, economical and easy to operate, the UASB reactor has been widely employed.

1.3.1 Upflow Anaerobic Sludge Blanket

UASB was developed in the 1970s by Lettinga et al. (1980) to treat rapidly degradable wastewaters, mainly carbohydrate based. There are some applications of UASB to treat wastewater rich in protein but minimal applications for wastewaters rich in fats, oils and grease. Solids in the waste stream can disrupt the system (Finstein, 2010), with UASB treatment applicable to pre-solubilised wastewaters (Shooshtari et al., 2012). A simplified example configuration of a UASB reactor is shown in Figure 1.2. The reactor is fed from the base. Feed and gas production enable mixing. Microbes consume substrate in the sludge

blanket zone. A three phase separator at the top of the reactor avoids the loss of sludge, and SRT in excess of 200d with HRT as low as 6 hours can be achieved (Hulshoff-Pol et al., 2004). Under these conditions, microorganisms spontaneously aggregate into larger particles (0.14mm to 5mm) called granules. Due to the high settling velocity of granules, high organic loading rates up to 50kg chemical oxygen demand (COD)·m^{-3}·d^{-1} can be applied without the risk of losing biomass as long as the upflow velocity does not exceed 2m·h^{-1} (Hulshoff-Pol et al., 2004).

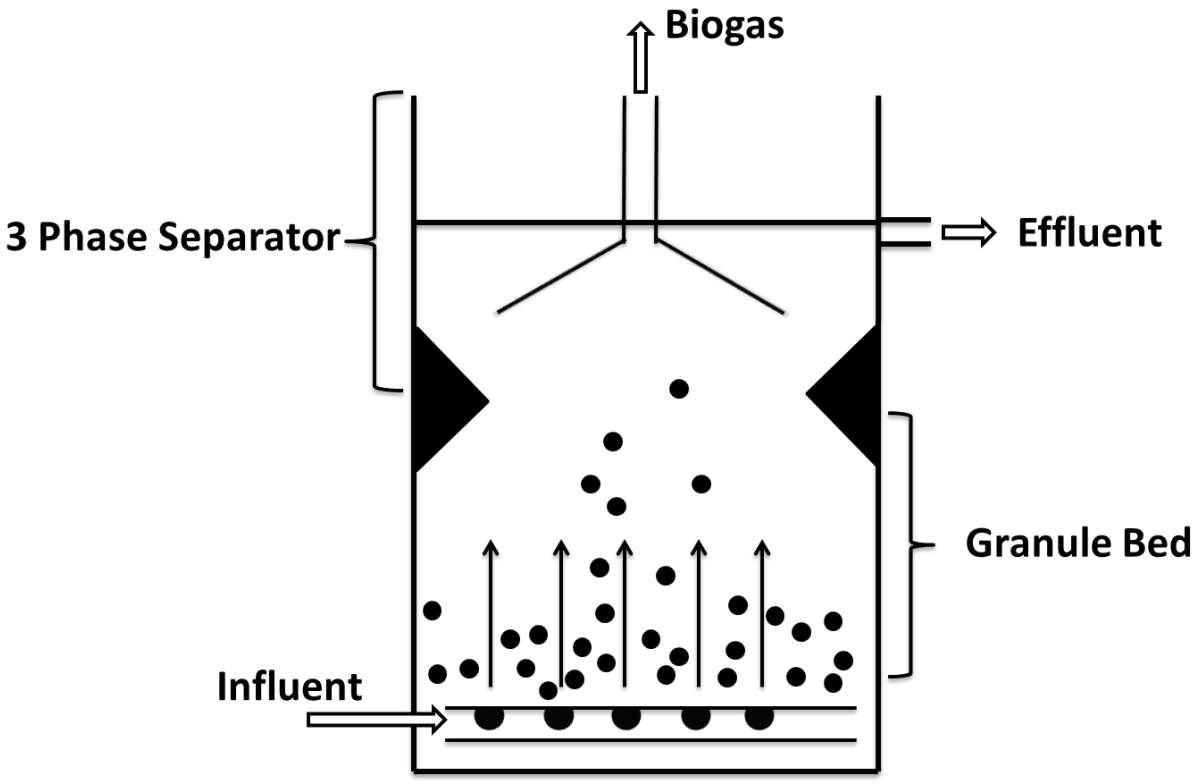


Figure 1.2 Configuration of upflow anaerobic sludge blanket reactor.

1.4 Anaerobic Granules

Successful operation of UASB reactors relies on the physical properties of anaerobic granules. Anaerobic granules normally have a disc or spherical shape with size varying from 0.14mm to 5mm. Factors affecting granule structure include wastewater type, reactor design and operational conditions (Kosaric et al., 1990; Pereboom, 1994; Schmidt and Ahring, 1996). Compared to dispersed individual micro-organisms, granules have a slightly higher density

ranging from 1.00 to 1.05kg·m^{-3} than water (Dolfing, 1986), the settling velocity is much higher due to the increased diameter. Settling velocity is associated with early stage granulation (Husshoff-Pol et al., 2004) and is an important factor that determines the organic loading rate and height of the reactor to prevent overflow. Reported settling velocities are in the range of 18-100m·h^{-1} with the general value below 50m·h^{-1} (Hulshoff-Pol, 1989; Schmidt and Ahring, 1996).

Under shear forces, granules can split into smaller non-symmetrical particles. As the size of granules is reduced, the settling velocity is also reduced and may lead to subsequent washout of the biomass. Shear strength is a critical property to maintain stable operation of UASB. Batstone and Keller (2001) suggested that high shear strength comes from granules with high cell density such as cannery-grown granules.

Most fermentative bacteria are tolerant to low pH due to their fermentative nature. For example, *Anaerolinea* requires a higher optimal pH range from 7.5-8.0 (Sekiguchi et al., 2003) and the growth is enhanced by co-cultivation with hydrogentrophic methanogens. Most methanogens prefer a pH range of 6.7 to 7.4. As the bicarbonate consumed by methanogens mitigates pH reduction caused by acid-producing bacteria (Liu and Tay, 2004), loss of methanogenic activity due to a low pH can lead to reactor souring.

The microbial study of anaerobic granule should consider three aspects: microbial architecture, microbial identification/abundance and microbial function.

1.4.1 Microbial Architecture

The microbial architecture of anaerobic granules was first identified based on morphology, with distinct layers apparent in scanning electron microscopy (McLeod et al., 1990). The driving force for different structures is thought to be due to the rate limiting kinetic steps, which depend on the primary substrate (Fang et al., 1995). It was proposed that fed with complex sugar and starch, anaerobic granule displays a layered structure with different organisms dominating on each layer. In these studies (McLeod et al., 1990; Guiot et al., 1992; Fang et al., 1995), *Methanosaeta* and *Methanosarcina* were frequently found in the inner layer of anaerobic granules, other methanogens such as *Methanococcales* and *Methanobacterium* were found on both the surface and inner layers. Large variations in

bacterial morphology have been commonly identified on the surface of anaerobic granules, but morphology based identification has limited accuracy.

A thin, presumed fermentative layer on the surface has been commonly observed with no association with archaea using Fluorescent *In Situ* Hybridization (FISH) (Harmsen et al., 1996b; Sekiguchi et al., 1999; Batstone et al., 2004; Sun et al., 2009). The inner layers generally consist of presumed syntrophic bacteria, acetoclastic methanogens such as *Methanosaeta,* and other archaea. The centre of the granule is seen to be an inactive core, with no binding to any FISH probes.

FISH analysis on methanogens has been extensively done. Diaz et al. (2003) found that *Methanosaeta* dominated in the inner layer under mixed VFAs substrate or low acetate concentration while *Methanosarcina* and members of *Methanobacteriales* dominated under high concentrations of acetate and formate respectively.

Bacterial community is highly diverse compared to the archaeal community due to the multiple functions bacteria mediate. Harmsen et al, (1996a and 1996b) located a syntrophic propionate oxidizing bacterium related to *Syntrophobacter wolinii* in the middle layer of sucrose-fed and VFA-fed granules and genus *Desulfobulbus* on the surface of methanogenic granules. Similar results were identified from a study on mesophilic granules by Sekiguchi et al. (1999). *Chloroflexi* has been commonly identified on the outer layer of anaerobic granules from mesophilic and thermophilic UASB reactors treating carbohydrate (Yamada et al., 2005) or synthetic medium containing powdered skim milk (Satoh et al., 2007; Satoh et al., 2012). Recently, by investigating the anaerobic granules treating sucrose-based wastewater at low temperature, Tsushima et al (2010) also found that both *Firmicutes* and *Chloroflexi* accumulated on the thin outermost layer. Diaz et al. (2003) also concluded that microbial diversity in anaerobic granules increased with the complexity of the substrate.

1.4.2 Microbial Community Identification and Abundance

The microbial community of anaerobic granules consists of a large diverse bacterial community (including fermentative bacteria such as *Bacteroidetes*, *Firmicutes* and *Chloroflexi*, syntrophic bacteria such as *Geobacter* and *Syntrophomonas*) and a relative simple archaeal community (including mainly acetoclastic methanogens such as *Methanosaeta* and *Methanosarcina*, and hydrogen-utilising methanogens such as

Methanobacterium and *Methanolinea*). Microbial identification and abundance shift between different types of granules has been mainly analysed by either FISH quantification or polymerase chain reaction (PCR) based molecular analysis or the combination of both previously.

Diaz et al. (2003) analysed granules grown on several different conditions by mainly FISH quantification and concluded that gram-positive bacteria dominated in all granules. In 2006, Diaz et al. also identified that *Firmicutes* was the dominant bacteria in anaerobic granules treating brewery wastewater. Both studies utilized a large set of group (phylum)-specific probes. However, the general distribution of bacteria and archaea was the only information provided with no further information on specific bacterial architecture determined. In a study on anaerobic granules treating ethanol, carbohydrates and protein respectively (Molina et al., 2008), it was found that *Methanosaetaceae* was the dominant archaea in all three types of granules while *Methanobacteriales* also co-dominated in granules fed with carbohydrate and protein.

Common PCR-based molecular techniques applied to anaerobic granules are denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (TRFLP) and clone libraries.

- DGGE, based on different denaturation properties of 16S Ribosomal Ribo-Nucleic Acid (rRNA) fragments due to sequence variations, provides a genetic profile representing the microbial diversity and identification of specific bands by excising, amplification, and sequencing. For example, a DGGE band affiliated to *Methanosarcina mazeii* was only detected in winery-fed granules while a band that affiliated to *Methanobacterium formicicum* was only detected in brewery fed granules (Keyser et al., 2006).
- TRFLP identifies populations based on the restriction site differences closest to a fluorescent labelled end of amplified 16S rRNA gene. It can monitor the composition of the community shift in response to changed conditions. Alternatively, TRFLP can be used in conjugation with clone library for *in silico* identification (Lu et al., 2011). The advantages of TRFLP include resolution power, as it allows gel-to-gel variation analysis, and suitability for routine analysis of large sample numbers, with reproducibility and potential for automatisation (Smalla et al., 2007). The drawback of TRFLP is that quantification is difficult. Absolute quantification data cannot be

obtained directly from TRFLP and relative quantification can be incorrect due to PCR bias. Thus other quantification methods such as FISH quantification or quantitative PCR should be applied to verify or correct results.

- The 16S rRNA gene clone library is a popular technique for investigating phylogenetic diversity. A collection of Deoxyribo-Nucleic Acid (DNA) sequences, usually derived from PCR amplification, is inserted into a plasmid vector and cloned into a bacterial host cell (for example *E.coli*). As nearly full 16S rRNA gene can be sequenced from clone library, thus it can provide taxonomy identification to species level. Members of genus *Bacillus* and *Pseudomonas* have been commonly detected in 16S rRNA clone libraries from different types of granules (Keyser et al., 2007). Hernon et al. (2006) conducted a study using 16S rRNA clone libraries on two lab-scale UASB treating synthetic wastewater containing glucose and sucrose at mesophilic and thermophilic conditions respectively. They found that *Bacteroidetes* and *Spirochaetes* dominated in mesophilic while *Clostridia* were the only dominating class in thermophilic reactors. *Alphaproteobacteria* were identified in granules treating synthetic powdered skim milk wastewater as the most frequently detected clone, which were closely related to *Sphingomonas rhizogenes* (Satoh et al., 2007). Recently, members in *Thermotogae*, *Synergistetes*, *Firmicutes, Chloroflexi* and *Proteobacteria* were observed in anaerobic granules treating syrup wastewater (Nakasaki et al., 2013). There are numerous limitations associated with these techniques (Head et al., 1998) including the choice of extraction methods, PCR bias plasmid selectivity, and long preparation periods (normally a week for picking 100 good quality clones in 16S rRNA clone library).

1.4.3 Microbial Function

Due to the difficulty in culturing, few microorganisms have been isolated from anaerobic granules. These are a group of filamentous bacteria including *Anaerolinea thermophila* (Sekiguchi et al., 2003) isolated from thermophilic UASB reactor treating soybean-curd manufacturing wastewater (Yamada et al., 2001). *Anaerolinea thermolimosa* was isolated from anaerobic granules treating wastewater from a factory producing shochu (Yamada et al., 2006); *Levilinea saccharolytica* was isolated from anaerobic granules in a mesophilic UASB reactor treating wastewater from a sugar-processing plant and *Leptolinea tardivitalis* was

isolated from anaerobic granules treating artificial wastewater mainly composed of sucrose and VFAs. They are classified as sugar-fermenting bacteria due to the ability to utilize a limited range of carbohydrates. Other than these, study of microbial function on anaerobic granules heavily relies on predicted function from phylogenetic alignment to previously cultured microorganisms.

The major drawback of all methods discussed so far is a lack of resolution power on rare species or even sub-dominated species. Many of these techniques are being replaced by more powerful next generation sequencing (NGS) techniques such as 16S rRNA pyrosequencing or metagenomic sequencing. For example, Rotaru et al., (2014) performed a metatranscriptomic analysis on anaerobic granules from a brewery UASB reactor. They identified *Geobacter* species expressed a large amount of genes for ethanol metabolism and extracellular electron transfer via electrically conductive pili.

1.5 The Next Generation Sequencing Approach

The properties of 16S rRNA (and 18S rRNA for eukaryotes), including its commonality with ribosomal RNA for complementary methods such as FISH and its characteristics in containing conserved and variable regions between species, make it favourable as a marker gene for microbial profiling (Pace, 1997). The recovery of marker gene sequences was conventionally done by cloning the PCR amplified fragments and sequencing each clone with capillary electrophoresis-based Sanger sequencing. NGS provides the opportunity to sequence small fragments in a parallel fashion with no cloning required. The ability to deeply sequence the community makes the detection of low abundance species possible and impacts on the interpretation of microbiological changes (Caporaso et al., 2012). There are several different platforms available for NGS (Shendure and Ji, 2008). Two most popular platforms: 454 and Illumina platforms (otherwise known as Solexa) are reviewed here.

1.5.1 Preparation for NGS

NGS has strict limit on read length (400-600bp in the 454 platform and 200-500bp in the Illumina platform) due to decreased base read quality with increasing length. The purpose of

library preparation is to prepare sequencing DNA concentrate (at an appropriate molecular length). Despite the diverse in sequencing biochemistry as well as in array generation, the library preparation is conceptually similar. DNA fragmentation can be done by nebulization, sonication or enzymatic digestion. Nebulization refers to that DNA is mechanically sheared by compressed nitrogen or air to produce random fragmentation. Alternatively, DNA is subjected to ultrasonic waves which generate resonance vibration as shear force. Finally, enzymatic DNA digestion, which utilizes restriction enzymes to randomly chop DNA into fragments, is the most popular method due to its consistent performance and straightforward methodology (Knierim et al., 2011). Sequencing then can be performed on one end of single strand (Single read) or on each ends of double strands (conventional pair-end reads). The paired-end reads contain positional information, allowing reconstruction of the whole fragment compared with single read. Based on conventional paired-end method, another strategy (called mate-pair by Illumina) was developed by Hong (1981) to extend positional information to a longer range. The 5' end and 3' end of longer fragments (2-5kb) are joined by a linker to form a circular fragment. It is then enzymatically digested to produce a ditag containing sequences corresponding to both ends of the original fragment. The ditag is then sequenced as double strands by the pair-end method. This further adds the scaffold information. Furthermore, to enable the application of NGS on multiple samples in a single run, multiplexing has been used in which a barcode is added to the template fragments from each sample in both platforms to enable multiple samples to be sequenced in a single run (Parameswaran et al., 2007; Gloor et al., 2010).

1.5.2 454 Pyrosequencing Platform

454 sequencing technology was first tested on sequencing whole genomes of single microorganism (Margulies et al., 2005). The sequence-ready DNA fragments are attached to beads by emulsion PCR (Dressman et al., 2003) (Figure 1.3A). The DNA beads, which contain millions copies of a single DNA fragment on each, are transferred to PicoTiter Plate for sequencing. During sequencing, the four bases are targeted sequentially and always in the same order. Nucleotide base identification is achieved by capturing the light signal generated from pyrophosphate release during the binding of correct base complementary to the template (Ronaghi et al., 1996; Ronaghi et al., 1998, Figure 1.3C).

1.5.3 Illumina Platform

The Illumina platform was originally invented by Turactii and colleages (Fedurco et al., 2006; Turactti et al., 2008). The throughput of Illumina can reach 50 times more than 454 pyrosequencing in one run but with a shorter read length. In two years, this technique has greatly improved by upgrading the read length to above 100bp with HiSeq 2000 system (Kircher and Kelso, 2010). The sequence-ready DNA fragments are attached to flow cells directly by bridging PCR (Figure 1.3B). During sequencing, the four bases, attached to different fluorescent labels, are added to flow cells at the same time. Once the correct base binds to the first position of a fragment, the nucleotide label severs as terminator to prevent the binding at the following position (Figure 1.3D). After capturing the signal image, the nucleotide label is enzymatically cleaved and sequencing moved to the next position. Compared to pyrosequencing, which requires light signal imaging of each base at one position, Illumina uses one multiple fluorescent single images at each position and it leads to less time consumed and more data generated. However, the large raw reads normally end with low usability due to errors from low quality and mismatched barcode (Degnan and Ochman, 2012).

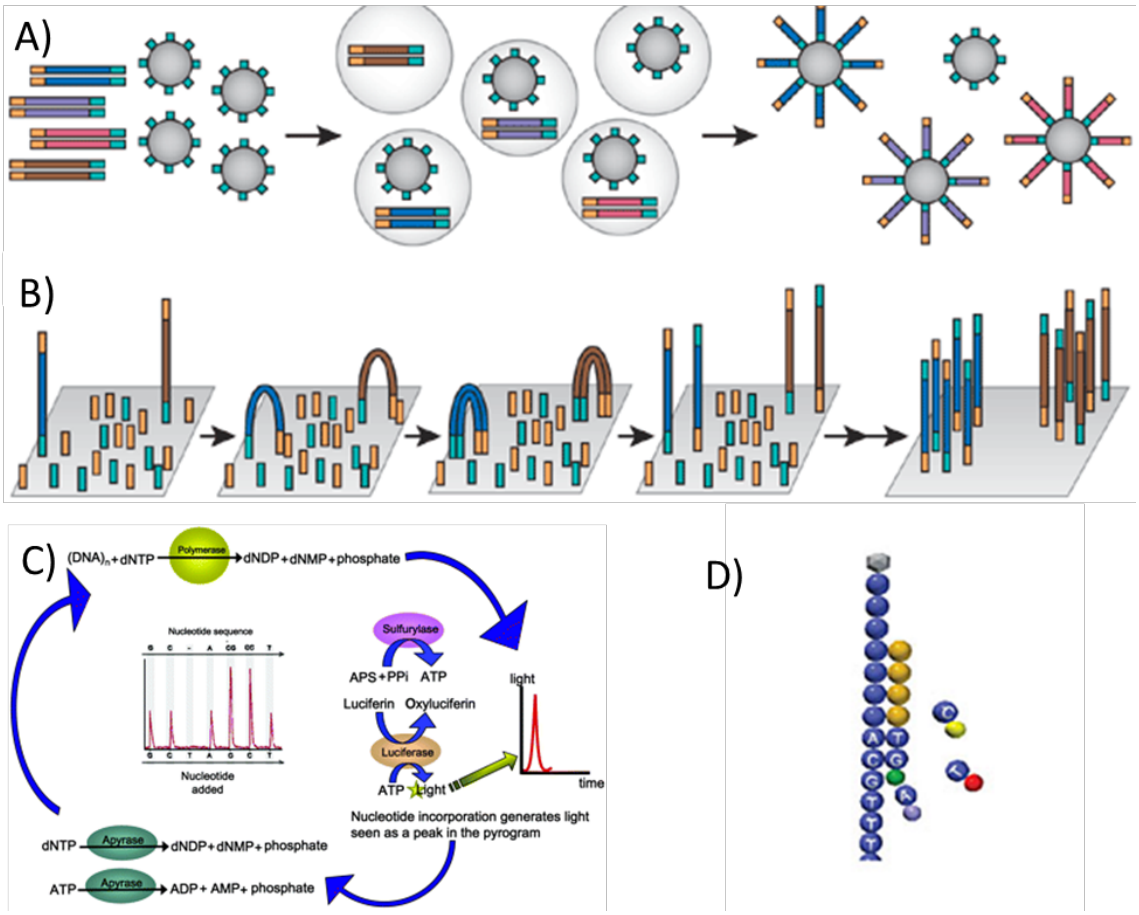


Figure 1.3 Comparing sequencing methods of 454 and Illumina platform. DNA fragments ligated to adaptor are attached to beads by emulsion PCR in 454 platform (A) or to follow cells by bridging PCR in Illumina platform (B). Base pairs are sequenced by light source generated from releasing of pyrophosphate in 454 platform (C) or by different fluorescent labelled dNTP (also acts as terminator) in Illumina platform (D) (modified from Sherdure and Li, 2008 and Marsh, 2007).

1.5.4 Comparison of platforms

Recent studies aim to identify the sequencing errors and artefacts specific to each NGS platform and develop algorithms that detect and correct these errors. High error rates in homopolymer regions caused by accumulated light intensity variance and up to 15% artificial amplification product have been reported in the 454 platform (Margulies et al., 2005; Quince et al., 2009). Systematic base calling biases due to non-stationary noise was identified with the Illumina platform (Erlich et al., 2008). In addition, sequencing quality variation at different tiles of the sequencing plate and increased single-base errors in association with GGC motifs were also observed (Dolan and Denver, 2008). This emphasises that NGS data should be carefully handled with incorporating appropriate algorithms into analysis.

Both platforms showed relative consistency with each other in terms of microbial profiling and whole genome sequencing. However, when it was used for resolving highly complex microbial composition on the 16S rRNA gene, shorter reads produced by Illumina platform, failed to be classified down to genus level due to their shorter length and higher error rates beyond 60bp (Claesson et al., 2010). Regarding to whole genome analysis, Luo et al., (2012) suggested that Illumina, and short-read sequencing, "may be a more appropriate method for metagenomic studies" by testing both methods on the same complex community DNA samples. They found that metagenomic data from 454 pyrosequencing contains large proportions of frameshift (insertions of deletions due to sequencing errors). To date, study on the microbial community of anaerobic granules using NGS is limited. Application of NGS to anaerobic granules should be capable for the detection of rare species due to its high resolution power and possibly provide potential functionalities.

1.6 Research Gaps

Archaea have been studied in terms of architecture and community identification and abundance due to the low diversity well developed molecular. However, bacteria, especially the fermentative bacteria, have not received substantial attention in either spatial distribution, community identification nor function studies.

To date, spatial information, i.e. the position of particular organisms in the granules, has only been studied with FISH. The targeted phylogenetic groups are quite limited per analysis due to logistical considerations (e.g., number of fluorochromes that can be visualized simultaneously, availability, and cost of suitable probes), and for gross community composition estimates, the choice of probes tends to target broader groups such as domains or phyla (Kunin et al, 2008).

PCR-based molecular sequencing is able to provide rapid high-resolution identification, but it can only be applied to the whole anaerobic granules. This can be used to compare the shifts between different granule sources. However, it fails to provide spatial distribution and possibly fails to detect sub-dominant microbes. Furthermore, identification based on FISH and identification from molecular techniques cannot be easily linked together, particularly

since FISH is based on a-priori knowledge, while bulk methods do not allow spatial resolution.

In contrast, molecular functional analysis has limited application on anaerobic granules to investigate the function of microbial community. Metagenomic analysis, which can provide the potential function of individual microorganisms, is a recent tool that can be applied to address this limitation.

1.7 Research Objectives and Approach

Based on the gap analysis above, key issues are investigation on microbial ecology of fermenters in anaerobic granules. This has been historically hampered by available tools to analyse and only limited depth of knowledge can be obtained. This thesis will address these gaps using a combined multidisciplinary approach that focuses on using next generation molecular technique to identify ecology and function of the fermentative community together with spatially orientated sampling of the granule.

Objective 1. Granular layers separation. To develop a method utilizing hydraulic shearing to sequentially remove layers from mature granules.

Approach: A shear method has been developed to remove microbes selectively from successive spatial layers through hydraulic shearing. The application has been demonstrated on three types of anaerobic granules collected from full-scale UASB reactor treating brewery, cannery and dairy wastewater.

Objective 2. Microbial community analysis. To assess microbial community and identify the potential microbial function in anaerobic granules.

Approach: Pyrosequencing was applied to successive granule layers obtained from objective 1 to study the microbial ecology in anaerobic granules of linked spatial, microbial identification and abundance information. Based on the microbial analysis, key anaerobic granules (with simplest microbial structure) were selected for further metagenomic analysis to provide potential function of reconstructed individual microorganism's sequences.

Objective 3. Location and function of the community. To investigate the impact of fermentative function on microbial community and external properties of anaerobic granules.

Approach: Three lab-scale UASB reactors were operated at 35°C by feeding with synthetic wastewater containing VFAs, glucose and gelatin respectively. Fermentative layers developed from each reactor were analysed and compared by shear method and molecular analysis.

Chapter 2. Material and Methods

2.1 Anaerobic Granules

The shear method and subsequent microbial analysis was carried out on anaerobic granules collected from three industrial wastewater treatment plants (Chapter 3 and 4); and anaerobic granules collected from three lab-scale UASB reactors inoculated with industrial granules (Chapter 5). Research methodologies used in this thesis are described in this chapter.

Anaerobic granules were collected from three full-scale industrial plants; two were UASB style reactors and one was a bulk volume fermenter (BVF, De Garie et al., 1987) treating industrial wastewater in south-east Queensland, Australia. The feedstock for the UASB plants are brewery and cannery wastewater. The feedstock for the bulk volume fermenter is cheese/butter processing wastewater. Detailed specification of these wastewater treatment plants are listed in Table 2.1. The granules collected from these full scale processes were referred as brewery granules, cannery granules and dairy granules respectively. Granules were transported at ambient for less than 2 hours (or send at 4°C by overnight freight for dairy granules) and stored at 4°C before further analysis. Influent of VFA and cannery granules were also collected from the wastewater plants and transported on ice for chemical analysis.

Table 2.1 Specification of three industrial wastewater treatment plants.

Type	OLR ($kgCOD \cdot m^{-3} \cdot d^{-1}$)	HRT (D)	Upflow velocity ($m \cdot h^{-1}$)	Reactor Size (m^3)	Major Contents	Gas production ($m^3 \cdot d^{-1} \cdot m^{-3}$)
Brewery (UASB)	9.1	0.3	0.5	580	Ethanol, Acetic acid and Propionic acid	8.2
Cannery (UASB)	4.5	2	0.2	1200	Glucose, Acetic acid and Ethanol	1.4
Dairy (BVF)	0.65	10	0.03	48000	Lactose, milk proteins and fats	0.27

2.2 Reactor Studies

Laboratory-scale reactors were used to grow differentiated granules as detailed in Chapter 5. Brewery granules studied in Chapter 4 were used as inoculum. Analysis from previous study (Chapter 4) demonstrated that there were minimal fermenters and none specifically on the surface of brewery granules. Granules were passed through a 200µm sieve as a pre-treatment prior to be used as inoculum. After sieving a minimum of 5 times, fines were discarded and approximately 100g granules (wet weight) with size >200µm were added to each reactor as inoculum.

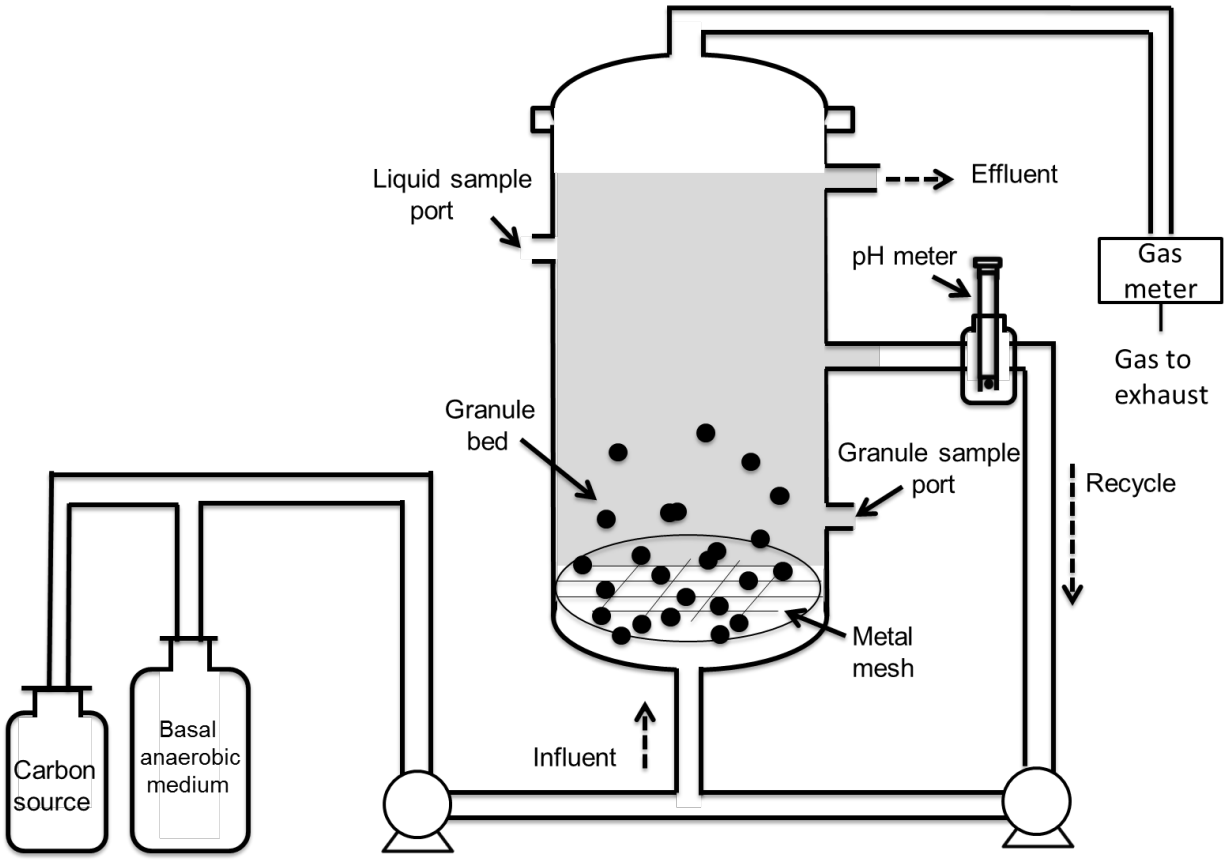


Figure 2.1 Lab-scale UASB reactor design (3 like reactors were used).

The experiments were carried out in three glass lab-scale reactors (Figure 2.1), each with 0.5L working volume, at mesophilic conditions (35°C) for approximately 60d. The three reactors were continuously fed with a common basal anaerobic medium and a separate synthetic carbon source. The dose rate of the carbon feedstock was controlled to achieve an

organic loading rate of 2gCOD·L^{-1}·d^{-1}. The synthetic wastewater was recirculated at 100mL·min^{-1} to achieve an upflow velocity of 1.5m·h^{-1}. The carbon source media for three reactors respectively were VFA (sodium acetate 1.73g·L^{-1}, sodium propionate 0.57g·L^{-1} in 2L bottle), glucose (D-glucose 1.87 g·L^{-1} in 2L bottle), and protein (gelatine 1.27g·L^{-1} in 1L bottle). The carbon media was stored in an ice bath during the experiment. All solutions were prepared using a concentrate solution (10×) filtered through a 0.2μm syringe filter into autoclaved MilliQ water. Basal anaerobic medium (in g·L^{-1}: NH_4Cl, 200; NaCl, 20; $MgCl_2 \cdot 6H_2O$, 20; $CaCl_2 \cdot 3H_2O$, 0.24; $K_2HPO_4 \cdot 3H_2O$, 400; trace metal and selenite solution and vitamins) were prepared according to Bastidas-Oyanedel et al. (2010). The HRT was approximately 0.5d. The pH of each reactor was monitored by an online pH transmitter (Hanna, Australia) in the recycle circuit. The volume of biogas produced was measured using a tipping bucket style gas meter (2mL per tip) and recorded online.

2.3 Shearing

Shear experiments were performed in a standard-geometry cell (1.3L, 120mm diameter) with a stainless steel 6 blade flat blade Rushton impeller (40mm diameter) as shown in Figure 2.2 (Holland and Chapman, 1966).

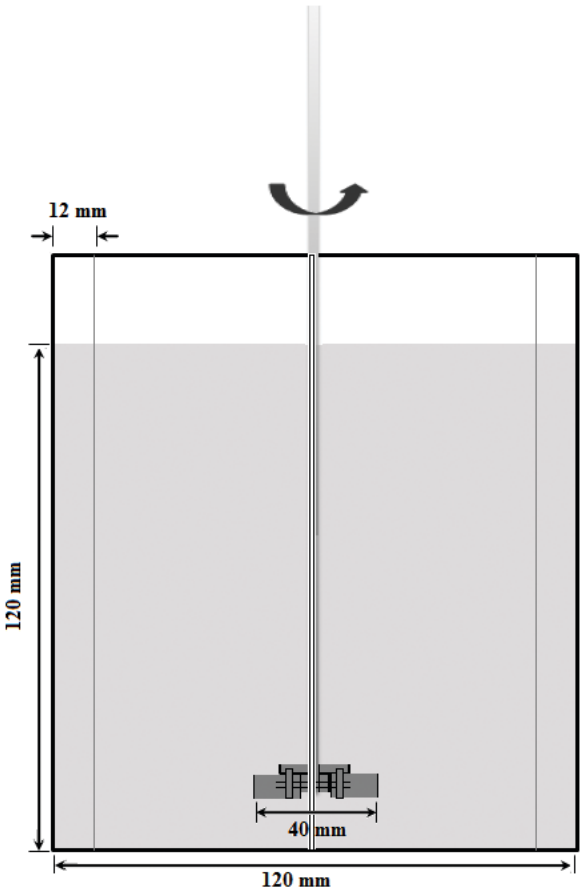


Figure 2.2 Shearing tank configuration. (Modified from Holland and Chapman, 1996).

Anaerobic granules were first gently washed with MilliQ water over a 200µm sieve (stainless steel mesh) to remove the native fines. Approximately 10g (wet weight) sieved granules were added to 1L of phosphate buffered saline (PBS, 130mM sodium chloride, 10mM sodium phosphate buffer, pH 7.2) at the beginning of the shear experiment and sheared at either 500rpm (brewery granules) or 1500rpm (cannery and dairy granules). Samples were collected after 5, 20, 90, 180, 270 and 360 minutes of shearing. At each sampling time, the contents of the cell were sieved through the 200µm sieve and rinsed with water gently to separate fines from 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 1L PBS and shearing continued. The shear strength was characterized according to the method developed by Pereboom (1997), which relates the growth of fines to shear rate squared as shown in Equation [15].

$$ln\left(\frac{X_0-X_F}{X_0}\right) = K_C \cdot \gamma^2 \quad [15]$$

Where X_0 is the total suspended solid (TSS) of granules at the beginning of each shear, X_F is the TSS of fines generated after each shear, γ is the shear rate and K_C is the characteristic shear coefficient. Fines were defined as particles smaller than 200μm and were collected through wet sieving.

The sheared depth was estimated based on the following calculations. Firstly, the volume of all granules (V) in 1L PBS was calculated based on TSS, density and moisture of anaerobic granules measured as shown in Equation [16].

$$V = \frac{TSS}{Desnsity} \times \frac{1}{1-moisture} \quad [16]$$

Secondly, the volume sheared (V_i') at each time point i was calculated based on the TSS measure from fines (TSS_i') as shown in Equation [17].

$$V_i' = \frac{TSS_i'}{Desnsity} \times \frac{1}{1-moisture} \quad [17]$$

Number of granules (N) in 1L 1 × PBS was estimated (as shown in Equation [18]) based on the volume of all granules obtained from Equation [16] and the average volume of all granules ($V_{average}$) obtained from granule size analysis (Section 2.1.1.4).

$$N = \frac{V}{V_{average}} \quad [18]$$

At last shear depth (D) was calculated in Equation [20] based on volume sheared on each granule (V_{each}' in Equation [19]) and average area of each granule ($A_{average}$) measured from granule image.

$$V_{each}' = \frac{V}{N} \quad [19]$$

$$D = \frac{V_{each}'}{A_{average}} \quad [20]$$

2.4 Analytical Methods

2.4.1 Bulk Property Test

2.4.1.1 Total Suspended Solid (TSS)

TSS was analysed by method 2540D and R in the Standard Methods (APHA, 1992). 10mL to 15mL (V_{sample}) samples were filtered through a pre-dried and weighed glass fibre filter paper (W_{filter}). The sample was then dried at 103°C for 12 hours and weighed after that ($W_{filter+sample}$). The total suspended solids (TSS) were calculated as Equation [21].

$$TSS = \frac{W_{filter+sample} - W_{filter}}{Vsample} \quad [21]$$

2.4.1.2 Density

Granule density was measured similar to that used by Bhatti et al. (1995) by displacement of pre-weighted drained granules in Milli-Q water in a 2L volumetric flask. The 2L flask was filled with Milli-Q water to the 1L mark and the temperature was recorded. Drained granules were then added to the flask. The water was pipetted from the flask to a pre-weighed container until the level returned to the 1L mark. The water was weighed and the equivalent volume was calculated from temperature-volume tables.

2.4.1.3 Drained Moisture

Drained moisture was estimated as described below. Approximately 100mL granules were first spread on a 200µm sieve. After cleaning the reverse side of the sieve with paper towel, the sieve was placed in a pre-weighed crucible and weighed. The sample was dried at 103°C and drained moisture was estimated using similar methodology to Equation [21].

2.4.1.4 Granule Size

Sieved granules (through 200µm sieve) were separated and placed in a flat glass dish on a white table. Granule images were taken by high resolution camera. Various characteristics such as surface area, length and width were analysed using Quantimet image analysis software (Leica, Australia).

2.4.2 Chemical Analysis

Wastewater samples (10mL) were collected from each lab-scale UASB reactor 3 times per week for chemical analysis including glucose, VFAs, protein and COD concentration.

2.4.2.1 High Performance Liquid Chromatography-UV

VFAs and glucose were measured by High Performance Liquid Chromatography (HPLC), which consisted of auto-injector (SIL-10ADVP), degasser (DGU-14A), LC pump LC-10ADVP), column oven (CTO-10ASVP), diode array detector (SPD-M10AVP), CLASS VP software and Shimadzu refractive index detector (RID-10A). Separation of the compounds was carried out on HPX-87H 300mm x 7.8mm ion exclusion column (Cat. No. 1240140, BioRad Aminex) by using 0.008N H_2SO_4 as the eluent at the flow rate of 0.4mL· min^{-1}. Prior to analysis, 0.9 mL sample was filtered into 2mL gas chromatograph vial containing 0.1mL 0.1% (v/v) azide.

2.4.2.2 Protein Analysis

Protein concentration of influent and effluent from lab-scale UASB fed with gelatine was measured by Bicinchoninic acid assay (BCA) (Smith et al., 1985). 0.1mL Liquid sample were mixed with 2mL prepared BCA working reagent from bicinchoninic acid assay kit (Sigma-Aldrich, USA) in a clean glass tube. 2mL prepared BCA reagent contains 2mL reagent A (bicinchoninic acid, sodium carbonate, sodium tartrate, and sodium bicarbonate in 0.1 N NaOH) and 0.04mL reagent B (4% (w/v) copper (II) sulfate pentahydrate). The tube was then incubated at 37 °C for 60 minutes. Reading at absorbance of 562nm is recorded and the protein concentration is determined by comparison to a standard curve.

2.4.2.3 Chemical Oxygen Demand (COD)

COD of influent and effluent from 3 lab-scale reactors were measured using a Merck Method potassium di-chromate/sulphuric acid method based on Standard Methods 5220D (APHA, 1998) with Merck Spectroquant® COD test kits (Cat No. 114540, ranging 10-150mgL^{-1} and Cat No. 114541, ranging 25-1500mgL^{-1}). Each sample were diluted with Milli-Q water to bring the concentration in the range of 10-150mg·L^{-1} or 25-1500 mg·L^{-1}. 1mL (for low range) or 3mL (for high range) diluted sample was added to a Merck Spectroquant® COD test cell,

where it is oxidised by sulphuric solution of potassium di-chromate and silver sulphate (catalyst). The tubes were incubated at 148°C for 2 hours. After cooling down, the concentration of Cr^{3+} was determined using spectrophotometer to reflect the COD concentration. Each COD measurement was carried out in triplicates.

2.4.2.4 Gas Analysis

Gas samples generated from each lab-scale UASB reactor were collected periodically using a precision gas tight syringe (SGE International Pty Ltd, Ringwood, Australia). A PerkinElmer AutoSystem gas chromatograph equipped with a thermal conductivity detector was used for measuring H_2, CO_2 and CH_4. The gas chromatograph was equipped with an Alltech (#8011/2) stainless steel column maintained at 40°C. The injector, detector, and column were maintained at 75,100 and 40°C (APHA, 1998). High-purity N_2 was used as carrier gas at a flow rate of 24.5mL·min^{-1} and a head pressure of 220kPa. Data were acquired and integrated using PerkinElmer GC Plus data station (Model 1022).

2.4.3 Microscopic Analysis - Cryosection-FISH

Granules were first fixed with 4% PFA overnight and then washed with PBS (130mM sodium chloride, 10mM sodium phosphate buffer, pH 7.2) and stored at 1:1 100% ethanol: PBS. 10-15 granules were placed in 15% sucrose for overnight, 3:1 15% sucrose : optimal cutting temperature compound (OCT) (Baxter Diagnostics, USA) for 2 d, 1:1 15% sucrose : OCT overnight, 1:3 15% sucrose : OCT for overnight and neat OCT for overnight (Batstone et al., 2004). Around 4-5 granules were transferred to a square mould and store at -20°C for overnight. The specimen was sliced on a Hyrax C60 cryostat machine (Zeiss, USA) with a knife temperature -19°C and specimen temperature -20°C to obtain a 5µm slice on Poly-L-Lysine coated slide (Polysciences, USA). The slide was then dehydrated for 3 minutes in ethanol series 50%, 70% and 98% respectively. After drying, the slide was stored at room temperature. FISH was performed according to the protocol described by Amann et al. (1995). Details of oligonucleotide FISH probes used are listed in Table 2.2. The slides were then viewed under Zeiss Axioscope LSM510 confocal microscope (Zeiss, USA).

Table 2.2 Details of oligonucleotide probes used for cryosection-FISH.

Target population(s)	FISH probe-fluorochrome	Probe sequences(5'-3')	References
Archaea	*ARC915-FITC*	GTG CTC CCC CGC CAA TTC CT	(Stahl et al., 1991)
Bacteria	*EUB338-Cy3*	GCT GCC TCC CGT AGG AGT	(Amann et al., 1990)
	EUB338II-Cy3	GCA GCC ACC CGT AGG TGT	(Daims et al., 1999)
	EUB338III-Cy3	GCT GCC ACC CGT AGG TGT	(Daims et al., 1999)
Methanosaeta	*MX825-Cy3*	TCG CAC CGT GGC CGA CAC CTA GC	(Raskin et al., 1994)
Methanococcales	*MC1109-Cy5*	GCA ACA TAG GGC ACG GGT CT	(Raskin et al., 1994)
Methanobacteriales	*MB1174-Cy5*	TAC CGT CGT CCA CTC CTT CCT C	(Raskin et al., 1994)
Methanomicrobiales	*MG1200-Cy5*	CGG ATA ATT CGG GGC ATG CTG	(Raskin et al., 1994)
Anaerolinea	*CFX784-Cy3*	ACC GGG GTC TCT AAT CCC	(Bjornsson et al., 2002)
Bacteroidetes	*CFB1082-Cy3*	TGG CAC TTA AGC CGA CAC	(Weller et al., 2000)
	CF319a-Cy3	TGG TCC GTG TCT CAG TAC	(Manz et al., 1996)
Candidatus Cloacamonas	*WWE1-Cy3*	CTT CCT CTG CGT TGT TAC	(Rakia et al., 2005)
Geobacter	*GEO1a-Cy5*	CTC ACG CAC TTC GGG ACC AG	(Demaneche et al., 2008)
	GEO1b-Cy5	CTC ACG CAC TTC GGG ACC AA	(Demaneche et al., 2008)

2.4.4 Bulk Molecular Analysis

2.4.4.1 DNA Extraction

Genomic DNA was separately extracted from generated fines and residual granules (on the sieve) collected at each sampling time from shearing experiment with the Power Soil DNA isolation kit (Mo Bio, USA) according to manufacturer's protocol with modification. Solution C1-C5, lysing matrix and spin filter tubes were provided by the kit. Generally, 0.3g sample was transferred to the cell lysing matrix tube supplied. Each tube was shaken on the bead beater instrument at speed 4,800rpm for 1 minute. The tubes were centrifuged at 13,000× *g*, for 5 minutes. All the supernatants were transferred to a 2mL tubes containing 300µL solution C2 and placed in 4°C for 5 minutes. After centrifuge at 13,000× *g*, for 2 minutes, the supernatant was transferred to 2mL tubes containing 300µL solution C3 and placed in 4°C for 5 minutes. After centrifuging at 13,000× *g*, for 2 minutes, supernatant was transferred to 1mL binding matrix in 15mL falcon tubes. After mixing well, all liquid was filtered through the spin filter tube and washed with 500µL solution C5. 40µL RNAnase-free water was added to the spin filter with new catch tube. The tube was then incubated at 50°C for 5 minutes before the final centrifuge elution at 13,000× *g* for 1 minute.

2.4.4.2 Terminal Restriction Fragment Length Polymorphism (TRFLP)

Bacterial and archaeal 16S rRNA genes (>1kb) were amplified by PCR using bacteria-specific primers 63F (5'-CAGGCCTAACACATGCAAGTC-3') with a fluorescent label on the 5' end, and 1389R (5'-ACGGGCGGTGTGTACAAG-3') (Marchesi et al., 1998) and archaea-specific primers Arc8F (5'-TCCGGTTGATCCTGCC-3') with a fluorescent label on the 5' end, and 1492R (5'-GGCTACCTTGTTACGACTT-3') (Smalla et al., 1993). The amplification protocol was based on that of Osborn et al. (2000) with adaptations. Each 50µL reaction contained 200µM each dNTP, 1× PCR buffer II (Applied Biosystems, USA), 3mM $MgCl_2$, 400nM each primer, 0.05U AmpliTaq Gold DNA polymerase (Applied Biosystems, USA) and 15ng genomic DNA. The PCR program consisted of an initial denaturation step at 95°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes, with a final extension step at 72°C for 10 minutes.

The PCR product was purified by QIAquick PCR purification kit (QIAGene, Australia) according to the manufacturer's protocol. Restriction digestions were carried out with *AluI*

(5'-AG^CT-3') (Fermentas, Canada), *MspI* (5'-C^CGG-3') (Fermentas, Canada) for bacteria and *AluI, MspI* and *HaeIII* (5'-GG^CC-3') (Fermentas, Canada) for archaea. Each 31μL restriction enzyme digestion reaction contained 150ng purified PCR products, 0.32U restriction enzyme and 1× relevant buffer. Each digestion was performed in duplicate. Ethanol precipitation of the digestion product was then performed. Samples were analysed using capillary electrophoresis in a DNA sequencer utilizing the AB3730 platform at the Australian Genomic Research Facility (Glen Osmond, Australia). The resulting electropherograms were analysed by GeneMarker software (SoftGenetics, USA). TRFLP data was then normalized to allow comparison between samples and standardized to remove errors associated with differential sample loading (Sait et al., 2003). Small and un-reproducible peaks were removed to reduce noise.

2.4.4.3 Clone Library and TRFLP Identification

Clone library was constructed to enable *in silico* TRFLP digestion on actual sequences from samples. As the archaeal community is relative simple, one archaeal 16S rRNA clone library was conducted on biomass collected from the whole brewery granules. Two bacterial 16S rRNA clone libraries were conducted on biomass collected from the whole brewery granules and the outer layer of brewery granules to increase the chance of picking fermenters. PCR was carried out with the same primers without fluorescent label and according to protocol described in TRFLP section (section 2.4.4.2). PCR products were cloned into the pGEM®-T Easy Vector (Promega, USA) through the ligation procedure described in the manufacturer's manual. Vectors were then transformed into JM109 high efficiency competent cells (Promega, USA) to construct the clone libraries. Twenty clones were randomly picked from each clone library. Clones were sequenced with primers T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') and SP6 (5'-TAT TTA GGT GAC ACT ATA G-3') (Pavco and Steege, 1991) with Sanger sequencing in Australian Genome Research Facility (Brisbane, Australia). Representative clone sequences were submitted to GenBank to obtain accession number. *In silico* TRFLP digestion and BLAST searches (Altschul et al., 1990) were performed on each clone in MEGA5 (Tamura et al., 2011).

2.4.4.4 Pyrosequencing and Result Analysis

DNA from each sample (300ng) was provided to Australian Centre for Ecogenomics (ACE) in the University of Queensland (Australia) for pyrosequencing analysis. Each 50μL reaction contained 200μM each dNTP, 1× PCR buffer II (Applied Biosystems, USA), 3mM $MgCl_2$, 400nM each primer, 0.05U AmpliTaq Gold DNA polymerase (Applied Biosystems, USA) and 15ng genomic DNA. The PCR program consisted of an initial denaturation step at 95°C for 10 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 1 minute and 30 seconds, with a final extension step at 72°C for 10 minutes. The primer used for pyrosequencing is 926F (5'-AAACTYAAAKGAATTGACGG-3') (Lane et al., 1985) and 1392R (5'-ACGGGCGGTGTGTAC-3') (Lane, 1991) with a Roche 454 GS FLX sequencer (Roche, Switzerland).

Pyrosequencing results were then analysed through the ACE Pyrosequencing Pipeline (https://github.com/minillinim/APP) in a local implementation. Basically, the sequences reads were split according to the barcode in QIIME (Caporaso et al., 2010b). De-multiplexed sequences were then trimmed to 250bp length and de-noised by ACACIA (Bragg et al., 2012). Sequences with > 97% similarity was assigned to same operational taxonomic units (OTUs) by CD-HIT-OTU (Wu et al., 2011; Li et al., 2012) and aligned by Pynast (Caporaso et al., 2010a). CD-HIT-OTU then grouped the OTUs with one base pair difference. OTUs with only 1 read were considered as background noise and removed. The most abundant sequences in each OTU were chosen as representative sequences. Each sequence was then assigned to the taxonomy with BlastTaxonAssigner in QIIME through greengenes database (2013_5 release). Finally the non-normalized OTUs table and rarefaction curve were generated by QIIME. Normaliser (https://github.com/minillinim/Normaliser) was used to find a centroid normalized OTUs table by randomly pick subsets with defined number from non-normalised OTUs table for 1000 repeats. Heatmap was generated in R version 3.0.1 (R Development Core Team, 2012) by function "pheatmap" in package pheatmap (Kolde, 2013).

2.4.4.5 Metagenomic Analysis

DNA of each sample (1ng) was used to prepare the library using Nextera DNA sample prep kit (Illumina, USA) according to manufacturer's protocol by ACE. The major library preparation is summarized in Figure 2.3.

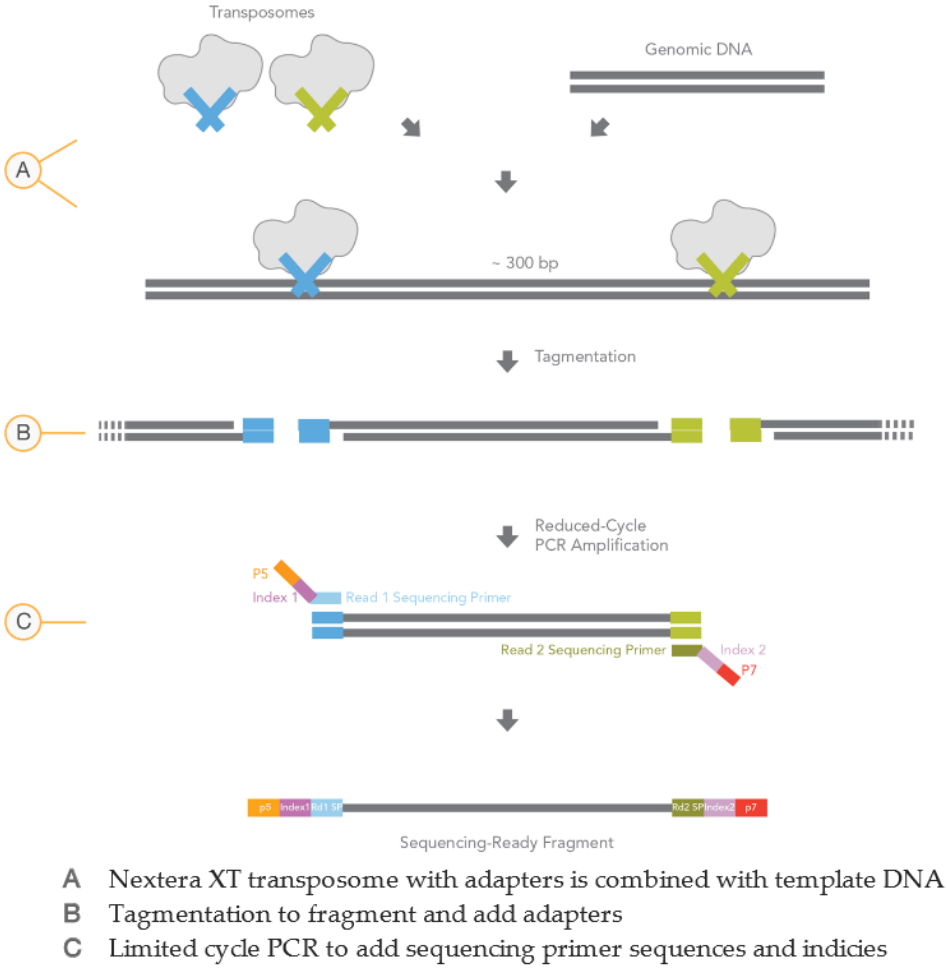
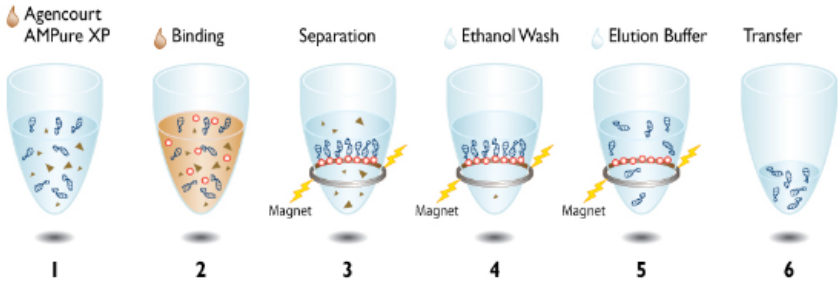


Figure 2.3 Library preparations for Illumina sequencing, modified from http://res.illumina.com/documents/products/datasheets/datasheet_nextera_dna_sample_prep.pdf**.**

Genomic DNA was fragmented enzymatically by the engineered transposome and blunted with adaptor that contains sequencing primer sites. Tagmented DNA was then amplified with provided primer containing both index (1 or 2 at each ends) and flow cell attachment site (P5 or P7 at each end). The PCR program consisted of an initial heating at 72°C for 3 minutes, denaturation step at 95°C for 30 seconds, followed by 12 cycles of denaturation at 95°C for 10 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds, with a final extension step at 72°C for 5 minutes. PCR products were then mixed with 1.8× AMPure beads. At magnet filed, PCR product (>100bp) bound to AMPure beads and separated from smaller fragments. Supernatant, along with small fragments, were removed and discarded. After washing with ethanol, long tagmented DNA fragments were eluted from AMPure beads (Figure 2.4).

Process Overview



1. Add 1.8ul AMPure XP per 1.0ul of PCR product
2. Bind PCR products to paramagnetic beads
3. Separation of beads + PCR from contaminants
4. Wash beads + PCR product 2x with 70% Ethanol to remove contaminants
5. Elute purified PCR product from beads
6. Transfer to new plate

Figure 2.4 AMPure beads purification modified from https://www.beckmancoulter.com/wsrportal/bibliography?docname=Protocol_000387v001.pdf.

Cleaned DNA fragments (20µL) were then mixed with 45µL from library normalization solution (800µL well mixed library normalization beads plus 4.4mL library normalization additives). Equal amount DNA fragments were then separated in magnetic field and captured. 5µL normalized DNA fragments from each sample were pooled together and mixed well. 15µL from pooled DNA fragments were mixed with 120µL hybridization buffer and sequenced by HiSeq in Queensland Centre for Medical Genomics in the University of Queensland (Australia).

Paired end de-multiplexed metagenomic data (provided from facility) were firstly process through SeqPrep (http://github.com/jstjohn/SeqPrep) to merge overlapping sequences into a single read according to the presence of adapters. Unable to be merged sequences were then trimmed to remove primer/adapter and filtered according to minimum quality of 10 and shortest length of 30bp by Nesoni (http://www.vicbioinformatics.com/software.nesoni.shtml). Merged sequences and quality controlled unmerged sequences were assembled using CLC workbench (Version 4.0, CLC bio) with default settings. Assembled sequences were mapped against the raw readings using BWA (http://bio-bwa.sourceforge.net/) with BWA-MEM algorithm.

Binning is the process to get the taxonomic diversity profile of a sample by classifying and clustering the reads (assembled contigs) into specific bins. Existing binning methods can be classified into taxonomy dependent and taxonomy independent.

The major driving force for taxonomy dependent method is the extend of similarity of read with existing sequences from reference database such as NCBI (ftp://ftp.ncbi.nih.gov/blast/db/), PFAM (http://pfam.sanger.ac.uk/) or pre-computed models (constructed based on reference databases). The comparison can be done by aligning reads to sequences or Hidden Markov Models (HMMs) corresponding to known taxonomic groups (e.g. BLAST, Altschul et al., 1990), comparing the compositional properties such as GC percentage, codon usage and oligonucleotide usage pattern between reads and sequences or HMMs (e.g. Phylopythia, McHardy et al., 2007), or a combination of both approaches (e.g. SPHINX, Mohammed et al., 2011). The classification of taxonomy dependent heavily relies on the genomic reference. As the majority reads of metagenomic studies are originated from genomes of hitherto unknown organisms, they are classified as unassigned by this approach.

The taxonomy independent category generates bins by utilising the properties of reads themselves without the need of reference database. These properties include the correlation between tetra-nucleotide usage patterns between reads (utilised by TETRA, Teeling et al., 2004), frequencies of tetra-nucleotide (utilised by SOMs, Ultsch and Morchen, 2005) or frequencies of k-mers with various lengths (utilised by CompostBin, Chatterji et al., 2008). The major drawback of these methods is the requirement of reads with sufficient length (>800bp). Abundance of reads is first utilised in recent published method called AbundanceBin which groups the reads with identical or similar abundance into bin and can handle short sequences such as 75bp (Wu and Ye, 2011). GroopM expanded this approach to utilising the abundance variance of species between samples, which are related in manners of time point or spatial positions etc. A manuscript is under preparation by the developer with detailed instruction for GroopM.

Binning was then performed with GroopM (http://minillinim.github.io/GroopM/) to group sequence reads with similar variation through different samples and generate a bin for each group. Bins were checked against marker genes against to 111 HMMs (Dupont et al., 2012) using checkM (https://github.com/Ecogenomics/CheckM) for completeness (whether all marker genes are presented) and contaminations (whether single marker genes presented more than once).

High quality bins (with >70% completeness and <10% contamination) were then translated to protein sequences using PhylogeneticM (https://github.com/Ecogenomics/PhylogeneticM) and used to generate phylogenetic tree by FastTree (Price et al., 2009; Price et al., 2010). OTUs were picked and assigned from raw reads of each sample against greengenes 16S database (2013_5 release, 97% similarity) by QIIME (Caporaso et al., 2010b).

2.4.5 Statistical Analysis

NGS technologies generate large data sets across a broad range of species. By utilising various statistical analyses, these data sets can be evaluated to provide concise information about the relationship between samples. The statistical analysis listed here was done on pyrotag sequencing, and metagenomic data.

2.4.5.1 Principle Component Analysis (PCA)

PCA is an Eigen analysis-based method that allows visualisation of the differences between samples in reduced dimensions (Chatfield & Collins, 1980). Briefly, PCA relies on Eigen decomposition of the Euclidian covariance matrix, which extracts both Eigenvalues (indicating contribution to variance of each principal component), as well as the Eigenvectors (indicating sample or site contribution to each principal component). The Eigenvector matrix can be thought as a projection or rotation of the original matrix and retains the same total variance. Eigenvectors (principal components) are sorted according to the eigenvalue (proportion of variance). The top 2 (sometimes 3) components are used to plot the ordination of samples as well as the direction of variable factors. The maximum variance is then expressed in minimum dimensions. However, PCA only analyses Euclidean covariance between samples. For microbial community data, many samples have closely related but different OTUs and this generates zeros in the covariance matrix. It is repeatedly argued that this distance measurement is inappropriate for raw species abundance data as null abundance can induce paradox (Orloci, 1978), which counts the null abundance as positive covariance samples. To overcome this, the community data is first transformed to relative abundance before calculating Euclidean distance as shown in Equation [22].

$$y'_{ij} = \frac{y_{ij}}{\sum y_{i+}} \qquad [22]$$

Where y'_{ij} is the relative abundance, y_{ij} is the raw reads of sequences in individual species, and $\sum y_{i+}$ is the total reads of all species in one sample. This transformation does not give extra weight on rare species and able to predict the differences based on the most abundant species. To enable the prediction based on the whole communities, species data can be further transformed as square root of Equation [22]. This is known as Hellinger transformation (Legendre and Gallagher, 2001) and highly recommended with species datasets. In this thesis, community data is analysed with both transformations on normalised OTUs table. The transferred dataset was imported into R, version 3.0.1 (R Development Core Team, 2012) to generate principle component analysis using Euclidean distance with function "rda" in package vegan (Oksanen et al., 2013). Graphs were produced with the function "biplot". In addition, PCA should be used to predict the variance, i.e. the abundance shift between samples, rather than ordinations, i.e. the cluster of samples based on the community data. This is because moderate or high beta diversity (which is normally seen in community data) can induce a horseshoe effect that twists the second axis and produces possible false ordination.

2.4.5.2 Multi-Dimensional Scaling (MDS)

MDS is classified as metric MDS (normally known as Principle coordination analysis (PCoA)) and non-metric MDS (NMDS). PCoA is an upgrade version of PCA as any distance measurement can be used (Borg and Groenen, 2005). It then reflects the maximum variance base on eigen-analysis of distance matrix in a low dimension space. While NMDS uses different approach, it is a projection of sample ordination from high dimension to a certain plane with defined number of dimensions based on the rank order of differences between samples. Thus it has following features: 1. Two apart points mean they are different but does not reflect the distance between them, 2. Unlike PCA or PCoA, in which the dimensions (eigenvectors) are uncorrelated, in NMDS the plot of low dimension is not a projection from a high dimension solution. There is no proportion of variation explained by each axis, as they are not eigenvectors. Instead, it uses the value called stress to evaluate the difference between predictions from the rank system and distance matrix. The flexibility of choice on different distance measurements gives advantage on MDS, for example, Bray Curtis dissimilarity measurements. It is commonly used for ecological studies over years due to the ability to distinguish two samples that have numerous zeros (Bray and Curtis, 1957). It is calculated as Equation [23].

$$S_{jk} = 100\left\{1 - \frac{\sum_{i=1}^{p}|y_{ij} - y_{ik}|}{\sum_{i=1}^{p}(y_{ij} + y_{ik})}\right\} \quad [23]$$

Where $\boldsymbol{S_{jk}}$ is the similarity between two samples j and k, $\boldsymbol{y_{ij(k)}}$ as the abundance of species $\boldsymbol{i}$ in sample $\boldsymbol{j}$ (or $\boldsymbol{k}$) of the community data set. The difference between samples is an absolute value. More important, as the similarity is expressed as a proportion of difference on the total abundance, the null abundance produces no effect on the similarity. However, transformation to relative abundance is also necessary to avoid extreme high values in the data sets. The transformed normalized OTUs table was imported into R, version 3.0.1 (R Development Core Team, 2012) to generate NMDS using Bray-Curtis dissimilarity method with function metaMDS in Package vegan (version 2.0-9) (Oksanen et al., 2013).

2.4.5.3 Ternary Plots

Ternary plots were originally used to graph the genotype frequencies of populations. It is recently used to show the shared and unique OTUs in pyrosequencing analysis on different samples (Pires et al., 2012; Bulgarelli et al., 2012). For example, Wilhelm et al., (2013) reported that glacier ice contributed only marginally to the microorganism in the stream water and benthic biofilm community as there are less commonality between glacier ice and later two samples. In this thesis, we applied ternary plots to distinguish the similarity and specificity of microbial community between different types of anaerobic granules. Only the OTUs present in all three types of granules were kept and imported into R. Ternary plots were constructed on pyrosequencing data with function ternaryplot in package "ggtern" (version 1.0.3.1) (Hamiton, 2014). Relative abundance (after normalization between samples) of each OTU in each sample is plotted as the proportion to the total abundance of that OTU in all three samples. The coordination of each species $\boldsymbol{P}$ (a,b,c) where a, b, c are the transformed proportion in each sample is $\boldsymbol{P}$ (b+c/2,c$\sqrt{3}$/2). The size of the points is enlarged according to the proportion of relative abundance of that species in all samples to the sum of relative abundance of all species in all samples.

In this thesis, the factors differentiating shared layers of granules and different types of granules are examined by PCA. The ordination of samples is obtained from NMDS, which reflects the similarity in a more accurate way. Community variation between different types of granules is focused by ternary plot.

Chapter 3. Shearing of Biofilms Enables Selective Layer Based Microbial Sampling and Analysis

3.1 Introduction

The UASB reactor (Lettinga et al., 1980) is one of the most widely applied reactor designs for anaerobic digestion of readily degradable wastewaters. In a UASB, functional microorganisms, including fermenters, acetogens and methanogens, aggregate into granules of 0.5–3 mm 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 (Fang et al., 1995; Guiot et al., 1992) depending on feedstock. Microbial identity is known to be important in determining reactor performance (Karakashev et al., 2005).

To date, the spatial information, i.e. the position of particular organisms within the granules, has only been studied with fluorescent *in situ* hybridization (FISH). However, this is highly dependent on the choice of probes, and the response of each organism to the FISH process. It is also limited to phylogenetic analysis based on 16S genes or similar, with limited opportunity for analysis of functional genes or metagenomics.

Small particles from the exterior of granules, or fines, can result when shear stress is applied. When this occurs in a UASB reactor, it can reduce the quality of reactor effluent. The granule shear strength is defined as the quantitative production rates of fines normalized by shear rate and can be useful to predict sensitivity to shear. Granule strength also has practical implications for effluent quality (Ghangrekar et al., 1996). Shear strength can be determined by an abrasion experiment designed by Pereboom (1997). The shear strength of granules appears related to the feed type, and possibly divalent cation availability. For example, simple substrates such as acetate (Fang et al., 1994) and mixed organic acids (Batstone and Keller, 2001) produce granules that have low shear strength and complex substrates, such as carbohydrate (Batstone et al., 2004) produce granules with higher shear strength. High calcium granules had very high shear strength (Batstone et al., 2002). Using abrasion modified version of this technique and information about a granule type's shear strength, it

may also be possible to sequentially remove granule layers, potentially allowing phylogenetic and functional characterization of each layer.

In order to sequentially remove layers from mature granules from full-scale UASB reactors, a modified version of Pereboom's shear strength measurement (Pereboom, 1997) was developed and evaluated by applying community profiling methods to successive biofilm layers.

3.2 Results

Most brewery and cannery granules were dark coloured with a smooth surface. The largest granule, brewery granules, had diameter ranging from 1.1-2.8mm (average 2±0.8mm), and with an oval or angular shape. Most cannery granules were spherical with diameter ranging from 0.5-0.8mm (average 0.6±0.2mm). Larger cannery granules had similar shape with brewery granules and smaller particles were possibly broken granules. Dairy granules were yellowish, and spherical with rough surface. Most dairy granules had diameters from 0.4-1.2mm (average 0.8±0.3mm). Particles measured above this size were observed as large aggregates of dairy granules and it was hard to separate the aggregate without damaging individual granules.

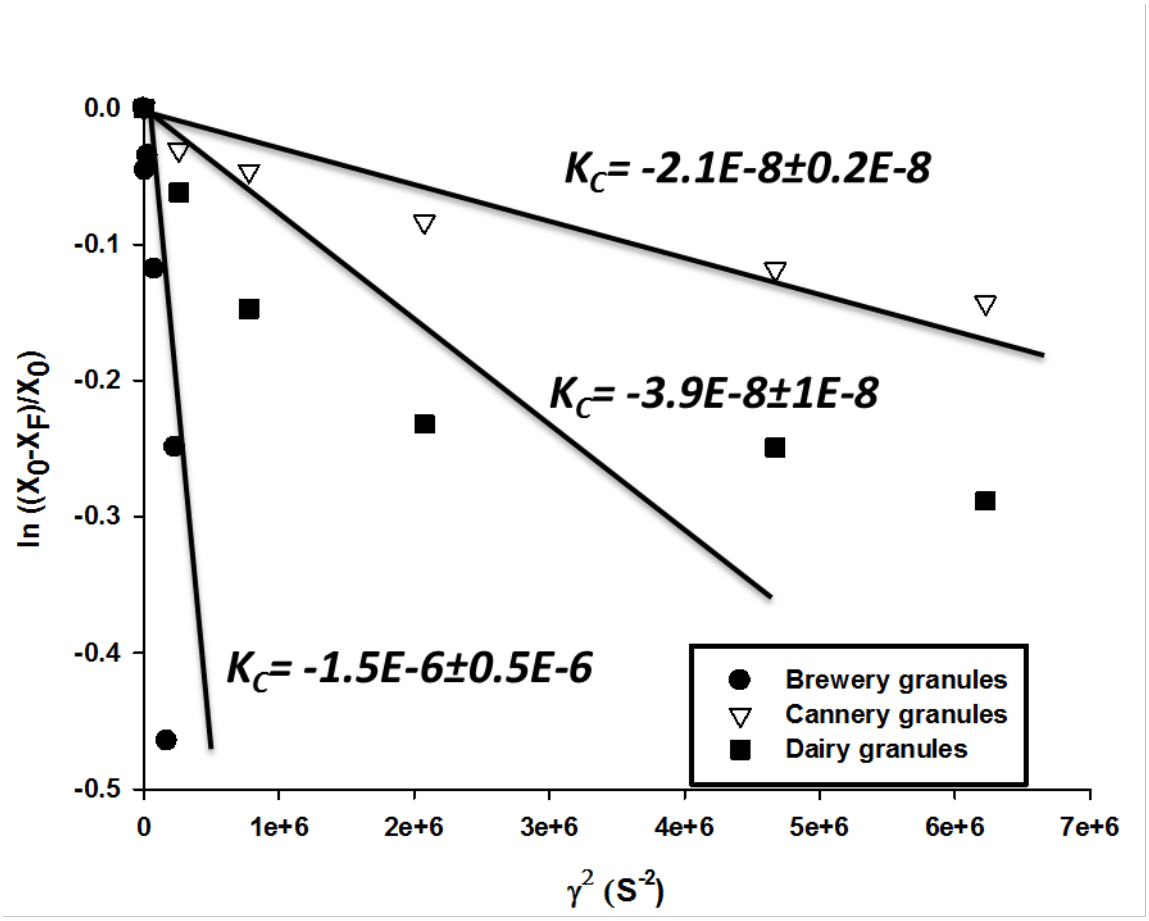


Figure 3.1 Estimation of characteristic abrasion coefficient (K_C). K_C for granules from full-scale reactors with different feedstock, including brewery, cannery and dairy were estimated according to the method described in materials and methods section. A lower K_C equates to a weaker granule. Brewery granules were sheared at 500 rpm, both carbohydrate and dairy granules were sheared at 1500 rpm.

The fines generated versus shear rate squared (γ^2) is shown in Figure 3.1. A lower K_C (characteristic shear coefficient from Equation [15] in Chapter 2), equates to a weaker granule (i.e., faster generation of fines). Cannery and dairy granule strengths were comparable (protein was slightly weaker), while brewery granules were far weaker. The amount of solids measured, together with the measured density allowed calculation of depth sheared as shown in Table 3.1.

Table 3.1 Shear depth (µm) of three types of anaerobic granules at each time point. Shearing was done at 500 rpm (brewery granules) and 1500 rpm (dairy and cannery granules).

Time (Minutes)	Depth (µm)		
	Brewery	**Dairy**	**Cannery**
5	14	9	5
20	24	28	16
60	58	53	42
180	117	79	72
270	196	98	86
360	N/A	127	99

Shearing was successful as demonstrated by FISH analysis of pre-sheared and post-sheared anaerobic granules. Bacteria, as shown in red in Figure 3.2, formed a distinct layer on the surface of cannery (Figure 3.2A), brewery (Figure 3.2C) and dairy granules (Figure 3.2E). This layer is completely removed as seen on cannery granules after 270 minutes shearing (Figure 3.2B), brewery granules after 5 minutes shearing (Figure 3.2D) and dairy granules after 180 minutes shearing (Figure 3.2F).

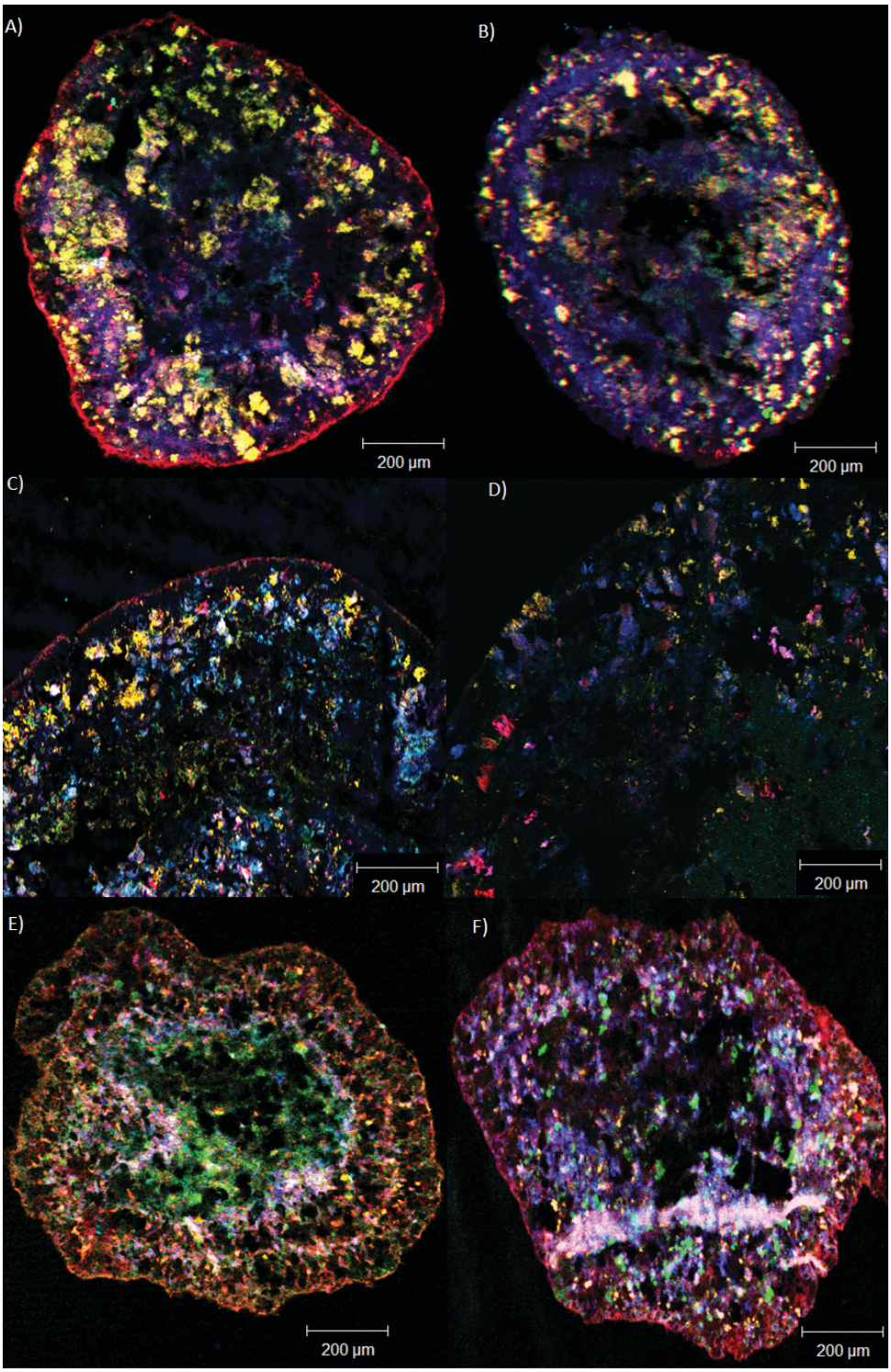


Figure 3.2 FISH images of the cannery granules before (A) and after (B) shearing, brewery granules before (C) and after (D) shearing and dairy granules before (E) and after (F) shearing. Bacteria are shown in red and form a distinct layer on the surface of cannery granules. This layer is completely removed after shearing. *Methanosaeta (MX825)* is shown in yellow. *Methanococcales* (*MC1109*), *Methanobacteriales* (*MB1174*) and *Methanobacteriales* (*MG1200*) are shown in cyan. Other Archaea (*ARC915*) are shown in green. Bacteria (*EUBmix*) are shown in red.

Based on *in silico* digestion of clone sequences from clone libraries, a number of TRFLP peaks were identified (Table 3.2).

Table 3.2 *In silico* and *in vitro* TRFLP peaks comparison.

Putative identification	GenBank Accession Number	*In silico*		*In vitro*	
		AluI	*MspI*	*AluI*	*MspI*
Methanosaeta	KC502890;KC502891	178	284	178	283
Methanolinea	KC502892;KC502893	188	129	189	126
Geobacter	KC502887	36	128	N/A	124
Syntrophomonas	KC502886	41	253	N/A	254
Anaerolinea	AP012029	201	485	205	471,479
Bacteroidetes	KC502888;KC502889	171	56,108	170	108

Quantitative analysis clearly shows progressive variation of TRFLP peaks through different layers (Figure 3.3A, B & C). Particular organisms were evident in outer but not inner layers, including members of the *Bacteroidetes* and the *Anaerolinea* (referred to as *Anaerolinea1* in Figure 3.3) in cannery granules (Figure 3.3A), *MspI* 126 and other members of the *Anaerolinea* (referred to as *Anaerolinea2* in Figure 3.3) in both brewery (Figure 3.3B) and dairy granules (Figure 3.3C). This is clearly shown in Figure 3.3D. As an example of this trend, the abundance of *Anaerolinea2* peak in the outer layer was seven times the size of the one in whole granules. On the other hand, members of the *Syntrophomonas* and *Geobacter* presented in inner layers in cannery (Figure 3.3A) and brewery granules (Figure 3.3B). There were 14, 10 and 16 additional peaks detected from layer samples than the one from whole cannery, brewery and dairy granules respectively.

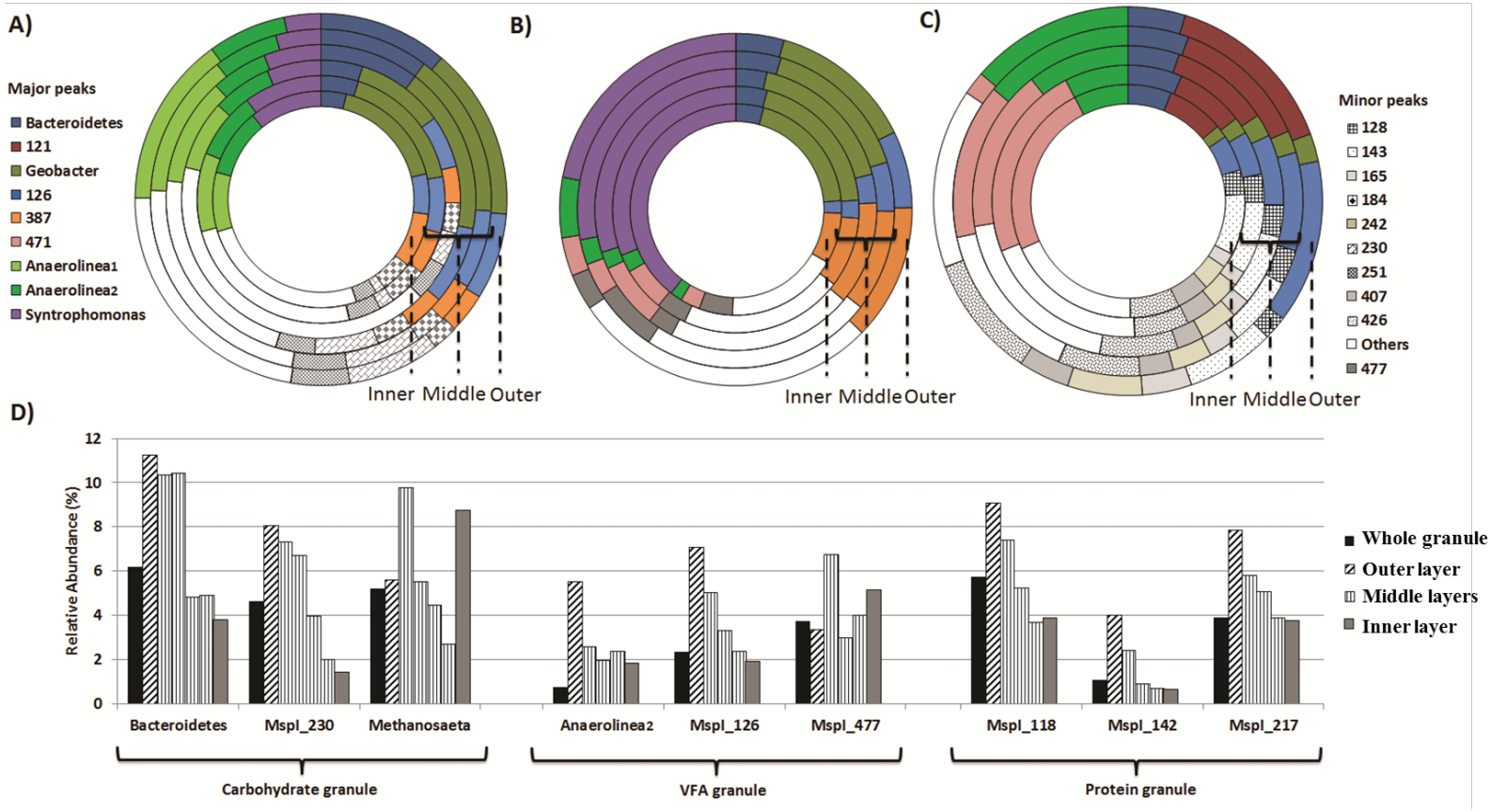


Figure 3.3 Relative abundance of different bacterial groups in outer, middle and inner layers of cannery granules (A), brewery granules (B) and dairy granules (C) as revealed by TRFLP. Peaks that were identified by BLAST searches and *in silico* TRFLP digestion of cloned 16S rRNA genes are labelled with their affiliated organism and other peaks are labelled with the peak number from enzyme digestion with *MspI*. The relative abundance of specific peaks can be greatly enhanced in individual layers compared to the average abundance detected in the whole granule (D).

3.3 Discussion

3.3.1Granule strength is variable

The granule strengths were highly variable as previously reported (Batstone and Keller, 2001). This is largely related to feedstock, with rapidly degradable primary substrates such as sugars resulting in dense and high-strength outer layers (Batstone et al., 2004). The importance of this is that the method as presented cannot be applied using fixed times and shear rates, as the amount sheared depth depends strongly on the granule strength (note that Figure 5.1 is a log relationship). However, the method does inherently return shear strength, and we recommend shearing initially at lower rates and shorter times to collect outer layers and characterize the shear strength, and to conduct additional tests at a higher shear rate as necessary.

3.3.2 Shearing combines the depth of microbial community profiling with localization capability

Compared with traditional microbial community profiling study, this method was able to analyse DNA from specific layers with a sub-millimetre resolution and detect the major microorganism on each layer. Applying FISH ideally requires a level of prior community knowledge, particularly where the system is phylogenetically diverse, where the population carrying out a specific function is relatively merged or where a single function has a diverse phylogeny. Anaerobic granules are a good example of this, with a high level of diversity, particularly amongst the fermenters (Werner et al., 2011). Without spatial enrichment, FISH hunting (i.e. systematically testing FISH probes based on clone library information) can be extremely time consuming and unproductive. However, when a particular organism is found to be enriched in an outer layer, it is an obvious fermentative candidate, and probes can be designed preferentially against that organism, making it more likely to result in a successful identification. Furthermore, the quality of DNA recovered from shearing is relatively good, as the shear force is relatively gentle compared to subsequent DNA extraction. We observed additional TRFLP peaks in the outer layer that were not found in whole granule samples. This is because in whole granules, organisms at relatively high abundance in the outer layer are not detected due to their far lower abundance in the granules as a whole. This spatial selection of organisms could also be used in conjunction with other community profiling

methods such as clone libraries and pyrosequencing, even where several thousand OTUs are identified. The localization by FISH and by shearing, in combination with microbial community profiling methods offers the possibility to assess the impact of factors influencing community profiling analysis such as PCR bias. We also believe it may be possible to extract whole live organisms in order to conduct further live culture analysis, including substrate screening, and enzymatic/proteomic analysis.

3.3.3 Complexity of outer layer

The outer layer has been regarded as being mainly fermentative (Batstone et al., 2004; Sekiguchi et al., 1999) in function. The results support it, with the presumptive fermenters, such as *Bacteroidetes* and *Anaerolinea*, found in granules with cannery and dairy wastewater. Importantly, the brewery granules were dominated by syntrophs such as *Geobacter* (ethanol), and *Syntrophobacter* (propionate). While the results support the findings from previous studies that the outer layers are dominated by fermenters, and additionally show that the outer layers are not dominated by a single population, but are quite complex. Microbial community profiling methods are the only way to attain the phylogenetic depth needed to identify and analyse communities at this depth. TRFLP was used here in order to readily assess the 3 whole granules samples and 17 sheared samples generated in this study, to demonstrate profiling through the depth of granules and hence demonstrate the utility of the method. However, future approaches would be to construct clone libraries or 16S pyrosequencing on individual layers, and fully characterize the community. This could be then followed by generation and application of FISH probes to confirm localization and suggest relationships between different organisms.

Chapter 4. Ecology of Anaerobic Granules

4.1 Introduction

Upflow Anaerobic Sludge Blanket (UASB) reactors are a high rate anaerobic technology commonly applied to treat wastewater from a range of industries. A key requirement of this technology is the formation of anaerobic granules containing microorganisms which convert the organic matter in the wastewater into methane and carbon dioxide through a series of complex biological reactions including interactions between multiple functional groups. Maintaining anaerobic granules and therefore maintaining stable operation of the process may be improved through increased understanding of microbial properties including community identification, spatial distribution and metabolic function.

Anaerobic granules are a key and readily observable form of large and layered biofilms. The structure of anaerobic granules has been generally regarded as a consequence of substrate, with functional distribution of primary substrate fermentation, oxidation of organic acids and alcohols, and methane production from acetate and H_2/CO_2.

Microbial community identification has been studied using PCR-based molecular techniques, particularly targeting 16S rRNA, on anaerobic granules degrading terephthalate (Wu et al., 2001), treating brewery wastewater (Chan et al., 2001, Diaz et al., 2006), municipal wastewater (Zhang et al., 2005), and paper mill wastewater (Roest et al., 2005). However, previous microbial identification studies lack spatial resolution, as sequencing based microbial analysis can only be applied to whole granules. This continues to limit fundamental understanding of anaerobic granules including the functional role of specific microorganisms. The distribution of functional groups can possibly guide links between microorganisms and physical properties of the granules thus provide opportunity for granules modification to shorten the reactor start up time and stability, and improve granule strength.

In order to improve the fundamental understanding of anaerobic granules by analysing and comparing the microbial community composition and spatial distribution within granules treating different industrial wastewaters, 454 pyrosequencing and metagenomic shotgun sequencing were applied to the anaerobic granule sheared in Chapter 3. To our knowledge, this is the first study providing high resolution in vivo evidence for spatial distribution and dominant organisms in anaerobic granules treating different kinds of wastewater.

4.2 Results

Pyrosequencing recovered 134,407 reads and grouped into 651 OTUs from total of 22 samples produced by shearing of three types of granules. There were 50,372 reads (average 7,196 reads ± 4762 standard deviation) across 5 layers and core of brewery granules grouped into 312 OTUs, 41,472 reads (average 5,184 reads ± 2503 standard deviation) across 6 layers and core of cannery granules grouped into 292 OTUs, and 42,562 reads (average 5,320 reads ± 3102 standard deviation) across 6 layers and core of dairy granules grouped into 263 OTUs.

4.2.1 Microbial Community of the Three Granules

Results according to cryosection-FISH and layered-pyrosequencing are shown in Figure 4.1 (bacterial) and 4.2 (archaeal). Brewery and cannery granules had similar bacterial niches consisted of *Bacteroidales*, *Anaerolinea*, *Geobacter*, *Syntrophomonas* and *SHA-114* with unique OTUs affiliated to *Candidatus Cloacamonas* and *Propionicimonas* in each granule respectively. Bacterial dominance shifted from *Bacteroidales.1*/*Candidatus Cloacamonas* in the outer layer to *Geobacter/Syntrophomonas* in the inner layers of brewery granules. *Geobacter* and *Syntrophomonas* were detected at low abundance in cannery and dairy granules. Unique OTUs, such as *Phycisphaerae* and *MAT-CR-H3-D11*, were found in dairy granules within > 1% relative abundance community. The archaeal communities in brewery and dairy granules were relatively simple and dominated by *Methanosaeta* and *Methanolinea* respectively. Other methanogens such as *Methanoregula* arose in cannery granules.

There is broad agreement between microbial measurements using phylogenetic analysis and FISH with specific probes. *Candidatus Cloacamonas* dominated in the outer layers of brewery granules by pyrosequencing (Figure 4.1) and were also detected in larger amount underneath the boarder of brewery granules by FISH (Figure 4.1A1). *Geobacter* were detected in the inner layers of brewery granules by pyrosequencing (Figure 4.1A) and FISH (Figure 4.1A2). *Bacteroidales.3* was the dominant microbes in the outer layers of cannery granules and mainly detected on the surface of cannery granules by FISH (Figure 4.1B2). Other microbes detected at high abundance in the outer layers of cannery granules were *Anaerolinea.1, Anaerolinea.2* and *Propionicimonas*. *Anaerolinea* were found in the middle layers of cannery granules by FISH (Figure 4.1B1). *Methanosaeta*, as a major archaeal OTU,

was evenly distributed in brewery granules (Figure 4.2A and A1) and gradually increased from outer layer towards the core in cannery granules (Figure 4.2B and B1). The abundance of *Methanobacterium* and *Methanolinea* accounted for a small proportion of the population in brewery granules, but the relative abundance of *Methanobacterium* rose up to 25% in each layer of cannery granules and *Methanolinea* became the dominant one in protein granule. Their growth was shown as closely associated with bacteria by FISH (Figure 4.2A2 and C1).

Anaerolinea.1 was the only common OTU detected in all granules. It accounted for a small proportion of brewery granules (0.8-2.2% of bacterial community). The abundance increased to 10% in cannery granules and *Anaerolinea.1* became the dominant OTU in dairy granules (30-60% of bacterial community).

In the bacterial community, there were three major trends identified regarding shifts in relative abundance. First trend referred to gradually decreasing relative abundance from outer layers to inner layers. This trend was observed on most dominant OTUs such as 3 groups of *Bacteroidales* (in all granules), genus *Candidatus Cloacamonas* and *Anaerolinea* in brewery granules (Figure 4.1A); *Propionicimonas and Anaerolinea.2* in cannery granules (Figure 4.1B); and *Phycisphaerae*, *Bacteroidales.3*, *MAT-CR-H3-D11* and *Candidatus Cloacamonas* in dairy granules (Figure 4.1C). The opposite trend i.e. gradually increasing from outer layers to inner layers, were found on OTUs affiliated to genus *Geobacter* and *Syntrophomonas* in both brewery and cannery granules (Figure 4.1A and B); and *Anaerolinea*.1 in dairy granules (Figure 4.1C). Lastly, some OTUs distributed evenly. For example, *SHA-114* in brewery granules (Figure 4.1A) and *Anaerolinea.1* in cannery granules (Figure 4.1B) showed no progressive changes.

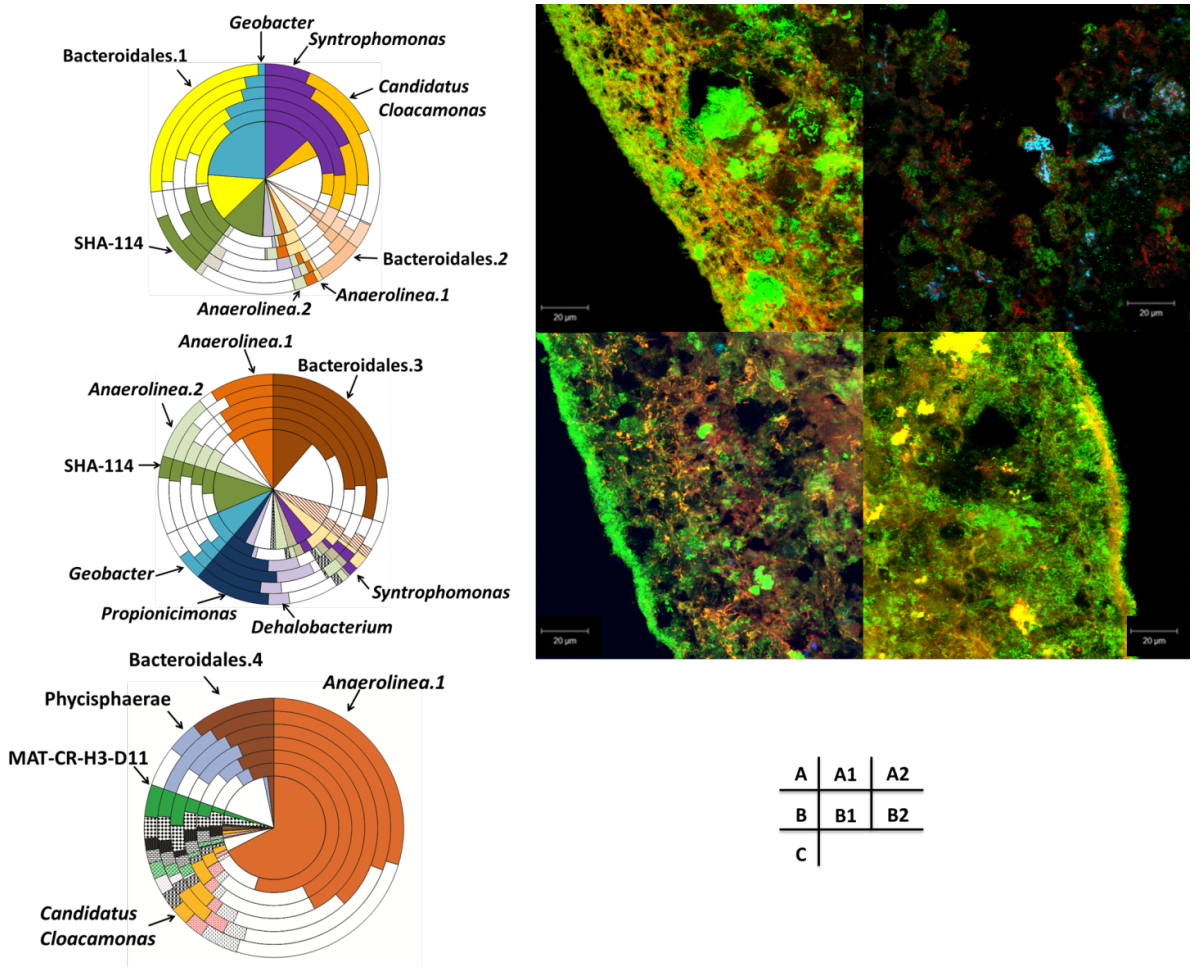


Figure 4.1 Relative abundance of bacterial community in each layer of brewery granules (A), cannery granules (B) and dairy granules (C) and FISH analysis on detecting *Candidatus Cloacamonas* (WWE1) in yellow (A1) and *Geobacter* (*GEO1*) in cyan (A2) in brewery granules, *Anaerolinea* (*CFX784*) in yellow (B1) and *Bacteroidetes* (*CF1082, CF319a*) in cannery granules (B2). Archaea (*ARC915*) is shown in green, other bacteria (*EUBmix*) is shown in red in all FISH pictures. OTUs with >1% in any sample are filled with colour or pattern. Major bacteria are labelled with affiliated taxonomy. Other OTUs (<1%) are shown as blank and presented as appropriate position in the donut chart.

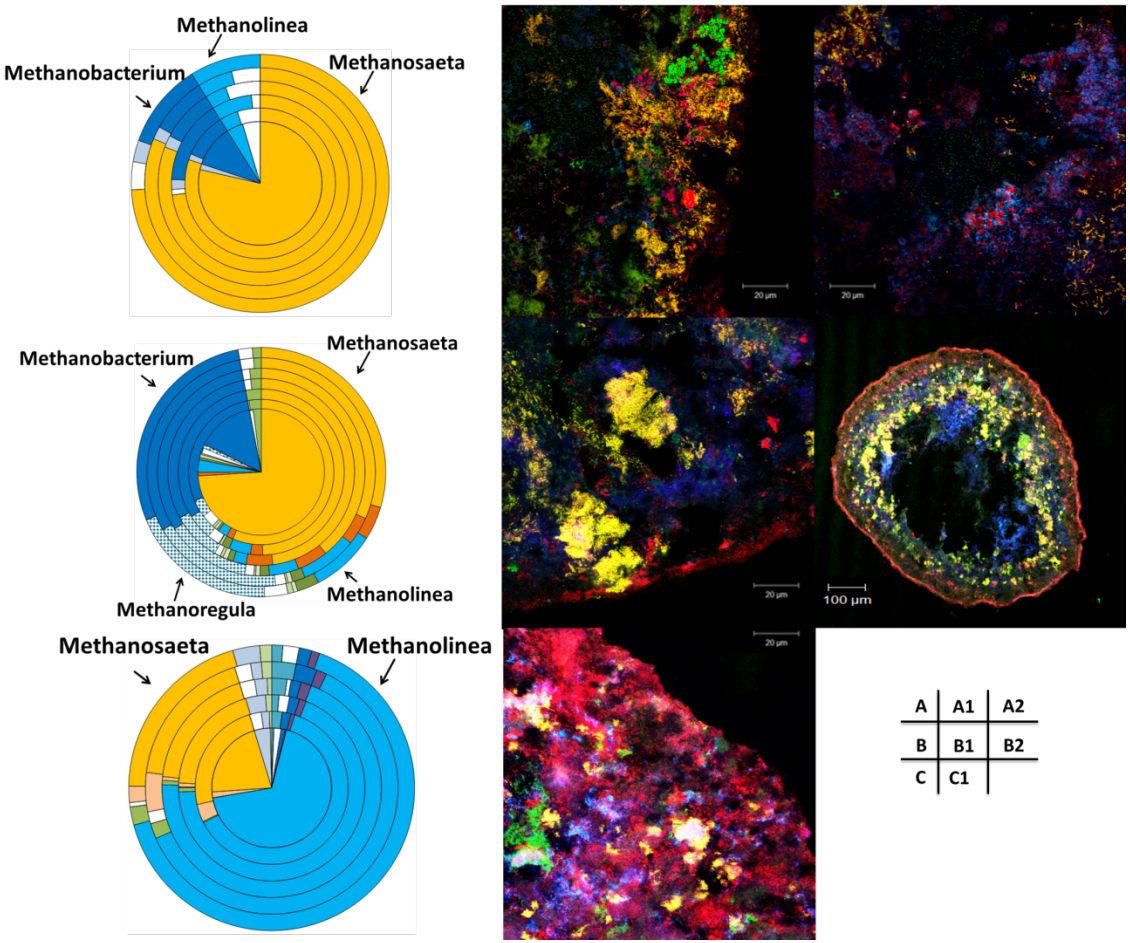


Figure 4.2 Relative abundance of archaea community in each layer of brewery granules (A), cannery granules (B) and dairy granules (C) and FISH analysis on detecting *Methanosaeta* (*MX825*) in yellow, *Methanolinea* (*MG1200*), *Methanobacterium* (*MB1174*) in cyan in brewery granules (A1 & A2), cannery granules (B1 & B2) and dairy granules (C1). Bacteria (*EUBmix*) are shown in red and other archaea (*ARC915*) are shown in green. OTUs with >1% in any sample are filled with colour or pattern. Major archaea are labelled with affiliated taxonomy. Other OTUs (<1%) are shown as blank and presented as appropriate position in the donut chart.

Regarding both archaeal and bacterial community, PCA analysis (Figure C1-C3 in Appendix C) showed that layers of granules could be divided into outer regions and inner regions according to PC1 (the most differences explained). The outer layer (first layer sample) was always located far away from others with respect to PC1 and PC2. The major differentiation factors were bacteria (presumed fermenters) dominated in the outer region while other bacteria (presumed acetogens) and archaea (methanogens) dominated in the inner region. In brewery granules (Figure C1), this outer region referred to 0-24μm from the granule surface with differentiation factors including dominant OTUs affiliated to *Candidatus Cloacamonas*, *Bacteroidales.1* and *Bacteroidales.2* in the outer region and dominant OTUs affiliated to *Methanosaeta*, *Geobacter* and *Syntrophomonas* in the inner region (PC1 41% of total variance). The 0-14μm and 14-24μm were differentiated by *Methanolinea* accumulating in outer layers (0-14μm according to PC2 with 16% explained). The outer region of cannery granules (Figure C2) consisted of three layers (0-42μm) with accumulation of OTUs affiliated to *Bacteroidales.2*, *Anaerolinea.1*, *Propionicimonas* and *Methanoregula*. The major factor driving the differentiation of inner region was the accumulation of *Methanosaeta* (PC1 54% explained). Due to the accumulation of *SHA-114/Bacteroidales.2* in 5-16μm and accumulation of *Methanoregula* in 0-5μm, the three outer layers were further separated (PC2 14% explained). Differentiation in dairy granules (Figure C3) were mainly driving by accumulation of bacteria including *Syntrophus*, *Phyhcisphaerae*, *Ruminococcaceae* and *Thermoplasmata* in the outer region (0-53μm) and the accumulation of *Methanolinea* in the inner region (PC1 30% explained). The outer layers were further separated due to the accumulation of *Anaerolinea.1* in 28-53μm, and low abundance OTUs accumulated in 9-28μm (PC2 19% explained).

4.2.2 Variation in Microbial Community across Different Granules

A ternary plot that indicates degree of commonality for microorganism within three types of granules is shown in Figure 4.3. To simplify, bacterial OTUs have been grouped at the phylum level and archaeal OTUs have been grouped order level except *Methanosaeta* (as it is the only genus detected in order *Methanosarcinales*). Granules were strongly differentiated with the cannery and brewery granules dominated by *Methanosaeta*, and *Methanobacteriales*, and the dairy granules dominated by *Methanomicrobiales*.

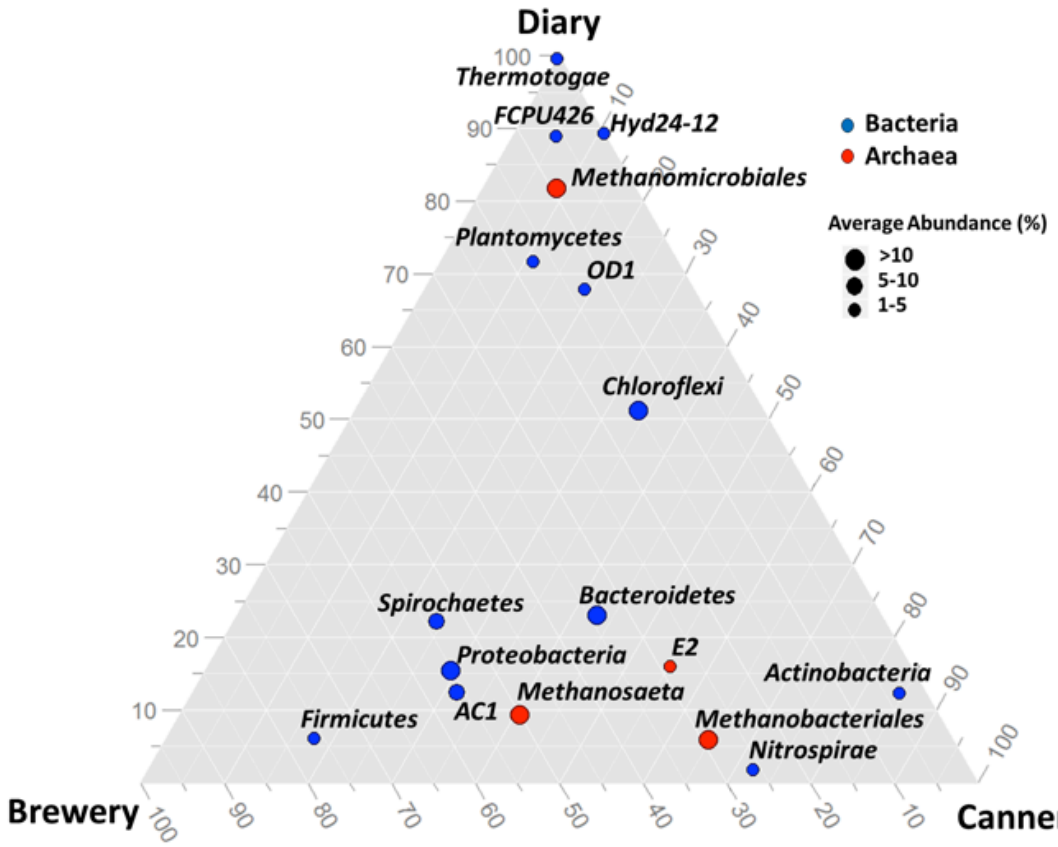


Figure 4.3 Ternary plot shows the distribution of bacterial (blue) and archaeal (red) OTUs with >1% average abundance among the tested granules based on pyrosequencing results. Size of each point was adjusted regarding to the average abundance of each microorganism in three types of granules.

Most high-abundance bacterial phyla were shared between all three types of granules to some extent. In particular, fully shared by all granules were *Bacteroidetes* (average abundance >10%), and *Spirochaetes* (average abundance 5-10%). *Proteobacteria* (>10%) and *AC1* (5-10%) were shared between brewery and cannery granules while *Chloroflexi* (>10%) was shared between cannery and dairy granules. Phylum *Firmicutes* and *Actinobacteria* were respectively found in brewery and cannery granules with average abundance of 1-5%, while they were rarely or not detected in dairy granules. There were unique OTUs detected in dairy granules only including *Thermotogae*, *FCPU426* and *Hyd24-12* as classified by greengenes.

A PCA biplot of the three granules with key phylogenetic classifications is shown in Figure 4.4. Layers are shown as different sized rings and the whole granules are shown as filled circles. The major predictor in differentiating profile is granule source, rather than subsamples (layers). The major differentiation is between brewery/cannery and dairy

granules along PC1 (57%), with the dairy granule dominated by unique OTUs compared to brewery/cannery, including *Methanolinea* and *Anaerolinea.1*. Interestingly, differentiation was due to both archaeal and bacterial communities.

Methanosaeta dominated in both brewery and cannery granules with more *Methanobacterium* presenting in cannery granules. Emergence of *Methanoregula* differentiated the outer layers from the whole cannery granules according to PCA. Cannery and brewery granules were differentiated along PC2 (20%), with differentiation mainly due to the bacterial community. Brewery granules consisted mainly of genera *Candidatus Cloacamonas, Syntrophomonas* and *Bacteroidales.1*, while genera *Propionicimonas*, *Baccteroidales.2* and *Anaerolinea.2* presented in cannery granules especially in the outer layers. The main differentiation factor between dairy granules and others was due to the dominance of *Methanolinea* and sub-dominance of *Anaerolinea.1*.

The spread within samples was also important. The only granules that had strong variation between layers were the cannery granules, indicating a strongly layered structure in line with the previous section and FISH results. This also indicated that differentiation between cannery and brewery granules was driven by the outer layer community, with samples at the furthest vertical extent being from the outer layer. Furthermore, the whole cannery granule sample (the red filled circle in Figure 4.4) was completely dominated by inner layer community, as it was located closely to the inner layers. This suggested that the outer layer community, especially the presumptively fermentative outer bacterial layer, could only be properly analysed by shearing as its abundance was very small in the whole granules.

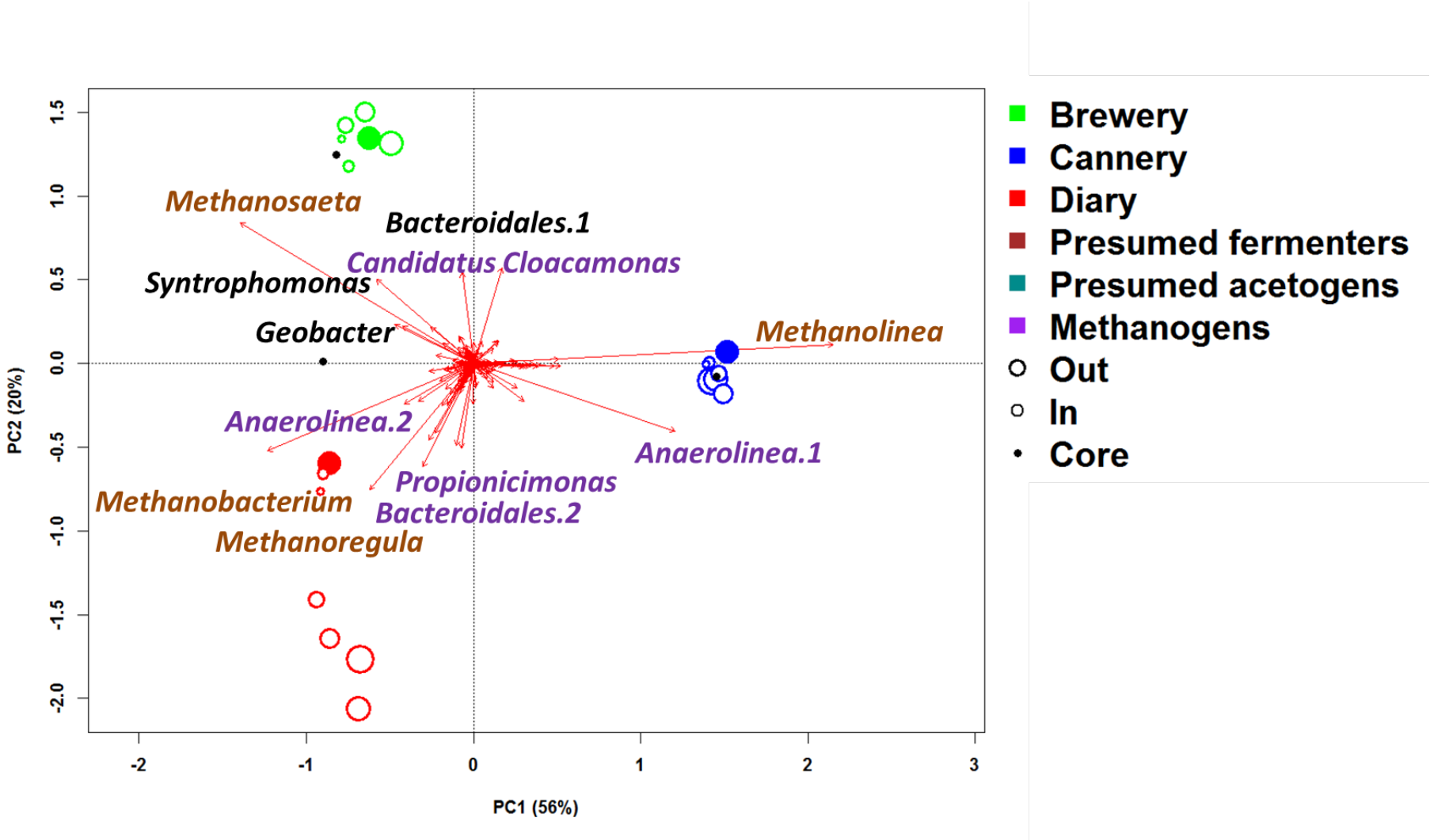


Figure 4.4 PCA analysis of pyrosequencing data on three types of anaerobic granules based on OTUs. Types of granules are distinguished by colour. Layer samples from outer to inner are distinguished by size of rings (larger as outer layer). Whole granules and the core are represented as filled colour circle and black circle respectively.

4.2.3 Metagenomic Analysis of Brewery Granules

Metagenomic analysis on the brewery granules was performed to (a) identify whether layer based (spatial) sampling would provide sufficient covariance based information (b) validate the pyrosequencing information, and (c) provide metagenomic information for further processing and functional analysis that is not applied to the full extent in this thesis due to time constraints. All three of these objectives are discussed further in this section.

4.2.3.1 Metagenomic Analysis on the Whole Genome

Metagenomic analysis on brewery granules identified total 378,718 contiguous sequences in 0.8Giga base pairs. They were clustered into bins based on PCA analysis of tetra-nucleotide signature (indicated by colour of each point and used as the bottom plane of Figure 4.5) and the normalised abundance (used to calculate the Euclidean distance as vertical axis in Figure 4.5). In total, 1 archaeal bin and 23 bacterial bins with high quality (>70% completeness and <10% contamination) were recovered from metagenomic analysis. Identification includes genus *Methanosaeta*, genus *Anaerolinea* and *Caldilinea* in phylum *Chloroflexi* (4 bins), phylum *Proteobacteria* (5bins) and *Bacteroidetes* (5bins) etc. (Figure 4.6). These include the key fermentative candidates identified through pyrosequencing. Identified bins with high to moderate abundance are shown in Figure 4.6. Bins, which were classified as *Methanosaeta concilii* (Bin953), *Syntrophomonas wolfei* (Bin168), *Geobacter* (Bin77) and *Candidatus Cloacamonas acidodaminovorans* (Bin58) had high abundance through all layers. Other high quality bins with moderate abundance are affiliated to phylum *Chloroflexi* (Bin 1086 & 138), *Bacteroidetes* (Bin190) and *Proteobacteria* (Bin 22 & 194).

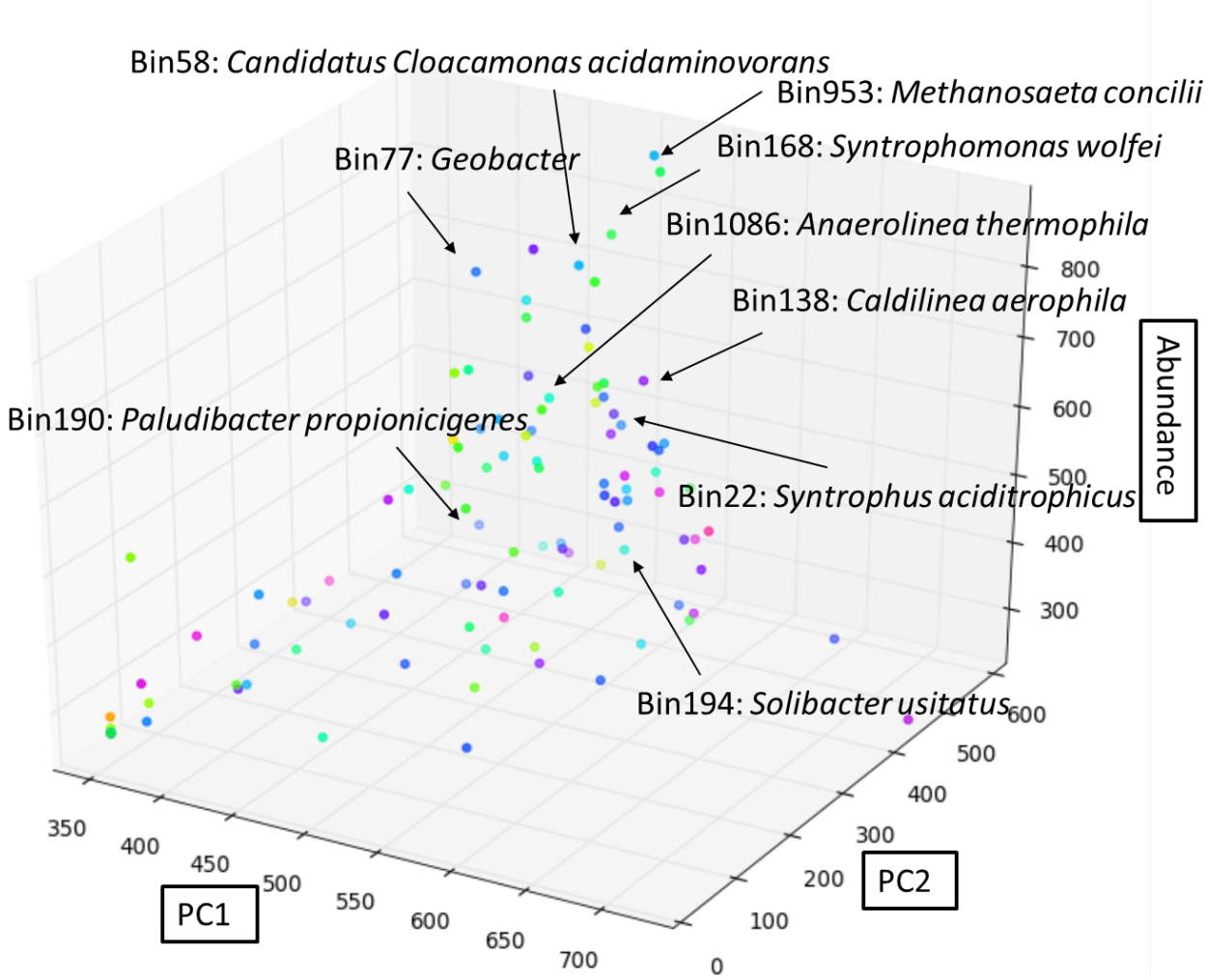


Figure 4.5 Major bins recovered from Metagenomic analysis. Bins are coloured according to K-mer and located based on PCA analysis between samples (PC1 and PC2 as X,Y axis) and the average abundance of all samples (as Z axis).

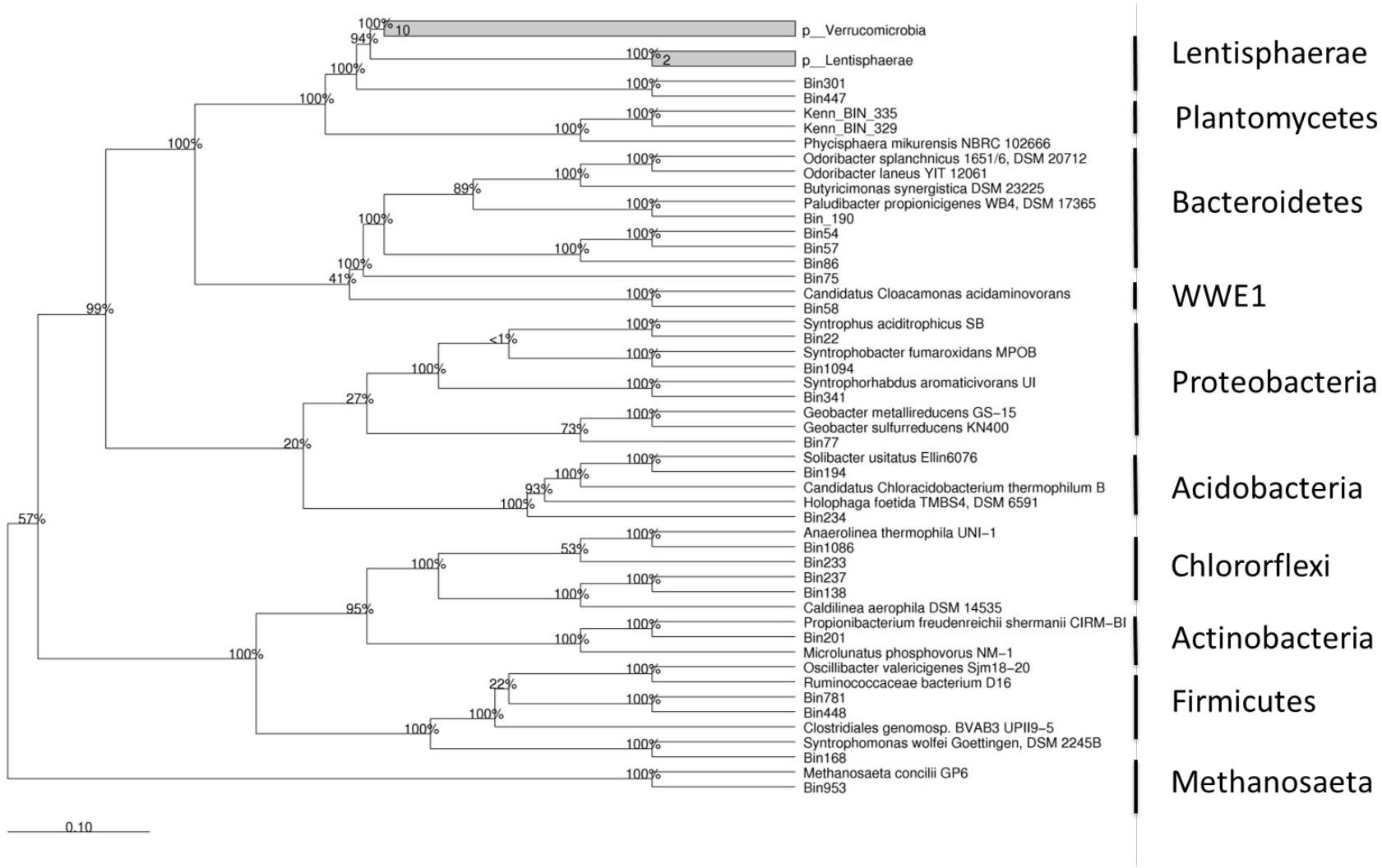


Figure 4.6 Phylogenetic tree generated based on protein sequences of good quality bins (labelled with bin number) from metagenomic data by FastTree. Bootstrap value at the branches indicates the confidence level with 100 replicates.

4.2.3.2 16S rRNA Gene Analysis from Metagenomic Data

16S rRNA analysis from metagenomic data retrieved 8,736 OTUs from total 152,618 reads from layered samples of brewery granule. Analysis of NMDS on layers from both pyrosequencing and metagenomic analysis generated less than 0.03 stress (Figure 4.7) indicating an excellent representation in 2 dimensions. Both NMDS plots showed that the outer layer was differentiated from the inner layers, whole granules and the core. This trend became clear when analysing the metagenomic result with high richness. In fact, the species distribution in NMDS formed a triangle shape with one corner (left top corner) affiliated with the outer layer and one corner (right top corner) affiliated to inner layers, the whole granule and the core. The formation of the corner is due to unique OTUs in those samples, and progressive differentiation across the different layers. The distance between the corner and sample, indicates that unique OTUs present at low abundance. There were 83 and 2,737 OTUs detected in the outer layer only with pyrosequencing and metagenomic analysis respectively. These unique OTUs had abundance in metagenomic analysis ranging from less than 0.001% to maximum 0.5% and were widely spread through around 50 different phyla including *Bacteroidetes*, *Firmicutes*, *Proteobacteria* etc. and recently discovered phyla including *OD1, OP1, TM7* etc.. The diversity of the outer layer was the highest in all granules (compared to subsequent layers) (Figure 4.8) with a Shannon index of 4 and 10 from pyrosequencing and metagenomic analysis respectively. The OTUs in the outer layer are unique compared with the whole granule community; there were 2,564 OTUs (data not shown) detected in the outer layer only, presented as spread dots at the top left corner of Figure 4.7A. The distribution of OTUs became denser in the region of all other layers which indicates that these OTUs including *Methanosaeta*, *Geobacter* etc., were shared among deeper layers but also dominated whole granule samples.

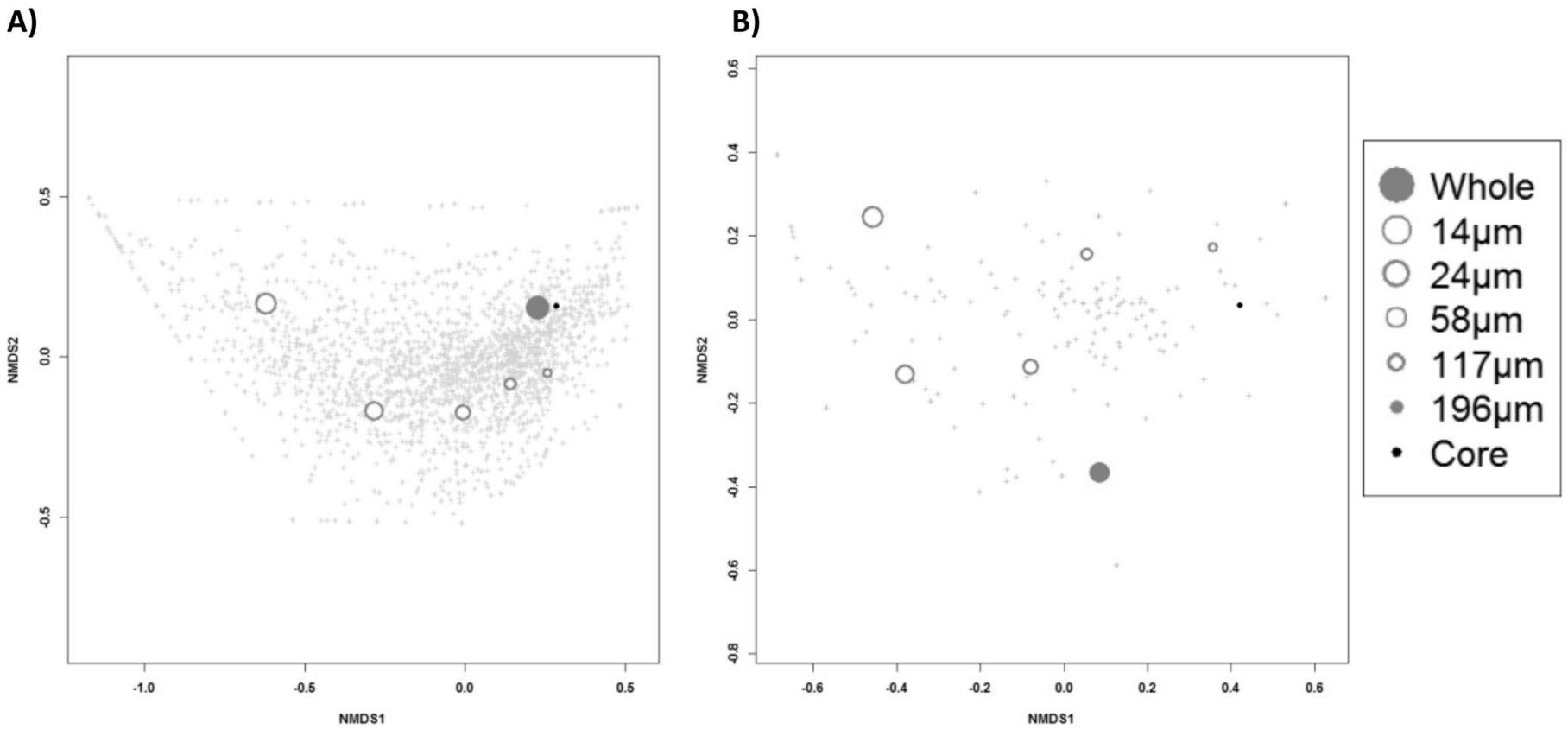


Figure 4.7 NMDS analysis of 16S rRNA from A) illumina shotgun metagenomic analysis and B) 454 pyrosequencing. OTUs are labelled as grey "+" in both graphes. Layer samples from outer to inner are distinguished by size of rings (larger as outer layer). Whole granules and the core are represented as filled circle and black circle respectively.

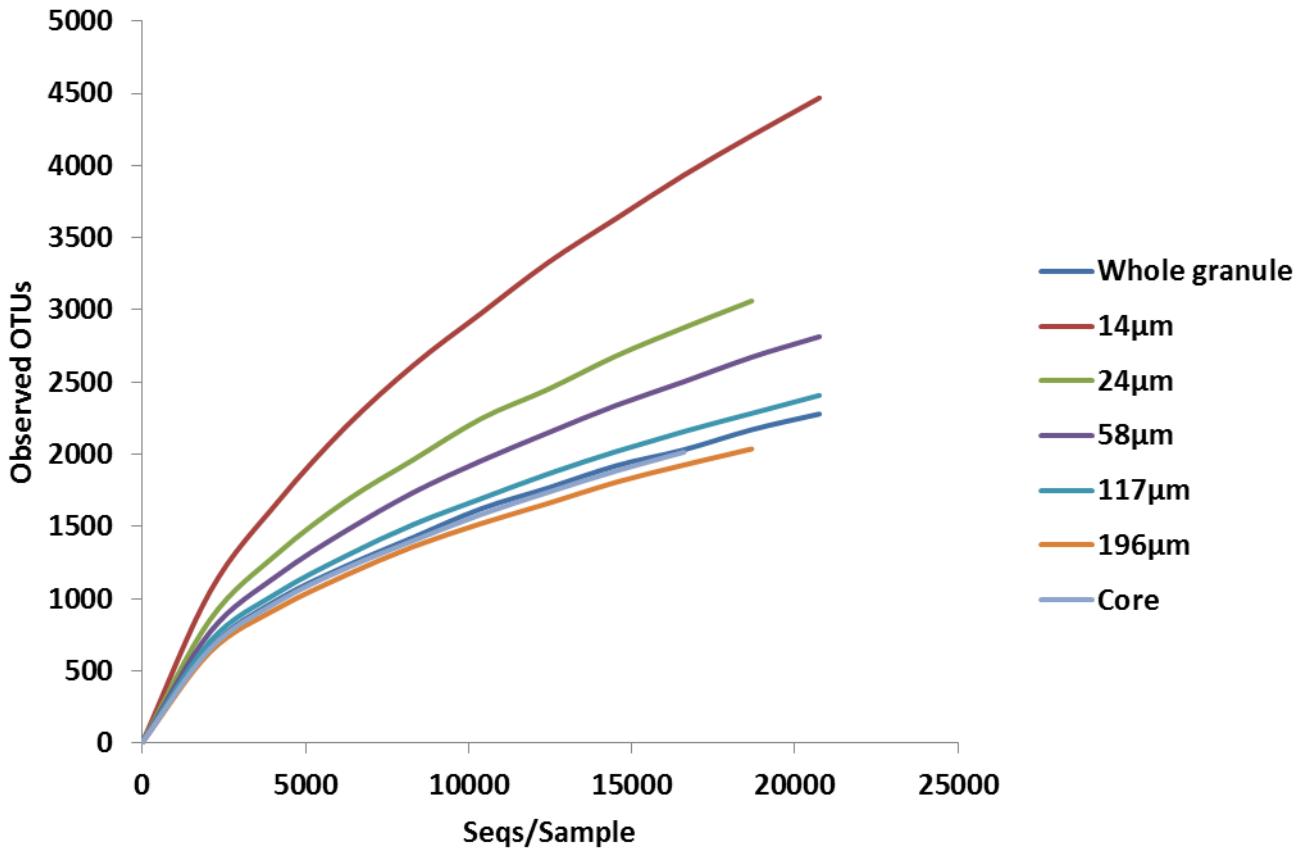


Figure 4.8 Rarefaction of each sample from metagenomic analysis on Brewery granules. Most OTUs are observed in the outer layer sample (14µm) than other samples after normalization.

PCA analysis based on pyrosequencing and metagenomic 16S rRNA gene data are shown in Figure 4.9. The proportion explained by first two PCs is similar between both methods and indicates that key differences are due to low richness of pyrosequencing. Major OTUs, which were consistently differentiated between outer layer and other samples, remained the same. Outer layers, which contained more *Bacteroidales* and *Candidatus Cloacamonas*, shifted to the left hand side and inner layers, which contained more *Methanosaeta* and *Geobacter*, shifted towards right hand side (Figure 4.9). These include key organisms decreasing in abundance towards the core such as *Candidatus Cloacamonas* and *Bacteroidales,* and those following an opposite trend such as *Geobacter* and *Syntrophomonas* (Figure 4.10). With higher sequencing effect, the dominance of *Anaerolinea* was found which was not identified by pyrosequencing.

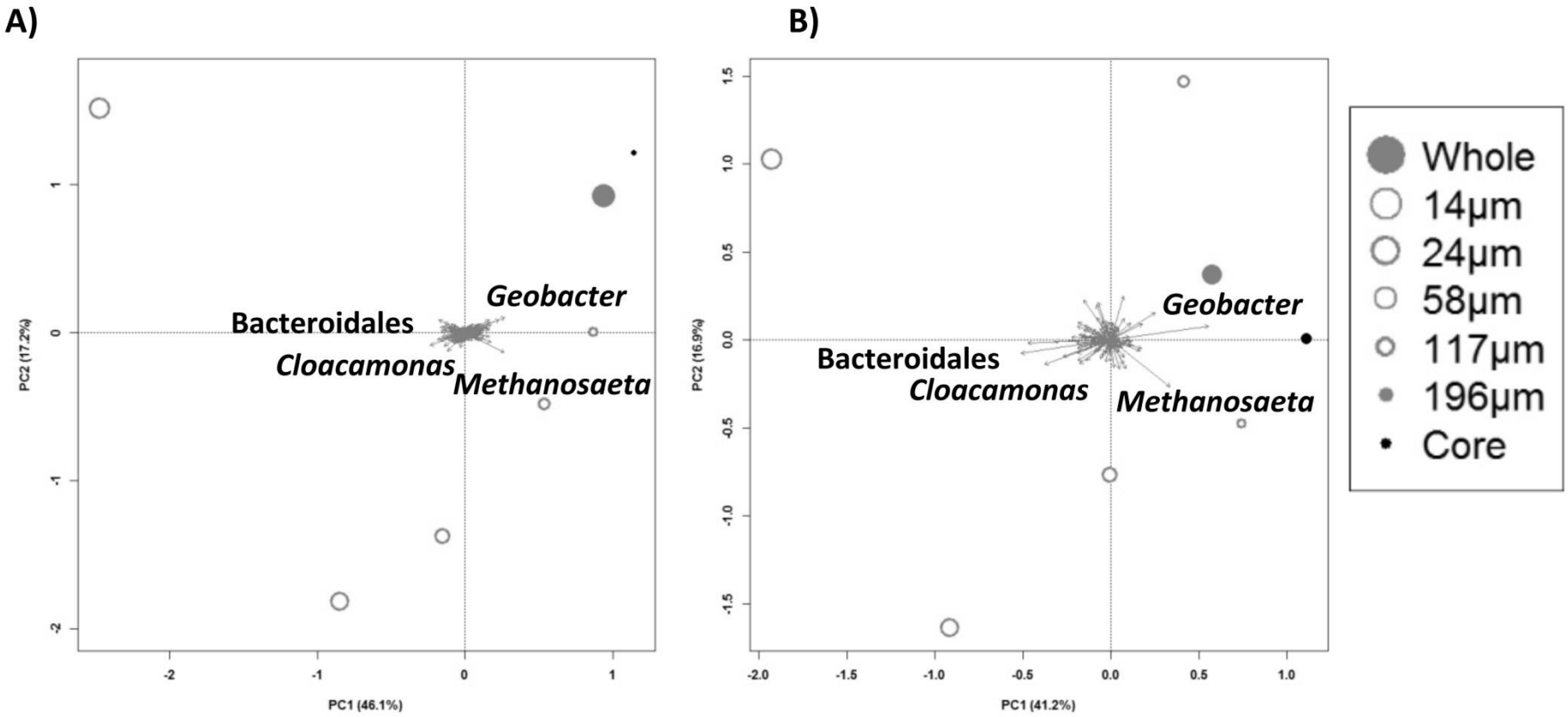


Figure 4.9 PCA analysis of 16S rRNA from A) illumina shotgun metagenomics and B) 454 pyrosequencing. Major differentiation OTUs are labelled with taxonomy. Layer samples from outer to inner are distinguished by size of rings (larger as outer layer). Whole granules and the core are represented as filled circle and black circle respectively.

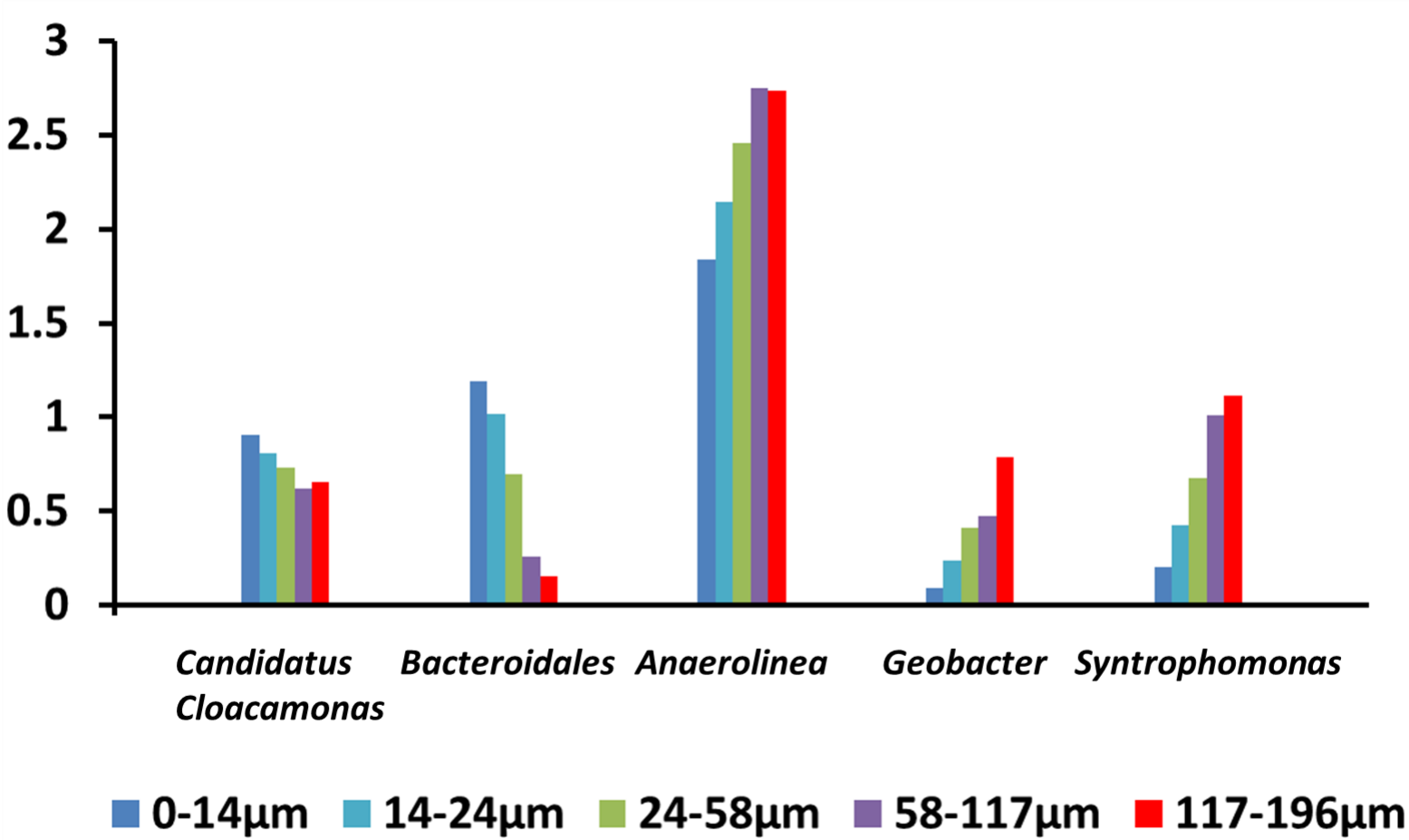


Figure 4.10 Major bacteria trend retrieved from 16S rRNA analysis of Metagenomic data. Representative OTUs are labelled with classification. The relative abundances of major bacteria are plotted according to the layers (indicated by different colours).

4.3 Discussion

4.3.1 Microbial Architecture

The traditional microbial architecture model (Guiot et al., 1992; Fang, 1995) of discrete communities in different layers can only be seen in brewery granules as a shift between two groups of presumed acetogens *Bacteroidales*/*Candidatus Cloacamonas*, and *Syntrophomonas*/*Geobacter*. The bacterial community was dominated by presumed fermenters in cannery and dairy granules, but with strong differentiation of the outer layers mainly in the cannery granules. Acetogenesis and methanogenesis appeared to be generally collocated through the whole granules rather than in sequential layers observed previously (Harmsen et al., 1996b; Batstone et al., 2004).

To illustrate general architecture and function, functionality and microorganism distribution is proposed in Figures 4.12 (and elsewhere in this discussion), where the x-dimension represents relative abundance (segregated into major phylogenetic or presumptive functional classifications), and the y-dimension represents granule depth.

4.3.1.1 Brewery Granules

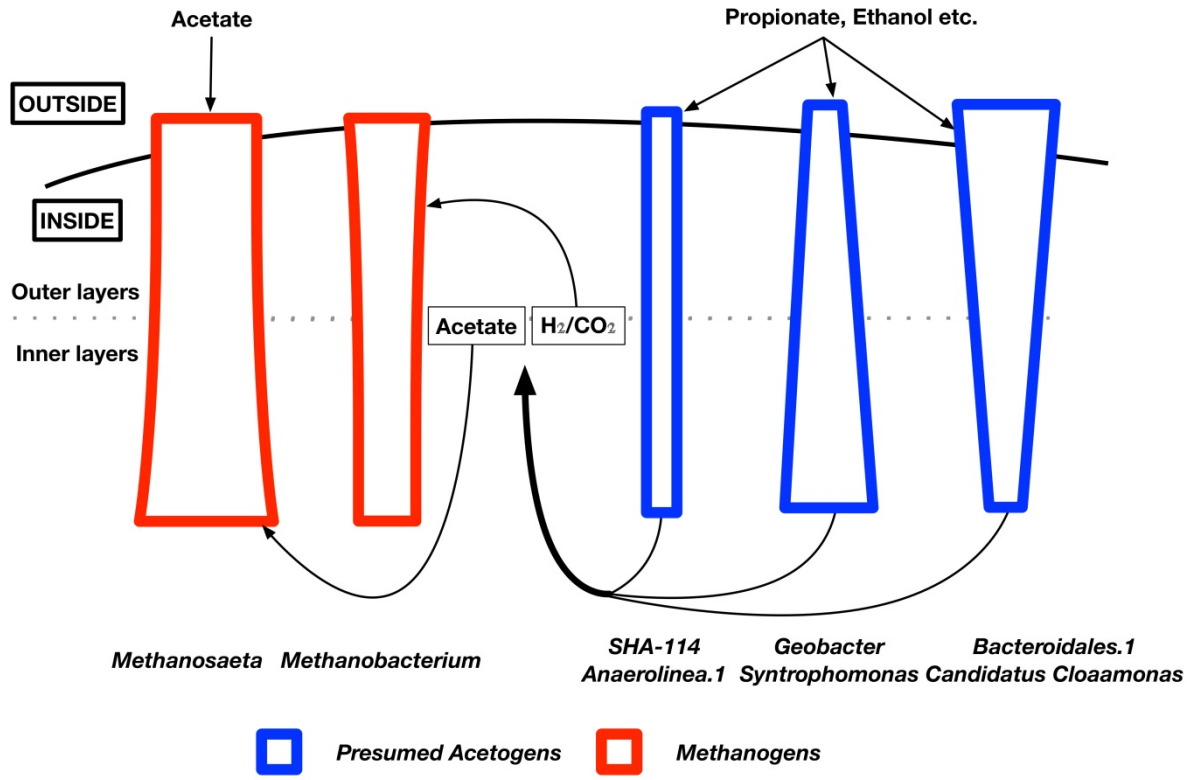


Figure 4.11 Proposed functionality and microorganism distribution in brewery granules. Arrows indicated the directions of substrate uptake and digestion products. Vertical dimension refers to depth. Horizontal dimension for each shape refers to relative abundance.

Methanosaeta dominated through the whole brewery granules (Figure 4.11). This archaea has high affinity to acetate (Berger et al., 2012) and is able to produce methane through acetoclastic methanogenesis. This indicates that acetate is supplied from both the bulk as well as by-products from acetogenesis (to the inner layers) carried out by two groups. The first group, which consisted of *Bacteroidales.1*, *Bacteroidales.2* and *Candidatus Cloacamonas*, was the main presumptive acetogen found on the outer layer. Based on *in silico* proteome study done by Pelletier et al. (2008), *Candidatus Cloacamonas* is able to gain energy from fermenting amino acid, sugar and certain aliphatic carboxylic acid (succinate, lactate and acetate), and also has the oxidative propionate degradation pathway to acetate and CO_2 under low H_2 pressure (Schink, 1997, Pelletier et al., 2008).

Bacteroidales is a highly diverse bacteria group in terms of phenotype and function. Commonly, they are recognised as a hydrolytic fermenter of carbohydrates in anaerobic habitats (Thomas et al., 2011; Rismani-Yazdi et al., 2013). Filamentous *Bacteroidetes* was

found in activated sludge and has the ability to take up glucose and propionate under aerobic conditions (Kragelund et al., 2008). *Proteiniphilum acetatigenes* belong to order *Bacteroidales* were isolated from propionate-degrading tri-cultures, consisting of *Syntrophobacter* and *Methanobacterium*, from a UASB treating brewery wastewater (Chen and Dong, 2005). It was proved that this specie is responsible for pyruvate and accelerates propionate degradation rate. *Bacteroidales* was more likely related to VFAs (except acetate) degradation as the major component in substrate of brewery granules were propionate, ethanol and acetate.

These organisms were completely replaced by another presumed acetogenic group including *Geobacter* and *Syntrophomonas* in the inner layers. This shift is possibly due to a shift in affinity with these organisms more able to degrade at lower concentrations, a focus on thermodynamically more limited substrates such as propionate, or better engagement with hydrogentrophic methanogens (e.g. propionate and butyrate, Pind et al., 2002). Both microorganisms appear to be associated with *Methanobacterium* (Figure 4.2A & A2) as hydrogen utilizer and possibly with *Methanosaeta* as acetoclastic methanogen throughout the granules. *Geobacter* is known to carry out oxidation of organic compounds to CO_2 (Nakasaki et al., 2013), and as anodic electro-active microbe in microbial fuel cells (Jia et al., 2013). A novel direct interspecies electron transfer was also discovered between *Geobacter* and *Methanosaeta* from anaerobic granules incubated in the lab-scale digesters fed with brewery wastewater (Morita et al., 2011; Rotaru et al., 2014). This supports its capability as a specialist in engagement with methanogenic electron accepting archaea. Other hydrogen-utilizing methanogens accounted for a small portion of the whole community possibly due to the low amount of H_2/CO_2 produced from acetogenesis.

High abundance of *Syntrophomonas* was generally detected with higher archaeal abundance in anaerobic reactors (Regueiro et al., 2012), engaging in obligate syntrophic propionate oxidation (Talbot et al., 2008). *Syntrophomonas* was also detected with high abundance in anaerobic sludge treating slaughterhouse wastewater with high fats level (Cammarota et al., 2013) and thought to be the major anaerobic bacterium oxidising butyrate and long-chain fatty acids to acetate and hydrogen through *β*-oxidation (Pereira et al., 2002).

4.3.1.2 Cannery Granules

Degradation of glucose (Figure 4.12) may follow a series of pathways including production of a mixture of organic acids by a mixed population of *Anaerolinea* and *Bacteroidales* (Sekiguchi et al., 2003); production of acetate and propionate by sub-dominant *Propionicimonas* (Akasaka et al., 2003); or production of acetate and lactate by *Bacteroidales.3* (Keyser et al., 2007). These microbes mainly accumulated in the outer layers of cannery granules (Figure C8 in appendix).

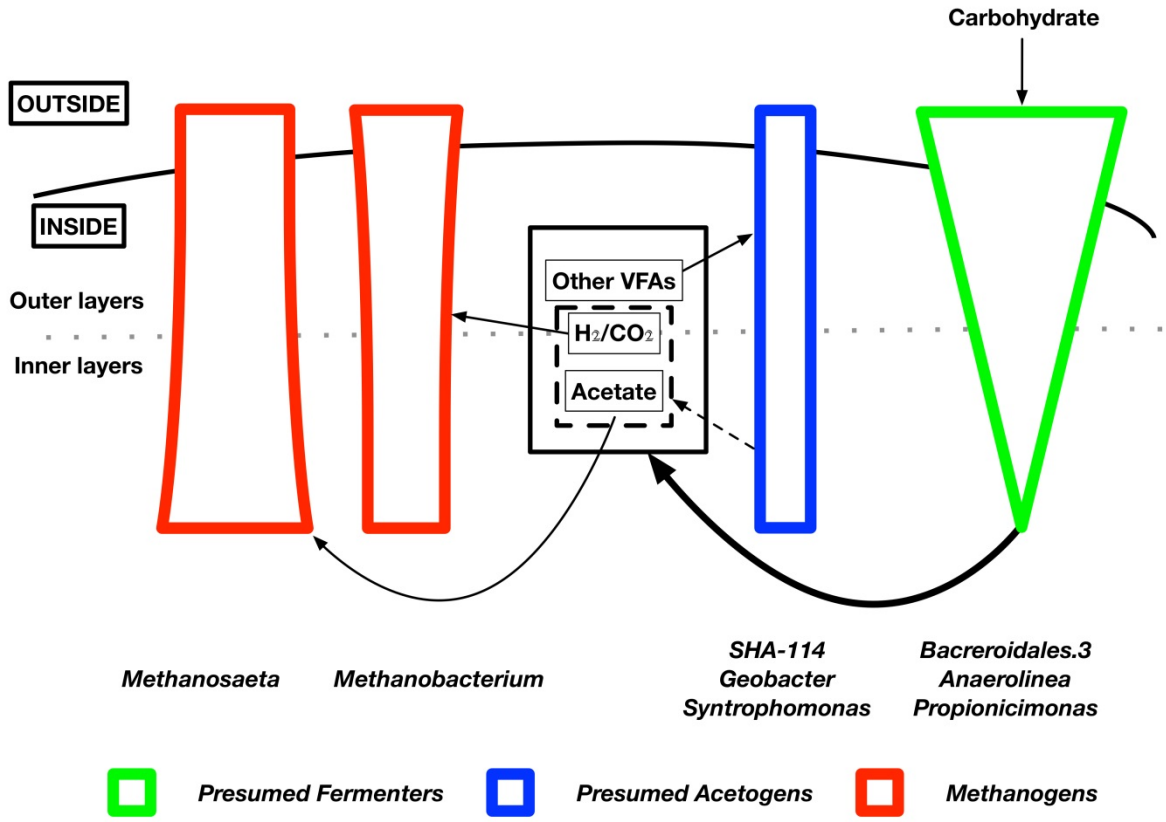


Figure 4.12 Proposed functionality and microorganism distribution in cannery granules. Arrows indicated the directions of substrate uptake and digestion products. Dotted arrow lines indicate the products from presumed acetogens. Vertical dimension refers to depth. Horizontal dimension for each shape refers to relative abundance.

Anaerolinea was first isolated from thermophilic granular sludge in an UASB reactor treating fried soybean-curd manufacturing wastewater (Sekiguchi et al., 2001). Growth of *Anaerolinea* is slow, but can be significantly stimulated by co-cultivation with hydrogentrophic methanogen (Sekiguchi et al., 2003). Carbohydrates are the favoured substrate with production of acetate, other VFAs and H_2/CO_2 (Ariesyady et al., 2007). Genus

Propionicimonas was detected in anaerobic sludge from a full-scale UASB reactor treating domestic wastewater (De Lucena et al., 2011) and identified as a major glucose-fermenting bacterium in a full-scale enhanced biological phosphorus removal plant by stable isotope probing (Nielen et al., 2012). The key species in this genus are *Propionicimonas paludicola* isolated from plant residues in irrigated rice-field soil (Akasaka et al., 2003) and *Propioniciclava tarda* isolated from a methanogenic reactor treating waste from cattle farms (Sugawara et al., 2011). Both species grow at an optimum of 35°C and produce acetate and propionate from glucose.

Geobacter and *Syntrophomonas* were distributed evenly through the layers of cannery granules. They are possibly the major acetogens and producing acetate for *Methanosaeta* and H_2/CO_2 for hydrogen-utilizing methanogens. The archaeal community shifted to a co-dominance of *Methanosaeta* and hydrogen-utilizing methanogens including *Methanobacterium* and *Methanoregula* with a higher relative proportion of hydrogentrophic methanogens:acetogens compared to the brewery granules. This is likely related to the H_2 flux from glucose, which provides a more diverse and abundant hydrogen utilising methanogenic community as compared to the brewery granules (where H_2 is only derived from acetogenesis). *Methanobacterium* and *Methanoregula* are hydrogentrophic methanogens. Some *Methanobacterium* (such as *Methanobacterium congolense* etc.) can only utilize H_2/CO_2 (Cuzin et al., 2001), while others in both genus (such as *Methanobacterium formicicum*, *Methanoregula boonei*) can utilize formate as well (Schauer & Ferry, 1982; Brauer et al., 2010). They are fed by both fermentation of glucose and further acetogenesis throughout the granules.

4.3.1.3 Dairy Granules

As the waste was milk based, the feed for dairy granules was a mixture of protein (largely milk proteins such as casein), and milk sugars, particularly lactose. *Anaerolinea.1* dominated the bacterial community throughout the depth (Figure 4.13). Sekiguchi et al. (2003) also reported that with casamino acid or tryptone, weak growth of *Anaerolinea* can be observed. However, the high relative abundance of *Anaerolinea.1* may suggest that the growth relies on its favoured substrate – carbohydrate rather than protein.

Genus *Phycisphaerae* contains the only specie *Phycisphaera mikurensis* isolated from a marine alga. The degradation of proteins such as starch, casein or gelatine is not observed

with this specie and only carbohydrates such as glucose or fructose are utilized. *Bacteroidales* was also an abundant bacterium, particularly growing on the outside. Abundance of *Bacteroidales* was found to be increased when casein were added to a biogas reactor treating manure (Kampmann et al., 2012). *Bacteroidetes* also dominated the microbial community during the digestion of bovine serum albumin in anaerobic reactors with synthetic wastewater (Tang et al., 2005). This suggests these organisms can ferment protein under anaerobic conditions.

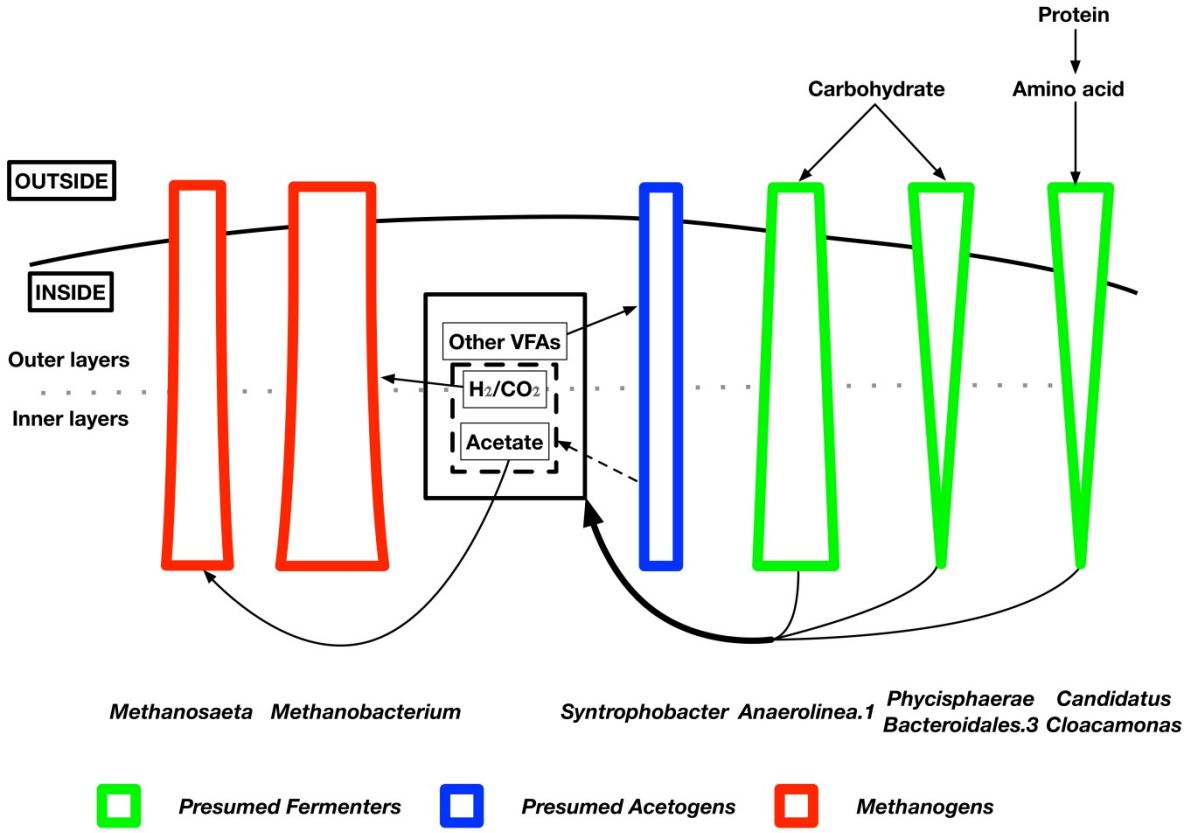


Figure 4.13 Proposed functionality and microorganism distribution in dairy granules. Arrows indicated the directions of substrate uptake and digestion products. Dotted arrow lines indicate the production from presumed acetogens. Vertical dimension refers to depth. Horizontal dimension for each shape refers to relative abundance.

Abundance of *Candidatus Cloacamonas* was relatively low throughout the dairy granules. It was reported that *Candidatus Cloacamonas* can ferment lysine (Pelletier et al., 2008). Through a metagenomic study on anaerobic digester of a municipal treatment plant, Perret et al., (2011) proposed *Candidatus Cloacamonas* ferments lysine through a variant route

involving 3-aminobutyryl-CoA aminotransferase. Thus *Candidatus Cloacamonas* is possibly associated to acetogenesis of amino acid.

Syntrophobacter was the only acetogen detected in dairy granules and presented at low abundance. Members of *Syntrophobacter* are generally recognized as propionate oxidizer by the methylmalonyl CoA pathway (Houwen et al., 1990; Plugge et al., 1993). *Syntrophobacter sulfatireducens* was also isolated from two mesophilic UASB treating brewery and bean curd wastewater respectively (Chen et al., 2005) and identified as a propionate oxidizing syntrophic bacterium.

Interestingly, the dominant methanogen in dairy granules was *Methanolinea* instead of *Methanosaeta*. This is a major issue, since it indicates a major proportion of the methane is coming from hydrogen rather than acetate. One possibility could be promotion of acetate oxidation through increased ammonia levels (inhibiting the growth of *Methanosaeta*, Calli et al., 2005; Karakashev et al., 2005) while retaining hydrogentrophs, but ammonia levels here are relatively low (<1000 $mgN \cdot L^{-1}$), and the dominance of hydrogentrophs may be due to different factors that may require more directed analysis to investigate.

4.3.1.4 Comparison to Previous Analysis

As identified in the introduction, previous analysis of methanogenic and acetogenic communities based on cryosection-FISH identified fermenters in the outer layer are thought to degrade organic compound in primary substrates and produce VFAs and H_2 etc. that are consumed by syntrophic bacteria and methanogens in the inner layers. Acetoclastic methanogens and syntrophs dominate the inner layer while the outer layer comprises a diverse array of fermentative bacteria (Harmsen et al., 1996, Sekiguchi et al., 1999, Batstone et al., 2004). The core may be inactive depending on the size of the granule, and structure may become more homogeneous where the primary substrate is slowly degradable (Batstone et al., 2004).

This chapter extends this microbial architecture analysis with high-resolution molecular analysis on separated layers of anaerobic granules. With FISH on cryosectioned granules, an inactive core without binding to any probes was commonly observed in all granules (e.g. Figure 4.2B2). Within the regions observed, micro-colonies of *Methanosaeta* were commonly found inside the granules as described by Sekiguchi et al., (1999). A distinct

bacterial outer layer can be distinguished on cannery and dairy granules but not on brewery granules. Similar result was reported by Batstone et al., (2004). However, the presence of archaea (e.g. *Methanosaeta* in Figure 4.2B1 or *Methanolinea* in Figure 4.2C1) on the outer layer can be observed under high magnification.

Based on the identification from pyrosequencing results, FISH probes can be identified to target dominant bacteria (especially the presumed fermenters) including *Candidatus Cloacamonas* and *Bacteroidales*/*Anaerolinea*. The dominance of these microbes in the outer layers can be observed which were also shown in molecular analysis. Similar result was only reported by Sekiguchi et al., (1999) and Sekiguchi et al., (2001) on *Chloroflexi* detected on the surface of thermophilic methanogenic granules fed with sucrose and low-molecular-weight fatty acids and treating fried soybean curd-manufacturing wastewater.

The molecular analysis on separated successive layers explores the microbial architecture under high resolution and shows that methanogens dominating the anaerobic granules. Fermentation, acetogenesis and methanogenesis may happen parallel with some functions dominating over others in specific layers. Bacterial community is much diverse and whether shifts between two groups of presumed acetogens (e.g. from *Bacteroidales* and *Candidatus Cloacamonas* to *Geobacter* and *Syntrophomonas* in brewery granules) or dominated by presumed fermenters (e.g. *Anaerolinea* in cannery and dairy granules) with other microorganisms (such as *Bacteroidales.3* and *Phycisphaerae*) varying between different layers. The presumed fermenters penetrate deep in the granules with complex substrate (e.g. carbohydrate or protein).

4.3.2 Microbial Differences across Granules

Apart from structural differences, the main differences in community between the different granules were generally within major phyla, and mainly differentiation in the bacterial (and presumptively fermenters) community. Between cannery and dairy granules, carbohydrate consumers such as *Anaerolinea* in phylum *Chloroflexi* are shared due to the presence of carbohydrate in both substrates. Between brewery and cannery granules, the shared phyla are mainly presumed acetogens including *Geobacter* in *Proteobacteria* and *Syntrophomonas* in *Firmicutes*, as the acetogenesis of VFAs are the major process in both granules. Phylum *Bacteroidetes* is shared between all granules due to their highly diverse capability. It is

important to note that *Bacteroidetes* presents consistently in the outer layers, indicating that they occupy a niche as primary fermenters. This is consistent with previous observations of granules from highly loaded food processing treatment systems (Narihiro et al., 2009). Novel unique phyla were frequently observed in the granules examined. However all unique phyla are uncultured (e.g. FCPU426 in dairy granules), and thus their actual ecophysiology remains unknown.

4.3.3 Comparison of Pyrosequencing and Metagenomic 16S rRNA Analysis

In the metagenomic analysis, 16S rRNA gene reads accounted for less than 0.01% of total reads. It generated 3 times more reads but identified 30 times more unique OTUs compared to pyrosequencing. The relative abundance of most dominant OTUs, which affiliated to *Methanosaeta*, dropped from 47-60% (pyrosequencing) to 5-7% (metagenomic). However, the dominance of *Methanosaeta* through the whole brewery granules remained. It may suggest that *Methanosaeta* were overestimated by pyrosequencing, indicating possible PCR bias affecting pyrosequencing. The opposite result was observed by Pinto *et al.,* (2012), with *Archaea* being under-represented against bacteria by separated bacterial and archaeal primers targeting V3-V5 region. The primers used in this thesis target V8 region and are universal (although there could be base-pairing exceptions in one or more lineages (Hugenholtz and Goebel, 2001)). Due to the higher sequence conservation in V8 region (Youssef et al., 2009), dominant OTUs can results a higher relative abundance (Engelbrektson et al., 2010). Despite this possible bias, the overall analysis, including microbial architecture shifts in terms of abundance of major microbes and major factors differentiating layers remains, validating the outcomes from pyrosequencing analysis.

Although the metagenomic analysis provided a deeper view on the microbial community than pyrosequencing and had minimum bias from PCR, there are several drawbacks or concerns on 16S rRNA analysis from metagenomic analysis.

First of all, pyrosequencing generates above 250bp per read. All pyrosequencing reads trimmed to 250bp for consistency prior to taxonomic analysis. However, the average read length in metagenomic analysis with Illumina platform is around 100bp, which is still appropriate for accurate taxonomy characterization (Liu et al., 2007). Read length can

possibly extended by newer platform (Hiseq 2500 produce 2x150bp) or merging paired-end reads (Rodrigue et al., 2010).

Metagenomic 16S rRNA reads comes from random regions of the 16S rRNA gene. Since each region of the 16S rRNA gene has different evolutionary rates (Hills and Dixon, 1991), reads from different regions (one from conserved and one from variable region) of the same 16S rRNA gene may be classified as different taxonomy. It was noted that, within the top 10 OTUs, 1 OTU in pyrosequencing analysis but 4 different OTUs in metagenomic 16S analysis were classified as *Methanosaeta*. It is possible that these OTUs affiliated to the same *Methanosaeta* if the 4 OTUs in metagenomic 16S analysis came from different region of 16S gene. In addition, the OTUs picking of metagenomic 16S analysis was done with closed reference databases that novel microorganism are ignored. Programs, such as EMIRGE (Miller et al., 2011) or Amplishot (https://github.com/ctSkennerton/Amplishot)**,** are developed to extract specific region from metagenomics or even *de novo* reconstruct the whole 16S rRNA genome.

Chapter 5. Impact of Primary Substrate Type on Development and Structure of Acidogen Layer on UASB Granules

5.1 Introduction

Anaerobic granules are macroscopic self-supporting biofilms that occur in high-rate anaerobic reactors. The microbial community structure of anaerobic granules consists of three major groups: fermenters, acetogens and methanogens. These microorganisms, due to their functional and substrate specificity, are spatially differentiated in anaerobic biofilms, including granules. Fermenters are key groups that carry out fermentative conversion of primary soluble substrates. The speed of the primary fermentation has been identified as a controlling factor in determining overall structure (Batstone et al., 2004). Fermenters are the major microbial clades presenting on the surface of the granule (Sekiguchi et al., 1999; Batstone et al., 2004; Satoh et al., 2007), and hence determining surface properties such as shear strength, microscopic texture, and density (Forster, 1991; Quarmby and Forster, 1995). A better understanding of the fermentative community offers the potential to enhance granule strength, density, functionality, and activity.

Compared to methanogens and acetogens, very limited work has been done on fermenters, particularly in granules (Sundh et al., 2003; Chackhiani et al., 2004). Functionality of fermenters (in terms of substrate capability) is diverse, due to the broad range of complex mono- and polymers, and phylogeny is also broad. Bacteria from the phyla *Chloroflexi* (Sekiguchi et al., 2003), *Firmicutes* (Song & Dong, 2008) and *Proteobacteria* (Ramons et al., 2010) were commonly identified from anaerobic granules and presumed as fermentative bacteria based on alignment to previous known fermenters. The microbial ecology of fermenters was thought to be closely related to the substrate content (Batstone et al., 2004). Satoh et al. (2012) monitored the distribution of radio-labelled substrate by beta-imaging and chemical profile by microsensor on single granules and concluded that *Chloroflexi* and *Smithellla spp.* are responsible for glucose fermentation and propionate oxidation respectively on granules grown on skim powdered milk. Due to the difference in operating

parameters (such as reactor volume, HRT or inoculum) and granule properties (strength and community), comparative studies have not been widely done.

This chapter aims to investigate the influence of differential formation of fermenters on community structure and particularly shear strength in a highly controlled comparative manner. Fermentative layers were grown on granules collected from the brewery digester (i.e., with a largely acetogenic functionality) by feeding synthetic carbohydrate and protein medium respectively and characterised by the combination of shear method (developed in Chapter 3), pyrosequencing and FISH.

5.2 Results

5.2.1 Reactor Performance

The reactors were operated for 52 d with relatively continuous feed during this period. They were cleaned on day 12 and day 46 to remove biofilm from the reactor wall that was causing volume and substrate loss. There was high COD load due to failure of the medium feed pump tube on day 25 (i.e. only substrate, not media was fed on that day). All three reactors received an average of $2 gCOD \cdot L^{-1} \cdot d^{-1}$ for other times and achieved high COD removal (>90%). Further details and operational results are summarised in Table D1-D3.

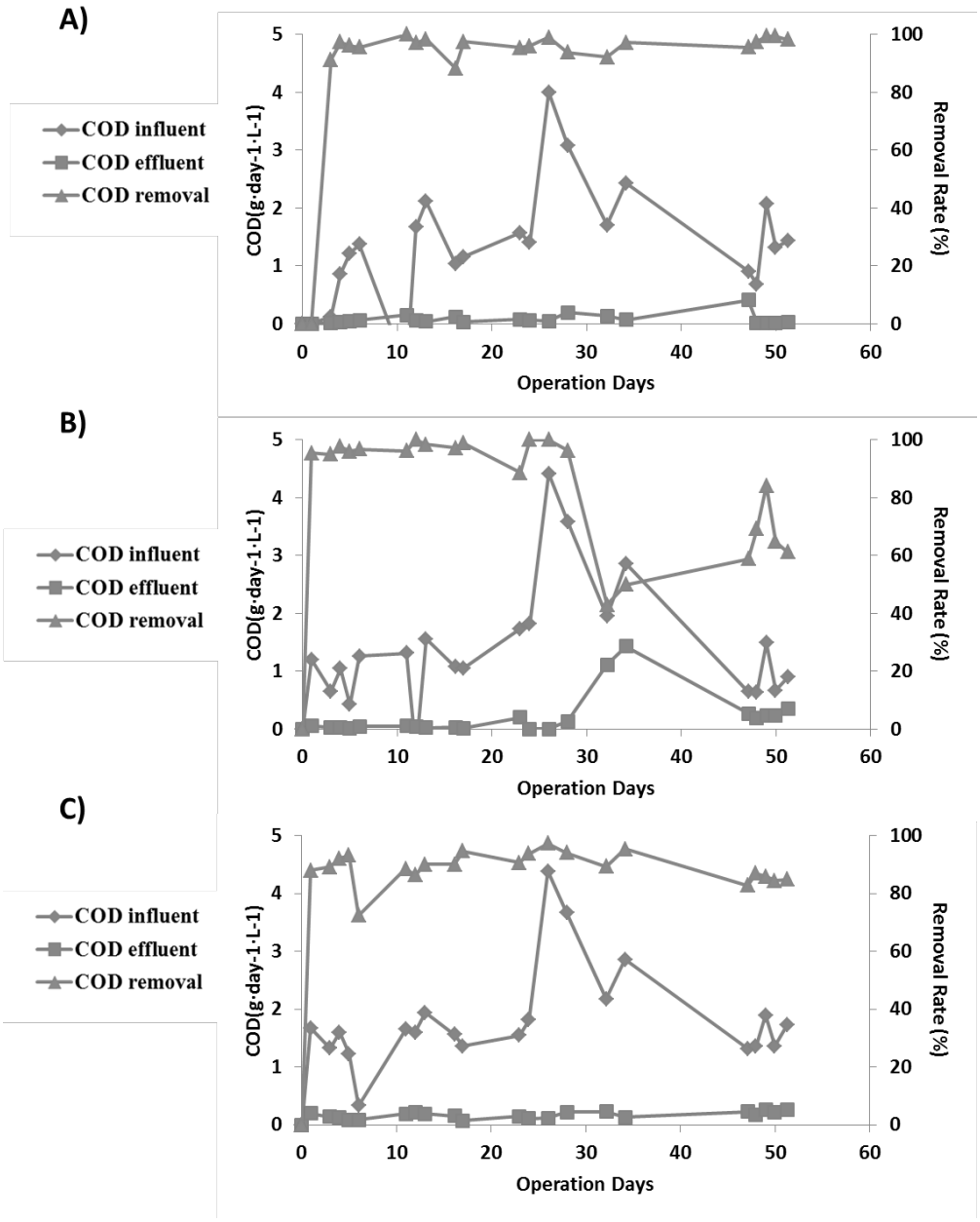


Figure 5.1 COD feed and effluent concentrations, and COD removal of A) control reactor, B) glucose reactor and C) gelatine reactor.

5.2.2 Shearing

All granules were black with a smooth surface. Granules were not significantly different in size, with an average diameter of 0.14mm for control granules with standard deviation of 0.03; 0.13mm for cannery granules with standard deviation of 0.03; and 0.15mm for gelatine granules with standard deviation of 0.02. The fines generated versus shear rate squared (γ^2) is shown in Figure 5.2. A lower (more negative) K_C, equates to a weaker granule (i.e., faster generation of fines). Gelatine granules was the slightly weaker (with lowest Kc=-5E-07s^2)

than other types of granules (with Kc=-4E-07s^2). The amount of solids measured, together with the measured density allowed calculation of depth sheared as shown in Table 5.1. Granules did not have significantly different strengths (p=0.47) based on ANCOVA of all data (mixed categorical-continuous ANCOVA against shear rate and granule source).

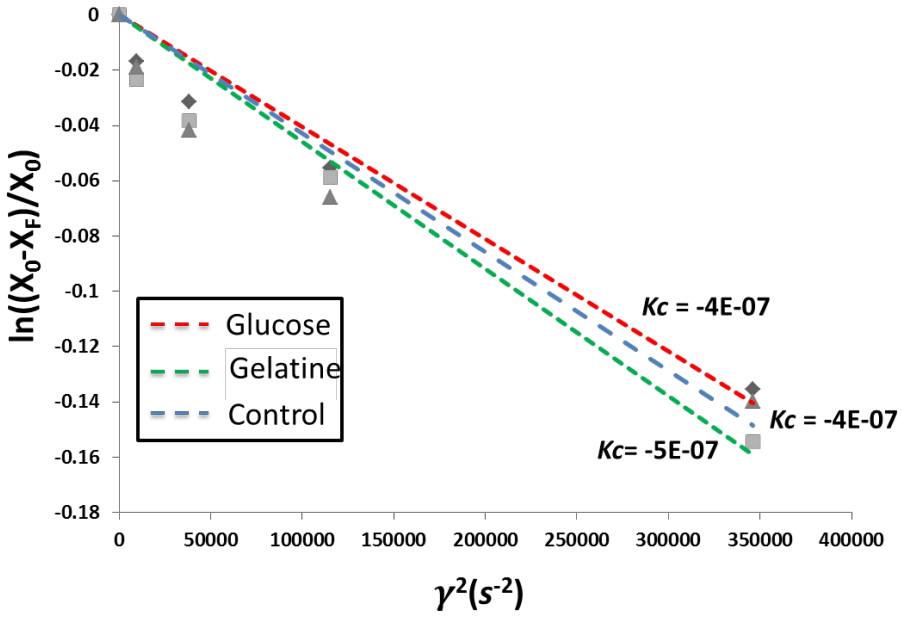


Figure 5.2 Estimation of characteristic abrasion coefficient (K_C). K_C for granules from lab-scale reactors with different feedstock, including glucose, gelatine and control were estimated according to the method described in materials and methods section. A lower K_C equates to a weaker granule.

Table 5.1 Shear depth (µm) of three types of anaerobic granules at each time point. Shearing was done at 1500 rpm.

Time (minutes)	Depth (µm)		
	Control	Glucose	Gelatine
5	4	4	6
20	13	10	15
60	26	20	27
180	51	43	57
270	73	59	86

5.2.3 Shift in Microbial Community – Outer Layers

A PCA plot demonstrating shift in population across layers and granules is shown in Figure 5.3. The largest shifts were the outer layers in glucose (blue dashed circle) and gelatine (green dashed circle) granules indicating that quantitatively distinguishable outer layers for gelatine and glucose granules were developed, particularly associated with 3-4 key OTUs in each case.

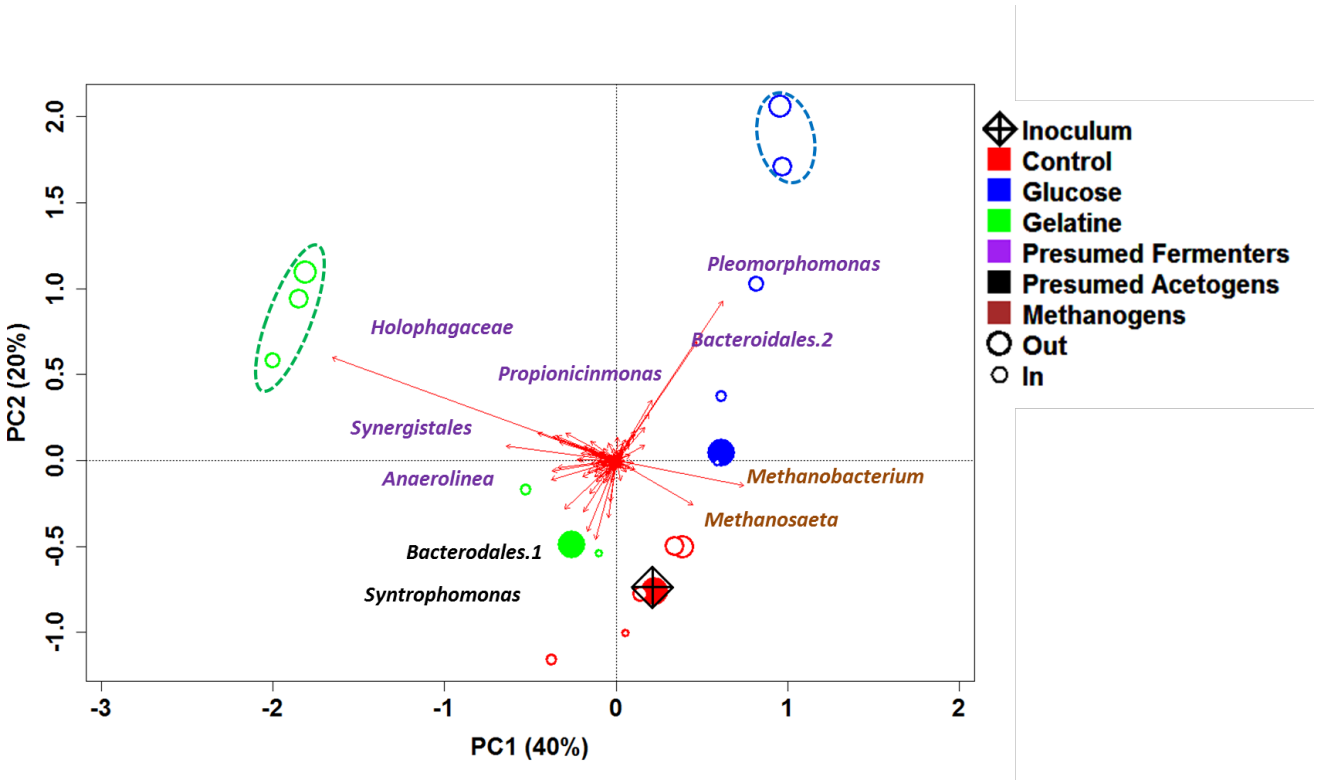


Figure 5.3 PCA biplot based on OTUs recovered to illustrate the differences between inoculum, harvested granules and sheared layers of each type of granules. Different types of granules were separated by colour. Layer samples from outer to inner were distinguished by size of rings (larger as outer layer). Whole granules were represented as filled colour circle and black circle respectively. Microorganisms were separated by colour based on the presumed functionality.

Layers from the control granules clustered with the initial inoculum sample due to the dominance by archaea including OTUs affiliated to *Methanosaeta* and *Methanobacterium*. The major factor differentiating glucose granules, especially those on the outer layer (0-10μm) were OTUs classified as genus *Pleomorphomonas*, *Propionicimonas* and order *Bacteroidales* (referred to *Bacteroidales.2* in Figure 5.3 and Figure C5). The layers from gelatine granules

formed two clusters. The outer layer group contained 0-27μm regions dominated by OTUs identified as family *Holophagaceae* (Figure C6) and order *Synergistales*. The inner layer group was mainly differentiated from other granules by OTUs affiliated to genus *Syntrophomonas*, *Anaerolinea* and one species in order *Bacteroidales* (refer to *Bacteroidales.1* in Figure 5.3).

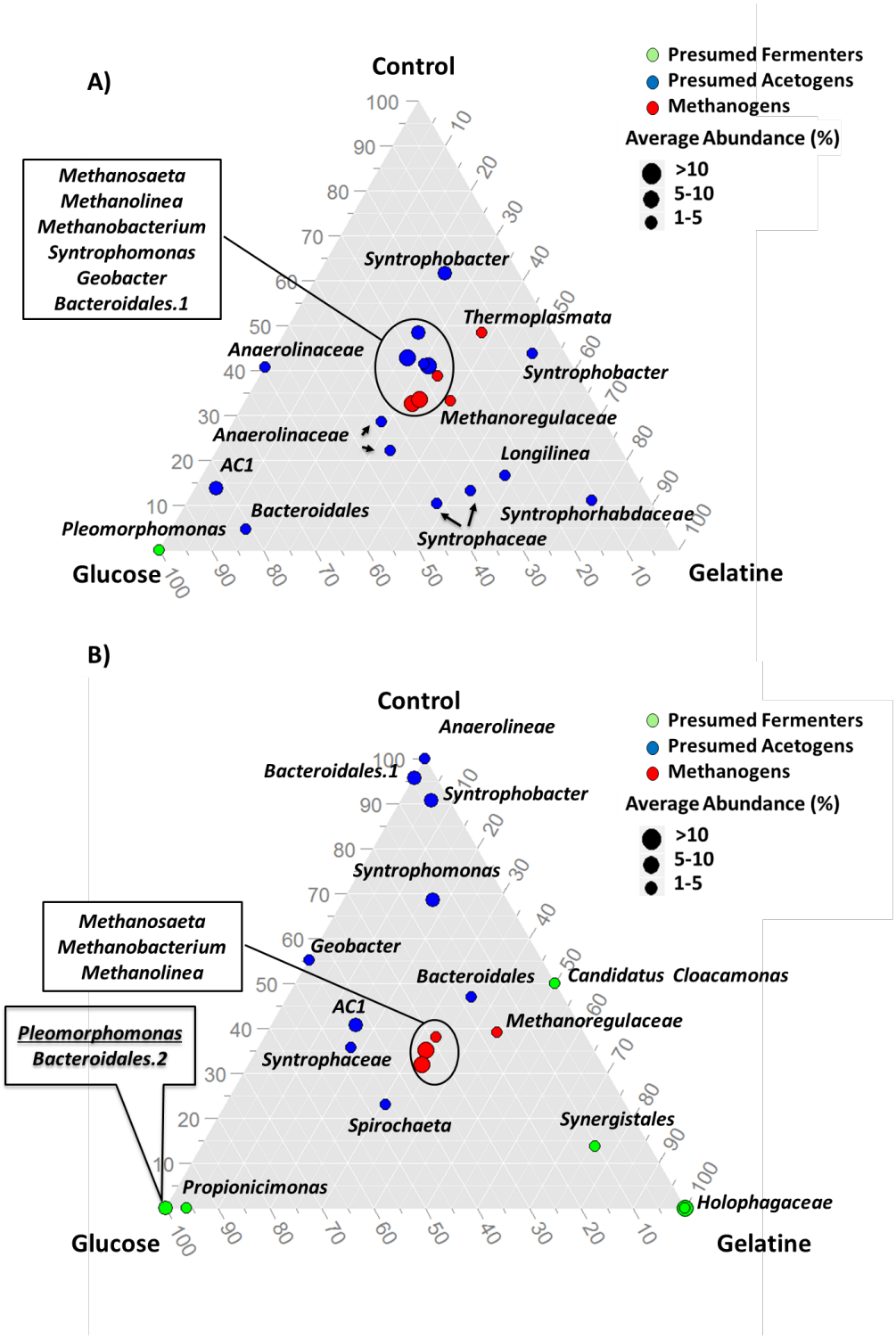


Figure 5.4 Ternary plots show the distribution of presumed fermenters (green circle), presumed acetogens (blue circle) and methanogens (red circle) among the whole granules samples (A) and the outer layer samples (B) of stimulated granules and control based on pyrosequencing results. Size of each point was adjusted regarding to the average abundance of each microorganism in three types of granules. OTUs with average abundance lower than 1% in three samples were excluded.

Within the microbial community of whole granules (Figure 5.4A), the shared OTUs among granules were mainly presumed acetogens or methanogens such as high average abundance OTUs (>10%) affiliated to *Syntrophomonas*, *Geobacter*, *Bacteroidales.1*, *Methanosaeta* and *Methanolinea* among all granules, low average abundance OTUs (1-5%) affiliated to *Anaerolinaceae* between control and glucose granules, *Syntrophaceae* between glucose and gelatine granules or *Syntrophobacter* and *Thermoplasmata* between control and gelatine granules. The only presumed fermenters can be predicted within the bacterial community of whole granules were the unique OTU in glucose granules affiliated to *Pleomorphomonas.* Differences of presumed fermenters can only be seen by examining the microbial community in the outer layers of granules (Figure 5.4B). The outer layers of glucose and gelatine granules contained a large group of highly differentiated organisms. Specifically, *Holophagaceae* dominated the outer layer of gelatine granules and genus *Pleomorphomonas* dominated the outer layer of glucose granules. Methanogens shared among three granules remained. Presumed acetogens with medium average abundance (5-10%) including *Bacteroidales.1*, *Syntrophobacter* and *Syntrophomonas*, were detected in the outer layer of control granules. OTUs with low average abundance (1-5%) affiliated to *Geobacter* (presumed acetogen) and *Candidatus Cloacamonas* (presumed fermenter) shared between control/glucose granules and control/gelatine granules respectively.

Microscopic images, and alignment to layer based pyrosequencing are shown in Figure 5.5. This also demonstrates microscopically the absence of a bacterial outer layer on inoculum granules and control granules as shown in Figure 5.5A1 and 5.5B1. In comparison, bacterial layers (shown in red) were found on both glucose granules (Figure 5.5C1) and gelatine granules (Figure 5.5D1) but with quite different morphology. Bacteria formed a thin layer in glucose, while they were in discrete colonies on the surface of gelatine granules. The distinguishable bacterial community shift from outer to inner layers of granules can be seen in both glucose and gelatine granules based on pyrosequencing results. Those decreasing in abundance from outer towards core were OTUs affiliated to family *Holophagaceae* in gelatine granules (Figure 5.5D2) and OTUs affiliated to order *Bacteroidales* (refer to *Bacteroidales.2*) and genus *Pleomorphomonas* in glucose granules (Figure 5.5D2). OTUs classified as genus *Geobacter, Syntrophomonas*, order *Bacteroidales* (refer to *Bacteroidales.1* in Figure 5.5C2 and 5.5D2) and phylum *AC1* were found mainly in the inner layer of both granules. The bacterial community of control granules was relatively simple with few bacteria dominating through whole granules including OTUs closely related to genus

Geobacter, Syntrophobacter, Syntrophomonas, class *Anaerolineae*, order *Bacteroidales* (refer to *Bacteroidales.1* in Figure 5.5B2) and phylum *AC1*. The archaeal community was much simpler with *Methanosaeta* and *Methanobacterium* distributed evenly through each granule (Figure 5.6). More PCA analyses on individual type of granules are shown in the Appendix (Figure C4-C6).

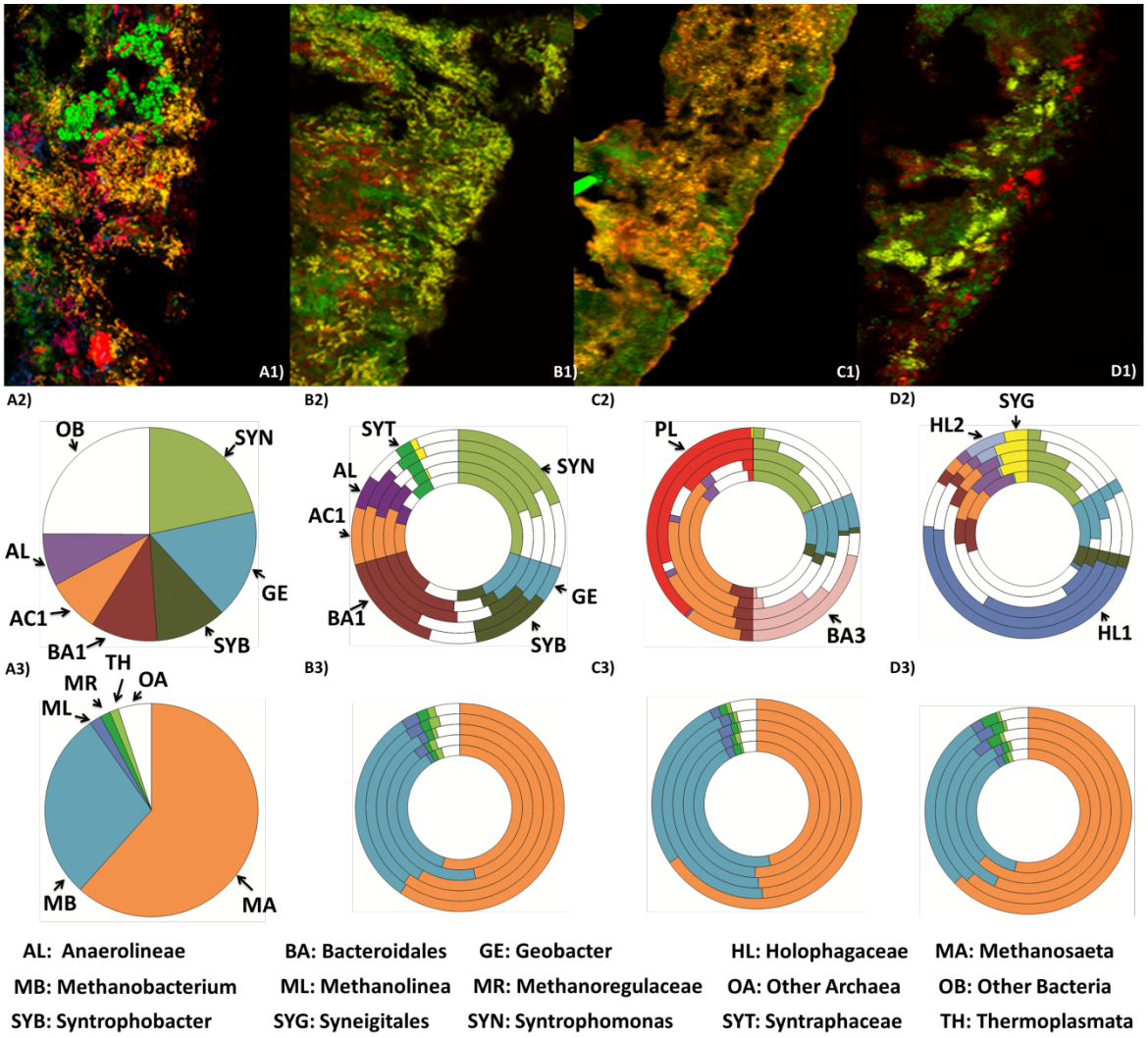


Figure 5.5 Cryosection FISH image shows the distribution of archaea (*ARC916*) in green, Methanosaeta (*MX826*) in yellow and bacteria (*EUBmix*) in red (image set 1) and relative abundance of bacterial community shift (graph set 2) and archaeal community shift (graph set 3) of inoculum (image set A), control granules (image set B), glucose granules (image set C) and gelatine granules (image set D).

5.3 Discussion

5.3.1 Development of Fermentative Layer

Microbial community differentiation among the outer layer of control and stimulated granules was mainly driven by the primary substrate. Both PCA and ternary plot on bacterial community of outer layer indicated that unique fermentative layers were developed on the surface of granules including major bacterial OTUs in genus *Pleomorphomonas* when fed with glucose and family *Holophagaceae* when fed with gelatine. Neither microbes were seen (in substantial numbers) in either the inoculum or in previous chapters in this thesis. The inner layers of stimulated granules were not affected substantially by the substrate. Acetogens and methanogens, including key OTUs affiliated to *Syntrophomonas, Methanosaeta* and *Methanobacterium*, dominated the inner layer of all three granules and seemed largely derived from the inoculum. Kovacik et al., (2010) found that with short term perturbations (substrate change from complex to simple feedstock), the microbial diversity of anaerobic granules is mainly related to changes in bacterial community while *Methanosaeta* and *Methanobacterium* remain as dominant archaea with a shift in abundance. However, the results strongly contrast with those in Chapter 4. The archaeal community was replaced with *Methanolinea* in dairy granules, and *Methanobacterium* and *Methanoregula* (to a lesser extent) in carbohydrate fed granules. This indicates that the top level fermentative community changes relatively rapidly, while the lower level community and possibly overall granule structure change on a longer time scale.

The physical properties of the granule, in terms of size distribution, granule strength, were not significantly impacted by the fermentative layer. This possibly indicates that physical and geometric characteristics, rather than those derived from the microbial community are more important. As an example, these granules were also not subject to hydrodynamic forces that may result in bulk properties such as surface texture (smoothness) and shear strength (Picioreanu et al., 2000; Nor-Anuar et al., 2012). In contrast, with a relatively thin and young fermentative layer formed, the effects on properties may be not strong enough to be detected.

5.3.2 Microbial Community within Granules

The microbial architecture of control granules was the same as the inoculum as previously analysed in Chapter 4 (separate sample from the same reactor) (Figure 4.12 in Chapter 4). Acetate is supplied from feedstock and via acetogenesis of propionate to be utilised by the dominant *Methanosaeta* (Figure 5.6A). Growth of *Methanosaeta* is normally favourable under low acetate concentrations due to the high substrate competition pressure from *Methanosarcina* under high acetate concentrations (Qu et al., 2009). However, *Methanosaeta* was always found as a dominant methanogen in anaerobic digesters (Regueriro et al., 2012). Van Haandel et al., (2013) also found the accumulation of *Methanosaeta* in COD overloading UASB reactors as *Methanosaeta* may be more readily incorporated granules th*en* *Methanosarcina* due to their filamentous structure. *Methanosaeta* are also favoured in granules and biofilms due to the concentration of acetate which is lower in-biofilm than the bulk as of biomass, which means in-biofilm acetate concentrations are lower than the bulk due to localised reaction exceeding diffusion (Batstone et al., 2004; 2006). *Methanosaeta* was commonly found in anaerobic digesters (Griffin et al., 1998) and anaerobic granules (McLeod et al., 1990; Harmsen et al., 1996b) and considered as a methanogen that uses only acetate. Many species were cultured in genus *Methanosaeta* including *Methanosaeta concilli* from sewage sludge (Patel and Sprott, 1990), *Methanosaeta thermophilia* (Kamagata et al., 1992) and *Methanosaeta harundinacea* from UASB treating beer-manufacturing wastewater (Ma et al., 2006) and were intensively studied (Smith and Ingram-Smith, 2007, Barber et al., 2011).

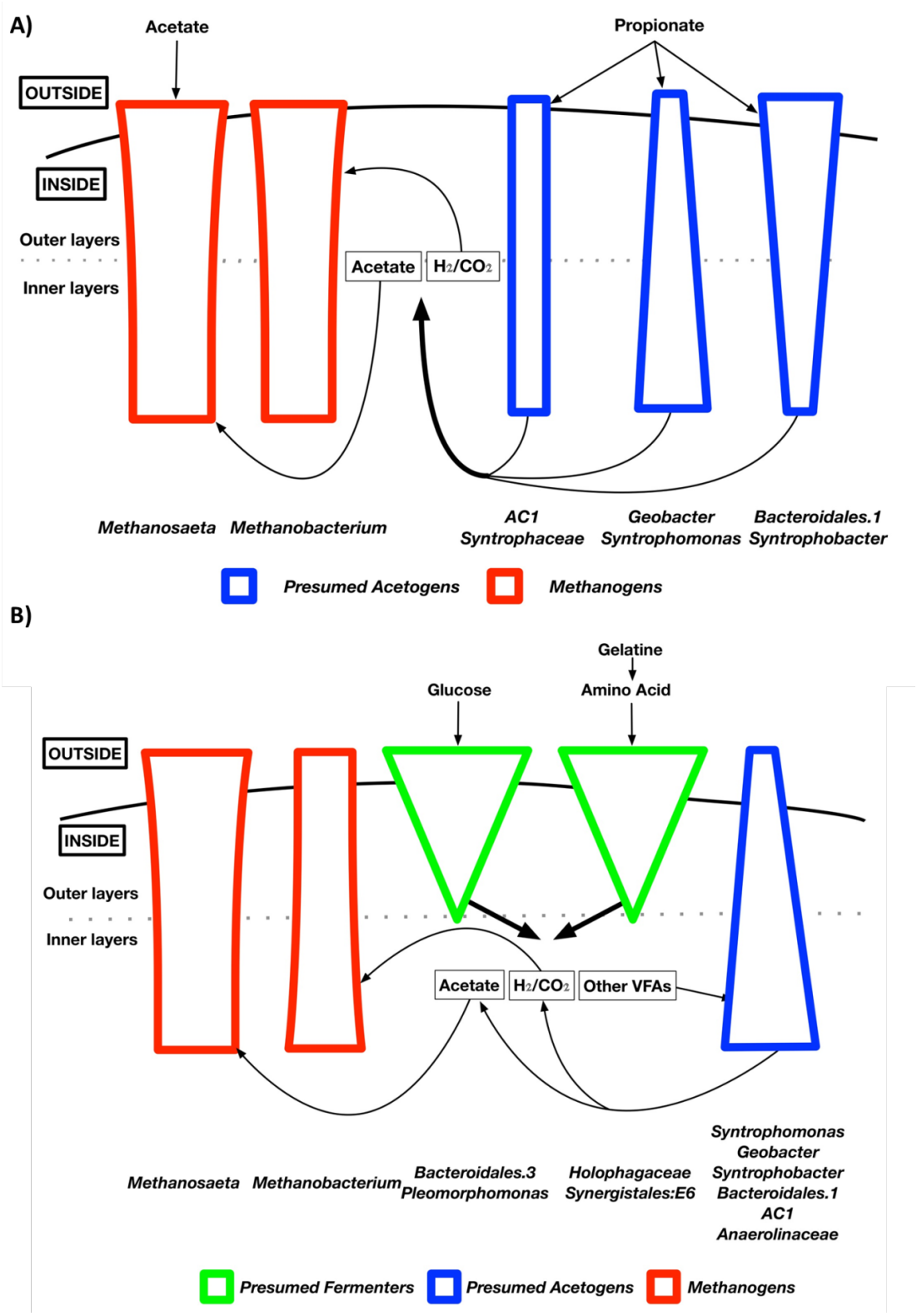


Figure 5.6 Proposed functionality and microorganism distribution in (A) control and (B) stimulated granules fed with glucose and gelatine respectively. Arrows indicated the directions of substrate uptake and digestion products. Vertical dimension refers to depth. Horizontal dimension for each shape refers to relative abundance. Glucose and gelatine are break down by presumed fermenters to produce VFAs. By presumed acetogens, VFAs are converted to acetate and H_2/CO_2 which in turn to be utilized by methanogens.

The bacterial community of control granules shifted from *Bacteroidales.1/Syntrophobacter* accumulated in the outer layer to *Geobacter/Syntrophomonas* in the inner layer. Both *Bacteroidales.1/Syntrophobacter* and *Geobacter/Syntrophomonas* are presumed acetogens indicating this function is distributed throughout the granules, but the role is filled by different consortia. This was also seen in the source industrial granules (refer to Brewery granules in Chapter 4). *Bacteroidales* and *Syntrophobacter* are known as propionate oxidizer (Kragelund et al., 2008; Shigematsu et al., 2006) and produce acetate plus H_2/CO_2 via the randomizing methyl-malonyl-coenzyme A pathway. The production of acetate and butyrate from propionate degradation through a dis-mutating pathway (Lens et al., 1996; Tholozan et al., 1990) has been identified in *Smithella* of family *Syntrophaceae*, which distributes evenly through the whole granules with low abundance. By tracing radioactive labelled propionate, Gan et al. (2012) proposed that under methanogenic condition, *Geobacter* contributes to propionate degradation and acetate production. The ability to degrade propionate and butyrate in genus *Geobacter* was also reported in several pure cultures (Coates et al., 2001; Aklujkar 2009). *Syntrophomonas* are well known butyrate oxidizer (Liu et al., 2011; Muller et al., 2010) via beta-oxidation pathway (Schink, 1997; McInerney et al., 2007). A membrane-bound protein complex (Schmidt et al., 2013) was recently found involved in reversed electron transport to overcome the redox potential difference in *Syntrophomonas*. The abundance shift between *Bacteroidales.1*/*Syntrophobacter* and *Geobacter/Syntrophomonas* is possibly due to the production of butyrate from propionate by *Syntrophaceae*, since butyrate is the only substrate for *Syntrophomonas*, or due to the syntrophic association between *Syntrophobacter* and methanogens outcompeting with association between *Syntrophomonas*/*Geobacter* and methanogens as butyrate oxidation is energetically unfavourable.

In contrast with acetogens and methanogens in which were the same in the glucose and gelatine fed granules, fermentative communities changed dramatically. The two key presumptive fermenters developed on stimulated granules were OTUs affiliated to genus *Pleomorphomonas* and family *Holophagaceae* (Figure 5.6B) in glucose and gelatine granules respectively.

Family *Methylocystaceae* has been most commonly identified as a key methanotroph (Bowman, 2006). However, genus *Pleomorphomonas* does not cluster (Xie and Yokota, 2006) with known methanotrophs such as genus *Methylobacterium* (Dedysh et al., 2004). The most closely related to the two identified OTUs is *Pleomorphomonas koreensis* (Figure 5.7) which

is a rod-shape nitrogen-fixing bacterium isolated from contaminated culture of the phototrophic bacterium *Rhodopseudomonas palustris*. Acids are produced from fermenting glucose by *Pleomorphomonas koreensis* (Im et al., 2006).

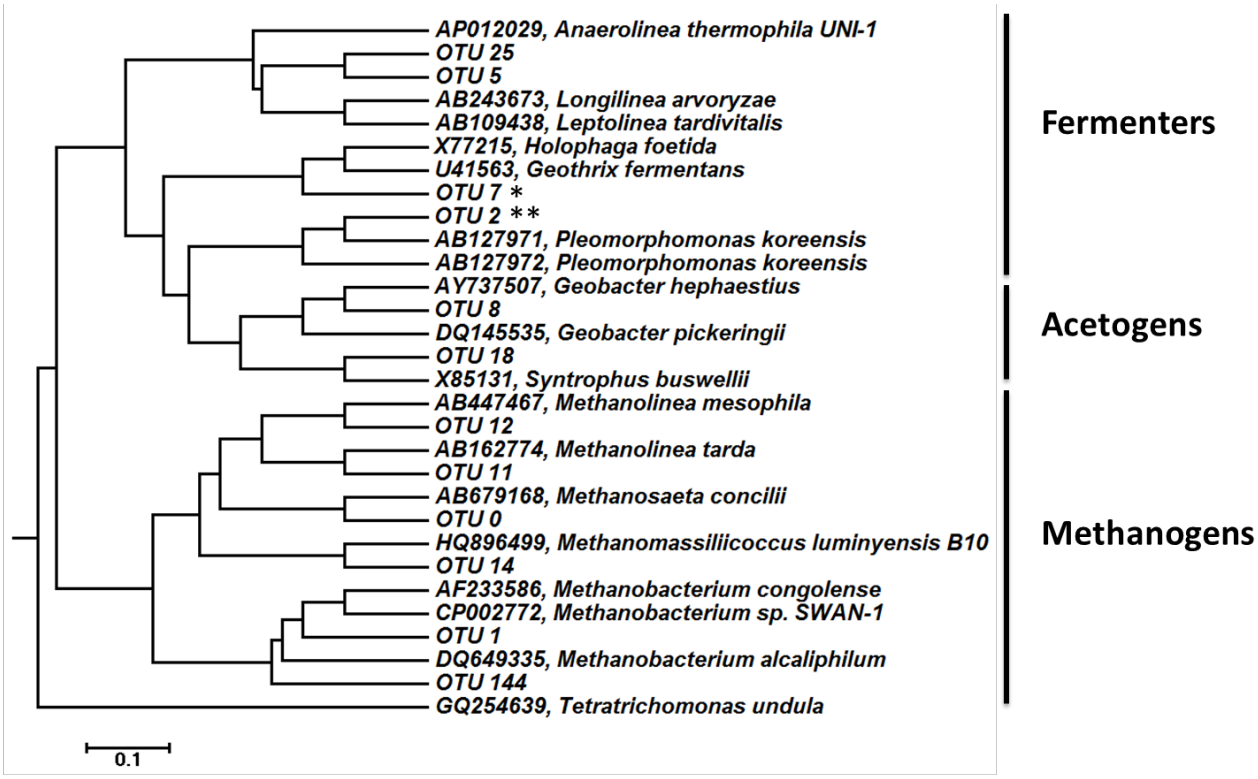


Figure 5.7 Phylogenetic tree of major archaea OTUs generated in Fasttree. *Tetratrichomonas undula* was set as out group.

Pleomorphomonas was not detected from previous studied cannery granules (refer to carbohydrate granule in Chapter 4) fed with large amounts of sugar, where the more commonly observed *Bacteroidales* and *Anaerolinea* occupied the fermentative niche. *Pleomorphomonas* completely displaced other acetogens in the outer layer and was not present in inner layers but as stated above, the deeper community appeared to be the same as control granules which consists of presumed acetogens including *Bacteroidales.1*, *Syntrophobacter* with *AC1*, *Geobacter* and *Syntrophomonas* dominating. Phylum *AC1* were first found in anaerobic marine sediment (Dhillon et al., 2003). The function of *AC1* in methanogenic bioreactors remains unclear. As *AC1* was frequently detected at high abundance in anaerobic granules treating brewery wastewater (Werner et al., 2011), it may possibly contribute to VFAs degradation and has been classified as presumed acetogens in this thesis. In industrial anaerobic granules treating similar types of wastewater, fermenters

penetrated to a much deeper extent (Figure 4.13 in Chapter 4). This is possible due to the relatively short timeline for growth, and possibly persistence of fermentative DNA in deeper layers.

Within the gelatine granules, *Holophagaceae* was detected with a similar shift in abundance as *Pleomorphomonas* in glucose granules. Family *Holophagaceae* were first identified by Fukunaga et al. (2008), which contains two genera *Holophaga* and *Geothrix*. The most affiliated organism to the OTU found in the gelatine granules is *Holophaga foetida*, isolated from a black anoxic freshwater mud sample (Liesack et al., 1994). It utilises methoxylated aromatic compounds to produce acetate through Phloroglucinol pathway. Pyruvate can also be oxidised to acetyl-CoA (and hence acetyl-CoA derived products such as butyrate, acetate, and ethanol). *Geothrix* is a strictly anaerobic chemo-organotroph that oxidises acetate with Fe (III) as electron acceptors (Coates et al., 1999). The type specie *Geothrix fermentans* can also use propionate, palmitate, lactate, fumarate or succinate as alternative electron donors, and can ferment citrate and fumarate in the absence of electron acceptors. There is hence no functional precedent for gelatine degradation in either related species.

Functionally, gelatine is hydrolysed by gelatinase (Malla et al., 2008) to amino acid and then degraded to produce acetate (Figure 5.7B), other VFAs and H_2/CO_2 by presumed fermenters. Gelatinase, as extracellular metallo- endopeptidase or metalloproteinase, are expressed in serveral bacteria including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Clostridium perfringens* (Baldassi et al., 2002), *Serratia marcescens* and *Bacillus* species (Balan et al., 2012). Whether *Holophagaceae* is hydrolytic or fermentative remained unclear due to that *Holophagaceae* was not previously identified in anaerobic granules. Evidence is strong that it is responsible for gelatine degradation, based both on location and differentiation from control granules. *Synergistales* (belong to phylum *Synergistes*) was found in a wide range of anaerobic habitats including rumen environment (Allison et al., 1992) and anaerobic digester treating proteinaceous waste (Ito et al., 2011; Delbes et al., 2001). Compared to all other bacterial genomes, highest average proportion of amino acid transport and metabolism genes were found in phylum *Synergistes* (Hugenholtz et al., 2009) thus amino acid fermentation may be a common feature within this group. To data, the first and only completed genome sequence in *Synergistes*, *Thermanaerovibrio acidaminovorans,* was isolated from anaerobic granules of sugar refinery UASB and found to carry out decarboxylation of succinate to propionate, a number of amino acid fermentation as well as carbohydrate fermentation (Chovatia et al., 2009). Acetogenesis was still carried out by the same acetogens group,

including *Geobacter*, *Syntrophomonas* and *AC1* found in control granules. *Syntrophobacter,* as the main acetogen found in dairy granules (in Chapter 4), was not found in substantial amounts in gelatine granules. The drop in relative abundance of *Methanosaeta* is possibly due to the inhibition of NH_4 produced during amino acid degradation. Similar change in abundance of *Methanosaeta* was seen in Chapter 4 (though it was replaced by *Methanolinea*).

Chapter 6. Discussions, Conclusions and Future Directions

The microbial community (specially the fermentative community) in different types of industrial granules were characterised in terms of not only the phylogenetic identification and shifts between different types of granules but also spatial distribution and shifts in relative abundance within each type of granules. The shifts in fermentative communities corresponding to substrate change were also examined in a controlled manner. This knowledge may now be applied to optimise existing granular processes or manipulate the composition of anaerobic granules to expand the functional capability (Guiot et al., 2002, Zhou et al., 2011) or boost granule properties such as strength or settling velocity (Macias and Carvajal, 2012). The following chapter reiterates the major findings on the characteristics of anaerobic granules, discusses concerns raised in this thesis and suggests recommendation for future research.

6.1 Shear Method

6.1.1 Applications of Shear Method

The shear method was developed to sequentially isolate successive layers of microorganisms within anaerobic granules and allow the application of molecular techniques to spatial layers separately rather than the whole granules. It provides the opportunity for community profiling on each layer of anaerobic granules and also provides additional spatial distribution information. This is the first time the microbial architecture and identification has been explored at such high resolution. Microbiology studies in anaerobic granules are no longer limited to whole granule molecular analysis and limited localization of FISH, which requires a prior knowledge, but have expanded to examine the shift in abundance through spatial layers and the localization of whole community. With additional information, it was found that the microbial architectures of industrial granules from processes fed different types of wastewater, are similar to results previously identified, but that particularly the fermentative community is substantially richer (in terms of both diversity and relative abundance) than previously identified using FISH (Harmsen et al., 1996; Batstone et al., 2004). The shear

method also successfully monitored the shift of fermenters on anaerobic granules under substrate change. An additional application of the shear method was developed in that it allowed development of covariance information for deep metagenomic sequencing which is the first time that covariance based analysis has been done on the layers of brewery granules. Normally it needs to be effected from multiple samples differentiated in either time (Fang et al., 2013) or space (Albertsen et al., 2013).

The application of the shear method can be extended to other systems including different types of anaerobic granules (fed on complex substrates), aerobic granules (to investigate the distribution of phosphate-accumulating organisms (Lemaire et al., 2008)), biofilms (such as the electrodes of a microbial fuel cell (Rabaey et al., 2007) or a sewer pipe (Satoh et al., 2009)). The technique may also be applied using a time series approach to investigate layering during the formation of granules or biofilms.

The shear method can also be used as an enrichment method for microorganisms of interests (particularly those forming in discrete layers such as fermenters). The results from this thesis showed that fermenters were found in the extracted outer layers of granules at a much higher relative abundance (up to 10-fold increase) than detected when examining the whole granules, this was due to the disproportionate dominance of fermenters in this outer layer, and possibly PCR bias (Pinto et al., 2012) favouring methanogens which were more concentrated in the granule core (see Chapter 4). The shear method enables the fermenters to be harvested at enriched levels. When considering potential applications for enrichment, Yamada et al. (2011) found that uncultivated microbes belonging to candidate bacterial phylum *KSB3* are associated with filamentous bulking in mesophilic UASBs and have been observed on the surface of bulking granules. The method developed in this thesis could be used to selectively extract these organisms for bulk nucleic acid analysis.

There is strong potential to combine the shear method with advanced metagenomic analysis techniques such as the GroopM method for genomic binning. GroopM generally requires at least 3 related samples, to recover the full genome of dominant microorganisms, and in this thesis, sufficient covariance information was extracted from a single sample that was processes into spatial layers. Brewery granules were split into 5 layers for metagenomic analysis and resulted in high quality bins on *Anaerolinea* (70% completeness, 1.8% contamination) and *Caldilinea* (90% completeness, 3.6% contamination). This level of

sequencing of fermenters has only previously been achieved by culture-dependent technique (Sekiguchi et al., 2009).

However, there are also limitations to the shear method, future development can address these limitations including:

1. **Physical contamination.** Granule sieving is performed prior to shear to ensure consistent initial granule size; and again at multiple time points during the shear method to separate the sheared layers (fines) and residual granules. Selection of sieve size is critical for this process as smaller granules may pass through the sieve and contaminant the fines. The effect of this possible contamination on molecular analysis is not clear, as it may be just a small portion compared to the layer samples.
2. **Estimated vs absolute shear depth.** The shear depth is estimated by measuring the mass of fines removed during shearing, rather than direct measurement of granule size after shear. The estimated depth can be used for microbial architecture construction. However, as the shear depth is not measured directly, the microbial layer information is difficult to compare with other depth-related information such as measurements from micro-sensors embedded in the granules or FISH quantification with absolute depth.

6.1.2 Future Directions

To overcome concerns with physical contamination, as well as variation in shear depth, granules may be selected by settling velocity prior to shear. Similar approach has been used to illustrate settling property of granular sludge (Nor-Anuar et al., 2007). The concept is size based stokes separation of granules. A separation rig, as shown in Figure 6.1 can be used to separate faster settling granules (larger mature granules) from slower settling granules (smaller young granules) and fines. This can be an iterative process with multiple treatments used to improve separation by shear method.

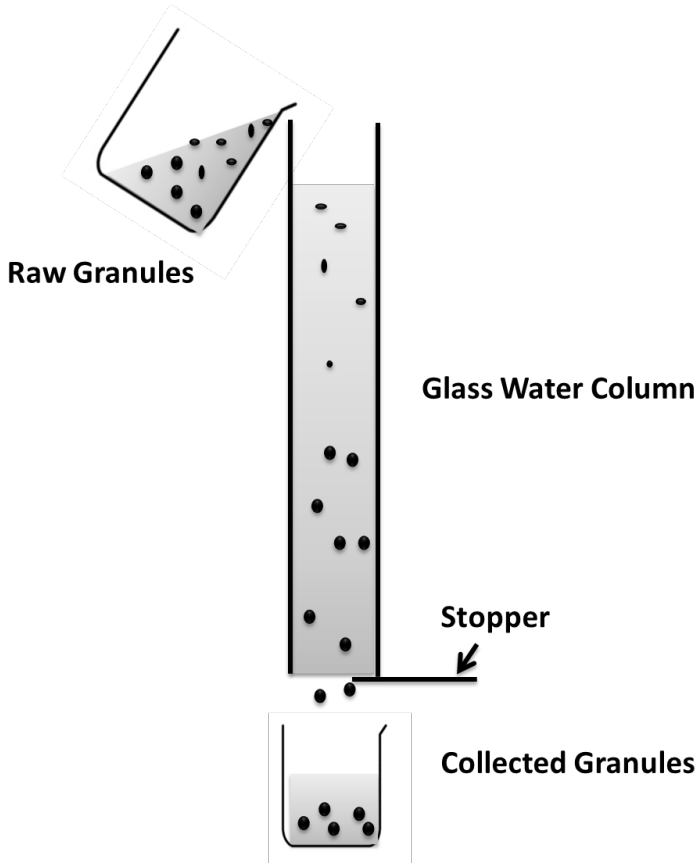


Figure 6.1 Sketch for granule separation by settling velocity.

Modification of shear method based on core sampling (Stone, 1991) and cryosectioning (Batstone et al., 2004) is proposed here. The objective of using a core sample is to reduce the complexity from a 3 dimensional granule to a 2 dimensional core as shown in Figure 6.2A using sampling tools (Abegg et al., 2008) or trimming the embedded sample by cryosection. The core can then be sliced vertically by cryosection to obtain layers from each granule (Figure 6.2B). Both molecular techniques and FISH can then be used to examine the layers.

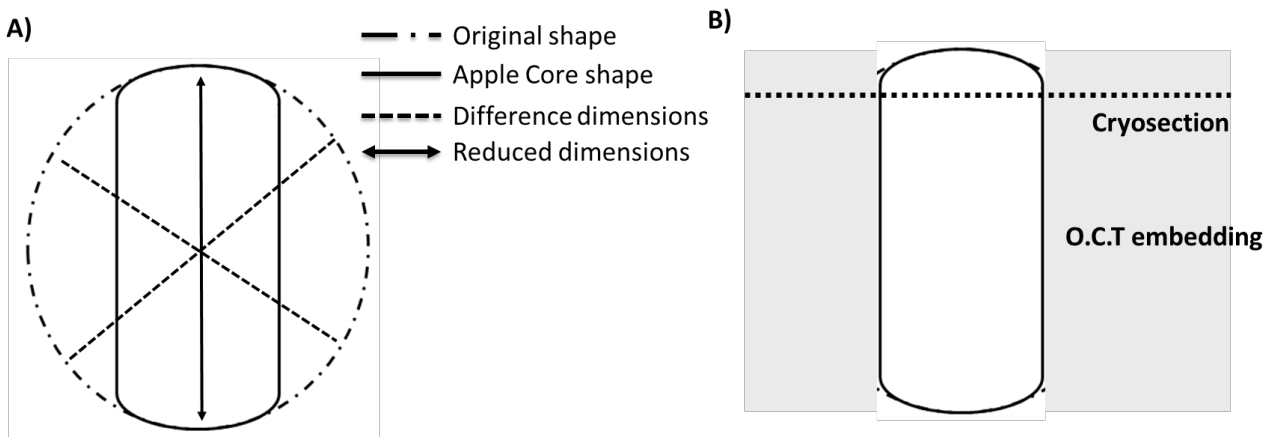


Figure 6.2 Granule core cryosectioning. A granule core sample is cut off from whole granules to reduce the layer difference to 2 dimensions (A). After embedding, it is cryosectioned vertically to separate the layers (B).

The advantages of examining a granule core sample over layers separated using the shear method are: (i) the slide thickness is consistent and can be controlled on a Cryostat machine, thus the absolute depth of each slide can be predicted based on slide thickness; (ii) the cryosection core method is based on assessment of an individual granule, this would reduce interference from smaller granules and prevent uncertainty due to variation between granules. In addition, multichannel moulds can be used to enable a large number of samples to be sectioned at the same time. It may allow analysis of the variation between granules and determine the representative populations within large reactors containing many granules. A variation of cryosection core technique has been applied successfully to sewer biofilms (unpublished) at the AWMC, UQ (Australia) and the technique allowed for pyrosequencing analysis on different layers within the biofilms.

6.2 Microbial Ecology

6.2.1 Influence of Substrate on Microbial Community Composition

A major outcome from this thesis is the determination that fermentative communities are far more complex than previously believed based on whole granule analysis (Keyser et al., 2006; Narihiro et al., 2009), FISH (Diaz et al., 2003; Batstone et al., 2004), and combined culture-based analysis and FISH (Sekiguchi et al., 2001; Yamada et al., 2007). Five OTUs detected in the outer layers are common to all types of granules investigated in this thesis (Figure 6.3). It is unknown as to whether these are synergistic or competitive; the latter is likely, and it is important to understand why stable complex communities could exist where there is strong competition. *Bacteroidales* was a key organism, which is diverse and the two key OTUs may have different functional contributions. *Bacteroidales.1* showed the highest relative abundance in the brewery granules (in Chapter 4) and possibly carried out acetogenesis as the substrate consisted mainly of propionate, ethanol, and acetate. *Bacteroidales.2* was the dominant group in cannery granules (refer to cannery granules in Chapter 4), and likely important for sugar fermentation. However *Bacteroidales.2* was not found in granules stimulated with glucose feed, indicating it may dominate in a more complex environment compared to the simple system in laboratory reactor.

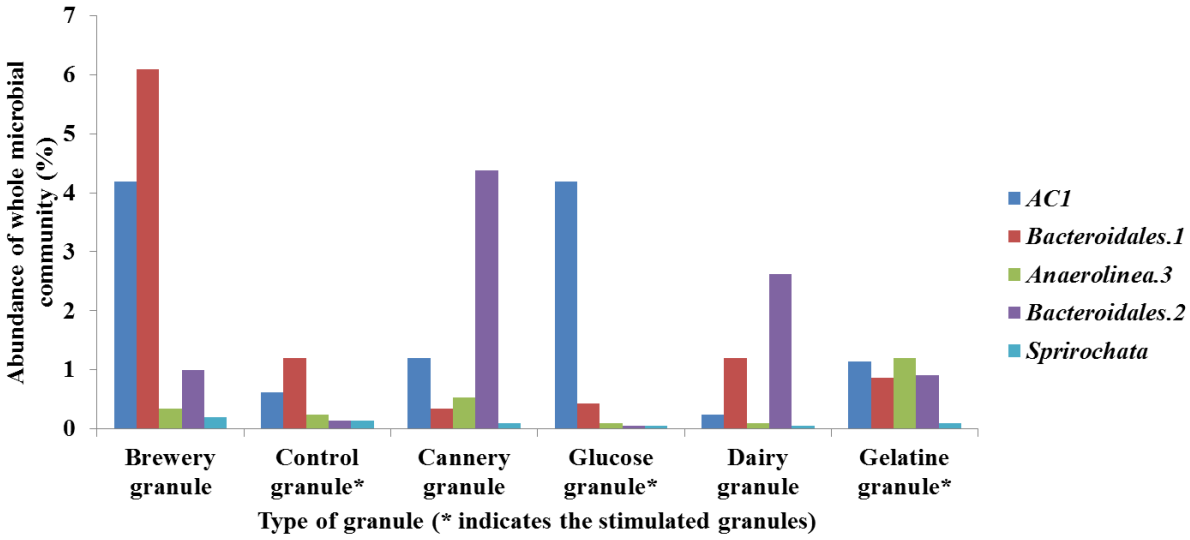


Figure 6.3 Relative abundance of common 5 OTUs present on the outer layer of all granules studied in this thesis.

According to spatial distribution, relative abundance and phylogenetic information, a draft relationship between substrate, organism and the location of each functional group is proposed in Figure 6.4. Fermentation likely occurs in the outer layer of granules, as this is the location that provides direct access to the more complex substrate and where key fermentative candidates are (based on relative phylogeny and differentiation from control in Chapter 5). In contrast, acetogenesis and methanogenesis are carried out by key and limited organisms (mostly known) distributed throughout the granules. The syntrophic relationship between acetogens and methanogens is well established in AD (Talbot et al., 2008; Gan et al., 2012). *Geobacter* and *Syntrophomonas* appear to be key VFA consuming/acetate producing microorganisms as they are associated with a high relative abundance of the acetoclastic methanogen, *Methanosaeta,* as well as the obligate syntrophic partner which may be *Methanobacterium* or *Methanolinea*. *Syntrophobacter* is the only presumed acetogen identified in the dairy granules, likely degrading propionate. The methanogenic community in the dairy granules shifted from *Methanobacterium to Methanolinea.* This is likely due to the lower affinities for H_2 by *Methanolinea* compared to *Methanobacterium* (Sakai et al., 2009) and is consistent with expectations that fermentation of proteins will result in a lower hydrogen concentration due to coupling of oxidation/reduction reactions through Stickland pairing (Ramsay and Pullammanappallil, 2001).

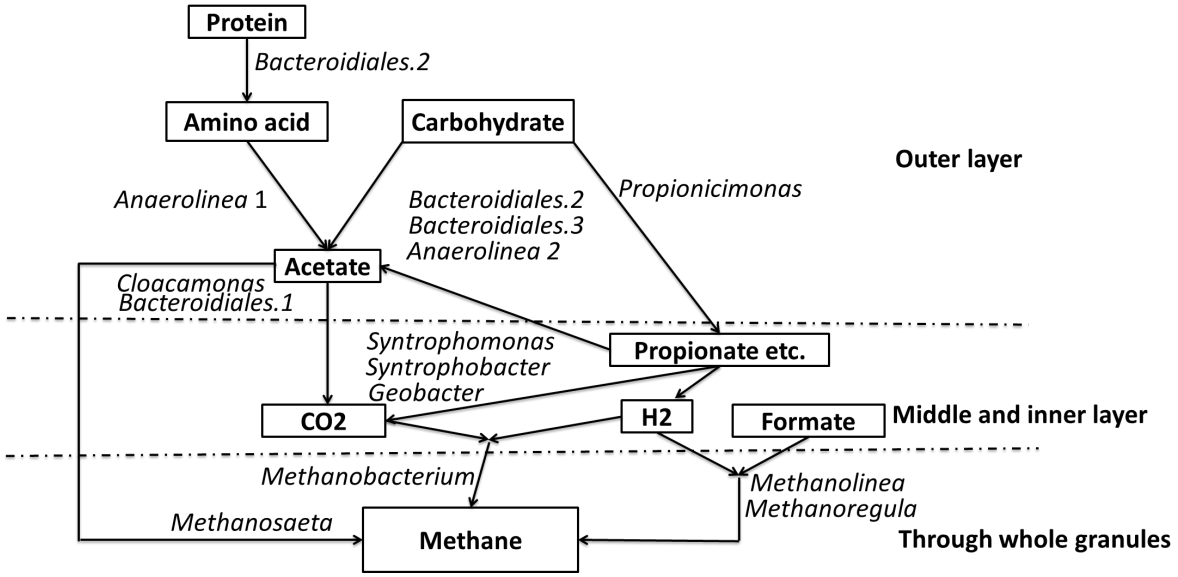


Figure 6.4 Proposed microbial functions and spatial distribution in anaerobic granules treating different substrate.

6.2.2 Relationship between Trophic Groups at Varying Granule Depth

In this thesis, the results showed that different trophic groups vary in relative abundance at different locations through whole granules (especially the industrial granules). The distinct shifts between fermenters and acetogens from outer to inner layers were only observed on brewery granules and the granules stimulated in the lab-scale UASBs (fed with low substrate loading rates). Compared to the traditionally proposed layer structure (Guiot et al., 1992; Fang, 1995), the microbial architecture was different with methanogens dominating through whole granules while other trophic groups were found to accumulate in the layers most suitable in terms of food source. A very important difference not traditionally observed using any previous method (including FISH) was the presence of acetoclastic methanogens such as *Methanosaeta* in the outer layers.

The accumulation of each microorganism has been analysed by a heatmap which shows the top 20 most abundance OTUs scaled and centred by each OTU. The accumulation is based on changes in an individual OTU relative to the same OTU at different layers and is not based on the relative abundance of the OTU within the population (i.e. not relative to other OTUs). *Methanosaeta* generally accumulated in the inner layers of granules (Figure C8, C9 & 6.5). As acetoclastic methanogenesis is generally favoured at high acetate concentrations (Zinder and Koch, 1984), it is likely that the acetate concentration is also high in this zone. Additionally, *Methanosaeta* accumulated in the outer layer of the control granules where acetate was a major component of the raw substrate (control granules in Figure C). This supports the conclusion that *Methanosaeta* accumulates in zones where acetate is available in abundance.

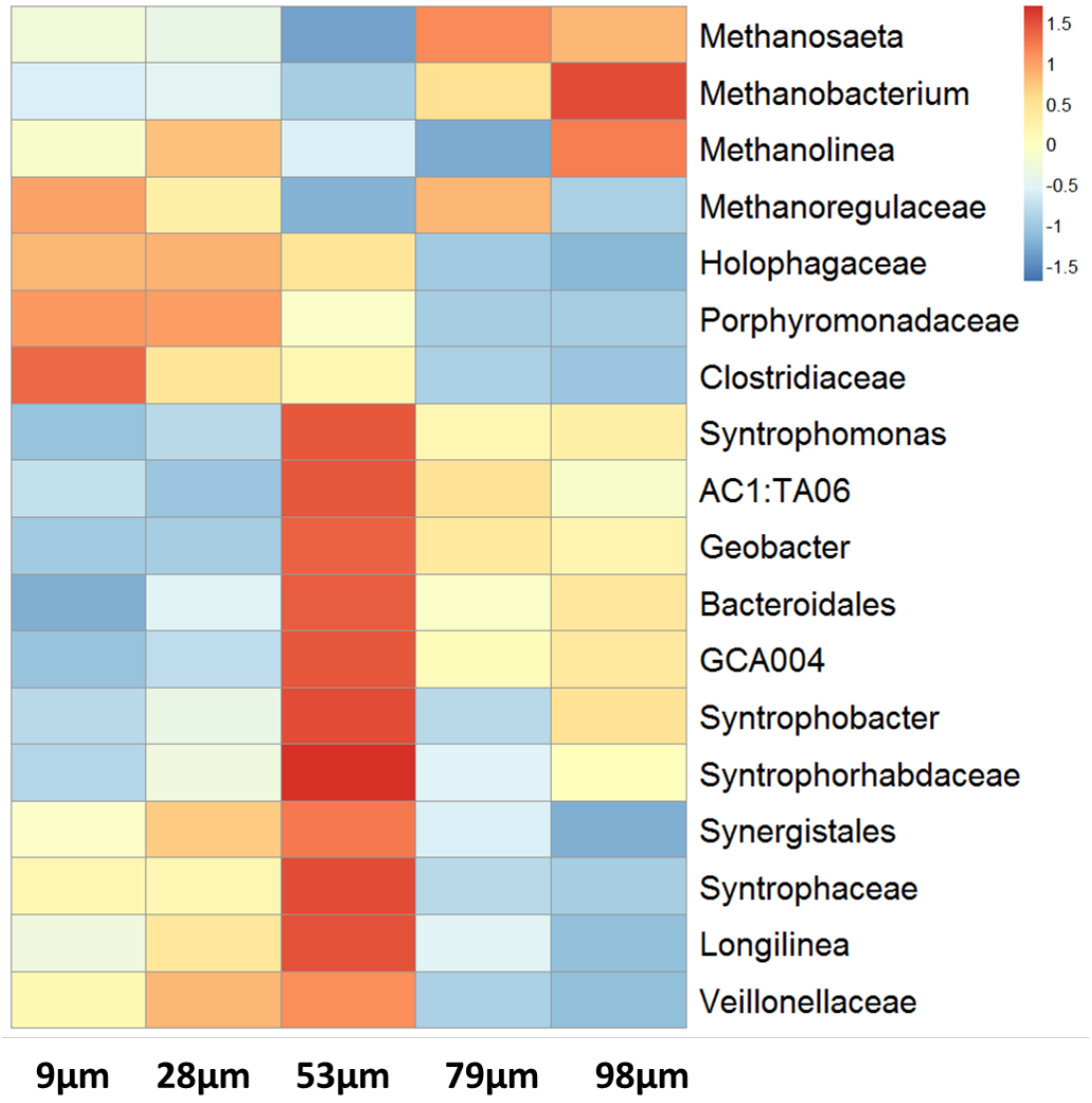


Figure 6.5 Heatmap shows the accumulation trend of each "top 20" OTU in gelatin granule through different layers. Accumulation trend reflects the relative abundance scaled and centered within range -1.5 to 1.5 of each OTU and indicates the abundance change of each OTU in different layers. Depth of each layer is labelled on the bottom of heatmap and the taxonomy of each OTU is labelled by the side of heatmap.

The heatmap shows a number of key features;

(a) Presumptive fermenters including *Holophagaceae*, *Porphyromonadaceae* and *Clostridiaceae* accumulate strongly in the outer layers (20µm). This was previously known from proportional analysis.

(b) Methanogens accumulate in mainly the inner layer (>57µm), but also in an outer zone.

(c) Presumptive syntrophs accumulate in a specific zone (27µm), which mainly excludes methanogens.

The last two observations were not readily seen in the proportional OTU analysis shown in Chapter 5, and only become evident once shifts are assessed as in the heatmap. The likely reasoning is that particularly syntrophs concentrate in layers immediately adjacent to fermenters where there are higher concentrations of organic acids; particularly those can be degraded without obligate electron transfer to a hydrogen utiliser (e.g., propionate to butyrate or ethanol). The depth of the acetogenic zone may be related to the fermentation rate of the raw substrate, e.g. gelatin may be degraded faster than glucose (Flotats et al., 2006; Garcia-Heras, 2003), therefore lead to a fast accumulation of organic acid. As a result the acetogen zone is found at 27µm in gelatin fed granules (Figure 6.5) compared to 43µm in glucose fed granules (Figure C10). Methanogens in the outer layer likely utilise acetate from both the bulk liquid and from fermenters in the granule, but methanogens in the centre mainly receive acetate and hydrogen from acetogens, including products from the outer layers that diffuse into the centre of the granule and products generated locally from oxidising propionate and butyrate in syntrophic associations.

Strong positive correlation between bacteria and archaea is found in the outer layers (upper levels of the acetogenic zone, Figure 6.6) in granules examined except cannery granules. This is possibly due to the growth of archaea in the outer layer of cannery granules being independent of acetogens, with substrate instead coming from fermentation or the bulk liquid. *Methanosaeta* links to presumed acetogens such as *Geobacter* and *Syntrophomonas* etc. in brewery granules but to only presumed fermenters such as *Candidatus Cloacamonas* in dairy granules, or *Holophagaceae* in gelatin granules. Such relationship is also observed with FISH, which shows the archaea grow in a close association with bacteria in the outer layer (Figure 6.7). This may suggest that *Methanosaeta* relies on acetate produced by acetogenesis under simple substrate (VFAs) but by fermentation under complex substrate (carbohydrate or protein). *Methanobacterium* and *Methanolinea* are also linked to presumed fermenters *Anaerolinea*/*Bacteroidales* in glucose granules due to the production of H_2/CO_2 from glucose hydrolysis (Sekiguchi et al., 2003; Thomas et al., 2011; Rismani-Yazdi et al., 2013). The correlation between bacteria and archaea in the inner layers (on the inside of acetogenic zone) may be more difficult due to that archaea are possibly related to multiple bacteria from different trophic groups and acetate and H_2/CO_2 are produced from fermentation and acetogenesis.

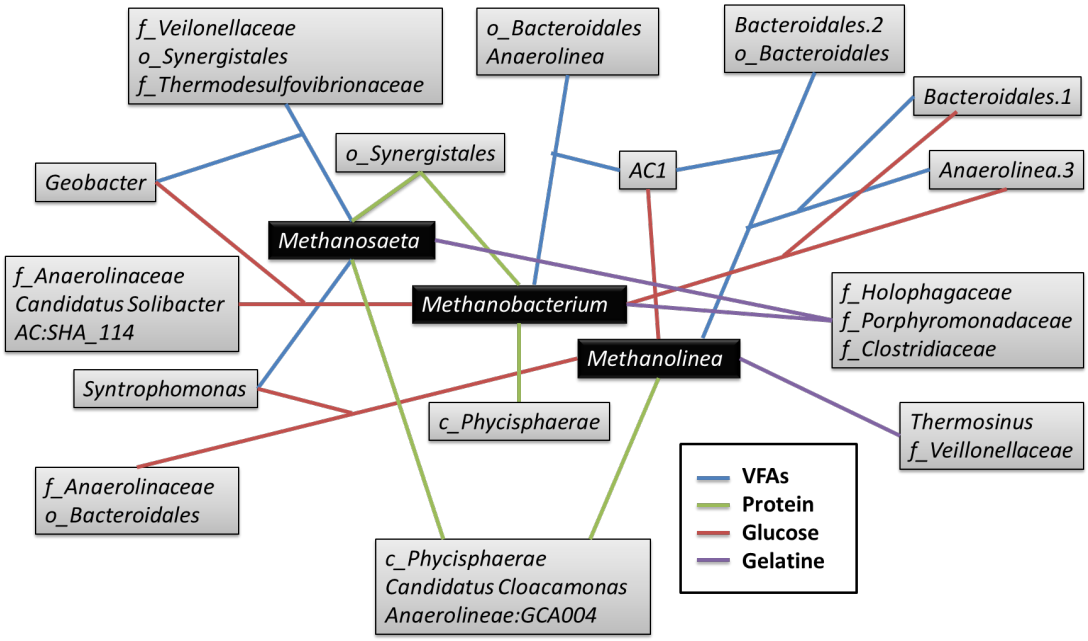


Figure 6.6 Strong positive correlation (*p*>0.8) between major archaea and bacteria (linked) based on the relative abundance in the outer layers (upper levels of the acetogenic zone). The relationships of different granules are indicated by colour codes.

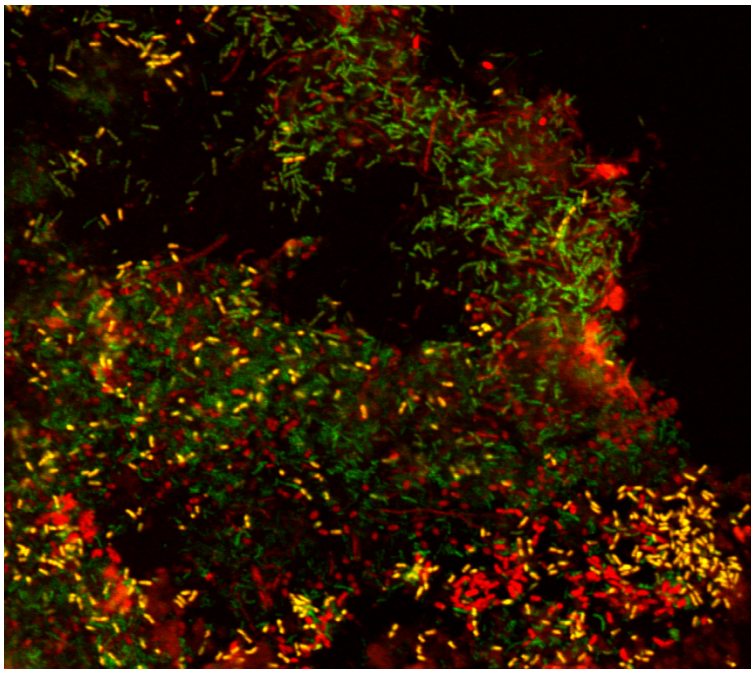


Figure 6.7 *Methanosaeta* and other archaea grow in association with bacteria on the outer layer of gelatin granules. *Methanosaeta* (*MX825*) are shown in yellow, Bacteria (*EUB338mix*) are shown in red, and other archaea (*ARC915*) are shown in green.

6.2.3 Further Directions

Recommended research on microbial ecology of anaerobic granules will focus on investigating the microbial relationships using functional analysis which has not been possible applied to the full extent in this thesis due to time limitations.

Although some fermenters were identified based on a combination of location, relative abundance and phylogenetic information obtained from NGS analysis in conjunction with the shear method, the function of many bacterial OTUs is presumed or remains unknown. Due to the success of the shear method, bacteria can be harvested at high abundance in specific layers thus it provides an opportunity to culture and isolate microorganism from anaerobic granules (for example 3 groups of order Bacteroidales mentioned in Chapter 4 and Chapter 5). Furthermore, comprehensive functional studies can be carried on cultured microorganism and possibly lead to novel application depending on the function.

Due to time constraints, metagenomic analysis of brewery anaerobic granules (Chapter 4) was reported directly after genomic binning. Further data processing including checking for assembly errors on high re, gene prediction to identify protein and RNA sequencing coded on the sample DNA, as well as functional annotation by comparing the predicted gene to existing, previously annotated sequence (Kunin et al., 2008) is still to be done. This is expected to identify the potential functions of the bacterial groups identified in the brewery granules to support the functional hypotheses in Section 6.2.

Additionally, the shear method can be applied to granules for meta-transcriptomic or meta-proteomic analyses (Vanwonterghem et al., 2014). This would require an additional step of preserving mRNA in the granules and hence make the shearing exercise much more complicated, but would allow detailed functional analysis including comparisons of active genes versus the complete functional potential of the whole granule, specific layers or individual microbes.

There are many environmental and process factors that may have contributed to the accumulation of microorganisms in specific layers (Davey & O'Toole, 2000). In this thesis the chemical distribution through the anaerobic granules (e.g. the accumulation of raw substrate, VFA or other intermediates) was assumed as a major contributing factor. To verify this assumption, detailed chemical analysis throughout different granules layers is required. This may be achieved using microsensor to detect VFAs, pH or dissolved gases in anaerobic

granules (Satoh et al., 2007, 2012). The chemical shift between layers can also provide additional support information for study of microbial architecture. This is also an extremely complicated procedure, requiring immobilisation of the granule in an anaerobic environment which can be accessed by microsensor (including the entire stepping motor apparatus).

6.3 Bio-stimulation of Anaerobic Granules

6.3.1 Microbial Community Affected by Substrate

16S rRNA analysis from pyrosequencing and metagenomics showed that the microbial community on the outer layer of anaerobic granules is diverse with many OTUs present at low abundance. This thesis illustrated that anaerobic granules would adapt to a substrate shift with a new community (in the case of stimulated granules), relatively narrow community. This is the first controlled comparative study on the fermentative community of anaerobic granules. OTUs affiliated to genus *Pleomorphomonas*, which is capable of glucose fermentation (Im et al., 2006), and *Holophagaceae*, which possibly contributes to gelatin degradation, emerged and dominated the bacterial community of anaerobic granules fed with glucose and gelatin respectively.

6.3.2 Compared to Industrial Granules

Interestingly the microbial community shift was limited to the fermenters, with only slightly changes in the relative abundance of acetogens and methanogens. The impact of substrate on the fermentative community is likely due to the diverse metabolic pathways available to degrade different complex substrates and the selection of specialized microbes (Kovacik et al., 2010; Zhou et al., 2011). This phenomenon is less likely to impact acetogens and methanogens as they grow on a limited range of intermediate products rather than the raw substrate. As seen from the field granules (Chapter 4), different reactors with different feeds did have different deeper communities but this may be partly due to reactor design and operational aspects with the designs of all three reactors being different.

Under low organic loading rate ($2 kgCOD \cdot L^{-1} \cdot d^{-1}$) compared to industrial organic loading rate (Batstone et al., 2004), the amount of fermenters stimulated is relatively low comparing to industrial high strength granules (such as cannery granules). The low amount and diversity of fermenters obtained on stimulated granules may be related to the low organic loading rate

(Cardinali-Rezende et al., 2013) and to the substrate type (Kovacik et al., 2010) or simply to stimulation time. Furthermore, physical properties such as granular strength and size are not affected by biostimulation due to the thin fermentative layer formed.

6.3.3 Further Directions

The possibility of enhance physical properties of anaerobic granules is a key direction. As the major microorganism located on the surface of anaerobic granules, fermenters are associated to surface properties such as shear strength, microscopic texture, and density (Forster, 1991; Quarmby and Forster, 1995). It was proposed that especially the filamentous bacteria (such as Anaerolinea, Sekiguchi et al., 2003) might be responsible for the preservation of granules structure (Uemura and Harada, 1993). In this thesis, biostimulation was applied to neutral granules with little or no fermentative layer, and it was found that the fermentative community did not significantly influence the shear strength. Future work could include longer stimulation time, higher organic loading rate and from low complexity to high complexity substrate (e.g. acetate to glucose to starch to cellulose), or possibly even to develop granules from crushed seed rather than a neutral granule. A future step may be to apply biostimulation to granules with an existing fermentative layer to treat wastewater with a different composition. This approach may allow granules with desired physical properties to be transferred and adapted to a new process. This work, suggests microbial community is highly associated to substrate type. If *Anaerolinea* is replaced by other microbes due to substrate change, property of high strength currently in cannery granules may not remain in stimulated granules. Alternatively, if the results indicating strength is independent of primary substrate type, it may be possible to achieve higher strength in poor strength granules using other means.

The stimulation experiment in this thesis illustrated new OTUs (rare species in base granules) can be stimulated to replace predominant OTUs in response to substrate change. By finishing the functional annotation on metagenomic analysis (future direction under section 6.2.3), the potential function of microorganisms (such as hydrolysis function) in anaerobic granules with little fermentative functionality (brewery granule) will be studied. If genes encoding hydrolysis present in the existing microbial community, it may provide opportunity to utilize anaerobic granules to treat more complex substrates which require hydrolysis. Microorganism with suitable functionality may be stimulated with corresponding substrate (Zhou et al., 2011) similar as the experiment carried out in this thesis. Alternatively,

exogenously grown microorganisms (pure culture or mixed culture) with known hydrolysis function may be introduced into UASB and test whether they can survive and incorporate into anaerobic granules (Guiot et al., 2002; Lanthier et al., 2002).

6.4 Conclusions

The results presented in this thesis investigated the microbial ecology of fermentative microbes in industrial and lab-scale anaerobic granules and elucidated the influence of fermentative layer. It provides fundamental understanding as well as appropriate methods for future microbial studies on anaerobic granules. Suggested future directions include an improved shear method, functional analysis and extended stimulation experiments. This should contribute to the understanding of anaerobic granules and offer possible opportunity to enhance this technology. Conclusions have been drawn as follows.

6.4.1 Shear method enables selective layer based microbial sampling and analysis

Microbes were successfully removed from spatial layers through hydraulic shearing. Molecular analysis can be applied to removed layers from anaerobic granules and provide spatial distributions which consents FISH analysis or potential function information from meta'omics analysis. Furthermore, this method may contribute to culture isolation or enzymatic/proteomic studies if whole live organisms or RNA/protein can be extracted.

6.4.2 Bacterial community in anaerobic granules are diverse and complex

The bacterial community shared between granules fed with different substrates is common to a substantial extent, but dominated by different groups. Based on the spatial distribution and molecular identification, microbes can be assigned to different functional groups. Fermentation, acetogenesis and methanogenesis may happen in parallel with some functions dominating over others in specific layers, particularly the outer layer.

6.4.3 Minor influences of fermenter layers on granule properties were observed.

In a controlled comparative lab-scale experiment, presumed fermentative layers were successfully stimulated on the surface of brewery granules with no disturbances on acetogenic and methanogenic community. The little or no impact on physical properties may be due to low organic loading rate or short operation period, or may be due to the fact that phylogeny has limited impact on bulk properties.

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Appendix A. Publication

ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Shearing of Biofilms Enables Selective Layer Based Microbial Sampling and Analysis

Yang Lu,[1] Frances Slater,[1] Ricardo Bello-Mendoza,[2] Damien J. Batstone[1]

[1]Advanced Water Management Centre, The University of Queensland, Level 4, Gehrmann Laboratories Building (60), Brisbane, QLD 4072, Australia; telephone: +61-7-33654739; fax: +61-7-3365-4726; e-mail: y.lu@awmc.uq.edu.au

[2]Department of Environmental Biotechnology, El Colegio de la Frontera Sur, Tapachula, Chiapas, Mexico

ABSTRACT: Granules are large, self-supporting biofilms that form naturally in high-rate anaerobic treatment systems and are extremely important to reactor functionality. Granules exhibit functional and phylogenetic layering, interesting to both scientists and technologists. Until now, it has only been possible to analyze layering through sectioning and microscopic analysis with fluorescent in situ hybridization, or to analyze the whole granule through DNA extraction and microbial community profiling methods. This means different functional and spatial layers cannot be analyzed separately, including next generation sequencing techniques, such as pyrotag sequencing. In this work, we describe a method to remove microbes selectively from successive spatial layers through hydraulic shearing and demonstrate its application on anaerobic granules of three different types (VFA-, carbohydrate-, protein-fed) in size ranges 0.6–2 mm. Outer layers in particular could be selectively sheared as confirmed by FISH. TRFLP was used as an example bulk DNA method on selectively sheared fractions. A shift in dominant population was found from presumptive acidogens (such as *Bacteroidetes* and *Anaerolinea*) in outer layers to syntrophs (such as *Syntrophomonas* and *Geobacter*) in inner layers, with progressive changes through the depth. The strength of the shear-bulk molecular method over FISH was that a deeper phylogenetic profile could be obtained, even with TRFLP, and that prior knowledge of the community is not required.

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The present address of Ricardo Bello-Mendoza is Department of Civil and Natural Resources Engineering, University of Canterbury, Private Bag 4800, Christchurch, 8140, New Zealand

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Author contributions: Y. Lu performed experiments; F. Slater, R. Bello-Mendoza, and D. J. Batstone supervised the study; Y. Lu wrote the manuscript. All authors were involved in devising the experiments and editing the paper.

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KEYWORDS: anaerobic granule; shear stress; UASB; granule layer; cryosection-FISH

Introduction

The upflow anaerobic sludge blanket (UASB) reactor (Lettinga et al., 1980) 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 0.5–3 mm 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 (Fang et al., 1995; Guiot et al., 1992) depending on feedstock. Microbial identity is known to be important in determining reactor 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 and Forster, 1995), and extensive interpretation was based on presumptive morphological identification. More recently, molecular techniques have been applied to identify the microbial ecology and structure of anaerobic granules (Kim et al., 2011; Werner et al., 2011). As microbial community profiling methods require processing whole granules, they do not distinguish spatial information. To date, the spatial information, that is, 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 the response of each organism to the FISH process. It is also limited to phylogenetic analysis based on 16S genes or similar, with limited opportunity for analysis of functional genes or metagenomics.

Small particles from the exterior of granules, or fines, can result when shear stress is applied. When this occurs in a

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UASB reactor, it can reduce the quality of reactor effluent. The granule shear strength defined as the quantitative fines production rate normalized by shear rate can be useful to predict sensitivity to shear and is an important practical property in determining effluent quality (Ghangrekar et al., 1996). Shear strength can be determined by an abrasion experiment designed by Pereboom (1997). The shear strength of granules appears related to the feed type, and possibly divalent cation availability. For example, simple substrates such as acetate (Fang et al., 1994) and mixed organic acids (Batstone and Keller, 2001) produce granules that have low shear strength and complex substrates, such as carbohydrate (Batstone et al., 2004) produce granules with higher shear strength. High calcium granules had very high shear strength (Batstone et al., 2002). Using modified version of this technique and information about a granule type's shear strength, it may also be possible to sequentially remove granule layers, potentially allowing phylogenetic and functional characterization of each layer.

In this paper, we describe a modified version of Pereboom's shear strength measurement technique (Pereboom, 1997) in which we aim to sequentially remove layers from mature granules from full-scale UASB reactors by applying shear stress. We then study microbial structure and distribution by applying community profiling methods to successive biofilm layers.

Materials and Methods

Shearing

Granules were collected from full scale UASB reactors at a brewery, a cannery and a dairy. These granules were referred to as VFA (volatile fatty acid) granules, carbohydrate granules, and protein granules, respectively due to the primary components of the reactor feeds. Shear experiments were performed in a standard-geometry cell (1.3 L, 120 mm diameter) with a stainless steel six blade flat blade Rushton impeller (40 mm diameter) as shown in Figure 1 (Holland and Chapman, 1966). Anaerobic granules were first gently washed over a 200 μm mesh sieve to remove the native fines. Approximately 10 g (wet weight) sieved granules were added to 1 L of 1× phosphate buffered saline (pH 7.2) at the beginning of the shear experiment and sheared at either 500 rpm (VFA granules) or 1,500 rpm (carbohydrate and protein granules). Samples were collected after 5, 20, 90, 180, 270, and 360 min of shearing. At each sampling time, the contents of the cell were sieved through the 200 μm sieve and rinsed with water gently to separate fines from 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 analyzed using Quantimet image analysis software (Leica, North Ryde, Australia) to determine the size of granules. The shear strength was characterized according to the method developed by Pereboom (1997), which relates the growth of fines to shear rate squared.

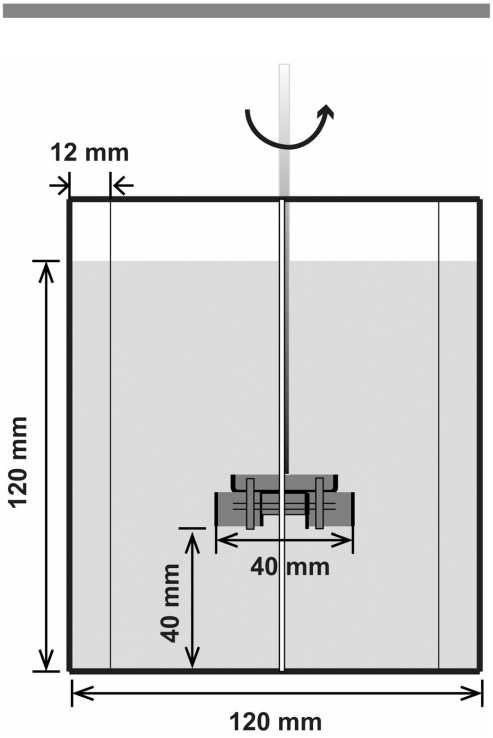

Figure 1. Shearing tank configuration including vessel geometry and six-bladed Rushton impeller (Holland and Chapman, 1966).

$$\ln\left(\frac{X_0 - X_F}{X_0}\right) = K_C \times \gamma^2 \quad (1)$$

where X_0 is the total suspended solid (TSS) of granules at the beginning of each shear, X_F is the TSS of fines generated after each shear, γ is the shear rate, and K_C is the characteristic shear coefficient. Fines were defined as particles smaller than 200 μm and were collected through wet sieving. TSS were analyzed by method 2540D and R in the 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.

TRFLP

Genomic DNA was separately extracted from generated fines and residual granules (on the sieve) collected at each sampling time with the Power Soil DNA isolation kit

(Mo Bio, Thebarton, Australia). Bacterial and archaeal 16S rRNA genes (>1 kb) were amplified by PCR using bacteria-specific primers 63F (5′-CAGGCCTAACACATGCAAGTC-3′) with a fluorescent label on the 5′ end, and 1389R (5′-ACGGGCGGTGTGTACAAG-3′) (Marchesi et al., 1998) and archaea-specific primers (Arc8F (5′-TCCGGTTGATCC-TGCC-3′) with a fluorescent label on the 5′ end, and 1492R (5′-GGCTACCTTGTTACGACTT-3′) (Smalla et al., 1993). The amplification protocol was based on that of Osborn et al. (2000) with adaptations. Each 50 μL reaction contained 200 μM each dNTP, 1× PCR buffer II (Applied Biosystems, Mulgrave, Australia), 3 mM $MgCl_2$, 400 nM each primer, 0.05 U AmpliTaq Gold DNA polymerase (Applied Biosystems), and 15 ng genomic DNA. The PCR product was purified by QIAquick PCR purification kit (QIAGene, Chadstone, Vic, Australia) according to the manufacturer's protocol. Restriction digestions were carried out with *AluI* (5′-AG^CT-3′) (Thermo Scientific, Scoresby, Australia), *MspI* (5′-C^CGG-3′) (Fermentas) for bacteria and *AluI*, *MspI*, and *HaeIII* (5′-GG^CC-3′) (Fermentas) for archaea. Each 31 μL restriction enzyme digestion reaction contained 150 ng purified PCR products, 0.32 U restriction enzyme, and 1× relevant buffer. Each digestion was performed in duplicate. Ethanol precipitation of the digestion product was then performed. Samples were analyzed using capillary electrophoresis in a DNA sequencer utilizing the AB3730 platform at the Australian Genomic Research Facility (Glen Osmond, Urrarae, Australia). The resulting electropherograms were analyzed by GeneMarker software (SoftGenetics, State College, PA). TRFLP data was then normalized to allow comparison between samples and standardized to remove errors associated with differential sample loading (Sait et al., 2003). Small and un-reproducible peaks were removed to reduce noise.

Cryosection-FISH

Granules were first fixed with 4% PFA overnight and then washed with 1× PBS and stored at 1:1 100% ethanol: 1× PBS. 10–15 granules were placed in 15% sucrose for overnight, 3:1 15% sucrose: optimal cutting temperature compound (OCT) (Sakura Finetek, Tokoyo, Japan) for 2 days, 1:1 15% sucrose: OCT overnight, 1:3 15% sucrose: OCT for overnight and neat OCT for overnight (Batstone et al., 2004). Around 4–5 granules were transferred to a square mould and stored at −20°C for overnight. The specimen was sliced on a Hyrax C60 cryostat machine (Zeiss, Oberkochen, Germany) with a knife temperature −19°C and specimen temperature −20°C to obtain a 5 μm slice on Poly-L-Lysine coated slide (Polysciences, Gymea, Australia). The slide was then dehydrated for 3 min in ethanol series 50%, 70%, and 98%, respectively. After drying the slide was stored at room temperature. FISH was performed according to the protocol described by Lee et al. (1999). Details of oligonucleotide FISH probes used are listed in Table SI. The slides were then viewed under Zeiss Axioscope LSM510 confocal microscope (Zeiss).

Clone Library and TRFLP Identification

One archaea 16S rRNA clone library was conducted on biomass collected from whole VFA granules. Two bacteria 16S rRNA clone libraries were conducted on biomass collected from whole VFA granules and the outer layer of VFA granules. PCR products amplified from previous section were cloned into the pGEM®-T Easy Vector (Promega, Alexandria, Australia) through the ligation procedure described in the manufacturer's manual. Vectors were then transformed into JM109 high efficiency competent cells (Promega) to construct the clone libraries. Twenty clones were randomly picked from each clone library. Clones were sequenced with primers T7 (5′-TAA TAC GAC TCA CTA TAG GG-3′) (Pavco and Steege, 1991) and SP6 (5′-TAT TTA GGT GAC ACT ATA G-3′) (Pavco and Steege, 1991). Representative clone sequences were submitted to GenBank to obtain accession number. In silico TRFLP digestion and BLAST searches (Altschul et al., 1990) were performed on each clone in MEGA5 (Tamura et al., 2011).

Results

Most VFA and carbohydrate granules were black with a smooth surface. The largest granule, VFA granules, had diameter averaging 2 mm (standard error of 0.8 mm), and with an oval or angular shape. Most carbohydrate granules were spherical with diameter ranging from 0.5 to 0.8 mm (average 0.65 ± 0.2 mm). Larger carbohydrate granules had similar shape with VFA granules and smaller particles were possibly broken granules. Protein granules were yellowish, and spherical with rough surface. Most protein granules had diameters from 0.4 to 1.2 mm (average 0.8 ± 0.3 mm). Particles measured above this size were observed as large aggregate of protein granules and it was hard to separate the aggregate without damaging individual granules.

The fines generated versus shear rate squared (γ^2) is shown in Figure 2. A lower K_C, equates to a weaker granule (i.e., faster generation of fines). Carbohydrate and protein granule strengths were comparable (protein was slightly weaker), while VFA granules were far weaker. The amount of solids measured, together with the measured density allowed calculation of depth sheared as shown in Table I.

Shearing was successful as demonstrated by FISH analysis of pre-sheared and post-sheared anaerobic granules. Bacteria, as shown in red in Figure 3, formed a distinct layer on the surface of carbohydrate (Fig. 3A), VFA (Fig. 3C), and protein granules (Fig. 3E). This layer is completely removed as seen on carbohydrate granules after 270 min shearing (Fig. 3B), VFA granules after 5 min shearing (Fig. 3D), and protein granules after 180 min shearing (Fig. 3F).

Based on in silico digestion of clone sequences from clone libraries, a number of TRFLP peaks were identified (Table SII). Quantitative analysis clearly shows progressive variation of TRFLP peaks through different layers (Fig. 4A–C). Particular organisms were evident in outer but not inner layers, including members of the *Bacteroidetes* and the

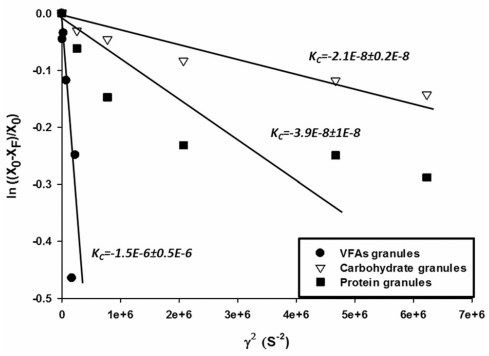

Figure 2. Estimation of characteristic abrasion coefficient (K_C). K_C for granules from full-scale reactors with different feedstock, including VFA, carbohydrate and protein were estimated according to the method described in materials and methods section. A lower K_C equates to a weaker granule. VFA granules were sheared at 500 rpm, both carbohydrate and protein granules were sheared at 1,500 rpm.

Anaerolinea (referred to as *Anaerolinea1* in Fig. 4) in carbohydrate granules (Fig. 4A), *MspI* 126 and other members of the *Anaerolinea* (referred to as *Anaerolinea2* in Fig. 4) in both VFA (Fig. 4B) and protein granules (Fig. 4C). This is clearly shown in Figure 4D. As an example of this trend, the abundance of *Anaerolinea2* peak in the out layer was seven times the size of the one in whole granules. On the other hand, members of the *Syntrophomonas* and *Geobacter* presented in inner layers in carbohydrate (Fig. 4A) and VFA granules (Fig. 4B). There were 14, 10, and 16 additional peaks detected from layer samples than the one from whole carbohydrate, VFA and protein granules, respectively.

Discussion

Granule strength is variable: The granule strengths were highly variable as previously reported (Batstone and Keller, 2001). This is largely related to feedstock, with rapidly degradable primary substrates such as sugars resulting in dense and high-strength outer layers (Batstone et al., 2004). The importance

Table I. Shear depth (μm) of three types of anaerobic granules at each time point.

Time (min)	VFA granules	Protein granules	Carbohydrate granules
5	14	9	5
20	24	28	16
60	58	53	42
180	117	79	72
270	196	98	86
360		127	99

Shearing was done at 500 rpm (VFA granules) and 1,500 rpm (protein and carbohydrate granules).

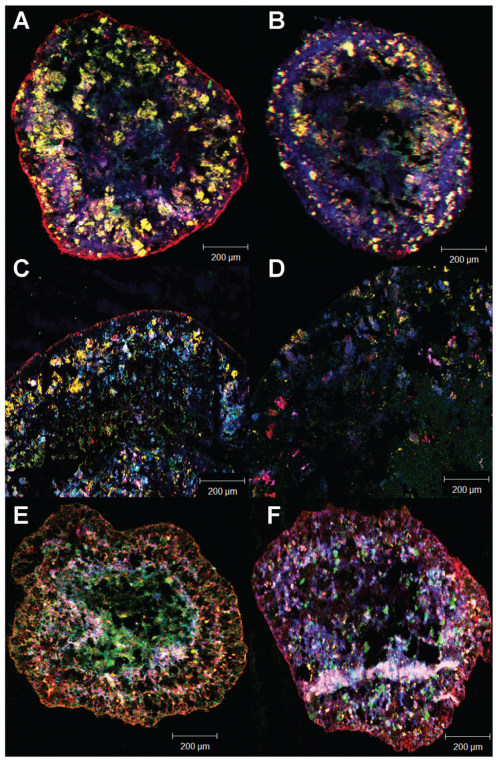

Figure 3. FISH images of the carbohydrate granules before (**A**) and after (**B**) shearing, VFA granules before (**C**) and after (**D**) shearing and protein granules before (**E**) and after (**F**) shearing. *Methanosaeta* (MX825) is shown in yellow. *Methanococcales* (MC1109), *Methanobacteriales* (MB1174), and *Methanobacteriales* (MG1200) are shown in cyan. Other Archaea (ARC915) are shown in green. Bacteria (EUBmix) are shown in red.

of this is that the method as presented cannot be applied blindly (i.e., for a fixed time and shear rate), as the amount sheared depends strongly on the strength (note that Fig. 2 is a log relationship). However, the method does inherently return shear strength, and we recommend shearing initially at lower rates and shorter times to collect outer layers and characterize the shear strength, and to conduct additional tests at a higher shear rate as necessary.

Shearing combines the depth of microbial community profiling with localization capability: Compared with traditional microbial community profiling study, this method was able to analyze DNA from specific layers with a sub-millimeter resolution and detect the major microorganism on each layer. Applying FISH ideally requires a level of prior community knowledge, particularly where the system is phylogenetically diverse, or where the population carrying out a specific function is

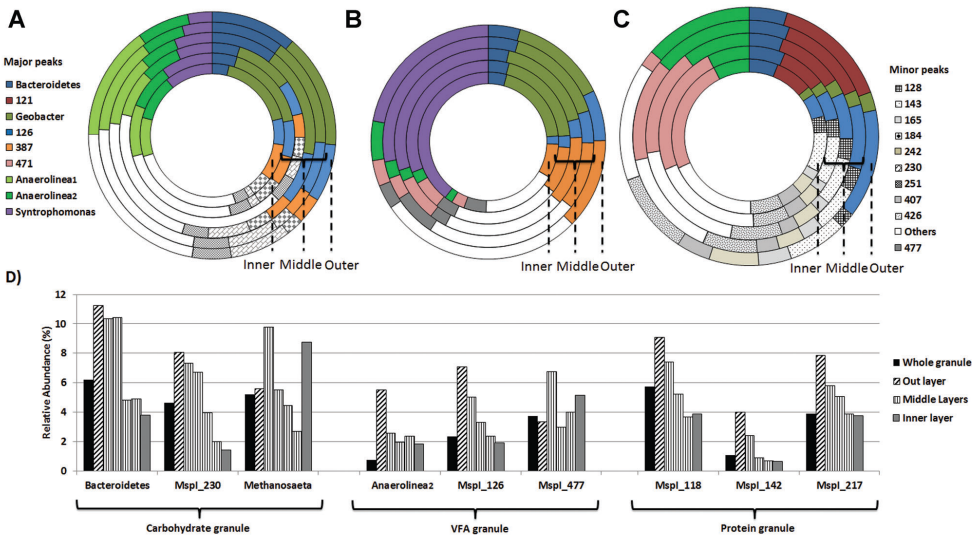

Figure 4. Relative abundance of different bacterial groups in outer, middle, and inner layers of carbohydrate granules (**A**), VFA granules (**B**), and Protein granules (**C**) as revealed by TRFLP. Peaks that were identified by BLAST searches and in silico TRFLP digestion of cloned 16S rRNA genes are labeled with their affiliated organism and other peaks are labeled with the peak number from enzyme digestion with *MspI*. The relative abundance of specific peaks can be greatly enhanced in individual layers compared to the average abundance detected in the whole granule (**D**).

relatively merged, or where a single function has a diverse phylogeny. Anaerobic granules are a good example of this, with a high level of diversity, particularly amongst the acidogens (Werner et al., 2011). Without spatial enrichment, FISH hunting (i.e., systematically testing FISH probes based on clone library information) can be extremely time consuming and unproductive. However, when a particular organism is found to be enriched in an outer layer, it is an obvious acidogenic candidate, and probes can be designed preferentially against that organism, making it more likely to result in a successful identification. Furthermore, the quality of DNA recovered from shearing is relatively good, as the shear force is relatively gentle compared to subsequent DNA extraction. We observed additional TRFLP peaks in the outer layer that were not found in whole granule samples. This is because in whole granules, organisms at relatively high abundance in the outer layer are not detected due to their far lower abundance in the granules as a whole. This spatial selection of organisms could also be used in conjunction with other community profiling methods such as clone libraries and pyrosequencing, even where several thousand OTUs are identified. The localization by FISH and by shearing, in combination with microbial community profiling methods offers the possibility to assess the impact of factors influencing community profiling analysis such as PCR bias. We also believe it may be possible to extract whole live organisms in order to conduct further live culture analysis, including substrate screening, and enzymatic/proteomic analysis.

Complexity of outer layer: The outer layer has been regarded as being mainly acidogenic (Batstone et al., 2004; Sekiguchi et al., 1999) in function. We confirm this, with the presumptive fermenters, such as *Bacteroidetes* and *Anaerolinea*, found in granules with carbohydrate and protein feed. Importantly, the VFA granules were dominated by syntrophs such as *Geobacter* (ethanol), and *Syntrophobacter*(propionate). While our results confirm the finding from previous studies that the outer layers are dominated by acidogens, we additionally show that the out layers are not dominated by a single population, but are quite complex. Microbial community profiling methods are the only way to attain the phylogenetic depth needed to identify and analyze communities at this depth.We used TRFLP here in order to readily assess the three whole granules samples and 17 sheared samples generated in this study, to demonstrate profiling through the depth of granules and hence demonstrate the utility of the method. However, future approaches would be to construct clone libraries or 16S pyrotag sequencing on individual layers, and fully characterize the community. This could be then followed by generation and application of FISH probes to confirm localization and suggest relationships between different organisms.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Appendix B. Anaerobic Digestion

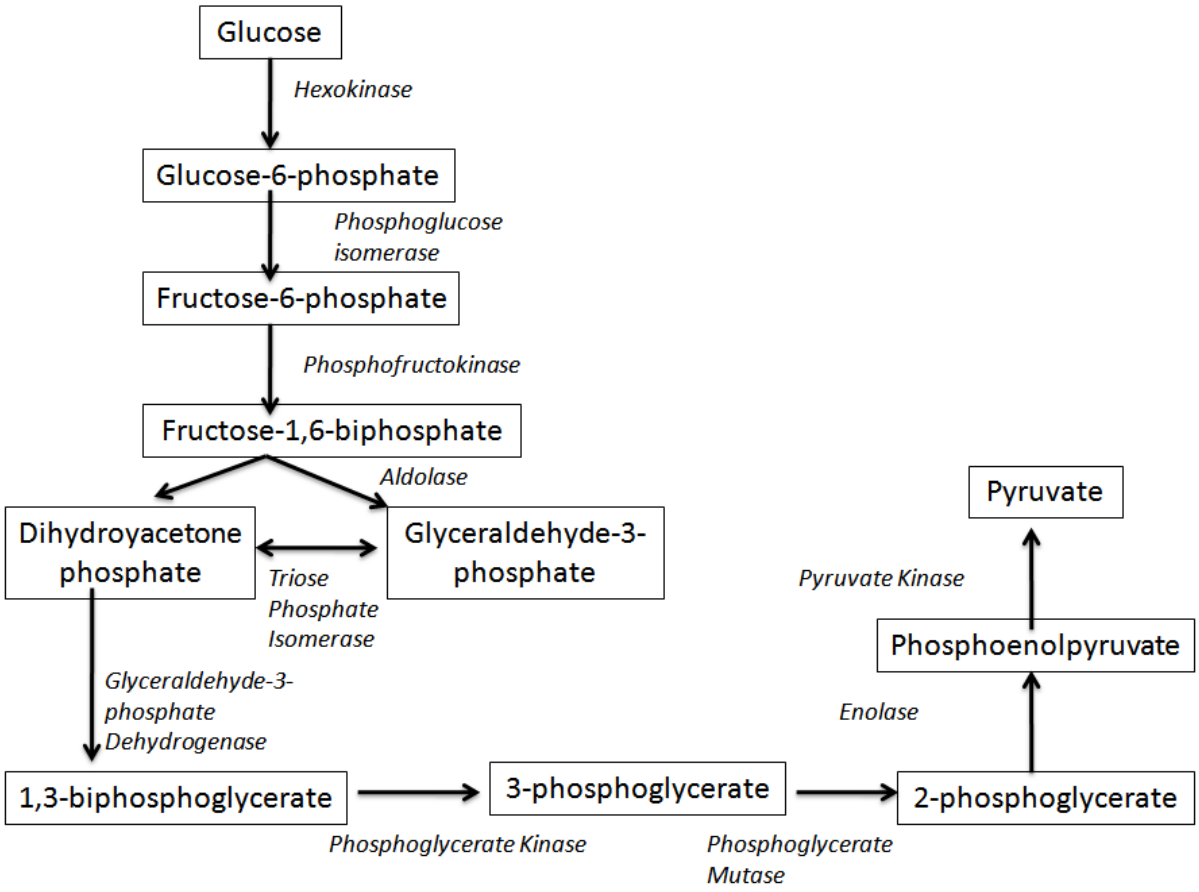

Figure B1. Embden-Meyerhof-Parnas pathway (EMP pathway) of glycolysis.

Table B1. Stoichiometric equations for major amino acid degradation pathway.

$C_6H_{13}O_2N$ (Leu) + 2 H_2O ⟶	$C_5H_{10}O_2$ (3-methylbutyrate) + NH_3 + CO_2 + $2H_2$ +ATP
$C_6H_{13}O_2N$ (Leu) + H_2 ⟶	$C_6H_{12}O_2$ (4-methylvalerate) + NH_3
$C_6H_{13}O_2N$ (Ile) + 2 H_2O ⟶	$C_5H_{10}O_2$ (2-methylbutyrate) + NH_3 + CO_2 + $2H_2$ +ATP
$C_5H_{11}O_2N$ (Val) + 2 H_2O ⟶	$C_4H_8O_2$ (3-methylpropionate) + NH_3 + CO_2 + $2H_2$ +ATP
$C_9H_{11}O_2N$ (Phe) + 2 H_2O ⟶	$C_8H_8O_2$ (phenylacetate) + NH_3 + CO_2 + $2H_2$ +ATP
$C_9H_{11}O_2N$ (Phe) + H_2 ⟶	$C_9H_{10}O_2$ (phenylpropanate) + NH_3
$C_9H_{11}O_2N$ (Phe) + 2 H_2O ⟶	C_6H_6 (phenol) +$C_2H_4O_2$ (acetate) + NH_3 + CO_2 + H_2 +ATP
$C_9H_{11}O_3N$ (Tyr) + 2 H_2O ⟶	$C_8H_8O_3$ (hydroxyphenyl acetate) + NH_3 + CO_2 + $2H_2$ +ATP
$C_9H_{11}O_3N$ (Tyr) + H_2 ⟶	$C_9H_{10}O_3$ (hydroxyphenyl propionate) + NH_3
$C_9H_{11}O_3N$ (Tyr) + 2 H_2O ⟶	C_6H_6 (cresol) + $C_2H_4O_2$ (acetate) + NH_3 + CO_2 + H_2 +ATP
$C_{11}H_{12}O_2N_2$ (Trp) + 2 H_2O ⟶	$C_{10}H_9O_2N$ (indole acetate) + NH_3 + CO_2 + $2H_2$ +ATP
$C_{11}H_{12}O_2N_2$ (Trp) + H_2 ⟶	$C_{11}H_{11}O_2N$ (indole propionate) + NH_3
$C_{11}H_{12}O_2N_2$ (Trp) + 2 H_2O ⟶	C_8H_7N (indole) + $C_2H_4O_2$ (acetate) + NH_3 + CO_2 + H_2 +ATP
$C_2H_5O_2N$ (Gly) + H_2O ⟶	$C_2H_4O_2$ (acetate) + NH_3
$C_2H_5O_2N$ (Gly) + ½ H_2O ⟶	¾ $C_2H_4O_2$ (acetate) + NH_3 + ½ CO_2 + ¼ ATP
$C_3H_7O_2N$ (Ala) + 2 H_2O ⟶	$C_2H_4O_2$ (acetate) + NH_3 + CO_2 + $2H_2$ +ATP
$C_3H_6O_2NS$ (Cys) + 2 H_2O ⟶	$C_2H_4O_2$ (acetate) + NH_3 + CO_2 + H_2S + ½ H_2 +ATP
$C_5H_{12}O_2NS$ (Met) + 2 H_2O ⟶	$C_3H_6O_2$ (propionate) + NH_3 + CO_2 + CH_4S + H_2 +ATP
$C_3H_7O_3N$ (Ser) + H_2O ⟶	$C_2H_4O_2$ (acetate) + NH_3 + CO_2 + H_2 +ATP
$C_4H_9O_3N$ (Thr) + H_2O ⟶	$C_3H_6O_2$ (propionate) + NH_3 + CO_2 + H_2 +ATP
$C_4H_9O_3N$ (Thr) + H_2O ⟶	2 $C_2H_4O_2$ (acetate) + NH_3
$C_4H_9O_3N$ (Thr) + H_2 ⟶	$C_2H_4O_2$ (acetate) + ½ $C_4H_8O_2$ (butyrate) + NH_3 +ATP
$C_4H_7O_4N$ (Asp) + 2 H_2O ⟶	$C_2H_4O_2$ (acetate) + NH_3 + 2 CO_2 + 2 H_2 +2 ATP
$C_5H_9O_4N$ (Glu) + H_2O ⟶	$C_2H_4O_2$ (acetate) + ½ $C_4H_8O_2$ (butyrate) + NH_3 + CO_2 + 2 ATP
$C_5H_9O_4N$ (Glu) + 2 H_2O ⟶	2 $C_2H_4O_2$ (acetate) + NH_3 + CO_2 + H_2 +2 ATP
$C_6H_9O_2N_3$ (His) + 4 H_2O ⟶	CH_3ON (formamide) + $C_2H_4O_2$ (acetate) + ½ $C_4H_8O_2$ (butyrate) + 2 NH_3 + CO_2 + 2 ATP
$C_6H_9O_2N_3$ (His) + 5 H_2O ⟶	CH_3ON (formamide) + $C_2H_4O_2$ (acetate) + 2 NH_3 + CO_2 + H_2 + 2 ATP
$C_6H_{14}O_2N_4$ (Arg) + 6 H_2O ⟶	2 $C_2H_4O_2$ (acetate) + 4 NH_3 + 2 CO_2 + 3 H_2 + 2 ATP
$C_6H_{14}O_2N_4$ (Arg) + 3 H_2O ⟶ + H2	½ $C_2H_4O_2$ (acetate) + ½ $C_3H_6O_2$ (propionate) + ½ $C_5H_{10}O_2$ (valerate) + 4 NH_3 + CO_2 + 2 ATP
$C_5H_9O_2N$ (Pro) + H_2O + H_2 ⟶	½ $C_2H_4O_2$ (acetate) + ½ $C_3H_6O_2$ (propionate) + ½ $C_5H_{10}O_2$ (valerate) + 4 NH_3 + CO_2 + ATP
$C_6H_{14}O_2N_2$ (Lys) + 2 H_2O ⟶	$C_2H_4O_2$ (acetate) + $C_4H_8O_2$ (butyrate) + 2 NH_3 + ATP

Table B2. Classification of anaerobic bacteria which degrade amino acid.

	Group Species	Enzyme Production	Amino Acids Utilised	Characteristics
I	*C.bifermentans*	proteo/saccharolytic	proline, serine, arginin, glycine, leucine, isoleucine, valine, ornithine, lysine, alanine, cysteine, methionine, aspartate, threonine, phenylalanine, tyrosine, tryptophan, and glutamate	organism that carry out Stickland reaction; proline utilised by all species; δ-aminovalerate, α-aminobutyrate and γ-aminobutyrate produced.
	C.sordellii	proteo/saccharolytic		
	C.botulinum types A,B,F	proteo/saccharolytic		
	C.caloritolerans	-		
	C.sporogenes	proteo/saccharolytic		
	C.cochlearium	-		
	C.difficile	saccharolytic		
	C.putrificum	proteo/saccharolytic		
	C.sticklandii	-		
	C.ghoni	proteolytic		
	C.mangenotii	proteolytic		
	C.scatologenes	saccharolytic		
	C.aerofoetidum	proteo/saccharolytic		
	C.butyricum	-		
	C.caproicum	saccharolytic		
	C.carnofoetidum	-		
	C.indolicum	-		
	C.mitelmanii	-		
	C.saprotoxicum	-		
	C.valerianicum	-		
II	*C.botulinum types C*	proteo/saccharolytic	glysine, arginie, histidine and lysine	glycine used by all species; δ-aminovalerate not produced.
	C.histolyticum	proteolytic		
	C.cochlearium	-		
	C.subterminale	proteolytic		
	C.botulinum types G	-		
	P.anaerobius	-		
	P.variabilis	-		
	P.micros	-		
III	*C.cochlearium*	-	glutamate, serine, histidine, arginine, aspartate, threonine, tyrosine, tryptophan and cysteine	δ-aminovalerate not produced; histidine, serine and glutamate used by all species.
	C.tetani	proteolytic		
	C.tetanomorphum	saccharolytic		
	C.lentoputrescens	-		
	C.limosum	proteolytic		
	C.malenomenatum	-		
	C.microsporum	-		
	C.perfringens	proteo/saccharolytic		
	C.butyricum	saccharolytic		
	P.asaccharolyticus	-		
	P.prevotii	-		
	P.activus	-		
IV	*C.putrefaciens*	proteolytic	serine and threonine	δ-aminovalerate not produced.
V	*C.propionicum*	-	alanine, serine, threonine, cysteine and methionine	δ-aminovalerate not produced.

Appendix C. Statistical Analysis on Pyrosequencing.

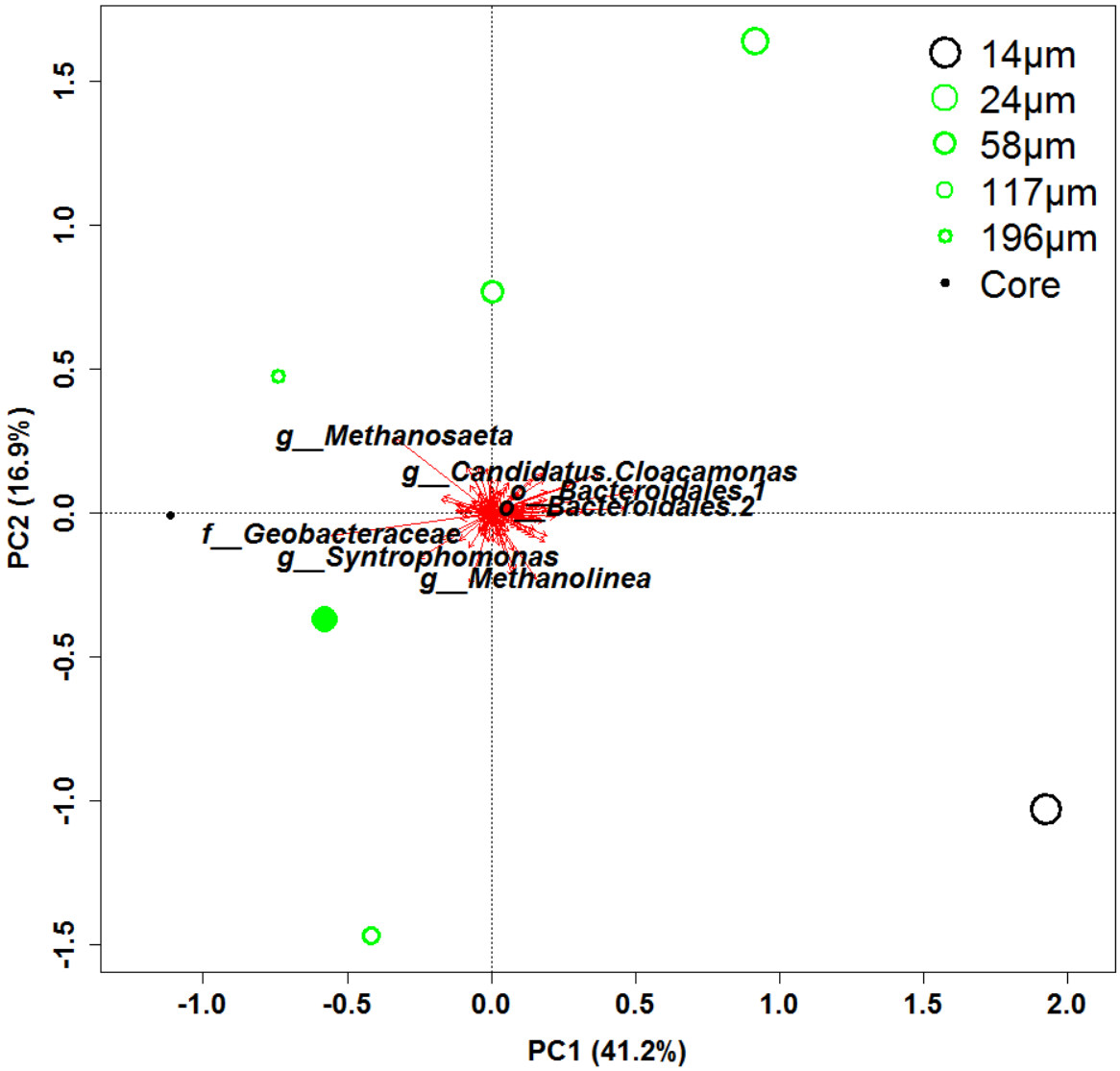

Figure C1. PCA analysis on pyrosequencing data from layers of brewery granules.

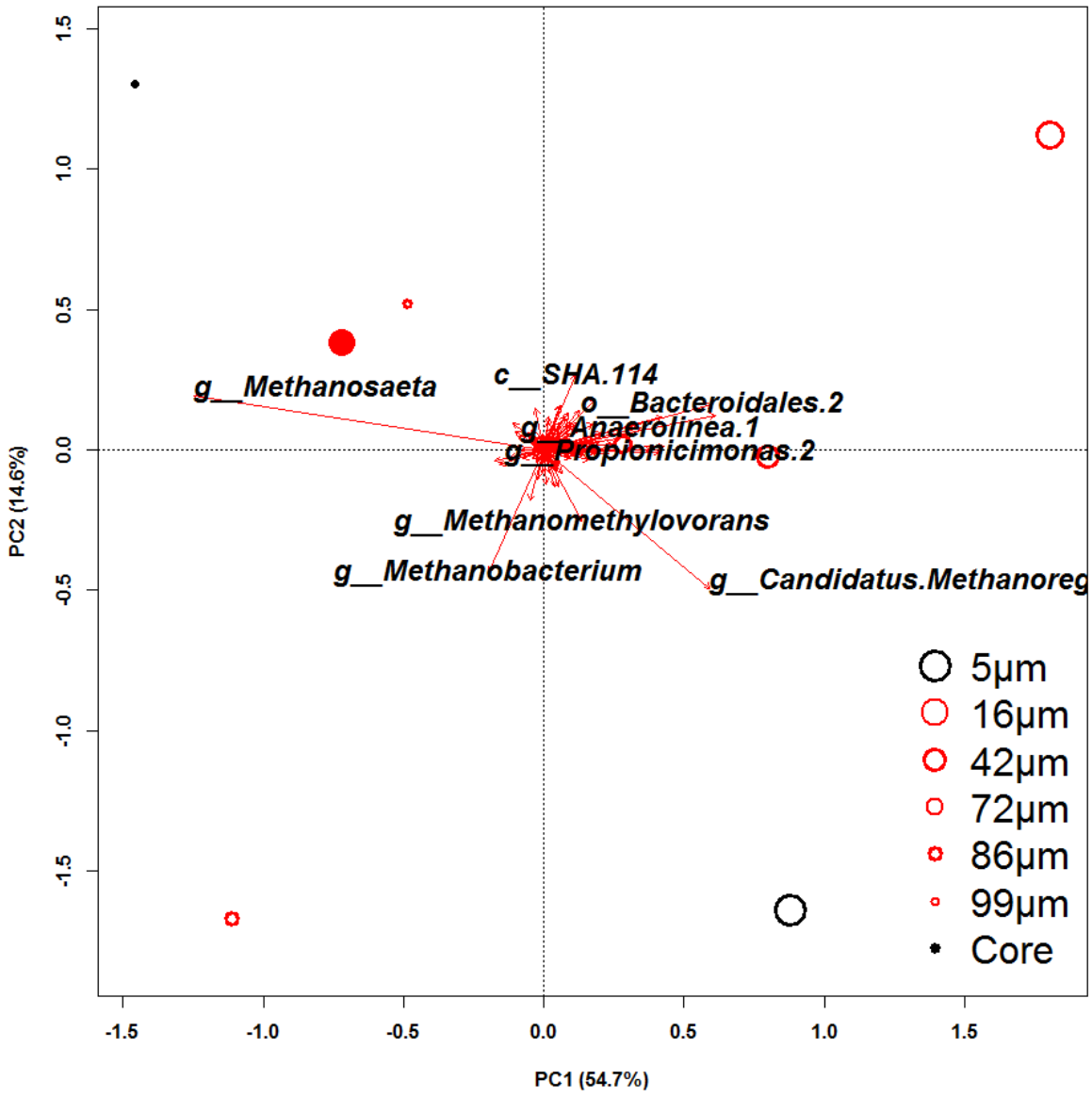

Figure C2. PCA analysis on pyrosequencing data from layers of cannery granules.

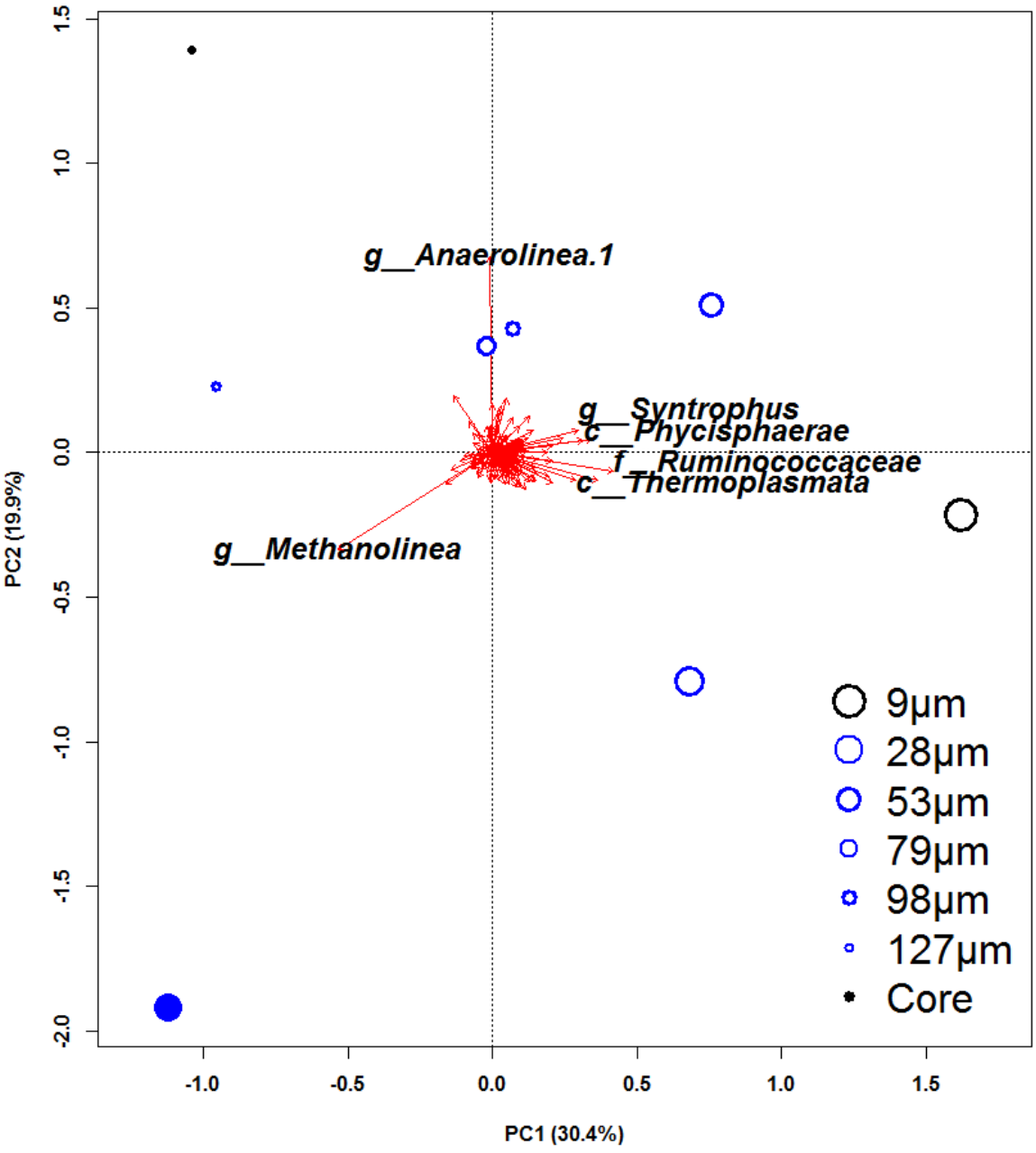

Figure C3. PCA analysis on pyrosequencing data from layers of dairy granules.

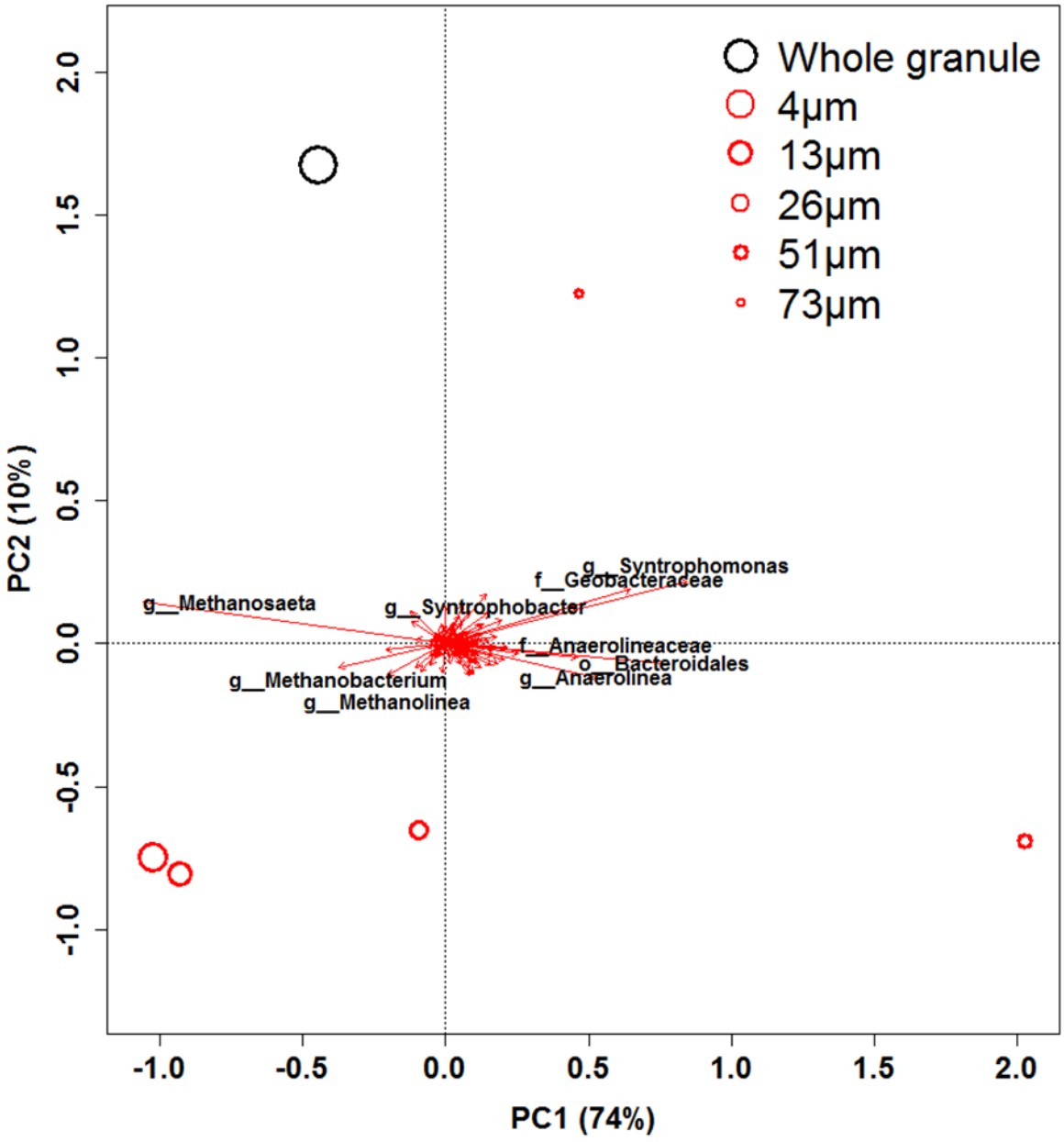

Figure C4. PCA analysis on pyrosequencing data from layers of control granules.

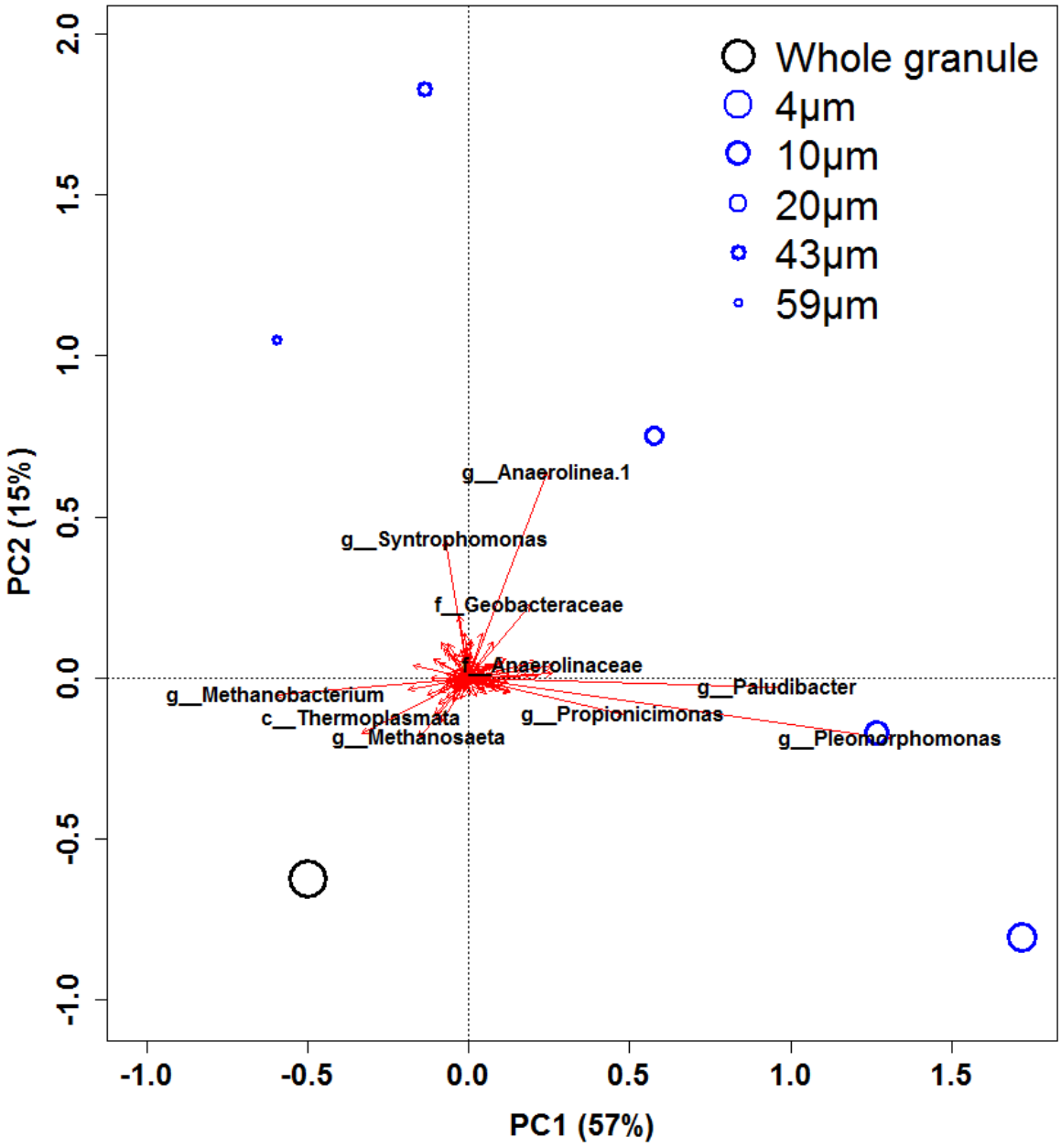

Figure C5. PCA analysis on pyrosequencing data from layers of glucose granules.

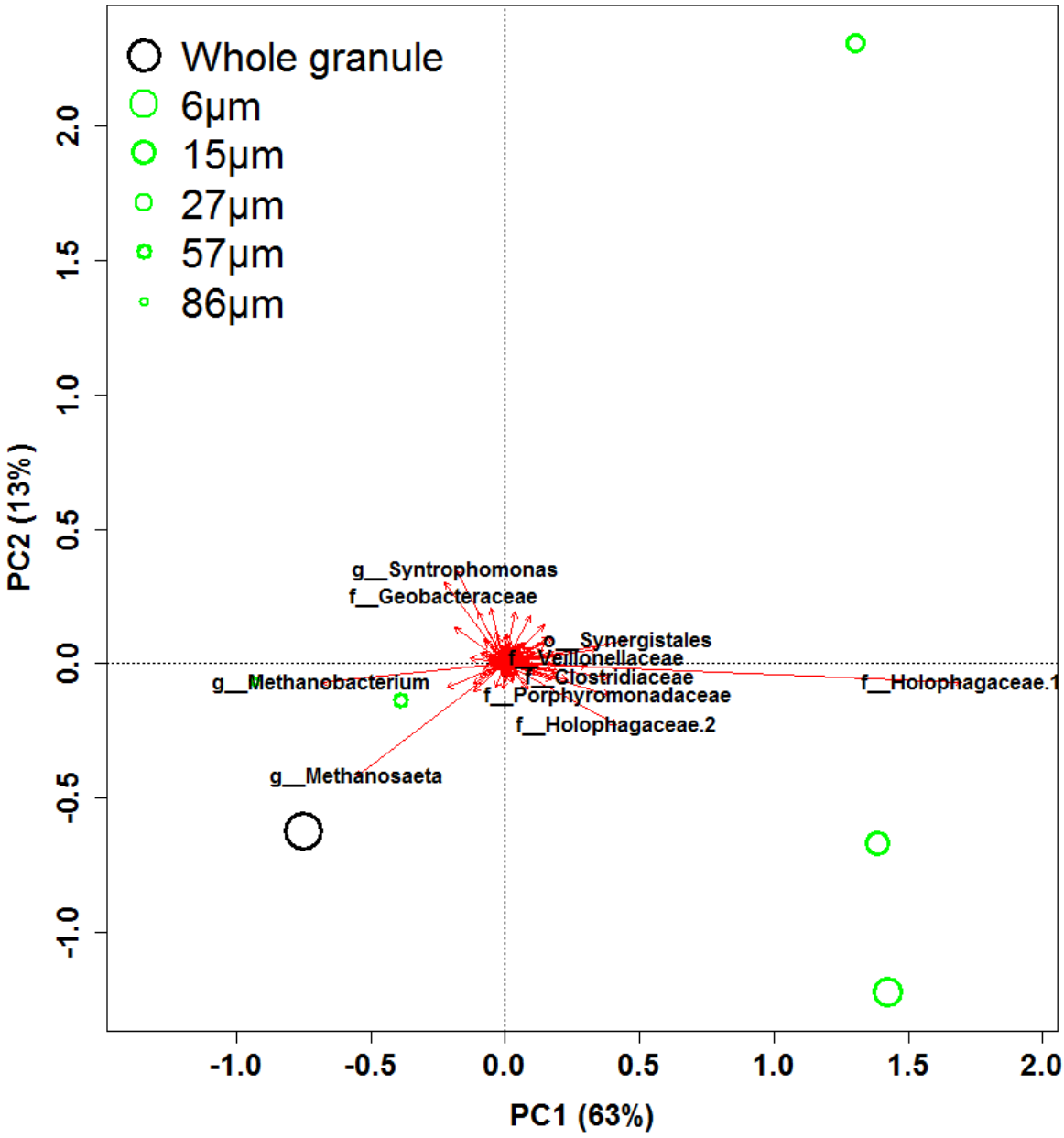

Figure C6. PCA analysis on pyrosequencing data from layers of gelatin granules.

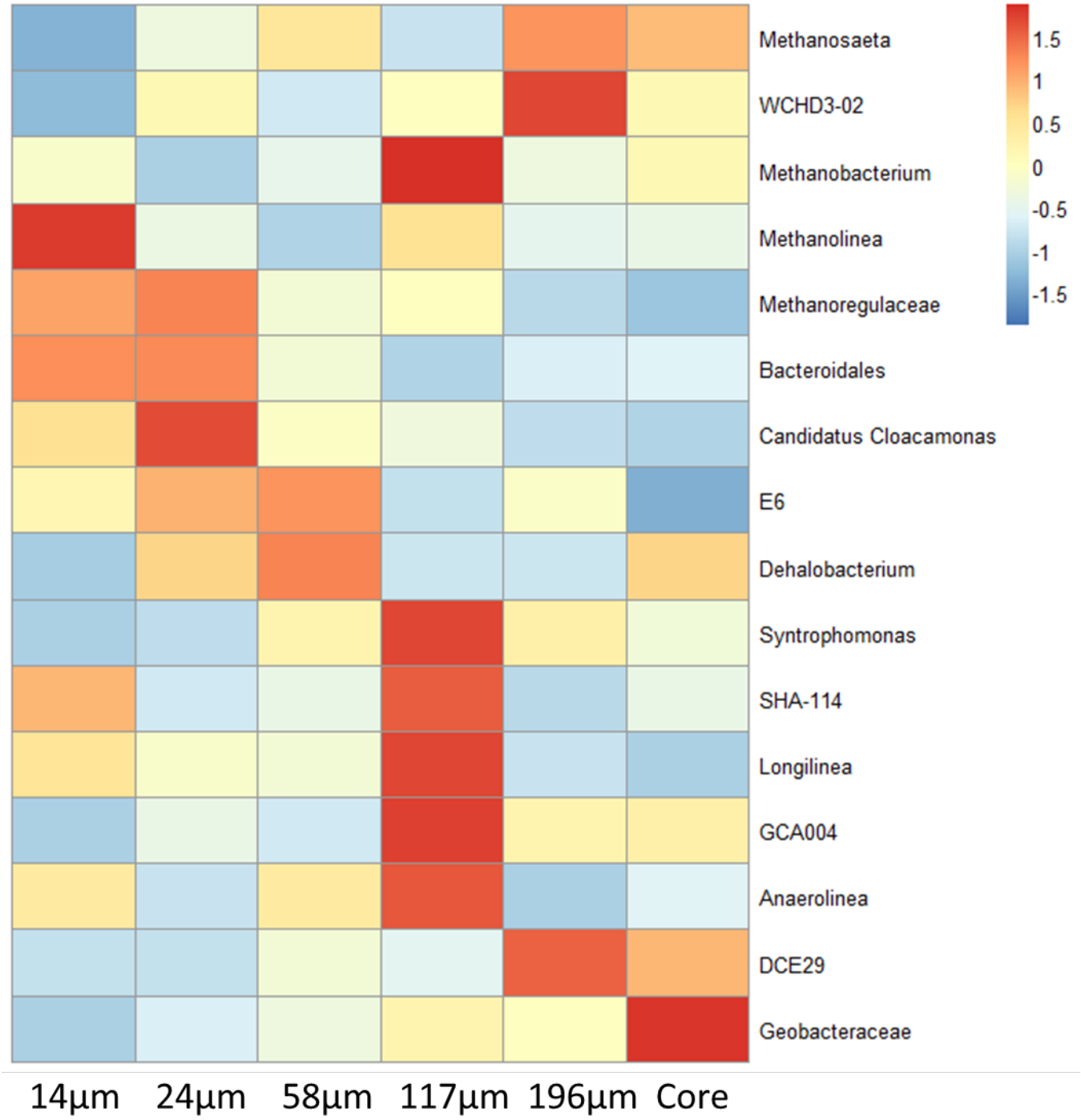

Figure C7. Heatmap analysis on top 20 OTUs of pyrosequencing data from layers of brewery granules.

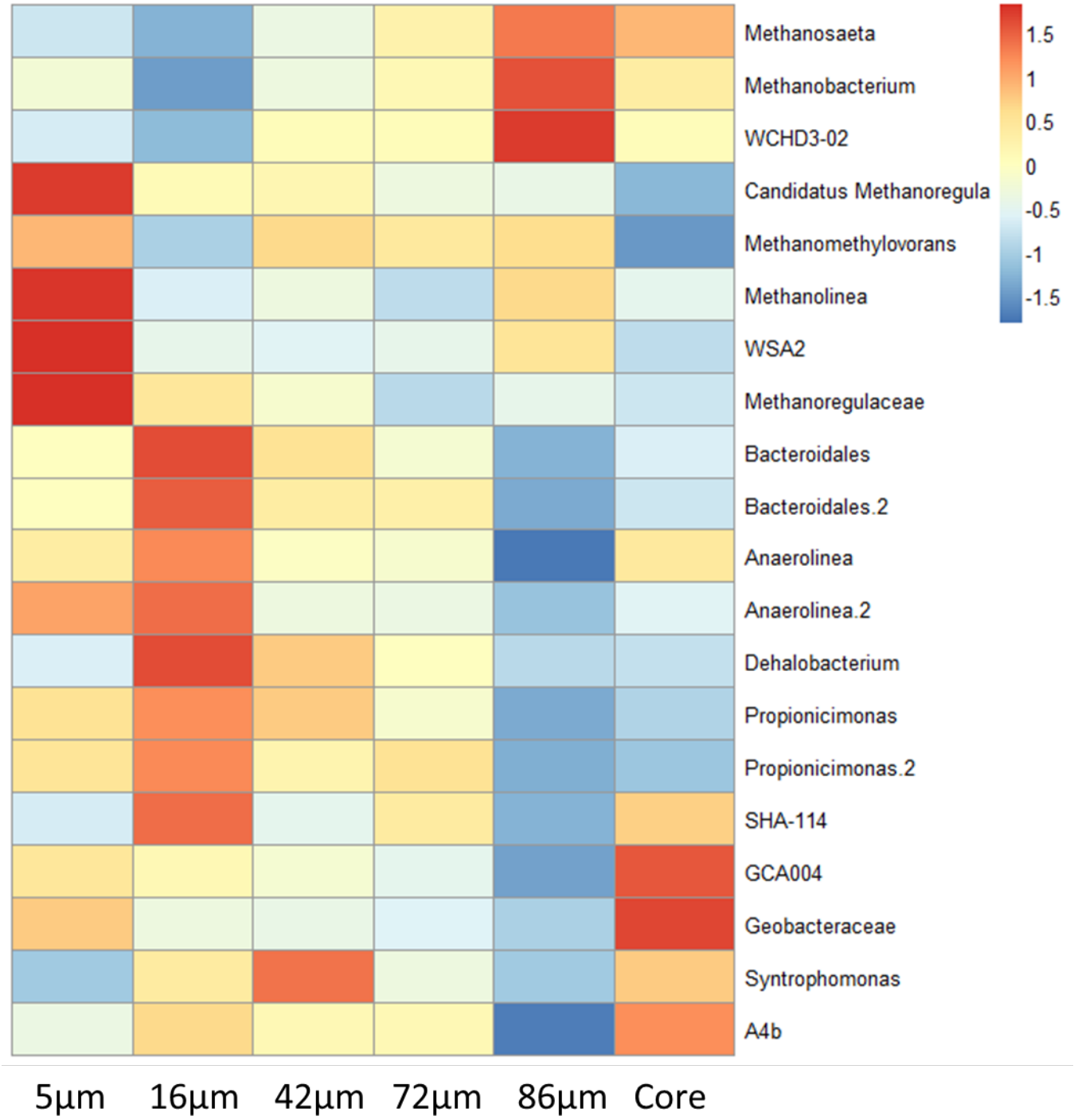

Figure C8. Heatmap analysis on top 20 OTUs of pyrosequencing data from layers of cannery granules.

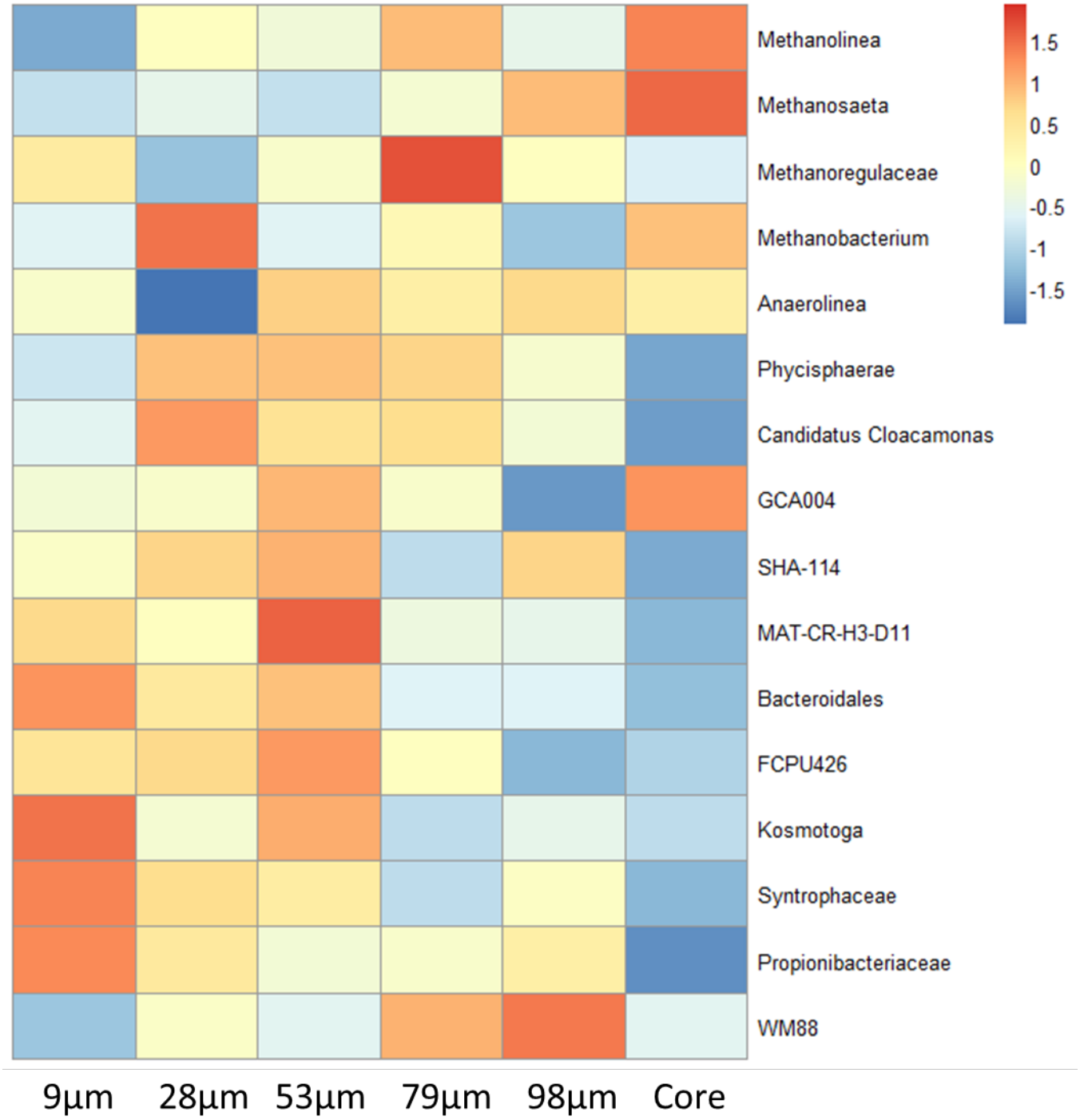

Figure C9. Heatmap analysis on top 20 OTUs of pyrosequencing data from layers of dairy granules.

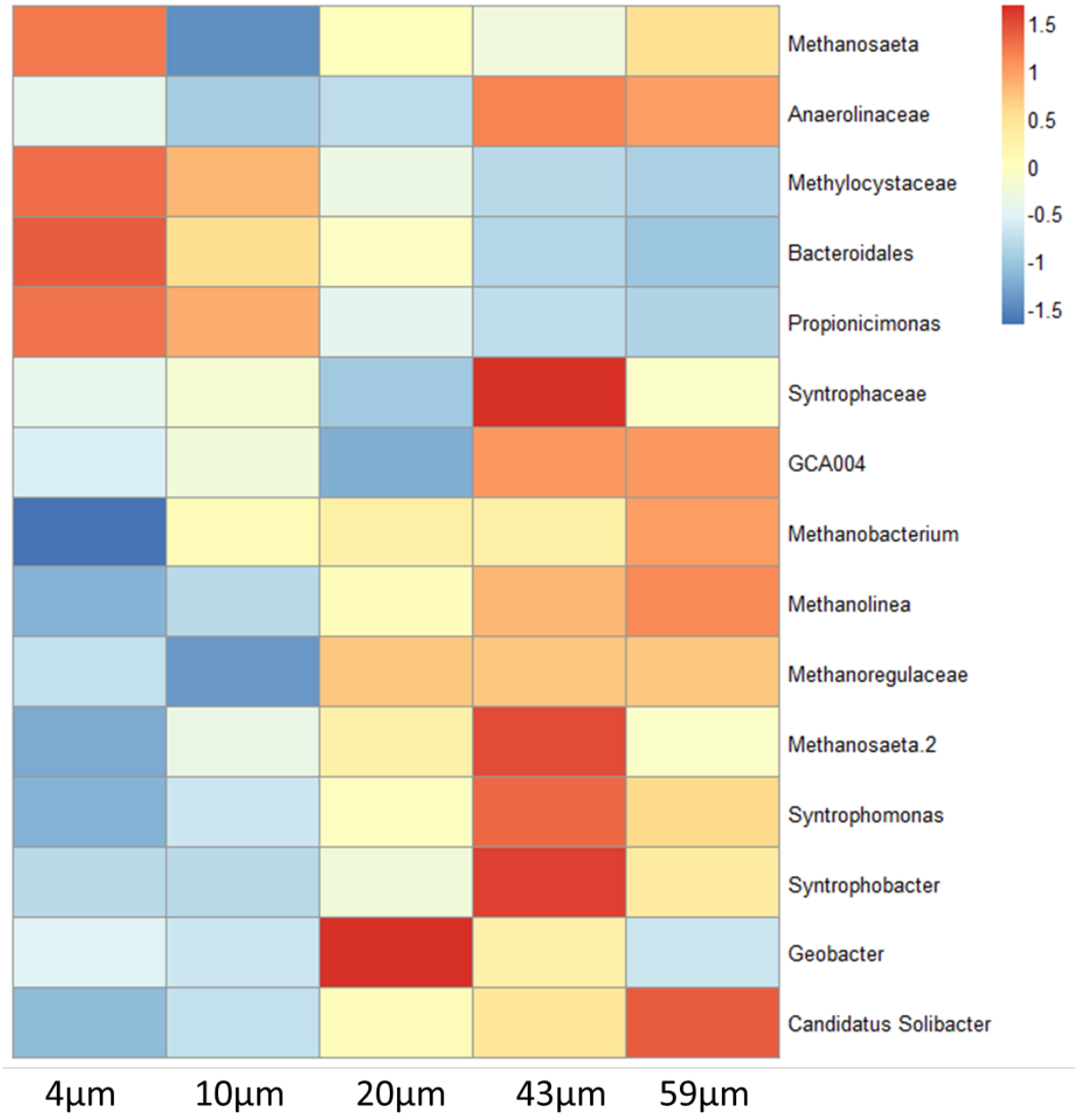

Figure C10. Heatmap analysis on top 20 OTUs of pyrosequencing data from layers of glucose granules.

Appendix D. Chemical analysis of lab-scale UASB operation.

Table D1. Chemical analysis of lab-scale UASB fed with acetic acid and propionic acid (control granules).

	FEED					Effluent					Biogas			
Days Operating	Volume	TCOD	sCOD	Acetic acid	Propionic acid	Volume	TCOD	sCOD	Acetic acid	Propionic acid	Volume	H2	CH4	CO2
	L	$mg \cdot L^{-1}$	$mg \cdot L^{-1}$	$mg \cdot L^{-1}$	$mg \cdot L^{-1}$	L	$mg \cdot L^{-1}$	$mg \cdot L^{-1}$	$mg \cdot L^{-1}$	$mg \cdot L^{-1}$	L	%	%	%
0														
1	0.500	0	0			0.50	0.00	0.00			0.000			
3	0.303	195	114	0.00	0.00	0.21	24.00	18.00	0.00	0.00	0.000			
4	0.398	1079	1055	648.58	229.20	0.32	35.00	22.00	0.00	0.00	0.000	0.2765	0.6778	0
5	0.593	1025	1027	603.86	217.35	0.53	44.00	17.00	0.00	0.00	0.000	0.1332	49.6035	2.2482
6	0.688	997	1006	571.77	206.01	0.58	52.00	16.00	1.21	0.00	0.000	0	73.2973	0
11	-0.713	1262	1227	638.54	282.55	0.62	121.00	20.00	1.63	0.00	0.112	0	72.6402	0
12	0.689	1208	1215	618.92	273.13	0.62	40.00	18.00	0.82	0.00	0.136	0	70.6774	2.3559
13	0.869	1214	1096	628.88	269.89	0.78	23.00	6.00	0.00	0.00	0.212	0.05	70.5576	0.8217
16	0.588	881	853	542.99	182.30	0.58	104.00	22.00	0.00	0.00	0.072	0	30.5729	4.6016
17	0.561	1027	961	624.18	209.72	0.33	44.00	15.00	1.52	0.00	0.224	0.0088	35.585	0
23	0.663	1184	1182	712.91	247.20	0.60	60.00	20.00	1.99	0.00	0.140	0	68.9859	0
24	0.614	1140	1168	682.99	239.90	0.57	50.00	20.33	1.88	0.00	0.032	0	69.9309	0
26	3.246	1029	1030	622.64	219.69	0.63	35.00	10.00	2.43	0.00	0.100	0.7939	71.9274	5.5038
28	1.603	961	964			0.78	123.00	15.00			0.148			
32	0.872	978	1085			0.84	79.00	14.00			0.192	0	74.2094	0
34	1.192	1018	1013			0.86	39.00	15.00			0.176	0	76.2507	0
47	0.545	827	793			1.81	112.00	9.00			0.168	0	43.3952	3.4877
48	0.379	895	1212			0.14	62.00	3.00			0.196	0	48.6517	1.8429
49	0.576	1802	1811			0.18	36.00	8.00			0.248	0	47.6664	0
50	0.436	1511	1972			0.08	51.00	10.00			0.260	0	45.6159	1.4587
51	0.713	1005	1693			0.25	52.00	15.00			0.336	0	54.3881	1.5895
52	0.362	1179	1845			0.08	52.00	16.00			0.344	0	54.5317	1.5589

Table D2. Chemical analysis of lab-scale UASB fed with glucose (glucose granules).

	FEED					Effluent						Biogas			
Days Operating	Volume	TCOD	sCOD	Glucose	Acetic acid	Volume	TCOD	sCOD	Glucose	Acetic acid	Propionic acid	Volume	H2	CH4	CO2
	L	$mg \cdot L^{-1}$	$mg \cdot L^{-1}$	$mg \cdot L^{-1}$	$mg \cdot L^{-1}$	L	$mg \cdot L^{-1}$	$mg \cdot L^{-1}$	$mg \cdot L^{-1}$	$mg \cdot L^{-1}$	$mg \cdot L^{-1}$	L	%	%	%
0															
1	0.50	1128.33	1081.67	897.46	0.00	0.50	52.00	32.00	0.00	0.34	0.00	0.134	0.5966	25.749	13.9426
3	0.29	1114.00	1129.67	875.81	3.93	0.27	61.00	40.00	0.00	0.85	0.00	0.002			
4	0.39	1339.00	1266.00	1038.94	3.81	0.31	41.00	28.00	0.00	0.00	0.00	0.008	0.082	36.0014	5.6132
5	0.29	728.33	150.00	72.85	0.00	0.26	32.67	27.00	0.00	0.00	0.00	0.008	0.0927	29.797	4.5988
6	0.53	1198.00	1093.00	837.91	36.02	0.50	41.33	30.00	0.00	0.00	0.00	0.000	0.0666	37.5413	6.7163
11	0.56	1164.00	1160.67	992.98	0.00	0.54	46.33	26.00	0.00	0.00	0.00	0.064	0	43.9503	10.9915
12	-0.15	1334.00	1307.00	1072.73	0.00	0.48	38.00	21.00	0.00	0.44	0.00	0.044	0.0572	46.2062	10.25
13	0.57	1374.67	1257.67	1058.38	0.00	0.47	27.00	10.00	0.00	0.00	0.00	0.024	0.0533	43.7534	8.8221
16	0.44	1228.33	1291.00	1138.62	0.00	0.38	40.00	25.00	0.00	2.72	0.46	0.002	0	66.9909	7.0536
17	0.41	1290.00	1231.33	1035.88	0.00	0.16	39.00	19.00	0.00	0.00	0.00	0.160	0.1132	6.4709	3.0308
23	0.73	1191.67	1274.33	957.49	21.67	0.69	144.00	81.00	0.00	0.00	0.00	0.146	1.275	57.5176	17.8273
24	0.77	1173.67	1219.67	964.75	0.00	0.68	0.00	0.00	0.00	40.08	3.73	0.090	0.3476	56.5925	17.2513
26	1.96	1126.33	1190.00	996.91	0.97	0.82	0.00	306.00	0.00	91.61	11.37	0.116	3.2311	38.7325	15.6218
28	1.60	1119.33	1174.33			0.77	86.00	36.00	0.00	169.37	29.01	0.196			
32	0.88	1112.33	1127.33			0.83	666.00	477.00				0.042	2.9292	51.1814	11.723
34	1.21	1185.67	1152.67			0.77	923.33	582.00				0.034	2.1864	36.9693	10.7074
36	0.63	516.33	543.00			0.60	220.50	116.00				0.004	0	18.1722	7.7295
37	0.57	558.00	523.33			0.68	144.00	68.00				0.078	0.0714	35.9797	10.5095
38	0.65	1145.00	1260.67			0.71	165.00	113.00				0.186	0.3005	51.0316	12.3056
39	0.50	660.00	847.00			0.53	222.00	110.00				0.286	0.8156	59.0849	12.9284
40	0.86	523.00	536.67			0.94	186.00	57.00				0.400	0.2565	61.5449	1.515
41	0.49	745.33	745.33			0.54	143.00	63.00				0.470	0.0845	63.4572	12.0411

Table D3. Chemical analysis of lab-scale UASB fed with gelatine (gelatine granules).

	FEED								Effluent								Biogas			
Days Operating	Volume	TCOD	sCOD	TKN	NH3	TP	TProtein	sProtein	Volume	TCOD	sCOD	TKN	NH3	TP	Tprotein	sProtein	Volume	H2	CH4	CO2
	L	$mg{\cdot}L^{-1}$	$mg{\cdot}L^{-1}$	$mg{\cdot}L^{-1}$ N	$mg{\cdot}L^{-1}$ N	$mg{\cdot}L^{-1}$ P	$ug{\cdot}ml^{-1}$	$ug{\cdot}ml^{-1}$	L	$mg{\cdot}L^{-1}$	$mg{\cdot}L^{-1}$	$mg{\cdot}L^{-1}$ N	$mg{\cdot}L^{-1}$ N	$mg{\cdot}L^{-1}$ P	$ug{\cdot}ml^{-1}$	$ug{\cdot}ml^{-1}$	L	%	%	%
0																				
1	0.500	1576.33	1932.67	0.22	5.81	0.00	103	39	0.500	202	40	0.65	6.91	3.63	96	87	0.022	0.105	55.824	10.294
3	0.634	1044.33	982.00	0.21	0.00	0.00	580	575	0.676	106	13	0.39	1.78	0.00	31	31	0.098	0.269	60.126	18.431
4	0.836	957.67	859.00	0.00	0.00	0.00	606	560	0.845	75	24	0.58	3.53	1.37	94	52	0.126	0.000	69.106	8.064
5	0.659	931.67	870.00	0.00	0.02	0.00	596	564	0.600	70	23	0.36	2.73	0.00	76	36	0.330	0.077	56.768	6.897
6	0.779	214.00	149.00	0.00	0.00	0.00	148	100	0.868	53	28	0.00	1.97	0.00	98	58	0.110	0.000	71.351	7.864
11	0.827	997.00	930.00	0.00	0.00	0.00	834	774	0.884	107	44	0.00	0.00	0.00	93	44	0.126	0.000	67.827	8.313
12	0.769	1039.67	1004.67	0.00	1.60	0.00	829	789	0.920	119	49	0.00	1.61	0.00	132	62	0.140	0.044	67.133	8.237
13	0.957	120.33	1012.67	0.00	0.00	0.00	840	664	0.970	99	0	0.00	1.77	0.79	31	31	0.124	0.000	66.744	8.207
16	0.774	1009.00	953.33	0.00	0.00	0.00	765	758	0.790	99	33	0.00	2.79	0.95	132	47	0.140	0.004	70.139	0.000
17	0.678	1003.00	968.00	0.00	16.57	0.00	866	841	0.557	66	29	0.00	2.50	0.00	91	59	0.074	0.000	38.467	5.778
23	0.698	1108.67	1096.00	0.00	0.00	0.00	639	607	0.736	99	43	0.00	2.72	0.83	94	49	0.186	0.150	49.207	8.533
24	0.841	1077.00	1031.67	0.00	26.20	7.19	691	668	0.819	69	24	0.00	5.24	0.00	99	42	0.136	0.000	56.511	8.211
26	2.029	1080.67	1023.67	0.00	0.00	0.00	648	620	0.858	68	23	0.00	2.49	0.96	101	39	0.156	0.000	53.672	7.871
28	1.675	1093.67	1049.00	0.00	0.00	0.00	780	718	0.851	129	23	0.00	3.01	2.00	156	38	0.142			
32	0.972	1118.33	1071.33				699	550	0.952	122	26				149	44	0.202	0.000	71.649	8.827
34	1.298	1102.33	1052.33				744	749	0.985	68	26				89	97	0.100	0.000	67.352	8.407
47	0.691	954.00	942.00				888	848	0.675	168	30				581	66	0.148	0	38.6903	5.0172
48	0.654	1035.00	1015.00				666	650	0.837	105	22				126	31	0.296	0	64.0452	7.5251
49	0.706	1340.67	1390.67				694	756	1.013	132	23				165	52	0.414	0	61.7716	8.6893
50	0.650	1047.67	927.33				699	701	0.818	129	25				216	79	0.574	0.0473	68.3155	8.1466
51	0.835	1031.33	968.67				774	775	1.097	118	28				113	31	0.726	0	66.7782	7.9546
52	0.578	1291.33	968.67				916	733	0.770	66	27				31	31	0.796	0	71.6418	8.0886