

UASB GRANULATION ENHANCEMENT BY MICROBIAL INOCULUM SELECTION AND PROCESS INDUCTION

by

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DECLARATION

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ABSTRACT

In the absence of anaerobic granules, anaerobically digested sewage sludge is frequently used to seed industrial upflow anaerobic sludge blanket (UASB) reactors. Because of its flocculent nature, start-up with digested sludge instead of granular sludge proceeds much slower and presents various operational problems. Any manner in which the granulation of digested sludge can be enhanced would benefit UASB reactor start-up and application in developing countries such as South Africa.

The main objective of this dissertation was to improve granulation and reduce UASB reactor start-up by using pre-treated digested sludge as seed. The sludge was pre-treated based on the batch granulation-enhancement model of Britz *et al.* (2002). The main aim of the model was to improve extracellular polymer (ECP) production of lactate-utilising populations by applying short-term controlled organic overloading in a mechanically agitated environment.

The batch granulation-enhancement (pre-treatment) process was applied to an ECP-producing digester strain, *Propionibacterium jensenii* S1. Non-methanogenic aggregates were formed when batch units were incubated on a roller-table instead of a linear-shake platform. Larger, more stable aggregates were obtained in the presence of apricot effluent medium.

Preliminary batch granulation-enhancement studies confirmed that using the roller-table as mixing system had a positive influence on batch granulation-enhancement. The roller-table showed the most potential for handling larger volumes in comparison to a linear-shake waterbath and linear-shake platform. The addition of 450 mg.L⁻¹ Fe²⁺ at the start of the study also influenced aggregate numbers positively. These studies revealed that pre-treatment results varied depending on the seed sludge source.

A denaturing gradient gel electrophoresis (DGGE) method was applied for the detection of *Archaea* in digested sludges and UASB granules. In addition, a methanogenic marker containing methanogens important to the granulation process was constructed to aid identification. The positive influence of DMSO and “touchdown” PCR on the elimination of artifactual double bands in DGGE fingerprints were also demonstrated. Results revealed that only one of the four digested sludges tested contained *Methanosaeta concilii* (critical to granular nuclei formation) while it was present in all the UASB granules regardless of substrate type.

Four digested sludges were obtained from stable secondary digesters. DGGE indicated the presence of *M. concilii* in all sludges. The Athlone 4Sb-sludge was the only sludge which exhibited measurable methanogenic activity during substrate dependent activity testing. The ST-sludge showed the highest increase in volatile suspended solids (VSS) particles $\geq 0.25 \text{ mm}^2$. Laboratory-scale UASB reactor start-up was done with both sludges and start-up proceeded better in the Athlone 4Sb-reactor.

Athlone 4Sb-sludge batches were pre-treated in a rolling-batch reactor in the presence of either lactate or sucrose and used to seed lab-scale UASB reactors B (sucrose seed) and C (lactate seed). Start-up efficiencies were compared to a control (Reactor A). Overall Reactor B was more efficient than the control. At the end of the study the Reactor B sludge had a higher methanogenic activity than the control reactor. It also had the highest increase in VSS $\geq 1.0 \text{ mm}^2$. Pre-treatment of digested sludge in the presence of sucrose, therefore, aided granulation and reduced UASB reactor start-up time.

OPSOMMING

Anaërobe verteerde rioolslyk word dikwels gebruik as inokulum vir industriële opvloei-anaerobe-slyklaag-(UASB) reaktors in die afwesigheid van anaërobe granules. Vanweë die vlokkige aard daarvan neem reaktoraanvang met verteerde slyk in die plek van granulêre slyk dikwels langer en hou dit verskeie bedryfsprobleme in. Enige metode om die granulasie van verteerde slyk te verbeter, sou voordelig wees vir UASB-reaktoraanvang en -toepassing in ontwikkelende lande soos Suid-Afrika.

Die doelwit van hierdie proefskrif was om granulasie te verbeter en die UASB-reaktoraanvangsperiode te verminder deur voorafbehandelde verteerde slyk as inokulum te gebruik. Die slyk is vooraf behandel gebaseer op die lotgranulasieverbeteringsmodel van Britz *et al.* (2002). Die hoofdoel van die model was om ekstrasellulêre-polimeer- (ECP) produksie van laktaatbenutters te bevorder deur die toepassing van korttermyn gekontroleerde organiese oorbelading in 'n meganies vermengde omgewing.

Die lotgranulasieverbeteringsproses (voorafbehandeling) is toegepas op 'n ECP-produiserende organisme, *Propionibacterium jensenii* S1. Nie-metanogeniese korrels het gevorm tydens inkubasie van die loteenhede op 'n roltafel in plaas van 'n lineêre skudtafel. Groter en meer stabiele korrels is verkry in die teenwoordigheid van appelkoosuitvloeiensmedium.

Voorlopige lotgranulasieverbeteringstudies het bevestig dat die gebruik van die roltafel as vermengingsmetode 'n positiewe uitwerking op die proses gehad het. Die roltafel het die grootste potensiaal getoon vir die hantering van groter volumes in vergelyking met 'n lineêre skudwaterbad en lineêre skudtafel. Die byvoeging van $450 \text{ mg.L}^{-1} \text{ Fe}^{2+}$ aan die begin van die studie het ook korrelgetalle positief beïnvloed. Hierdie studies het getoon dat voorafbehandelingsresultate gewissel het, afhangend van die slykbron.

'n Denaturerende gradiënt jeelektroforese (DGGE) metode is aangewend vir die opsporing van *Archaea* in verteerde slyke en UASB-granules. Daar is ook 'n metanogeniese merker, wat metanogene bevat wat belangrik is in die granulasieproses, saamgestel om identifikasie te bevorder. Die positiewe invloed van DMSO en "touchdown"-PKR op die voorkoming van vals dubbelbande in DGGE-patrone is ook aangetoon. Resultate het bevestig dat slegs een van die vier getoetste verteerde slyke

Methanosaeta concilii (krities vir granulekernformasie) bevat het, terwyl dit aanwesig was in al die UASB-granules, ongeag die substraat tipe.

Vier verteerde slyke is verkry vanaf stabiele sekondêre verteerders. DGGE het die teenwoordigheid van *M. concilii* in al die slyke getoon. Die Athlone 4Sb-slyk was die enigste slyk wat meetbare metanogene aktiwiteit tydens substraatafhanklike aktiwiteitstoetsing getoon het. Die ST-slyk het die grootste vermeerdering in vlugtige gesuspendeerde vastestowwe (VSS) partikels $\geq 0.25 \text{ mm}^2$ getoon. Albei slyke is aangewend as inokulums tydens laboratoriumskaal UASB-reaktorstudies en die reaktoraanvangsproses het beter verloop in die Athlone 4Sb-reaktor.

Athlone 4Sb-slyklotte is vooraf behandel in 'n rol-lotreaktor in die aanwesigheid van óf laktaat óf sukrose en is gebruik om laboratoriumskaal UASB-reaktors B (sukrose-slyk) en C (laktaat-slyk) te inokuleer. Aanvangsdoeltreffendhede is vergelyk met 'n kontrole (Reaktor A). In geheel was Reaktor B meer doeltreffend as die kontrole. Aan die einde van die studie het die Reaktor B-slyk 'n hoër metanogene aktiwiteit gehad as die kontrolereaktor. Dit het ook die grootste toename in VSS $\geq 1.0 \text{ mm}^2$ getoon. Voorafbehandeling van verteerde slyk in die aanwesigheid van sukrose het dus granulasie bevorder en die UASB-reaktoraanvangstyd verminder.

Dedicated to my husband

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Language and style used in this dissertation are in accordance with the requirements of the *International Journal of Food Science and Technology*. This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

CHAPTER 1

INTRODUCTION

It has been estimated, based on population growth projections by the United Nations, that South Africa will face an absolute water scarcity by 2025. This implies that less than 1 000 m³ of renewable water per person per year will be available (Stikker, 1998; Seckler *et al.*, 1999). Within this water-stressed environment, reports about the deterioration of the South African water supply and sanitation infrastructure have recently been published (SAICE, 2006). It is inevitable that renewal of the public water and sanitation infrastructure will lead to substantial increases in the costs associated with water supply and effluent discharge.

The development of food production technologies leads to increases in water consumption as well as wastewater production. If these wastewaters are not managed and disposed of properly, further pollution of South Africa's scarce water resource might be unavoidable. Government regulations have been designed to impose restrictions on effluent disposal, and non-compliance might result in high disposal charges (Kroyer, 1995; Coetzee *et al.*, 2004). In order to reduce costs, South African food industries will need to consider investing in waste treatment strategies that would ultimately minimise waste, save water, recycle wastewater and, in light of the current national electricity shortages (Eskom, 2007, 2008a, 2008b), save electricity. Thus, food industries should strive for 'zero effluent discharge' and in the process reduce their ecological footprint.

Implementing anaerobic digestion technology as part of a waste treatment strategy has advantages as it is a relatively simple and compact technology with high COD removal efficiencies and low excess sludge production (which also has a market value). The methane produced can be used as a source of energy (potentially generating about 1.5 kWh electric energy per kg COD removed, assuming a 40% electric conversion efficiency from methane energy) (Kassam *et al.*, 2003; Van Lier, 2007). The combination of anaerobic technologies with a variety of pre- and post treatments can furthermore ensure stable and effective treatment and reuse of food industry waste streams (Van Lier, 2007; Britz *et al.*, 2008).

Internationally, granular sludge bed-based anaerobic technologies, such as the upflow anaerobic sludge bed (UASB) reactor, the expanded granular sludge bed reactor (EGSB) and the internal circulation (IC) reactor, are the preferred industrial applications

of anaerobic digestion (Van Lier, 2007). The successful operation of these installations is highly dependent on the self-aggregation of anaerobic bacteria into active and compact sludge granules.

Granules and granular seed sludge have several advantages over flocculent seed sludge. Granules have higher settling velocities and can withstand elevated gas and liquid shear rates due to high physical strength. Granules would, therefore, be better retained in an anaerobic reactor as the upflow liquid loading rate is increased. In granules, anaerobic bacteria are immobilised within a permanent extracellular polymer (ECP) matrix, which allows the development of syntrophic associations between microbial partners due to optimum positioning for substrate supply and assimilation. Sensitive bacteria such as methanogens are better protected against environmental shocks and toxins within this matrix. Granular sludge, therefore, has a higher specific methanogenic activity than flocculent sludge, which would result in a larger conversion of waste to methane (Wirtz & Dague, 1996; Batstone *et al.*, 2004). Reactors inoculated with granular seed sludge would thus be able to process larger volumes of wastewaters with higher organic loads than reactors inoculated with flocculent seed sludge.

Unfortunately, availability of suitable granular seeding material to ensure the rapid start-up of industrial installations is limited and purchase costs are high (Liu & Tay, 2004). The current international cost of good-quality granular sludge is EUR130–150 per m³ (D. Piet, Biothane Systems International, the Netherlands, personal communication, 2008). In South Africa, surplus granular sludge can occasionally be purchased from a limited number of anaerobic industrial installations at a current cost of around R800 per m³ (G.O. Sigge, Department of Food Science, University of Stellenbosch, Stellenbosch, South Africa, personal communication, 2008). These costs exclude transport and handling fees, which can be high if one considers that a 450-m³ industrial UASB reactor would need between 80 and 100 m³ good-quality granular sludge for start-up. The occasional process failure might also result in excessive granular sludge losses, and re-inoculation with granular inoculum would have additional financial implications.

In the absence of suitable granular biomass, digested sewage sludge is frequently used as seeding material because it is affordable and widely available. Reactor start-up with digested sludge proceeds very slowly, mostly as a result of sludge washout which can require re-inoculation. Granulation of digested sludge can take anything from three to eight months (Singh *et al.*, 1998; Liu & Tay, 2004; Zhou *et al.*,

2006). Any manner in which anaerobic granulation of municipal digested sludge can be enhanced could, therefore, promote the industrial application of granule-based anaerobic technologies in industrial effluent treatment not only in South Africa but all over the world.

It has also been reported that the rate of anaerobic sludge granulation is related to the microbial populations initially present in the seed sludge (Singh *et al.*, 1998). While certain species have been associated with initial granular nuclei formation, other members of the anaerobic consortium are responsible for ECP production, which is necessary for granule growth and maturation. Anaerobic granulation of digested sludge might be accelerated simply by manipulating the anaerobic populations present in the digested seed sludge (Liu & Tay, 2004). The batch granulation-enhancement model of Britz *et al.* (2002) is one such example where the authors focused specifically on enhancement of the lactate-utilising populations in order to increase ECP production and enhance granulation of digested sewage sludge in small batch units.

The primary objective of this dissertation was to enhance the anaerobic granulation of digested sewage sludge prior to UASB reactor seeding based on the batch granulation-enhancement model of Britz *et al.* (2002). To achieve this objective, different mixing systems for future scale-up were assessed, the methanogenic content and activity of digested seed sludge were considered and UASB reactor start-up efficiency with enhanced seed sludge was evaluated.

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CHAPTER 2

LITERATURE REVIEW*

A. BACKGROUND

Anaerobic digestion has successfully been implemented all over the world to treat various wastewaters of industrial and domestic origin (Verstraete *et al.*, 1996). Compared to conventional aerobic methods and as a result of the development of sustainable technologies, anaerobic wastewater treatment has several important advantages. These include the following: a) instead of consuming energy, useful energy is produced in the form of methane (CH₄) (about 13.5 MJ CH₄ energy per kg COD removed); b) the volume of surplus sludge produced is significantly lower (between 5 and 10 times less), the sludge has a high dewatering capacity and is well stabilised. The dry solids content of the anaerobic sludge varies between 2% (digester) to over 8% (upflow anaerobic sludge blanket [UASB] reactor), which is higher than the 0.5 - 2% of aerobic sludge; c) the sludge can generally remain stable when preserved unfed for long periods, without serious deterioration; d) anaerobic processes can be applied practically anywhere and at any scale. Very high space loading rates (up to 20 - 35 kg COD.m⁻³ reactor volume per day) can be applied to modern UASB treatment systems so that the space requirements of the system are relatively small; e) the digestion process may be implemented at relatively low costs because technologically simple and relatively inexpensive reactors, that can operate with little energy, can be used; and f) anaerobic wastewater treatment can be combined with post-treatment methods by which useful products such as ammonia or sulphur can be recovered (Lettinga, 1995; Verstraete *et al.*, 1996; Anon., 2002; Van Lier, 2007).

Despite these advantages, however, anaerobic digestion cannot provide a complete treatment. The known disadvantages of anaerobic treatment include the

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following: a) the relatively high susceptibility of methanogens and acetogens to a variety of toxic substances; b) the presumed low stability of anaerobic treatment, which in the past was mainly the result of a lack of fundamental knowledge by the supervising engineers and operators; c) the slow start-up period (at least 3 - 5 months) of new full-scale installations (Lettinga, 1995); and d) low nutrient removal (nitrogen and phosphorous) as a result of the low biosolids production (Anon., 2002). According to Lettinga (1995), these drawbacks are not serious and will gradually disappear. Long start-up periods can be reduced significantly if large quantities of highly active anaerobic granular sludge from existing industrial reactors are used as seed sludge. Although this is an obvious choice in developed countries where industrial-scale reactors are fairly common, access to high-quality granular sludge still poses a serious problem in developing countries (Britz *et al.*, 1999). The application of anaerobic technology in developing countries would, therefore, greatly benefit from any technology that could reduce the start-up times of anaerobic digesters inoculated with seeding material other than granular sludge.

B. HISTORY OF UASB REACTOR TECHNOLOGY

The development of the UASB reactor by Prof. Gatzke Lettinga and the Wageningen research group was the most important development in the field of anaerobic wastewater treatment. In this reactor type, wastewater is pumped upwards through a sludge blanket at a rate of between 0.5 and 1.5 m.h⁻¹. Under these anaerobic conditions a selection process occurs that can result in the growth of anaerobic bacteria as aggregates, pellets or granules. These granules are effective biocatalysts that can rapidly convert biodegradable organic matter into biogas.

The principle of internal settling of suspended and granular sludge was initially reported in South Africa (Hemens *et al.*, 1962; Ross, 1984; Verstraete *et al.*, 1996), where reversed-flow Dorr-Oliver clarigesters were successfully applied since the late 1950s to treat industrial effluents arising from starch manufacture and wine distillation (Hemens *et al.*, 1962; Cillié *et al.*, 1969). In these clarigesters, raw feed introduced into the lower digester compartment was mixed with sludge containing a "dense culture of acclimatised organisms", which resulted in the stabilisation of the organic component of the feed (Cillié *et al.*, 1969). The incoming feed displaced an equal amount of sludge liquor into the clarifier compartment directly above the digester where the sludge was

intended to settle and returned to the digester by means of a scraper system. The clarified supernatant liquid left the clarifier as stabilised effluent. Chemical oxygen demand (COD) reduction varied between 70% and 97%, depending on the type of effluent treated (Cillié *et al.*, 1969). The main disadvantage in terms of the loading rate was the sludge washout that occurred with the 'clarified' effluent (Lettinga *et al.*, 1980).

The breakthrough in UASB technology development occurred in the 1970s in the Netherlands (Lettinga *et al.*, 1980; Verstraete *et al.*, 1996). The UASB process resembled the anaerobic contact process described by the South African researchers (Hemens *et al.*, 1962; Cillié *et al.*, 1969; Pretorius, 1971) with the exception that mechanical sludge recirculation was greatly reduced or even completely omitted and the reactor was equipped with a suitable gas solids separation system at the top (Lettinga *et al.*, 1980). Research investigating the UASB concept and its applicability to industrial effluent treatment was conducted at various institutes, including the Agricultural University of Wageningen, the Technical University of Delft, the University of Amsterdam and Dutch research institutes (IBVL and NIKO) as well as by various consulting and industrial firms. The commercialisation of the concept by about half a dozen competing Dutch consulting companies unfortunately resulted in a decrease in the open exchange of research information (De Zeeuw, 1988). The UASB concept itself was never patented, although various patents concerning the design of UASB gas solid separators were published. International interest in the UASB concept since the early 1980s has, however, broadened this field of research tremendously.

The basic principles of the UASB process were, firstly, that the anaerobic sludge would obtain and maintain superior settling characteristics if chemical and physical conditions were favourable for sludge flocculation. Secondly, a sludge blanket may be considered as a separate, more or less fluid but stable phase with its own specific characteristics. Thirdly, the washout of flocculent sludge particles could be minimised by creating a quiescent zone within the reactor where these particles can settle and become part of a secondary sludge blanket present in the settler compartment (Lettinga *et al.*, 1980). Based on early laboratory experiments Lettinga *et al.* (1980) reported that although the sludge loading rate (SLR) was kept at levels of between 0.1 and 0.2 kg COD.kg total solids⁻¹.d⁻¹ during start-up, a maximum SLR of 1 kg COD.kg total solids⁻¹.d⁻¹ and a maximum organic loading rate (OLR) of between 10 and 14 kg COD.m⁻³.d⁻¹ were eventually achieved in laboratory-scale UASB reactors. The UASB design has successfully been used to treat effluents from the food and beverage, dairy, distillery,

pulp and paper, petrochemical and slaughterhouse industries (Singh *et al.*, 1998). Investigations into the use of UASB technology in the treatment of wastewaters containing phenols and cresols have also been conducted (Veeresh *et al.*, 2005).

The washout of seed sludge during UASB reactor start-up remained a major concern. In an attempt to overcome the washout-associated problems more advanced UASB-related reactor types have since been developed, such as the internal circulation (IC) reactor and the expanded granular sludge bed (EGSB) reactor (Liu & Tay, 2004). Both these reactor types can accommodate higher volumetric loads than the more traditional UASB reactor (Liu & Tay, 2004).

C. ANAEROBIC SLUDGE GRANULATION

Anaerobic sludge granulation is commonly found in anaerobic wastewater treatment systems such as UASB reactors and modified UASB-type reactors such as IC reactors and EGSB reactors. Anaerobic granulation has also been reported in other anaerobic reactors such as the continuously stirred tank reactor (CSTR), the anaerobic sequencing batch reactor (ASBR), the anaerobic migrating blanket reactor (AMBR) and the anaerobic baffled reactor (ABR) (Liu & Tay, 2004; She *et al.*, 2006). As previously mentioned, the slow first start-up of an anaerobic reactor, especially if sludge granulation is pursued, is still considered a major disadvantage of the anaerobic treatment technology. Lettinga (1995 & 1996) emphasised that a granular sludge is not always required for UASB systems, as was found for installations treating domestic wastewater with well-settled, flocculent sludge types. Other researchers, however, see the development of a dense granular sludge as crucial to the optimum performance of anaerobic wastewater treatment systems such as UASB reactors, especially in the treatment of high-strength wastewaters (Schmidt & Ahring, 1996; El-Mamouni *et al.*, 1998; Tay *et al.*, 2000). Significantly higher loading rates can be accommodated in granular sludge reactors in comparison with flocculent sludge reactors. According to Rajeshwari *et al.* (2000), the maximum loading potential of a typical flocculent UASB system would be in the range of 1 - 4 kg COD.m⁻³.d⁻¹ while typical loading rates reported for granular UASB systems were between 10 and 30 kg COD.m⁻³.d⁻¹.

The granulation process itself is not fully understood (Schmidt & Ahring, 1996; Liu *et al.*, 2002; Hullshoff Pol *et al.*, 2004; Huang *et al.*, 2005). It is believed to be a physical, chemical and biological process that can best be described as a conglomeration of biomass that results from the self-immobilisation of anaerobic micro-

organisms under hydrodynamic conditions (Tay *et al.*, 2000). Sludge granulation mechanisms have been elucidated sufficiently for their practical application (Lettinga, 1995 & 1996). However, much still needs to be done before controlled in-reactor granular growth can be achieved during the first start-up (Verstraete *et al.*, 1996).

A description of the difference between granular sludge and flocculent sludge was given by Dolfig (1987) who distinguished between three different types of anaerobic microbial aggregates: flocs, pellets and granules. *Flocs* and *flocculent sludge* are described as conglomerates with a loose structure that forms one homogenous macroscopic layer upon settling. *Pellets*, on the other hand, are aggregates with a denser structure than flocs and are still visible as separate entities after settling, while *granules* are best described as dense pellets that have a well-defined, granular appearance with a shape that does not rely on the presence of water and can withstand a certain amount of pressure.

The fact that finely dispersed matter such as viable bacterial biomass will be decreasingly retained in a working UASB reactor reinforces aggregate/biofilm formation (Hulshoff Pol *et al.*, 1983). The actual size of a granule is dependent both on the intrinsic strength of the granule as well as the external forces exerted on it. First-generation aggregates, described by Hulshoff Pol *et al.* (1983) as “voluminous, filamentous aggregates”, are more floc than granule. In due time they will fall apart and give rise to a next generation of growth nuclei, which will in turn grow in size and density. These aggregates will eventually mature to form dense, compact granules of high quality (Hulshoff Pol *et al.*, 1987).

Granules and granular sludge have two main advantages over flocculent sludge: Firstly, granular sludge has a higher settling velocity than flocculent sludge and is better retained in anaerobic digesters. Secondly, granular sludge has a higher specific methanogenic activity than flocculent sludge, which results in a higher conversion of waste to methane (Wirtz & Dague, 1996).

D. GRANULATION MODELS

In the search for clarification of the granulation mechanism several anaerobic sludge granulation theories and models have been developed over the years, none of which has been accepted unanimously (Zhou *et al.*, 2007). Various reviews have classified these theories into groups such as “thermodynamic models” vs. “structural models” (as

described by Liu *et al.* (2002) and Tay *et al.* (2000)) or “physico-chemical models” vs. “structural models” (as described by Liu *et al.* (2003)). Hulshoff Pol *et al.* (2004) placed granulation models in three main groups: “physical” vs. thermodynamic” vs. “microbial”, with the following subdivisions under “microbial”: “physiological”, “growth” and “ecological”.

Anaerobic granules have, however, been described as spherical biofilms, and according to the review of Schmidt & Ahring (1996), similarities exist between biofilm formation and the granulation process. They have reviewed the four steps involved in the development of a biofilm or granule, which include:

- a) the transport of cells to the surface of the ‘substratum’ (other bacterial cells as well as uncolonised inert organic or inorganic material such as precipitates or even straw);
- b) the initial reversible adsorption to the substratum by physico-chemical forces (such as ionic, dipolar and hydrogen bonds, hydrophobic interactions, etc.). Initial adhesion is dependent on the surface characteristics of the individual cells;
- c) the permanent adhesion of cells to the substratum by means of microbial polymers (extracellular polymers or ECPs) or appendages (such as fimbria); and
- d) the multiplication of the cells and the development of granules.

In my opinion the best way to classify the various granulation theories would be to use the basic four-step biofilm formation process proposed by Schmidt & Ahring (1996) to describe four different classes under which the different models could be grouped. These are the following: Stage 1: cell transport to substratum; Stage 2: initial reversible adsorption; Stage 3: permanent adhesion; and Stage 4: cell multiplication. For this study, the different granulation theories are thus grouped into one of the four above-mentioned stages and reviewed below.

Stage 1: Cell transport to substratum

Selection pressure theory

In 1983 Hulshoff Pol *et al.* postulated that the trigger for anaerobic granulation is the continuous selection for sludge particles with a high settleability during reactor operation. The selection pressure can be manipulated by controlling the upflow velocity in a UASB reactor, using the hydraulic loading rate and the gas-production rate (which

is dependent on the SLR). It has been reported that under conditions of high selection pressure, finely dispersed sludge will be washed out of the reactor, thereby limiting the number of organic and inorganic particles to heavier growth nuclei that the remaining anaerobic bacteria can attach to. These particles would, through normal bacterial growth, increase in size and density (Hulshoff Pol *et al.*, 1983).

On the other hand, under conditions of low selection pressure, bacterial adhesion and aggregation would not be advantageous and most of the influent will be metabolised by dispersed sludge. These conditions have also been reported to promote the growth of the filamentous form of *Methanothrix* (synonym *Methanosaeta*), which leads to the formation of a bulking type of anaerobic sludge with poor settling properties (Hullshoff Pol *et al.*, 1987).

Inert nuclei theory

During the development stages of the UASB system in the 1970s it was thought that the inert matter content of granules initiated the granulation process by acting as nuclei for anaerobic bacteria to attach to, thereby forming initial embryonic granules (Lettinga *et al.*, 1980). Once these embryonic granules were formed, subsequent granular growth occurred through a process that could best be described as biofilm formation around these inert carriers or suspended matter. The development of a mature granular sludge bed was then considered to be the result of an increase in biofilm thickness (Yu *et al.*, 1999).

There have, however, also been reports that granules could be developed without the addition of any inert material and that high concentrations of finely suspended matter in the influent could be detrimental to granulation (Tay *et al.*, 2000; Hulshoff Pol *et al.*, 2004). The beneficial effect that added inert particles may have on the granulation process in anaerobic processes has, however, been recognised more than once (Hulshoff Pol, 1989; Wirtz & Dague, 1996; Yu *et al.*, 1999) and will be discussed in more detail in the 'Granulation enhancement' section of this chapter.

Methanogens as nucleation centres for granulation

Around 70% of the total methane produced in anaerobic digesters is derived from acetate, and the majority of acetate is metabolised by species of the two acetoclastic (acetotrophic) genera *Methanosarcina* and *Methanosaeta* (known previously as *Methanothrix*) (Gerardi, 2003). Several granulation theories involving the acetoclastic

methanogens have been developed. Various researchers postulated that *Methanosaeta* and/or *Methanosarcina* species form precursors or granular nuclei that facilitate the start of the granulation process (De Zeeuw, 1988; Wiegant, 1988; Chen & Lun, 1993; Zhu *et al.*, 1997).

The first of these theories was the so-called 'spaghetti' model for granulation that Wiegant (1988) proposed based on scanning electron microscopy (SEM) observations. According to this theory, filamentous *Methanosaeta* spp. would, through a multidirectional branched-growth process, form spaghetti-like structured aggregates that would entrap other bacteria, thereby serving as precursors in the overall granulation process. These aggregates would in time increase in size and density due to bacterial growth while at the same time becoming more spherically shaped as a result of hydraulic shear forces. Wiegant (1988), however, stressed the importance of careful manipulation of loading rates during UASB reactor start-up to prevent high acetate levels that would promote the growth of species of *Methanosarcina*.

Hulshoff Pol (1989) also reported the presence of two types of aggregates during the initial stages of granulation in a UASB reactor fed with a volatile fatty acid (VFA) solution. The two types observed were filamentous aggregates (presumably *Methanosaeta*) as well as smaller *Methanosarcina*-dominated granules. It was found that the smaller *Methanosarcina* granules washed out of the reactor more easily while the filamentous *Methanosaeta* aggregates remained.

Various other researchers have also supported the idea of the filamentous *Methanosaeta* structures serving as granulation nuclei to which other bacteria would strategically attach and colonise for optimum substrate transfer and utilisation – as the layered structure of certain mature granules would suggest (Dubourgier *et al.*, 1988; Macleod *et al.*, 1990; Morgan *et al.*, 1991a; Zhu *et al.*, 1997; Fang, 2000; Baloch *et al.*, 2008). Some authors have also highlighted the potential importance of ECPs during this process (Dubourgier *et al.*, 1988; Morgan *et al.*, 1991a; Baloch *et al.*, 2008).

Chen & Lun (1993) also supported the hypothesis of acetoclastic methanogens functioning as granulation nuclei by suggesting (based on SEM and transmission electron microscopy (TEM) observations) that species of both *Methanosarcina* and *Methanosaeta* are involved in nucleus formation. At higher acetate levels (as observed during start-up) *Methanosarcina* spp. grow in clumps and produce ECPs. As acetate levels drop, *Methanosaeta* growth would be favoured, and because *Methanosaeta* spp. can easily attach to different surfaces, they colonise *Methanosarcina* clumps and grow

on the surface, forming young small granular nuclei with *Methanosarcina* cores and *Methanosaeta* spp. on the surface. Although only the acetoclastic methanogens are involved in the formation of granular nuclei according to this theory, the authors highlighted the importance of other anaerobic bacterial species that are syntrophically associated with the methanogens and play a very important role in nuclei growth and maturation (Chen & Lun, 1993).

De Zeeuw (1988) indicated the possible colonisation of *Methanosarcina* clumps by *Methanosaeta* under specific start-up conditions, but in contrast with Chen & Lun (1993), *Methanosaeta* spp. were believed to colonise the central cavities of *Methanosarcina* clumps, forming young granules with *Methanosaeta* cores and *Methanosarcina* spp. near the periphery of the granules. According to De Zeeuw (1988), the outer *Methanosarcina* layer is not believed to be involved in granule maturation later on and is supposedly easily lost once granules obtain macroscopic dimensions.

Other researchers have also emphasised the importance of *Methanosaeta* species in anaerobic granulation (Wu *et al.*, 1996; McHugh *et al.*, 2003; Zheng *et al.*, 2006; Molina *et al.*, 2007). Morgan *et al.* (1991b) reported that the addition of a *Methanosaeta* supplement to digested sewage sludge improved granulation and reactor performance. He also suggested that this type of biosupplementation may only be necessary when the basic inoculum is deficient of *Methanosaeta* spp. According to Hulshoff Pol *et al.* (2004), *Methanosaeta concillii* can be considered as the key organism in granulation.

Stages 2 and 3: Initial reversible adsorption and permanent adhesion

Secondary minimum adhesion model

This model is based on the assumption that bacterial cells can be considered as nothing more than living colloidal particles and as such will obey the laws of physical chemistry and interact with surfaces in the same manner as normal colloidal particles (Van Loosdrecht & Zehnder, 1990). As a result, bacterial adhesion could be explained by colloid chemical theories such as the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory that describes the change in Gibbs energy as a function of the distance between particle surfaces. According to this model, reversible adhesion takes place in the secondary minimum of the DLVO free energy curve. At a separation distance (H) of 5 - 20 nm, the Gibbs energy involved in this reversible process is relatively small since

there is no direct contact between two surfaces/cells. This type of 'long-range' interaction is considered to be the summation of Van der Waal's forces of attraction and electrostatic forces of repulsion (Van Loosdrecht & Zehnder, 1990; Van Loosdrecht *et al.*, 1990).

Reversible adhesion could change to irreversible adhesion if a particle or cell can overcome the energy barrier to reach the primary minimum at small separation distances ($H < 1$ nm). The strength of adhesion at the primary minimum is determined by short-range interactions such as hydrogen bonding and ion pair formation (according to the DLVO theory) or in the case of bacteria, ECP or fimbriae bridging (Van Loosdrecht *et al.*, 1990).

The main disadvantage of this model is that bacterial cells cannot be considered 'classic colloidal particles' since they have no simple geometry or uniform molecular surface composition. Intracellular metabolic reactions may result in molecular changes on the inside and the outside surfaces of the cell, both before and after adhesion. Therefore, to consider bacterial adhesion only as a physico-chemical phenomenon would be to over-simplify the process.

Influence of hydrophobic interactions on granulation

It is known that under normal pH conditions, the outside surface of bacterial cells has a net negative charge that results in the formation of a hydration layer on the outside of the cell due to hydrogen bonding with water molecules. This hydration layer does not influence initial adhesion in the secondary minimum (as stated by the DLVO theory), since bacterial cells are still far enough from each other (5 - 20 nm according to the DLVO theory). The hydration layer might, however, hinder permanent adhesion since it can be the cause of strong hydration-repulsion forces. The strength of these forces is influenced by various factors such as the ionic strength of the medium (which determines the thickness of the hydration layer), the presence of bacterial surface appendages and, very importantly, the hydrophobicity of the bacterial cell or attachment surface. Van Loosdrecht & Zehnder (1990) stated that various studies indicated that adhesion increases with increasing hydrophobicity of the bacterial cells or solid surfaces. Experimental observations also supported the theory that the primary minimum of adhesion can be reached when both surfaces are hydrophobic, causing very strong Van der Waal's attraction forces.

The generally accepted method for measuring bacterial surface hydrophobicity is by using water contact angle measurements (Van Loosdrecht & Zehnder, 1990). Using this contact angle technique, Daffonchio *et al.* (1995) assumed that the cut-off contact angle between hydrophobicity and hydrophilicity is 45° and accordingly found that acetogens and methanogens are more hydrophobic (with water contact angles $>45^\circ$, indicating lower energy surfaces). Acidogens, on the other hand, are more hydrophilic (with water contact angles $<45^\circ$, indicating higher energy surfaces). According to Thaveesri *et al.* (1995), various studies have indicated that in an aqueous environment, hydrophobic cells would first stick together, after which they would associate with more hydrophilic cells to form aggregates with hydrophilic properties.

Based on the above considerations and their own experimental data, Thaveesri *et al.* (1995) proposed the “liquid surface tension (γ_{LV}) model” that is based on the thermodynamic consideration that hydrophilic and hydrophobic bacteria get their maximal free energies at different γ_{LV} values. According to this model, the development of a layered granule structure would be favoured under conditions of low γ_{LV} ($<50 \text{ mN}\cdot\text{m}^{-1}$), while granules with uniform distributed structures would develop under high γ_{LV} conditions ($>55 \text{ mN}\cdot\text{m}^{-1}$). Neither type of granulation would be favoured under conditions where intermediate γ_{LV} exist ($50 - 55 \text{ mN}\cdot\text{m}^{-1}$). Granulation could thus be controlled by manipulating the γ_{LV} through the choice of reactor feed and the addition of effective surfactants (Thaveesri *et al.*, 1995; Grootaerd *et al.*, 1997).

Divalent cation-bridge model

The positive effect that divalent cations such as calcium (Ca^{2+}), magnesium (Mg^{2+}) and iron (Fe^{2+}) can have on the granulation process has led some researchers to suggest that these cations may play a key role in initial granule formation. Divalent cations could stimulate granulation either by neutralising negative charges on bacterial cell surfaces, or by functioning as cationic bridges between bacteria (Goodwin *et al.*, 1990). It has also been reported that ECPs prefer to bind multivalent metals, resulting in the formation of more stable polymer complexes (Goodwin *et al.*, 1990; Yu *et al.*, 2000). According to Tay *et al.* (2000), reports on membrane fusion studies have also indicated that Ca^{2+} might cause conformational changes of some surface proteins and polypeptides, which could then interact with different surfaces and induce membrane fusion.

On the other hand, it has also been reported that Ca^{2+} did not trigger sludge granulation (Guiot *et al.*, 1988) and that they could even be detrimental to granulation under certain conditions (Van Langerak *et al.*, 1998; Batstone *et al.*, 2001). The negative effects that high concentrations of Mg^{2+} and Fe^{2+} can have on the granular biomass have also been reported (Schmidt & Ahring, 1993; Yu *et al.*, 2000).

Under optimal conditions and concentrations, the presence of Ca^{2+} , Mg^{2+} and Fe^{2+} have been reported to influence anaerobic granulation and granule stability positively, and this observation will be discussed in detail in the 'Granulation enhancement' section of this chapter.

Extracellular polymer bonding model

Extracellular polymers (ECPs) or extracellular polymeric substances (EPSs) are generally believed to be responsible for the cohesive forces that keep aggregates such as biofilms, flocs and granules together (Jia *et al.*, 1996; Flemming & Wingender, 2001). In doing this EPSs and ECPs play a critical role in the operation of biological wastewater treatment processes (Shin *et al.*, 2001). ECP was defined by Schmidt & Ahring (1994) as any polysaccharide-containing structure of bacterial origin lying outside the bacterial cell, either as a result of excretion or cell lyses. It is now recognised that, in addition to capsular material and peripheral slime, other macromolecules such as proteins, nucleic acids and other polymeric substances can also occur in the intercellular space of microbial aggregates, representing significant quantities of ECP in many environments (Flemming & Wingerder, 2001; Shin *et al.*, 2001).

ECP accumulation has widely been associated with biological aggregation processes and according to Tay *et al.* (2000) it has been reported that the metabolic blocking of exopolysaccharide synthesis prevented microbial adhesion. It has been hypothesised that ECP changes the surface negative charge of bacteria, thereby bonding bacterial cells and other inert matter together to form bacterial aggregates or flocs (Ross, 1984 & 1989; Schmidt & Ahring, 1996). Although it has been reported that ECPs are critical in maintaining the structural integrity of granular sludge (MacLeod *et al.*, 1995), it has also been observed that too much ECP can cause deterioration in floc formation (Schmidt & Ahring, 1996). The amount of ECP produced is usually influenced by the growth temperature, carbon source, as well as the excess or limitation of nutrients, substrate loading rate and hydrodynamic shear force (Schmidt & Ahring, 1994

& 1996; Veiga *et al.*, 1997; Puñal *et al.*, 2000; Liu *et al.*, 2004). ECP production is substantially enhanced under environmentally stressful conditions (Liu *et al.*, 2004). It has also recently been reported that organic overloading (Zhou *et al.*, 2006 & 2007) as well as high concentrations of sodium (Vyrides & Stuckey, 2007) increased ECP production in anaerobic sludge.

Various granulation theories have been developed in an attempt to explain which bacteria are responsible for ECP production. Some researchers favour the methanogens as main ECP producers. The “Cape Town Group” (Sam-Soon *et al.*, 1987) suggested that *Methanobacterium* strain AZ, a hydrogen-utilising methanogen, secreted ECP mainly consisting of long-chain polypeptides under conditions of high hydrogen partial pressure and limited cysteine. The ECP produced would then enmesh any other anaerobic bacteria present, thus leading to the formation of anaerobic pellets that settle rapidly (Sam-Soon *et al.*, 1990). According to this model, granulation will not occur in UASB reactors treating wastewaters containing acetate, propionate or butyrate due to inadequate hydrogen partial pressure (Sam-Soon *et al.*, 1990). Sludge granulation has, however, been reported in UASB systems treating effluents containing organic acids such as acetate (Ahring *et al.*, 1993). Veiga *et al.* (1997) compared ECP extracted from methanogenic granules fed with a VFA mixture with ECP extracted from pure cultures of *Methanobacterium formicicum* and *Methanosacina mazei* and concluded that both methanogens contributed significantly to the production of ECPs in granules and played an important role in granulation.

In contrast, several studies indicated that, although it is not clear whether ECPs are produced by one specific species or by a variety of different species, hydrolytic and acidogenic populations have a greater influence on ECP production than do methanogenic and acetogenic populations (Schmidt & Ahring, 1994 & 1996; Zhou *et al.*, 2006). It was found that the ECP content of granules and anaerobic sludge fed with acetogenic and methanogenic substrates was significantly lower than for granules and sludge fed with acidogenic substrates (Schmidt & Ahring, 1994; Zhou *et al.*, 2006). Fukuzaki *et al.* (1995) reported that starch- and sucrose-grown UASB granules were larger in size and had higher concentrations of ECP than granules cultivated on ethanol or VFAs. It was also reported that ECP-like materials cross-linking bacterial cells were observed in lactate-grown granules but not in propionate-grown granules that consisted mainly of propionate-degrading acetogens and methanogens (Fukuzaki *et al.*, 1991). The same study indicated that individual granules that consisted of large numbers of

lactate-degrading acidogens also had higher ECP contents. These studies did, however, also highlight the importance of *Methanosaeta* in the formation of high-quality granules (Fukuzaki *et al.*, 1991 and 1995).

Stage 4: Cell multiplication

Syntrophic micro-colony model

This theory is based on the fact that it would be more advantageous for the different anaerobic bacteria involved in anaerobic digestion to live in a close synergistic relationship where efficient transfer of product intermediates can occur among the respective groups. This kind of association would then eventually lead to the formation of stable micro-colonies, which would serve as initial granules (Tay *et al.*, 2000). According to Fang (2000), the layered structure of certain granules suggested that the granulation process is not just a random aggregation of suspended bacteria but that microbial associations are highly specific so as to create syntrophic associations between bacteria for optimum substrate supply and removal of metabolic products.

Syntrophic colonies of acetogens and hydrogenotrophic methanogens in anaerobic granules have been observed before and it was reported that these micro-colonies were major structural components in granules (Dubourgier *et al.*, 1988; Wu *et al.*, 1991). It was reported that the distances between the syntrophic acetogens (such as *Syntrophobacter* and *Syntrophomonas* spp.) and the methanogens were very small (in some instances less than 50 nm), which would favour the interspecies transfer of hydrogen (Dubourgier *et al.*, 1988) and thus ensure efficient substrate conversion.

Wu *et al.* (1996) investigated the aggregate-forming behaviours of various defined methanogenic and syntrophic fatty acid-degrading cultures alone and in combination in order to determine potential granule-forming syntrophic associations. Although they showed that a butyrate-degrading syntrophic strain produced ECP that could contribute to granulation, their study proved that the methanogens *Methanosaeta* and *Methanobacterium formicicum* play key roles in granulation. El-Mamouni *et al.* (1995) reported that granulation proceeded rapidly on syntrophic-enriched nuclei as well as on *Methanosaeta*-enriched nuclei. Larger granules over a shorter time period were, however, observed in the reactor seeded with the syntrophic-enriched nuclei. A similar observation was made by Zheng *et al.* (2006) who reported that granulation starts with *Methanosaeta concilii* cells as growth nuclei but that syntrophic acetogens attach to the

M. concilii backbone. They reported that granule size could be enhanced by the enrichment of the propionate degrading syntrophic consortia.

H⁺ translocation-dehydration theory

The only model that attempts to explain granulation in terms of all the steps involved in basic biofilm development is the H⁺ translocation-dehydration theory of Tay *et al.* (2000). In their opinion, the key factors to be considered in sludge granulation other than upflow hydraulic stress and wastewater composition are, firstly, that highly negatively charged bacterium surfaces facilitate hydrogen bonding with water molecules, which would lead to strong repulsive hydration interactions between the hydration layers of two approaching bacteria, preventing further attachment. Secondly, it has been shown that the initiation of bacterial adhesion can be improved when the hydrophobicity of adhering surfaces is increased (Verrier *et al.*, 1988; Van Loosdrecht *et al.*, 1990; Pereira *et al.*, 2000). Therefore, another important factor that should be considered according to Tay *et al.* (2000) is that proton translocation activity across the bacterial membrane may also cause dehydration of the cell surfaces by breaking the hydrogen bonds between negatively charged groups and water molecules, thereby increasing surface hydrophobicity.

The above-mentioned factors led these researchers (Tay *et al.*, 2000) to formulate a new model for the molecular mechanism of anaerobic sludge granulation: the proton translocation-dehydration theory. According to this theory, the four stages included in overall sludge granulation are as follows:

- i. Dehydration of bacterial surfaces, as a result of proton translocation activity across bacterial membranes. This proton translocation activity is activated when reduced coenzymes (the result of the energy-yielding respiration chain) are reoxidised.
- ii. Embryonic granule formation. Due to the weakened hydration repulsion, the relatively hydrophobic bacteria adhere by the action of external hydraulic forces. Effective metabolite transference promotes further dehydration of bacterial surfaces, thereby strengthening initial aggregate formation. ECP production is also induced.
- iii. Granule maturation. Intermediate transference determines the distribution of the different bacterial groups, which eventually leads to a well-structured microbial community in the mature granules.

- iv. Post-maturation. The granule structure is continuously maintained by the mechanisms of proton translocation activity. ECP production, in turn, causes hydration of the outside surface of the granule and prevents attachment of gas bubbles to the granule.

This model can theoretically explain some of the phenomena observed during the practical operation of anaerobic digesters such as granular sludge washout. For instance, it is generally believed that for faster reactor start-up and enhanced granulation, part of the reactor feed should be easily digestible sugars such as glucose or sucrose. According to the H⁺ translocation-dehydration theory (Tay *et al.*, 2000), this observation is the result of the quicker digestion and uptake of the readily degradable substrate, which leads to the more rapid activation of the proton pumps on the acidogenic cell membranes, which, in turn would lead to quicker dehydration of the bacterial surface and subsequent bacterial adhesion.

Another example is the spontaneous washout of granular sludge that has been reported in circumstances where the carbon source of a reactor has been changed suddenly. According to the model of Tay and his co-workers, the enzymes involved in substrate degradation are substrate specific, which means that sudden substrate change would lead to a new lag phase in bacterial growth during which a new set of enzymes is synthesised. The respiration process slows down, resulting in a decrease in proton pumping activity, which in turn leads to a decrease in bacterial adhesion. This decrease in adhesion eventually results in granule disintegration and washout (Tay *et al.*, 2000).

Although Tay *et al.* (2000) attempted to explain the granulation theory at molecular level, it does not, in my opinion, take into account that bacterial metabolism (including proton translocation activity) can also be influenced by a variety of internal factors. These would include the growth phase of the bacterial cell as well as changes in the internal and surface chemical composition of the bacterial cell as a result of the presence or absence of nutrients or toxic substances. I would also agree with Hulshoff Pol *et al.* (2004) that bacterial adhesion is a complex process that cannot only be explained as a physical chemical process, since bacteria do not have sharp surface boundaries, simple geometries or uniform molecular surface compositions.

E. ENVIRONMENTAL FACTORS INFLUENCING GRANULATION

It should be kept in mind that the anaerobic granulation process strongly depends on bacterial growth since organic and inorganic compounds excreted during bacterial metabolism can promote nuclei formation. The structural maintenance of mature granules (or spherical anaerobic biofilms) is furthermore influenced by the interactions between the individual microbial species involved in anaerobic digestion (Gonzalez *et al.*, 1998). Methanogens have the longest generation times of all the microbial species present in granules (from three days (d) at 35°C to 50 d at 10°C) (Gerardi, 2003). Since the acetoclastic methanogens in particular have been implicated in granular nuclei formation, it is logical to assume that just by manipulating the environmental conditions so as to create the optimal growth conditions for these slow-growing bacteria, granulation could be enhanced significantly (Hulshoff Pol *et al.*, 2004).

Wastewater type

It is generally accepted that the wastewater type has a greater effect on granulation and granule quality than reactor design or operational conditions (Jhung & Choi, 1995; Batstone & Keller, 2001; Zhou *et al.*, 2006).

It has been reported that anaerobic granulation proceeds faster in UASB reactors fed with soluble carbohydrate-rich substrates (Hulshoff Pol, 1989). Typical carbohydrate-rich substrates would include effluents originating from industries such as breweries, yeast factories and sugar beet- and potato-processing factories (Lin & Yang, 1991). Batstone & Keller (2001) examined granules from full-scale reactors treating different effluent types and found that granules fed with fruit and vegetable cannery effluent had excellent shear strength, settling properties and high cell densities and were ideal for UASB applications. Brewery-fed reactors also produced granules of acceptable quality, but the granules fed with a complex protein-based effluent were of poor strength and had low settling velocities and cell densities. According to the authors, the lower shear strength of the protein-fed granules also coincided with a higher polypeptide to carbohydrate ratio in the ECPs extracted from these granules while ECPs from cannery and brewery-fed granules had higher polysaccharide contents. It was suggested that a qualitative correlation might exist between loading rate, ECP content or substrate type and granule density that may in turn correspond

with better granule shear strength and settling velocity (Batstone & Keller, 2001). As can be expected, bacterial distribution in anaerobic granules is also influenced by the wastewater type. Granules fed with carbohydrate- or protein-based effluents showed higher acidogenic bacterial colonisation than granules cultivated in ethanol-based effluent.

Differences also exist in the dominant methanogen populations. The methanogenic component of granules used in the treatment of carbohydrate- and protein-based effluents were reported to be dominated by members of *Methanosaetaceae* and *Methanobacteriales* while ethanol-cultivated granules were only dominated by members of *Methanosaetaceae* (Molina *et al.*, 2007).

According to Zhou *et al.* (2006), the surface charge of wastewaters is one of the most important triggering forces for anaerobic granulation. Their study revealed that different substrates had different surface properties, which could also be manipulated by employing different operating conditions. ECP production was also found to be closely related to substrate type and running conditions. The authors supported the DLVO theory according to which bacterial adhesion could be promoted by neutralising the repulsion forces that exist between similarly charged particles (in the case of anaerobic granulation, bacteria with negative surface charges) by the addition of oppositely charged ions. According to them, the substrates functioned as external electrolytes while ECPs acted as high-molecular flocculants (Zhou *et al.*, 2006).

Macro- and micronutrients

In addition to a carbon and energy source, anaerobic bacteria also have nutritional requirements for certain macro- and micronutrients. The macronutrients critical for sustained growth and optimal granulation are nitrogen and phosphorous while cobalt, iron and nickel are obligatory micronutrients necessary for the optimal functioning of methanogenic enzyme systems. Other micronutrients that can contribute to methanogenesis and granulation include calcium, magnesium, molybdenum, tungsten, selenium, potassium, barium, manganese, zinc, sulphur, copper and aluminium (Nel *et al.*, 1985; Guiot *et al.*, 1988; Goodwin *et al.*, 1990; Singh *et al.*, 1999; Sharma & Singh, 2001; Gerardi, 2003).

The macronutrient requirements of an anaerobic digester are determined by the COD:N:P ratio of the reactor feed. As the COD increases so does the need for nitrogen

and phosphorous. Gerardi (2003) recommended a minimum COD:N:P ratio of 100:3–4:0.5–1 to ensure adequate nitrogen and phosphorous levels while Souza (1986) suggested a COD/N ratio below 70 and a COD/P below 350 (which corresponds to a minimum COD:N:P ratio of 100:1.4:0.3). Using digested sludge as inoculum and sugar molasses as feed, González *et al.* (1998) obtained granulated anaerobic sludge at N:COD and P:COD ratios of 0.018 and 0.0028 respectively. This corresponds to a minimum COD:N:P ratio of 100:2:0.3. They also reported that the addition of nutrients could be suspended after granule formation without changes in process performance.

Yeast extract is a good source of amino acids, minerals and B vitamins such as biotin and folic acid, which can be added to improve digester performance. Yeast extract has rarely been found to be inhibitory to anaerobic organisms (Singh *et al.*, 1999; Gerardi, 2003). Shen *et al.* (1993) reported that the absence of Ni, Co and Fe supplements did not affect the COD conversion rates of UASB reactors as long as the feeds contained yeast extract. Although the trace mineral content of the yeast extract used was much lower than the impurities of the other chemicals used in the feed, the COD conversion rates were reduced once yeast extract supplementation was ceased. According to the authors, yeast extract also enhanced the ability of anaerobic bacteria to collect essential trace elements from other sources.

Choice of seed sludge

In theory, any sludge with the proper anaerobic bacterial composition can be used as seed sludge for UASB reactor start-up. The choice of seed sludge is mostly determined by cost and availability. Any of the following materials could be used: manure, fresh water sediments, septic tank sludge, aerobic activated sludge, digested sewage sludge or surplus granular sludge from functioning UASB treatment plants (De Zeeuw, 1988; Lin & Yang, 1991; Liu *et al.*, 2002). Surplus granular sludge is the material of choice since it would significantly reduce start-up periods and quickly reach high removal efficiencies (Goodwin *et al.*, 1992; Van Lier, 2007). Unfortunately, availability is limited and purchase costs are high (approximate cost: \$500 - \$1 000 per ton, as reported by Liu *et al.* (2002)). The seed material most commonly used in start-up studies other than granular sludge is digested sewage sludge (Hulshoff Pol *et al.*, 1983; De Zeeuw, 1988; Guiot *et al.*, 1988; Lin & Yang, 1991; Yan & Tay, 1997; Francese *et al.*, 1998; Show *et al.*, 2004; Ghangrekar *et al.*, 2005; She *et al.*, 2006; Zhou *et al.*, 2006 & 2007).

According to the review of Lin & Yang (1991) it was reported in the early 1980s that thinner digested sewage sludge types (30 - 40 kg TS.m⁻³) generally had a higher specific methanogenic activity compared with thicker digested sludge (>60 kg TS.m⁻³). A thicker, inactive sludge would, however, contribute to sludge retention during UASB reactor start-up since thinner, more active sludge could easily cause excessive sludge bed expansion, resulting in sludge washout (Hulshoff Pol *et al.*, 1983; De Zeeuw, 1988). The optimum amount of seed sludge to be used during UASB reactor start-up must also be determined. Excessive sludge washout could be minimised while adequate contact between sludge and influents should still be possible. According to the review of Lin & Yang (1991), reports by Gatzke Lettinga and his co-researchers specified that for mesophilic reactors the optimum amount of thick sludge should be between 12 and 15 kg VSS.m⁻³ while the concentration of thinner sludges should be 6 kg VSS.m⁻³. Singh *et al.* (1998) suggested a suitable seed concentration ranging between 6 and 25 g VSS.L⁻¹ depending on the type of waste treated as well as the specific activity of the seed material.

UASB granules have also been added to digested sewage sludge in an attempt to improve methanogenic activity and granulation of the digested sewage sludge during UASB reactor start-up (Hulshoff Pol *et al.*, 1983; Hulshoff Pol, 1989; Goodwin *et al.*, 1992). Hulshoff Pol *et al.* (1983) reported that sludge bed granulation was influenced positively by the addition of small amounts of crushed granular sludge (8 - 9% of the volatile suspended solids [VSS] content). It was furthermore reported that although the addition of crushed granular sludge at concentrations of 8 - 15% of the VSS content had more or less the same enhancing effect on granulation, the observed effect was higher than that caused by the addition of only 2% crushed granular sludge (Hulshoff Pol, 1989). The fact that granular sludge was crushed before addition in the above-mentioned studies seemed to be important since Goodwin *et al.* (1992) could not find any conclusive evidence that sludge granulation could be enhanced by the addition of whole granular sludge at concentrations of 25 - 75% (v/v).

It has been reported that the rate of granulation is related to the microbial populations initially present in the seed sludge (Singh *et al.*, 1998). The improvement that the addition of crushed granules had on sludge granulation was probably the consequence of supplying (or increasing) a specific inoculum that is responsible for granulation, and therefore it is possible that anaerobic granulation can be expedited simply by manipulating the microbial composition of the seed sludge (Liu & Tay, 2004).

Consensus as to which species in anaerobic seed sludge contribute the most to anaerobic granulation has, however, not been reached. As previously discussed, many researchers consider acetoclastic *Methanosaeta* spp. to be the key organisms in granulation, although other methanogens such as *Methanobacterium formicicum* and *Methanosarcina mazei* have also been shown to contribute to granule formation (Wu *et al.*, 1996; Veiga *et al.*, 1997; McHugh *et al.*, 2003; Zheng *et al.*, 2006; Baloch *et al.*, 2008).

Upflow velocity and hydrodynamic shear

It is a well-known fact that the selective washout of flocculating matter with the simultaneous retention of heavier particles within the reactor contributes to sludge granulation. This can be achieved by manipulating the degree of mixing or hydrodynamic shear that occurs as a result of the liquid upflow velocity as well as the biogas load (O'Flaherty *et al.*, 1997). Biogas production is mainly influenced by feed composition and sludge metabolic properties and will be discussed in the next section.

Rising biogas bubbles normally do not provide adequate mixing of the sludge bed during start-up (Lin & Yang, 1991), which proves the need for the simultaneous manipulation of the upflow velocity to find the optimum degree of mixing within the reactor. If the degree of mixing is too low, channelling can easily occur and the selective washout of flocculant matter with a low settleability will be minimised. If the degree of mixing is too high, granules or flocs can disintegrate as a result of the abrasive action of reactor shear forces or washout of the reactor (De Beer *et al.*, 1996).

Gentle or intermittent agitation would preserve the microstructure of anaerobic aggregates and promote granulation (Lin & Yang, 1991). O'Flaherty *et al.* (1997) reported that granulation proceeded well in a VFA/ethanol-fed anaerobic upflow hybrid reactor operated at a liquid upflow velocity of $0.5 \text{ m}\cdot\text{h}^{-1}$ in contrast with the flocculent sludge that developed at a low liquid upflow velocity of $0.01 \text{ m}\cdot\text{h}^{-1}$. The same trend was observed by Alphenaar *et al.* (1993) who reported that granulation in an acetate/propionate/sucrose-fed UASB reactor preceded better at an upflow velocity of $0.5 \text{ m}\cdot\text{h}^{-1}$ than at $0.05 \text{ m}\cdot\text{h}^{-1}$. Both these studies illustrate that a minimum amount of hydrodynamic shear should be present so that the selection pressure applied is high enough to encourage bacterial granule formation.

Organic loading rate (ORL) and sludge loading rate (SLR)

Although it has been reported that the wastewater type rather than the loading rates applied determine the bulk characteristics of granular sludge (Batstone & Keller, 2001), Ghangrekar *et al.* (2005) found that sludge characteristics differ for the same wastewater and inoculum under different OLRs and SLRs. The OLR (expressed as $\text{kg COD} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$) remains one of the most important operating parameters in anaerobic granulation as it defines the capacity of a reactor to convert organic substrate per unit volume. The SLR (expressed as $\text{kg COD} \cdot \text{kg VSS}^{-1} \cdot \text{d}^{-1}$) defines the capacity of the anaerobic population present in the reactor to convert organic substrate per unit mass.

In the early 1980s, it was reported that granulation was only observed at SLRs of $>0.6 \text{ kg COD} \cdot \text{kg VSS}^{-1} \cdot \text{d}^{-1}$ (Hulshoff Pol *et al.*, 1983). Francese *et al.* (1998) reported that granulation in laboratory scale reactors seeded with non-granular sewage sludge was observed at an ORL of $8 \text{ g COD} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$ when the upflow liquid velocity was $0.5 - 2.0 \text{ m} \cdot \text{h}^{-1}$. According to Ghangrekar *et al.* (2005), the development of a granular sludge bed with good characteristics can be promoted by keeping loading rates within favourable ranges, such as an OLR of $2.0 - 4.5 \text{ kg COD} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$ and an SLR of $0.1 - 0.25 \text{ kg COD} \cdot \text{kg VSS}^{-1} \cdot \text{d}^{-1}$. Singh *et al.* (1998) suggested that the SLR should be $0.07 - 0.49 \text{ kg COD} \cdot \text{kg VSS}^{-1} \cdot \text{d}^{-1}$ (depending on the type of waste treated) at the beginning of reactor start-up. The SLR should then be increased very gradually in a stepwise manner once a steady increase in methane production and VFA degradation is observed so that a final rate of $0.2 - 0.86 \text{ kg COD} \cdot \text{kg VSS}^{-1} \cdot \text{d}^{-1}$ can be reached at the time of granulation.

Zhou *et al.* (2007) investigated the effect of overloading on granulation time and methanogenic population activity by comparing three UASB reactors (A, B and C) operated under different conditions and found that OLR, ECP content and granulation were closely related. In reactor A, which was operated at low loading rates (an OLR below $1 \text{ kg COD} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$ for the first 40 d), the start of granulation (characterised by the appearance of white, flocculent nuclei-type materials) was observed for the first time on the 48th day of operation at an OLR of $2 \text{ kg COD} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$. Mature granules eventually produced under these conditions were of excellent quality and the activity of the acetate-consuming methanogens improved greatly during operation. Reactor B was operated under conditions of slight overloading and the start of granulation was observed for the first time on the 7th day at an OLR of $2.5 \text{ kg COD} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$ and mature granules were observed on day 39 at an OLR of $30 \text{ kg COD} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$. The activity of the

acetate-consuming methanogens did improve slightly during operation but not to the same extent as in reactor A. Reactor C was operated under conditions of extreme overloading, reaching an OLR of $15 \text{ kg COD} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$ on day 14. Granulation proceeded very poorly under these conditions since extensive sludge washout and acidification occurred. The authors reported that an increase in ECP content was observed in all three reactors each time the OLR was increased. This was followed by a slight decrease in ECP content as the OLR was fixed at a certain level for a period of time, indicating bacterial consumption of part of the ECP content under stable reactor conditions. Since the OLR was increased more often in reactor B than in reactor A, ECP concentrations reached higher levels, which, according to the authors, enhanced the process of granulation efficiently (Zhou *et al.*, 2007).

Zhou *et al.* (2007) also confirmed the findings of Jhung & Choi (1995) that anaerobic granulation was not observed at a low OLR (below $1.0 \text{ kg COD} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$).

Temperature

Methanogenic growth and activity (just like microbial activity in general) are strongly influenced by environmental temperature. The temperature range for most mesophilic methanogens is reported to be between 30 and 35°C with an optimum temperature of 35°C (Gerardi, 2003). Mesophilic *Methanosaeta* species grow optimally at temperatures between 35 and 40°C , while the optimum range for *Methanosarcina* species is greater ($30 - 40^\circ\text{C}$) (Patel, 2001; Boone & Mah, 2001). Temperature fluctuations rather than the operating temperature itself can affect methanogenic activity greatly and should be kept to a minimum ($2 - 3^\circ\text{C}$ per day for mesophilic methanogens) (Gerardi, 2003).

The population composition of granules strongly depends on operational temperature. Since an efficient anaerobic process is the result of more than one microbial population, temperature fluctuations could be advantageous for some microbial groups while the growth of other species could be restricted. Methanogens are generally more sensitive than acidogenic populations. For instance, hydrolytic activity is not greatly influenced by temperature and VFA production can still proceed rapidly at low temperatures while the methane-production rate, on the other hand, is directly proportional to the operating temperature. Careful monitoring of the VFA to alkalinity ratio is thus recommended if the operating temperature decreases to below 32°C (Gerardi, 2003; Tiwari *et al.*, 2006).

Alkalinity and pH

Anaerobic digestion proceeds well within a pH range of 6.8 - 7.2, although the optimum range is within 7.0 - 7.2. Within the anaerobic community acidogenic populations are significantly less sensitive to pH fluctuations than are methanogens. Methanogenic activity can be severely restricted at pH values below 6.2 and above 8.0 (Gerardi, 2003; Tiwari *et al.*, 2006). The optimum growth pH of *Methanosaeta* species is slightly higher than for the *Methanosarcina* species. Although both these genera metabolise acetate, *Methanosarcina* spp. cannot consume acetate at very low concentrations and can only dominate at acetate concentrations above 3 mM. As a result, *Methanosarcina* spp. are more acid tolerant and might thus be expected to dominate in digesters and environments with pH values below 7.0 while *Methanosaeta* spp. will probably dominate in environments with pH values above 7.0 (Patel, 2001; Boone & Mah, 2001).

Alkalinity serves as buffer to prevent rapid pH fluctuations. Therefore, sufficient alkalinity in a digester is essential for proper pH control (Gerardi, 2003). Under stable reactor conditions VFAs produced by acidogenic populations are consumed by methanogens that produce methane and CO₂. The release of CO₂ results in the production of bicarbonate alkalinity, carbonate alkalinity and carbonic acid (the equilibrium between these compounds is a function of the digester pH). A decrease in alkalinity normally precedes a rapid change in pH and could serve as an early indication of methanogenic inhibition and process failure (Gerardi, 2003). It has been suggested that the ratio of VFAs (as acetic acid) to total alkalinity (as calcium carbonate) should remain above 0.1 to ensure a stable anaerobic digestion process. Various chemicals such as lime, sodium hydroxide and sodium carbonate can be used to increase alkalinity and restore the pH balance in a reactor (Bitton, 1999).

UASB granule structure can also be influenced by reactor pH. Teo *et al.* (2000) measured granule strength in terms of turbidity change and found that granule strength decreased between pH 8.5 and 11.0 and between pH 5.0 and 3.0 while granule structure remained more or less stable in the pH range 5.5 - 8.0. It has been reported that acetotrophic methanogens are located in the centre of UASB granules treating carbohydrate substrates while acidogenic bacteria are located on the outside of the granules. This observation could explain why methanogens in granules have a higher resistance to various toxicants and low pH when compared to those present in suspended form in methanogenic sludge (Chen & Lun, 1993).

F. IN-REACTOR GRANULATION ENHANCEMENT

Various different strategies have been employed to shorten the long start-up periods associated with UASB reactor start-up. These include the following:

Addition of inert nuclei, synthetic and natural polymers

In the late 1980s and early 1990s various researchers reported the positive influence that addition of substances such as powdered zeolite, polyurethane foam, hydro-antracite and other inert support materials such as sepiolite and diabase can have on aspects such as granule development, retention of methanogenic bacteria (sepiolite for acetotropic and diabase for hydrogenotropic methanogens) and increased methane production (Yoda *et al.*, 1989; Fukuzaki *et al.*, 1990; Hulshoff Pol, 1989; Sanchez *et al.*, 1994). More recent studies have indicated that granulation of anaerobic sludge can be enhanced by the addition of:

- a 'cationic polymer' (described as a hetero-polymer of acrylamide and acryloyloxethyl-trimethylammonium chloride) (Wirtz & Dague, 1996);
- a water-absorbing polymer (WAP) (a pulverulent resin) (Imai *et al.*, 1997);
- Percol 736 (a cationic synthetic acrylamide polymer) (El-Mamouni *et al.*, 1998);
- Chitosan (a natural polymer) (El-Mamouni *et al.*, 1998);
- powdered activated carbon (PAC) and granular activated carbon (GAC) (Wirtz & Dague, 1996; Yu *et al.*, 1999);
- a water extract of *Moringa oleifera* seeds (effective in flocculating organic matter) (Kalogo *et al.*, 2001);
- Kymene SLX-2 (a cationic polymine-epichlorohydrin polymer) (Uyanik *et al.*, 2002);
- the cationic polymer 'AA184H' (Show *et al.*, 2004); and
- hybrid polymers (Jeong *et al.*, 2005).

The mechanisms of enhancement by synthetic or natural polymers differ slightly from those of classical inert nuclei (such as PAC, GAC or hydro-antracite). While inert nuclei are believed to only provide sites to which anaerobic bacteria can adhere to under conditions of high selection pressure, polymers can either promote cell aggregation by

forming bridges between individual cells or provide a stable three-dimensional matrix that bacteria can colonise (Wirtz & Dague, 1996; Liu *et al.*, 2002)

An important drawback of heavier inert particles (such as sand) is that the specific gravity of these particles is greater than biomass. This may result in an accumulation of the inert particles at the bottom of the reactor while the biomass accumulates higher up in the reactor. This minimises the chance of contact between the particles and the biomass and subsequently reduces microbial attachment growth. The addition of inert matter could not, under such circumstances, cause any significant enhancement of granulation. In contrast, polymeric substances such as WAP not only have lower densities, which promote contact between these particles and the biomass, but also provide more attachment sites due to their matrix structure (Imai *et al.*, 1997). Disadvantages of using synthetic polymers to promote sludge granulation include the possible high cost of application in industrial-scale reactors, pipe blockages, precipitation as well as the reduction of the active volume of reactors (Zhou *et al.*, 2007).

Addition of divalent ions

Various reports have highlighted the positive influence that divalent cations can have on the granulation process and on granule stability. Most of the research conducted investigated the effect of different concentrations of calcium ions (Ca^{2+}) on granulation. The stabilising effect that Ca^{2+} has on granule strength, settleability and solid retention time has been well documented (Hulshoff Pol *et al.*, 1983; Goodwin *et al.*, 1990; Grotenhuis *et al.*, 1991; Guiot *et al.*, 1992). However, the importance of controlling the Ca^{2+} dosage to achieve an optimum concentration has also been proved. Hulshoff Pol *et al.* (1983) observed a definite improvement of sludge retention in UASB reactors at lower Ca^{2+} concentrations (150 mg.L^{-1}) while higher sludge washout was observed at higher Ca^{2+} concentrations (450 mg.L^{-1}); Mahoney *et al.* (1987) found improved granulation at levels between 100 and 200 mg.L^{-1} . Yu *et al.* (2001) reported that the optimum Ca^{2+} concentration is dependent on the influent COD concentration and stressed the importance of finding the optimum ratio of Ca^{2+} to COD concentration rather than only focusing on the Ca^{2+} concentration. They found that the optimum concentration for calcium was between 150 and 300 mg.L^{-1} at a constant influent COD

concentration of 4 000 mg.L⁻¹ while lower COD removal rates and higher effluent VSS concentrations were observed at calcium ion concentrations ≥ 450 mg.L⁻¹.

The main problem associated with high Ca²⁺ concentrations in anaerobic digesters is the excessive precipitation of calcium carbonate. The consequences of this type of precipitation include decreased efficiency as a result of sludge washout, scaling of reactor walls, pipes and granule surfaces, high calcium accumulation and decreased substrate diffusion inside granules, loss of buffer capacity, reduced methanogenic activity and space occupation by precipitates (Van Langerak *et al.*, 1998; Batstone *et al.*, 2001; Liu *et al.*, 2003).

Although most of the studies on divalent metal ions have focused on the effect of Ca²⁺, the effects of other divalent ions have also been investigated. It was reported by Schmidt & Ahring (1993) that although concentrations of 0.5 - 10 mM magnesium ions (Mg²⁺) enhanced UASB reactor performance, the addition of 100 mM or more Mg²⁺ caused disintegration and biomass loss as a result of washout. Yu *et al.* (2000) and Vlyssides *et al.* (2009), on the other hand, examined the role of ferrous iron (Fe²⁺) in sludge granulation. Yu *et al.* (2000) found that Fe²⁺ enhanced granulation at concentrations of 300 and 450 mg.L⁻¹, while Vlyssides *et al.* (2009) reported improved COD conversion rates and significant increases in granular sludge quality at a Fe²⁺ load range of 14 to 100 mg.L⁻¹.d⁻¹. Yu *et al.* (2000), however, found that further increases in Fe²⁺ concentration in the feed resulted in large mineral deposits within the granules, as well as a significant decrease in the water content of the granules. This, along with the possible toxicity of high concentrations of Fe²⁺ that could have accumulated within the granules, could be the reason for the decrease in the specific activity of the granules that was observed at higher Fe²⁺ dosages (Yu *et al.*, 2000).

Influence of pulsation of influent

Interesting studies by Franco *et al.* (2002 & 2003) investigated the application of external pulsing of the influent to promote hydrodynamic stress during laboratory-scale (lab-scale) UASB reactor start-up. According to the authors, the low OLR applied during the first phases of reactor start-up is too low to cause proper hydraulic mixing and mass transfer. This can result in poor degassing of the sludge bed, which, in turn, can lead to biomass washout and extended reactor start-up periods. According to the authors, the application of pulsation would result in the better release of gaseous metabolites and

improved mass transfer while it prevents channelling in the sludge bed. The improved hydrodynamic stress would also promote aggregation by creating conditions of high selection pressure. The results of this study showed that the reactors of which the influent was pulsed had higher COD removal rates and higher specific methanogenic activity (SMA) values. In addition, granules from the pulsed reactors were smaller and had a well-defined shape with evidence of channels on the granule surface while no channels were observed in the granules from the non-pulsed reactor. According to the authors, these channels proved that the granules had higher porosity, which increased biogas release and might also have improved nutrient transport to the inner core of the pulsed granules (Franco *et al.*, 2002 & 2003).

Although the study again illustrated the importance of proper hydrodynamic shear force in anaerobic granule development, the technology was only tested in 0.8 L lab-scale units. The application of pulsation in larger units as well as in industrial-scale installations have not been investigated and would almost certainly have extra cost implications that should be taken into account when the advantages of the technology are considered.

G. PRE-TREATMENT OF SEED SLUDGE TO ENHANCE GRANULATION

Methanol-cultured seed sludge

In an attempt to accelerate UASB reactor start-up, Xu & Tay (2002) developed a biotechnological method that involved the cultivation of digested seed sludge in a methanol-based substrate prior to UASB reactor inoculation. Methanol is a common substrate for the methylotrophic methanogens and certain other methanogens such as the acetoclastic *Methanosarcina* spp. that can grow faster on methanol than on acetate in pure culture. The authors argued that since methanogens are slow growers under normal anaerobic conditions, the methanogenic content of the UASB seed sludge can be increased during pre-incubation of the seed sludge in the presence of methanol. UASB reactor inoculation with this seed sludge could then reduce UASB reactor start-up. In this study, 75% of an anaerobic column was filled with digested sludge, fed a synthetic substrate (with a total COD content of 1 000 mg.L⁻¹ that contained methanol [60% of the COD content] and acetate [15% of the COD content]) and operated as an

anaerobic chemostat for 15 d. After incubation, a UASB reactor was inoculated with the methanol-cultured seed sludge (Reactor 1) and, along with a control UASB reactor inoculated with ordinary digested sludge (Reactor 2), was operated for 100 d to study the effect that the pre-treatment had on start-up and granulation. Granular particle size measurements showed that embryonic granule formation did not occur in the methanol-cultured seed sludge during the 15-day incubation period prior to UASB reactor inoculation. During UASB reactor operation it was, however, observed that embryonic granule formation occurred about 20 d earlier in Reactor 1 than in Reactor 2 and that the granulation process in Reactor 1 reached the post-maturation phase 15 - 20 d ahead of Reactor 2. At the end of the 100-day operation period the granules from both reactors had similar particle sizes. Reactor 1 was slightly more efficient than Reactor 2 in terms of biogas production and COD removal rates during start-up, but at the end of the 100-day operation period very little difference could be observed between the efficiency of the reactors.

All things considered, the advantages gained during pre-incubation of the seed sludge in the methanol-based substrate might not be that significant. Initial granulation was noticed 15 - 20 d earlier in Reactor 1 when compared to the control (Reactor 2), but if the pre-incubation period for the methanol-cultured seed sludge is subtracted, this 'time advantage' gained by pre-incubation is only 8 - 13 d (the methanol-cultured seed sludge of Reactor 1 was incubated for 15 d before start-up while Reactor 2 was recirculated for one week before start-up).

According to Switzenbaum *et al.* (1988), methanol has been used before as initial substrate to reduce reactor start-up time in anaerobic fixed film reactors before gradually changing the feed to the wastewater to be treated, but although methanol-utilising methanogens (methylotrophic methanogens) grow faster than acetate-utilising methanogens (acetoclastic methanogens), it was not clear whether these organisms remained at high levels after the reactor feed was changed. Hydrogenotrophic methanogens as well as most of the methylotrophic methanogens use hydrogen during methane production and although more energy is gained during this process than during acetate degradation, less than 30% of the methane produced in an anaerobic digester is produced by this manner because the supply of hydrogen is limited. Approximately 70% of methane is derived from acetate by the acetoclastic methanogens from the *Methanosarcina* and *Methanosaeta* genera (Gerardi, 2003). Of these two genera only *Methanosarcina* spp. can also utilise substrates other than

acetate, such as methanol, methylamines and H_2/CO_2 (Schmidt *et al.*, 2000), and might thus also be enhanced during pre-incubation in the presence of methanol. *Methanosaeta* spp., which, as discussed before, are considered by many to be the key organisms in granulation, can only utilise acetate and might only have been slightly enhanced during pre-incubation since the substrate of Xu & Tay (2002) contained a small percentage of acetate.

Enhancement of lactate-utilising populations

Riedel & Britz (1993) isolated 60 *Propionibacterium* strains from anaerobic digesters and were the first authors to present a hypothesis as to the role of propionic acid-producing bacteria during the anaerobic granulation process. Based on this hypothesis, Britz *et al.* (2002) developed a method to enhance granulation of digested sludge in shaking batch systems by incubating digested sludge in the presence of lactate, glucose and sucrose substrates for a period of 14 - 26 d. Increases in granular counts were observed in the lactate and glucose units but not in the sucrose units. In all units, a drop in pH followed by a subsequent increase and stabilisation of pH levels preceded the increases in granular content. Britz *et al.* (2002) argued that by manipulation of environmental conditions lactate-utilising ECP-producing acidogenic bacteria could temporarily gain a competitive advantage and enhance granulation. The application of the enhanced seed sludge during UASB reactor start-up was, however, not evaluated as part of their study.

Lactate is one of the common products of fermentation processes in anaerobic digesters and can be produced by a variety of anaerobic to aerotolerant bacteria such as *Bifidobacterium*, *E. coli*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Clostridium* spp. (Gerardi, 2003). Most of the lactate-producing bacteria are highly saccharolytic and ferment sugars such as glucose, fructose, galactose, mannose, saccharose, lactose, maltose and pentose. Lactate is the preferred substrate of propionate-producing bacteria such as *Propionibacterium*, *Selenomonas* and *Veillonella* (Gerardi, 2003). Several strains of these genera isolated from anaerobic processes have been shown to produce excessive amounts of ECP and displayed clumping characteristics, which could ultimately contribute to the anaerobic granulation process (Riedel & Britz, 1993; Slobodkin & Verstraete, 1993).

The possible involvement of lactate-degrading acidogens in granulation development has also been investigated by Fukuzaki *et al.* (1991) who reported that granules that had large populations of lactate-utilising bacteria also had high ECP contents and cell densities. The study by Britz *et al.* (2002) was the only one that attempted to manipulate the indigenous lactate-utilising population in digested sludge to enhance granulation.

H. DISCUSSION

The international awareness of the importance of anaerobic granulation is not new considering the amount of literature available that dates back to the 1980s. The GASMAT workshop on anaerobic granulation (Lettinga *et al.*, 1988), in particular, is a good example of the international interest in the subject since the list of participants included not only attendees from most European countries but also delegates from countries such as China, Brazil, Indonesia, the USA and Yugoslavia. Although research on the subject has never ceased during the past 20 - 30 years, the number of reviews of anaerobic granulation in recent years demonstrated a renewed international interest in the subject (Rajeshwari *et al.*, 2000; Tay *et al.*, 2000; Liu *et al.*, 2002; Liu *et al.*, 2003; Hulshoff Pol *et al.*, 2004; Liu & Tay, 2004; Tiwari *et al.*, 2006). As discussed in this chapter, a great deal of research has been conducted in the area of artificial granulation enhancement by the addition of synthetic polymers. Questions have been raised regarding the successful application of such granules in industrial installations, and good-quality granular sludge that developed naturally is still regarded as superior in quality to those developed by the addition of polymers (Zhou *et al.*, 2007).

Judging from the large amount of recent research done by authors from countries such as India, Singapore, China, Japan, Malaysia and Korea, the interest in anaerobic digestion (including anaerobic granulation) seems to be very prominent in the Far East. These countries with their high population densities (and, by implication, their large volumes of food industry and agricultural effluents) seem to be very aware of the advantages that anaerobic digestion can offer with its relatively compact systems that can treat large volumes of concentrated waste at low cost and energy requirements and with the added benefit of generating energy from waste.

In contrast, a market study by Kassam *et al.* (2003) on anaerobic wastewater systems in Western societies has shown that while the European market was found to

be relatively mature, the North American market was considered volatile, with no indication of future growth at that stage. The main reasons given for this observation were that in North America energy was still easily obtainable at a reasonably low cost, land availability was not much of a concern, and North America also had more lenient environmental legislation regarding discharged waste.

Internationally, however, there is renewed interest in the energy-generating aspects of anaerobic wastewater treatment, which is the direct result of increasing energy prices as well as the overall concern about global warming. The Kyoto Protocol on greenhouse emissions in particular contains clauses that call for the limitation and/or reduction of methane emissions through recovery and use in waste management, as well as for research on and development of new and renewable forms of energy (Kassam *et al.*, 2003; Van Lier, 2007).

Van Lier (2007) reported that annual installations of anaerobic high-rate reactors by renowned companies such as Paques, Biothane, Biotim, Enviroasia, ADI, Waterleau, Kurita, Degremont, Envirochemie, GWE and Grontmij gradually increased between the mid-1970s and 2007. An analysis of the international market history of commercial anaerobic models has also indicated a phasing out of the more traditional UASB models in favour of large-capacity high-rate systems such as the Biothane EGSB reactor and the Paques IC systems (Kassam *et al.*, 2003; Van Lier, 2007). According to Van Lier (2007), granular sludge bed-based treatment technologies still dominated the anaerobic wastewater treatment market between 2002 and 2007 with 89% of all new industrially implemented installations being granule based (UASB: 34%; IC: 33%; EGSB: 22%).

These statistics indicate that although no granulation hypothesis has unanimously been accepted, the wealth of information available on the subject has contributed to the increased industrial implementation of anaerobic treatment technologies worldwide. As discussed in this chapter, the availability of good-quality granular seed sludge remains the limiting factor in the industrial application of anaerobic granule-based technologies. The enhancement of granulation of flocculent anaerobic sludge would therefore remain an important field of research in the efficient anaerobic treatment of wastewaters.

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CHAPTER 3

EXTRACELLULAR POLYMER PRODUCTION AND POTENTIAL FOR AGGREGATE FORMATION BY CLASSICAL PROPIONIBACTERIA*

Summary

Nineteen *Propionibacterium* strains were screened for extracellular polymer (ECP) production. The best producer, *P. jensenii* S1, was introduced into two different media: yeast extract lactate medium (YELM) and apricot effluent medium (AEM). The YELM samples were incubated in different mixing systems (a roller-table and a linear-shake platform) for 24 days at 35°C. According to the volatile fatty acids and pH profiles no real differences could be detected between the two mixing systems. Bacterial aggregates were, however, only observed in the roller-table samples. The process was repeated with AEM on the roller-table. Larger and more stable flocs were observed in the AEM samples. Scanning electron microscopy and polymerase chain reaction analysis confirmed the presence of propionibacteria in these flocs even after five months of storage at 4°C. It was concluded that ECP-producing *Propionibacterium* strains could be manipulated to form bacterial flocs under certain environmental conditions, which might be enhanced in the presence of fibrous material occurring naturally in food industry effluents.

Introduction

The upflow anaerobic sludge blanket (UASB) process is one of the most extensively applied anaerobic high-rate wastewater treatment systems (Lettinga *et al.*, 1997) but the process depends on the upward movement of soluble matter through a blanket of granules. The major problems in operating the UASB system are the long start-up times as a result of the slow process of granulation as well as the need for a speedy replacement of granules once they have been washed out of the system. These limitations clearly restrict the general application of this system, unless the granulation

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reaction can be enhanced. The precise nature of the mechanisms involved in the formation of granules and the reason for their stability are still not fully understood (Schmidt & Ahring, 1993).

It was hypothesised by Riedel & Britz (1993) that, through the implementation of environmental 'stress' conditions, a shift in the population dynamics of the anaerobic community can be obtained. Subsequently, ECP-producing *Propionibacterium* strains were isolated under the stress conditions where granule formation was stimulated (Riedel & Britz, 1993).

According to Riedel & Britz (1993) and Britz *et al.* (2002), an increase in the intermediate lactic acid concentration, as a result of unbalanced loading conditions, could result in an orderly shift to a predominant lactate-utilising population in response to the gradual decrease in the pH and increase in H₂ partial pressure. The propionic acid-producing strains could then gain a competitive advantage, as they are more acid tolerant and obtain a maximum of ATP per mol of lactate fermented. Once they have the advantage at the lower pH, they start producing extracellular polysaccharides, which could contribute to aggregate formation while serving as a hydrogen sink mechanism (Riedel & Britz, 1993). However, this hypothesis must still be validated and may have far-reaching implications for the food and dairy industries using the UASB bioreactor system to treat wastewaters.

The classical propionibacteria are used in a variety of natural dairy fermentations but their ECP-producing ability makes them of special interest to the food wastewater management industry. Propionibacteria have previously been isolated from anaerobic processes (Riedel & Britz, 1993; Qatibi *et al.*, 1990) and it has been argued that *Propionibacterium* could be one of the major genera present in granules. The ECPs produced by propionibacteria have also been indicated to play a role in the initial granule formation in bioreactors (Britz *et al.*, 2002).

Before the precise role of lactate-utilising, propionic acid-producing organisms such as propionibacteria could be determined in a mixed anaerobic population, it will be necessary to know whether these organisms actually do exhibit aggregate-forming abilities. Therefore, the aim of this study was to determine whether propionibacteria could be manipulated in a pure culture to produce bacterial flocs.

Materials and methods

Bacterial strains and culture conditions

The 19 'classical' *Propionibacterium* strains as indicated in Table 1, 10 from anaerobic digesters and nine from dairy products, were obtained from the University of Stellenbosch Food Science Culture Collection and cultured anaerobically at 30°C in yeast extract lactate medium (YELM) prior to ECP isolation and viscosity measurement.

After being identified as the best ECP-producing strain, *P. jensenii* strain S1 was again cultured in YELM and duplicate samples were incubated at 35°C on a linear-shake platform (130 rpm) (Labotec, Johannesburg, South Africa) and on a roller-table (30 rpm) (specifically manufactured by the Process Engineering Workshop, University of Stellenbosch). The roller-table was designed as a multi-roller-tumbler driven by an electric motor via sprockets and chain. The variable speed was obtained with the use of an inverter connected to the electric motor. A tenth of the total volume was replaced every day for 24 days, the pH of the growth media was monitored and the samples were visually checked for aggregate formation. Once the best agitation system had been determined, which in this case was the roller-table method, the whole process was repeated in duplicate on the roller-table system with apricot effluent medium (AEM) as an alternative cheaper growth substrate.

The YELM (pH 7.0) consisted of the following (g.L⁻¹): yeast extract (Biolab) 5.0; sodium lactate (Sigma) 20.0; peptone (Biolab) 2.0; KH₂PO₄ (BDH) 10.0; and Tween 80 (Merck) 1.0 ml. The AEM (pH 7.0) consisted of sieved (0.5 mm²) sterilised apricot cannery effluent obtained from RFF Foods (Pty) Ltd., Groot Drakenstein, South Africa and diluted to a final chemical oxygen demand (COD) value of 2 000 mg.l⁻¹ (Standard Methods, 1998). Urea (0.2 g.l⁻¹) and KH₂PO₄ (0.2 g.l⁻¹) were also added to the AEM.

Viscosity determination and ECP isolation and characterisation

The viscosity was measured at 30°C using a Brookfield viscometer (Model RTN 55878) with a number one spindle at a rotation of 100 rpm according to the method of Racine *et al.* (1991). The ECP was isolated using a modification of the method described by Ludbrook *et al.* (1997), which involved the precipitation of ECP with cold ethanol at 4°C after which it was dialysed, lyophilised and the mass determined. The

Table 1 *Propionibacterium* strains examined for ECP production

Strain no.	Species	Isolated from
S1	<i>P. jensenii</i>	Anaerobic digester
S2	<i>P. thoenii</i>	Anaerobic digester
S3	<i>P. freudenreichii</i> ss. <i>shermanii</i>	Anaerobic digester
S4	<i>P. acidipropionici</i>	Leerdammer cheese
S5	<i>P. freudenreichii</i> ss. <i>freudenreichii</i>	Swiss cheese
S6	<i>P. jensenii</i>	Anaerobic digester
S7	<i>P. jensenii</i>	Anaerobic digester
S8	<i>P. jensenii</i>	Dairy products
S9	<i>P. jensenii</i>	Cheese
S10	<i>P. jensenii</i>	Anaerobic digester
S11	<i>P. jensenii</i>	Anaerobic digester
S12	<i>P. acidipropionici</i>	Anaerobic digester
S13	<i>P. freudenreichii</i> ss. <i>shermanii</i>	Anaerobic digester
S14	<i>P. thoenii</i>	Anaerobic digester
S15	<i>P. freudenreichii</i> ss. <i>shermanii</i>	Leerdammer cheese
S16	<i>P. jensenii</i>	Leerdammer cheese
S17	<i>P. jensenii</i>	Leerdammer cheese
S18	<i>P. thoenii</i>	Dairy products
S19	<i>P. acidipropionici</i>	Dairy products

presence of proteins in the ECP was determined using the Pierce Bicinchonic acid (BCA) protein assay kit (Pierce, USA) according to the manufacturer's instructions. The sugar content of the ECP produced by *P. jensenii* S1, the best overall ECP producer, was analysed by one-dimensional ascending thin layer chromatography (TLC) using silica gel G plates (Merck) according to the method of Cato *et al.* (1970).

Volatile fatty acid (VFA) analysis

VFAs produced in the different growth media were determined using a Varian (model 3700) gas chromatograph equipped with a flame ionisation detector and a 30 m x 0.53 mm Nukol, 0.5 μm fused silica capillary column (Supelco, USA). The column temperature was initially held at 105°C for 2 min and then increased at a rate of 8°C.min⁻¹ to 190°C where it was held for 10 min. The detector and inlet temperatures were set at 300°C and 130°C respectively and nitrogen was used as carrier gas at a flow rate of 2.5 ml.min⁻¹. An aqueous stock solution was prepared as standard and contained 0.5 ml.l⁻¹ hexanol (internal standard) and 1 ml.l⁻¹ of each of the following short-chain fatty acids: acetic, formic, propionic, iso-butyric, butyric, iso-valeric and valeric acid (Sigge, 2005).

Scanning electron microscopy (SEM)

Bacterial aggregates from the AEM samples (AE aggregates) were fixed, dehydrated in an ascending series of ethanol concentrations and 'critical point dried' with CO₂. The aggregates were mounted on a stub with double-sided tape and observed with a JEOL JSM 6100 scanning electron microscope at an acceleration voltage of 7.0 kV after being coated with a 10 nm gold layer (R. van Zyl, ARC-Infruitec, Stellenbosch, South Africa, personal communication, 2001).

Polymerase chain reaction (PCR) detection

DNA was isolated from the AE aggregates using the method of Van Elsas *et al.* (1997). *Propionibacterium*-specific amplification was achieved by using PCR primers and reaction conditions as developed by Schoeman (2001). The primer designs were based

on aligned 16S ribosomal RNA (rRNA) gene sequences of specific classical *Propionibacterium* reference strains (Riedel *et al.*, 1998).

Results and discussion

ECP production by propionibacteria

The concentration of ECP produced by the 19 different *Propionibacterium* strains in the YELM was determined visually during the first part of the study. Six of the strains (S1 - S6) that produced a reasonable amount of ECP were identified and used in the subsequent ECP production and viscosity measurement studies in YELM (Fig. 1). The ECP method was optimised until a variation of less than 10% was obtained between repetitions. TLC was used in the preliminary identification of the carbohydrates present in the extracted ECP from *P. jensenii* strain S1. It was found to contain a complex polysaccharide which, upon further hydrolysis, resulted in major amounts of mannose and lesser amounts of glucose and galactose. Using the BCA protein kit, no proteins could be found in the ECP and it was concluded that the ECP probably only consisted of carbohydrate complexes.

P. jensenii strain S1 produced the most ECP and showed the highest medium viscosity under the applied growth conditions (Fig. 1). The lack of correlation between viscosity and ECPs of the other strains such as S2 and S3 could probably be attributed to a difference in ECP composition and structure. Viscosity is generally a good indication of ECP production, but it is dependent on factors such as ECP linearity and structure as well as polymer molecular mass and type, the presence and physical state of the carbohydrates and the presence of proteins and other metabolites (Cerning, 1990). Strains S1, S2, S3 and S6 were all originally isolated from anaerobic digesters while strains S4 and S5 were isolated from Swiss-type cheeses. When considering only the amount of ECP produced by the six strains, it is furthermore interesting to note that the three best ECP producers (S1, S2 and S3) were all originally isolated from anaerobic digesters. It could be that the ability to produce ECPs might be more advantageous for classical propionibacteria naturally living in wastewater environments than for strains adapted to typical dairy fermentation conditions.

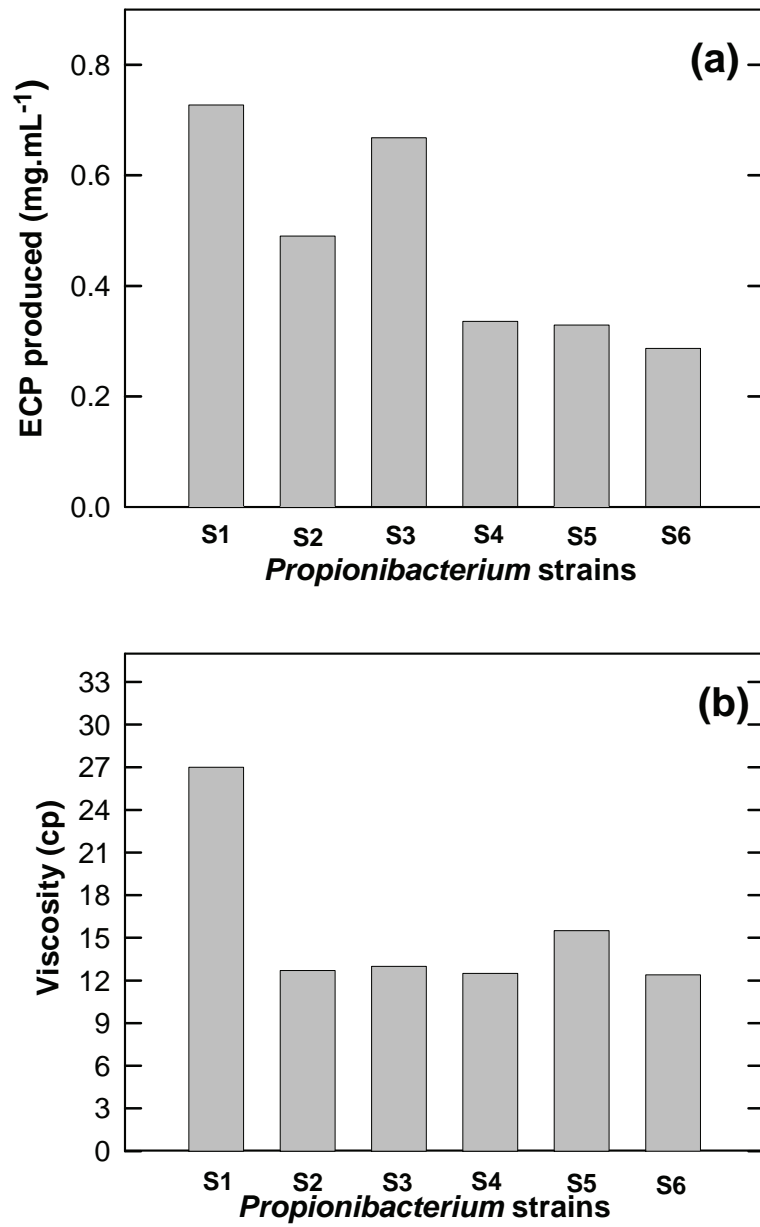


Figure 1 ECP production (a) and viscosity (b) changes in YELM inoculated with different *Propionibacterium* strains.

Aggregate formation

The overall best ECP producer, *P. jensenii* S1, was inoculated once again into YELM, and the samples were incubated both on a roller-table and on a shake platform. The process was also performed with AEM on the roller-table. VFA production (Fig. 2) and pH changes (Fig. 3) in the YELM samples were monitored to determine what influence the different mixing systems would have on the metabolism of *Propionibacterium*, but no distinct differences were noted between the roller-table and the shake platform. The pH of the AEM samples (Fig. 3) was found to drop to a minimum of 5.8 and then stabilised at a pH level of 6.2, which was lower than the pH values of the YELM samples, which dropped to a minimum of 6.6 after which they stabilised (pH 6.7 - 6.8). It is also known that propionibacteria produce high amounts of propionic acid and lesser amounts of acetic acid during the fermentation of lactate (Vorobjeva, 1999). This same trend was observed in all the YELM samples, regardless of the mixing system used. Propionic and acetic acids were also the only VFAs produced in the AEM samples, although not in the same ratio or at the same levels as in the YELM samples. These observations can probably be attributed to the basic differences in substrate composition and environmental pH.

After 18 days, bacterial flocs were observed in all the roller-table YELM and AEM samples (Fig. 4a and 4b). Larger, more stable flocs were observed in the AEM. Scanning electron microscopy of the flocs in the AEM (Fig. 5 and Fig. 6) and PCR analysis were successfully used to positively confirm the presence of propionibacteria in these aggregates by the amplification of a 720 base pair (bp) fragment specific to propionibacteria. It was also found that the flocs in the AEM remained stable for at least five months of storage at 4°C.

Although there were no noticeable differences between the pH and VFA profiles (Fig. 2) of the shake platform and the roller-table YELM samples, visible bacterial flocs were only observed in the roller-table samples (Fig. 4a). This suggested that the mixing action and speed are important to the formation of bacterial aggregates, as was also observed by Bossier & Verstraete (1996) who reported that shear forces were instrumental in providing cells growing in aggregates with a selective advantage over free suspended cells. In this study it appeared as if the gentle rolling motion of the roller-table was more advantageous for bacterial aggregation than the more vigorous mixing of the linear-shake platform.

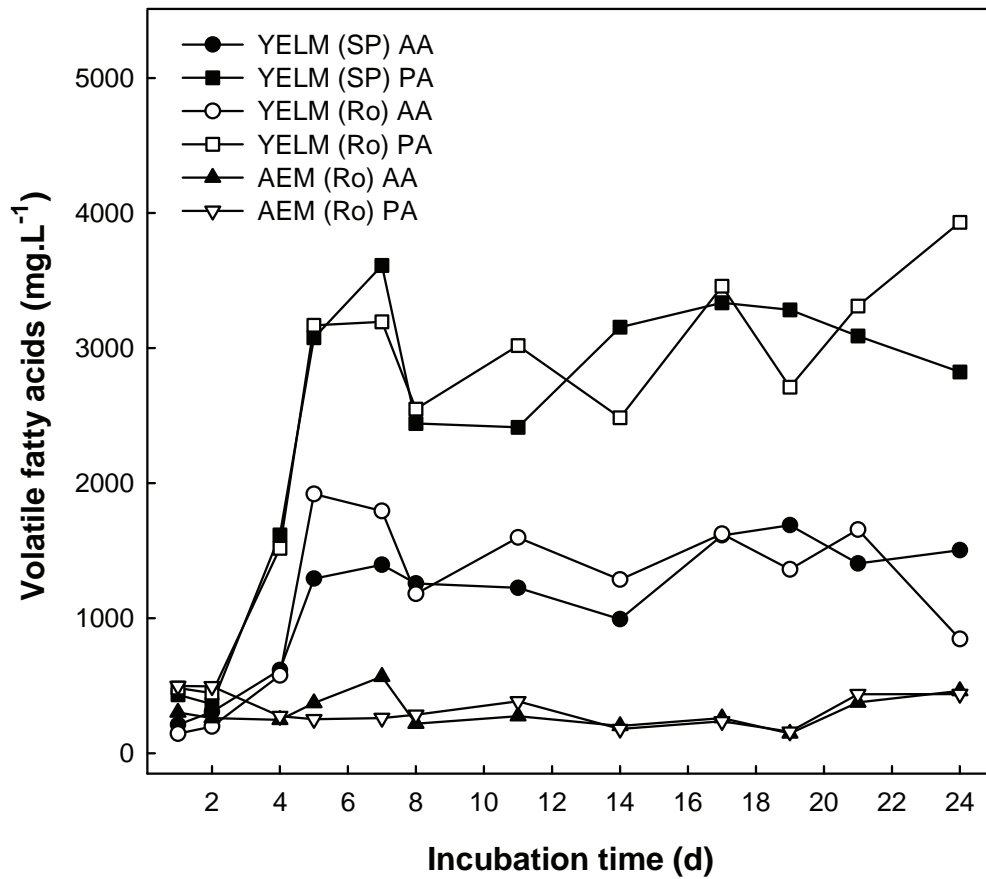


Figure 2 Volatile fatty acids produced by *P. jensenii* S1 in the YELM and AEM during the 24 d incubation period. SP - shake platform; Ro - Roller-table; AA - Acetic Acid; PA - Propionic Acid.

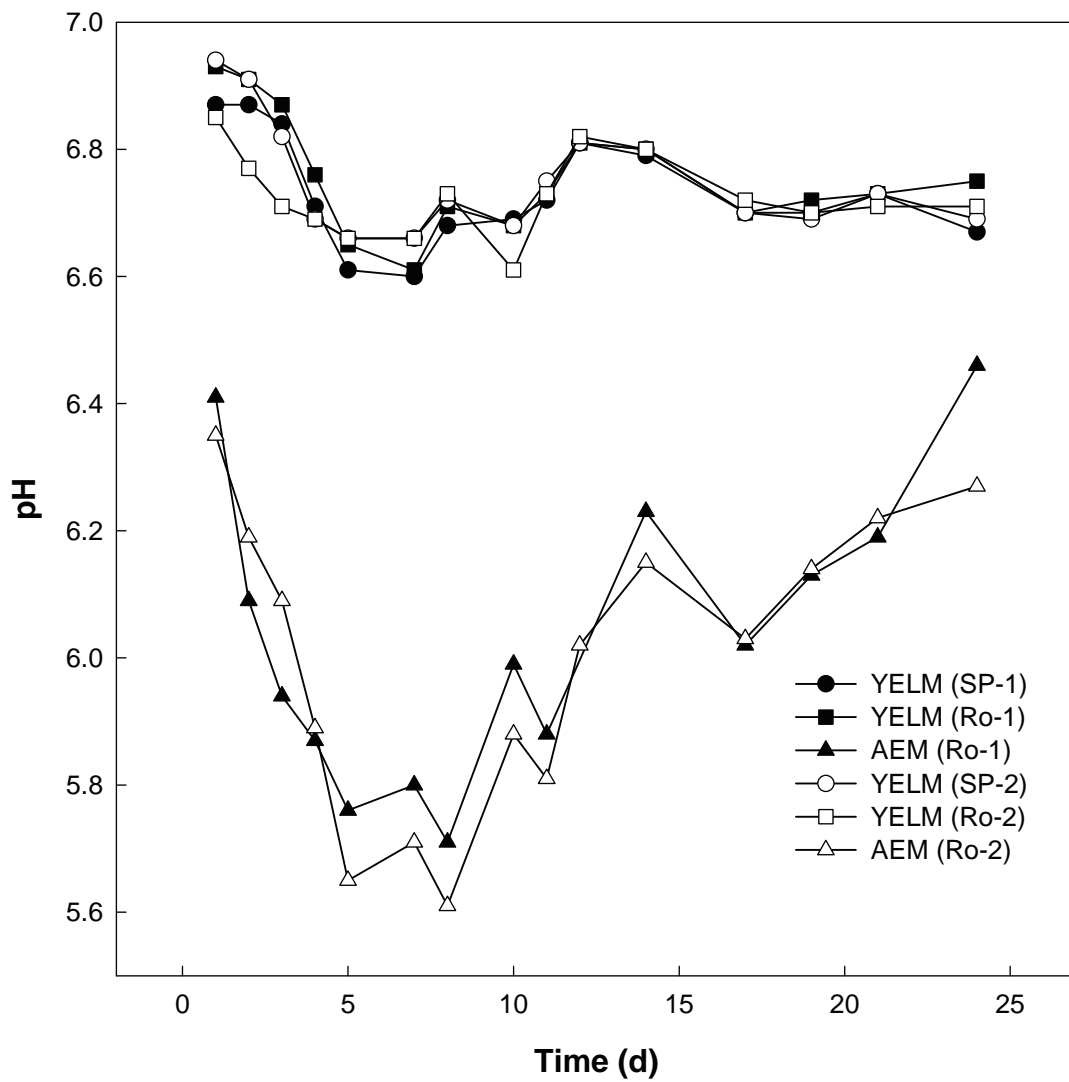


Figure 3 pH profiles of duplicate sets of the YELM samples (incubated on the shake platform [SP] and the roller table [Ro]) and AEM samples (incubated on the roller table [Ro]) during the 24 d incubation period.

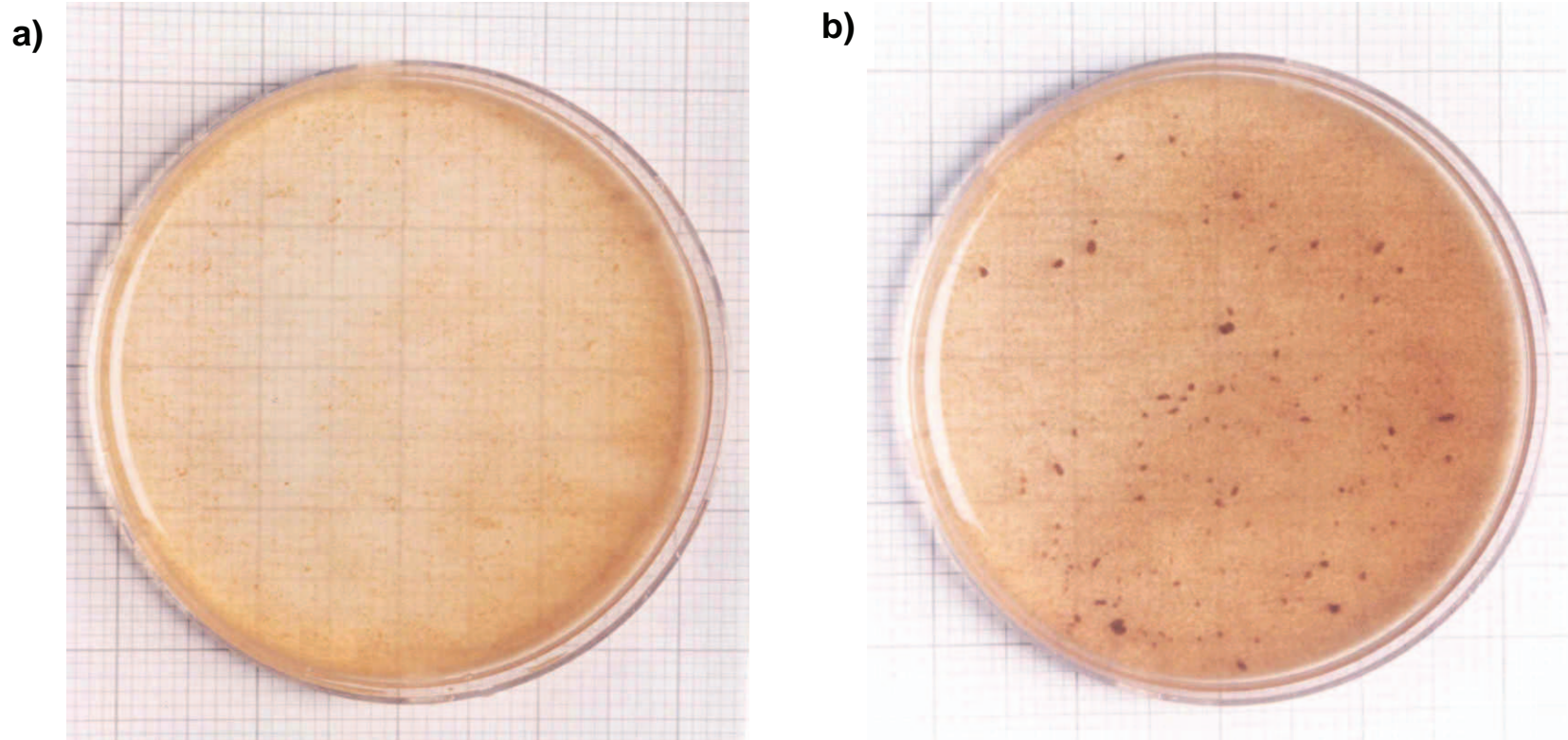


Figure 4 Bacterial flocs produced by *P. jensenii* strain S1 in YELM (a) and in AEM (b)

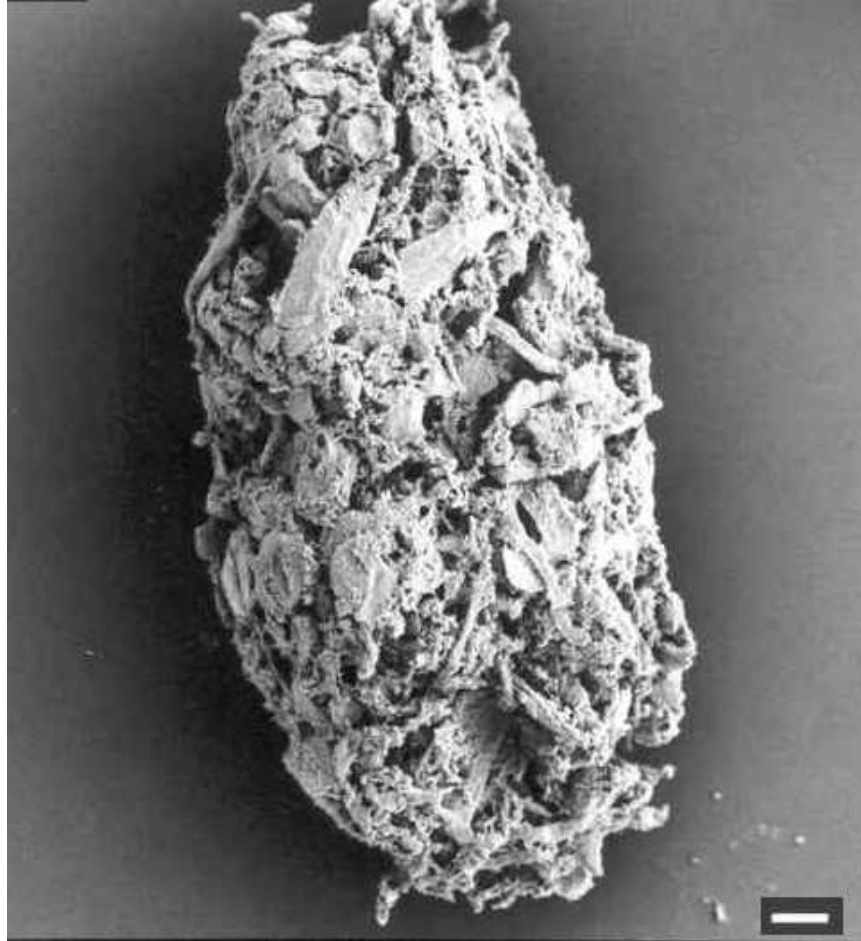


Figure 5 Scanning electron micrograph of a *P. jensenii* strain S1 aggregate formed in AEM. (Magnification x 40; Bar = 100 μm).

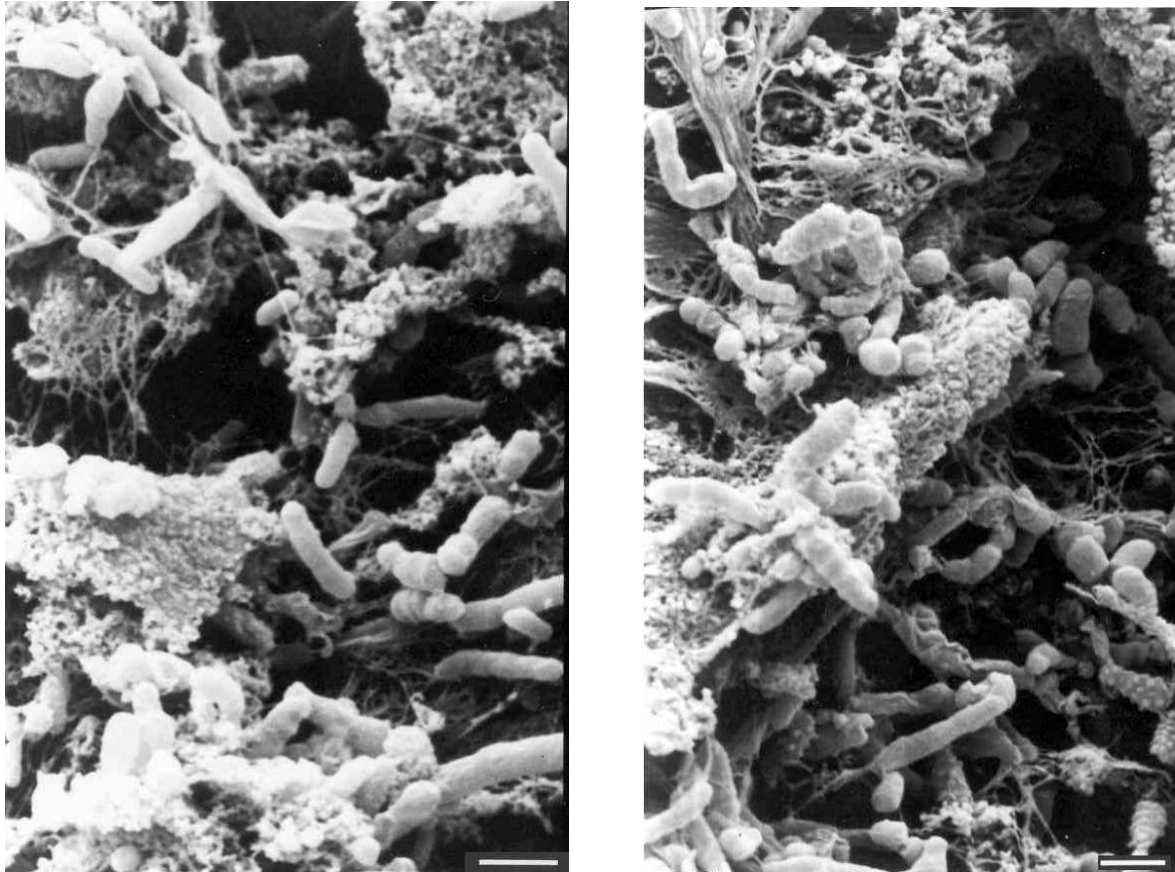


Figure 6 Scanning electron micrograph of *P. jensenii* strain S1 aggregates formed in AEM. (Magnification x 10 000; Bar = 1 μ m)

It is known that industrial UASB digesters treating fruit cannery effluent generally produce very good-quality granules, even in the presence of high shear forces, compared to digesters treating other types of wastewater, such as breweries effluents and protein-containing wastewaters (Batstone & Keller, 2001). It could be that the fruit particles naturally present in this type of effluent may have an additional stabilising effect when integrated in digester granules during the granulation process. In this study it was also observed that bacterial flocs produced in the AEM samples were larger and more stable (Fig. 4b and Fig. 5). This was possibly as a result of the fine fruit fibres being present in the cannery medium, which provided an excellent attachment matrix for the propionibacteria. As can be seen in Fig. 6, the whole fruit fibre-propionibacteria composite was held together by the ECP matrix produced by *P. jensenii* strain S1, which remained stable even after five months of storage.

Conclusions

In conclusion, this study showed that classical propionibacteria, such as *P. jensenii* strain S1 (originally isolated from an anaerobic digester), have a strong ECP-producing ability. *P. jensenii* strain S1 was furthermore successfully manipulated in pure culture to produce bacterial aggregates. This was achieved under the specific conditions of hydrodynamic shear provided by incubation of test samples on the roller-table. The bacterial aggregates were further stabilised by the presence of fruit fibres in the AEM, which provided a lattice to which the bacteria could attach via their ECPs.

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CHAPTER 4

PRELIMINARY EVALUATION OF DIFFERENT ENHANCING SYSTEMS IN THE BATCH GRANULATION-ENHANCEMENT PROCESS*

Summary

In this study three batch granulation-enhancement studies were conducted as part of a preliminary investigation of factors impacting the batch granulation-enhancement process. The first study compared three different mixing systems (a linear-shake waterbath, a linear-shake platform and a roller-table) for application in batch granulation-enhancement. The second study investigated the effect of *Propionibacterium* addition on granulation-enhancement while also determining the optimum sieve size for the screening of digested sludge prior to inoculation. Lastly, the aim of the third study was to determine whether the addition of Fe^{2+} (450 mg.L^{-1}), Ca^{2+} (100 mg.L^{-1}) or powdered activated carbon (1.5 g.L^{-1}) at the start of the study could aid aggregation.

Study 1 demonstrated that the highest volatile solids (VS) were retained by the batch units incubated on the roller-table. The roller-table also showed the most potential for handling larger volumes during batch granulation-enhancement. The results presented in Study 2 did not conclusively prove that the addition of *Propionibacterium* contributed to anaerobic granulation. Aggregate enumerations did, however, indicate that the digested sludge screened with a 1.0 mm^2 mesh showed higher increases in aggregate numbers than the 0.7 mm^2 -screened sludge. Study 3 showed that the addition of Fe^{2+} at the start of the study had a more positive influence on aggregate numbers than the addition of Ca^{2+} or powdered activated carbon. The positive influence of the roller-table on the granulation-enhancement process was also further demonstrated during this study.

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Introduction

The operational efficiency and performance of anaerobic systems, such as the upflow anaerobic sludge blanket (UASB) reactor, for the treatment of agricultural and food industry wastewaters depend on the development of an effective granular sludge bed. Anaerobic granules are formed naturally when anaerobic bacteria aggregate. These aggregates have higher settle abilities and methanogenic activities than flocculent sludge and are more resistant to environmental shocks and toxins (Batstone *et al.*, 2004). Reactor start-up with granular biomass is less problematic than sludge-based start-ups, and high organic loading rates can be reached fairly quickly (Van Lier, 2007). The availability of suitable granular inoculum is, however, limited, especially in developing countries, and purchase and transport costs are high (Britz *et al.*, 1999; Liu & Tay, 2004). In the absence of granular biomass, digested sewage sludge is commonly used as seeding material for reactor inoculation. Unfortunately granulation of digested sludge can take anything from three to eight months (Singh *et al.*, 1998; Liu & Tay, 2004; Zhou *et al.*, 2006). This clearly restricts the general application of anaerobic reactor technologies in developing countries unless the granulation of digested sludge can be enhanced.

In order to address this problem a South African Water Research Commission project (Britz *et al.*, 1999) was started in 1995 to investigate the anaerobic granulation process. A batch granulation-enhancement model was developed based on the hypothesis of Riedel & Britz (1993). According to their hypothesis intermediate lactate concentrations increase along with H_2 partial pressure under unbalanced loading conditions. Under these conditions acid-tolerant lactate-utilising propionate-producing bacteria such as *Propionibacterium* gain a competitive advantage. As the environmental pH decreases, these bacteria produce high concentrations of extracellular polymers (ECPs) as a hydrogen sink mechanism. Higher ECP levels would temporarily contribute to the growth of granular nuclei under the hydrodynamic conditions typical to the UASB process.

This hypothesis is also supported by the following: i) ECPs are a major component of anaerobic granules and contribute to the strength and stability of granules (Quarmby & Forster, 1995; Liu *et al.*, 2004); ii) most of the ECPs in an anaerobic community are produced by fermentative acidogenic populations, which explains why granules treating carbonaceous effluents are generally larger in size than granules used

in the treatment of volatile fatty acid (VFA)-containing effluents (Fukuzaki *et al.*, 1991); iii) ECPs affect the surface properties of bacterial cells and help cells to adhere (Zhou *et al.*, 2007); iv) microbial ECP production is substantially enhanced under stressful culture conditions (Liu *et al.*, 2004; Zhou *et al.*, 2007); v) increases in anaerobic granulation have been reported after short periods of organic overloading (Riedel & Britz, 1993; Zhou *et al.*, 2007); vi) lactate is one of the common fermentation products in anaerobic digesters and is the preferred substrate of propionic acid-producing bacteria (Gerardi, 2003); vii) propionic acid is an important intermediate in anaerobic digestion and is known to accumulate under conditions of environmental stress (Riedel & Britz, 1993; Gerardi, 2003); and viii) strains from the propionic acid-producing genera *Propionibacterium*, *Selenomonas* and *Veillonella*, which also produced high levels of ECPs, were previously isolated from UASB reactors (Slobodkin & Verstraete 1993; Riedel & Britz, 1993).

The aim of the biological model developed by Britz *et al.* (1999) was therefore to simulate a UASB reactor environment under organic overloading conditions in 500 mL batch units. By changing the environmental conditions on batch scale, the propionic acid producers were given a competitive advantage that led to enhanced granulation. The granular sludge was cultured in batch units using different carbon sources (including lactate) (Britz *et al.*, 1999). The upflow shear forces that normally exist in a UASB reactor environment were also considered since these are instrumental in providing cells growing in granules with a selective advantage over free suspended cells (Vanderhaegen *et al.*, 1992). In the study conducted by Britz *et al.* (1999), these shear forces were simulated by incubating the 500 mL batch units in linear-shake waterbaths at 35°C. A granulation enhancement of between 400 and 1 000% was reported.

As part of a preliminary evaluation of factors impacting the batch granulation-enhancement process, the following aspects were investigated. Firstly, before the batch granulation-enhancement process could be of industrial value, it has to be evaluated on larger scale. One of the limiting factors is the manner in which larger-volume batch units can be agitated to provide the same type of mixing as was provided by the linear-shake waterbaths used by Britz *et al.* (1999). The first aim of this study was, therefore, to evaluate the effect of three mixing systems (a linear-shake waterbath, a linear-shake platform and a roller-table) in the up-scaling of the biological model of Britz *et al.* (1999) from a batch unit volume of 500 mL to a batch unit volume of 5 L.

Secondly, ECP-producing *Propionibacterium* strains have been isolated from anaerobic processes (Riedel & Britz, 1993). It has also previously been demonstrated that these strains can be manipulated in pure culture to form aggregates (Van Schalkwyk *et al.*, 2003). The biological model of Britz *et al.* (1999) aims at providing lactate-utilising populations in general with a competitive advantage. It is, however, not yet clear whether addition of, specifically, *Propionibacterium* cultures to digested sludge during batch granulation-enhancement could further contribute to the granulation-enhancement process. Therefore, the second aim of this study was to determine whether the addition of an ECP-producing *Propionibacterium* strain to the sludge batch units during batch granulation- enhancement could enhance sludge granulation.

The third aim of this study was to determine whether the addition of Fe^{2+} (450 mg.L^{-1}), Ca^{2+} (100 mg.L^{-1}) or powdered activated carbon (1.5 g.L^{-1}) at the start of the study could aid aggregation during the batch granulation-enhancement process. It has been reported in the literature that the presence of certain divalent ions or inert nuclei could contribute to granulation in UASB reactors (Mahoney *et al.*, 1987; Yu *et al.*, 1999; Yu *et al.*, 2000). Adding these agents during batch granulation-enhancement might thus also be advantageous.

Materials and methods

Digested sludges

Anaerobically digested sewage sludges were obtained from both the 'Athlone 1 Primary B' and the 'Athlone 1 Secondary B' anaerobic digesters at the Athlone municipal wastewater treatment works (WWTW) and used, respectively, as seed sludge in the first and second batch granulation-enhancement studies. For the third batch granulation-enhancement study anaerobically digested sewage sludge was obtained from the anaerobic digester at the Kraaifontein WWTW.

Study 1 – Comparison of mixing systems for application in granulation-enhancement

Batch units of three different volume sizes (500 mL, 1 L, and 5 L), with operational volumes of 400 mL, 800 mL and 3.5 L, respectively, were inoculated with a 1:4 part ratio

of sludge:lactate (Lac) medium. The Lac medium consisted of the following (g.L⁻¹): sodium lactate (Sigma) 20.0; yeast extract (Biolab) 5.0; peptone (Biolab) 2.0; KH₂PO₄ (BDH) 10.0; and Tween 80 (Merck) 1 mL. The pH was adjusted to 7.0 using a 2M NaOH solution after which the medium was sterilised. The 500 mL and 1 L units were glass containers while the 5 L containers were plastic.

Three mixing systems were used: i) a linear-shake waterbath (manufactured by Scientific Manufacturing, Paarden Eiland, Cape Town) with a shaking speed of 130 rpm; ii) a linear-shake platform (Labotec) with a shaking speed of 130 rpm; and iii) a roller-table (manufactured by the Process Engineering Workshop, University of Stellenbosch) with a constant roller speed. This roller speed resulted in the 1 L containers 'rolling' at a speed of 30 rpm and the 5 L containers rolling at a speed of 17 rpm. The temperature of the linear-shake waterbaths was maintained at 35°C while the linear-shake platform and the roller-table were placed in a temperature-controlled incubator room at 35°C.

Triplicate sets of the 500 mL units were incubated in the linear-shake waterbath (Wa500mL samples) and on the linear-shake platform (Sp500mL samples). Triplicate sets of the 1 L units were incubated in the linear-shake waterbath (Wa1L samples), on the linear-shake platform (Sp1L samples) and on the roller-table (Ro1L samples), and duplicate sets of the 5 L units were incubated on the linear-shake platform (Sp5L samples) and on the roller-table (Ro5L samples). Each day, for a period of 30 d, 80 mL, 160 mL and 800 mL were, respectively, removed from the different batch units (500 mL, 1 L and 5 L) and replaced with sterile substrate to simulate UASB reactor feeding operations.

Study 2 – Effect of *Propionibacterium* addition on aggregation during granulation-enhancement

The sludge used as seed in this study was divided into two parts and screened separately through one of two sieves. The one sieve had a 700 µm² mesh and the sludge screened with this sieve was designated as sludge A. Sludge B was screened through a 1 mm² mesh sieve. After sieving both sludges A and B were left to settle overnight and the sludges separated into two phases: a heavy, concentrated sludge phase with a more watery phase at the top. Before inoculation of the batch units, the watery phases were decanted.

The *Propionibacterium* (PAB) inoculum was prepared using *P. jensenii* strain S1, which was originally isolated from anaerobic processes and has also previously been identified as an ECP producer (Van Schalkwyk *et al.*, 2003). The PAB inoculum was prepared by inoculating 300 mL of sterile Lac medium with 25 mL of a *P. jensenii* S1 active culture and incubating it for 5 d at 30°C. The resulting culture had a concentration of approximately 10^8 colony forming units per mL. The culture was stored at 4°C and was used directly as PAB inoculum for the batch granulation-enhancement study.

The batch units for this study (Table 1) had a total volume of 500 mL, with an operational volume of 400 mL. The Lac medium for this study had the same composition as the Lac medium used during the first batch granulation-enhancement study (Study 1). Duplicate sets of batch units were prepared for both sludge A and sludge B. These batch units contained 300 mL Lac medium, 80 mL sludge (either A or B) and 20 mL PAB inoculum (A.1, A.2, B.1 and B.2). Control units that contained no PAB were also prepared for each sludge (A-control and B-control). All units were incubated on a linear-shake waterbath with a shaking speed of 130 rpm for a period of 24 d at 35°C, similar to the conditions used by Britz *et al.* (1999). One mL of PAB inoculum was also added daily to batch units A.1, A.2, B.1 and B.2 for the duration of the study.

Study 3 – Influence of Fe^{2+} , Ca^{2+} and powdered activated carbon on aggregation during granulation-enhancement

The batch units in this study had a total volume of 500 mL, with an operational volume of 450 mL (100 mL sludge and 350 mL Lac medium) (Table 1). The Lac medium for this study consisted of the following (g.L⁻¹): sodium lactate (Sigma) 10.0; yeast extract (Biolab) 5.0; peptone (Biolab) 2.0; KH₂PO₄ (BDH) 10.0; and Tween 80 (Merck) 1 mL. The pH was adjusted to 7.0, using a 2 M NaOH solution, after which the medium was sterilised. Fe^{2+} (450 mg.L⁻¹), Ca^{2+} (100 mg.L⁻¹) or powdered activated carbon (ActC) (1.5 g.L⁻¹) were added on day 0 (D0), each in duplicate, before the sludge was inoculated. Control units (Con samples) were also included. One unit of each duplicate set was incubated in a linear-shake waterbath (130 rpm) (Wa samples) similar to the conditions used by Britz *et al.* (1999) while the other unit was incubated on a roller-table (35 rpm) (Ro samples) for a period of 20 d at 35°C.

Table 1 Summary of all batch unit abbreviations used in the three batch granulation-enhancement studies

Batch unit	Volume	Agitation system	Additions
Study 1			
Sp500mL	500 mL	Linear-shake platform	
Wa500mL	500 mL	Linear-shake waterbath	
Sp1L	1 L	Linear-shake platform	
Wa1L	1 L	Linear-shake waterbath	
Ro1L	1 L	Roller-table	
Sp5L	5 L	Linear-shake platform	
Ro5L	5 L	Roller-table	
Study 2			
A-control	500 mL	Linear-shake waterbath	Sludge (0.7 mm ²); no PAB
A.1	500 mL	Linear-shake waterbath	Sludge (0.7 mm ²) + PAB
A.2	500 mL	Linear-shake waterbath	Sludge (0.7 mm ²) + PAB
B-control	500 mL	Linear-shake waterbath	Sludge (1.0 mm ²); no PAB
B.1	500 mL	Linear-shake waterbath	Sludge (1.0 mm ²) + PAB
B.2	500 mL	Linear-shake waterbath	Sludge (1.0 mm ²) + PAB
Study 3			
Fe-Ro	500 mL	Roller-table	Fe ²⁺ added on D0
Fe-Wa	500 mL	Linear-shake waterbath	Fe ²⁺ added on D0
Ca-Ro	500 mL	Roller-table	Ca ²⁺ added on D0
Ca-Wa	500 mL	Linear-shake waterbath	Ca ²⁺ added on D0
ActC-Ro	500 mL	Roller-table	ActC added on D0
ActC-Wa	500 mL	Linear-shake waterbath	ActC added on D0
Con-Ro	500 mL	Roller-table	No Fe ²⁺ , Ca ²⁺ or ActC
Con-Wa	500 mL	Linear-shake waterbath	No Fe ²⁺ , Ca ²⁺ or ActC

Aggregate enumeration

Different sludge-withdrawal strategies were employed for each batch granulation-enhancement study. During the first batch granulation-enhancement study sludge was allowed to settle for 10 min before 10 mL sludge samples were withdrawn from each of the batch units at five-day intervals. One mL sludge was then removed from each of these 10 mL samples and mixed with 20 mL of gelatine (30 g.L⁻¹) in the base of a round glass container (diameter = 14 cm) and left to set at 4°C. The remaining sludge samples were returned to their respective batch unit containers.

During the second batch granulation-enhancement study each batch unit was sampled on D0, D10 and D24. Before sampling, each unit was shaken after which two 1 mL sludge liquor samples were withdrawn. From each 1 mL sample, 250 - 500 µL sludge liquor was transferred to a petri dish (diameter = 9 cm) with gelatine solution after which it was left to set at 4°C.

For the third batch granulation-enhancement study the same sampling procedure was followed as for the first batch granulation-enhancement study, except that all batch unit containers were well mixed before the sludge liquor was sampled.

A graded grid was placed underneath each sludge-gelatine container and 10 fields, each with a diameter of 10 x 6 mm, were scanned into the computer using the Matrox Intellicam Interactive (Version 2.0) frame-grabber program (Matrox Electronic Systems Ltd) and a Nikon SMZ800 stereoscopic microscope with an online Panasonic CP410 video camera. The number of aggregates present in the sludge was counted using the Scion Image program (release Beta 3b) (Scion Corporation, Maryland USA).

Analytical procedures

The following parameters were monitored using the methods specified in Standard Methods (1998): pH; total solids (TS); and volatile solids (VS).

VFAs were determined using a Varian (model 3700) gas chromatograph equipped with a flame ionisation detector and a 30 m x 0.53 mm Nukol, 0.5 µm fused silica capillary column (Supelco, USA). The column temperature was initially held at 105°C for 2 min, before it was increased at a rate of 8°C per min to 190°C where it was held for 10 min. The detector and inlet temperatures were set at 300°C and 130°C respectively and nitrogen was used as carrier gas at a flow rate of 2.5 mL.min⁻¹ (Sigge, 2005).

BorwinTM computer software (JMBS Developpements, France) was used to quantitatively determine the presence of VFAs by integrating the peak areas, using internal standard calibration. Identification of the unknown compounds was achieved by comparing their retention times to those of analytical grade standard VFAs. An aqueous stock solution was prepared as standard and contained 0.5 mL.L⁻¹ of hexanol (BDH) as internal standard and 1 mL.L⁻¹ of each of the following fatty acids: acetic (Merck), propionic (Merck), iso-butyric (Sigma), butyric (Merck), iso-valeric (Hopkin & Williams Ltd.) and valeric acid (Aldrich).

Results and discussion

Study 1 – Comparison of mixing systems for application in granulation-enhancement

The main aim of this preliminary investigation was to determine which one of three different mixing systems (a linear-shake waterbath, a linear-shake platform and a roller-table) would be the agitation method of choice when different-sized batch units (500 mL to 5 L) are incubated during the batch granulation-enhancement of digested sewage sludge.

Aggregate numbers

The average aggregate numbers of the different batch units, as determined using the Scion Image program, can be seen in Fig. 1. The percentage increase in aggregate numbers was calculated after 20 d and after 30 d. The results were compared to determine the optimum period for batch granulation-enhancement. The four units that showed the highest increase in aggregate numbers over the first 20 d were the following: Sp1L (D20: 862.4%); Ro5L (D20: 695.1%); Sp500mL (D20: 645%); and Ro1L (D20: 455.6%). The four units that had the highest increase in aggregate numbers after 30 d were Sp1L (D30: 968.0%); Sp500mL (D30: 506.5%); Ro5L (D30: 460.9%); and Wa500mL (D30: 419.7%). All these increases were within the range of granulation increase of 400% - 1 000% reported by Britz *et al.* (1999) for 500 mL units incubated in a shaking waterbath. Only two of the six unit sets showed an increase in aggregate numbers between D20 and D30 while all the other unit sets showed a decrease. This is

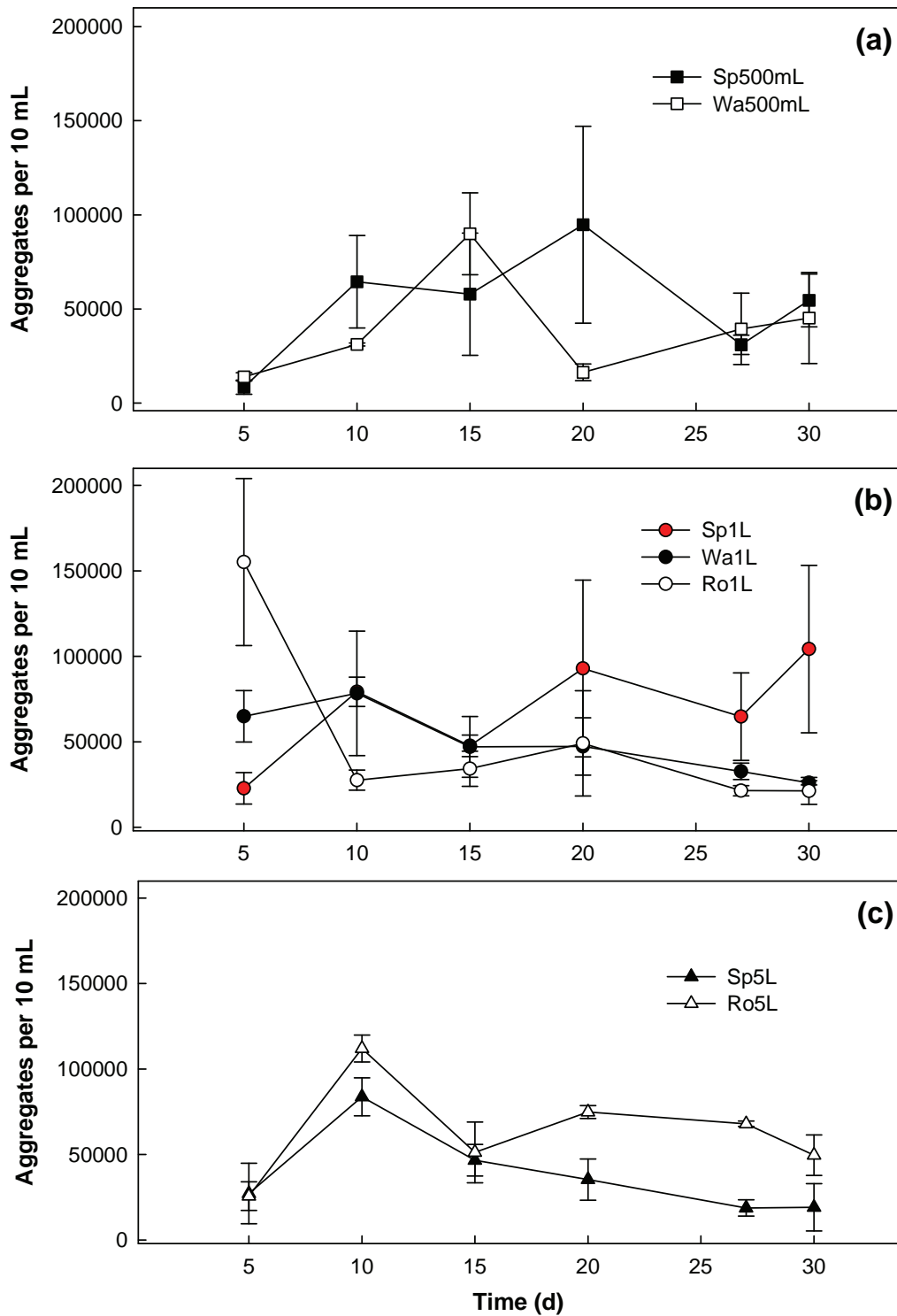


Figure 1 Study 1: Averages of aggregate numbers in the batch units incubated on a linear-shake platform (Sp), in a linear-shake waterbath (Wa) or on a roller-table (Ro). Die different unit volumes were: (a) 500mL (Sp500mL and Wa500mL); (b) 1 L (Sp1L, Wa1L and Ro1L); and (c) 5 L (Sp5L & Ro5L). Each data point of the 500 mL and 1 L units represent triplicate values, while the 5 L unit data points represent duplicate values. The standard deviation was used as the error bar.

in agreement with Britz *et al.* (1999) who reported that the optimum batch granulation enhancement period is between 14 and 26 d.

The data clearly showed large variations (Fig. 1.), which made proper evaluation of aggregate numbers difficult. This indicated that the counting method needed to be improved for application in future studies. Although increases were noted overall, visible increases in granulation were, however, not observed to the same extent (granule size: pinpoint to 3 mm) as was reported by Britz *et al.* (1999). One of the major differences between this study and that of Britz *et al.* (1999) was the digested sludge used as seed sludge. Britz *et al.* (1999) used a thick, digested sludge from the Kraaifontein WWTW. At the start of this study, however, anaerobic sludge from the Kraaifontein digester was unavailable due to mechanical problems, and Athlone primary anaerobically digested sludge was used instead. This anaerobic sludge was more watery, although it was visibly active.

Sampling of the sludge during the study was also problematic and difficult to standardise as a result of sludge flotation in certain units, which might explain the 'zig-zag' effect seen for units such as Sp1L and Ro5L. Biofilm formation inside the Ro5L containers was also observed, which might further have influenced granule numbers.

An important consideration is the fact that this method only measures aggregate numbers without taking into account the size distribution of the aggregates. It was, therefore, not possible to determine from the aggregate numbers whether increases in granule numbers were the result of increased aggregate formation or the result of the disintegration of larger aggregates into smaller pieces. The intensity of agitation that occurred in the different units was more vigorous on the linear-shake platform (Sp units) and in the linear-shake waterbath (Wa units) than the agitation provided by the roller-table (Ro units). It could be that larger aggregates present in the Wa and Sp units might have disintegrated under these conditions. There is, therefore, an urgent need for an enumeration method that would be able to distinguish between increases in both aggregate numbers and aggregate size.

Total solids

The percentage TS and VS of the different units after 30 d (D30) is compared to the D0 sample in Table 2. In all the units decreases in the TS and VS contents were observed over 30 d. This was probably the result of sludge washout, which might have been exaggerated by the excessive gas production that was observed in all the units. The Ro

Table 2 Total solids (TS) and volatile solids (VS) content of the different units of Study 2 on D30 compared with the TS and VS content on D0

Batch units	TS (g.L⁻¹)	VS (g.L⁻¹)
D0 (all)	47.0	29.8
D30 Sp500mL	36.2	18.7
D30 Wa500mL	39.7	22.4
D30 Sp1L	42.5	23.5
D30 Wa1L	39.9	21.2
D30 Ro1L	42.6	25.7
D30 Sp5L	34.3	18.7
D30 Ro5L	45.9	28.1

units (Ro1L and Ro5L) had the highest TS and VS content, which may indicate that the sludge is retained better in this agitation system.

It was observed that the units with higher TS and VS retention did not necessarily correspond with the units with the highest aggregate numbers (Fig. 1). Although the Ro5L units (with the highest TS and VS values) also had the second highest aggregate number on D20 and the third highest on D30, the Sp500mL units, which showed the lowest VS content on D30, had the second highest aggregate numbers on D30. This might be the result of problematic sludge sampling during counting, as discussed previously. This again emphasises the need for an improved counting method in order to distinguish between total sludge biomass and microbial aggregate content.

Another fact that should be considered is that the VS content determined in this study consists of both volatile suspended solids (VSS) and volatile dissolved solids (VDS). Since the VSS content would be a more accurate indicator of microbial biomass than the VS content, it is recommended that VSS rather than VS determinations should be used to determine anaerobic sludge retention during batch granulation-enhancement studies and UASB start-ups.

pH profiles

The pH profiles of all the units can be seen in Fig. 2. The 5 L units (Sp5L and Ro5L) and the Ro1L units all exhibited the typical pH profiles reported by Britz *et al.* (1999). A typical pH profile during start-up of an anaerobic digester or after an organic overload would show a rapid decrease to a pH minimum within the first five d after which the pH profile recovered to levels between 6.7 and 6.9 within 10 d. The rapid pH decrease would be the direct result of an accumulation of VFAs produced by fast-growing acidogenic populations in response to the organic overload. Under controlled conditions the pH decrease would not result in permanent acidification as the accumulated VFAs are consumed by the slower-growing acetogens and methanogens and methane and alkalinity are produced. This would result in a recovery and stabilisation of the pH profile to more neutral pH levels (Gerardi, 2003).

According to Britz *et al.* (1999) a rapid pH decrease in response to a lactate overload may be the result of a temporary population shift to a predominant lactate-utilising population. Ro5L and Sp5L showed the best recovery of all the units with the pH profiles decreasing to 6.5 within the first 3 d after which they recovered to values above 6.7 after 5 d, then stabilised and gradually increased further to a pH value of 6.94

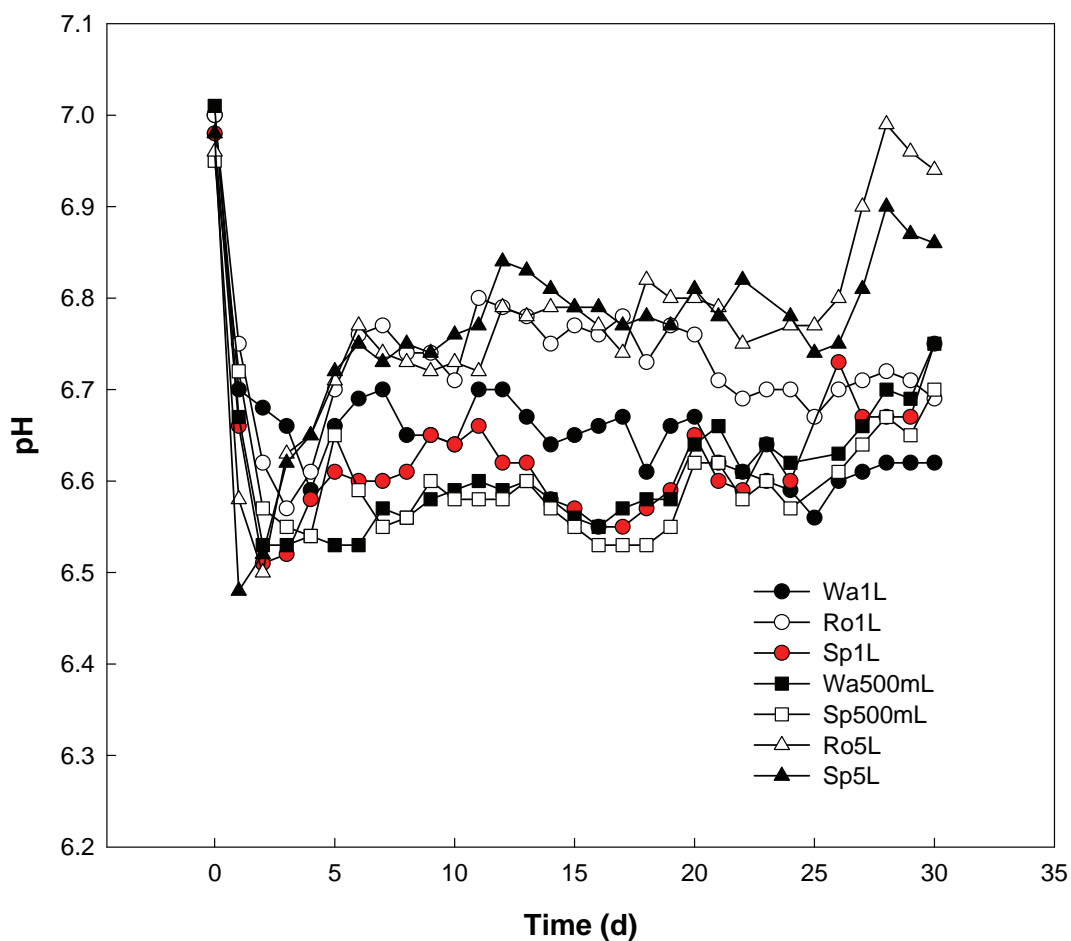


Figure 2 Study 1: Averages of pH values of batch units incubated in a linear-shake waterbath (Wa), on a roller-table (Ro), or on a linear-shake platform (Sp) at 35°C. The different unit volumes were: (a) 500mL (Sp500mL and Wa500mL); (b) 1 L (Sp1L, Wa1L and Ro1L); and (c) 5 L (Sp5L & Ro5L).

on D30. The Ro1L units showed a similar pH decrease and recovery but only remained stable until D20. The pH profiles of the other two 1 L units (Sp1L and Wa1L) both showed a rapid pH decrease and slight recovery within the first few days, but neither could stabilise at pH levels above 6.7. The 500 mL units (Sp500mL and Wa500mL) exhibited a more gradual recovery in pH over the 30 d and reached a maximum pH around 6.7 only after D28. Neither the 1 L nor the 500 mL units recovered to the same extent as the 5 L samples.

Volatile Fatty Acid profiles

The VFAs produced by the 500 mL, the 1 L and the 5 L units can be seen in Figs. 3, 4 and 5, respectively. Iso-valeric and iso-butyric acid were also produced by all the units but the concentrations of these fatty acids were very low ($<250 \text{ mg.L}^{-1}$) and remained so for most of the experiment.

From the VFA profiles (Figs. 3, 4 and 5) it is clear that there is a difference after 30 d between the anaerobic communities in the Ro units (1 L and 5 L) and Sp5L units (Group 1), when compared to the populations in the Sp and Wa (500mL and 1 L) units (Group 2). In the first group of units (Ro1L; Ro5L; and Sp5L), the typical profile of a lactate-utilising population (propionic acid [PA] $>$ acetic acid [AA]) can only be seen during the first 3 d, after which the PA concentration decreased to levels below a $1\ 000 \text{ mg.L}^{-1}$ for the Ro units and to below $1\ 500 \text{ mg.L}^{-1}$ for Sp5L. Along with this, there was an increase in butyric acid (BA) production to levels above $1\ 500 \text{ mg.L}^{-1}$ within the first 8 d. AA remained the most abundant VFA in this group (above $2\ 000 \text{ mg.L}^{-1}$ after D3).

In Group 2 (Sp500mL; Wa500mL; Sp1L; and Wa1L), PA and AA mostly remained at levels above $2\ 500 \text{ mg.L}^{-1}$ while BA production remained below $1\ 500 \text{ mg.L}^{-1}$. In the three units (Sp500mL; Wa500mL; and Sp1L) that also showed increases in aggregate numbers on D20 and D30 (Fig. 1), the PA concentrations were higher than the AA concentrations for the largest part of the study. In contrast to the observations of Britz *et al.* (1999), none of the units showed a simultaneous drop in PA and AA concentrations after the first few days.

Differences in VFA profiles between the two groups were also reflected in their pH profiles. In the first group the simultaneous decrease in PA and increase in BA levels after D3 coincided with a rapid recovery in pH of these units, as was observed in Fig. 2. In the second group the high PA concentrations over a longer period resulted in lower pH profiles, which only recovered to levels above 6.7 on D30.

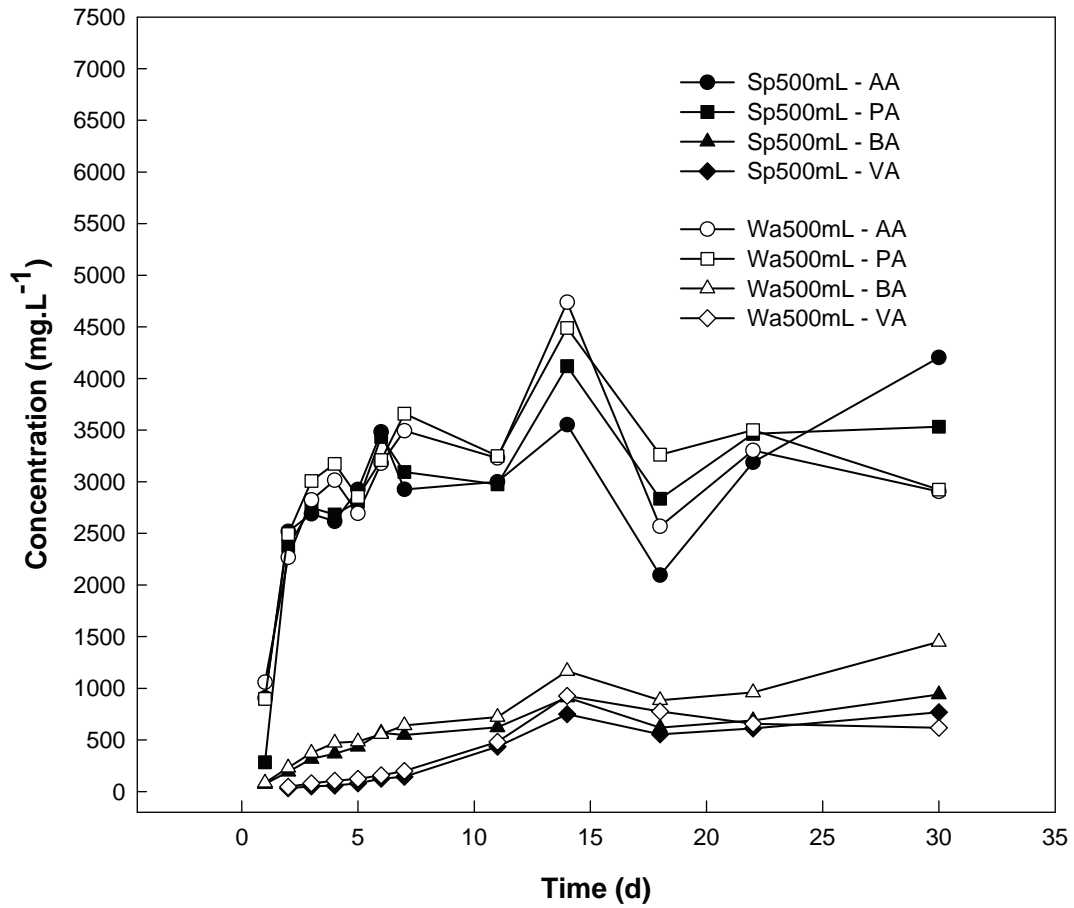


Figure 3 Study 1: Averages of volatile fatty acids produced by triplicate sets of 500 mL batch units incubated at 35°C for 30 d on a linear-shake platform (Sp500mL) and in a shaking waterbath (Wa500mL). AA - acetic acid; PA - propionic acid; BA - butyric acid; VA - valeric acid.

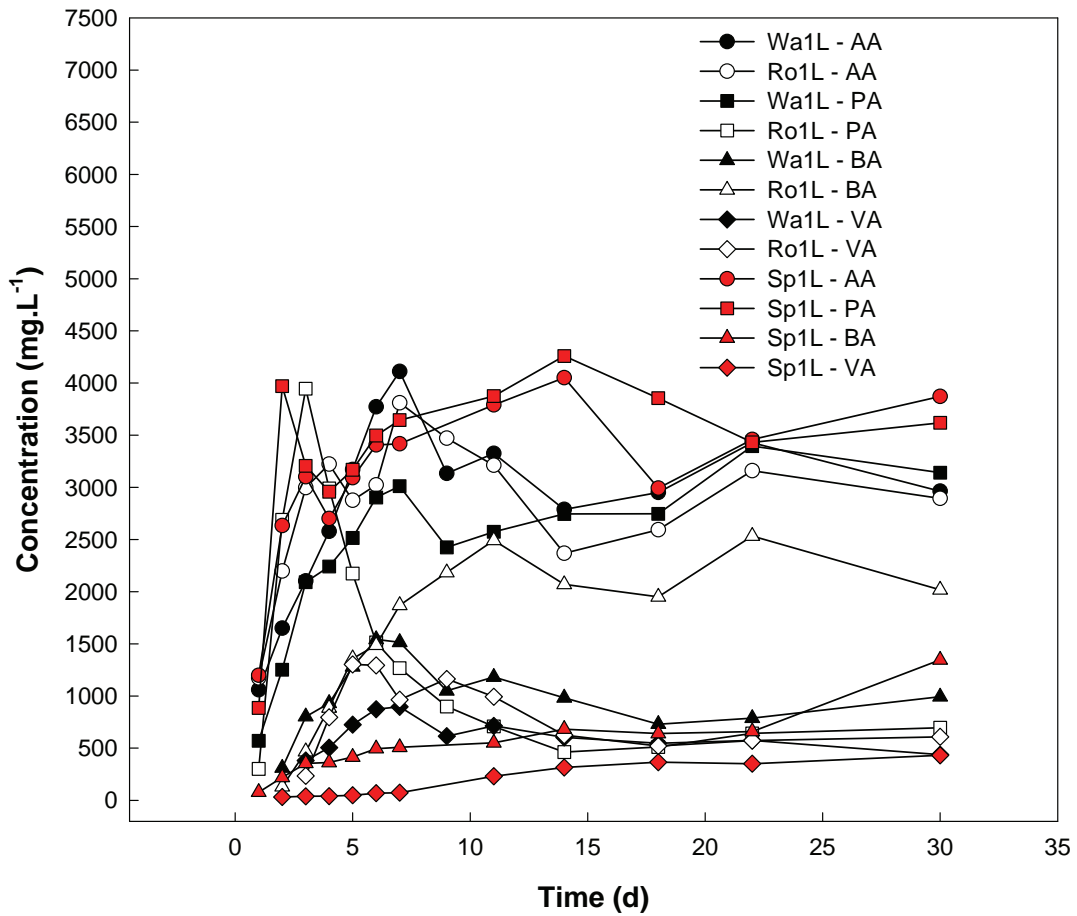


Figure 4 Study 1: Averages of the volatile fatty acids produced by triplicate sets of 1 L batch units incubated in a linear-shake waterbath (Wa1L), on a roller-table (Ro1L) and on a linear-shake platform (Ta1L) at 35°C for 30 d. AA - acetic acid; PA - propionic acid; BA - butyric acid; VA - valeric acid.

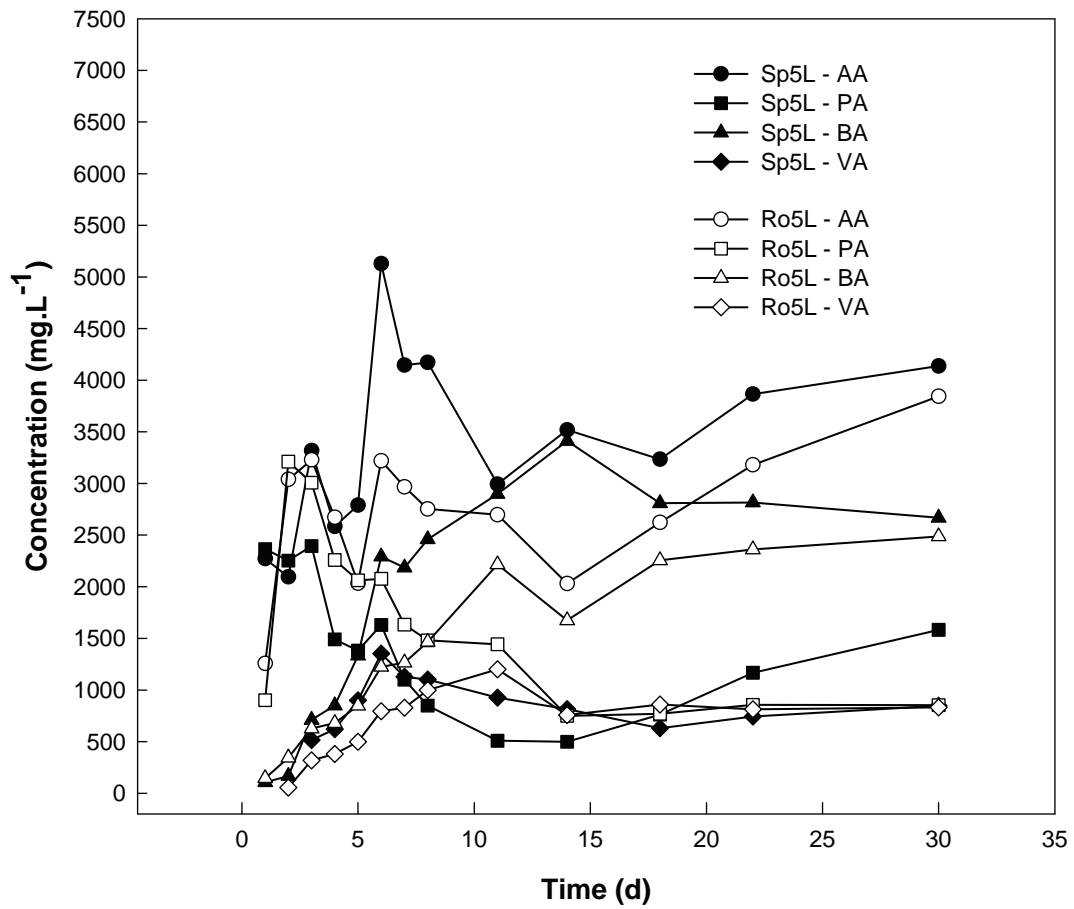


Figure 5 Study 1: Averages of volatile fatty acids produced by duplicate sets of 5 L batch units incubated on a linear-shake platform (Sp5L) and on a roller-table (Ro5L) at 35°C for 30 d. AA - acetic acid; PA - propionic acid; BA - butyric acid; VA - valeric acid.

The difference in pH and VFA profiles between Group 1 and Group 2 could be the indirect result of the different mixing actions (the gentle rolling motion vs. the more vigorous linear-shaking motion), or it could be the result of daily biogas build-up in all the Group 1 units (Ro units [1 L and 5 L] and Sp5L units). As a result of excessive gas production during the experiment, the caps of the glass containers used for the Group 2 units (Sp units [500 mL and 1L] and Wa units [500 mL and 1L]) were slightly loosened to allow constant biogas release for safety reasons. It could be that the atmospheric conditions within the Group 2 units were slightly less reduced since a small amount of oxygen could have entered the units and, as a result of the mixing action of the linear-shake platform and linear-shake waterbath, dissolved in the sludge liquor. In contrast, all the Ro units had to be closed well to prevent leakage on the roller-table, which made constant biogas release impossible and might have resulted in a more anaerobic environment. The 5 L plastic containers used on the roller-table were the same container type used for the Sp5L units. The caps of these specific containers were clamp-on caps that could not be loosened without removing them completely, which also resulted in daily biogas build-up in the Sp5L units.

The anaerobic condition of an aquatic environment is defined in terms of its oxidation-reduction potential (ORP). Although most facultative anaerobes grow well in aquatic environments with an ORP of between -200 and +200 mV, methanogens function optimally at an ORP of -300 mV or less (Gerardi, 2003). Therefore, any amount of dissolved oxygen would have raised the ORP and could have slowed down VFA removal by syntrophic acetogens and methanogens and delayed pH recovery. This might explain why PA levels remained high in the Sp units (500 mL and 1L) and the Wa units (500 mL and 1L). Propionate is fermented by the acetogenic *Syntrophobacter wolinii*, which is an obligate anaerobe and grows in a close symbiotic relationship with hydrogenotrophic methanogens. Any environmental stress that would reduce methanogenic activity would, therefore, also indirectly influence PA removal. Since the caps of the Ro units (1 L and 5 L) and the Sp5L units were tightly closed during agitation, oxygen was less likely to enter these units.

It could also be that daily biogas build-up within the Ro units (1 L and 5 L) and in the Sp5L units might have increased alkalinity levels more rapidly in these units, which could have contributed to the rapid pH recoveries observed during the first few days of the study. Alkalinity is present primarily in the form of bicarbonates that are in

equilibrium with carbon dioxide in the biogas at a given pH, as can be seen in the following equation (Gerardi, 2003):



Biogas produced during the first few days would have mostly consisted of CO₂ since CO₂ is also produced during acidogenic activity. Optimum methane production would not have been probable at the low initial pH observed directly after start-up (Gerardi, 2003). Since constant biogas release would not have been possible in these closed containers, excessive CO₂ production could have increased the CO₂ content of the unit atmosphere to such an extent that some of the CO₂ would have been forced to remain in solution as carbonic acid (H₂CO₃). This might explain why the pH profiles of the Sp5L and Ro5L units decreased the most rapidly of all the units. In addition, the initial hydrogen partial pressure would also have been high as a result of the unbalanced conditions. The hydrogen partial pressure can usually be lowered in anaerobic systems by hydrogen-consuming organisms (such as hydrogenotrophic methanogens) (Gerardi, 2003) or as a result of ECPs acting as a hydrogen sink (Riedel & Britz, 1993). At the low pH values observed during the first few days, it is probable that the hydrogen partial pressure would have been decreased mostly by interaction with ECP components, which would have forced the conversion of carbonic acid to bicarbonate alkalinity and carbonate alkalinity (equation 4.1). After the initial pH recovery to more acceptable levels, hydrogenotrophic methanogens would have further contributed to the lowering of hydrogen partial pressure by producing methane from hydrogen and CO₂. The carbonic acid concentrations would now have been rapidly converted to CO₂, bicarbonate alkalinity and carbonate alkalinity (equation 4.1), resulting in stable anaerobic environments in all the Ro units (1 L and 5 L) as well as in the Sp5L units.

Under these conditions it could be that the syntrophic populations necessary for propionate removal (*Syntrophobacter wolinii*) as well as the methanogenic populations were more active in all the Ro units (1 L and 5 L) and in the Sp5L units. This would explain why the pH profiles of these three unit sets stabilised at levels above 6.7 in spite of increases in BA concentrations. It is also probable that in this specific anaerobic environment a second population shift towards a BA-producing population occurred. Butyrate is a major fermentative product of anaerobic bacteria such as *Clostridium*, which also produce acetate during fermentation (Gerardi, 2003). Furthermore, the

analysis of the *Propionibacterium* ECP in the previous chapter showed that it contained a complex polysaccharide that consisted mostly of mannose and to a lesser extent glucose and galactose. *Clostridium* spp. ferment a variety of sugars and could thus also have utilised the excessive amount of ECPs produced by lactate-utilising propionibacteria under conditions of organic overloading. This means that the enhancing effect that these ECPs could have on aggregation would be for a limited period only. This would suggest that the optimum period of batch granulation-enhancement would be closer to 14 - 20 d than 20 - 26 d, considering the 14 - 26 d period reported by Britz *et al.* (1999). It might explain why the aggregate numbers in Ro units (1 L and 5 L) and in the Sp5L units reached a maximum within the first 5 - 10 d, after which they decreased.

Zhou *et al.* (2007) also reported short-term increases in the ECP content of glucose-fed UASB reactor sludge directly after an organic overload, which resulted in increased granulation. The ECP content also decreased after the organic loading rate was fixed for a longer period, which might have been the result of ECP consumption by the anaerobic community (Zhou *et al.*, 2007).

The fact that the AA levels remained high in all units in spite of the varying levels of pH recovery suggested that unbalanced conditions persisted to a degree in all units. Butyrate is usually degraded to acetate and methane by acetogenic bacteria and acetoclastic methanogens (Gerardi, 2003). The BA accumulation in the Ro units (1 L and 5 L) as well as in the Sp5L units can, therefore, also be considered an indicator of stress. Although it is probable that hydrogenotrophic methanogens would have contributed to pH recovery in all units, the levels of active acetoclastic methanogens in the seed sludge used in this study were possibly not high enough to facilitate acetate removal. Although acetoclastic methanogens reproduce more slowly than hydrogenotrophic methanogens and are adversely affected by the accumulation of hydrogen, 70% of all methane produced during anaerobic digestion is derived from acetate (Gerardi, 2003). The acetoclastic methanogen *Methanosaeta* has also been implicated in granular nuclei formation (Zheng & Raskin, 2000, Baloch *et al.*, 2008). The presence of active acetoclastic methanogens in any digested seed sludge used for batch granulation enhancement should, therefore, be of the utmost importance for future studies.

Study 2 – Effect of *Propionibacterium* addition on aggregation during granulation-enhancement

The main aim of this study was to determine whether addition of an active *Propionibacterium* culture before and during the batch granulation-enhancement of digested sewage sludge would improve the anaerobic granulation process.

Culturing conditions

As seed sludge for this study, digested sludge was obtained from the '1 Secondary B' anaerobic digester instead of the '1 Primary B' anaerobic digester at the Athlone WWTP. The seed sludge was size standardised by screening before inoculation. The main aim of the screening process was to remove larger impurities. The effect of the two different screening sizes (0.7 mm² and 1.0 mm²) was also evaluated by inoculating batch units with either sludge A (0.7 mm²) or sludge B (1.0 mm²).

Aggregate numbers

The average aggregate numbers of the different batch units (Table 1), as determined using the Scion Image program, can be seen in Fig. 6. The units inoculated with sludge B (including the control) all showed a greater increase in aggregate numbers than the units inoculated with sludge A over the 23 d incubation period. This suggests that it could be more advantageous to the batch granulation-enhancement process if digested seed sludge is screened with a slightly larger sieve mesh (1.0 mm² instead of the 0.7 mm² mesh).

At the end of the study, batch unit B.1 showed the highest increase in numbers (562%) when compared with unit B.2 and B-control. Based on average values unit B.2 (increase of 426%) showed a slightly higher increase than B-control (385%), but both values were within the standard deviation range for B-control, and therefore the difference between B.2 and B-control cannot be considered significant.

The A-control batch unit, with an increase of 327%, performed better than units A.1 (increase of 214%) and A.2 (increase of 271%) over the 23 d incubation period. This suggests that the sludge A units did not benefit from the PAB addition during the course of the study.

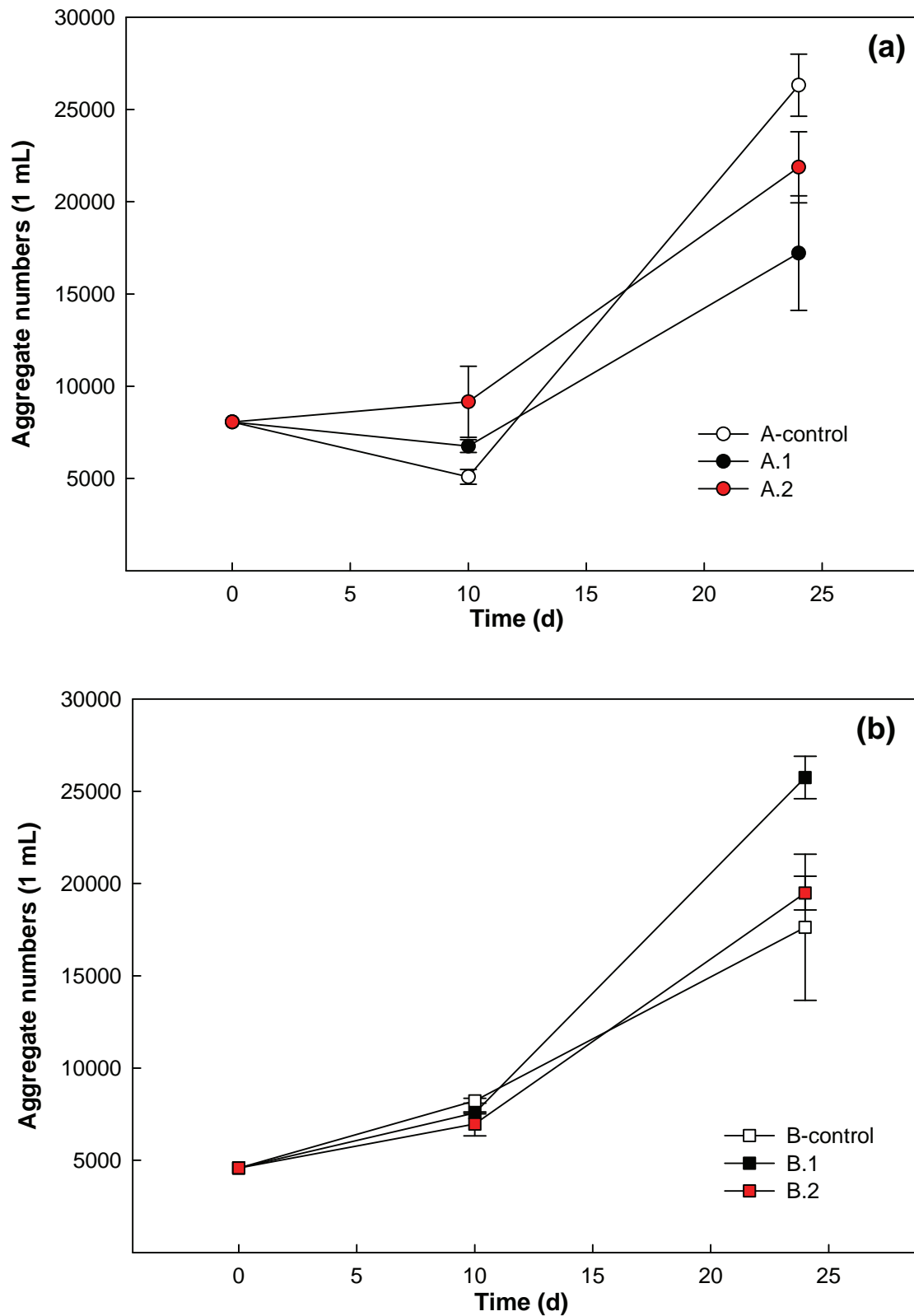


Figure 6 Study 2: Averages of aggregate numbers ($n=2$) in the 500 mL PAB-sludge batch units inoculated with either (a) sludge A, or (b) sludge B. A-control and B-control (control units inoculated with sludge A or sludge B but which no added PAB); A.1 and A.2 (500 mL batch units inoculated with sludge A and PAB); and B.1 and B.2 (500 mL batch units inoculated with sludge B and PAB).

pH profiles

The pH profiles of all units are presented in Fig. 7. Within a day all units decreased to a pH minimum between 6.4 and 6.5 and then recovered to levels above 6.7 by D12. These profiles are comparable to the profiles observed in the previous batch granulation-enhancement studies. This once again showed that a controlled organic overload could be induced using the Lac medium of Britz *et al.* (1999) regardless of the digested sludge used as seed sludge for batch granulation-enhancement.

The B-control unit had a slightly higher pH profile than the PAB units B.1 and B.2 while the pH profile of A-control was lower than the other two PAB units A.1 and A.2. It was, therefore, not possible to determine whether PAB additions had any influence on the pH profiles of the batch units.

Volatile Fatty Acid profiles

The VFA profiles of the control units (A-control and B-control) as well as the PAB units (A.1, A.2, B.1 and B.2) are presented in Fig. 8. In all units an initial shift occurred to lactate-utilising and propionate-producing populations following the organic overload induced at the start of the batch granulation-enhancement study. These profiles showed for the first 10 d that the PA levels were slightly higher than the AA levels, which is typical of propionate-producing populations such as *Propionibacterium*.

An important observation was that PA levels were not higher in the units to which the PAB inoculum were added daily (units A.1, A.2, B.1, B.2) in comparison with the control units that had no PAB inoculum (A-control and B-control). This suggested that the seed sludge already had inherent lactate-utilising PA-producing populations and that the added PAB inoculum did not contribute significantly to the PA-producing capacity of the seed sludge.

After 12 d a second population shift occurred in all units as PA and AA levels decreased and BA levels increased. These profiles were similar to the VFA profiles discussed for the Ro units (1 L and 5 L) and Sp5L units in the previous study (Study 1) in the respect that they coincided with increases in the pH profiles (Fig. 7). The profiles were, however, different from the Ro units (1 L and 5 L) and Sp5L unit profiles since the shift occurred later (after D12) and AA levels decreased and VA levels increased in this study. These dissimilarities are probably the direct result of the different anaerobic population compositions of the seed sludges used in this and the previous batch granulation-enhancement study. PA levels in both studies were below 5 000 mg.L⁻¹,

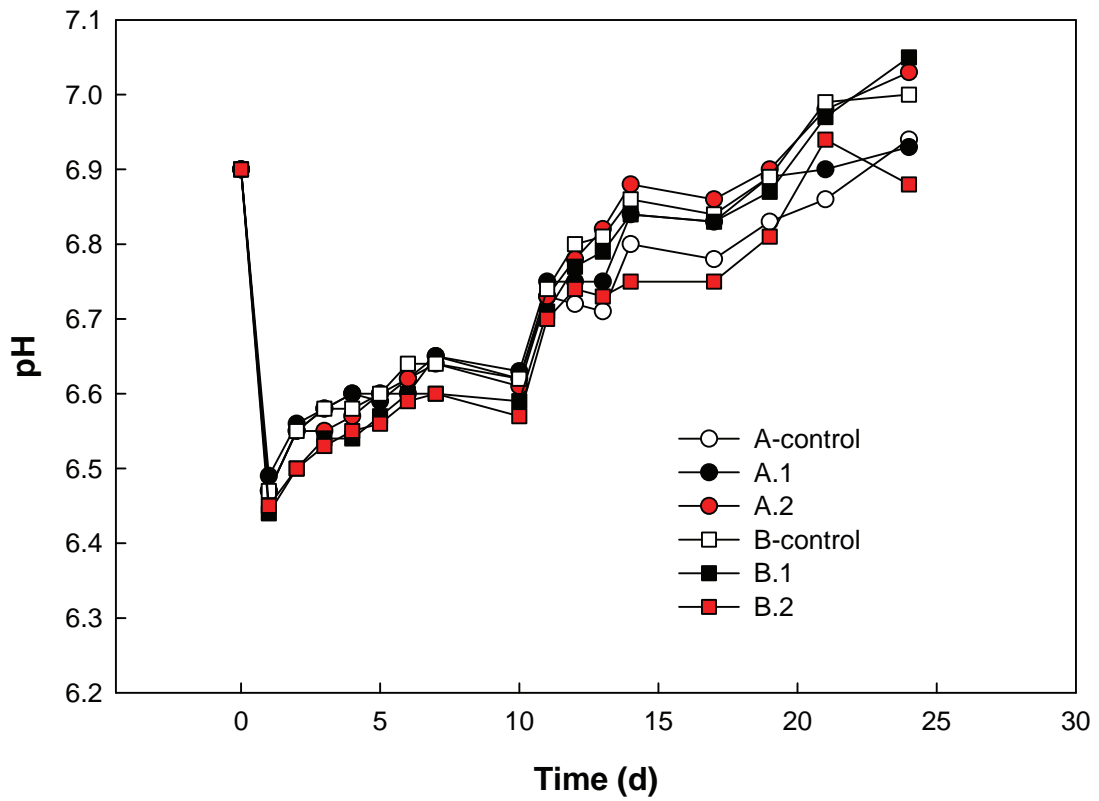


Figure 7 Study 2: pH profiles of 500 mL PAB-sludge batch units. A-control and B-control (control units inoculated with sludge A or sludge B but with no added PAB); A.1 and A.2 (500 mL batch units inoculated with sludge A and PAB); B.1 and B.2 (500 mL batch units inoculated with sludge B and PAB).

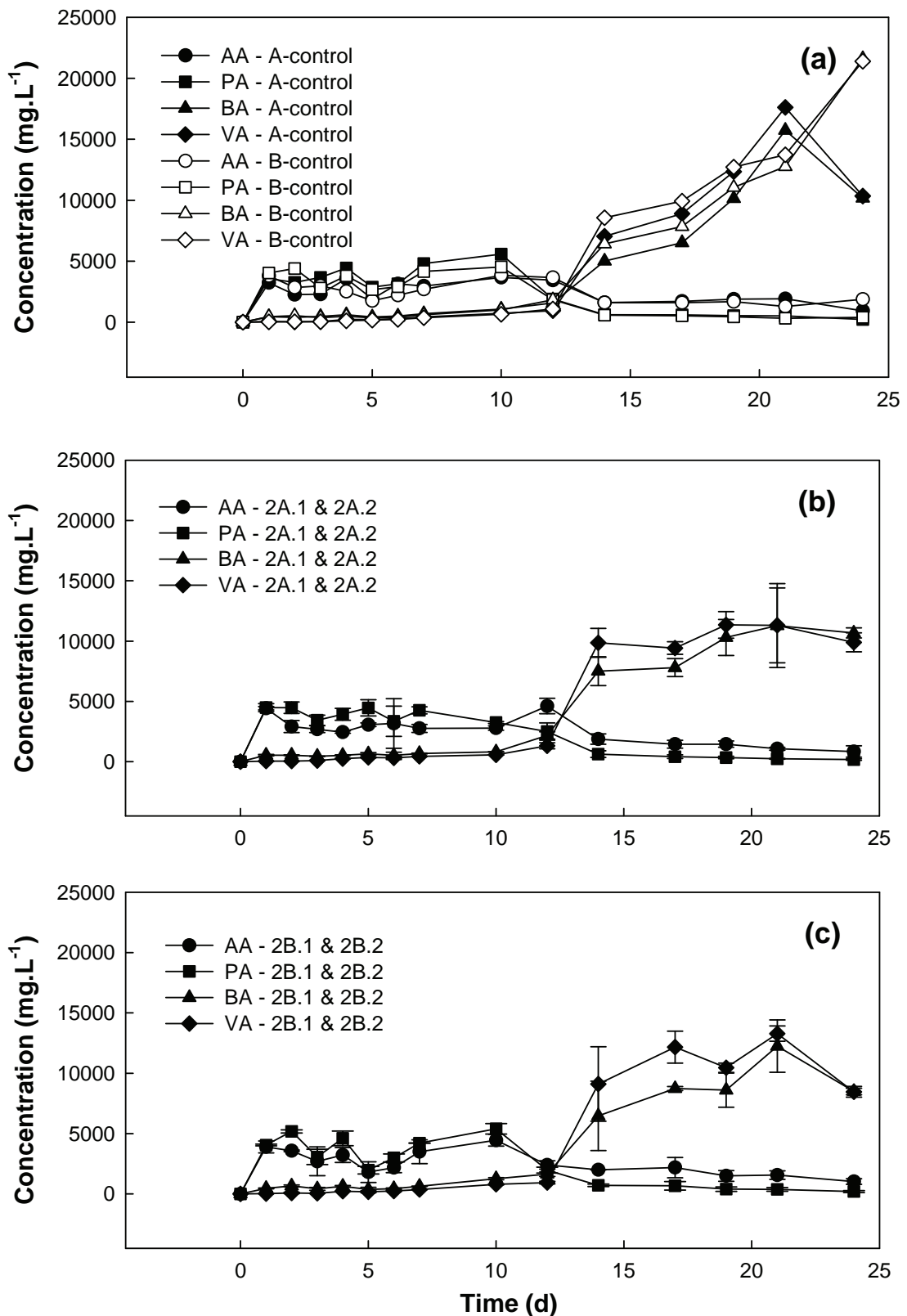


Figure 8 Study 2: Volatile fatty acid profiles of **(a)** control units inoculated with sludge A or sludge B with no added PAB (A-control and B-control); **(b)** averages ($n=2$) of 500 mL batch units inoculated with sludge A and PAB; and **(c)** averages ($n=2$) of 500 mL batch units inoculated with sludge B and PAB. AA - acetic acid; PA - propionic acid; BA - butyric acid; VA - valeric acid.

which could indicate that the lactate-utilising populations of both seed sludges had the same activity. The batch units in this study were incubated in a similar manner as the Wa500mL units in the previous section where the caps were slightly loosened to facilitate biogas release. In contrast with the Wa500mL units in the previous section, PA levels decreased after D10, which showed that the batch units in this study contained more active propionate-utilising acetogenic and methanogenic populations than the Wa500mL units in the previous section. Because of their higher activity, they would have been better able to cope with less favourable conditions, such as a slightly less reduced environment.

The decreased AA levels in all units in this study after D10 also indicated that the acetoclastic methanogen populations in the seed sludge, which are responsible for acetate removal, were more active than the seed sludge population in the previous study. The increased BA and VA levels after D12 indicated that the syntrophic acetogenic populations responsible for BA and VA removal were not active enough to metabolise the increased levels of BA and VA.

Study 3 – Influence of Fe²⁺, Ca²⁺ and powdered activated carbon on aggregation during granulation-enhancement

The aim of this study was to determine whether addition of Fe²⁺ (450 mg.L⁻¹), Ca²⁺ (100 mg.L⁻¹) or ActC (1.5 g.L⁻¹) at the start of the study could aid aggregation during granulation-enhancement of digested sludge in batch systems.

Culturing conditions

For this batch granulation-enhancement study the lactate content of the growth medium was reduced by 50% (to 10 g.L⁻¹) so as to reduce excessive gas production, which was previously found to encourage sludge flotation and washout. The Lac medium still had a COD of 10 300 mg.L⁻¹ (to which the lactate carbon source contributed 3 200 mg COD per litre) and was considered to provide a satisfactory organic overload during start-up.

Aggregate numbers

The aggregate numbers of the different batch units (Table 1), as determined using the Scion Image program, can be seen in Fig. 9. The Fe-Ro unit gave the highest increase in aggregate numbers (818%) over 20 d. This was also confirmed during the physical

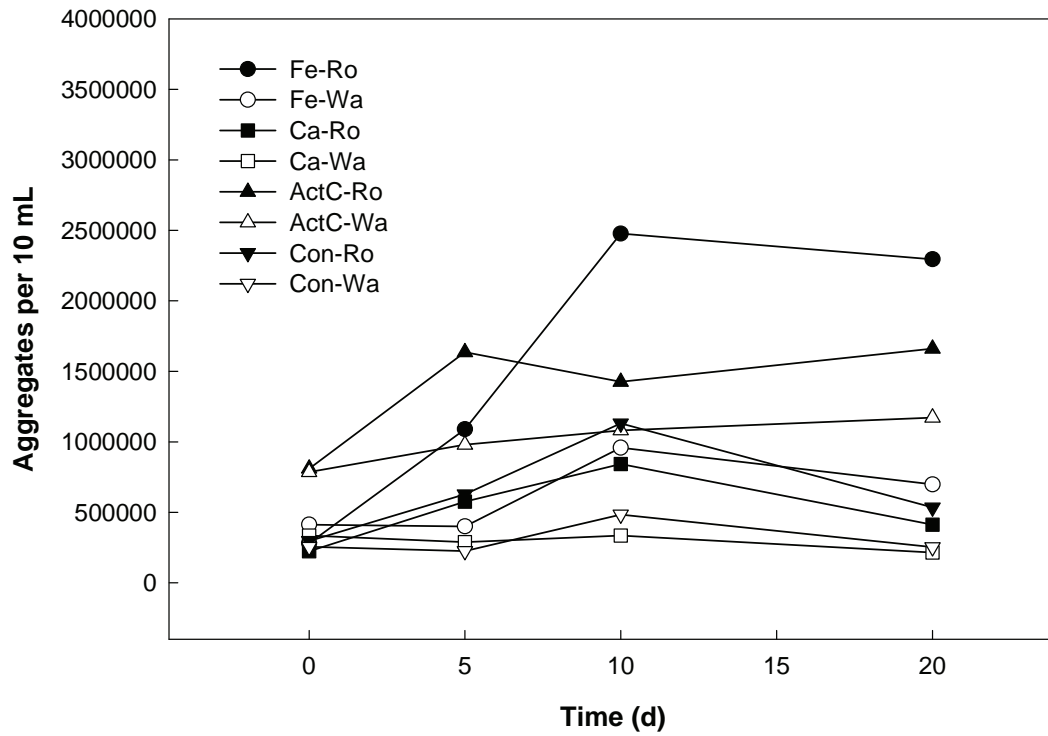


Figure 9 Study 3: Aggregate numbers in 500 mL batch units inoculated with digested sludge from the Kraaifontein WWTW and Lac medium and incubated on a linear-shake waterbath (Wa) or on a roller-table (Ro). Con - control; Fe - Fe²⁺ added on D0; Ca - Ca²⁺ added on D0; ActC - Activated carbon added on D0.

examination under a microscope at 10 x magnification where small aggregates could be distinguished in both the Fe-Ro and the Fe-Wa (increase: 169%) units.

The aggregate numbers were also high for the ActC samples, but it was clear from the physical examination that this was due to the activated carbon particles being present. This also explains the high numbers for ActC-Ro and ActC-Wa on D0. There was, however, a steady increase in aggregate numbers over the 20 d incubation period (205% and 149% for ActC-Ro and ActC-Wa, respectively). This demonstrates the limitation of the enumeration method used in this study as it fails to distinguish between aggregates and inorganic particles.

Increases in aggregate numbers of the Con-Ro unit (179%) and the Ca-Ro unit (183%) were very similar while both the Con-Wa and the Ca-Wa samples showed an overall decrease in numbers over the 20 d incubation period.

Overall, the Ro samples performed better than their corresponding Wa samples, indicating that the gentle rolling motion might be more advantageous to aggregate formation than the vigorous shaking action. Most of the samples (all except the ActC samples) showed the highest counts on D10, where after the counts decreased slightly on D20. Whether this is the result of the aggregates clumping together and increasing in size, as was speculated by Britz *et al.* (1999), or an indication of aggregate washout is not yet clear. Once again it might be valuable to study the size distributions of the aggregates over the 20 d period to clarify this observation.

pH profiles

The pH profiles of the units are given in Fig. 10. All the units showed a rapid pH decrease and recovery to levels above 6.7 within the first 10 d, which proved that the 10 g.L⁻¹ lactate content of the Lac medium provided adequate overloading conditions. During the stabilisation period the Ro units did stabilise at a slightly higher pH levels than their corresponding Wa units.

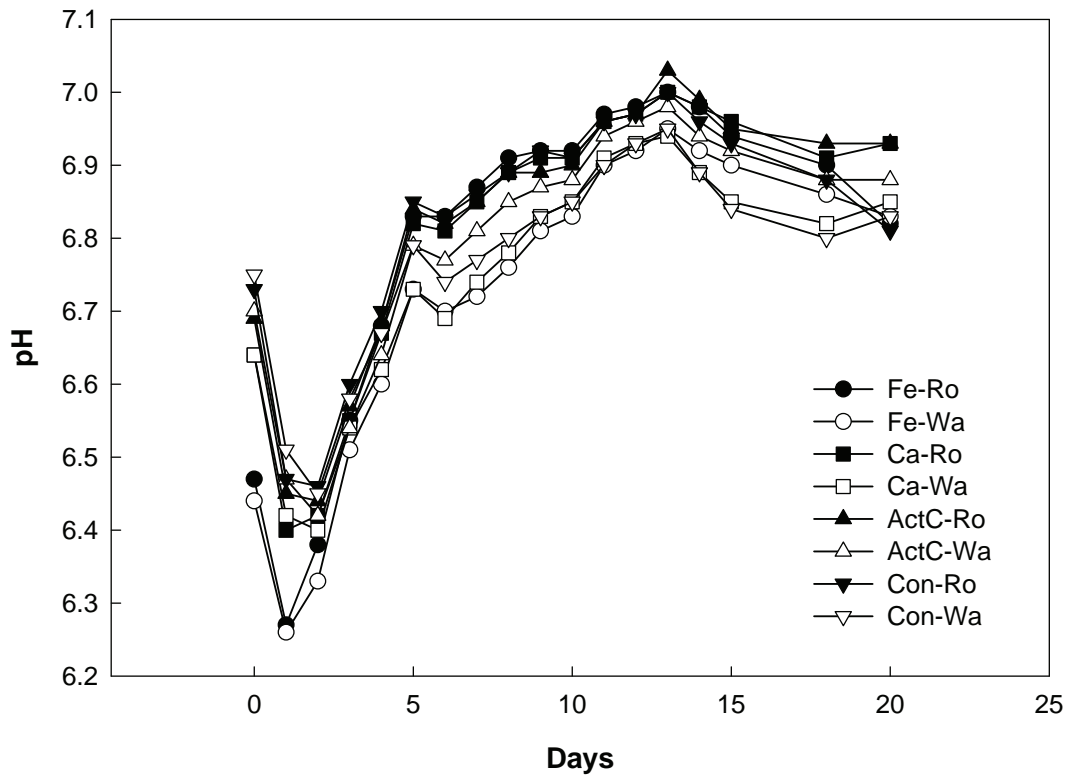


Figure 10 Study 3: pH profiles of 500 mL batch units inoculated with digested sludge from the Kraaifontein WWTW and Lac medium and incubated on a roller-table (Ro) or in a linear-shake waterbath (Wa). Con - control; Fe - Fe^{2+} added on D0; Ca - Ca^{2+} added on D0; ActC - Activated carbon added on D0.

Conclusions

Study 1 – Comparison of mixing systems for application in granulation-enhancement

Based on the results it was concluded that a population shift towards a lactate-utilising and propionic acid-producing population occurred in all units under the controlled organic overloading conditions that were applied during this batch granulation-enhancement study. In the 5 L units (Ro and Sp) and the Ro1L units a second population shift towards a butyric acid-producing population occurred, probably as a result of the biogas build-up that occurred in the closed containers. Rapid decreases in propionic acid concentrations also coincided with more rapid recovery in the pH profiles of these units in spite of increased butyric acid levels. This might indicate that the syntrophic populations necessary for propionate removal (*Syntrophobacter wolinii*) and its symbiotic methanogenic partner were more active under these conditions. In contrast, the high acetic acid levels that persisted in all units throughout the study indicated that the seed sludge used in this study did not have very active acetoclastic methanogen populations present.

Aggregate numbers showed that the units reached their maximum numbers at different times throughout the study. The 5 L units (Ro and Sp) and Ro1L units reached their maximum numbers between 5 and 10 d while the other units all reached their maximum numbers after D14. Due to limitations of the enumeration method used in this study, it was not possible to determine whether the decreases in aggregate numbers observed in all units after they reached their maximum values were the result of sludge washout or the result of smaller aggregates combining to form larger aggregates. The TS and VS values did show that the Ro samples (1 L and 5 L) retained the most solids during the 30 d incubation period. Therefore, the decreases observed in numbers might have been the result of the formation of larger aggregates. Of the three mixing systems the roller-table also showed the best potential for handling larger volumes and might be a valuable tool in the application of the batch granulation-enhancement model on larger scale.

The same degree of granulation enhancement as was reported by Britz *et al.* (1999) was, however, not observed. The main difference between this study and that of Britz *et al.* (1999) was that a different digested sludge was used as inoculum, which

might have influenced the degree of granulation observed. VFA data also indicated that the seed sludge lacked a balanced methanogenic community. This fact emphasises the importance and need for proper characterisation of digested seed sludge populations prior to batch granulation-enhancement studies in order to determine their suitability as anaerobic inoculum for future studies. Other factors that will need to be taken into consideration are the cost of lactate as a substrate in this process as well as safe biogas release from closed containers agitated on the roller-table.

Study 2 – Effect of *Propionibacterium* addition on aggregation during granulation-enhancement

Overall the units seeded with sludge B (1.0 mm² sludge) showed higher increases in aggregate numbers than the units seeded with sludge A (0.7 mm²). The B.1 unit showed the highest increase in aggregate numbers of all the units, although no significant difference could be detected between the other PAB-supplemented unit (B.2) and the control unit (B-control). A-control also showed higher increases in aggregate numbers than PAB-supplemented units A.1 and A.2. As reported in Study 1, due to the limitations of the enumeration method, it could not be determined whether the increases in aggregate numbers also coincided with increases in aggregate size. From results presented in this section it cannot be determined whether PAB addition during batch granulation-enhancement contributed to digested sludge granulation.

Study 3 – Influence of Fe²⁺, Ca²⁺ and powdered activated carbon on aggregation during granulation-enhancement

The addition of Fe²⁺ on D0 contributed to a larger increase in aggregate numbers than the addition of Ca²⁺ or ActC. Aggregation did not, however, occur to the same extent as was reported by Britz *et al.* (1999). The aggregate numbers in all the Ro units were higher than the numbers in their corresponding Wa units, which might indicate that the gentle rolling motion of the units incubated on the roller table could be more advantageous to the aggregation process.

General comments

Several concerns emerged during the three preliminary batch granulation-enhancement studies described in this chapter.

First, the requirements for an ideal digested sludge that can be used as inoculum for batch granulation-enhancement will have to be addressed later in this dissertation since this aspect can have a significant influence on the effectiveness of the enhancement process. In the 1999 study (Britz *et al.*, 1999) the digested seed sludge was obtained from the Kraaifontein WWTW and applied successfully in the batch granulation-enhancement process. Since then the Kraaifontein facility's anaerobic digester has had a series of breakdowns and is also well known for its history of instability. Because of this, Athlone digested sludge was used as an alternative inoculum for the first and second batch granulation-enhancements described in this chapter. Increases in aggregate numbers were noted, but granulation was not observed to the same extent as was reported by Britz *et al.* (1999). This can possibly be ascribed to differences in the microbial communities of the different digested seed sludges.

It is known that a variety of factors influence anaerobic digestion and granulation in UASB reactors. One of the most important factors is the presence of specific methanogenic populations that may serve as essential nuclei for granulation. An example of such a population is the acetoclastic *Methanosaeta concilii*, which has been reported to be essential for granule formation (MacLeod *et al.*, 1990; Grotenhuis *et al.*, 1991; Zheng & Raskin, 2000; Raskin, 2001, Baloch *et al.*, 2008). It is thus logical to assume that the populations important to granulation in UASB reactors are the same populations that should be present in the digested sludge used to seed the batch granulation-enhancement process. The aim of Britz *et al.* (1999) was not to induce anaerobic granulation but to enhance the naturally occurring granulation process by temporary manipulation of environmental conditions. Therefore, the presence of essential methanogens in any digested sludge must be confirmed before it is used as seed sludge.

Secondly, the method used for the determination of aggregate formation should be addressed. To measure the effectiveness of enhancement, two factors need to be measured: retention of the anaerobic microbial biomass under conditions of organic overloading and aggregate size increases instead of aggregate numbers. As an alternative to the aggregate enumeration method used in this study, the method of

Laguna *et al.* (1999) will be applied in further studies. This method involves separating the digested sludge into different size fractions and determining the VSS content of each fraction. Although it is an indirect method of measurement, comparison of the VSS size distribution at the start and end of the batch granulation-enhancement process should give an indication of increases in the granular content of the sludge.

Thirdly, although VFA profiles of all batch units in studies 1 and 2 showed the presence of PA-producing populations typical to *Propionibacterium*, it was not conclusively proved in Study 2 that addition of *Propionibacterium*-cultures to the batch granulation-enhancement process would enhance the granulation process.

Fourthly, from the results presented in this chapter and the previous chapter (Chapter 3) it was concluded that the gentle rolling motion provided by the roller-table could be more advantageous to aggregate formation than the linear-shake motion originally employed by Britz *et al.* (1999). Although the roller-speed was constant, the containers incubated on the roller-table were rolled at different speeds based on the different diameters of the containers (Study 1: Ro1L [30 rpm] and Ro5L [17 rpm]; Chapter 3 and Study 2 of this chapter: 500 mL units [35 rpm]). Comparison of the results obtained with the different-sized containers could therefore not be made without considering the flow dynamics inside the containers, which resulted not only from the rolling speed but also from the specific container design. This was investigated further by Els *et al.* (2005) who designed and built three bench-scale reactors with different flow patterns. Their results confirmed the observation of this study that the flow pattern induced by the rolling motion is, indeed, the most advantageous to aggregate formation. The 5.4 L reactor they designed to simulate the rolling motion was also equipped with 12 baffles to minimise biofilm formation. Biogas was allowed to exit continuously through a biogas vent that was connected to a water-filled U-tube, which ensured an anaerobic environment in the cylindrical reactor during agitation. This reactor will also be used for batch granulation-enhancement studies later in this dissertation.

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CHAPTER 5

DGGE FINGERPRINTING OF *ARCHAEA* PRESENT IN ANAEROBIC DIGESTED SEWAGE SLUDGE AND MATURE UASB GRANULES*

Summary

In this study, a polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE) method for the molecular fingerprinting of the archaeal populations of digested sewage sludges and mature upflow anaerobic sludge blanket (UASB) granules was optimised. A DGGE marker was constructed from pure methanogen strains for quick identification of specific bands in the DGGE fingerprints. The positive influence of dimethyl sulfoxide and 'touchdown' PCR on the elimination of artifactual double bands in DGGE fingerprints was also demonstrated. From the results, it was apparent that all the mature UASB granules scanned contained *Methanosaeta concilii* while only one of the four digested sludges tested positive for this bacterium. *Methanosaeta concilii* is reported to be critical for granule formation, and its absence in digested sludge used to seed UASB reactors can be detrimental to the sludge granulation process in the reactor. It is, therefore, recommended that the presence of *M. concilii* in a digested sludge be confirmed before it is used as seed sludge.

Introduction

Anaerobic digestion is recognised as an energy-efficient technology for the treatment of a variety of high-strength industrial wastewaters. The two main phases of this treatment are acidogenesis and methanogenesis. Acidogenesis is the process whereby complex organic matter is reduced to organic acids by aerobic, facultative anaerobic and anaerobic *Bacteria*. During methanogenesis methane-forming *Archaea* (methanogens) convert these organic acids to biogas, consisting of a mixture of methane and carbon dioxide (Leclerc *et al.*, 2001; Casserly & Erijman, 2003).

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Although different species of methanogens contribute to the complex process of anaerobic digestion, researchers are in agreement that acetoclastic *Methanosaeta* species are important for stable anaerobic digester function and anaerobic granulation (McHugh *et al.*, 2003; Hulshoff Pol *et al.*, 2004; Zheng *et al.*, 2006). McHugh *et al.* (2003) reported that the dominance of *Methanosaeta* species in general are important in the stable and efficient operation of anaerobic digesters while most theories on anaerobic granulation recognise that *Methanosaeta concilii* plays a key role during anaerobic granulation, mostly by acting as nucleation centres or precursors to granulation (Chen & Lun, 1993; Wu *et al.*, 1996; El-Mamouni *et al.*, 1997; Hulshoff Pol *et al.*, 2004; Zheng *et al.*, 2006; Baloch *et al.*, 2008).

An assessment of the composition of the methanogenic community in digested sewage sludge, in particular the distribution of *Methanosaeta* species, could provide valuable information regarding the quality and suitability of digested sewage sludge before it is used to seed anaerobic digesters. Unfortunately, methanogens are notoriously difficult to culture since they require an environment with a reducing potential of less than -330 mV (Lange & Ahring, 2001). Molecular techniques based on the DNA sequences of the small subunit ribosomal RNA (rRNA) gene have been used in recent years to study the microbial community structure in various natural environments, such as soil and anaerobic digesters (Oude Elferink *et al.*, 1998; Torsvik & Øvreas, 2002; McHugh *et al.*, 2003; Fornay *et al.*, 2004). These cultivation-independent methods enabled researchers to determine microbial community structure and identify important but not necessarily culturable microbial species (Oude Elferink *et al.*, 1998; Fornay *et al.*, 2004).

Molecular hybridisation methods, such as membrane (dot blot) hybridisation and fluorescent *in situ* hybridisation (FISH) of rRNA genes with oligonucleotide rRNA probes, have been used to detect and quantify anaerobic microorganisms. FISH has also been used to visualise the spatial organisation of microorganisms in granular sludge (Zheng & Raskin, 2000; Briones & Raskin, 2003; Casserly & Erijman, 2003). Detection limits of these fluorescent-based methods are, however, influenced by various factors such as the relative size and metabolic state of the target population, cell shape and permeability as well as the presence of autofluorescent particles (Oude Elferink *et al.*, 1998). The phylogenetic relationships that exist between anaerobic species in the same environment can also be explored by the construction and sequence analysis of clone libraries after the total community DNA has been extracted from environmental

samples. This method is, however, time consuming and costly and is not well suited to the analyses of numerous samples or for any investigation into community structure changes that might occur in response to environmental changes (Fornay *et al.*, 2004).

Fingerprinting techniques such as amplified ribosomal DNA restriction analysis (ARDRA), randomly amplified polymorphic DNA (RAPD) and more recently single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) can simultaneously provide information on community heterogeneity of a large number of samples. DGGE and TGGE utilise the different melting properties of DNA fragments of identical lengths as the basis of separation in either denaturing gradient or temperature gradient gels (Muyzer & Smalla, 1998; Oude Elferink *et al.*, 1998; Chan *et al.*, 2001). Separation is based on differences in nucleotide sequence and since each band is likely to be derived from a phylogenetically distinct population, an estimation of species number and abundance can be made (Muyzer *et al.*, 1993; Øvreas *et al.*, 1997). Once an effective DGGE method has been established, further phylogenetic information can be obtained by excising and sequencing specific bands or by hybridising banding patterns with oligonucleotide probes (Muyzer & Smalla, 1998; Oude Elferink *et al.*, 1998; Heuer *et al.*, 2001).

As with all methods, DGGE and TGGE are not free from errors and biases. Limitations include the separation of only smaller fragments (≤ 500 bp) and possible overestimation of species number as a result of PCR-introduced biases or underestimation because of the difficulty of electrophoretically separating fragments that differ only two to three nucleotides (Muyzer & Smalla, 1998; Heuer *et al.*, 2001). Band intensity might also not be representative of the natural abundance of the amplified sequence in a mixed population, since it can be influenced by rRNA gene copy number, PCR-cycling parameters as well as the preferential amplification of certain sequences during PCR (Murray *et al.*, 1998; Gelsomino *et al.*, 1999). Studies have also indicated that predominant microbial species (more than one per cent of a mixed population) can be visualised with PCR-based DGGE (Muyzer *et al.*, 1993; Murray *et al.*, 1996). Nevertheless, substantial information about microbial diversity can be obtained from very complex environmental samples with DGGE and TGGE analysis (Murray *et al.*, 1998; Heuer *et al.*, 2001).

The aim of this study was to optimise and establish an effective PCR-based DGGE method for the molecular fingerprinting of the archaeal population of anaerobic

digested sewage sludges and mature UASB granules. Furthermore, a DGGE marker was constructed from methanogen strains (*Archaea*) for the quick identification of specific bands in the archaeal DGGE fingerprints.

Materials and methods

Anaerobic digested sludges and mature UASB granules

The sources and types of the anaerobically digested sludges and mature UASB granules analysed in this study are listed in Table 1. The digested sludges were all obtained from anaerobic digesters at municipal wastewater treatment works. Granule samples were obtained from various industrial-scale as well as laboratory (lab)-scale UASB reactors treating a variety of effluents.

Methanogen reference cultures

Various pure methanogen reference cultures were chosen to be included in the DGGE reference marker based on literature reports on their possible importance during anaerobic granulation. The methanogenic cultures, as listed in Table 2, were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) as live cultures in growth medium.

DNA isolation from digested sludges and mature UASB granules

DNA was isolated from each of the sludge samples and mature UASB granules by using a modified method of Van Elsas *et al.* (1997). Sludge samples were either sampled directly for DNA isolation or sieved before sampling (mesh size = 1 mm²), similar to the sieving performed to remove larger impurities and debris before the sludge is used in batch granulation-enhancement. Thirty µL of each sludge was transferred to a sterile centrifuge tube containing 0.6 g sterile glass beads (0.2 - 0.3 mm diameter) (Sigma) for DNA isolation. For the mature granules, one granule was transferred to a sterile centrifuge tube and grinded with a small sterile pestle before the contents were also transferred to sterile centrifuge tubes containing 0.6 g sterile glass beads.

Table 1 Anaerobic digested sludges and mature UASB granules analysed in this study

Sample code and type	Digester description	Digester influent
P-sludge (digested sludge)	Municipal anaerobic digester (Paarl WWTW)	Sewage
K-sludge (digested sludge)	Municipal anaerobic digester (Kraaifontein WWTW)	Sewage
A1Pb-sludge (digested sludge)	Municipal primary anaerobic digester (Athlone WWTW)	Sewage
A1Sb-sludge (digested sludge)	Municipal secondary anaerobic digester (Athlone WWTW)	Sewage via primary anaerobic digester
B (anaerobic granules)	Commercial UASB reactor	Brewery wastewater
F (anaerobic granules)	Commercial UASB reactor	Fruit juice processing wastewater
A (anaerobic granules)	Commercial UASB reactor	Apple processing wastewater
W (anaerobic granules)	Commercial UASB reactor	Winery wastewater
M (anaerobic granules)	Municipal anaerobic digester (Pietersburg/ Polokwane WWTW)	Sewage
L (anaerobic granules)	2.4 L lab-scale UASB reactor	Lye-containing fruit canning wastewater
P (anaerobic granules)	2.4 L lab-scale UASB reactor	Apple pomace composting leachate

WWTW – municipal wastewater treatment works

Table 2 Methanogenic species used in the construction of the methanogenic DGGE marker

Species	Culture no. ¹	Literature reference
<i>Methanosaeta concilii</i> (Synonyms <i>Methanotherix concilii</i> and <i>Methanotherix soehngenii</i>)	DSM 3671 (type strain) DSM 3013	Chen & Lun, 1993; Wu <i>et al.</i> , 1996; McHugh <i>et al.</i> , 2003; Zheng <i>et al.</i> , 2006; Baloch <i>et al.</i> , 2008
<i>Methanosaeta thermophila</i>	DSM 4774 (type strain)	McHugh <i>et al.</i> , 2003; Baloch <i>et al.</i> , 2008
<i>Methanosarcina barkeri</i>	DSM 800	Chen & Lun, 1993; Wu <i>et al.</i> , 1996
<i>Methanosarcina mazei</i>	DSM 2053	Wu <i>et al.</i> , 1996; Veiga <i>et al.</i> , 1997
<i>Methanobacterium formicicum</i>	DSM 1535	Wu <i>et al.</i> , 1996; Veiga <i>et al.</i> , 1997

¹ DSM – Deutsche Sammlung von Mikroorganismen und Zellkulturen

The following reagents were added to each tube: 800 μL phosphate buffer (1 part 120 mM NaH_2PO_4 [Saarchem] mixed with 9 parts 120 mM Na_2HPO_4 [Merck]; pH 8); 700 μL phenol (pH 4.3) (Fluka); and 100 μL of 20% (m/v) sodium dodecyl sulphate (Merck). The tubes were vortexed for 2 min and incubated in a 60°C waterbath for 20 min. This procedure was repeated twice, after which the samples were centrifuged for 5 min at 1 500 x g. The aqueous phase of each sludge sample was transferred to new sterile centrifuge tubes and proteins and other cell debris were removed by extracting with 600 μL phenol (pH 4.3) (Fluka), followed by repeated extractions with 600 μL phenol/chloroform/isoamylalcohol (25:24:1) until the interphase of each sample was clean. The DNA was precipitated with 0.1 x vol 3 M sodium acetate (NaAc) (pH 5.5) (Saarchem) and 0.6 x vol isopropanol (Saarchem) on ice for at least 1 h. The precipitated DNA was collected by centrifugation for 10 min at 15 000 x g. The pellet was washed and air-dried for 20 min after which it was redissolved in 100 μL TE buffer (10 mM Tris, 1 mM EDTA; pH 8).

PCR amplification

The PCR was performed as described by Øvreas *et al.* (1997) for the amplification of archaeal gene sequences from environmental samples. This method involved the nested PCR amplification of the V3 variable region of the 16S rRNA gene, followed by DGGE analysis. A 1 072 base pair (bp) fragment was amplified, which was then used as template in a nested PCR (referred to as dgge-PCR) to produce a 179 bp fragment that was resolved using DGGE.

The primers used for the initial PCR amplification were PRA46f (5' (C/T)TA AGC CAT GC(G/A) AGT 3') and PREA1100r (5' (C/T)GG GTC TCG CTC GTT (G/A)CC 3') (Øvreas *et al.*, 1997). The PCR reaction volume was 50 μL and contained 0.5 μM of each of the primers, 100 μM of dNTPs (Promega), 2.0 mM MgCl_2 , 2.5 U *Biotaq*TM DNA polymerase (Bioline), 1 x reaction buffer (Bioline) and 1 - 2 μL of isolated DNA. Acetamide to a final concentration of 5% (m/v) was added to minimise nonspecific annealing of the primers. A routine negative control was included every time PCR was performed to confirm that contamination of the PCR mastermix with foreign DNA did not occur during PCR sample preparation. This control contained the same reagent mix, but 2 μL sterile water was added instead of DNA template.

The primers used in the nested PCR (dgge-PCR) reaction were PARCH340f (5' CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG CCC TAC GGG G(C/T)G CA(G/C) CAG 3') (GC-clamp sequence underlined) and PARCH519r (5' TTA CCG CGG C(G/T)G CTG 3') (Øvreas *et al.*, 1997). The reaction volume of the dgge-PCR was 50 µL and contained 0.5 µM of each of the primers, 100 µM dNTPs (Promega), 0.5 mM MgCl₂, 2.6 U of *Expand* DNA Polymerase (Roche), 1 x reaction buffer (Roche) and 0.25 - 0.5 µL of the initial PCR as template. A routine negative control was included every time PCR was performed. The GC-clamp sequence included on the 5' end of the forward primer was identical to that reported by Chan *et al.* (2001) and enabled the separation of the dgge-PCR amplicons, using DGGE.

The reaction conditions were the same for both primer sets and amplicons were generated using an initial denaturation step of 2 min at 92°C, followed by 31 cycles of denaturation at 92°C for 60 s, annealing between 53.5 and 55°C for 30 s, and chain elongation at 72°C for 60 s. A single final elongation step at 72°C for 6 min was also included. PCR reactions were performed in an Eppendorf Mastercycler Personal (Eppendorf AG, Germany) and the presence of PCR products was confirmed on either 1% (m/v) agarose gels (for initial PCR products) or 1.5% (m/v) agarose gels (for dgge-PCR products). All agarose gels contained ethidium bromide and the separated fragments were visualised under UV light (Vilber Lourmat).

DGGE analysis

DGGE was performed with a BioRad DCode™ Universal Mutation Detection System (BioRad Laboratories, USA). Separation of the archaeal-specific PCR amplicons was obtained using 8% (m/v) polyacrylamide gels in a 0.5 x TAE buffer containing a linear denaturing gradient of between 35 and 70%. The 100% denaturing solution used in the preparation of this gradient contained 40% (v/v) formamide (Saarchem) and 7.0 M urea (Merck). After electrophoresis was performed at a constant voltage of 130 V for 5 h at 60°C, gels were stained with 10% (v/v) ethidium bromide and visualised under UV light (Vilber Lourmat).

A DGGE reference marker was developed. The marker contained equal amounts of PCR amplicons of the five methanogenic species listed in Table 2. During the study, nested PCR amplification and DGGE analysis were repeated twice for all DNA isolates.

The resulting DGGE gels were then compared to confirm reproducibility of the DGGE banding patterns.

Results and discussion

PCR optimisation

Initially the PCR-amplification method of Chan *et al.* (2001) was evaluated. According to this method, the primers ARC622f (with a GC-clamp) and ARC915r are used directly to generate 293 bp fragments that are then resolved using DGGE. However, with this method, single PCR-amplification products could only be obtained for pure cultures. PCR amplification of the DNA isolated from the digested sludges and granules was unsuccessful. Some of the granule and sludge samples produced no PCR products while the PCR products that were generated from other samples contained fragments of various lengths.

The two-step PCR-amplification method of Øvreas *et al.* (1997) was then tried and proved to be more useful in the amplification and identification of the methanogens from the sludges and granules. Two DNA polymerase systems were used: *Biotaq*TM DNA polymerase for the initial PCR and the *Expand* High Fidelity PCR system for the dgge-PCR reaction. The *Expand* polymerase system is a mixture of a *Taq* DNA polymerase and *Tgo* DNA polymerase with proof-reading activity. The optimal MgCl₂ concentrations for both polymerases were determined by using MgCl₂ gradients (Fig. 1a and b) with the A1Pb-sludge DNA as template. Optimum MgCl₂ concentrations of 2 mM for 2.5 U of *Biotaq*TM and 0.5 mM for 2.6 U of the *Expand* polymerase mixture were chosen based on a high PCR product yield and low concentrations of non-specific amplification products. Specificity was increased further by increasing the annealing temperatures of both PCR reactions from 53.3°C to 55°C (Fig. 1c).

Examples of typical PCR products generated from other isolated DNA using the optimal MgCl₂ concentrations and annealing temperature are presented in Fig. 1d (for the initial PCR) and Fig. 1e (for the dgge-PCR). Although the initial PCR products of some DNA isolates still showed non-specific amplification, dgge-PCR products of high specificity could be generated for each DNA isolate by using different volumes of the initial PCR as template.

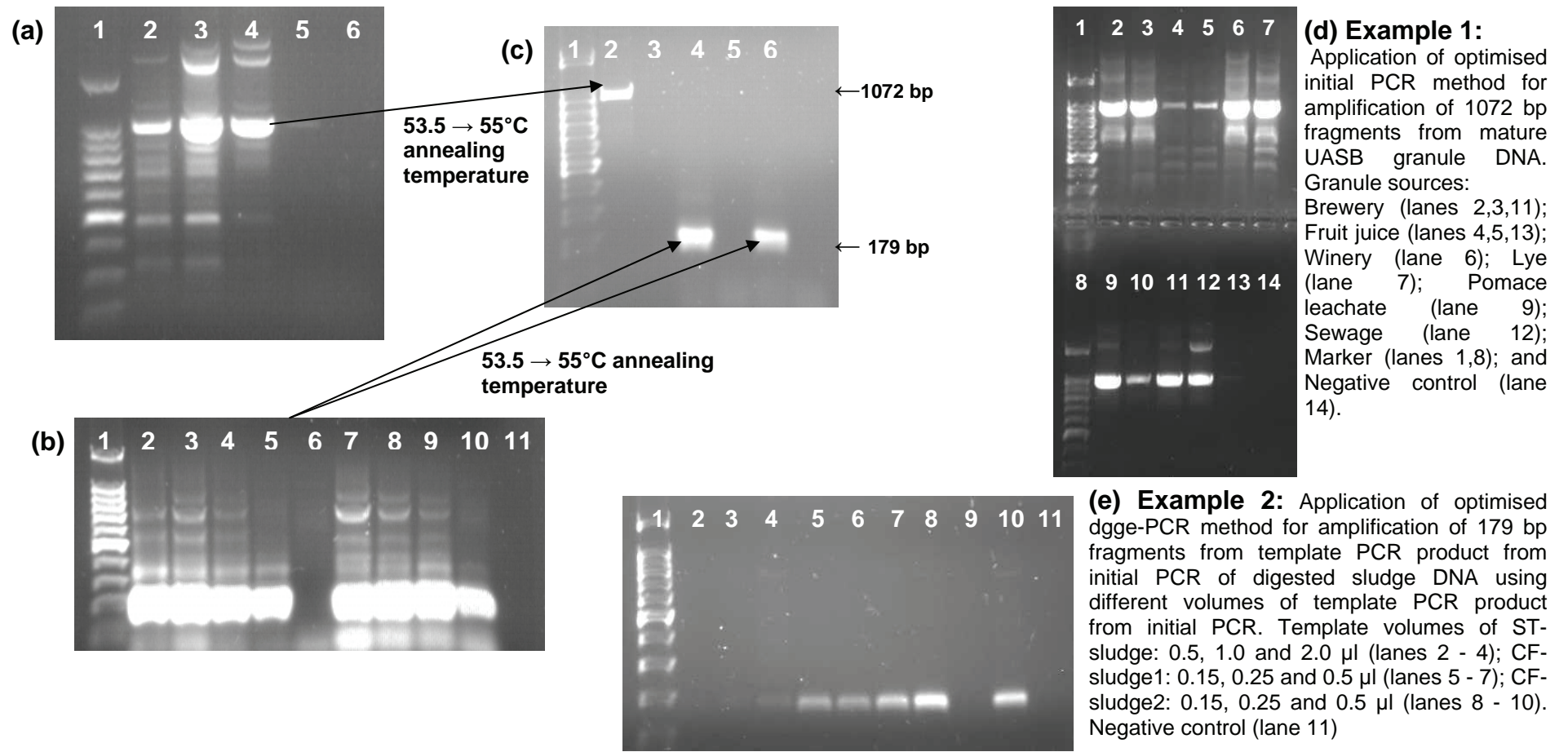


Figure 1 (a) - (c) Optimisation of two-step PCR amplification of part of the V3 region of the 16S rRNA gene of the archaeal content of the A1-sludge: (a) MgCl₂ gradient for *Biotaq*TM polymerase. MgCl₂ concentrations: 8 mM (lane 2); 4 mM (lane 3); 2 mM (lane 4 = optimum); 1 mM (lane 5); Marker (lane 1); and Negative control (lane 6). (b) MgCl₂ gradient for *Expand* polymerase. Enzyme concentrations tested were 2.6 U (lanes 2 – 6) and 5.2 U (lanes 7 – 10). MgCl₂ concentrations tested were 1.25 mM (lane 2); 1 mM (lanes 3 and 7); 0.75 mM (lanes 4 and 8); 0.5 mM (lanes 5 (optimum) and 9); 0.25 mM (lanes 6 and 10); Marker (lane 1); and Negative control (lane 11). (c) Effect of increased annealing temperature (from 53.5°C to 55°C) for both the initial PCR (lane 2) and the dgge-PCR: lane 4 (amplified from 1 µl template PCR product) & lane 6 (amplified from 0.5 µl template PCR product). Negative controls (lanes 3, 5, 7). (d) Example of initial PCR product (Example 1). (e) Example of dgge-PCR product (Example 2).

DGGE fingerprints of digested sludges

The DGGE fingerprints of the P, K, A1Pb and A1Sb digested sludges (Table 1) are presented in Fig. 2. Reproducibility of the nested PCR amplifications as well as DGGE banding patterns was confirmed by repeating both the amplification process and DGGE separation (data not shown). Since acetoclastic methanogens from the *Methanosaeta* and *Methanosarcina* genera are responsible for 60 - 70% of all methane produced during anaerobic degradation, it was decided to include the type species for both genera, *Methanosaeta concilii* (DSM 3671) and *Methanosarcina barkeri* (DSM 800), in the DGGE marker to aid DGGE band identification. *Methanosarcina mazei* (DSM 2053) was also included since literature reference has been made to its ability to produce ECP that might aid anaerobic granulation (Veiga *et al.*, 1997).

The differences in archaeal banding patterns between the sludges indicated the presence of different archaeal populations in the sludges. The DGGE fingerprints showed that band **c** was the only band present in the DGGE fingerprints of all four the sludges tested. Bands **d** and **g** were detected in three of the four sludges while bands **a**, **f** and **g** were observed in two of the four sludge fingerprints. Bands that were each detected in only one of the four sludges were **b**, **e**, **h1**, **h2**, **i**, **k**, **n** and **j**. Only one of the four sludges tested (A1Sb-sludge) contained *Methanosaeta concilii* (Fig. 2, band **m**).

The presence of *M. concilii* in only one of the four sludges is in contrast with the conclusions of Straub *et al.* (2006) who reported that most anaerobic digesters at municipal wastewater treatment facilities are operated under conditions favouring the presence of *Methanosaeta* spp. Although all the municipal digesters sampled in this study (Table 1) were operating efficiently at the time of sampling, the long-term stability and operational history of these specific digesters were not considered at the time of sludge sampling. However, it is known that the Kraaifontein digester (K-sludge source) had a history of operational instability before the sampling for this study took place. The absence of *Methanosaeta concilii* in the K-sludge might have been a direct result of the operational problems experienced with digester stability.

The A1Pb and A1Sb sludges were also acquired from a two-stage municipal anaerobic sludge digester with the A1Pb-sludge being obtained from the primary anaerobic digester and the A1Sb-sludge from the secondary anaerobic digester. Differences between the banding patterns of these two sludges indicated that although they were linked, different archaeal populations dominated in each digester. This was

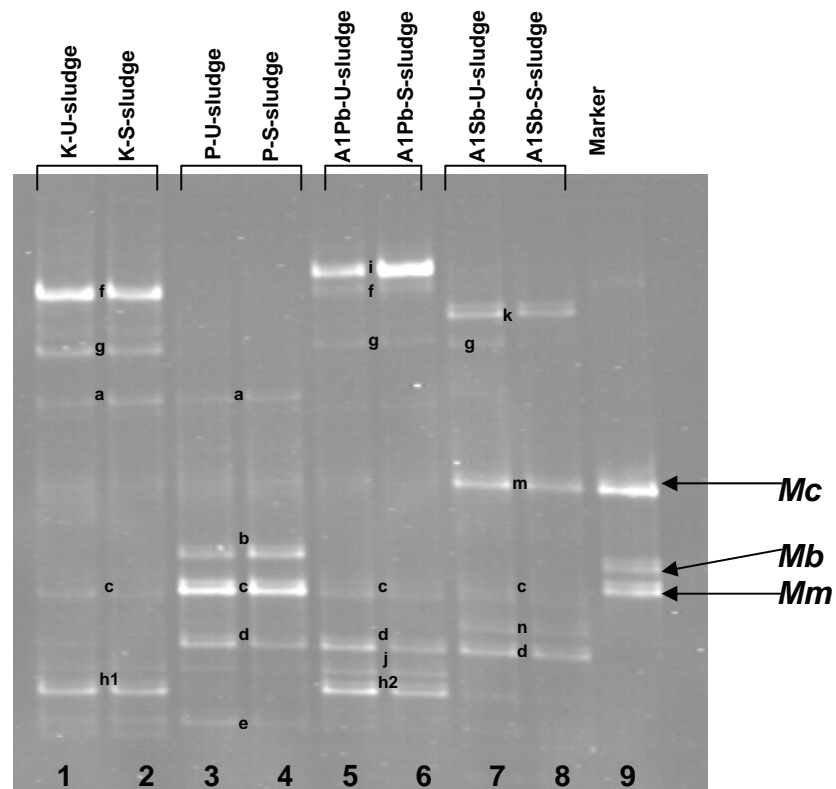


Figure 2 DGGE fingerprints from DNA isolates of sieved (S) and unsieved (U) anaerobic sludges (K, P, A1Pb and A1Sb). Pure cultures included in lane 9 were *Methanosaeta concilii* DSM 3671 (**Mc**), *Methanosarcina barkeri* DSM 800 (**Mb**), and *Methanosarcina mazei* DSM 2053 (**Mm**). For discussion purposes small letters indicate positions of bands.

probably a direct consequence of the diverse environmental conditions that existed in each digester as a result of their separate principal operational functions. The fact that *Methanosaeta concilii* was only present in the A1Sb-sludge suggests that the conditions in the secondary digester, which is mainly used for sludge storage and thickening, were more favourable for *Methanosaeta* growth than were the conditions in the primary digester.

Methanosaeta concilii is generally considered to be crucial for a part of the core structure formation during granulation (Chen & Lun, 1993; Wu *et al.*, 1996; McHugh *et al.*, 2003; Zheng *et al.*, 2006; Baloch *et al.*, 2008). It is, therefore, recommended that further batch granulation-enhancement studies be seeded with digested sludge containing *Methanosaeta concilii*. It would, in the future, be of value to do a study to determine whether the long-term stability of municipal digesters has any influence on the *Methanosaeta concilii* population content of the digested sludge.

The DGGE profiles of the sludges (Fig. 2) showed that not one contained either *Methanosarcina barkeri* or *Methanosarcina mazei*. It could, however, be that the sludges contained other members of the *Methanosarcina* genus since this genus includes more than seven different species.

Bands **g**, **j** and **n** in the A1Pb and A1Sb sludges were the only bands that could not be confirmed during DGGE reproducibility testing. The low band intensity of these bands might be an indication that these bands were either PCR artefacts (which usually result from point mutations, deletion mutations or chimera formation) or that the concentration of these sequences in the DNA isolates was so low that they might not always have been amplified during PCR. However, the primers used in this study were designed to amplify the shorter highly variable V3 region of the 16S rRNA gene in order to avoid chimera formation (Øvreas *et al.*, 1997). Similarly, the possibility of PCR artefacts could be ruled out. This was done by testing the repeatability of DGGE banding patterns since the probability of producing identical chimeras in two or more independent PCR reactions was reported by Hugenholtz & Goebel (2001) to be very low. Since these bands (**g**, **j** and **n**) appeared in the DNA profiles of at least two sludges, it was concluded that the bands were not the result of chimera formation. If the unpredictable emergence of bands **g**, **j** and **n** in the A1Pb and A1Sb sludges was the result of low DNA template concentrations, this could be addressed in future DGGE studies by enlarging the sludge sample used for DNA isolation. Tsai & Rochelle (2001) proposed a sample size of between 0.5 and 1 g of material for DNA isolation from soils,

sediments and faecal material, which is far greater than the 30 µl of sludge used for DNA isolation from the P, K, A1Pb and A1Sb digested sludges in this study. Therefore, for the DGGE studies conducted in the next research chapter, it was decided to concentrate larger sludge samples (1.5 - 2 mL sludge) by centrifugation before DNA isolation.

The DGGE fingerprints of the sieved (S) and unsieved (U) DNA isolates of the P, K, A1Pb and A1Sb digested sludges, as given in Fig. 2, were found to be identical, except for the very faint bands **g** and **c** in the A1Sb-U-sludge fingerprint. This would suggest that the archaeal content of the sludge is not affected by screening the sludge through a 1 mm² sieve. This sieving step is performed routinely as part of the sludge-preparation process for batch granulation-enhancement studies.

Construction of methanogenic DGGE marker and the DGGE fingerprints of granules

The inclusion of a DGGE marker on DGGE gels can facilitate the rapid identification of individual bands in complex DGGE banding patterns. Methanogenic *Archaea* are believed to be involved in the anaerobic granulation process, so for this study a methanogen-containing DGGE marker was constructed. Identification of methanogens present in digested sludge as well as in mature granules that play a role during granulation may lead to a better understanding of their importance to the granulation process. It will also provide insight into population shifts that may occur during reactor start-up. In a follow-up study, a more comprehensive methanogenic marker was constructed by adding *Methanosaeta thermophila* as well as *Methanobacterium formicicum* to the three methanogenic strains used as marker organisms in the previous section (*Methanosaeta concilii*, *Methanosarcina barkeri* and *Methanosarcina mazei*). Selection of these five methanogens was based on literature references describing their role in granulation. These literature reports are discussed below.

Various reports have indicated that *Methanosaeta* spp. are important for stable digester function and efficient operation of anaerobic digesters (McHugh *et al.*, 2003; Hulshoff Pol *et al.*, 2004; Zheng *et al.*, 2006) and that *Methanosaeta concilii* plays a key role in granulation (Chen & Lun, 1993; Wu *et al.*, 1996; El-Mamouni *et al.*, 1997; Hulshoff Pol *et al.*, 2004; Zheng *et al.*, 2006; Baloch *et al.*, 2008). *Methanosaeta concilii* was, therefore, included in the DGGE marker. No specific species reference was made

to the role of *Methanosaeta thermophila* in granulation. For this study it was, however, decided to include *M. thermophila* in the marker since the *Methanosaeta* genus at present includes only two species: *M. concilii* and *M. thermophila*.

Other methanogens might, however, also contribute to the granulation process. Chen & Lun (1993) studied the composition of different types of granules as well as the growth of *Methanosaeta concilii* and *Methanosarcina barkeri* in pure culture and postulated that both species might be involved in initial nucleus formation. According to these authors, *Methanosarcina barkeri* would typically dominate at high acetate levels during start-up, growing in clumps. As the acetate levels decrease and the hydraulic loading rate increases, *Methanosaeta concilii* would grow faster and attach to and colonise some of the initial *M. barkeri* clumps while the rest of the *M. barkeri* clumps would be washed out. The hypothesis of Chen & Lun (1993) also supports reports of De Zeeuw (1988) and Hulshoff Pol (1989). De Zeeuw (1988) indicated the possible colonisation of *Methanosarcina* clumps by *Methanosaeta* spp. Hulshoff Pol (1989) reported the presence of both *Methanosaeta*-dominated aggregates and *Methanosarcina*-dominated aggregates during start-up and found that the *Methanosarcina*-dominated aggregates washed out more easily. Although the study of Chen & Lun (1993) was the only study that made specific species reference to *Methanosarcina barkeri*, it was decided to include *Methanosarcina barkeri* in the methanogenic marker since it is the type species for the *Methanosarcina* genus.

Two other methanogens, *Methanobacterium formicicum* and *Methanosarcina mazei*, were also included in the marker based on the reports of Veiga *et al.* (1997) and Wu *et al.* (1996). Veiga *et al.* (1997) reported that the ECP produced by pure cultures of *M. formicicum* and *M. mazei* is similar in composition to ECP extracted from granules fed with a volatile fatty acid (VFA) mixture. Based on this observation they postulated that both these strains probably contribute to anaerobic granulation. Wu *et al.* (1996) studied granule formation by defined species and concluded that *M. formicicum* interacted with *Methanosaeta* species during initial granulation and that *M. mazei* contributed to the development of the granule structure.

The positions of the DGGE bands of the species of methanogens included in the marker (listed in Table 2) are presented in Fig. 3. The two different *Methanosaeta concilii* strains (DSM 3671 and DSM 3013) were loaded separately in lanes 1 and 2 and then combined in lane 3. The results indicated that this DGGE method was species specific and not strain specific since the bands in lanes 1, 2 and 3 are in the same

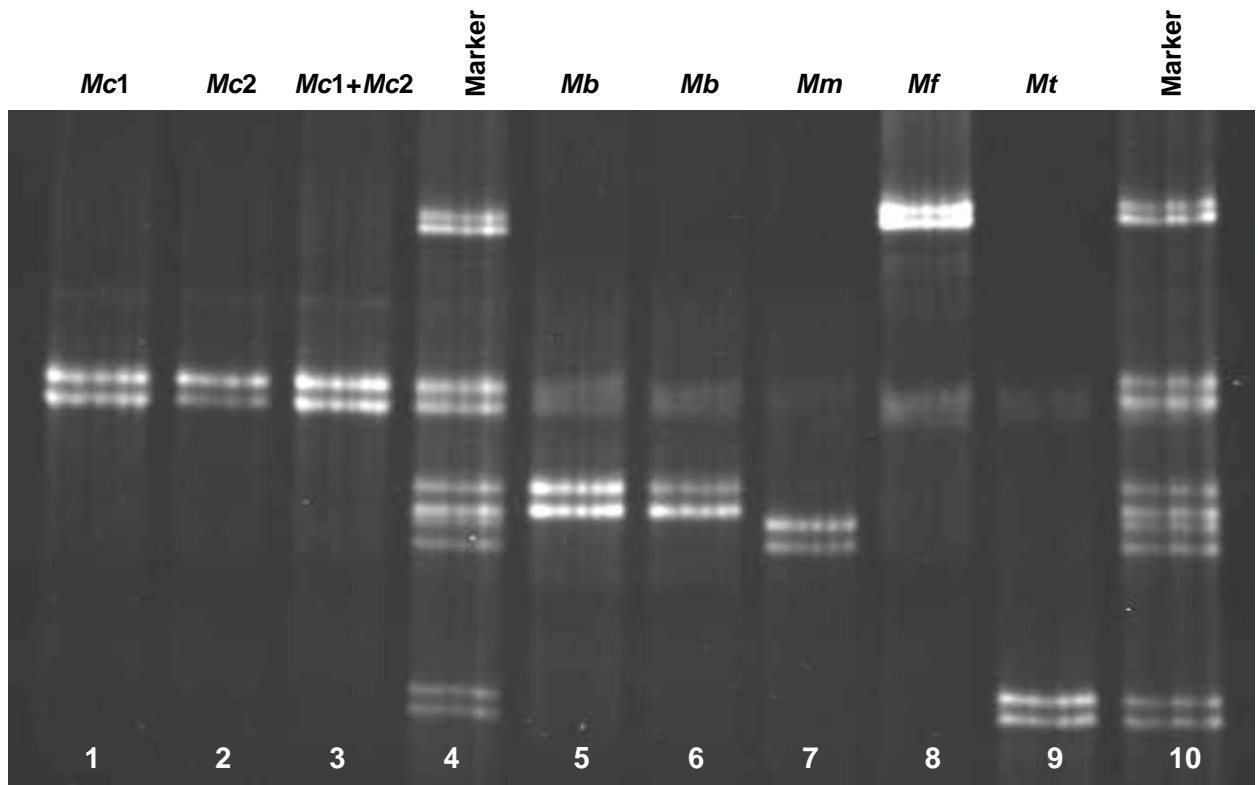


Figure 3 DGGE fingerprints of the pure methanogenic strains used to in the construction of methanogenic marker. *Methanosaeta concilii* DSM 3671 (**Mc1** - lane 1); *M. concilii* DSM 3013 (**Mc2** - lane 2); DSM 3671 and DSM 3013 combined (**Mc1+ Mc2** - lane 3); *Methanosarcina barkeri* DSM 800 (**Mb** - lanes 5 and 6); *Methanosarcina mazei* DSM 2053 (**Mm** - lane 7); *Methanobacterium formicicum* DSM 1535 (**Mf** - lane 8); and *Methanosaeta thermophila* DSM 4774 (**Mt** - lane 9). For the DGGE marker all the strains (except for DSM 3013) were combined (lanes 4 and 10).

position. Thus, for the rest of the study, only the type strain (*Methanosaeta concilii* DSM 3671) was used for the final marker construction. For the marker equal volumes of PCR product of each methanogen were combined and loaded in lanes 4 and 10.

The comparison of the marker to the DGGE fingerprints of mature granules could further help to identify important methanogens that are or might be present in the fingerprints of digested sludges. The DGGE fingerprints of the mature UASB granules are presented in Fig. 4. In contrast to the digested sludges tested, all the granule types (B, F, A, W, L, M and P) contained *Methanosaeta concilii* (band **b**). The lab-scale reactors from which the L and P granules were obtained were both originally seeded with brewery granules, which may explain why the banding patterns of the L, P and B granules exhibited similarities. The occurrence of band **g** in the fingerprint of the L-granule as well as the variation in banding patterns observed overall for granule types B, F, W and M suggested that the archaeal populations of mature granules are influenced by differences in substrate composition. This is in spite of the fact that methanogens are usually situated at the centre of granules used for the treatment of carbonaceous substrates (Fang, 2000). Band **f** (shared by the F and W granules) and band **g** (shared by the L and M granules) are the only bands other than **b** (*Methanosaeta concilii*) that were present in more than one mature granule type. The presence of bands **f** and **g** might be substrate related (i.e. the organisms represented by these bands might metabolise similar derivatives produced during anaerobic digestion). It is at this stage unclear whether the *Archaea* represented by bands **f** and **g** have any influence on sludge granulation.

For every species tested a prominent band accompanied at close distance by another band was observed (Fig. 3). These 'double bands' were also observed in the DGGE fingerprints of mature UASB granules (Fig. 4). The appearance of artifactual double bands after DNA amplification from pure cultures as well as from plasmids has been reported, although no explanation for their formation could be given (Janse *et al.*, 2004). It is, however, necessary to minimise the formation of double bands since it could lead to an overestimation of sequence diversity and the increased overlap of bands could also hinder identification (Janse *et al.*, 2004). In an attempt to minimise double band formation during PCR of the pure methanogen species, two techniques were evaluated. The first technique involved the addition of dimethyl sulfoxide (DMSO) to the PCR reaction mixture (2% (v/v) final concentration) (Shen & Hohn, 1992). In the

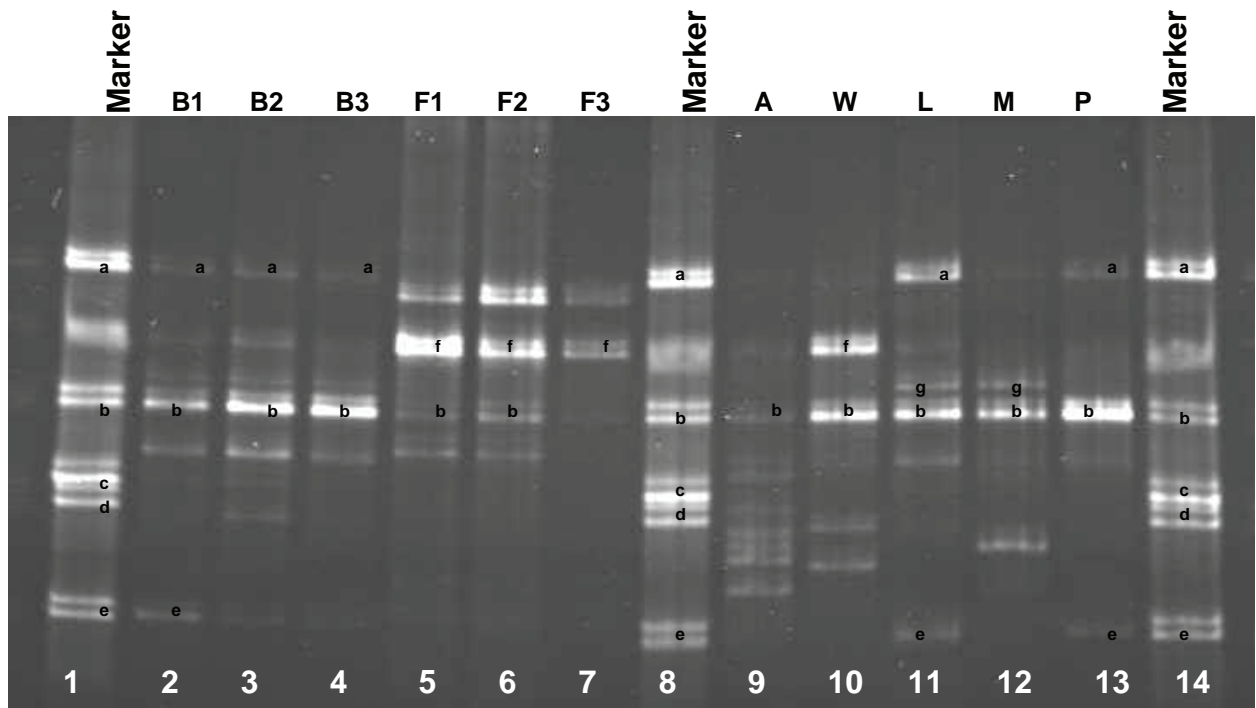


Figure 4 DGGE fingerprints of mature UASB granules. Granule sources were six UASB reactors treating a variety of food processing wastewaters as well as one anaerobic digester treating municipal wastewater. Granules presented were used in the treatment of the following wastewaters: brewery (B1 – B3) (lanes 2 – 4); fruit juice processing (F1 – F3) (lanes 5 – 7); apple processing (A) (lane 9); winery (W) (lane 10); lye (L) (lane 11); municipal (M) (lane 12); and apple pomace leachate (P) (lane 13). The methanogenic marker was included in lanes 1, 8 and 14. The marker bands represent the following methanogens: a - *Methanobacterium formicicum* DSM 1535; b - *Methanosaeta concilii* DSM 3671; c - *Methanosarcina barkeri* DSM 800; d - *Methanosarcina mazei* DSM 2053; and e - *Methanosaeta thermophila* DSM 4774.

second approach, the influence of 'touchdown' PCR on double band formation was determined according to the method of Don *et al.* (1991). After the initial denaturation step, touchdown PCR was achieved by decreasing the annealing temperature of the PCR reaction by 1°C every second cycle, starting at 65°C and ending at 55°C. This was then followed by the 31 PCR cycles described.

The effect that DMSO and touchdown PCR had on double band formation can be seen in Fig. 5. Compared to the double bands observed for the methanogens in Fig. 3, an improvement was evident in the samples to which DMSO had been added (D-samples) as well as in the samples that underwent touchdown PCR (T-samples). Overall the T-samples did appear better than the D-samples. Complete elimination of double bands was, however, not obtained in the T-samples, as can be seen for *Methanosaeta thermophila* in lane 12. DMSO addition and touchdown PCR were also combined to improve the appearance of double bands in the DGGE fingerprints of mature granules (Fig. 6). Comparison of granule banding patterns of Figs. 4 and 6 revealed that DMSO and touchdown PCR resulted in improvements in the appearances of bands **a** and **b** (present in granules L, B, F and P). A slight improvement in the appearance of band **f** (granule F) was also observed. This shows that double band formation can be reduced during PCR amplification from pure methanogenic species as well as from DNA extracts of environmental samples containing different microorganisms.

Conclusions

The optimised DGGE method was successfully used to obtain DGGE fingerprints of both digested sludges and mature UASB granules. The appearance of artifactual double bands in archaeal fingerprints was minimised successfully by using touchdown PCR. The DGGE fingerprints of the digested sludges showed that only one of the four sludges tested (A1Sb-sludge) contained *Methanosaeta concilii*. This is in contrast to the findings for mature UASB granules, which all contained *Methanosaeta concilii*, regardless of substrate type.

Methanosaeta concilii is generally considered to be critical for granule formation (Chen & Lun, 1993; Wu *et al.*, 1996; McHugh *et al.*, 2003; Zheng *et al.*, 2006; Baloch *et al.*, 2008). Choice of seed sludge for further batch granulation-enhancement should thus be based on the presence of a *Methanosaeta concilii* population. Long-term stability of

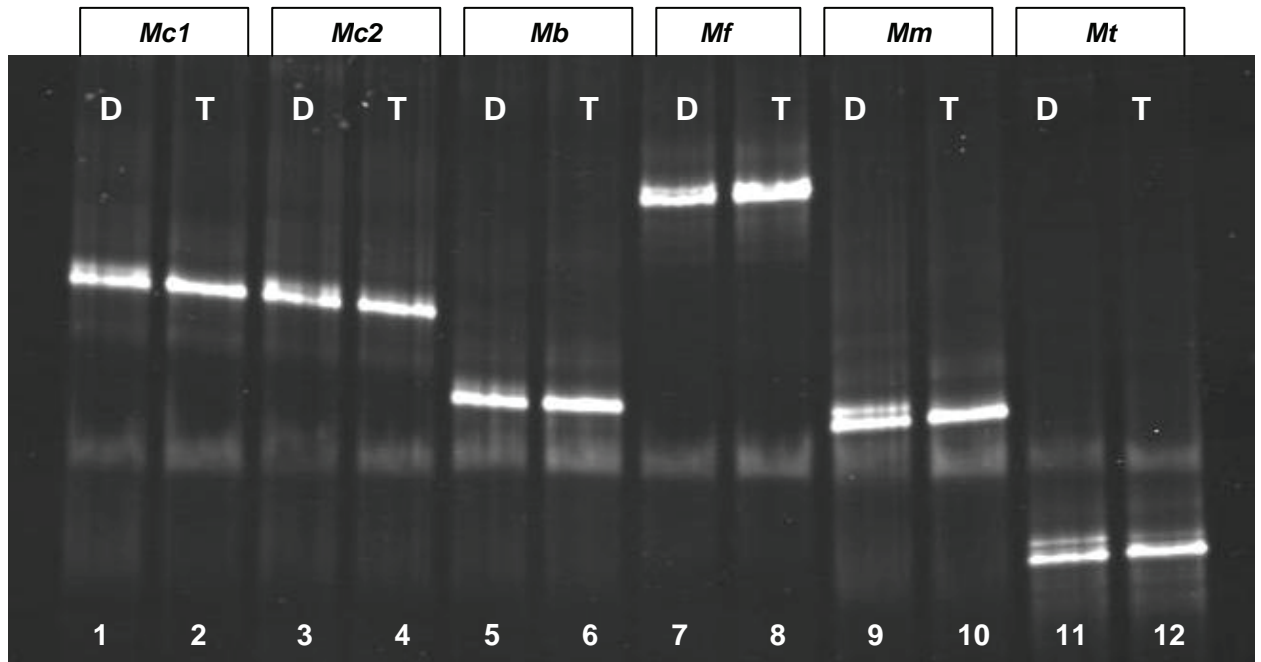


Figure 5 The influence of DMSO addition (D) and ‘touchdown’ PCR (T) on the elimination of artifactual double bands during PCR amplification of 16S rRNA gene sequences from the pure methanogenic spp. used for the construction of the methanogenic DGGE marker. The methanogens were:
Methanosaeta concilii DSM 3671 (**Mc1** – lanes 1 and 2);
Methanosaeta concilii DSM 3013 (**Mc2** - lanes 3 and 4);
Methanosarcina barkeri DSM 800 (**Mb** - lanes 5 and 6);
Methanobacterium formicicum DSM 1535 (**Mf** - lanes 7 and 8);
Methanosarcina mazei DSM 2053 (**Mm** - lanes 9 and 10); and
Methanosaeta thermophila DSM 4774 (**Mt** - lanes 11 and 12).

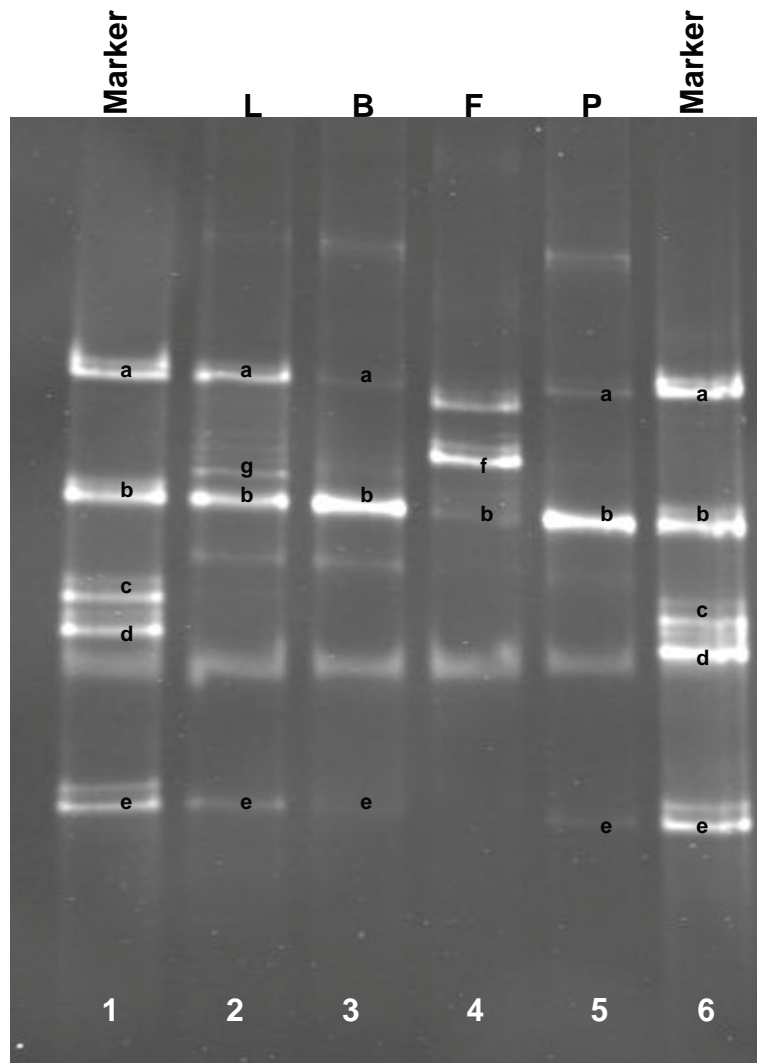


Figure 6 DGGE fingerprints of four mature granules after DMSO addition and “touchdown” PCR. Granules were obtained from facilities treating the following wastewaters: lye (L) (lane 2); brewery (B) (lane 3); fruit juice processing (F) (lane 4); and apple pomace leachate (P) (lane 5). The methanogenic marker was included (lanes 1 and 6) and the marker bands represent the following methanogens:

- a - *Methanobacterium formicicum* DSM 1535;
- b - *Methanosaeta concilii* DSM 3671;
- c - *Methanosarcina barkeri* DSM 800;
- d - *Methanosarcina mazei* DSM 2053; and
- e - *Methanosaeta thermophila* DSM 4774.

the municipal digesters might have a direct influence on the *Methanosaeta concilii* population content of the digester sludge, which, in turn, might have an effect on the degree to which granulation can be enhanced. It is, therefore, recommended that digested sludges be obtained from municipal digesters that show stable digester behaviour over a period of at least five months. The DGGE method and marker developed in this study could then be used to screen digested sludges for the presence of *Methanosaeta concilii* as well as other important methanogens. It might then be determined whether stable digester performance can be linked to the presence of *Methanosaeta concilii*, and the best seed sludge for further batch granulation-enhancement studies might then be identified.

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CHAPTER 6

EVALUATION OF THE GRANULATION POTENTIAL OF MUNICIPAL DIGESTED SLUDGES AS SEED INOCULUM FOR UASB REACTOR START-UP

Summary

Digested sludge is frequently used as seed material during upflow anaerobic sludge blanket (UASB) reactor start-up. In the previous chapter of this dissertation it was shown that digested sludges from different sources vary in terms of acetoclastic methanogen content. Stable digester performance was, however, not taken into account when the sludges were collected. In this study four anaerobic digested sludges (Athlone 4Sb, Athlone 2Sb, CF and ST) were chosen based on stable digester performance. In order to identify the most suitable seed sludge for further studies in enhancing UASB reactor start-up, the anaerobic population of each sludge was compared in terms of substrate-dependent biogas production, granulation-enhancement potential in batch systems as well as archaeal population composition, using polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE) fingerprinting.

The Athlone 4Sb-sludge had the highest biogas-production potential and was the only sludge that exhibited measurable methanogenic activity by both hydrogenotrophic and acetoclastic methanogen populations. The ST-sludge showed the highest increase in VSS particles $\geq 0.25 \text{ mm}^2$ during the 17 d batch granulation-enhancement study in lactate medium (LacM) and in apricot effluent medium (AEM). DGGE and PCR-detection results confirmed the successful enhancement of *Methanobacterium formicicum* as well as *Propionibacterium* in the ST-sludge during this time, which might have contributed to an increase in VSS $\geq 0.25 \text{ mm}^2$. The ST-sludge also had the highest concentration of divalent ions (Ca^{2+} and Mg^{2+}) of the four sludges tested, which might also have contributed to the increase in VSS $\geq 0.25 \text{ mm}^2$ observed during the batch granulation-enhancement study. DGGE results showed that all the sludges contained *Methanosaeta concilii* before the 17 d batch granulation-enhancement study. *M. concilii* was present in the LacM and AEM samples of the Athlone 4Sb, CF and ST sludges after 17 d but the band intensity was very faint for all the AEM samples. Low band intensity was attributed to the inhibition of *M. concilii* as result of the unstable pH conditions in the AEM samples during batch granulation. Since *M. concilii* is reported to

be crucial to anaerobic granulation it is recommended that a substrate such as LacM (where the pH is easier to control) be used instead of AEM in future batch granulation-enhancement studies. It is furthermore recommended that normal UASB reactor start-up be done with both Athlone 4Sb and ST sludges to determine which of the parameters measured above is the most accurate indicator of how suitable a digested sludge is as seed material for UASB reactor start-up.

Introduction

UASB reactor start-up times can be greatly reduced if surplus granular sludge from functioning UASB digesters is used as seed material (Goodwin *et al.*, 1992), but access to high-quality granular sludge is limited in developing countries. In the absence of granular biomass, digested sludge is commonly used as inoculum for UASB reactors. This does, however, present a variety of problems, the most common of which include long start-up times to minimise sludge washout. The ultimate aim of the batch granulation-enhancement model (BGEM) developed by Britz *et al.* (2002) was to reduce UASB reactor start-up periods by enhancing sludge granulation before inoculating an UASB reactor. According to their hypothesis, lactate-utilising populations such as *Propionibacterium* could produce ECPs under unfavourably low pH conditions to act as a hydrogen sink mechanism in order to gain a competitive advantage. The ECPs in turn, may then promote bacterial aggregation, which could ultimately enhance anaerobic granulation. According to Britz *et al.* (2002), a very important factor in the success of this type of granulation enhancement is the granulation potential of the inoculum sludge to be used.

It should be kept in mind that the aim of the BGEM (Britz *et al.*, 2002) was only to enhance the natural anaerobic granulation process, which could in itself be influenced by a variety of other factors. Researchers agree, however, that the key organism to the natural anaerobic granulation process is *Methanosaeta concilii*, an acetoclastic methanogen, which reportedly acts as granular nuclei around which a more mature UASB granule could develop through the process of microbial population aggregation and growth (Hulshoff Pol *et al.*, 2004; Baloch *et al.*, 2008). The data presented in the previous chapter of this dissertation (Chapter 5) showed that digested sludges from different sources do differ in their methanogenic microbial content. Denaturing gradient gel electrophoresis (DGGE) screening also revealed that *Methanosaeta concilii* was not present in some of the sludges tested. Ultimately it would seem that the choice of the

most suitable digested sludge to be used as inoculum during industrial UASB reactor start-up is more complex than originally thought and the inoculum choice should be based on more than just the mere proximity of a wastewater treatment facility.

The aim of this study was to characterise the anaerobic populations of four 'healthy' well-digested and stabilised digested sludges in order to, firstly, determine what differences exist in biogas production activity, granulation-enhancement potential in batch systems and archaeal population composition and, secondly, to identify the most suitable seed sludge for further UASB reactor start-up and granulation-enhancement studies.

Materials and methods

Choice and preparation of digested sludges and industrial granules

Four anaerobically digested sludges from local municipal wastewater treatment works (WWTW) in the Cape Town metropolitan area were chosen for further studies. The choice was based on advice from Dr W.R. Ross (Ross Consultancy, South Africa, personal communication, 2002) and the cooperation of Mr R. Moolan and Mr H. Rus of the Scientific Services Department of the Cape Metropolitan Council (CMC). The main selection criteria were that each sludge should be a well-stabilised homogenous sludge sample taken from secondary anaerobic digesters of which the control parameters and routine analyses indicated a 'healthy' digester operation.

With the kind cooperation of the CMC the following anaerobic digesters were identified and sampled by the respective WWTW plant operators:

- Cape Flats digester 3 (= CF);
- Athlone 2 Secondary B digester (= Athlone 2Sb);
- Athlone 4 Secondary B digester (= Athlone 4Sb); and
- Simonstown secondary digester (= ST).

The Cape Flats, Athlone 2Sb and Simonstown digesters were, at the time of sampling, all used for the anaerobic digestion of primary settling tank (PST) sludge while the Athlone 4Sb digester was fed with a mixture of PST and activated sludge (H. Rus, CMC, South Africa, personal communication, 2002).

Before the sludge samples were used as inoculum for the batch granulation-enhancement experiment, the sludges were 'size-standardised' by sieving them through a 1 mm² Endocott sieve (Merck) to remove larger pieces of sludge aggregates and

other debris. Duplicate samples of each sludge type were also taken for the DNA isolation studies.

Samples of mature UASB granules for the DNA isolation studies were obtained from a 2.4 L laboratory-scale UASB reactor treating lye-containing (L) fruit canning industry effluent ($\text{COD}_{\text{in}} = 2\,500 \text{ mg.L}^{-1}$; $[\text{Na}^+] = 2\,000 \text{ mg.L}^{-1}$; $\text{pH} [\text{influent}] = 8.5$) as well as from three industrial-scale mesophilic UASB reactors treating apple pomace (P), brewery (B) and fruit juice (F) industry effluents.

Activity tests

The activity of anaerobic granular sludge can either be measured overall, which would give an indication of the total process activity at, for example, a specific digestion stage, or the activity of the different populations present can be measured individually. The latter method of activity testing is helpful in the identification of possible unbalanced situations between different sludge populations (Soto *et al.*, 1993; O'Kennedy, 2000).

The activity test method procedure developed by O'Kennedy (2000) was used to determine and compare the microbial activity of the four different digested sludges before the granulation-enhancement experiment was conducted. Since the original method was developed with anaerobic granules as inoculum, the method was modified for this study in that centrifuged digested sludge was used instead. The method was as follows: 3 g of centrifuged sludge was incubated with 13 mL of basic test medium (BTM) (Valcke & Verstraete, 1993) supplemented with different carbon sources (Tables 1 and 2), in 20 mL test vials sealed with butyl rubber septa and aluminium crimp caps. Control vials containing 13 mL of BTM without any additional carbon source were also prepared in triplicate for each digested sludge. The vials were incubated at 35°C for 25 h.

Biogas production was measured (in mL) at 5, 10 and 25 h intervals by using a free-moving 5 mL syringe equipped with a 25-gauge needle. Biogas measurements were made by holding the syringe vertically, inserting it into the rubber stopper and allowing the plunger to move freely until equilibrium was reached. The methane and CO_2 contents were determined by injecting biogas samples into a Varian 3300 gas chromatograph (Varian Inc., Palo Alto, CA) equipped with a thermal conductivity detector and 2.0 x 3.0 mm i.d. column packed with Hayesep Q (Supelco, Bellefonte, PA) 80/100 mesh. The oven temperature was set at 55°C and helium was used as carrier gas at a flow rate of 30 mL.min^{-1} . Activity was expressed in terms of cumulative

Table 1 Composition of the Basic Test Medium (BTM) (pH 7.0) (Valkce & Verstraete, 1993)

Compound	Concentration (g.L ⁻¹)
Glucose (BDH)*	2.0
K ₂ HPO ₄ (Saarchem)	1.0
KH ₂ PO ₄ (BDH)	2.6
Urea (BDH)	1.1
NH ₄ Cl ₂ (Saarchem)	1.0
Na ₂ S (Saarchem)	0.1
MgCl ₂ .6H ₂ O (Merck)	0.1
Yeast Extract (Biolab)	0.2

* Additional to the medium of Valcke & Verstraete (1993)

Table 2 The different test media used for activity testing

Test media	Composition
BTM	As presented in Table 1, pH = 7.0
GTM	BTM + 2 g.L ⁻¹ glucose, pH = 7.0
LTM	BTM + 4 g.L ⁻¹ lactic acid (60% v/v), pH = 7.0
ATM	BTM + 1 g.L ⁻¹ acetic acid, pH = 7.0
FTM	BTM + 1 g.L ⁻¹ formic acid, pH = 7.0
PTM	BTM + 0.5 g.L ⁻¹ propionic acid, pH = 7.0

biogas and methane production (in mL) over 25 h and in terms of tempo of biogas production in $\text{mL}\cdot\text{h}^{-1}$ (S_b) or tempo of methane production in $\text{mL}\cdot\text{h}^{-1}$ (S_m).

Batch granulation-enhancement study

A modified version of the method developed by Britz *et al.* (2002) for granulation enhancement in a laboratory batch system was used. The modification involved the use of a roller-table (35 rpm) (specifically manufactured by the Process Engineering Workshop, University of Stellenbosch) for agitation instead of the original shake waterbaths. The roller-table was designed as a multi-roller-tumbler, driven by an electric motor via sprockets and chain. The variable speed was obtained with the use of an inverter connected to the electric motor.

Each one of the four digested sludges was introduced into a LacM (all in duplicate) and into an AEM (all in duplicate). The LacM was a defined chemical medium that included only lactate as carbon source and was used by Britz *et al.* (2002) to provide the lactate-utilising anaerobic bacteria with a competitive advantage in the anaerobic environment. The AEM was included in this study as an example of an industrial effluent that could serve as a more economical carbohydrate source during batch granulation-enhancement. Chapter 3 of this dissertation describes its successful use to produce microbial aggregates of pure *Propionibacterium* cultures, which demonstrated that although fruit cannery effluent does not typically contain lactate, lactate-utilising bacteria such as *Propionibacterium* could still utilise it as carbon source. It has also been reported in literature that fruit cannery effluent-fed UASB granules exhibit excellent shear strengths, settling distributions and densities compared to granules fed with brewery or complex protein-based effluents (Batstone & Keller, 2001), a fact that supported the decision to include it as a more economical substrate in this batch granulation-enhancement study.

The LacM consisted of ($\text{g}\cdot\text{L}^{-1}$): sodium lactate (Sigma) 10.0; yeast extract (Biolab) 5.0; peptone (Biolab) 2.0; KH_2PO_4 (BDH) 10.0; and Tween 80 (Merck) 1.0 mL. The pH was adjusted to pH 7.0, using a 2M NaOH solution, after which the medium was autoclaved. A trace element solution, specified by Nel *et al.* (1985), was also prepared and added to the medium directly before use. The LacM had a COD of $10\,300\text{ mg}\cdot\text{L}^{-1}$ to which the lactate carbon source contributed $3\,200\text{ mg COD per litre}$.

For the preparation of the AEM, effluent was collected at a nearby canning factory (RFF Cannery, Klein Drakenstein) during the apricot-processing season. The

COD of the undiluted effluent was in the range of 12 000 - 15 000 mg total COD per litre. The AEM was sieved (1.0 mm^2) to remove most of the larger suspended solids and prepared by diluting the effluent to a concentration of $2\,000 \text{ mg.L}^{-1}$ total COD. The following was added to the AEM in (mg.L^{-1}): KH_2PO_4 (BDH) 200; CaCO_3 (Saarchem) 200 and urea (Saarchem) 200. A trace element solution, specified by Nel *et al.* (1985), was prepared and added to the medium directly before use.

For all the samples the ratio of sludge to medium was 1:3.5. The 500 mL units were incubated on the roller-table for 17 d at 35°C and 25% of the liquid after settling was replaced daily. The pH of the medium was measured directly before and after the sludge was added to the samples on D0 and then measured every day for 17 d. At the end of the study, duplicate sludge samples from each bottle were also collected for DNA isolation.

Size distribution

Size distribution determinations were conducted on the sludge samples at the start and at the end of the granulation-enhancement study. The method was based on that developed by Laguna *et al.* (1999) for determining the granulometry of UASB reactor sludge and was as follows: Four Endocott sieves with mesh openings of 1.0, 0.71, 0.50 and 0.25 mm^2 were stacked with the largest mesh opening at the top. The sample was well mixed before a representative 25 mL was added to the 1.0 mm^2 sieve. The top sieve was rinsed with distilled water to remove particles smaller than 1.0 mm^2 and then removed. The washing procedure was repeated for each sieve. The particles retained on each sieve were recovered by backwashing with distilled water and then the total suspended solids (TSS) and volatile suspended solids (VSS) contents were determined. The TSS and VSS of each sludge sample and its respective sieve fractions were determined using the method specified in Standard Methods (1998).

Mineral analysis

All four of the digested sludges were analysed for the presence of inorganic elements in solution. The screened elements included Ca, Fe, K, Mg, Na and Ni ions. A Varian Liberty II radial emission inductively coupled plasma (ICP) spectrometer was used at various wavelengths specific to the individual elements analysed and emissions were

compared to those of standardised solutions (R. Rossouw, Central Analytical Facility, University of Stellenbosch, personal communication, 2002).

DNA isolation

DNA was isolated from each of the day 0 (D0) and day 17 (D17) sludge samples as well as from mature UASB granules using the modified method of Van Elsas *et al.* (1997). From each of the four digested sludges, 1.5 mL of a well-mixed sample was transferred to a centrifuge tube (done in duplicate) and centrifuged at 10 000 x *g* for 10 min. The supernatant was discarded and the pellet of each tube was transferred to a sterile centrifuge tube containing 0.6 g sterile glass beads (0.2 - 0.3 mm diameter) (Sigma). The following reagents were added to each tube: 800 μ L phosphate buffer (1 part 120 mM NaH₂PO₄ mixed with 9 parts 120 mM Na₂HPO₄; pH 8); 700 μ L phenol (pH 4.3) (Fluka); and 100 μ L of 20% (m/v) sodium dodecyl sulphate (Merck). The tubes were vortexed for 2 min and incubated in a 60°C water bath for 20 min. This procedure was repeated twice, after which the samples were centrifuged for 5 min at 1 500 x *g*.

The aqueous phase of each sludge sample was transferred to a new sterile centrifuge tube and proteins and other cell debris were removed by extracting with 600 μ L phenol (pH 4.3) (Fluka), followed by repeated extractions with 600 μ L phenol/chloroform/isoamylalcohol (25:24:1) until the interphase of each sample was clean. The DNA was precipitated with 0.1 x vol 3 M sodium acetate (NaAc) (pH 5.5) (Saarchem) and 0.6 x vol isopropanol (Saarchem) on ice for at least 1 h. The precipitated DNA was collected by centrifugation for 10 min at 15 000 x *g*. The pellet was washed with 70% (v/v) cold ethanol (Merck) and air-dried for 20 min after which it was redissolved in 100 μ L TE buffer (10 mM Tris, 1 mM EDTA; pH 8).

PCR amplification

The examination of the archaeal community structure of the different digested sludges and mature UASB granules involved the nested PCR amplification of the V3 variable region of the 16S rRNA gene followed by DGGE analysis. PCR amplification of the isolated DNA samples was performed as described by Øvreas *et al.* (1997). A 1 072 base pair (bp) fragment was amplified and used as template in a nested PCR (referred to as dgge-PCR) to produce a 179 bp fragment with a GC-clamp that was resolved using DGGE.

The primers used for the initial PCR amplification were PRA46f (5' (C/T)TA AGC CAT GC(G/A) AGT 3') and PREA1100r (5' (C/T)GG GTC TCG CTC GTT (G/A)CC 3') (Øvreas *et al.*,1997). The PCR reaction volume was 50 µL and contained 0.5 µM of each of the primers; 100 µM dNTPs (Promega); 2.0 mM MgCl₂; 2.5 U *Biotaq*TM Polymerase; 1 x reaction buffer (Bioline); and 1 - 2 µL isolated DNA. Acetamide was also added to a final concentration of 5% (m/v) to minimise non-specific annealing of the primers.

The primers used in the dgge-PCR amplification were PARCH340f (5' CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG CCC TAC GGG G(C/T)G CA(G/C) CAG 3') (GC-clamp sequence underlined) and PARCH519r (5' TTA CCG CGG C(G/T)G CTG 3') (Øvreas *et al.*,1997). The GC-clamp sequence included on the 5' end of the forward primer was identical to that reported by Chan *et al.* (2001). The dgge-PCR had a reaction volume of 50 µL and contained 0.5 µM of each of the primers; 100 µM dNTPs (Promega); 0.5 mM MgCl₂; 2.6 U *Expand* Polymerase (Roche); 1 x reaction buffer (Roche) and 0.25 - 0.5 µL of the initial PCR as template.

The reaction conditions were the same for both primer sets and amplicons were generated using an initial denaturation step of 2 min at 92°C followed by 31 cycles of denaturation at 92°C for 60 s, annealing at 55°C for 30 s, and chain elongation at 72°C for 60 s. A single final elongation step at 72°C for 6 min was also included. PCR amplification was performed in an Eppendorf Mastercycler Personal (Merck) and the presence of PCR products was confirmed on either 1% (m/v) agarose gels (for initial PCR products) or 1.5% (m/v) agarose gels (for dgge-PCR products). All agarose gels contained ethidium bromide and the separated fragments were visualised under UV light (Vilber Lourmat).

DGGE analysis

The 179 bp fragments generated during nested PCR analysis of the different sludges and granules were resolved using DGGE. Band separation was based solely on differences in nucleotide sequence.

DGGE was performed with a Biorad DcodeTM Universal Mutation Detection System (Bio-Rad Laboratories, USA). Separation of the archaeal-specific PCR amplicons was obtained using 8% (m/v) polyacrylamide gels in a 0.5 x TAE buffer containing a linear denaturing gradient of between 35 and 70%. The 100% denaturing solution used in the preparation of this gradient contained 40% (v/v) formamide

(Saarchem) and 7.0 M urea (Merck). After electrophoresis was performed at a constant voltage of 130 V for 5 h at 60°C, gels were stained with a 10% (v/v) ethidium bromide solution. The archaeal population fingerprints were then visualised under UV light (Vilber Lourmat).

A DGGE reference marker containing equal amounts of PCR amplicons of the five methanogenic species listed in Table 3 was also applied to DGGE gels before electrophoresis. The choice of these species was based on literature references as to the importance of these methanogens to the anaerobic granulation process (Table 3).

Propionibacterium genus-specific PCR

PCR detection was used to determine the presence of *Propionibacterium* species in the isolated DNA of both the D0 and D17 sludge samples obtained during the batch granulation-enhancement experiment. The *Propionibacterium*-specific PCR primers (Prop 1 and Prop 2) developed by Schoeman (2001) based on aligned 16S rRNA gene sequences of classical *Propionibacterium* species were applied. The PCR method was also tested against the *Propionibacterium* strains listed in Table 4.

The PCR reactions had a reaction volume of 50 µL and contained 50 µM dNTPs (Promega); 4.0 mM MgCl₂; 2.5 U *Biotaq*TM Polymerase; 1 x reaction buffer (Bioline); and 0.5 - 2 µL of isolated DNA. Each reaction also contained 0.1 µM of both Prop 1 (5'-GAT ACG GGT TGA CTT GAG G-3') and Prop 2 (5'-GTA ATC GCA GAT CAG CAA CGC-3') (Schoeman, 2001).

Amplicons, 720 bp in size, were generated using an initial denaturation step of 60 s at 95°C, followed by 35 cycles of denaturation at 95°C for 35 s, annealing at 60°C for 60 s, and chain elongation at 72°C for 60 s. A final elongation step at 72°C for 10 min was also included.

Results and discussion

Characteristics of selected digested sludges

The results that were provided by the CMC of the routine analyses done on the four selected digesters over the five-month period before sampling are summarised in Table 5.

Table 3 Methanogenic species present in DGGE marker

Species	Culture ¹	Literature reference
<i>Methanosaeta concilii</i> (Synonyms <i>Methanotherix concilii</i> and <i>Methanotherix soehngenii</i>)	DSM 3671	Chen & Lun, 1993; Wu <i>et al.</i> , 1996; McHugh <i>et al.</i> , 2003; Zheng <i>et al.</i> , 2006; Baloch <i>et al.</i> , 2008
<i>Methanosaeta thermophila</i>	DSM 4774	McHugh <i>et al.</i> , 2003; Baloch <i>et al.</i> , 2008
<i>Methanosarcina barkeri</i>	DSM 800	Chen & Lun, 1993; Wu <i>et al.</i> , 1996
<i>Methanosarcina mazei</i>	DSM 2053	Wu <i>et al.</i> , 1996; Veiga <i>et al.</i> , 1997
<i>Methanobacterium formicicum</i>	DSM 1535	Wu <i>et al.</i> , 1996; Veiga <i>et al.</i> , 1997

¹ DSM – Deutsche Sammlung von Mikroorganismen und Zellkulturen

Table 4 *Propionibacterium* reference cultures

Species	Group	Culture ^{1, 2, 3}
<i>Propionibacterium acnes</i>	Cutaneous	ATCC 6919
<i>Propionibacterium parvum</i>	Cutaneous	ATCC 11829
<i>Propionibacterium jensenii</i>	Classical	DSM 20535
<i>Propionibacterium acidipropionici</i>	Classical	ATCC 25562
<i>Propionibacterium thoenii</i>	Classical	NCFB 568
<i>Propionibacterium freudenreichii</i> ss <i>freudenreichii</i>	Classical	ATCC 6207
<i>Propionibacterium freudenreichii</i> ss <i>shermanii</i>	Classical	Lab isolate 434

¹ ATCC – American Type Culture Collection; ² DSM – Deutsche Sammlung von Mikroorganismen und Zellkulturen;

³ NCFB – National Collection of Food Bacteria c/o NCIMB Ltd. Aberdeen, Scotland, United Kingdom

Table 5 Summary of digester performance based on the measurement of weekly parameters over five months for the four anaerobic digesters from which anaerobic sludges were obtained for this study. Data was kindly provided by the Scientific Services Department of the CMC. All the values (except for the % methane) are the data averages ($n = 4$) \pm standard deviations (SD). For the % methane, only one value was determined in the final month before the sludge was collected

Parameters	CF digester ¹	Athlone 2Sb digester ¹	Athlone 4Sb digester ¹	ST digester ¹
pH	6.93 \pm 0.20	7.11 \pm 0.02	7.10 \pm 0.07	7.03 \pm 0.04
Alkalinity (mg.L ⁻¹)	3748 \pm 985	3241 \pm 106	4203 \pm 507	2523 \pm 173
Total solids (%)	3.42 \pm 1.12	3.36 \pm 0.73	3.23 \pm 0.17	5.48 \pm 0.23
Volatile solids (%)	75.20 \pm 1.3	69.90 \pm 1.62	74.44 \pm 1.48	68.64 \pm 0.96
Temperature (°C)	36.5 \pm 2.1	27.4 \pm 1.8	26.7 \pm 1.1	INA ²
Methane (%)	62.1%	60.3%	INA ²	INA ²

¹ CF - Cape Flats WWTW

Athlone 2Sb - Athlone WWTW

Athlone 4Sb - Athlone WWTW

ST - Simonstown WWTW

² INA – Information not available

Activity tests

It is well known that certain factors influence the operation of a UASB digester. These factors include the activity status of the granular sludge, the presence and concentration of specific carbon sources as well as several other environmental factors. These directly impact on the anaerobic digestion efficiency, which in turn affects biogas production. Thus, the biogas and methane production can be considered one of the most important indicators of operational performance, since methane formation is the final step of the anaerobic digestion process. The specific activity, quality and quantity of the biomass involved influence the efficiency of the wastewater treatment process. Subsequently, the activity of a specific sludge sample can be measured to obtain an overall activity measurement. Thus, for this study the activity of the sludge samples was taken as substrate-dependent biogas or methane production in a standardised biomass system containing specific substrates.

Biogas production and composition of the digested sludges were determined before the batch granulation-enhancement study. Measurements were done after 5, 10 and 25 h and the concentrations of CO₂ and CH₄ were determined gas chromatographically. Biogas production activity was expressed as cumulative production (in mL) over 25 h (Fig. 1) and in terms of tempo of biogas production in mL.h⁻¹ (S_b) (Fig. 2).

From the results it was concluded that of the four sludges that were evaluated, the CF-sludge produced the lowest cumulative biogas volumes (Fig. 1) and was the least active after 25 h (Fig. 2) for all the activity substrates tested. This was followed by the Athlone 2Sb-sludge samples. The Athlone 4Sb-sludge produced the most biogas after 25 h (Fig. 1) in all the test substrates with the exception of the GTM, where the ST-sludge produced more. The ST-sludge performed the second best overall in all the other test substrates. Similar profiles were observed when evaluating the tempo of biogas production in mL.h⁻¹ (S_b), as the Athlone 4Sb-sludge also had the highest S_b after 25 h (Fig. 2).

Methane was produced only by the Athlone 4Sb-sludge. The methane production data were presented as the methane production (in mL) (Fig. 3) as well as the tempo of methane production (S_m) by the Athlone 4Sb-sludge in mL.h⁻¹ (Fig. 4). Since the Athlone 4Sb-sludge was the only sludge in which methane was detected using the techniques described in this study, it could be concluded that this was a digested sludge with a more active methanogenic population (per gram of centrifuged sludge). With the

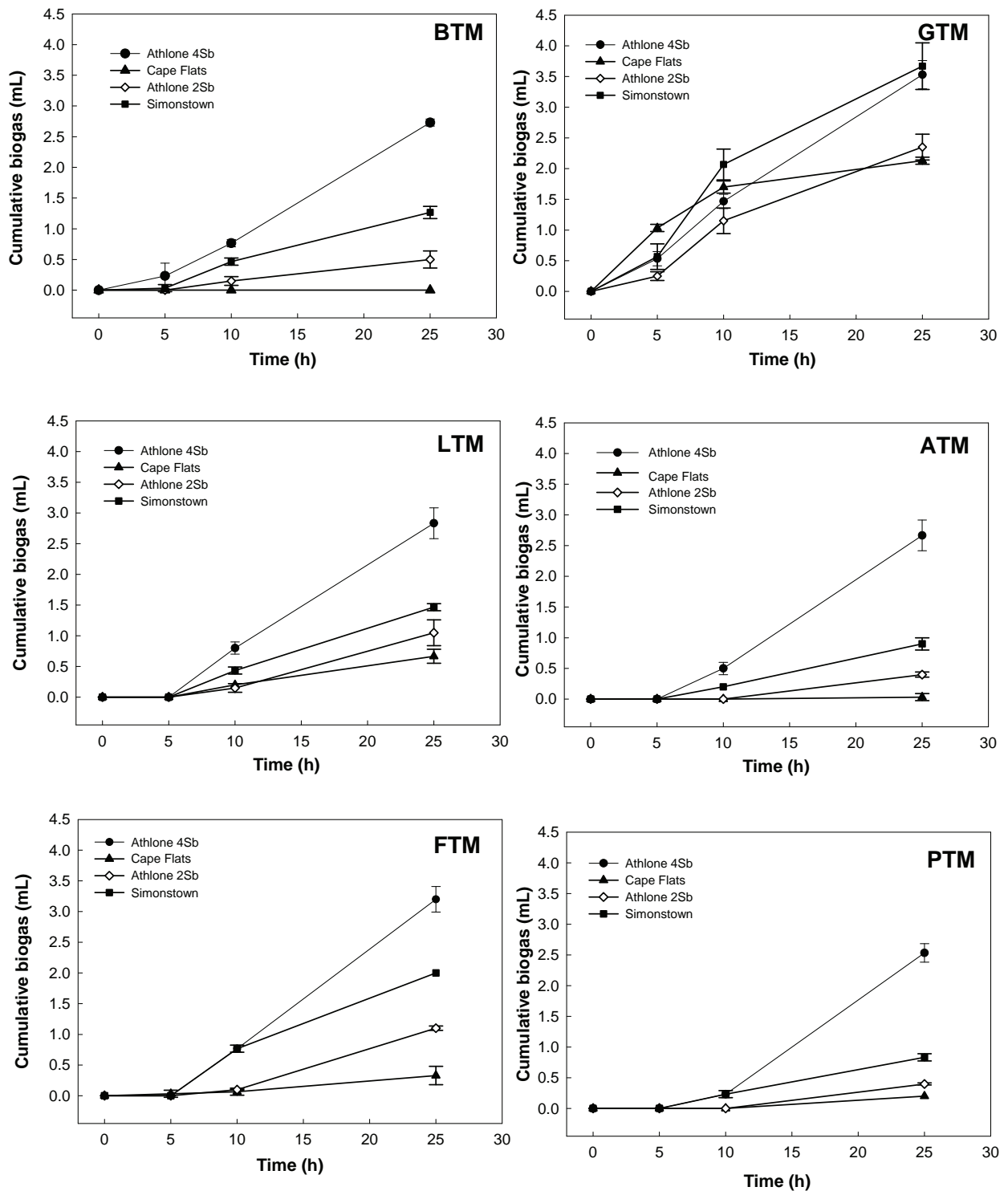


Figure 1 Cumulative biogas production over 25 h by each of the four digested sludges in the six different activity test substrates (Each data point represents triplicate values. The standard deviation was used as the error bar). BTM - basic test medium; GTM - glucose test medium; LTM - lactate test medium; ATM - acetate test medium; FTM - formate test medium; PTM - propionate test medium.

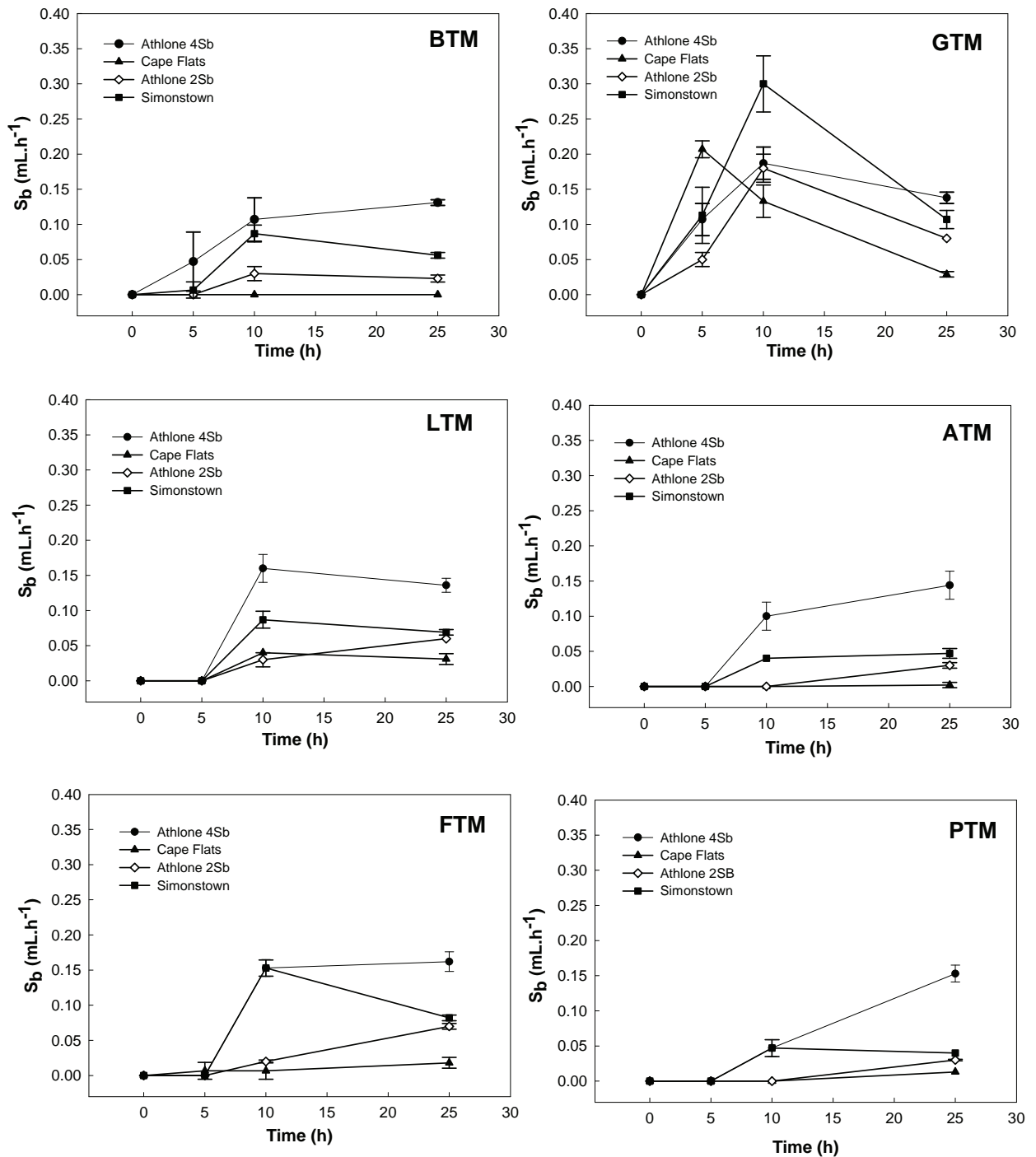


Figure 2 Tempo of biogas production (S_b) in $\text{mL}\cdot\text{h}^{-1}$ achieved by each of the four digester sludges in the six different activity test substrates (Each data point represents triplicate values. The standard deviation was used as the error bar). BTM - basic test medium; GTM - glucose test medium; LTM - lactate test medium; ATM - acetate test medium; FTM - formate test medium; PTM - propionate test medium.

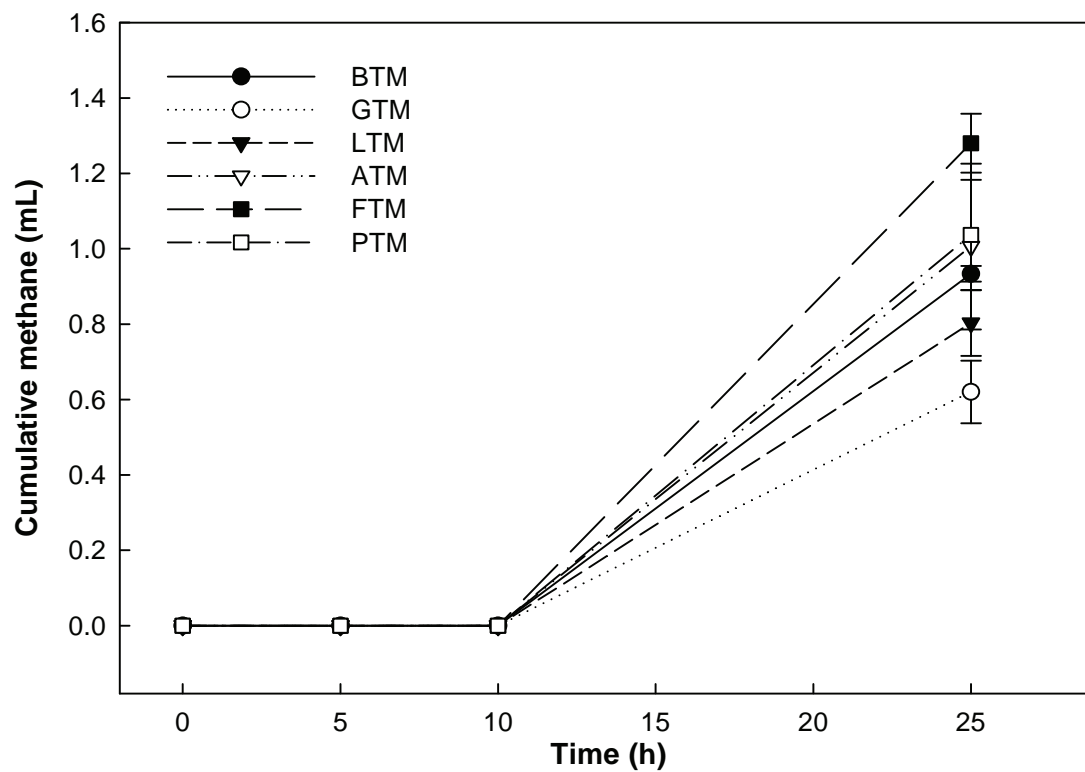


Figure 3 Cumulative methane production achieved by the Athlone 4Sb-sludge during activity testing over 25 h in the six different test media. (Each data point represents triplicate values. The standard deviation was used as the error bar). BTM - basic test medium; GTM - glucose test medium; LTM - lactate test medium; ATM - acetate test medium; FTM - formate test medium; PTM - propionate test medium.

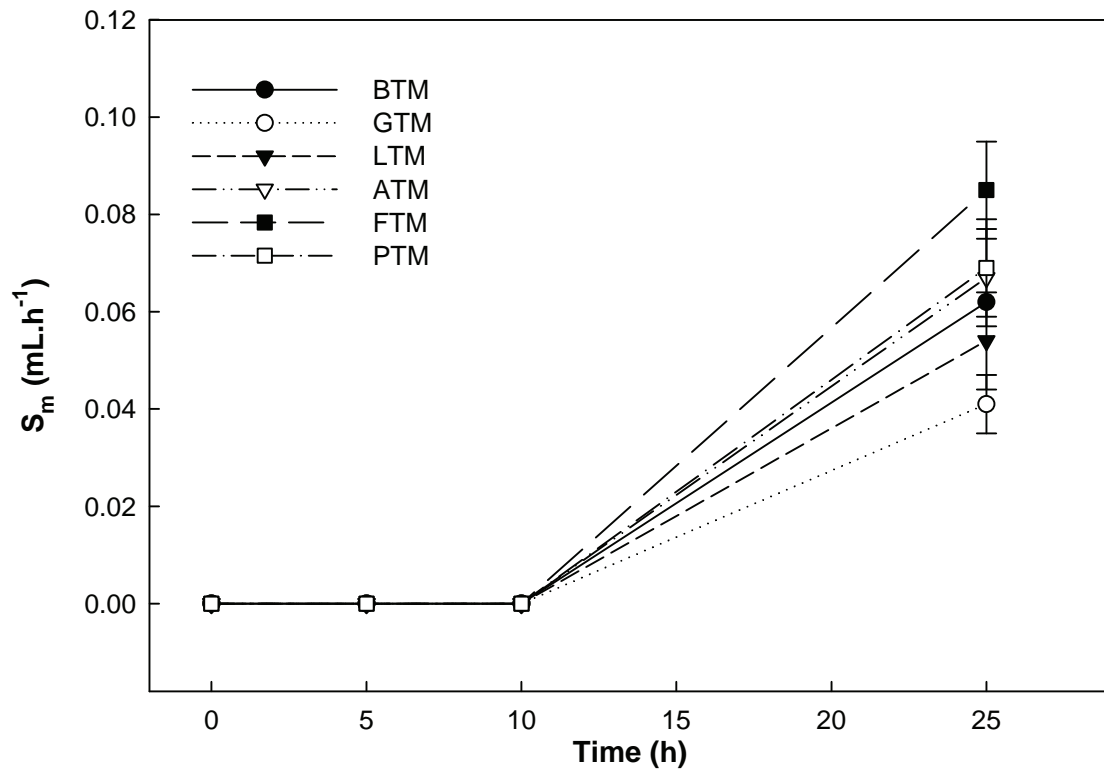


Figure 4 Tempo of methane production (S_m) in $\text{mL}\cdot\text{h}^{-1}$ achieved by the Athlone 4Sb-sludge during of activity testing over 25 h in the six different test media. (Each data point represents triplicate values. The standard deviation was used as the error bar). BTM - basic test medium; GTM - glucose test medium; LTM - lactate test medium; ATM - acetate test medium; FTM - formate test medium; PTM - propionate test medium.

exception of the GTM, all the other test substrates had the same glucose content as the control sample (2 g.L^{-1}). The higher methane production in the formate, propionate and acetate test samples when compared to that of the control sample could be respectively attributed to the presence of active formate-utilising hydrogenotrophic methanogens, propionate-utilising syntrophic acetogenic organisms and acetate-utilising acetoclastic methanogens.

Both GTM and LTM had a lower tempo of methane production (S_m) than BTM (Fig. 4). The GTM and LTM samples of the Athlone 4Sb-sludge also showed a decreased tempo of biogas production (S_b) (Fig. 2) between 10 and 25 h while the BTM samples of the Athlone 4Sb-sludge showed an increase in S_b . These trends were probably due to carbon source depletion. It was assumed that the higher concentration of readily digestible sugars present in GTM and LTM would result in a higher initial growth rate of the fast-growing acidogenic population, which could lead to faster carbon source depletion than in the BTM samples. Since glucose can be metabolised by a larger microbial consortium than lactate, carbon source depletion might also occur more rapidly in the GTM samples than in the LTM samples.

Interestingly, the Athlone 4Sb-sludge was obtained from an anaerobic digester used for the digestion of a mixture of primary settling tank (PST) sludge and activated sludge while the three other sludges were collected from digesters used for the digestion of PST sludge only. Zeikus (1979) reported that methanogens are already present in aerobic activated sludge at slightly lower levels (1×10^8 per gram suspended solids [SS]) than in anaerobic digested sludge (2.5×10^{10} per gram SS). Therefore, it could be that an anaerobic digester treating a mixture of PST sludge and activated sludge could have a higher methane-producing potential than a digester only treating PST sludge, mostly as a result of the methanogenic content of its activated sludge feed. This might explain why methane was only produced by the Athlone 4Sb-sludge and not in the three other digested sludges that originated from digesters treating only PST sludge.

Batch granulation-enhancement study

Each of the four sludge samples was introduced, in duplicate, into LacM and AEM and incubated on a roller-table for 17 d at 35°C . The LacM had a COD of $10\,300 \text{ mg.L}^{-1}$ (to which the lactate carbon source contributed $3\,200 \text{ mg COD per litre}$) and 25% of the

sample volume was replaced daily. The aim of this feeding strategy was to maintain a constant state of 'stress' in a lactate-abundant environment in order to improve ECP production by lactate-utilising populations, thereby enhancing sludge granulation, as was hypothesised by Britz *et al.* (2002). The AEM had a COD of 2 000 mg.L⁻¹ and 25% of the AEM sludge sample volume was replaced daily. The pH profiles (duplicate sets) are presented in Fig. 5 (for the LacM samples) and in Fig. 6 (for the AEM samples).

It was observed from the results presented in Fig. 5 that the LacM samples of Athlone 2Sb, Athlone 4Sb and CF dropped to a minimum pH of about 6.6 by D2, where after the pH profiles stabilised and recovered slightly to reach levels of between pH 6.85 and 6.90 (for Athlone 2Sb and CF) and a pH of 6.75 (for Athlone 4Sb) on the final day of the study. The ST-LacM sample reached its minimum pH of 6.57 only on D4, where after it stabilised and recovered slightly to a final pH of 6.70. The pH profile of ST was still the lowest of all four the sludge profiles. The pH profiles of the LacM samples of the Athlone 4Sb, Athlone 2Sb, CF and ST sludges were fairly close and were all within 0.2 pH units throughout the study.

More prominent differences were observed in the pH profiles of the sludges incubated in AEM, especially for the PST/activated sludge-fed Athlone 4Sb digester sludge when compared to the PST-fed ST, Athlone 2Sb and CF digester sludges (Fig. 6). The pH profile of Athlone 4Sb samples dropped to a minimum of about 6.4 on D5, after which it stabilised. All the PST-fed sludges eventually stabilised at levels below 6.3 with the ST samples reaching the lowest levels (between 6.0 and 6.1). In an attempt to stabilise the decreasing pH levels, 200 mg.L⁻¹ of CaCO₃ was added to the following samples: ST on D7 and Athlone 2Sb on D10. In both instances the pH increased with 0.2 pH units the following day, but the higher pH-levels were not sustainable. This was probably due to the high concentration of readily degradable carbohydrates naturally present in AEM. In terms of pH control the choice of AEM as substrate might thus be problematic if applied on a larger scale.

Biofilms on the inside of the AEM-fed containers were also observed for all four the sludges at the end of the experiment. To increase granulation it would be recommended that a way be found to decrease biofilm formation. This could possibly be accomplished by inserting baffles in the containers used for incubation during the batch granulation-enhancement study.

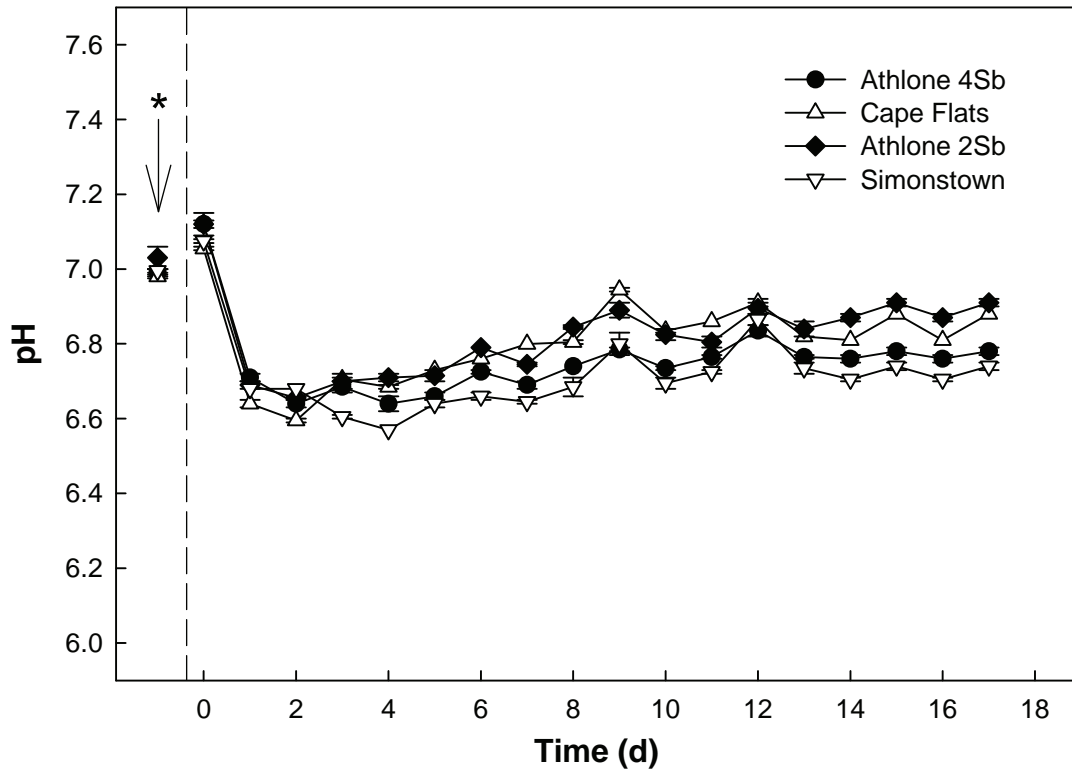


Figure 5 Changes in pH values of the four different sludges during the 17 d batch granulation-enhancement incubation period in LacM. (Each data point represents duplicate values. The standard error was used as the error bar).
* pH of LacM before addition of sludge

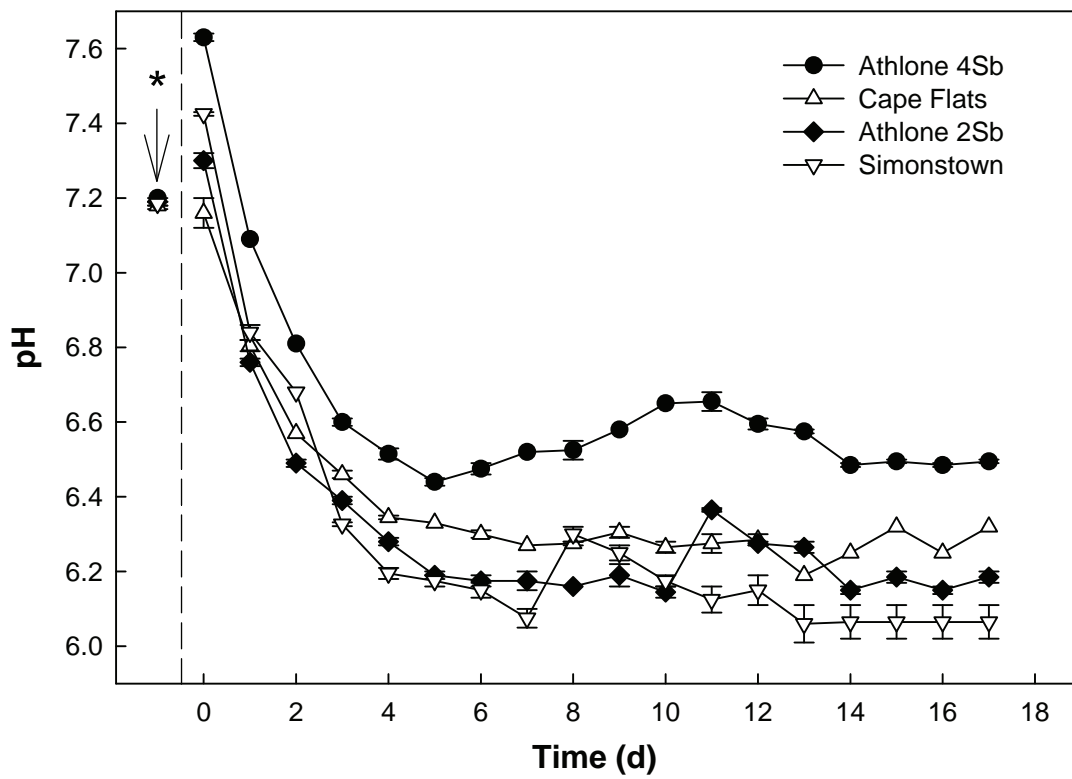


Figure 6 Changes in pH values of the four different sludges during the 17 d batch granulation-enhancement incubation period in AEM. (Each data point represents duplicate values. The standard error was used as the error bar).
* pH of AEM before addition of sludge

Size distribution determinations

Size distributions of the sludge samples at the beginning, D0, and the end, D17, of the batch granulation-enhancement study were determined using the sieve-based fractionation method of Laguna *et al.* (1999). For the purpose of this discussion 'retained VSS' referred to all the particles large enough to be retained on any of the sieves (i.e. all VSS particles $\geq 0.25 \text{ mm}^2$) while 'total VSS' referred to the amount of VSS present in each sludge-liquor sample before it was fractionated into different sizes (i.e. all VSS particles $\geq 0 \text{ mm}^2$).

In Fig. 7, the amount of VSS retained on all the sieves (i.e. VSS particles $\geq 0.25 \text{ mm}^2$) was compared to the total amount of VSS present in the individual diluted sludge samples. When considering only total VSS, it is clear that the ST samples had the highest total VSS on D0. It was also the only sludge in which the total VSS did not decrease over 17 d in either the LacM or the AEM samples when measured on the final day (D17). For both the Athlone 4Sb and CF sludges the total VSS of the LacM samples collected on D17 decreased while increases in the values for the AEM samples were observed. The opposite was observed for the Athlone 2Sb LacM and AEM samples.

The values for the VSS retained on all the sieves (i.e. VSS particles $\geq 0.25 \text{ mm}^2$) did not reflect the same trends observed for the total VSS, as discussed in the previous paragraph. No clear decreases in the VSS $\geq 0.25 \text{ mm}^2$ of any of the sludge samples (incubated in LacM and AEM) on D17 of the study were observed. Increases, however slight, were observed for all samples with the ST samples showing the greatest increases (more than 100%) for both the LacM and AEM samples, with the VSS $\geq 0.25 \text{ mm}^2$ of the LacM samples being slightly more than that of the AEM samples.

In Fig. 8 the amount of VSS $\geq 0.25 \text{ mm}^2$ for the duplicate samples of the four different sludges on each individual sieve is presented. From the data it was concluded that the largest part of the VSS $\geq 0.25 \text{ mm}^2$ on both D0 and D17 was between 0.25 mm^2 and 0.49 mm^2 for all the sludges.

The influence that the solids and COD content of the original substrate could have had on the VSS content of each of the test samples should also be considered. The LacM was a clear substrate with no suspended matter while the AEM contained a fair amount of fruit fibres that could also have contributed to the higher VSS content of all the AEM samples. The substrates also differed in two other respects: firstly, the

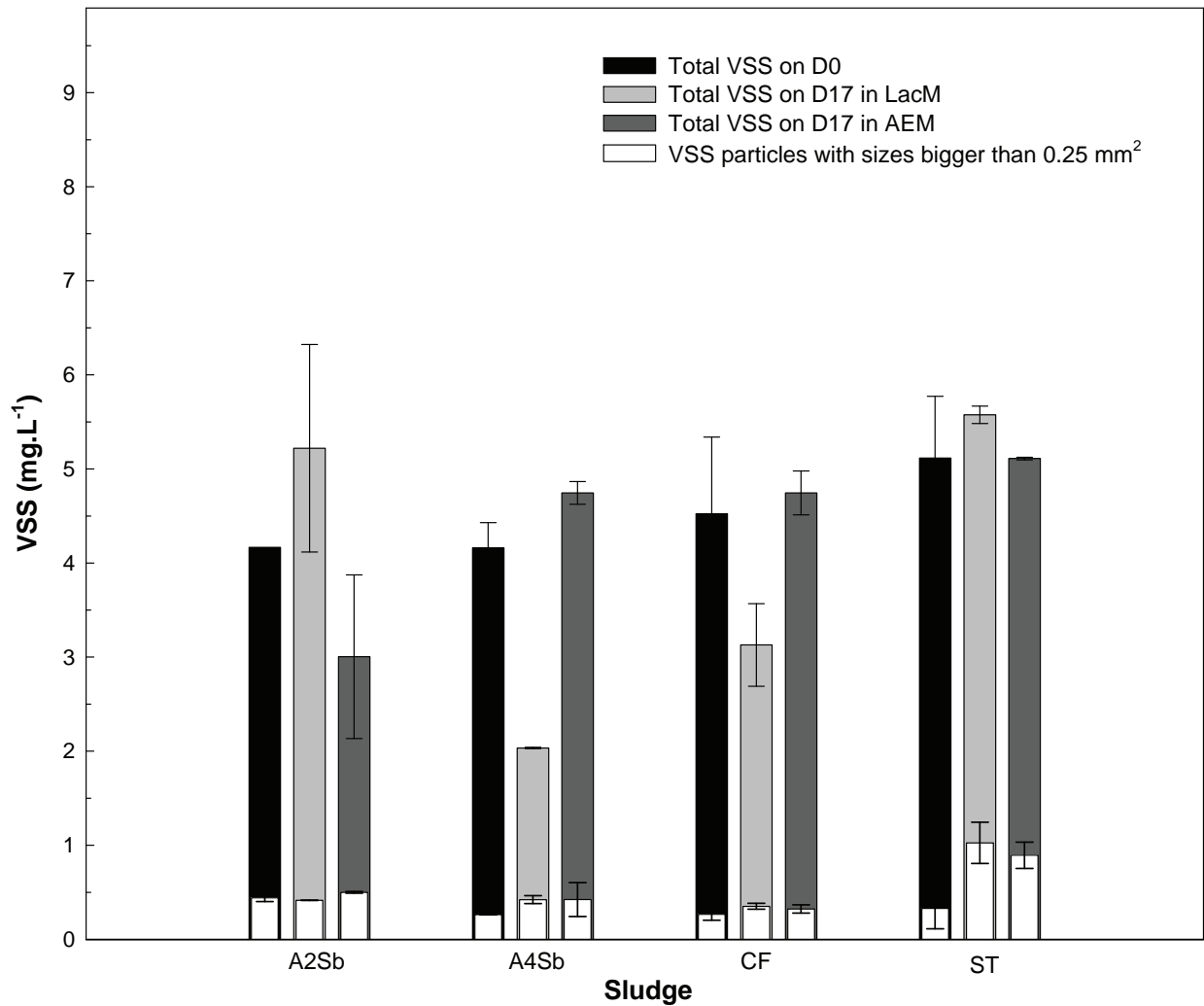


Figure 7 Total VSS (mg.L^{-1}) as well as all VSS particles retained on sieves (VSS content $\geq 0.25 \text{ mm}^2$) measured for the different sludges on day 0 (D0) and on day 17 (D17) of the batch granulation-enhancement study in both the LacM and the AEM samples. (Each data point represents duplicate values. The standard error was used as the error bar).

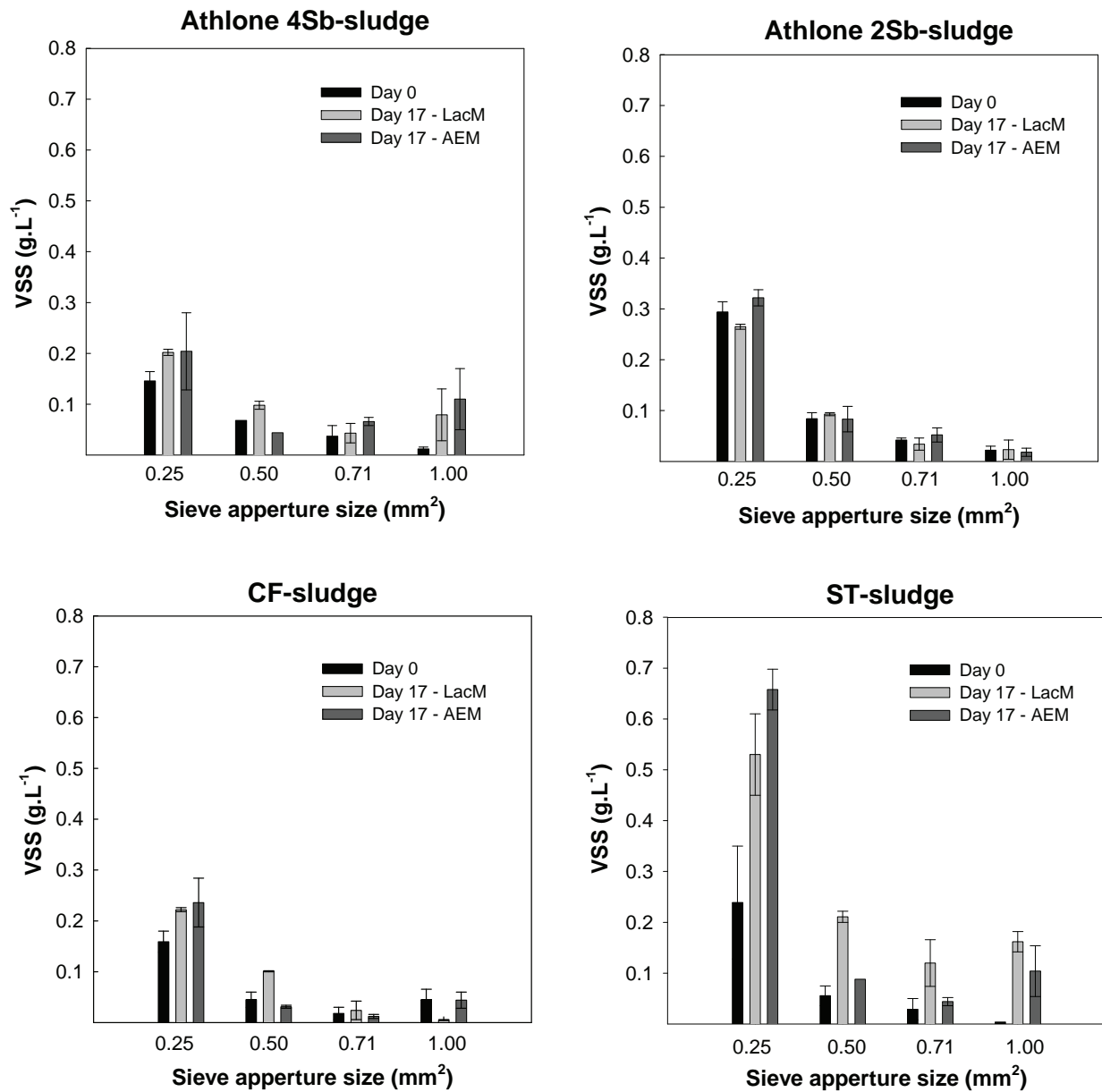


Figure 8 Size distribution fractions of the VSS content ≥ 0.25 mm² as determined for the four sludges before and after the batch granulation-enhancement study. (Each data point represents duplicate values. The standard error was used as the error bar).

LacM had a much higher COD content than the AEM, and secondly, the sugar content of the AEM (mostly fructose) had a higher degradability than the LacM (which contained only lactate). Since biogas production was not measured during this particular batch granulation-enhancement study, it is unknown how these two factors would influence biogas production as well as the resultant sludge washout (which might increase as a result of high biogas production, resulting in a lower VSS content). In order to determine the efficiency of the anaerobic digestion process during batch granulation-enhancement, biogas production and composition values were determined in all granulation-enhancement experiments conducted as part of later chapters of this dissertation.

Another factor that might also have influenced the size distribution results is the sludge activity of the LacM samples compared to the AEM samples. From the pH graphs (Figs. 5 and 6) it was clear that during the experiment the AEM samples had lower pH values than the LacM samples. As discussed previously, the AEM samples of the ST-sludge in particular had pH values below 6.2 for the largest part of the experiment. According to Gerardi (2003), pH values below 6.2 can severely restrict general methanogenic activity. This, in turn, might have resulted in lower biogas production. Higher washout, particularly of smaller sludge particles, could have occurred if the sludge was more active, and vice versa. Therefore, it could be that the VSS retention of AEM sludge samples in general (and the AEM samples of ST-sludge in particular) was positively influenced for two reasons: Their overall activity was lower, which resulted in a lower sludge washout, and the high fibre content of the substrate replaced each day of the experiment might also have increased the VSS content during the course of the experiment.

Mineral analysis

The levels of certain elements present in the four digested sludges were determined with ICP analysis before the batch granulation-enhancement experiment. The data are presented in Table 6.

Both Ca^{2+} and Mg^{2+} ions have been indicated as contributing to anaerobic sludge retention and granulation when present at specific levels. The positive influence of Ca^{2+} on granulation was reported to be at levels of 150 mg.L^{-1} (Hulshoff Pol *et al.*, 1983), between 100 and 200 mg.L^{-1} (Mahoney *et al.*, 1987) and between 150 and 300 mg.L^{-1} for a constant COD concentration of $4\,000 \text{ mg.L}^{-1}$ (Yu *et al.*, 2001). Mg^{2+} ions have been

Table 6 Mineral composition (average of duplicate samples) (in mg.L⁻¹) of the four sludges, as determined by ICP spectrometry

Sludge origin	Mineral (mg.L ⁻¹)					
	Ca	Fe	K	Mg	Na	Ni
Athlone 4Sb digester	56.65	10.05	186.00	5.90	164.25	0.25
ST digester	122.15	1.05	65.10	30.35	151.45	0
CF digester	43.00	0.88	187.05	8.00	108.90	0.15
Athlone 2Sb digester	226.95	2.15	71.95	28.40	164.75	0.15

shown to contribute to granulation at levels between 12 and 240 mg.L⁻¹ (or 0.5 and 10 mM) (Schmidt & Ahring, 1993). Interestingly, the two sludges (ST and Athlone 2Sb) with concentrations of these two divalent ions (Ca²⁺ and Mg²⁺) within the reported contributing ranges are also the only sludges where there were no significant losses in total VSS content (particle size 0 – 1.0 mm²) from D0 to D17 of the batch granulation-enhancement study in the LacM samples (Fig. 7). Although no significant increase in the retained VSS (particle size ≥0.25 mm²) for the Athlone 2Sb-sludge was observed, the significant increase in the retained VSS bigger than 0.25 mm² for the ST-sludge has already been discussed in the previous section.

DGGE analysis of *Archaea* populations

The *Archaea* population composition profile of each of the four sludges was evaluated before the batch granulation-enhancement study (Fig. 9) and on D17 of the batch granulation-enhancement study (Figs. 10 and 11). These fingerprints were also compared with the archaeal populations of four types of mature UASB granules obtained from industrial UASB digesters as well as with pure methanogenic strains (Table 3). Reproducibility of the DGGE results was also investigated by not only comparing duplicate DNA isolations but also by comparing repetitive initial PCR and dgge-PCR amplifications (see Chapter 5 of this dissertation).

It was concluded from the fingerprints in Fig. 9 that *Methanosaeta concilii* was present in all four of the granules tested as well as in the D0 sludge samples of ST, Athlone 4Sb (both in the sludge and in the aggregates found in the sludge) and Athlone 2Sb (only in the aggregates found in the sludge). *Methanobacterium formicicum* was detected only in the mature UASB granule types P and L, with a very faint band in the type B granules. The two *Methanosarcina* strains were not found to be present in any of the four granule types screened in this study. In contrast, *Methanosarcina mazei* was present in both the Athlone sludges (Athlone 2Sb and Athlone 4Sb). The presence of *Methanosaeta thermophila* was also observed in two of the granules (types P and L) as well as in the sludges from ST, CF and Athlone 2Sb. The two prominent bands present in the profile of the type F granules (in the region between *Methanobacterium formicicum* and *Methanosaeta concilii*) did not correspond with any of the bands present in the selected pure cultures or the other granule and sludge profiles.

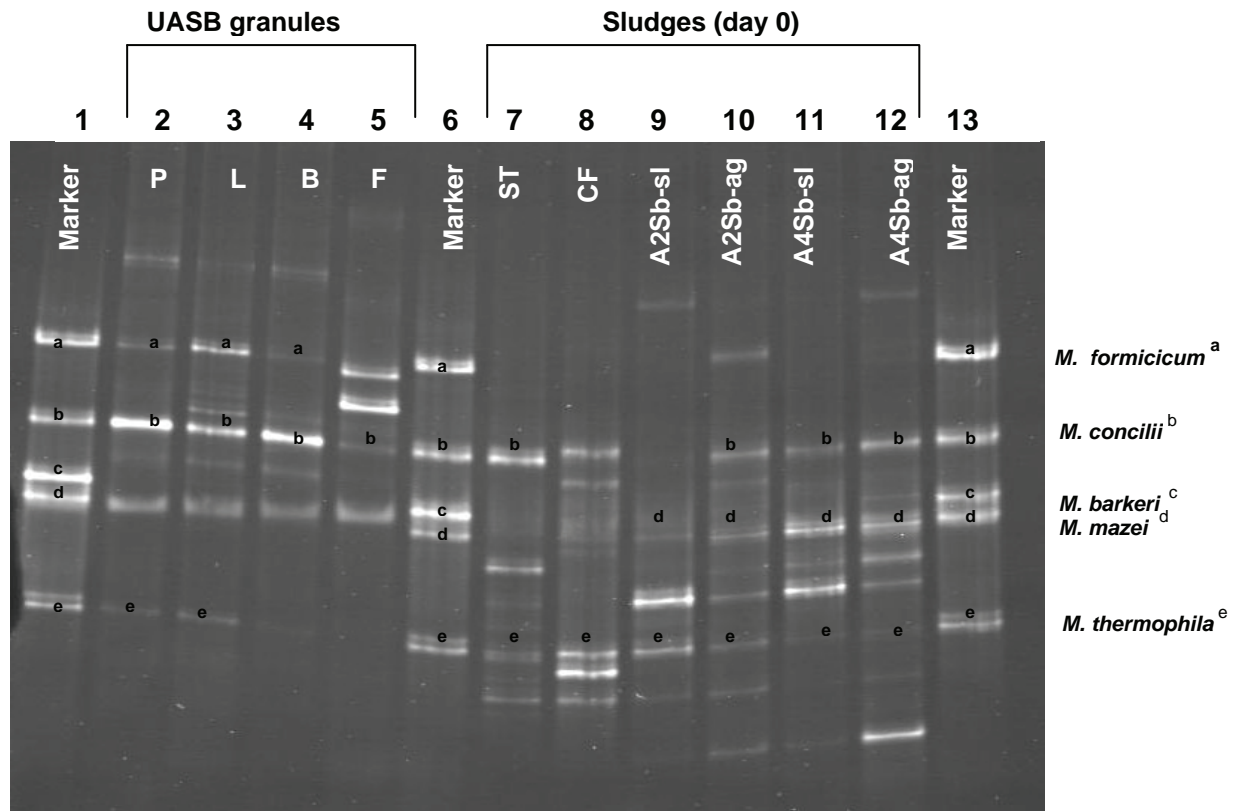


Figure 9 DGGE profiles of mature UASB granules from industrial scale UASB reactors treating different effluents [P - apple pomace (lane 2); L - lye (lane 3); B - brewery (lane 4); F - fruit juice (lane 5)] and the four digested sludges [CF (lane 7); ST (lane 8); Athlone 2Sb (A2Sb) (lanes 9-sludge, 10-aggregate)*; and Athlone 4Sb (A4Sb) (lanes 11-sludge, 12-aggregate)*] before the start of the batch granulation-enhancement study. The methanogenic marker was loaded in lanes 1, 6 and 13.

* Small aggregates were present in the digested sludge from the Athlone 2Sb and Athlone 4Sb-digesters. For PCR-based DGGE analysis DNA was extracted from the "total sludge" (which was a centrifuged mixture of small aggregates and suspended matter, marked 'sl'), as well as from the washed aggregates (marked 'ag').

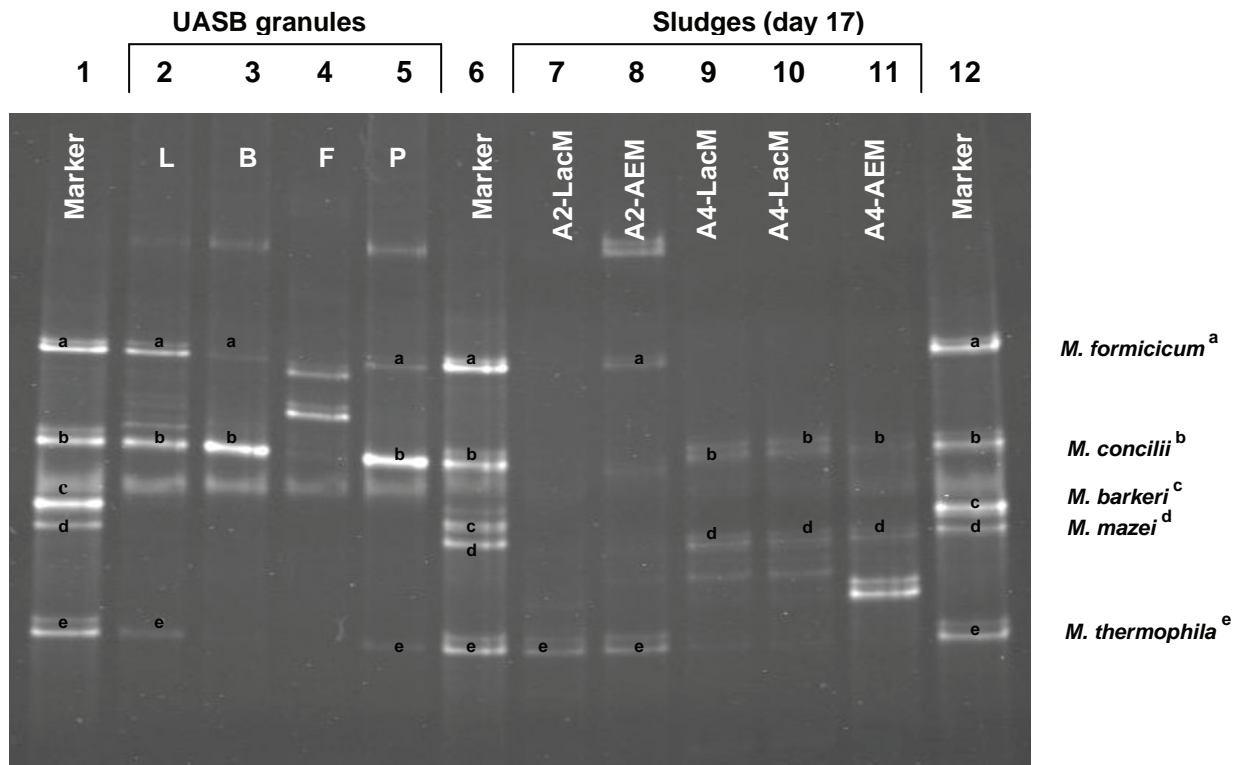


Figure 10 DGGE profiles of mature UASB granules from industrial scale UASB reactors treating different effluents [L - lye (lane 2), B - brewery (lane 3), F - fruit juice (lane 4), P - apple pomace (lane 5)] and the Athlone 2Sb-sludge (A2-samples) and the Athlone 4Sb-sludge (A4-samples) on day 17 of the batch granulation-enhancement study in either LacM [A2-LacM (lane 7), A4-LacM (lanes 9 and 10)] or AEM [A2-AEM (lane 8), A4-AEM (lane 11)]. The methanogenic marker was loaded in lanes 1, 6 and 12.

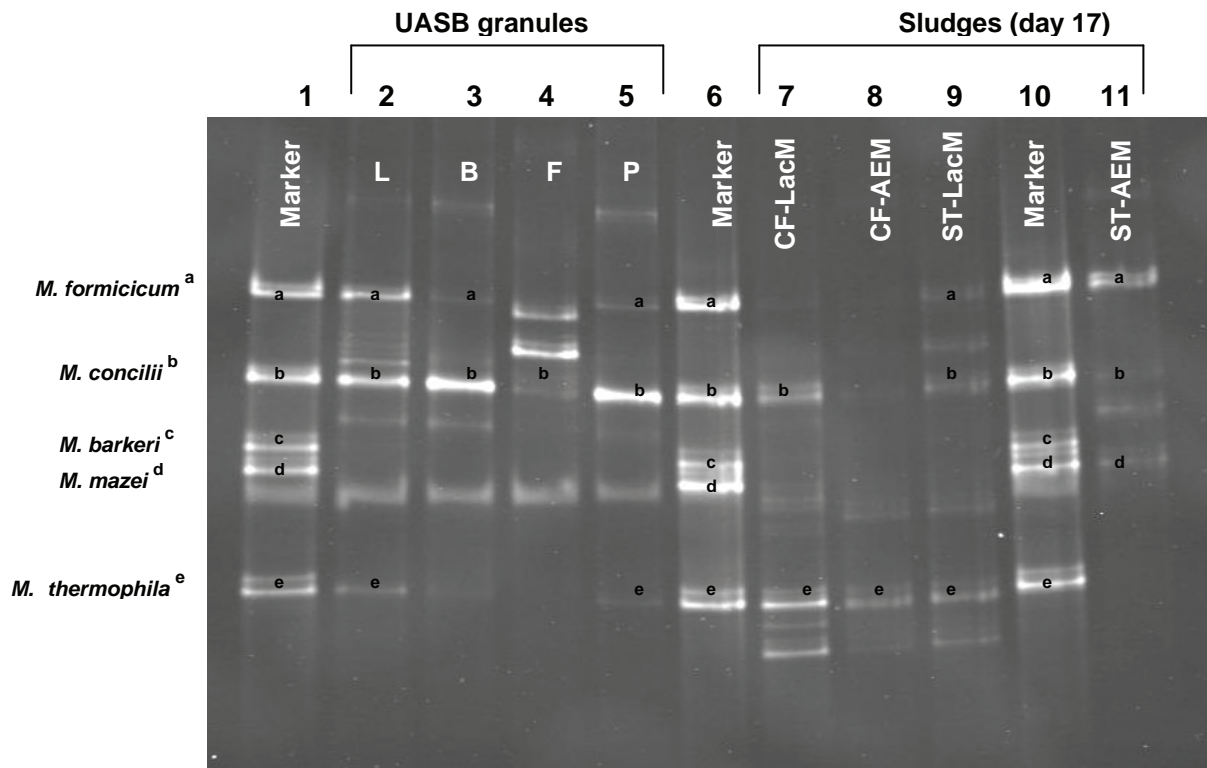


Figure 11 DGGE profiles of mature UASB granules from industrial scale UASB reactors treating different effluents [L - lye (lane 2), B - brewery (lane 3), F - fruit juice (lane 4), P - pomace (lane 5) and the CF-sludge samples and the ST-sludge samples on day 17 of the batch granulation-enhancement study in either LacM [CF-LacM (lane 7), ST-LacM (lane 9)] or AEM [CF-AEM (lane 8), ST-AEM (lane 11)]. The methanogenic marker was loaded in lanes 1, 6 and 10.

It was interesting to observe that more complex banding patterns were present in the sludge fingerprints than in the fingerprints of the mature UASB granules. In the two digesters in which small (pinpoint) aggregates were present in the digested sludge (in Athlone 4Sb and Athlone 2Sb), it was found that the archaeal fingerprints of the washed aggregates isolated from the digested sludge of Athlone 4Sb and Athlone 2Sb (marked as A4Sb-ag and A2Sb-ag in Fig. 9) were more complex (i.e. had more bands) than the 'total sludge' of Athlone 4Sb and Athlone 2Sb (which was a centrifuged mixture of small aggregates and suspended matter) (A4Sb-sl and A2Sb-sl in Fig. 9).

The advantages that aggregation holds for the bacteria present in anaerobic digesters have well been documented and include reasons such as strategic positioning within the aggregate for improved substrate supply and metabolite removal, as well as an increased resistance to detrimental environmental factors (Bae & Lee, 1999; Fang, 2000). Methanogens are especially sensitive to pH fluctuations and chemical toxicity, and since these organisms are mostly located near the centre of mature anaerobic granules, they will be better protected when present in granular form rather than just in suspended liquid (Fang, 2000). It could be that the more complex DGGE fingerprints of the aggregate DNA of the Athlone 2Sb and Athlone 4Sb sludges were the result of a larger archaeal variety in the DNA isolated from the washed small aggregates compared to the 'total sludge' DNA samples.

Comparison of the DGGE fingerprints of the digested sludges before (Fig. 9) and after (Fig. 10 and Fig. 11) the batch granulation-enhancement study showed that definite archaeal population shifts occurred during the 17 d incubation of the sludges in the LacM and AEM. The appearance and disappearance of bands indicated species concentration increased above or decreased below the PCR-detection limits. *Methanosaeta concilii* was still present in all the sludges with the exception of the Athlone 2Sb samples, *Methanosarcina mazei* was still present in the Athlone 4Sb samples and *Methanosaeta thermophila* was still present in all samples except the Athlone 4Sb samples (LacM and AEM) and the ST samples incubated in AEM.

The most noticeable difference between the D0 and the D17 samples was the appearance of *Methanobacterium formicicum* in the D17 samples of the ST sludge, which also coincided with the appearance of an unknown band in the region between *Methanobacterium formicicum* and *Methanosaeta concilii* in the ST-LacM fingerprint. The latter unidentified band can also be seen in the type F granule fingerprint, but further speculation as to its possible role in the anaerobic community was excluded from

this discussion since it was not within the scope of this investigation to identify the unknown organisms represented by these bands. The fact that *Methanobacterium formicicum* was present at PCR-detectable levels after the batch granulation-enhancement in the ST-sludge might be significant. It has been reported that pure cultures of *Methanobacterium formicicum* exhibited aggregate-forming behaviour in the presence of syntrophic butyrate-degrading cultures and also with propionate-degrading syntrophic cultures in the presence of *Methanosaeta* spp. (Wu *et al.*, 1996). The presence of *Methanobacterium formicicum* at the end of the batch granulation-enhancement study might thus have contributed to the ST-sludge having the highest increase in VSS $\geq 0.25 \text{ mm}^2$ (Fig. 7) when compared to other sludges. The question that arises now is whether *Methanobacterium formicicum* could contribute to granulation in the ST-sludge during normal UASB start-up if there is no prior population enrichment, as was observed during batch granulation-enhancement.

Differences between the D17 LacM and AEM samples were also apparent. The band intensity of *M. concilii* was much weaker in the AEM samples of the Athlone 4Sb, CF and ST sludges than in the LacM samples. It was stated before that since DGGE band intensities could be influenced by the amount of DNA template that was available during PCR amplification, band intensity might also be proportional to the initial species concentration (Øvreas *et al.*, 1997; Gelsomino *et al.*, 1999). During the optimisation of the PCR method, reproducibility of the results was confirmed. The primers that were chosen for this study were also originally designed to reduce the chance of chimera formation (Øvreas *et al.*, 1997). Should any bias in the amplification process exist it could well be constant. Appearance and disappearance of bands in the DGGE fingerprints might, therefore, be considered to give an indication of the original shifts in the archaeal community structure (Øvreas *et al.*, 1997). One of the differences between the LacM and the AEM samples was the difference in pH during the batch granulation-enhancement study. Since methanogens are known to be pH sensitive, differences in band intensity might be attributed to the inhibition of methanogen growth under certain less favourable conditions. It has been reported that the presence of *M. concilii* is crucial to the anaerobic granulation process and, therefore, it is recommended that a substrate such as LacM (of which the pH is easier to control) be used instead of AEM in future batch granulation-enhancement studies.

Propionibacterium-specific PCR

Propionibacterium strains have previously been isolated from anaerobic processes (Qatibi *et al.*, 1990; Riedel & Britz, 1993) and ECP-producing propionibacteria have also been indicated to play a role in the granulation process in anaerobic bioreactors (Britz *et al.*, 2002). Furthermore, pure cultures of ECP-producing *Propionibacterium* strains have also been manipulated to produce bacterial aggregates in LacM and AEM (Van Schalkwyk *et al.*, 2003) (Chapter 3). The possible presence of propionibacteria in the four sludge samples used in this study might, therefore, be of importance when comparing the granulation potential of the four digested sludges. *Propionibacterium*-specific PCR primers (Prop 1 and Prop 2) were thus used to determine whether *Propionibacterium* species were present in the mature UASB granules tested in this study as well as in the four digested sludge samples on D0 and D17 of the batch granulation-enhancement experiment.

The concentration of *Propionibacterium* DNA present in the total sludge DNA was unknown and, therefore, different volumes of isolated DNA (0.5, 1.0 and 2.0 µl) were tested. A positive control (*Propionibacterium shermanii* culture) and negative control (Millipore water) were also included in all PCR reactions. After visualisation of PCR products on agarose gels under UV light (Vilber Lourmat), the presence of 720 bp fragments in the PCR samples was recorded either as + (strong bands) or (+) (weak bands). An example of the intensity of + and (+) bands is presented in Fig. 12. All the results observed are summarised in Table 7. If a positive result was obtained for any of the three DNA volumes tested, neither of the other two volumes was tested.

The original PCR-detection method was developed for the determination of added 'classical' *Propionibacterium* strains to fermented milk products (Schoeman, 2001). The method's sensitivity to recognise 'cutaneous' *Propionibacterium* gene sequences was never investigated because cutaneous strains are not indigenous to fermented milk products. It was thus necessary to determine whether the method of Schoeman (2001) could be used to distinguish between classical and cutaneous *Propionibacterium* strains. The PCR method used for the detection of *Propionibacterium* strains in both the D0 and D17 sludge samples was thus also applied to the *Propionibacterium* strains listed in Table 4. The results are given in Fig. 13 and it was concluded that the PCR method amplified both classical and cutaneous gene sequences.

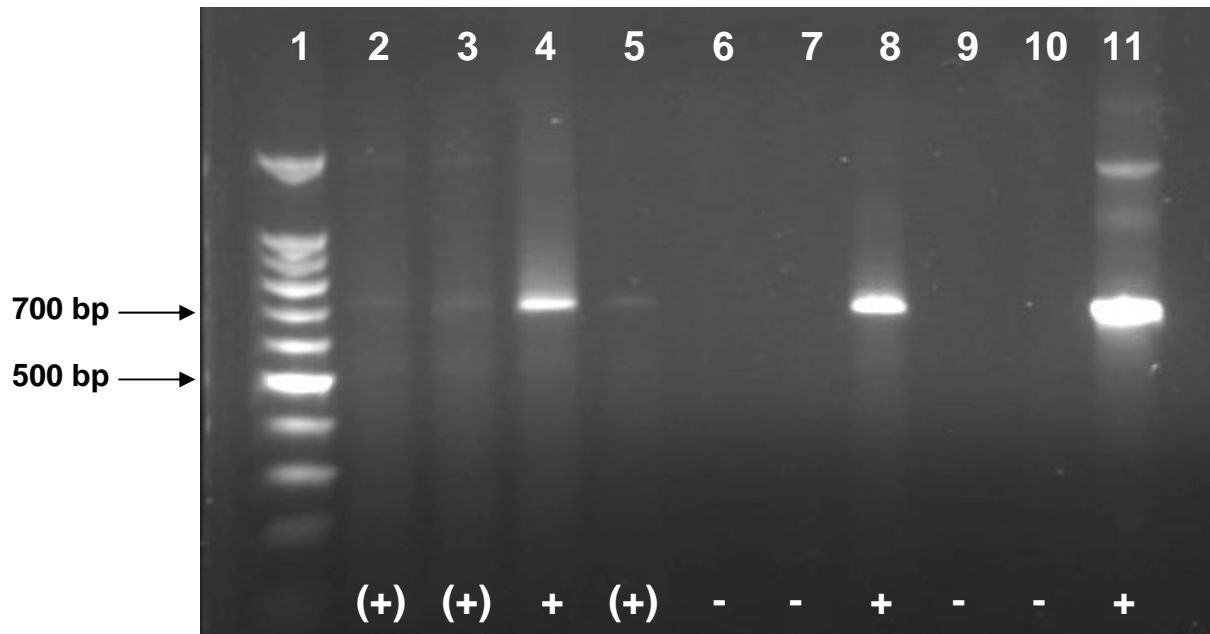


Figure 12 A 1% (m/v) agarose gel showing the 720 bp PCR fragments that indicate the presence of *Propionibacterium* 16S rRNA gene sequences in the isolated DNA of some of the sludge samples on D17 of the batch granulation-enhancement experiment.

Lanes 1, 100 bp ladder (Promega); 2, Simonstown (LacM 1); 3, Simonstown (LacM 2); 4, Cape Flats (AEM 2); 5, Cape Flats (AEM 1); 6, Simonstown (AEM 2); 7, negative control; 8, Simonstown (AEM 1); 9, Cape Flats (LacM 2); 10, Cape Flats (LacM 1); 11, positive control.

[“+” indicates strong bands, “(+)” indicates weak bands and “-” indicates that *Propionibacterium* is not present at detectable levels.]

Table 7 Summary of results after *Propionibacterium*-specific PCR detection of DNA from mature UASB granules and from the four digested sludges (on D0 and D17 of the batch granulation-enhancement study). Duplicate DNA samples were isolated from each sludge before PCR analysis and are referred to as either '1' or '2'

Mature anaerobic digester granules			
Granule type	PCR detection	Granule type	PCR detection
Brewery	(+)	Brewery 2	+
Sewage	+	Fruit juice 2	+
Fruit juice	+		
Digested sludges on D0			
Sludge type	PCR detection	Sludge type	PCR detection
Simonstown 1	-	Athlone 2Sb aggregate 1	-
Simonstown 2	-	Athlone 2Sb aggregate 2	-
Cape Flats 1	(+)	Athlone 2Sb sludge 1	-
Cape Flats 2	+	Athlone 2Sb sludge 2	-
Athlone 4Sb aggregate 1	-		
Athlone 4Sb aggregate 2	-		
Athlone 4Sb sludge 1	+		
Athlone 4Sb sludge 2	+		
Digested sludges on D17 (incubated in either LacM or AEM)			
Sludge type	PCR detection	Sludge type	PCR detection
Simonstown LacM 1	(+)	Athlone 2Sb LacM 1	-
Simonstown LacM 2	(+)	Athlone 2Sb LacM 2	-
Simonstown AEM 1	+	Athlone 2Sb AEM 1	(+)
Simonstown AEM 2	(+)	Athlone 2Sb AEM 2	-
Cape Flats LacM 1	-	Athlone 4Sb LacM 1	-
Cape Flats LacM 2	-	Athlone 4Sb LacM 2	-
Cape Flats AEM 1	(+)	Athlone 4Sb AEM 1	-
Cape Flats AEM 2	+	Athlone 4Sb AEM 2	+

[“+” indicates strong bands; “(+)” indicates weak bands; “-” indicates *Propionibacterium* not present at detectable levels.]

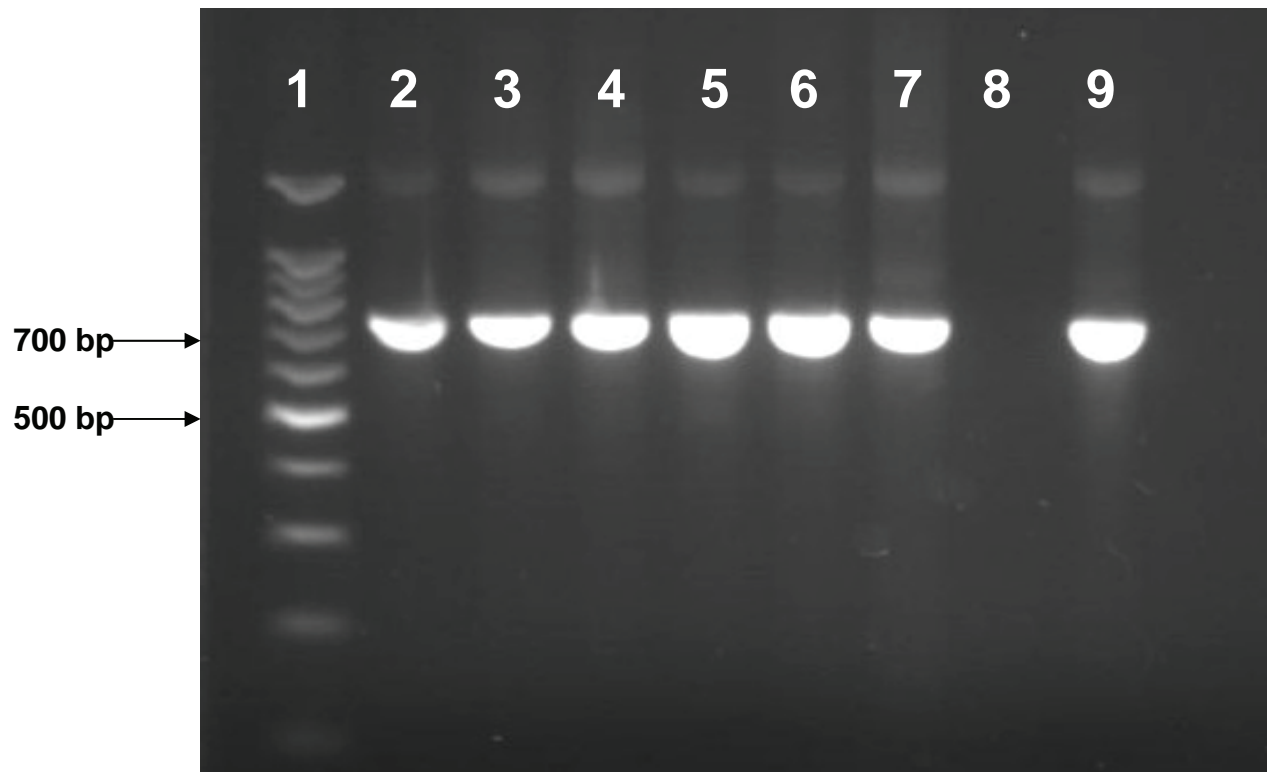


Figure 13 A 1% (m/v) agarose gel showing the 720 bp fragments generated from part of the 16S rRNA gene sequences of the selected propionibacterial strains. **Lanes 1**, 100 bp ladder (Promega); **2**, *Propionibacterium freudenreichii* ss. *shermanii* 434-FS; **3**, *Propionibacterium freudenreichii* ss. *freudenreichii* ATCC 6207; **4**, *Propionibacterium acidipropionici* ATCC 25562; **5**, *Propionibacterium jensenii* DSM 20535; **6**, *Propionibacterium parvum* ATCC 11829; **7**, *Propionibacterium acnes* ATCC 6919; **8**, negative control; **9**, *Propionibacterium thoenii* NCFB 568.

It was also concluded from the results in Table 7 that all the DNA samples isolated from the mature granules contained *Propionibacterium* gene sequences. This was expected since *Propionibacterium* species were previously isolated from anaerobic environments using traditional bacteriological culturing methods (Riedel & Britz, 1993). The presence of these gene sequences appeared to be unaffected by substrate composition if the various granule sources are considered. It could be that in mature UASB granules, propionibacteria are part of the more permanent granule population and are not directly dependent on the digester substrate.

Only two of the four digested sludges tested (Athlone 4Sb and CF) contained *Propionibacterium* gene sequences at detectable levels before the batch granulation experiment (Table 7). After batch granulation *Propionibacterium* gene sequences were detected in the AEM samples of all four sludges. The only LacM samples that tested positive for *Propionibacterium* on D17 were the LacM samples of the ST-sludge (which did not initially have *Propionibacterium* gene sequences at detectable levels). The propionibacteria were thus successfully enhanced in the ST sludge and might have contributed to the increased VSS retention observed in the ST test samples after the batch granulation-enhancement study.

Conclusions

It can be concluded that of the four sludges tested, both the Athlone 4Sb-sludge and the ST-sludge might serve as good UASB reactor start-up inocula for different reasons. From the activity test results it was concluded that the Athlone 4Sb-sludge had the highest biogas-production potential and was the only sludge that exhibited measurable methanogenic activity. Methane production was observed in all six the test media but was the highest in the basic test medium (BTM) supplemented with formate (FTM samples), followed by the test media containing propionate (PTM samples) and acetate (ATM samples). Formate and acetate conversion into methane are single-step processes that only involve methane-producing bacteria. Formate would typically be converted by hydrogenotrophic methanogens (such as *Methanobacterium formicicum*) while acetate can be utilised by acetoclastic methanogens, which only include members from the *Methanosaeta* and *Methanosarcina* genera (Fang *et al.*, 1995a). All the hydrogenotrophic methanogens that can degrade formate can also convert H₂ and CO₂ to methane (Boon & Whitman, 1988). The higher activity observed in the supplemented

test media (the FTM and ATM samples) in comparison with the control samples (the BTM samples) could thus be attributed only to methanogen activity. Therefore, it was concluded that an active methanogen population, consisting of both hydrogenotrophic and acetoclastic methanogens, was present in the Athlone 4Sb-sludge. Active methanogens are essential for the anaerobic digestion process to function efficiently. About a third of the methane produced in nature is derived from CO₂ reduction with electrons derived from H₂ and formate while two-thirds of all methane produced are derived from acetate (Ferry, 1992). Therefore, the overall rate of conversion of substrate to methane during anaerobic digestion could be limited by the degradation of acetate (Zinder, 1988; Ferry, 1992). The presence of active acetate-consuming or acetoclastic methanogens is, therefore, principally important to anaerobic digestion efficiency. *Methanosaeta* spp. in particular are also considered essential for granular nuclei formation during the natural anaerobic granulation process (Fukuzaki *et al.*, 1991a; Fang *et al.*, 1994; McHugh *et al.*, 2003; Zheng *et al.*, 2006; Baloch *et al.*, 2008).

Propionate is one of the major intermediates of anaerobic digestion of soluble carbohydrates (Fang *et al.*, 1994). Propionate conversion is a multi-step process that involves syntrophic acetogens (which degrade propionate to acetate, CO₂ and H₂), hydrogenotrophic methanogens as well as acetoclastic methanogens (Fukuzaki *et al.*, 1991a). Since propionate degradation is thermodynamically unfavourable unless the two primary metabolites (H₂ and acetate) are constantly removed, the propionate-degrading acetogens have to grow in the close vicinity of both groups of methanogens (Fang *et al.*, 1994). The immobilisation of these populations in granules can only enhance their synergistic association by permanently minimising interspecies distance as can be concluded from previous reports of juxtapositioned syntrophic microcolonies in mature UASB granules (Fang *et al.*, 1994; 1995a; 1995b). The higher methane production observed in the PTM samples of the Athlone 4Sb-sludge in comparison with the Athlone 4Sb control samples (BTM samples) shows an active propionate-degrading population, which might, in turn, indicate the existence of a synergistic association between the propionate-consuming acetogens and the H₂- and acetate-consuming methanogens. The very small aggregates that were observed in the Athlone 4Sb-sludge might have provided these populations with a more permanent matrix to promote interspecies transfer of metabolites and contribute to the stability of the synergistic relationship. The capacity of the Athlone 4Sb-sludge to optimally degrade propionate might thus also contribute to its efficiency as seed sludge during UASB reactor start-up.

On the other hand, the ST-sludge showed the most potential during the granulation enhancement experiments based on the ECP-production and granulation-enhancement model of Britz *et al.* (2002). While no *Propionibacterium* strains could be detected in the ST-sludge at the beginning of the batch granulation-enhancement experiment, positive *Propionibacterium* PCR results indicated the presence of *Propionibacterium* strains on the final day. This confirmed that these lactate-utilising organisms were successfully enhanced during the batch granulation-enhancement study. It has been reported that UASB granules that had large populations of lactate utilisers also had higher ECP contents and cell densities. These granules also had high ash contents (between 44 and 53%) as a result of mineral accumulation due to local alkaliphilic conditions caused during the conversion of fatty acids to methane (Fukuzaki *et al.*, 1991b). It is also known that ECPs promote intercellular bridging in microbial aggregates (Costerton *et al.*, 1981). ECP produced by the lactate-utilising population present in the ST-sludge could thus have improved cell adhesion and consequently contributed to the increased VSS retention observed in the ST test samples after the batch granulation-enhancement study. Coincidentally, the ST-sludge was also one of the two sludges with the most favourable divalent ion (Ca^{2+} and Mg^{2+}) concentrations, which might also have contributed to VSS retention during the batch granulation-enhancement experiment by promoting divalent-bridging between cells. One might argue that, based on the above-mentioned aspects, the ST-sludge could, therefore, be a good choice as seed sludge for UASB reactor start-up.

It should, however, be noted that although the ST-sludge showed the highest increase in VSS content $\geq 0.25 \text{ mm}^2$ in both the LacM and AEM during the batch granulation-enhancement study, it does not guarantee that the same trend would be observed when the ST-sludge was used (without prior granulation enhancement) during normal UASB reactor start-up where a different wastewater is treated. *Propionibacterium* strains could not be detected by PCR in the ST-sludge before the batch granulation-enhancement study, which might indicate very low initial levels of *Propionibacterium* that may not be able to contribute significantly to ECP production during normal UASB reactor start-up if in-reactor conditions, such as the accumulation of lactate and H_2 under unbalanced conditions as discussed by Riedel & Britz (1993), do not allow propionibacteria to gain a competitive advantage.

Another factor that needs investigating is how batch granulation-enhancement affects the methanogenic activity of the digested sludge treated. Although biogas

production was observed visually in all units during the batch granulation-enhancement study, it is recommended that activity be measured quantitatively both during and after batch granulation-enhancement studies in terms of volume of methane to determine whether it is a viable option to improve both granulation and anaerobic digestion during UASB reactor start-up.

The differences between the methanogenic activities observed for the four sludges also showed that although the DGGE method can give a fingerprint as to which *Archaea* may be dominant in the different sludges, it does not give an indication of the actual activity status of the different methanogenic populations present. The DGGE results did confirm that all four the sludges contained *Methanosaeta* species (which play an important part in granulation). This is in contrast with the DGGE results of the sludges analysed in the previous chapter of this dissertation. It may well be that these organisms are present in all stable anaerobic systems. Straub *et al.* (2006) reported that most anaerobic digesters at municipal WWTW are normally operated under conditions favouring *Methanosaeta* spp. Therefore, although it is of value to determine at a molecular level whether the important acetoclastic populations are present, it might be more practical for future large-scale UASB reactor start-ups, especially if molecular biology facilities are not available, to base the choice of digested sludge inoculum firstly on stable digester performance at the source and secondly on population-specific activity determinations based on substrate-dependent biogas- or methane-production analyses.

It is, however, also clear that differences do exist between stable well-digested sludges, especially as shown in this study in terms of activity and VSS retention in the granulation-enhancement experiment. It is still unclear which of these two parameters measured (activity or VSS-retention potential) gives the most accurate indication of which sludge would perform best during normal UASB reactor start-up. It is, therefore, recommended that normal UASB reactor start-up be performed with both the Athlone 4Sb-sludge (with the highest methanogenic activity), and the ST-sludge in order to determine whether VSS-retention potential during batch granulation-enhancement could serve as a possible third indicator (after municipal sludge digester stability and population-specific activity determinations) to differentiate between stable digested sludges.

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CHAPTER 7

COMPARISON OF THE START-UP EFFICIENCY OF TWO ANAEROBIC DIGESTED SLUDGES USED FOR THE SEEDING OF UASB REACTORS

Summary

In the previous chapter, two stable digester sludges (Athlone 4Sb and ST) were chosen for application in this study in order to determine the most suitable seed sludge for further application in accelerated UASB start-up studies. The choice was based on the highest activity in substrate-dependent activity tests and the highest increase in volatile suspended solids (VSS) particles in batch granulation-enhancement studies (digester sludge). These sludges were subsequently used to seed two 2.4 L UASB reactors (A4Sb-reactor and ST-reactor) and then evaluated in terms of UASB reactor efficiency. At the end of the evaluation period, sludge from both reactors were compared in terms of VSS retention, size distribution and methanogenic activity.

During start-up, the A4Sb-reactor reached COD-removal efficiencies above 90% earlier than the ST-reactor. As the organic loading rates were increased (1.3 – 2.14 kg COD.m⁻³.d⁻¹), it became evident that the A4Sb-reactor consistently had higher COD-removal rates than the ST-reactor. The A4Sb-reactor also had higher pH levels and biogas volumes as well as lower VSS washout than the ST-reactor. At the end of the study only the A4Sb-reactor showed an increase in VSS content. The VSS fraction larger than 0.25 mm² also increased in the A4Sb-reactor sludge during the course of the study, which was not observed for the ST-reactor sludge. Substrate-dependent activity testing also revealed that the A4Sb-reactor sludge had higher methanogenic activities.

It was concluded that start-up proceeded better in the A4Sb-reactor and it is, therefore recommended that the Athlone 4Sb digester sludge be used as seed sludge for enhanced UASB reactor start-up. It is also recommended that substrate-dependent activity testing information regarding population interactions be considered in addition to municipal digester performance during seed sludge selection for future UASB reactor start-ups.

Introduction

The development of a dense, well-settled granular sludge bed is critical to the optimum performance of anaerobic wastewater treatment systems such as UASB, IC and EGSB reactors (Schmidt & Ahring, 1996; El-Mamouni *et al.*, 1998; Tay *et al.*, 2000; Ghangrekar *et al.*, 2005). During industrial-scale UASB reactor start-up, surplus granular sludge from functioning UASB reactors would thus be the seed material of choice since it would significantly reduce reactor start-up periods and quickly reach high removal efficiencies (Goodwin *et al.*, 1992; Van Lier, 2007). Although this is an obvious choice in developed countries where industrial-scale reactors are fairly common, access to high-quality granular sludge still poses a serious problem in developing countries (Britz *et al.*, 1999). The availability of granular inocula is limited and international purchase and transport costs can be high. In the absence of granular inoculum, anaerobically digested sewage sludge is the most commonly used seed material (Liu & Tay, 2004).

Sludge granulation is indicative of a successful UASB reactor start-up, but start-up with digested sludge as seed can be problematic since it might take 3 - 8 months for the anaerobic consortium to granulate (Liu *et al.*, 2004; Ghangrekar *et al.*, 2005; Zhou *et al.*, 2006). Granulation of flocculent sludge is also highly dependent on the microbial population composition of the seed sludge. It is generally believed that the acetoclastic *Methanosaeta concillii* acts as nuclei for granule development by providing growth support for other anaerobic bacteria (Zheng *et al.*, 2006). The absence of this organism in digested seed sludge might, therefore, have a detrimental effect on reactor start-up. Various researchers have also commented on the positive influence of other methanogens as well as certain acidogenic and syntrophic acetogenic populations on the granulation process, provided that *M. concillii* is already present (Colleran, 1988; Fukuzaki *et al.*, 1991; Slobodkin & Verstraete, 1993; El-Mamouni *et al.*, 1995; Wu *et al.*, 1996; Veiga *et al.*, 1997; Britz *et al.*, 2002; Xu & Tay, 2002; Zheng *et al.*, 2006). Theoretically, digested sludge should contain all the appropriate organisms for anaerobic digestion, albeit in flocculent form (Liu & Tay, 2004). In practice, the proximity of the municipal wastewater treatment facility is usually the most decisive factor when seed sludge is chosen. Although the stability of the full-scale municipal anaerobic digester might also be a consideration, the presence and activity of specific populations in the digested sludge is not usually taken into account in the choice of seed sludge.

In the previous chapter of this dissertation (Chapter 6) it was demonstrated that differences do exist between the anaerobic populations of stable well-digested anaerobic sludges from different municipal wastewater treatment facilities. Substrate-dependent activity testing and batch granulation-enhancement studies indicated that although the four anaerobic sludges tested in Chapter 6 all contained acetoclastic *M. concilii* (identified using DGGE screening), one performed best in substrate-dependent activity tests (Athlone 4Sb digester sludge) while another showed the highest batch granulation-enhancement potential (Simonstown digester sludge). It was, however, not clear which of the two main parameters tested would give the best indication of seed sludge efficiency for UASB reactor start-up. The specific aim of this study was to identify the most suitable seed sludge for accelerated UASB reactor start-ups. The most suitable seed sludge was thus determined by comparing the two best-digested sludges identified in the previous chapter, in terms of UASB reactor efficiency during start-up as well as volatile suspended solids (VSS) retention, size distribution and methanogenic activity at the end of the evaluation period.

Materials and methods

Choice and preparation of digested seed sludge

With the kind cooperation of Mr R. Moolan and Mr H. Rus of the Scientific Services Department of the Cape Metropolitan Council (CMC), well-stabilised homogenous sludge samples were obtained from the 4-Secondary-b digester at the Athlone municipal wastewater treatment works (WWTW) (= Athlone 4Sb-sludge) and from the secondary digester at the Simonstown WWTW (= ST-sludge). Control parameters and routine analyses from both these digesters indicated 'healthy' digester operations (see Chapter 6).

The Simonstown digester was, at the time of sampling, used for the anaerobic digestion of primary settling tank (PST) sludge while the Athlone 4Sb digester was fed with a mixture of PST and waste activated (WA) sludge (H. Rus, CMC, South Africa, personal communication, 2002).

Before reactor inoculation, both sludges were sieved (mesh size = 1 mm²) to remove larger pieces of debris and inert impurities. Each sludge type was used to seed a separate laboratory-scale UASB reactor (the UASB reactor inoculated with Athlone

4Sb sludge = A4Sb reactor and the reactor inoculated with ST sludge = ST reactor). Seeding volumes were adjusted so that both reactors started with a VSS concentration of 10 g.L^{-1} .

UASB reactor set-up and analyses

The two identical laboratory-scale UASB reactors were set up to operate under similar conditions. Both reactors had operational volumes of 2.4 L (height = 830 mm and internal diameter = 50 mm) and were equipped with open gas/solids separators at the top, similar to the UASB reactor design used by Trnovec & Britz (1998) and Sigge (2005). Both reactors were insulated and reactor temperatures were maintained at 35°C with heating tape and an electronic control unit (Meyer *et al.*, 1985). A Watson-Marlow 323 peristaltic pump (Watson-Marlow Bredel Inc., Wilmington, MA), fitted with twin pumpheads and controlled by an electronic timer, was used to feed both reactors semi-continuously. Substrate was introduced at the bottom of each reactor while reactor overflow exited through a water-filled U-tube in order to maintain an anaerobic environment. A recirculation upflow velocity of 1.0 m.h^{-1} was maintained in both reactors by means of a second Watson-Marlow 323 peristaltic pump fitted with twin pumpheads.

The biogas volume for each reactor was determined daily with a wet-type gas meter equipped with an electronically controlled counter. Each gas meter had a built-in water-filled U-tube, and biogas left each reactor as single air bubbles via this route once the internal air pressure exceeded atmospheric pressure. Effluent pH (measured daily), bicarbonate alkalinity (measured every second day), total suspended solids (TSS) content and volatile suspended solids (VSS) content (measured weekly) were determined according to Standard Methods (1998). The effluent COD content was determined colorimetrically with a Hach DR2000 spectrophotometer (Hach Co., Loveland, CO) and was measured twice a week.

The methane (CH_4) content of the biogas was determined with a Varian 3300 gas chromatograph (Varian Inc., Palo Alto, CA) equipped with a thermal conductivity detector and a $2.0 \times 3.0 \text{ mm}$ i.d. column packed with Hayesep Q (Supelco, Bellefonte, PA) 80/100 mesh. The oven temperature was set at 55°C and helium was used as carrier gas at a flow rate of 30 mL.min^{-1} . The injection volume of each biogas sample was 0.2 mL and the percentage CH_4 was determined with a Varian 4290 integrator (Sigge, 2005).

Reactor feed

Both reactors were fed a sterilised synthetic sucrose-based substrate with a COD value of 2 500 mg.L⁻¹ and pH of 7.2. The substrate consisted of the following (g.L⁻¹): sucrose 1.36; sodium lactate (Sigma) 0.2; peptone (Biolab) 0.4; beef extract (Biolab) 0.28; NH₄Cl (Saarchem) 0.32; KH₂PO₄ (BDH) 0.08; FeSO₄.7H₂O (Saarchem) 0.02; and NaHCO₃ (Saarchem) 1.2. This substrate was based on the substrate composition of Show *et al.* (2004), with the difference that glucose was replaced with sucrose and the addition of a small amount of lactate. During the first part of Phase 1, the pH of the sterilised substrate was adjusted to 7.2 with a phosphoric acid solution (2 M). During the start-up, a CaCO₃ solution (average concentration 4.2 mM per litre reactor volume [Lrv]) was added directly to both reactors to facilitate pH stability. The addition of CaCO₃ to facilitate pH control became unnecessary after Phase 1. The trace mineral component of the substrate described by Show *et al.* (2004) was excluded in favour of the trace mineral solution of Nel *et al.* (1985) of which 1 mL.(Lrv)⁻¹ was added directly to each reactor once a week.

Reactor operation

After inoculation, both reactors were operated for nine months. The reactors were both started on diluted substrate and the organic loading rate (OLR) was increased by increasing the COD content of the substrate in steps until substrate concentration reached full strength. During the first five months the hydraulic retention time (HRT) was maintained at 45 h. Once steady state (less than 10% variation in percentage COD removal) (Sigge, 2005) was reached on full-strength substrate, the OLR of both reactors was increased further by changing the HRT.

Size distribution

The TSS and VSS contents of the sludge from both reactors were monitored at the start and the end of the study. Size distribution determinations were also done on the VSS fraction larger than 0.25 mm² for both sludges at the start and end of the study, using the method of Laguna *et al.* (1999). Four sieves with mesh openings of 1.0, 0.71, 0.50 and 0.25 mm² (Endecotts Ltd., London, UK) were stacked with the largest mesh

opening at the top, after which a well-mixed representative 25 mL sludge sample was added to the top sieve. The sieve was rinsed with distilled water to remove particles smaller than 1.0 mm², after which the sieve was removed. The washing procedure was repeated for each sieve and the particles retained on each sieve were recovered by backwashing with distilled water. The VSS content of each fraction was determined using the method described in Standard Methods (1998).

Activity tests

The methanogenic activity of the sludges from both reactors was compared at the end of the study by using the activity test method of O’Kennedy (2000). According to this method, 3 g of centrifuged sludge was incubated in 13 mL of test medium. The samples were incubated in 20 mL test vials sealed with butyl rubber septa and aluminium crimp caps. Vials were prepared in triplicate for each test substrate and were incubated at 35°C for 24 h. Four different test media, supplemented with different carbon sources, were used in this study:

1. basic test medium (BTM) (Valcke & Verstraete, 1993), which contained 2 g.L⁻¹ glucose as carbon source;
2. glucose test medium (GTM) = BTM + 2 g.L⁻¹ glucose (Saarchem);
3. lactate test medium (LTM) = BTM + 4g.L⁻¹ lactate (Sigma); and
4. acetate test medium (ATM) = BTM + 1 g.L⁻¹ acetate (Merck).

Biogas volume was measured (in mL) at 5 h, 10 h and 25 h intervals by using a free-moving 5 mL syringe equipped with a 25-gauge needle. Biogas measurements were made by holding the syringe vertically, inserting it into the rubber stopper and allowing the plunger to move freely until equilibrium was reached. The percentage CH₄ was determined gas chromatographically as previously described, and used to calculate the CH₄ volume (in mL).

Results and discussion

Phase 1: Reactor seeding and start-up (D0 – D170)

Both reactors were seeded and left to recirculate for 48 h for the sludge to acclimatise to a reactor temperature of 35°C. Initial feed introduction during the acclimatisation phase resulted in a sharp decrease in the effluent pH, especially in the ST-reactor. On D0,

additional CaCO_3 was added to the ST-reactor at a concentration of $0.0125 \text{ M} \cdot (\text{Lrv})^{-1}$ to stabilise the pH of the ST-reactor.

During Phase 1, the HRT was kept constant at 45 h and the OLR was set by changing the COD concentration of the reactor feed. Full-strength substrate was fed to both reactors from D95 onwards, giving an OLR of $1.3 \text{ kg COD} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$. This OLR was maintained for a further 75 d to confirm reactor stability. The operating efficiencies of both reactors during Phase 1 are presented in Figs. 1 (% COD removal), 2 (pH and alkalinity) and 3 (effluent TSS and VSS content).

The A4Sb-reactor reached COD removal rates higher than 92% by D48, ahead of the ST-reactor, and remained at this level during the rest of Phase 1 (Fig. 1). The ST-reactor reached 90% COD removal by D65. In the case of the pH profiles, both reactors showed similar trends (Fig. 2). In terms of solids, the ST-reactor effluent mostly had a higher VSS content than the A4Sb-reactor effluent (Fig. 3), which probably was the reason for the slightly higher COD content found in the ST-reactor effluent (Fig. 1). Although the CaCO_3 addition to the reactors was identical, the ST-reactor had higher alkalinity values between D40 and D80 (Fig. 2). The higher alkalinity observed for the ST-reactor did, however, not influence the reactor pH profile noticeably.

It can be concluded from the COD removal data for the period up to D95 that the COD load increases, especially for the A4Sb-reactor, could have been performed earlier. The reactor set-up in this study was, however, done in such a way that both reactors could be treated identically in all respects. Both reactors were fed the same amount of substrate at the same concentration using the same feed pump controlled by the same timer. The set-up did not allow for any flexibility with regard to additional organic loading in one reactor without affecting the other, and increases in organic loading were only done once a reasonable stable-state was achieved in both reactors. It is, therefore, recommended that the reactor set-up and/or feeding strategies be adapted for further start-up studies to allow for the application of separate OLRs in the separate reactors.

The data also showed that between D95 and D170 both reactors were stable at an OLR of $1.3 \text{ kg COD} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$. Under these conditions, VSS washout of flocculent matter was not excessive and did not have a detrimental effect on reactor efficiency. No re-inoculation was necessary.

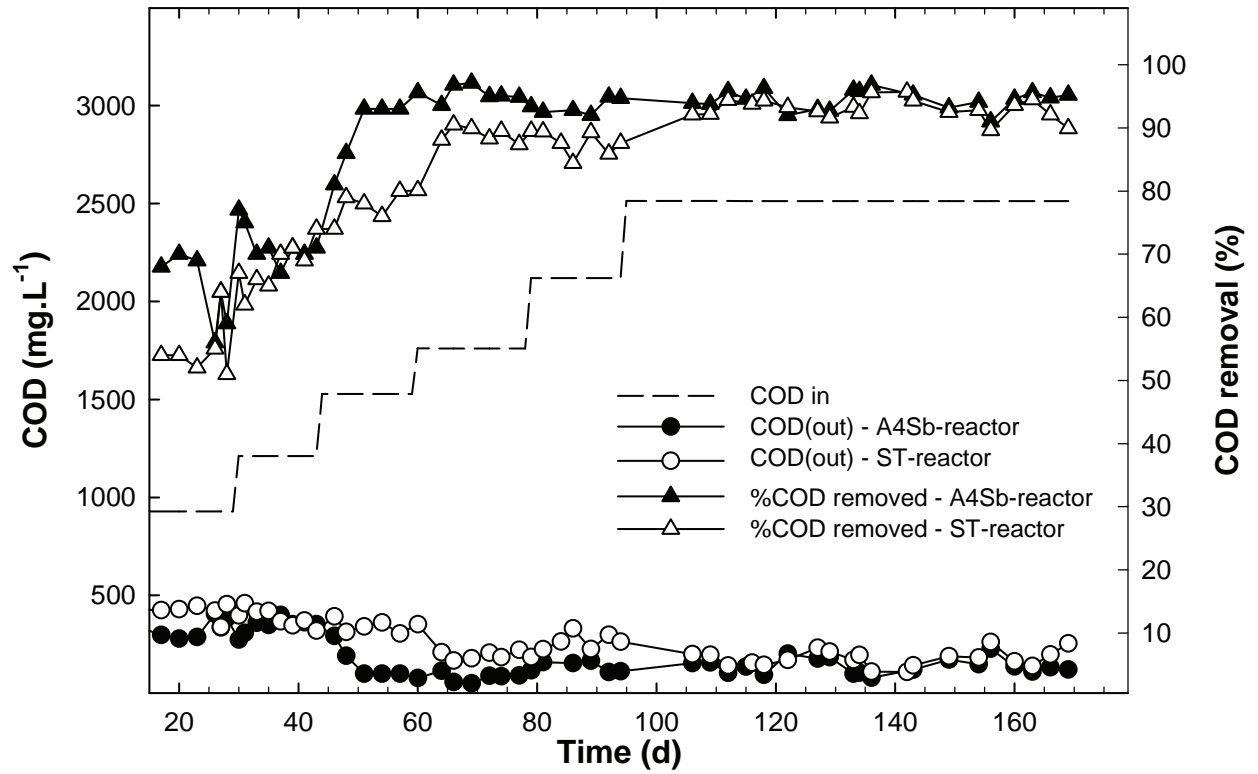


Figure 1 Performance of the UASB reactors during Phase 1 (HRT= 45 h) in terms of feed concentration (COD_{in}), effluent COD content (COD_{out}) and COD removal efficiency (% COD removed).

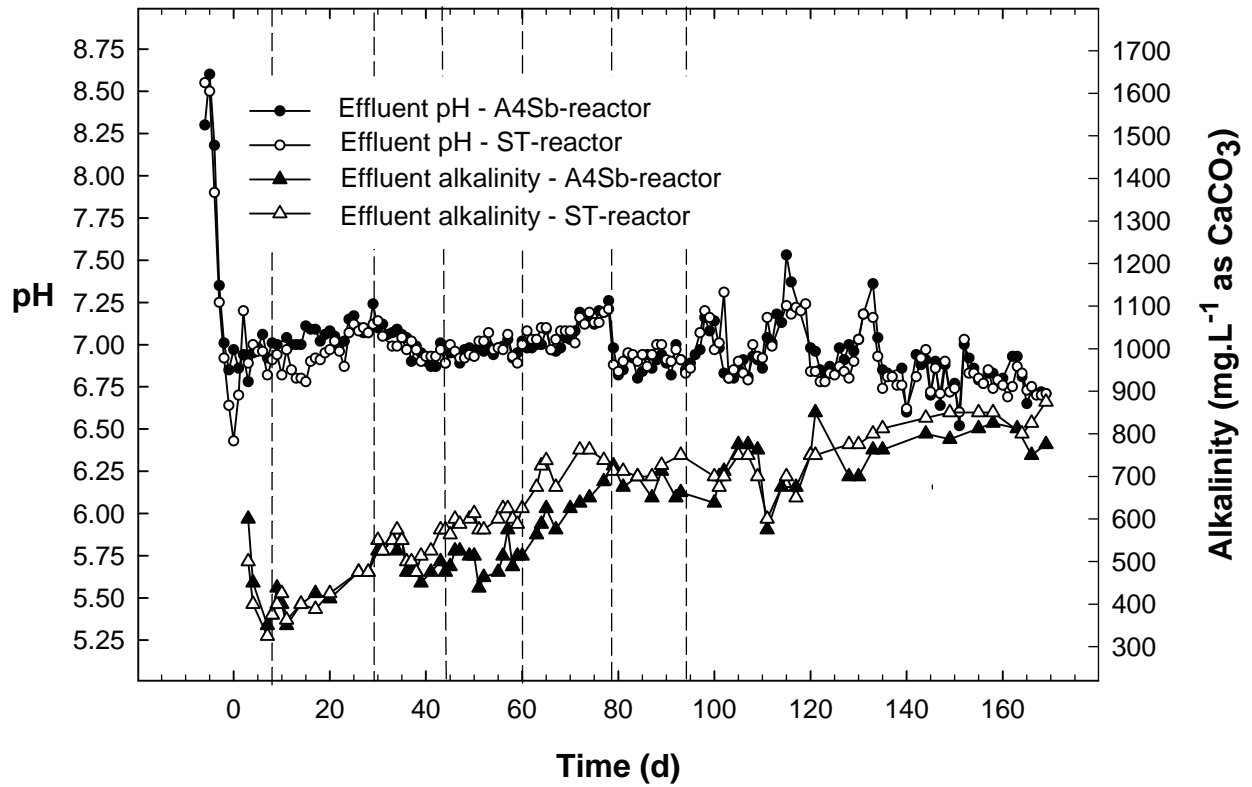


Figure 2 Effluent pH and alkalinity profiles of both reactors during Phase 1 (HRT= 45 h). Dashed lines indicate substrate COD increases until full strength (and an OLR of 1.3 kg COD.m⁻³.d⁻¹) were reached on day 95.

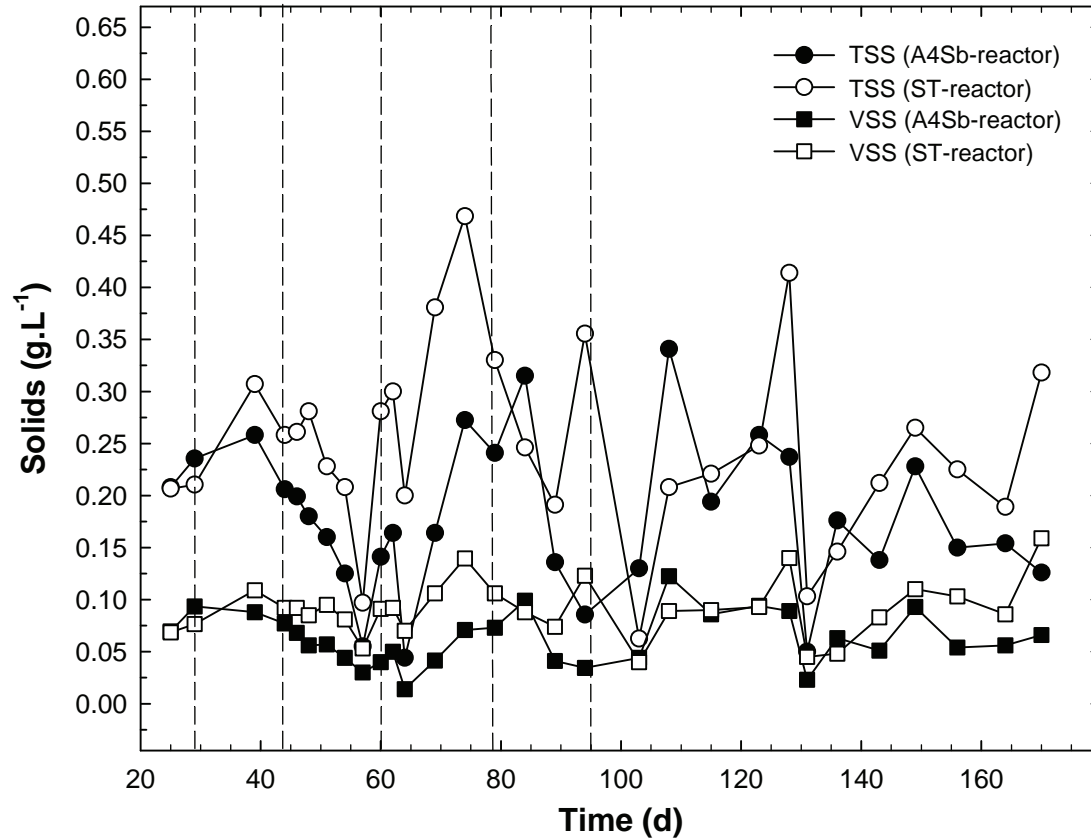


Figure 3 Total suspended solids (TSS) and volatile suspended solids (VSS) contents of the effluents of both reactors during Phase 1 (HRT = 45 h). Dashed lines indicate substrate COD increases until full strength was reached on day 95.

Phases 2 – 4: Reactor efficiency (D170 – D283)

During the second part of this study both reactors were fed full-strength substrate and changes in the HRT were used to change the OLR of the reactors. The operating efficiencies of both reactors during this part of the study are presented in Table 1. In this table the HRT and OLR changes are presented in four separate phases where each phase represents an increase in OLR.

During Phase 2a both reactors entered a period of pH instability. The decrease in HRT from Phase 1 to Phase 2a might have been too rapid for the slower-growing acetogenic and methanogens populations of both reactors, which were not active enough or present at high enough numbers to deal with the increased COD load. In Table 1, the average pH at the end of Phase 2a does not differ to a large extent from the average pH presented at the end of Phase 1. This might be misleading. When the pH of one or both reactors decreased to pH levels ≤ 6.60 , the reactors were not fed and were left to recirculate for 24 h in an attempt to allow the slower-growing acetogenic and methanogenic populations to metabolise the high levels of free volatile fatty acids (VFAs) (produced by fast-growing acidogenic populations) that caused the decrease in pH levels. Phase 2a was 14 d long and during the last 10 d, the reactors were left to recirculate five times. After each recirculation session the pH levels were higher, and since these higher pH values were also used to calculate the average pH values presented for Phase 2a in Table 1, these values are not a true indication of reactor efficiency. Although the percentage COD removal was above 90% and the percentage methane above 60% for both reactors, the pH values for the last day of Phase 2a were 6.59 (for the A4Sb-reactor) and 6.62 (for the ST-reactor).

During the first two weeks of Phase 2b the OLR was decreased and both reactors were frequently recirculated in an attempt to stabilise pH levels, before the OLR was restored at $1.57 \text{ kg COD}\cdot\text{m}^{-3}\cdot\text{d}^{-1}$. This stabilisation period proved to be long enough for the A4Sb reactor sludge population to adapt to the higher OLR, as can be seen from the improvement in pH, alkalinity, COD-removal efficiency and biogas production between Phase 2a and 2b. The same trend was not observed in the ST-reactor. All these variations suggest that the acetogenic and methanogenic populations of the ST-sludge were not large enough to remove the VFA produced by the acidogenic populations in response to the increased COD load in Phase 2.

Table 1 Operating conditions and efficiency of the UASB reactors with HRT reductions. Data represent average of values taken over the last 5 – 8 d of each specific phase

Parameters	Phase 1		Phase 2a		Phase 2b		Phase 3		Phase 4a		Phase 4b	
HRT	45 h		38 h		38 h		32 h		28 h		28h	
OLR	1.3		1.57		1.57		1.88		2.14		2.14	
Operational period	170 d		14 d		33 d		12 d		After 19 d		After 50 d	
Reactor	A4Sb	ST	A4Sb	ST	A4Sb	ST	A4Sb	ST	A4Sb	ST	A4Sb	ST
pH (effluent)	6.71	6.71	6.70	6.68	6.99	6.77	6.92	6.85	6.89	6.71	6.86	6.81
Alkalinity	771	871	700	763	1266	1225	1163	1119	1108	1083	1138	1138
COD removal	95.2%	90.6%	94.4%	91.9%	98.0%	53.6%	95.8%	62.8%	93.4%	73.7%	92.8%	86.7%
VSS	0.061	0.123	0.075	0.166	0.065	0.178	0.094	0.253	0.068	0.135	0.094	0.134
Biogas	1165	1431	1281	1498	1626	1454	1935	1578	1939	1575	2028	2266
CH ₄	62%	62%	61%	63%	55%	49%	58%	55%	58%	54%	58%	57%
CH ₄ yield	0.237	0.306	0.219	0.272	0.242	0.352	0.260	0.306	0.234	0.225	0.246	0.290

OLR = kg COD.m⁻³.d⁻¹ ; Alkalinity = mg.L⁻¹ CaCO₃ ; VSS = g.L⁻¹ ; Biogas = mL.d⁻¹ ; CH₄ yield = m³.kg⁻¹ COD_{removed}

From Phase 2b onwards, the HRT was decreased in steps to a final value of 28 h. As the OLR was increased from 1.57 to 2.14 kg COD.m⁻³.d⁻¹ during phases 2b, 3 and 4a, the pH of the A4Sb-reactor consistently stabilised at higher levels than the pH of the ST-reactor. The alkalinity and methane production of the ST-reactor during this time were lower than that of the A4Sb-reactor, which indicated that these lower pH levels were probably the result of the slower removal of free VFAs by acetogenic and methanogenic populations present in the ST-reactor sludge.

The A4Sb-reactor also consistently had COD removal rates above 92% and effluent VSS levels below 0.1 g.L⁻¹ for the duration of this part of the study. In contrast, the ST-reactor had higher levels of VSS washout during phases 2b, 3 and 4a, which was probably the reason for the lower percentage COD removal observed for the ST-reactor. The ST-reactor did, however, manage to recover during Phase 4b, as can be seen from the increases observed in the final average values for pH, percentage COD removal and methane production. The ST-reactor also had a higher methane yield per kg COD_{removed} for the most part of the study (Table 1), which might indicate that in the A4Sb-reactor a larger portion of the COD load was converted to biomass instead of methane. It was also concluded from the data presented in Table 1 at the end of phases 2b, 3 and 4a that these OLR increases might also have been executed earlier in the A4Sb-reactor than in the ST-reactor.

Suspended solids content and size distribution

In Fig. 4 it can be seen that although the TSS concentration of the ST-reactor sludge increased from the start to the end of the study, the higher solids washout observed in the ST-reactor during the course of this study resulted in an overall VSS loss. In contrast a decrease was observed specifically for TSS and VSS particles $\geq 0.25\text{mm}^2$ in the ST-reactor sludge. This was in contrast to the A4Sb-reactor sludge that showed increases in TSS and VSS contents, which also included increases in TSS and VSS particles $\geq 0.25\text{mm}^2$.

Although the sieving procedure used in this study does not give an indication of the number of granules formed, information on the size distribution of the suspended solids content can be obtained (Laguna *et al.*, 1999). The VSS fraction represents all the organic suspended matter, including the microbial biomass. Comparison of the size distribution of the VSS contents of the reactor sludges at the beginning and end of the

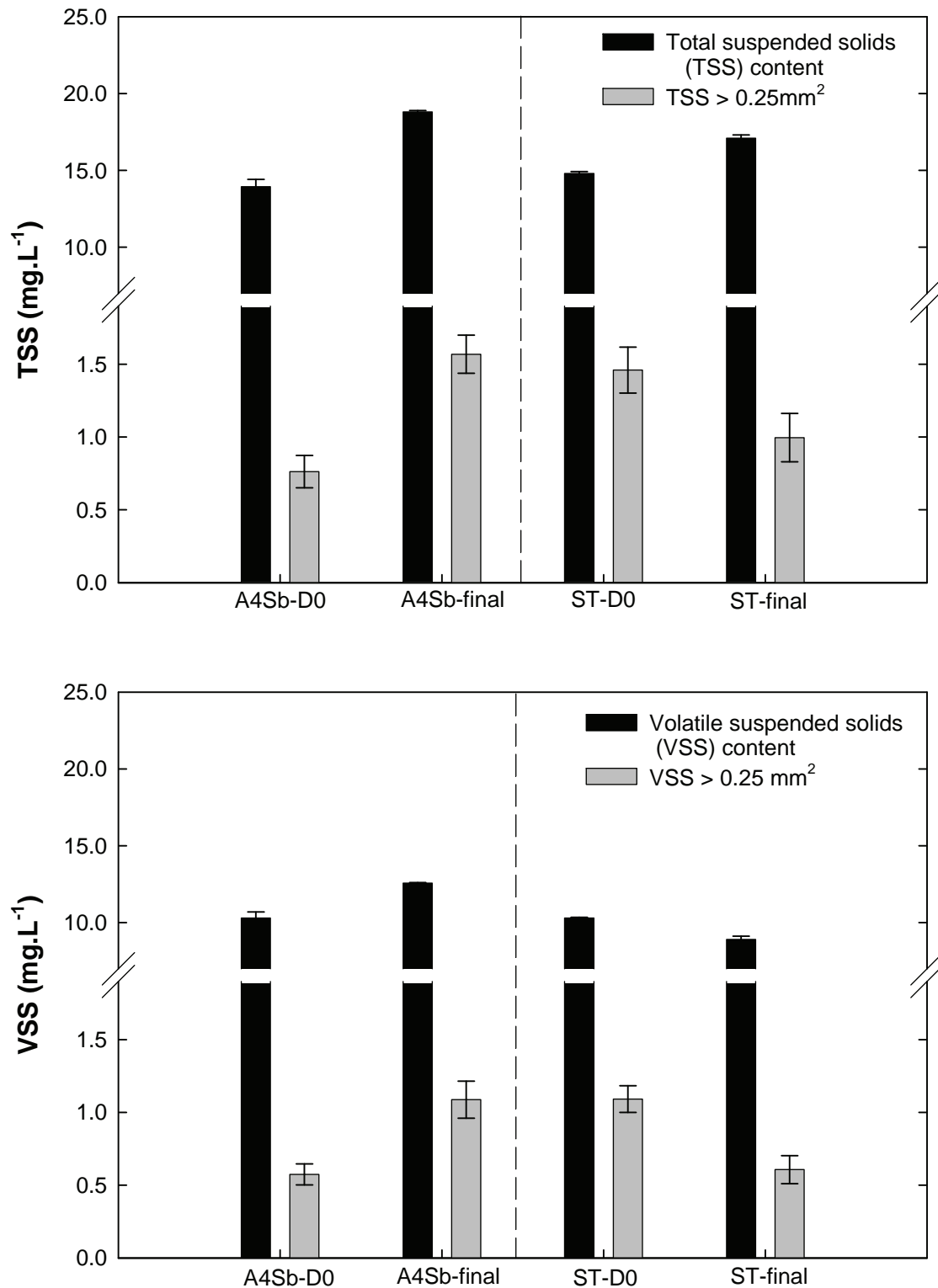


Figure 4 Total suspended solids (TSS) and volatile suspended solids (VSS) contents of both reactor sludges at the start (D0) and end (final) of the study. Grey bars indicate the contents of TSS and VSS particles $\geq 0.25 \text{ mm}^2$. Each data point represents triplicate values. The standard deviation was used as the error bar.

study could indicate whether increases in the flocculation and subsequent granulation of the suspended matter occurred.

Size distribution determinations of VSS contents $\geq 0.25\text{mm}^2$ in the A4Sb-reactor sludge and the ST-reactor sludge are presented in Figs. 5 and 6, respectively. The VSS content increases observed between 0.25 mm^2 and 0.99 mm^2 in the A4Sb-reactor sludge were mostly within the size range $0.25\text{ mm}^2 - 0.49\text{ mm}^2$, which indicates the formation of granular nuclei (Fig. 7). No increases in VSS content between 0.25 mm^2 and 0.99 mm^2 were observed in the ST-reactor sludge.

Activity tests

Methane formation is the final step in the anaerobic digestion process and optimal production requires the balanced interaction of acidogenic, acetogenic and methanogenic populations. In the previous study it was shown that substrate-dependent activity testing provides valuable information about these population interactions.

The biogas- and methane-production activities of the reactor sludges at the end of the study were determined using standardised biomass systems containing four different test substrates. Results are presented as cumulative biogas production (Fig. 8) and cumulative methane production (Fig. 9). The BTM substrate, which contained 2g.L^{-1} glucose as carbon source, served as control to measure overall methanogenic activity. GTM and LTM were used to measure the effect that different acidogenic populations (glucose utilisers and lactate utilisers) might have on methanogenic activity while ATM was used to measure the methanogenic activity of the acetoclastic methanogens.

In all the substrates tested, the A4Sb-reactor sludge showed higher methanogenic activities than the ST-reactor sludge per gram of centrifuged sludge. From the results presented on the TSS contents of both sludges in Fig. 4, it was concluded that while the TSS content of the ST-reactor sludge increased during reactor operation, the VSS content decreased. This resulted in the ST-reactor sludge having a VSS/TSS ratio of 0.52, which was lower than the VSS/TSS ratio of 0.67 of the A4Sb-reactor sludge at the end of the study. The ST-reactor sludge thus had a lower viable sludge concentration per gram of centrifuged sludge, which might have contributed to the lower methanogenic activity observed in the ST-reactor sludge samples during activity testing.

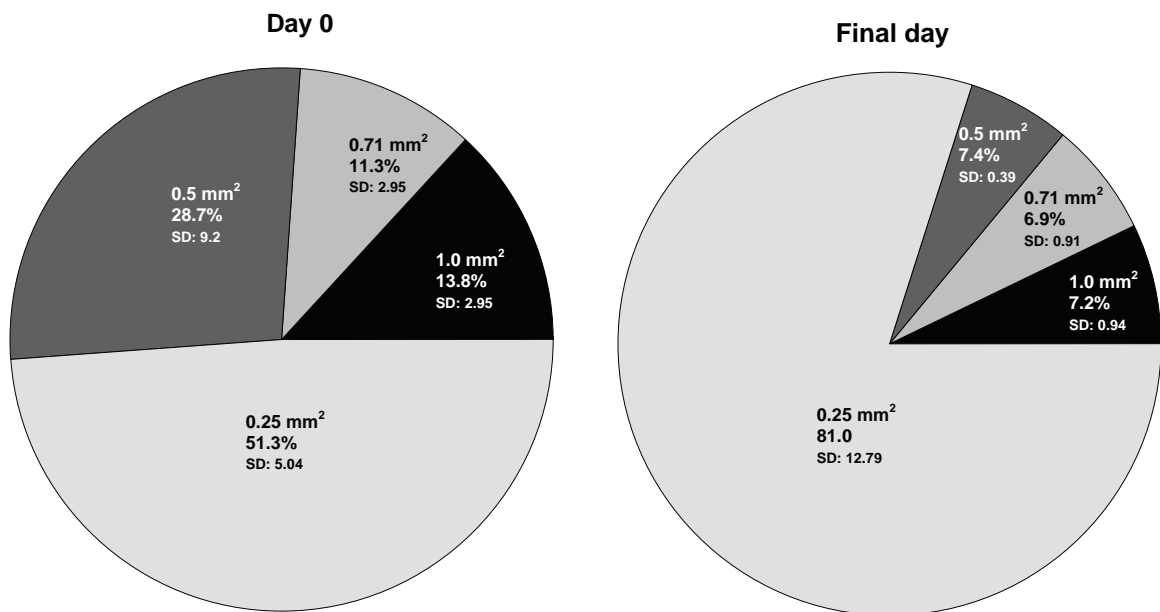


Figure 5 Size distributions of the VSS contents ≥ 0.25 mm² of the A4Sb-reactor sludge at the start (Day 0), and end (Final day) of the study. Aperture size is presented in mm², followed by the VSS content (in %) and standard deviation (SD) values (calculated from triplicate VSS values).

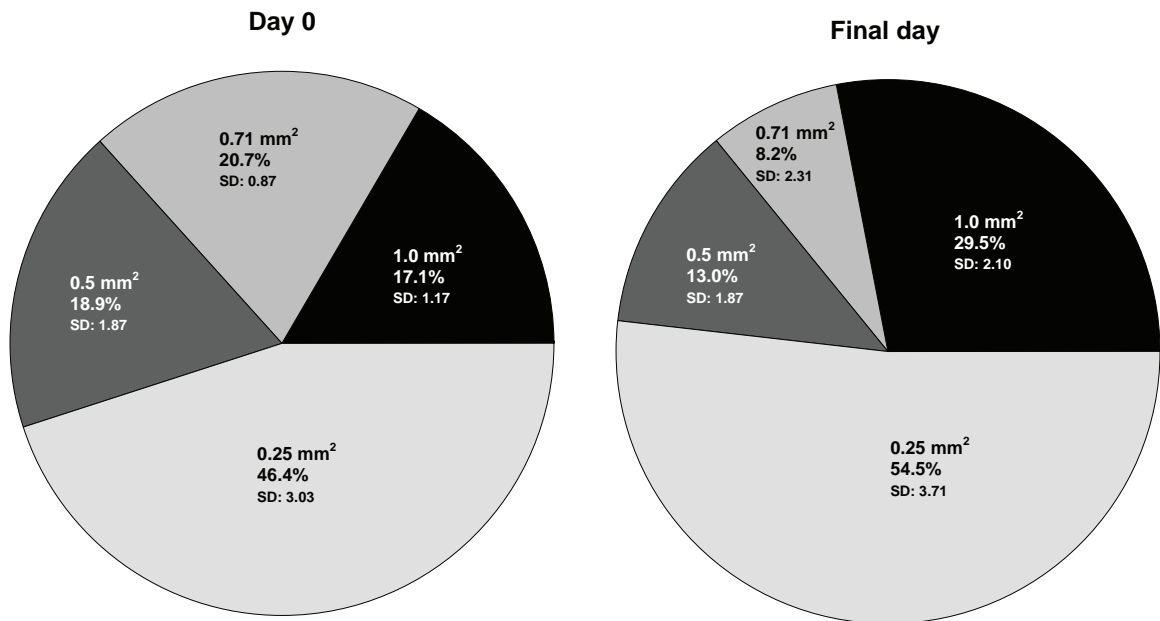
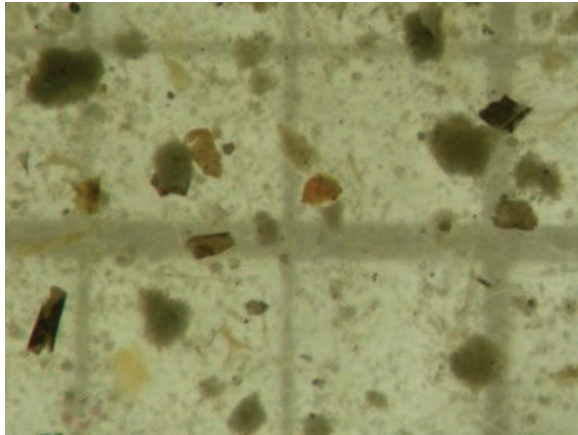
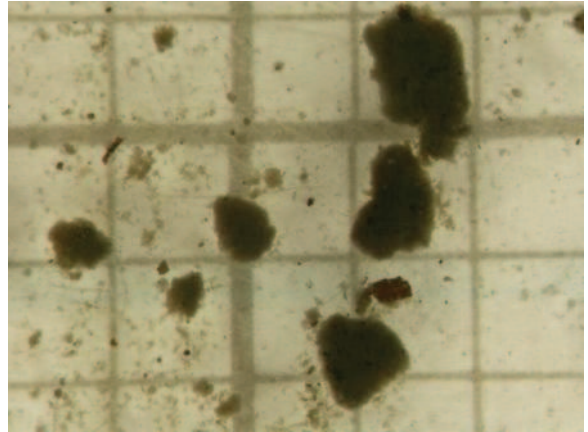


Figure 6 Size distributions of the VSS contents $\geq 0.25 \text{ mm}^2$ of the ST-reactor sludge at the start (Day 0), and end (Final day) of the study. Aperture size is presented in mm^2 , followed by the VSS content (in %) and standard deviation (SD) values (calculated from triplicate VSS values).



(a) $0.25 - 0.49 \text{ mm}^2$



(b) $\geq 1.00 \text{ mm}^2$

Figure 7 Suspended solids size fractions of the A4Sb-reactor sludge at the end of the study: (a) $0.25 - 0.49 \text{ mm}^2$, and (b) $\geq 1.00 \text{ mm}^2$. (Grid size = $2 \times 2 \text{ mm}$)

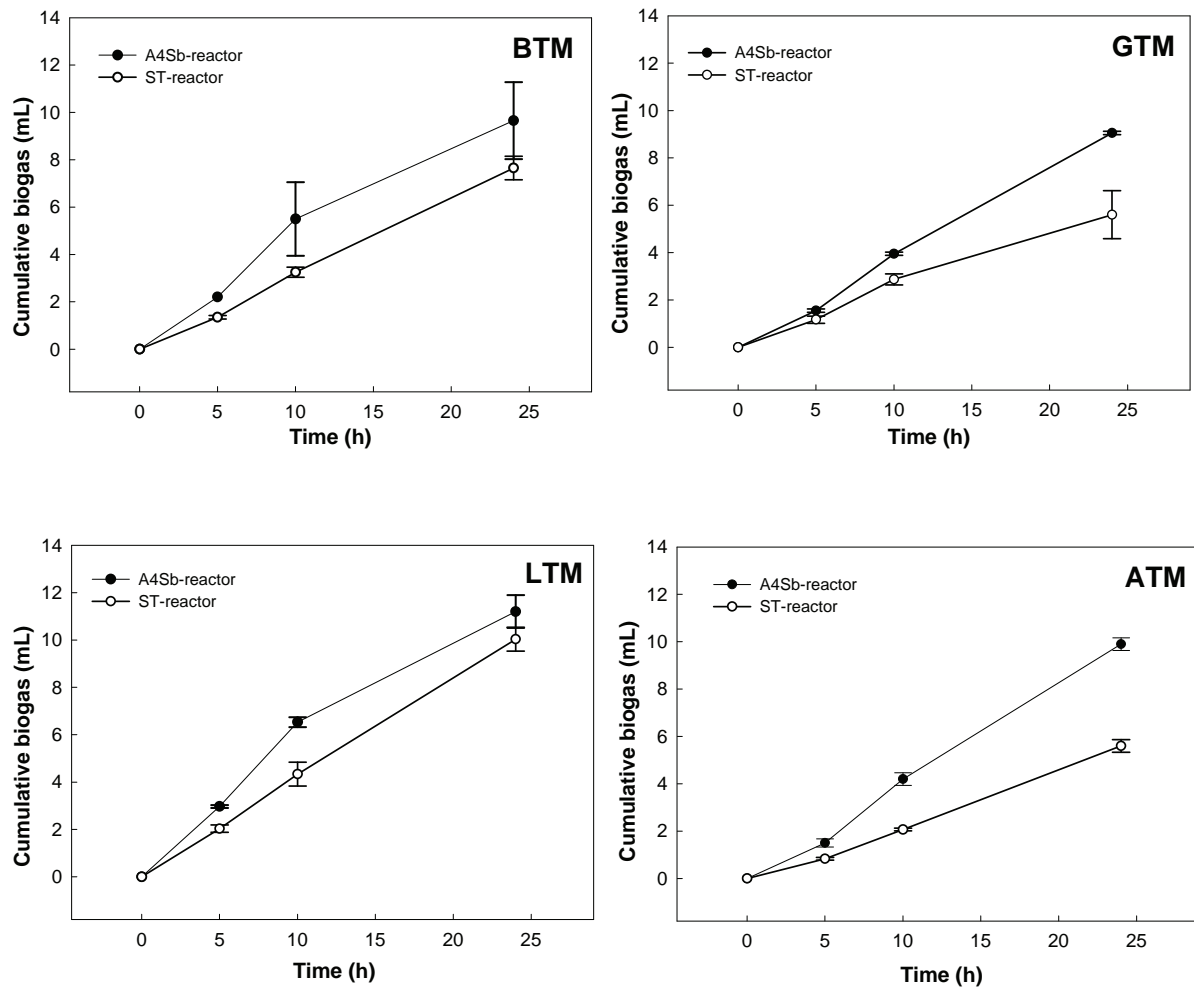


Figure 8 Cumulative biogas production over 24 h by each reactor sludge determined at the end of the reactor start-up. Each data point represents triplicate values, and the standard deviation was used as the error bar. BTM - basic test medium; GTM - glucose test medium; LTM - lactate test medium; ATM - acetate test medium.

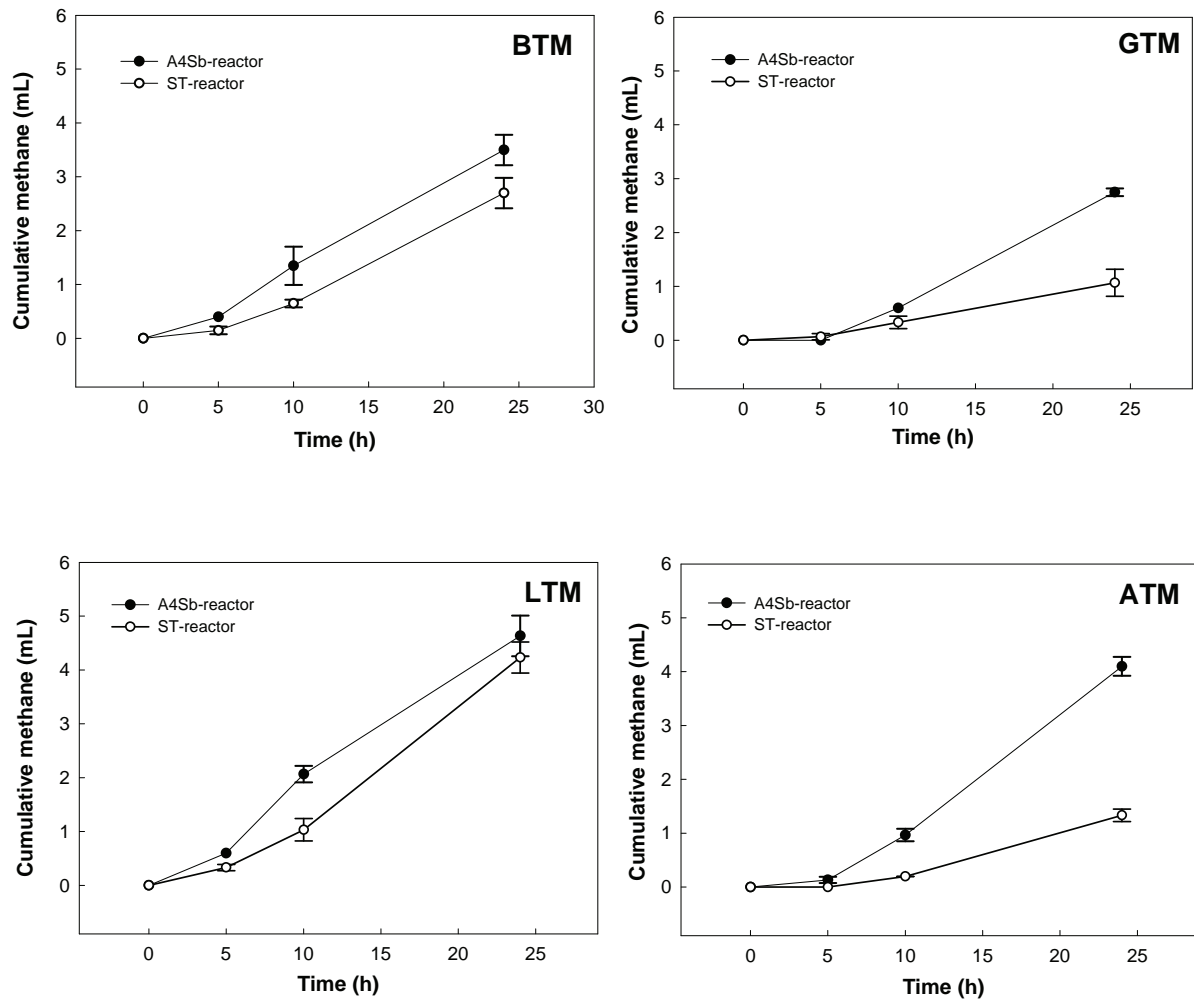


Figure 9 Cumulative methane production over 24 h by each reactor sludge determined at the end of the reactor start-up. Each data point represents triplicate values, and the standard deviation was used as the error bar. BTM - basic test medium; GTM - glucose test medium; LTM - lactate test medium; ATM - acetate test medium.

The cumulative biogas production of the A4Sb-reactor sludge for GTM, LTM and ATM was within the range of biogas production activity indicated by the standard deviation values of the biogas production observed in BTM after 24 h (Fig. 8). Differences were, however, observed in the cumulative methane values of the A4Sb-reactor sludge (Fig. 9) in which a higher methanogenic activity was observed in both LTM and ATM compared to the cumulative methane production in BTM. Since acetate can only be metabolised by acetoclastic methanogens such as *Methanosaeta* and *Methanosarcina* spp., the higher methane concentrations observed for the A4Sb-reactor sludge in ATM indicated the presence of an active acetoclastic methanogen population.

The production of methane from lactate is a multi-step process that involves lactate-utilising acidogens as well as propionate- and butyrate-consuming acetogens that are usually closely associated with hydrogenotrophic and acetoclastic methanogens. More methane was produced in LTM than in BTM by both the A4Sb-reactor sludge and the ST-reactor sludge. This indicated that in both sludges, all the populations involved in the methane production from lactate functioned optimally and that the individual population numbers were, in relation to each other, well balanced and optimally positioned. The small amount of lactate added to the reactor feed of both reactors might have contributed to the development of these synergistic relationships.

In addition to being less active than the A4Sb-reactor sludge, the ST-reactor sludge also showed lower methanogenic activity in the GTM and ATM test samples in comparison with the BTM samples. This pointed to an inability of the acetogenic and methanogenic populations of the ST-reactor sludge to metabolise the higher VFA concentrations caused by the addition of more glucose (GTM) and more acetate (ATM). In the GTM samples the acetogens and methanogens in general might not have been active enough (or present at high enough levels) to remove the VFA produced by acidogenic populations fast enough to prevent pH inhibition of methanogenesis in the test samples during the 24 h incubation period of the activity test. In the ATM samples the acetoclastic methanogen population in particular might not have been active enough or present at high enough levels to metabolise the added acetate as well as the acetate produced naturally by acidogenic populations from the 2 g.L⁻¹ glucose also present in the test substrate.

The higher methanogenic activity observed in the Athlone 4Sb-sludge might also be attributed to the fact that the Athlone municipal digester from which the sludge was obtained was fed a combination of WA as well as PST sludge. This is in contrast to the

ST municipal digester that was fed only PST sludge. PST sludge in general refers to the settled solids from primary clarifiers at municipal WWTW as well as any colloidal wastes associated with these solids. WA sludge, on the other hand, consists of sludge of which the organic content has already been stabilised by aerobic bacterial growth, in other words waste has been converted to aerobic biomass (Gerardi, 2003). Almost 80% of the bacteria involved in aerobic processes are facultative anaerobes, which are also found in relatively high numbers in anaerobic processes (Gerardi, 2003). It has also been reported that WA sludge contains a considerable amount of both hydrogenotrophic and acetoclastic methanogens, which are situated at the centre of aerobic activated sludge flocs where they are protected from oxygen. WA sludge is also reportedly rich in nutrients and contains little sand or soil that might decrease the viable volume of the digester sludge (Wu *et al.*, 1987; Lens *et al.*, 1995; Kim & Speece, 2002). The addition of WA sludge to the feed of the A4Sb anaerobic digester at the Athlone WWTW might, therefore, not only have contributed to the diversity of the acidogenic population content but might also have improved the methanogenic population density and diversity in the Athlone 4Sb-sludge used as seed sludge in this study.

Conclusions

The data on UASB reactor start-up studies revealed that the Athlone 4Sb-sludge was overall the best choice as seed sludge. During reactor operation the A4Sb-reactor was more efficient than the ST-reactor. The A4Sb-reactor showed higher COD removal rates and had lower VSS washout than the ST-reactor throughout the reactor operation period. At higher OLRs ($\geq 1.57 \text{ kg COD}\cdot\text{m}^{-3}\cdot\text{d}^{-1}$) the A4Sb-reactor also had higher methane production rates and pH profiles.

Activity tests performed on the reactor sludges after the reactor runs revealed that the A4Sb-reactor sludge had a higher methanogenic activity per gram of centrifuged sludge than the ST-reactor reactor sludge in all the substrates tested. VSS analyses also confirmed that in the A4Sb-reactor there was an increase in VSS content (including an increase in VSS particles $\geq 0.25 \text{ mm}^2$) that was not observed in the ST-reactor sludge.

Based on these results and the fact that the Athlone 4Sb-sludge performed the best in the substrate-dependent activity testing described in the previous chapter, it was concluded that there is probably a link between the inherent methanogenic activity of a

digested sludge and the efficiency of the anaerobic reactor it is used to seed. Valuable information regarding the activity status of the indigenous anaerobic sludge populations (as well as their activity in relation to each other) can be obtained with substrate-dependent activity testing. Since the efficiency of the anaerobic digestion process during reactor start-up is dependent on a well-balanced anaerobic community, it is recommended that seed sludge selection (of digested sludges) be based not only on stable digester performance at the source but also on substrate-dependent activity testing.

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CHAPTER 8

UASB REACTOR START-UP: EVALUATION OF THE EFFICIENCY AND GRANULATION OF PRE-TREATED DIGESTED SLUDGE*

Summary

The aim of this study was to shorten upflow anaerobic sludge blanket (UASB) reactor start-up by using pre-treated digested sludge as inoculum to improve granulation and anaerobic digestion in the UASB reactor. Pre-treatment of the inoculum sludge involved the incubation of digested sludge in a rolling-batch reactor for 17 days (d) while feeding it daily with either a lactate-based substrate or a sucrose-based substrate. Three 2.4 L UASB reactors were seeded with the following: digested sludge without any pre-treatment (control = reactor A); sucrose-fed batch-incubated digested sludge (reactor B); and lactate-fed batch-incubated digested sludge (reactor C). All the UASB reactors were fed with a synthetic sucrose-based substrate initially. Severe foaming occurred in reactor C and the feed of this reactor was changed to a lactate-based feed. Start-up was continued in reactor C in spite of low COD removal rates, which was the direct result of the low residual sludge content of the reactor. Results showed that reactor B operated more efficiently with higher COD removal rates, higher methane production volumes and lower hydraulic retention time (HRT) values than the control reactor A. At the end of the study reactor B had a higher biogas production capacity as well as methanogenic activity per gram of centrifuged sludge than the control reactor. Size distribution results also showed that reactor B had a larger portion of volatile suspended solids (VSS) $\geq 1.0 \text{ mm}^2$ than the sludge from reactors A and C. It was concluded that batch granulation-enhancement of the inoculum sludge of UASB reactor B in the presence of sucrose did enhance anaerobic digestion as well as sludge granulation during UASB reactor operation, thereby shortening start-up.

* A condensed version of this chapter was presented at the 11th IWA World Congress on Anaerobic Digestion. Brisbane, Australia, September 23–26, 2007.

Introduction

The UASB reactor is considered internationally as one of the most effective and economical high-rate anaerobic systems, especially in the treatment of high-strength organic effluents (Zhou *et al.*, 2006 & 2007). Optimum performance is, however, dependent on the development of a dense, well-settleable granular sludge bed (Schmidt & Ahring, 1996; El-Mamouni *et al.*, 1998; Tay *et al.*, 2000; Ghangrekar *et al.*, 2005; Tiwari *et al.*, 2006; Zhou *et al.*, 2007). Although UASB reactors can be operated with flocculent sludge, granulation of flocculent sludge offers significant operational advantages. Granular sludge has higher settling velocities, shear strength and resistance to environmental shocks and toxins as well as reduced limitations on inter-species metabolite transfer between syntrophic groups (Wirtz & Dague, 1996; Ghangrekar *et al.*, 2005; Tiwari *et al.*, 2006; Zhou *et al.*, 2007). The use of surplus granular sludge from functioning UASB reactors as seed sludge for reactor start-up would, therefore, result in better sludge retention under higher loading rates, higher methanogenic activities as well as better process stability. Reactor start-up periods would be significantly reduced and high removal efficiencies would be reached in a short time (Goodwin *et al.*, 1992).

Although this is the preferred option in developed countries where industrial-scale reactors are fairly common, access to high-quality granular sludge is still limited in developing countries (Lamprecht & Britz, 2007). Under these conditions the most commonly used seed material is digested sewage sludge, but this usually results in longer start-up periods due to the increased washout of finely dispersed flocculent sludge particles as the upflow velocity is increased. Continuous washout of flocculent sludge would ultimately require continuous reactor reinoculation. Problems associated with this kind of start-up could be reduced by improving digested sludge retention and ultimately enhancing sludge granulation in a UASB reactor.

Effective short-term treatment of digested sludge to enhance granulation in batch systems has been reported by Britz *et al.* (2002), but their study did not include an evaluation of the performance of the enhanced digested sludge during UASB reactor start-up. The aim of this study was to determine whether short-term pre-treatment of digested sludge in laboratory batch granulation-enhancement systems prior to UASB inoculation could reduce UASB reactor start-up by improving anaerobic digestion efficiency, sludge retention as well as sludge granulation.

Materials and methods

In this study three identical laboratory (lab)-scale UASB reactors were seeded with either enhanced or un-enhanced digested seed sludge before reactor start-up proceeded.

Preparation of UASB reactor inocula

The results presented in the previous chapter (Chapter 7) of this dissertation showed that the digested sludge collected from the Athlone 4Sb digester at the Athlone Municipal Wastewater Treatment Works (WWTW) was the best seed sludge choice during the normal UASB reactor start-up studies conducted. The same digested sludge was, therefore, used in this study. The sludge was screened through a 1-mm² sieve and used directly as inoculum for UASB reactor A (control reactor). However, for UASB reactors B and C, the sieved sludge was pre-treated for 17 d in a cylindrical batch reactor (with an operational volume of 5.4 L) that was designed by the Department of Process Engineering, University of Stellenbosch (Fig. 1). The cylindrical reactor rotated around a horizontal axis and was designed to simulate a rolling motion (speed: 2 rpm). Twelve baffles were mounted on the inside of the cylinder to minimise biofilm formation (Els *et al.*, 2005). Biogas exited continuously through a biogas vent that was connected to a wet gas meter equipped with an electronically controlled counter. The gas meter had a built-in water-filled U-tube, and biogas left the reactor as single air bubbles via this route once the internal air pressure exceeded atmospheric pressure. The methane (CH₄) content of the biogas was determined using a gas chromatograph. All the batch granulation-enhancement experiments were conducted in a temperature-controlled room at 35°C. In order to achieve an organic loading rate (OLR) of 2 kg COD.m⁻³.d⁻¹, 1.2 L of the operational volume was replaced daily with fresh substrate after a short period of sludge settling had been allowed. The effluent pH and biogas production were monitored daily to confirm process stability.

The seed sludge for UASB reactor C was prepared by combining three batches of batch granulation-enhanced (pre-treated) lactate-fed digested sludge (lactate seed sludge). During batch granulation-enhancement each of the sludge batches was incubated in the presence of a yeast extract-lactate substrate in the rolling-batch reactor for 17 d. The yeast extract-lactate substrate consisted of the following (g.L⁻¹): sodium



Figure 1 Cylindrical reactor used in this study for the pre-treatment of the UASB reactor inocula for reactors B and C.

lactate (Sigma) 10.0; yeast extract (Biolab) 5.0; peptone (Biolab) 2.0; KH_2PO_4 (BDH) 10.0; NaHCO_3 (Saarchem) 1.2; and Tween 80 (Merck) 1.0 mL. The pH was adjusted to 7.0. The substrate had a total COD of 10 300 mg.L^{-1} to which the lactate carbon source contributed 3 200 mg.L^{-1} . Initial inoculation for all three batches was at a ratio of 1:3.5 of sludge:substrate.

The seed sludge for UASB reactor B was prepared by combining three batches of pre-treated sucrose-fed digested sludge (sucrose seed sludge). Each sludge batch was incubated in the presence of a yeast extract-sucrose substrate in the rolling-batch reactor for 17 d. The yeast extract-sucrose substrate consisted of the following (g.L^{-1}): sucrose 2.5; yeast extract (Biolab) 5.0; peptone (Biolab) 2.0; KH_2PO_4 (BDH) 10.0; NaHCO_3 (Saarchem) 1.2; and Tween 80 (Merck) 1.0 mL. The pH was adjusted to 7.0. The substrate had a total COD of 9 700 mg.L^{-1} to which the sucrose carbon source contributed 3 200 mg.L^{-1} . The initial inoculation of the first batch was at a ratio of 1:3.5 of sludge:substrate.

Volatile fatty acid analysis

The volatile fatty acid (VFA) content of the effluent was determined during each of the batch granulation-enhancement experiments. This was done using a Varian (model 3700) gas chromatograph equipped with a flame ionisation detector and a 30 m x 0.53 mm Nukol, 0.5 μm fused silica capillary column (Supelco, USA). The column temperature was initially maintained at 105°C for 2 min before it was increased at a rate of 8°C per min to 190°C where it was maintained for 10 min. The detector and inlet temperatures were set at 300°C and 130°C respectively and nitrogen was used as carrier gas at a flow rate of 2.5 mL.min^{-1} (Sigge, 2005).

BorwinTM computer software (JMBS Developpements, France) was used to quantitatively determine the presence of VFAs by integrating the peak areas, using an internal standard calibration. Identification of the unknown compounds was achieved by comparing their retention times to those of analytical-grade standard VFAs. An aqueous stock solution was prepared as standard and contained 0.5 mL.L^{-1} of hexanol (BDH) as internal standard and 1 mL.L^{-1} of each of the following fatty acids: acetic (Merck), propionic (Merck), iso-butyric (Sigma), butyric (Merck), iso-valeric (Hopkin & Williams Ltd.) and valeric acid (Aldrich).

UASB reactor start-up

Three identical lab-scale UASB reactors (A, B and C), with operational volumes of 2.4 L, were operated under similar conditions for at least 80 d. Sludge inoculation volumes were adjusted so that all the reactors had a starting concentration of 7.8 g.L⁻¹ VSS. Reactor temperatures were maintained at 35°C. The upflow velocity of all the UASB reactors was 1.0 m.h⁻¹ at the start and then increased during the study to reach a final upflow velocity of 2.0 m.h⁻¹.

All the UASB reactors were initially fed a sterilised synthetic sucrose-based substrate with a COD value of 2 500 mg.L⁻¹ and pH of 7.2. The substrate consisted of the following (g.L⁻¹): sucrose 1.36; sodium lactate (Sigma) 0.2; peptone (Biolab) 0.4; beef extract (Biolab) 0.28; NH₄Cl (Saarchem) 0.32; KH₂PO₄ (BDH) 0.08; FeSO₄.7H₂O (Saarchem) 0.02; and NaHCO₃ (Saarchem) 1.2. The reactor feed of UASB reactor C was, however, changed during the study to a lactate-based feed (3 200 mg.L⁻¹ COD) with the following composition (g.L⁻¹): sodium lactate (Sigma) 5.62; peptone (Biolab) 0.4; beef extract (Biolab) 0.28; NH₄Cl (Saarchem) 0.32; KH₂PO₄ (BDH) 0.08; FeSO₄.7H₂O (Saarchem) 0.02; and NaHCO₃ (Saarchem) 1.2. A trace mineral solution (Nel *et al.*, 1985) was added directly to each reactor once a week at a concentration of 1 mL per litre reactor volume.

The HRT was reduced individually for each reactor once stable-state conditions (less than 10% variation in percentage COD removal) (Sigge, 2005) had been reached. Efficiency parameters were pH, COD (total and centrifuged), alkalinity as well as total suspended solids (TSS) and VSS content. These were determined according to Standard Methods (1998). The biogas volume produced (mL. d⁻¹) for each UASB reactor was determined daily with a wet gas meter equipped with an electronically controlled counter. The methane content of the biogas was determined a gas chromatograph (Sigge *et al.*, 2005).

Sludge analysis

The UASB reactor inoculum sludges as well as sludge from each of the three UASB reactors (A, B and C) at the end of the study were compared in terms of total VSS content, biogas production activity per gram of centrifuged sludge as well as the size distribution of the VSS content with a particle size range of 0.25 – 1.0 mm². Size

distribution (Laguna *et al.*, 1999) involved the use of four sieves (0.25, 0.50, 0.71 and 1.00 mm²) to separate the anaerobic sludge into fractions. The VSS content of each fraction was determined.

Activity tests

Biogas production activity of the UASB reactor inoculum sludges as well as the UASB reactor sludge at the end of the operational period was determined by using the activity test method developed by O'Kennedy (2000). The method was as follows: 3.0 g of sludge was incubated with 13 mL of basic test medium (BTM) (medium of Valcke & Verstraete (1993)) plus 2.0 g.L⁻¹ glucose and pH set at 7.0, in 20 mL test vials sealed with butyl rubber septa and aluminium crimp caps.

In addition to the BTM, four other test media were also prepared for this study: glucose test medium (GTM) (BTM + 2 g.L⁻¹ glucose); lactate test medium (LTM) (BTM + 4 g.L⁻¹ lactate); acetate test medium (ATM) (BTM + 1 g.L⁻¹ acetate); and formate test medium (FTM) (BTM + 1 g.L⁻¹ formate). The vials were prepared in triplicate for each test medium and incubated at 35°C for 24 h.

Biogas production (in mL) was measured at 5 h, 10 h and 25 h intervals by using a free-moving plunger in a 5 mL syringe equipped with a 25-gauge needle. The methane and CO₂ compositions were determined with a Fisons 3300 gas chromatograph (Fisons Instruments, USA) equipped with a thermal conductivity detector and 2.0 x 3.0 mm i.d. column packed with Hayesep Q (Supelco, Bellefonte, PA) 80/100 mesh. The oven temperature was set at 55°C and helium was used as carrier gas at a flow rate of 30 mL.min⁻¹.

Results and discussion

Preparation of UASB reactor inocula

Culturing conditions

The purpose of the short-term (17 d) pre-treatment process in the rolling-batch reactor was to improve seed sludge characteristics prior to UASB reactor inoculation. This was done by incubating digested sludge under conditions of slight organic overloading (organic loading rate [OLR] = 2 kg COD.m⁻³.d⁻¹ from the first day) in a controlled,

mechanically agitated environment. Although the sludge was allowed to settle daily before substrate replenishment, washout still occurred but never to such an extent that reinoculation was necessary to ensure process stability.

The substrates used during batch granulation-enhancement were nutrient rich. Yeast extract was included as it is an excellent source of Vitamin B and organic nitrogen and promotes cell growth (Singh *et al.*, 1999). As carbon source either lactate or sucrose was included. The choice of lactate as carbon source was based on the fact that UASB granules with large populations of lactate-utilising bacteria also had high extracellular polymer (ECP) contents and cell densities (Fukuzaki *et al.*, 1991). Several strains of lactate-utilising, propionate-producing bacteria that were isolated from anaerobic processes have also been shown to produce excessive amounts of ECP (Riedel & Britz, 1993; Slobodkin & Verstraete, 1993). An ECP-producing *Propionibacterium* strain has also been shown to have aggregate-forming abilities (Chapter 3). It has been hypothesised that these organisms can gain a competitive advantage if lactate is used to induce an organic overload, which would ultimately contribute to the process of granulation (Riedel & Britz, 1993; Britz *et al.*, 2002). Sucrose (di-saccharide consisting of two monosaccharides, glucose and fructose) was also included in this study since it has been reported that sucrose and glucose-fed UASB granules are typically large and have high concentrations of ECP (Fukuzaki *et al.*, 1995; Zhou *et al.*, 2006). Sucrose is also a cheaper carbon source than glucose or lactate, which would be of importance for future scaling-up of the process.

Lactate-fed batch granulation-enhancement

The pH and alkalinity profiles of three sludge batches pre-treated in the rolling-batch reactor in the presence of the lactate-containing substrate (batches 1 - 3) are presented in Fig. 2. Batch 2 reached its minimum pH on day 2 (D2) after which it recovered to pH levels above 6.75 from D4 onwards. In contrast, batches 1 and 3 reached minimum levels on days 4 and 5 and recovered to pH levels above 6.75 only after D11. Alkalinity profiles for all three batches remained stable between 6 000 and 7 000 mg CaCO₃.L⁻¹. No additional pH-controlling measures (such as additional NaHCO₃) were necessary since all three batches recovered after the initial organic overload applied at the start of each batch study (inoculation ratio 1:3.5 of sludge:substrate) in spite of the constant daily organic load of 2 kg COD.m⁻³.d⁻¹.

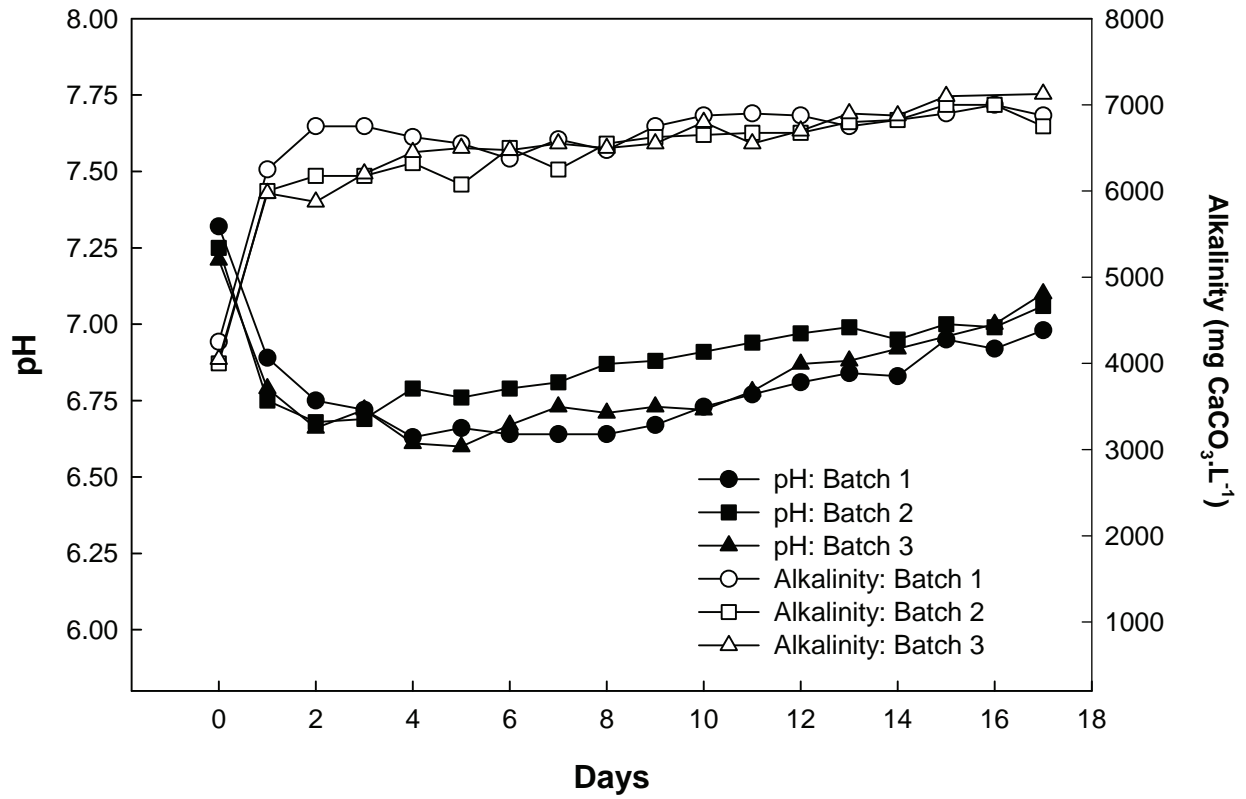


Figure 2 Alkalinity and pH profiles of the lactate-fed batch granulation-enhancement batches (nos. 1 - 3) conducted in the rolling-batch reactor.

The VFA content in the effluents of all three batches (Fig. 3) showed that acetic acid was the most abundant VFA until D8, after which levels decreased rapidly. Propionic acid levels increased slightly and propionic acid was the dominant VFA in the three batches between D10 and D17. Although the ratio of propionic acid > acetic acid could be typical of lactate-utilising, propionate-producing species such as *Propionibacterium* (Holdeman *et al.*, 1977), a definite shift towards a propionate-producing population was not evident in any of the three batches. Other VFAs were detected at low concentrations (iso-butyric acid <200 mg.L⁻¹; iso-valeric and valeric acids <300 mg.L⁻¹) and were not presented.

The pre-treated sludges from batches 1, 2 and 3 were combined and used as inoculum for UASB reactor C.

Sucrose-fed batch granulation-enhancement

The pH and alkalinity profiles of the three sludge batches pre-treated in the rolling-batch reactor in the presence of the sucrose-containing substrate (batches 5 - 7) are presented in Fig. 4. In contrast to the lactate-fed batches, pH control for these batches was problematic with only batch 7 recovering to pH levels above 6.75 after 7 d.

The first sucrose batch (batch 5) was initially inoculated using the same inoculation ratio as the lactate batches (1:3.5 of sludge: substrate). This proved to be too high for the system to recover by itself and the reactor was not fed on days 1 - 3, 7 and 9 to allow for VFA removal by acetogenic and methanogenic populations. In addition, NaHCO₃ had to be added to prevent system failure. These additions were 1.2 g per litre reactor volume (Lrv) on days 2 - 4, decreasing to 0.4 g.(Lrv)⁻¹ after D5 and 0.2 g.(Lrv)⁻¹ after D9. This probably resulted in the higher alkalinity profile observed for batch 5 in spite of it showing the lowest pH profile of all three the sucrose-fed batches. For batches 6 and 7 the initial inoculation ratio was adjusted to 1:1:2.5 of sludge:substrate:water. The reactor was not fed on days 3, 5 and 8 (for batch 6) and on D3 for batch 7. Batches 6 and 7 also required NaHCO₃ addition but not to the same extent as was required for batch 5. For batch 6, 0.4 g.(Lrv)⁻¹ was added on the days that the reactor was not fed, and 0.2 g.(Lrv)⁻¹ was added on all the other days. Batch 7 required only 0.2 g.(Lrv)⁻¹ NaHCO₃ and only on days 1 - 8. After batch 7 a thick biofilm was observed inside the reactor, as illustrated in Fig. 5. A part of this biofilm was probably already established after batch 6 and might account for the better pH recovery observed for batch 7 (Fig. 4).

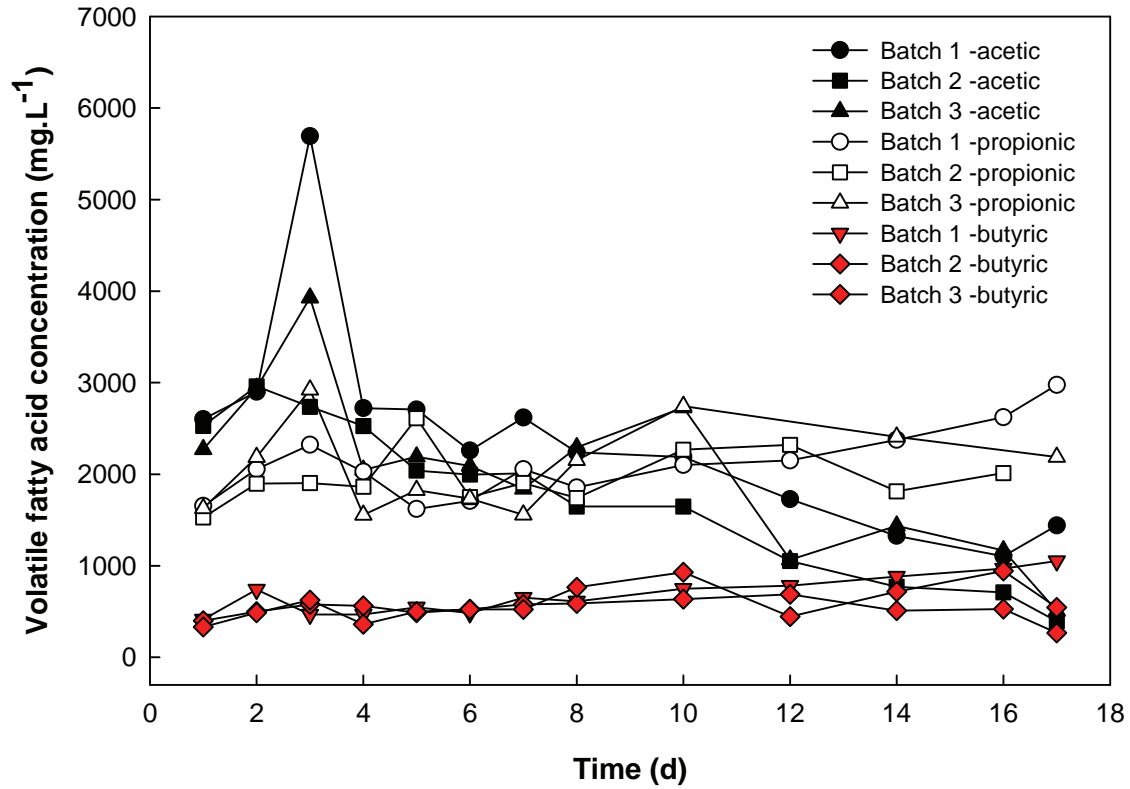


Figure 3 Volatile fatty acid content (in particular Acetic, Propionic and Butyric acids) in the daily effluents collected during the lactate-fed batch granulation-enhancement batches (nos. 1 - 3) conducted in the rolling-batch reactor.

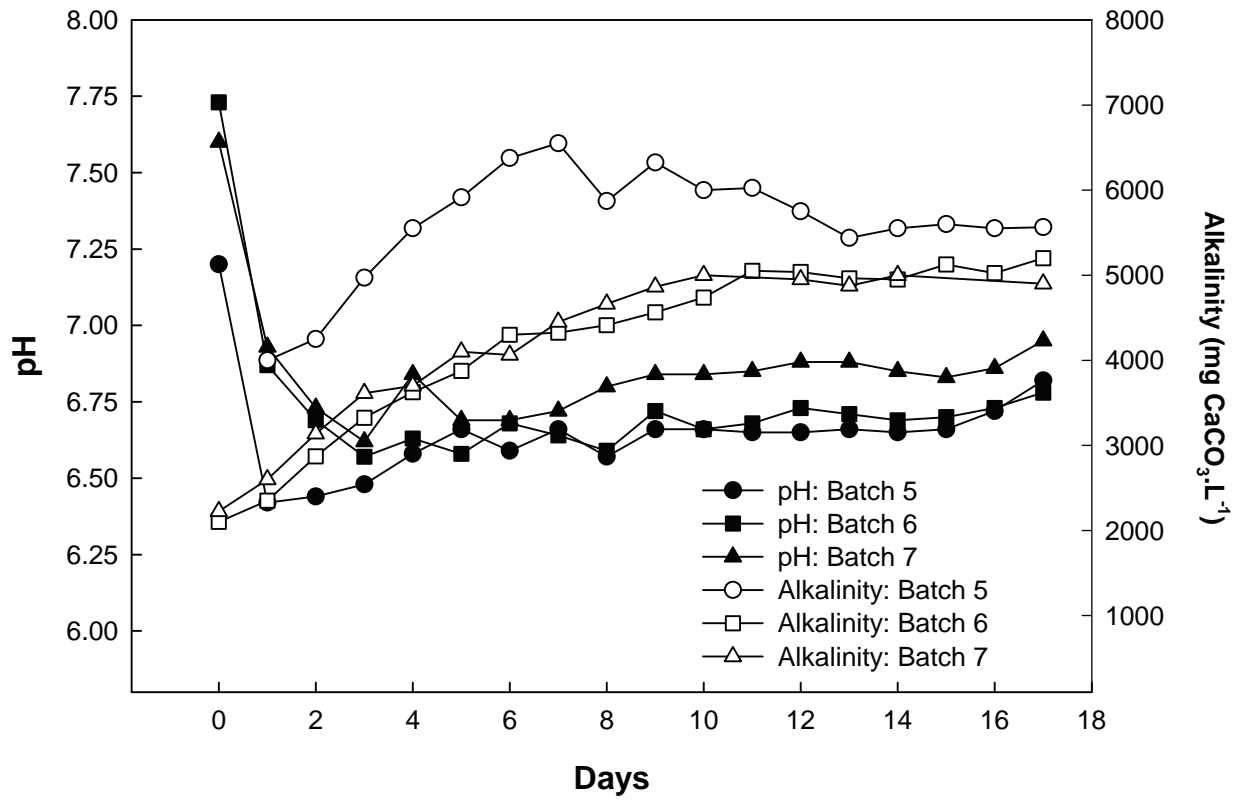


Figure 4 Alkalinity and pH profiles of the sucrose-fed batch granulation-enhancement batches (nos. 5 - 7) conducted in the rolling-batch reactor.



Figure 5 Biofilm formation observed on the inside of the rolling batch reactor after batch granulation-enhancement batch 7.

The VFA content of the sucrose-fed batches is presented in Figs. 6a and b. Overall, the VFA content of the sucrose-fed sludge effluents proved to be more complex than the VFA content of the lactate-fed sludge effluents (Fig. 3). This may probably be an indirect indication of the larger number of acidogenic species able to metabolise sucrose. An excessive amount of acetic acid ($>4\ 000\ \text{mg.L}^{-1}$) was present up to D10 in batch 5. In comparison with the other two sucrose-fed batches, the batch 5 effluent had the highest concentrations of acetic acid (up to D14) as well as the highest concentrations of butyric, iso-butyric and valeric acids (up to D17), which shows why pH control was problematic.

Propionic acid was detected during all the sucrose-fed batches (batches 5 - 7) but, in contrast to the lactate-fed batches, only at levels below $1\ 200\ \text{mg.L}^{-1}$. Although propionate-producing bacteria can utilise glucose, lactate is the preferred substrate for these bacteria (Gerardi, 2003). Lactate is also a common product of anaerobic fermentation since lactate-forming bacteria are highly saccharolytic (Gerardi, 2003). Therefore, it could be that lactate was produced as fermentation product during the sucrose-fed pre-treatment batches (batches 5 - 7) and that this lactate was utilised by propionate-forming bacteria.

Batch 7 was the only sucrose-fed batch during which the propionic acid levels were higher than the acetic acid levels for part of the study (D12 - D17). This was not considered to be the result of a population shift to a propionate-producing population such as *Propionibacterium*. The propionic acid levels did not increase during D12 - D17 and remained at the same levels as observed earlier during batch 7 (D4 - D10), which did not indicate any increase in activity of the propionate-producing populations. There was, however, a rapid decrease in the acetic acid concentration in batch 7 during D12 - D17, which may be attributed to an increase in acetoclastic methanogenic activity under the environmental conditions that prevailed during the latter stages of batch 7.

Biogas and methane production

Biogas volumes produced as well as the methane content of the biogas are presented in Figs. 7a and b, respectively. Overall increases in biogas volumes were observed for all the sludge batches. The highest volume of biogas and the highest methane content were observed in batch 2 (for the three lactate batches) and in batch 7 (for the three sucrose batches). Since biofilm formation was observed (Fig. 5) the higher biogas and

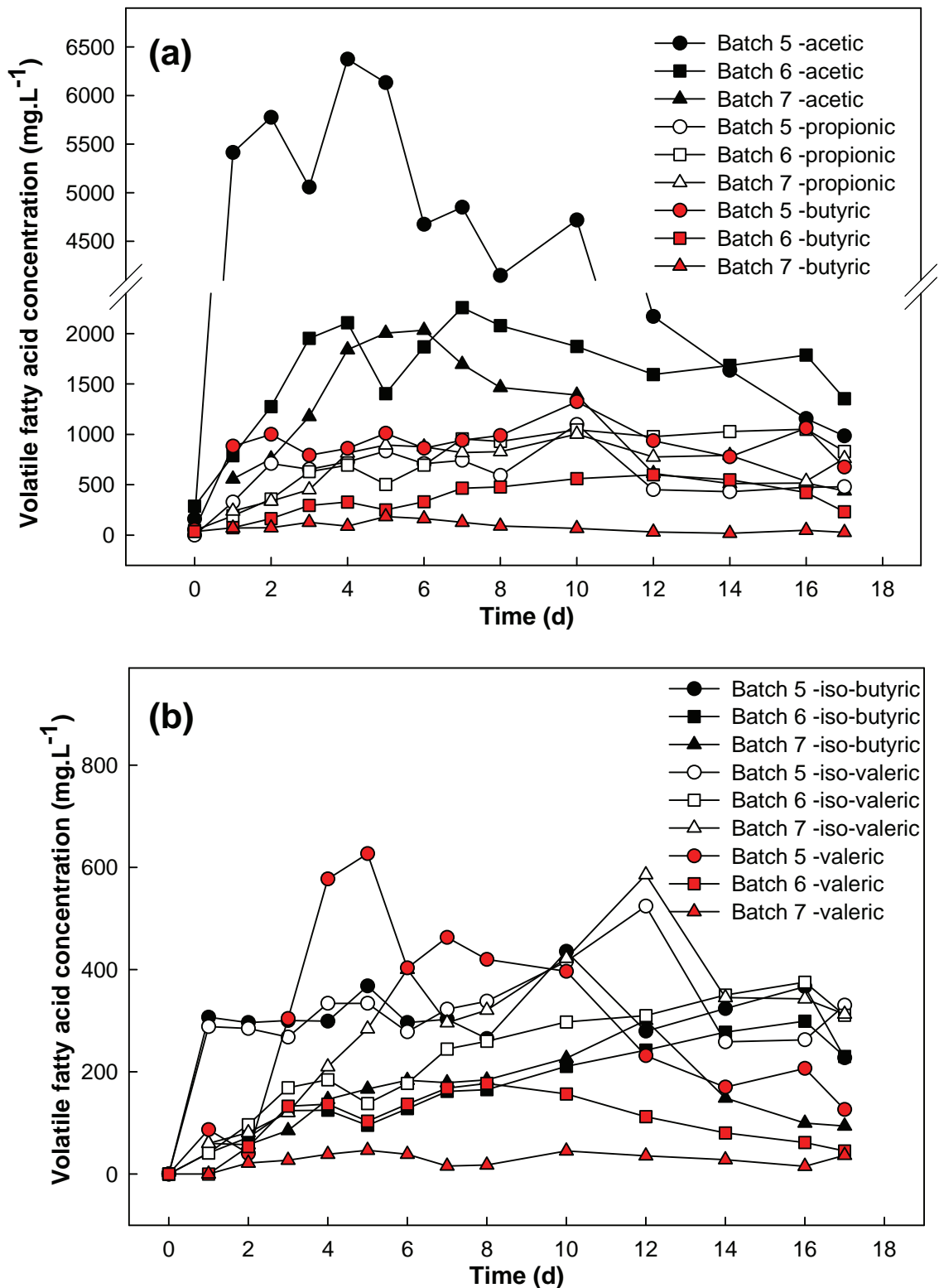


Figure 6 Volatile fatty acid content in the daily effluents collected during the sucrose-fed batch granulation-enhancement batches (nos. 5 - 7) conducted in the rolling batch reactor. (a) Acetic, propionic and butyric acid concentrations; and (b) iso-butyric, iso-valeric and valeric acid concentrations.

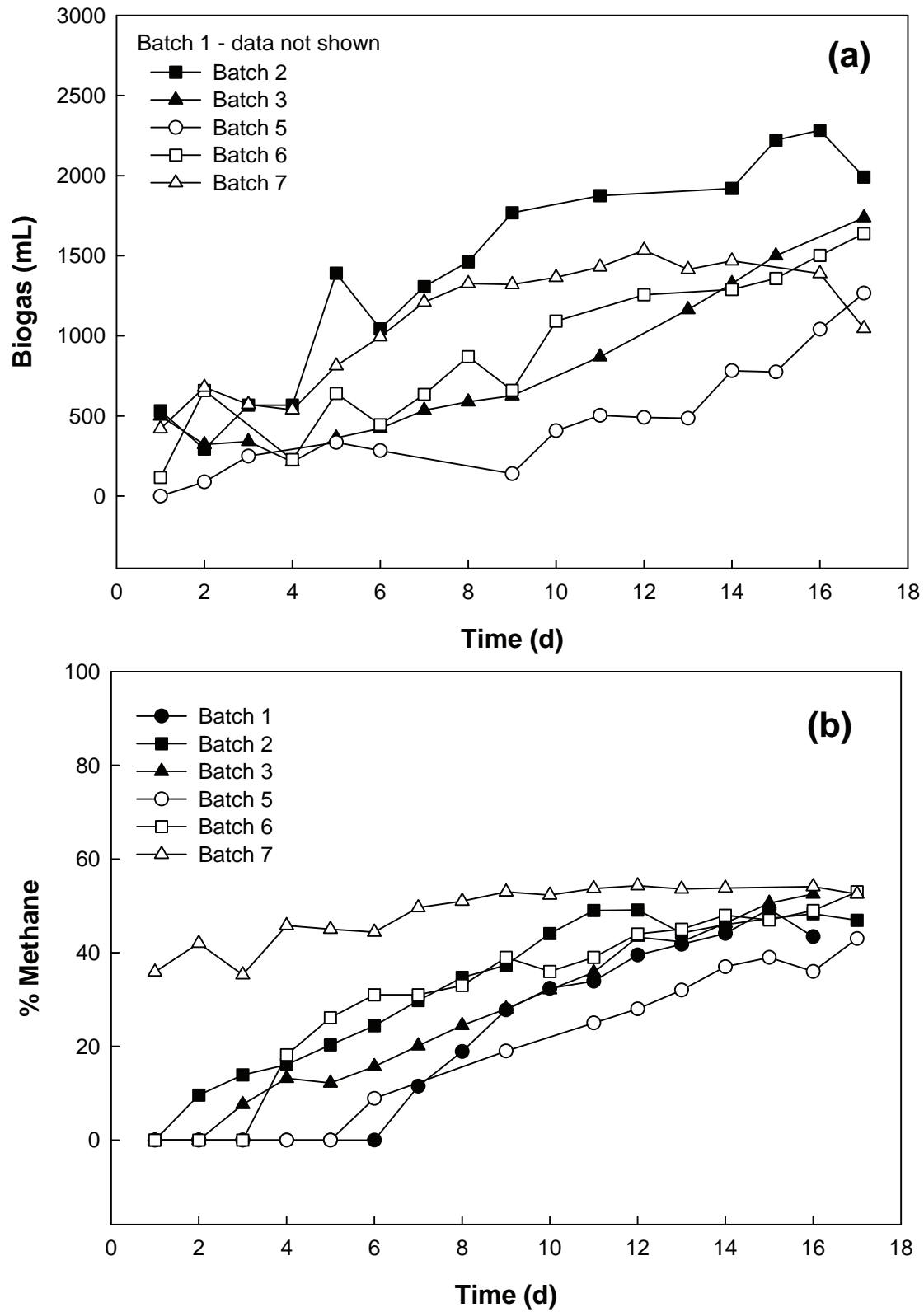


Figure 7 Biogas production volumes (a) and methane content (b) of all the batch granulation-enhancement experiments in the rolling-batch reactor.

methane production might be ascribed to a well-established methanogenic population in the biofilm.

Activity testing

Biogas and methane production activities of the inoculum sludges for UASB reactors B (sucrose seed sludge) and C (lactate seed sludge) were determined using the substrate-dependent activity test method of O'Kennedy (2000). (For data on reactor A inoculum sludge see Chapter 6.) The results clearly show that the sucrose seed sludge showed higher cumulative biogas and methane production than the lactate seed sludge in all the substrates tested (Figs. 8 and 9).

In order to determine the activities of specific populations the activity measured in the BTM samples (that contained 2 g.L⁻¹ glucose) was compared to the activities measured in the other four test media (that contained 2 g.L⁻¹ glucose and one other carbon source). Methanogenic activity in the BTM samples gave an indication of the overall activity of the sludge while activity in the other test media provided more information regarding specific anaerobic populations. Higher activities in the presence of a test medium containing glucose and additional carbon source, when compared to the activity measured in the BTM samples, would indicate that the specific population necessary for the metabolisation of the additional carbon source is active and present at high enough levels in the sludge so that the anaerobic community can manage to metabolise 2 g.L⁻¹ glucose as well as the added carbon source.

The sucrose seed sludge showed higher concentrations of methane in ATM and FTM (Fig. 9). This indicated that both the acetoclastic methanogens (ATM) and the hydrogenotrophic methanogens (FTM) in the sludge were active enough to metabolise the added acetate (ATM) and formate (FTM) as well as the acetate and formate that would result from the normal anaerobic digestion of glucose. The lactate seed sludge only showed a slightly higher activity in the FTM, which shows that pre-treatment of the sludge in the presence of the lactate-containing substrate did not contribute to the enhancement of the acetoclastic methanogenic population, as was observed for the sucrose seed sludge.

The sucrose seed sludge also showed much higher biogas (Fig. 8) and methane (Fig. 9) production activities in LTM when compared to BTM than what was observed for the lactate seed sludge. The production of methane from lactate is a multi-step process that involves lactate-utilising acidogens as well as propionate- and butyrate-consuming

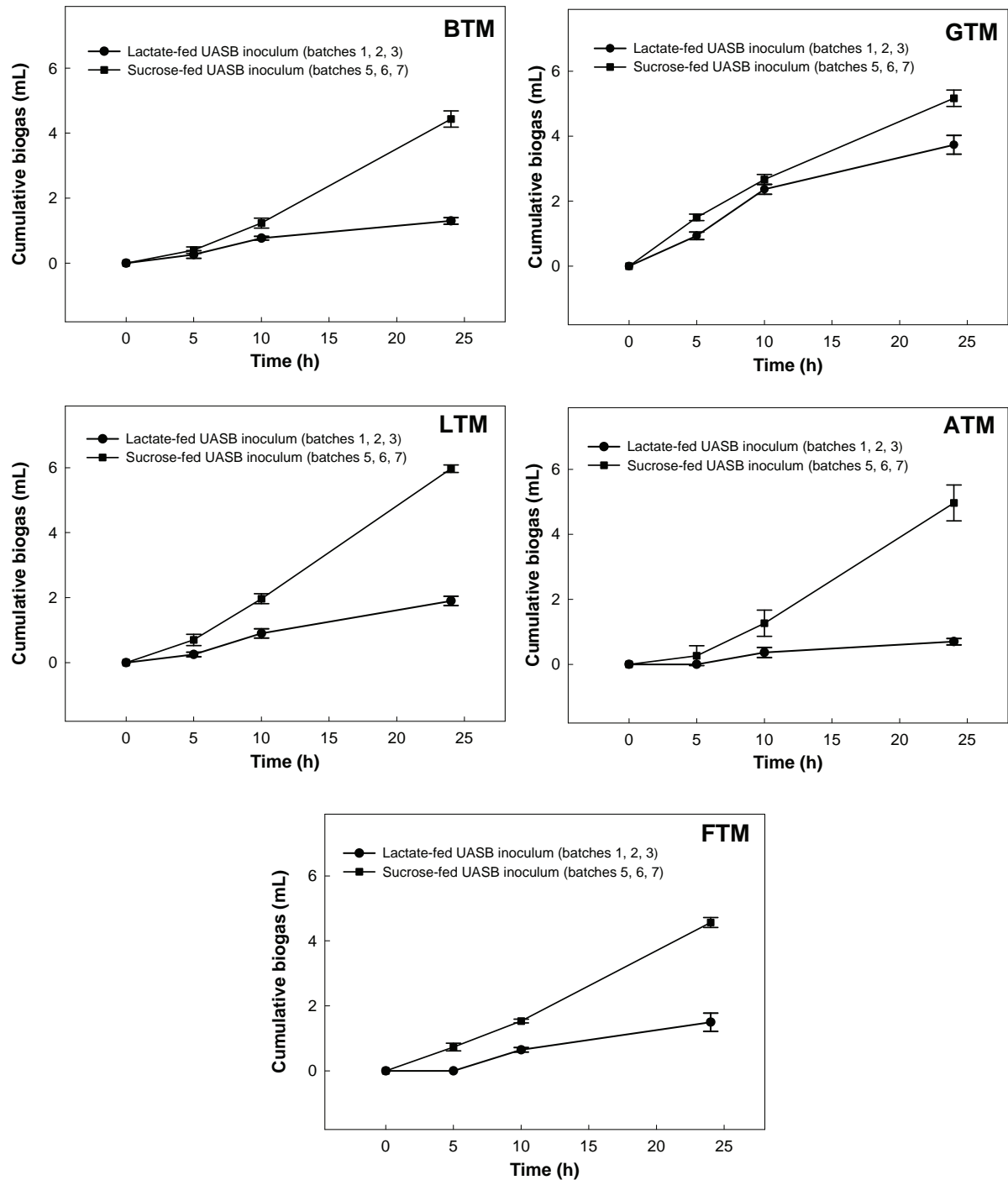


Figure 8 Cumulative biogas production over 24 h by lactate-fed UASB seed sludge (averages of batches 1 - 3) and sucrose-fed UASB seed sludge (averages of batches 5 - 7) before UASB reactor inoculation. Each data point represents triplicate values, and the standard deviation was used as the error bar. BTM - basic test medium; GTM - glucose test medium; LTM - lactate test medium; ATM - acetate test medium; FTM - formate test medium.

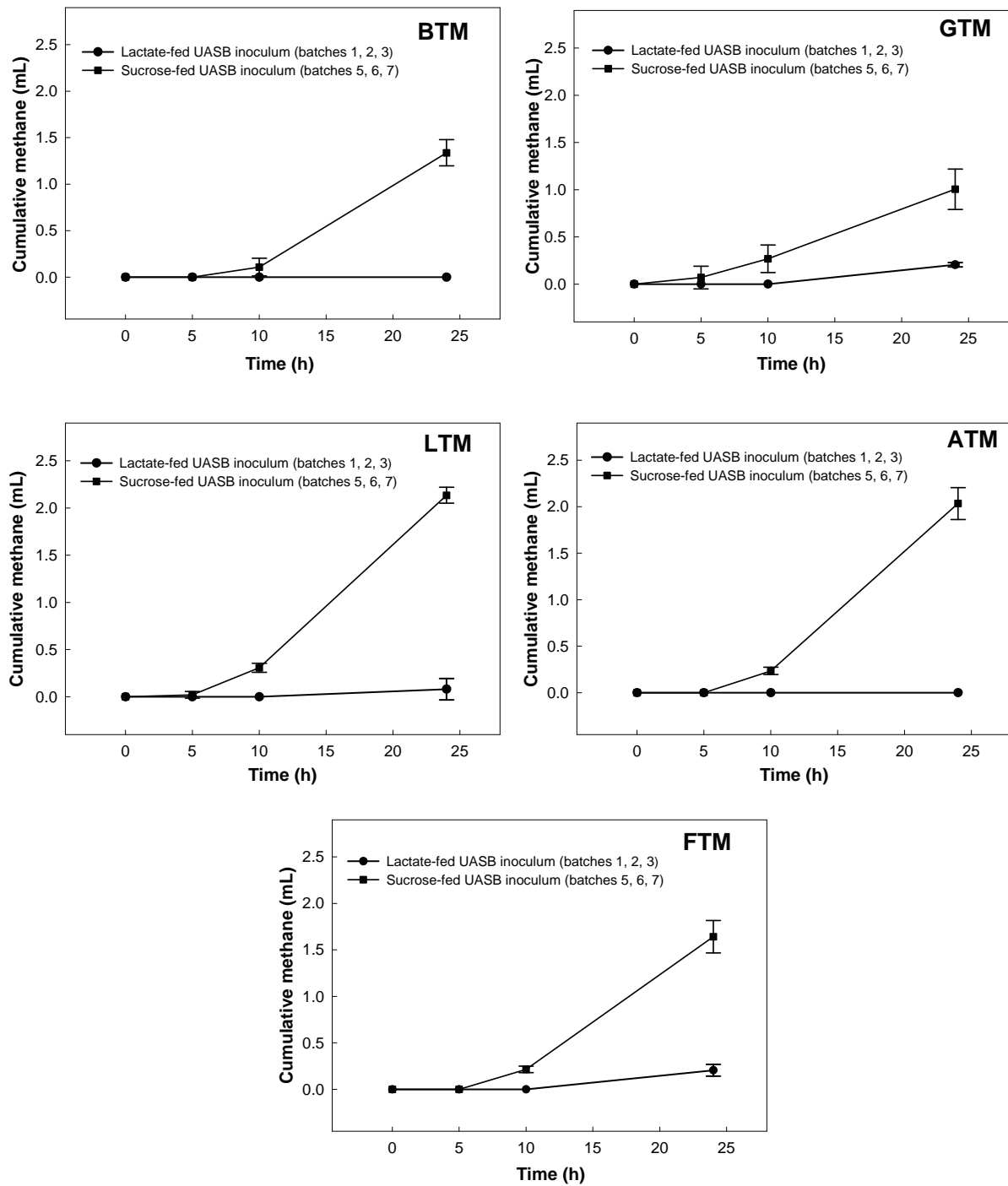


Figure 9 Cumulative methane production over 24 h by lactate-fed UASB seed sludge (averages of batches 1 - 3) and sucrose-fed UASB seed sludge (averages of batches 5 - 7) before UASB reactor inoculation. Each data point represents triplicate values, and the standard deviation was used as the error bar. BTM - basic test medium; GTM - glucose test medium; LTM - lactate test medium; ATM - acetate test medium; FTM - formate test medium.

acetogens that are usually closely associated with hydrogenotrophic and acetoclastic methanogens. The higher activities observed in the sucrose seed sludge in the presence of lactate, therefore, suggest that all the populations involved, including the lactate-utilising acidogens, must have been enhanced during the pre-treatment of the sludge batches in the presence of sucrose.

The lactate seed sludge did show slightly higher biogas volumes after 24 h in LTM when compared to BTM, although no increases were observed in methane volumes. Lactate-utilising acidogens such as *Propionibacterium*, *Veillonella* and *Clostridium* ferment lactate and produce metabolic products such as propionate, acetate, butyrate and CO₂ (Gerardi, 2003). Biogas volumes that increased while no increases were noted in methane volumes might, therefore, be the direct result of the CO₂ produced by these acidogens. It further indicates that although active lactate-utilising populations were present, all the acetogenic and methanogenic populations necessary for removal of the fermentation products from both the lactate and glucose present in the LTM were not active enough.

UASB reactor inoculation

After the batch granulation-enhancement phase was completed, UASB reactor start-up with the enhanced sludges followed. Reactor C was inoculated with the combined sludge from the lactate-fed batches (batches 1 - 3), reactor B was inoculated with the combined sludge from the sucrose-fed batches (batches 5 - 6), and reactor A, the control reactor, was inoculated with the raw and un-enhanced digested sludge. Sludge inoculation volumes were adjusted so that all the reactors had a starting concentration of 7.8 g.L⁻¹ VSS.

The TSS and VSS content of the original sludge batches as well as the TSS and VSS concentrations in UASB reactors A, B and C at start-up are presented in Fig. 10. Although all the reactors had the same starting VSS concentration, differences still existed with regard to TSS concentrations. Since TSS consists of both VSS and fixed (non-volatile) suspended solids (FSS), higher TSS:VSS ratios would indicate that the sludge had a higher FSS content. The inoculum of the control reactor (reactor A) had a higher TSS:VSS ratio than the enhanced inocula used for reactors B and C. This could indicate that during the batch granulation-enhancement process either more FSS than

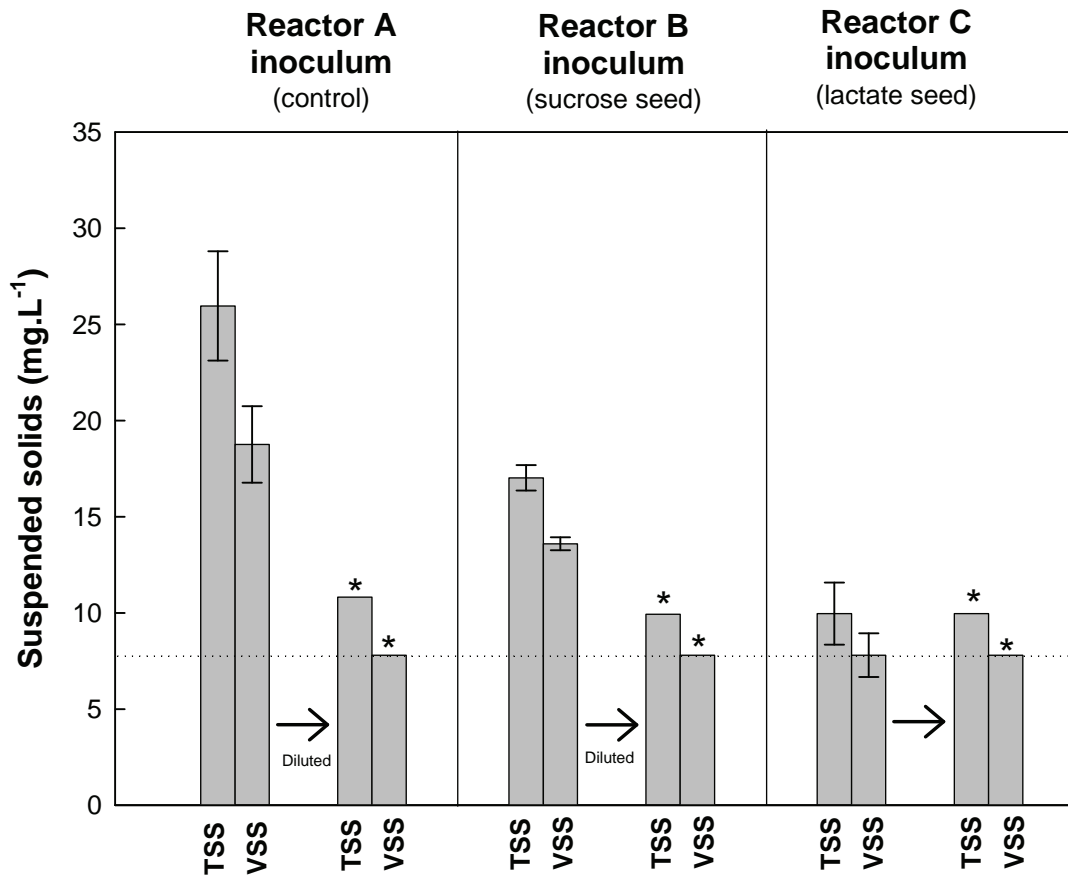


Figure 10 Total suspended solids (TSS) and volatile suspended solids (VSS) content of the different inocula. The (*) indicates the TSS and VSS concentrations at start-up of the individual reactors. The dotted line indicates the standardised VSS starting concentrations in UASB reactors A, B and C. Each data point represents duplicate values, and the standard error was used as the error bar.

VSS was lost due to washout or that the VSS content of both the lactate and sucrose seed sludges had increased.

The lactate seed sludge had the lowest TSS and VSS concentrations and required no dilution for the inoculation of reactor C. The sucrose seed sludge used to inoculate reactor B had to be diluted at start-up since it had higher TSS and VSS concentrations than the lactate seed sludge. This indicated that more suspended solids were retained during batch granulation-enhancement in the presence of sucrose than in the presence of lactate. It could be that more ECPs were produced during the sucrose-fed batches, which would have contributed to the biofilm formation observed (Fig. 5). The biofilm might, in turn, have contributed to higher TSS and VSS contents in the sucrose seed sludge.

The size fractions of the VSS content $\geq 0.25 \text{ mm}^2$ present in each UASB reactor at start-up are presented in Fig. 11a. The reactor B inoculum (sucrose seed) clearly showed higher concentrations in the four size fractions analysed than both the inoculums for reactors A and C. Most of the VSS particles $\geq 0.25 \text{ mm}^2$ were in the size range $0.25 - 0.49 \text{ mm}^2$.

Although the VSS size fractions of the lactate seed sludge used as inoculum for reactor C were all within the standard deviation range of the fractions present in the control reactor (reactor A), the reactor C values were slightly higher than those of reactor A. This could indicate that the VSS washout that occurred during the batch granulation-enhancement processes was mostly of smaller VSS particles ($< 0.25 \text{ mm}^2$). In fact, the significant increase observed in the size range $0.25 - 0.49 \text{ mm}^2$ of the sucrose seed sludge during batch granulation-enhancement could be the result of the selection pressure applied in the mechanically agitated environment in the presence of the sucrose-based substrate. These specific conditions might have improved the clumping of smaller VSS particles ($< 0.25 \text{ mm}^2$) to form slightly larger aggregates within the $0.25 - 0.49 \text{ mm}^2$ range.

UASB reactor efficiency

The UASB reactors were operated under similar conditions and were all initially fed with a sucrose-based substrate. In reactors B and C, sludge floatation or foaming occurred during the first few days (Fig. 12). In reactor B this continued for only 2 - 3 d, but in reactor C foaming led to excessive sludge washout. Foaming decreased in reactor C

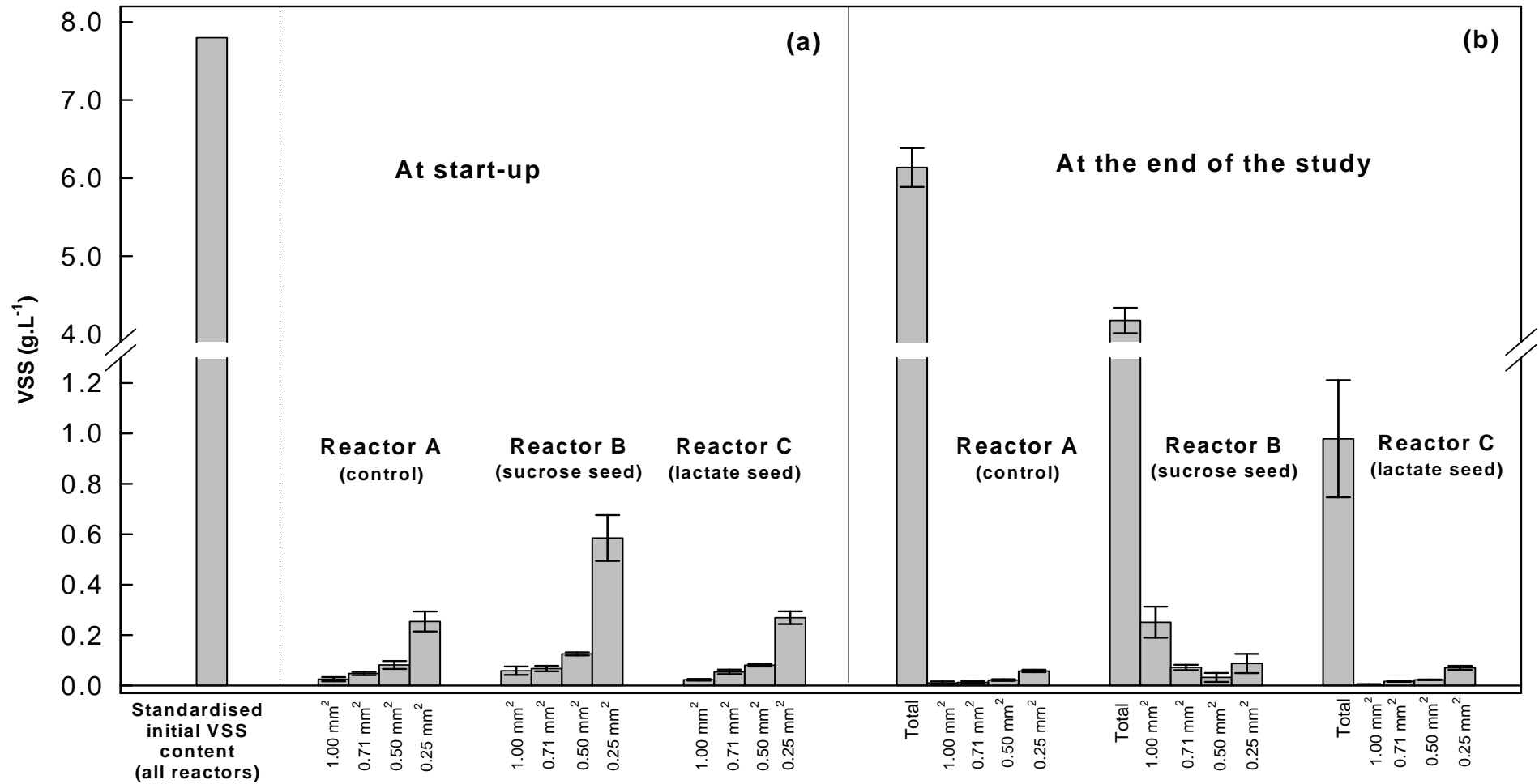


Figure 11 VSS content (a) at start-up and (b) at the end of the study of the three UASB reactors (A, B and C), as well as the size distribution fractions of all VSS particles ≥ 0.25 mm². Each data point represents triplicate values and error bars indicate standard deviation values.

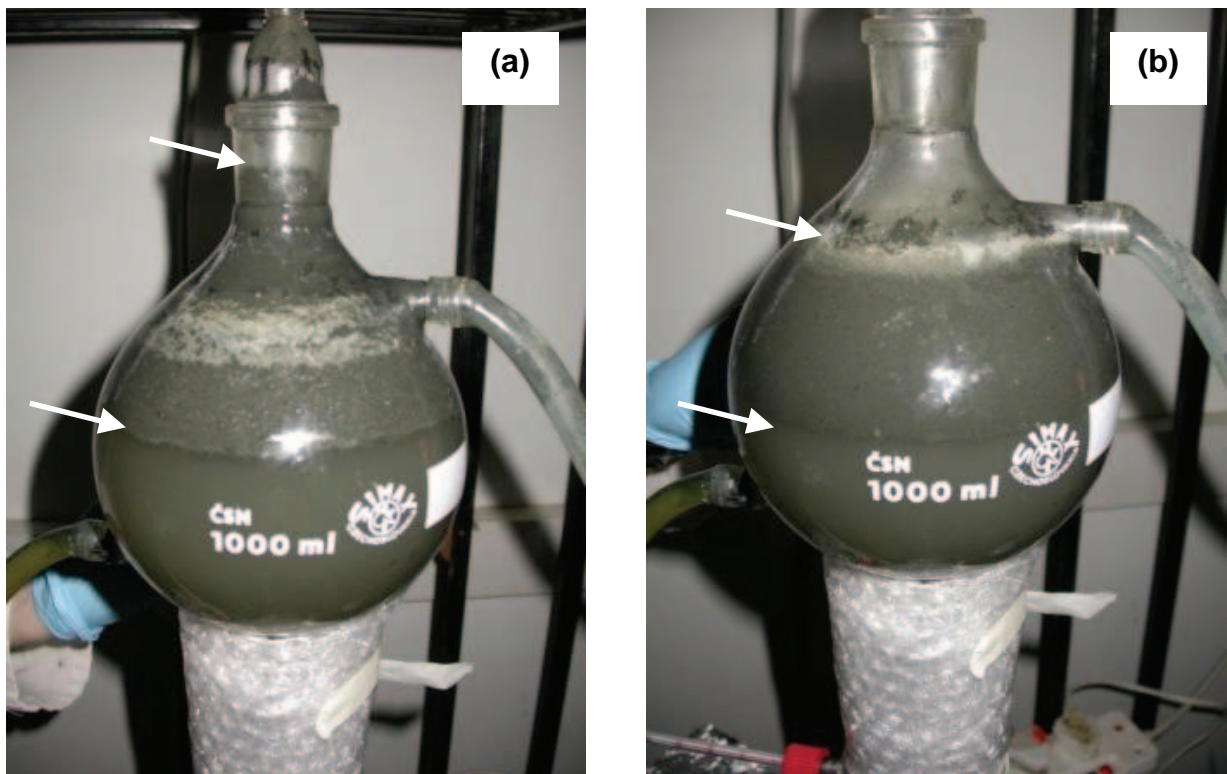


Figure 12 Example of sludge foaming that was observed during the first days of start-up in both reactors B and C. (a) Typical sludge layer before stirring; and (b) the sludge layer after stirring and settling.

once the reactor feed was changed to a lactate-based feed instead of a sucrose-based feed.

According to Gerardi (2003) sludge foaming in anaerobic digesters is the result of a reduction in the surface tension of the reactor sludge, which leads to the entrapment of biogas within the sludge. Foaming usually occurs during start-up, system imbalance and overloading, and other adverse operational conditions such as a change in wastewater composition or temperature fluctuations. Various factors can cause sludge foaming, such as excessive increases in VFAs, CO₂ and alkalinity, as well as low levels of total solids (Gerardi, 2003).

In this study, the change in wastewater composition (from the batch granulation-enhancement media to the UASB reactor substrate) might have been responsible for the foaming observed. The anaerobic community present in the digested sludge would have been conditioned specifically based on the composition of the substrate used during batch granulation-enhancement. Since the UASB reactor feed was sucrose-based, the foaming observed in reactor C (seeded with lactate seed sludge) could have been the result of the unbalanced conditions caused by sudden increases in VFA and CO₂ production by the sucrose-utilising populations, combined with a decline in the (previously dominant) lactate-utilising population. The sucrose seed sludge population used to inoculate reactor B would have been better able to metabolise the UASB reactor feed, although changes in the other media components might also have caused a temporary imbalance. Bacteria involved in the hydrolysis of yeast extract during batch granulation-enhancement might, for instance, not have been able to survive in its absence. Subsequent cell lysis and release of amines and polymers might have contributed to a reduction in the surface tension of the reactor sludge.

The efficiency values of UASB reactors A, B and C are presented in Table 1. Since the feed composition of UASB reactor C was changed on D19 from a sucrose-based feed to a lactate-based feed, no comparison will be made of the efficiency of reactor C to that of the control reactor (reactor A). As mentioned, a large amount of sludge in reactor C was lost initially due to extensive sludge foaming, which stopped once the reactor feed was changed to a lactate-based feed. Start-up was continued in spite of the low remaining sludge content. As expected, COD removal remained low, although it improved towards the end of the study. OLRs for reactor C were thus increased based on the stability of pH, alkalinity, biogas and CH₄ percentage values.

Table 1 Operating conditions and efficiency of UASB bioreactors: **A** (control reactor - inoculated with anaerobic sludge that did not receive any prior treatment), **B** (inoculated with sucrose pre-treated anaerobic sludge) and **C** (inoculated with lactate pre-treated anaerobic sludge) while reducing HRT after stable state conditions had been reached. COD_{in} of sucrose-based feed (reactors B and C) = 2 500 mg.L⁻¹, and COD_{in} of lactate-based feed (reactor A) = 3 200 mg.L⁻¹

Days in operation	40			61			81			86			91	
	A	B	C	A	B	C	A	B	C	A*	B	C	B*	C*
HRT (h)	75	56	56	45	45	38	45	38	32	45	32	32	28	32
OLR	0.8	1.1	1.4	1.3	1.3	2.0	1.3	1.6	2.4	1.3	1.9	2.4	2.1	2.4
pH (effluent)	7.09	7.14	7.18	6.65	6.85	7.15	6.67	6.87	7.15	6.70	7.00	7.15	7.00	7.21
Alkalinity	829	1129	1975	809	808	2075	788	750	1953	793	788	1967	909	2050
COD removal:														
- total	45%	70%	17%	56%	60%	24%	57%	74%	25%	61%	73%	36%	79%	34%
- centrifuged	72%	80%	39%	64%	72%	34%	65%	85%	31%	68%	82%	45%	85%	39%
TSS in effluent	0.337	0.107	0.152	0.083	0.073	0.063	0.085	0.050	0.057	0.131	0.053	0.064	0.035	0.055
VSS in effluent	0.287	0.095	0.129	0.074	0.064	0.058	0.070	0.040	0.048	0.088	0.051	0.053	0.033	0.051
Biogas	434	932	366	774	802	480	833	594	752	919	886	760	1292	737
CH ₄	53%	55%	56%	48%	51%	58%	50%	55%	61%	51%	56%	60%	57%	60%
CH ₄ yield	0.266	0.513	0.366	0.207	0.213	0.238	0.228	0.116	0.318	0.240	0.151	0.215	0.181	0.225

* = last day of operation; OLR = kg COD.m⁻³.d⁻¹; Alkalinity = mg.L⁻¹ CaCO₃; TSS and VSS = g.L⁻¹; Biogas = mL.d⁻¹; CH₄ yield = m³.kg⁻¹ COD_{removed}

Given time, the COD removal would probably have recovered as anaerobic population numbers increased further.

Reactor A (the control reactor) was operated for 85 d and could not stabilise at HRT values lower than 45 h. At an HRT of 45 h pH values remained below 6.70 and total COD removal rates reached a maximum of 60%.

In contrast, reactor B reached an HRT of 24 h after 91 d of operation. Reactor B also had higher COD-removal rates and lower TSS and VSS washout in the effluent and was, therefore, considered the most efficient of the three UASB reactors. Since the seed sludges that the reactors were inoculated with were diluted to the same VSS concentration (7.8 g.L^{-1} VSS) at the start of the study, the differences in performance efficiency were attributed to the impact of the pre-treatment of the anaerobic sludge that reactor B had been inoculated with.

UASB reactor sludge analyses

Suspended solids content and size distributions

The data presented in Fig. 11b show the VSS content of each of the UASB reactor sludges at the end of their respective operational periods. Size distributions of the VSS contents $\geq 0.25 \text{ mm}^2$ are also presented for each reactor sludge. From the results it can be seen that although the amount of VSS $\geq 0.25 \text{ mm}^2$ sludge particles present in the reactor sludges is less than 1.0 g.L^{-1} VSS, the fractions retained on the sieves for the reactor B sludge are significantly more than the sieve fractions of reactor C or the control reactor A. In Reactor B the highest VSS fraction was obtained from the sieve with the biggest aperture size (1 mm^2) while the opposite was true for reactors A and C. Photos of the different size fractions of reactor B can be seen in Fig. 13. Granule-like aggregates are clearly visible in the three larger-size fractions (a to c) while numerous floc-like aggregates can be observed in the smaller-size fractions (d and e). It would appear as if pre-treatment of digested sludge in the presence of sucrose, prior to UASB reactor inoculation, contributed strongly to aggregate formation during start-up of UASB reactor B and is clearly advantageous to UASB reactor start-up.

Interesting to note is that although reactor C had a markedly lower VSS content than both reactors A and B at the end of the study, it had approximately the same (if not slightly higher) VSS component $\geq 0.25 \text{ mm}^2$ than that present in the control reactor (reactor A) (Fig. 11b). It would appear as if switching to the lactate-based feed might

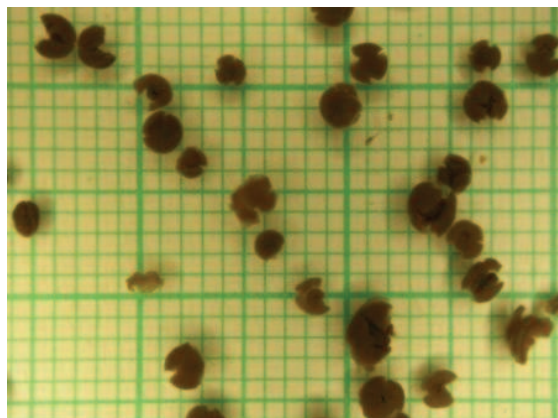
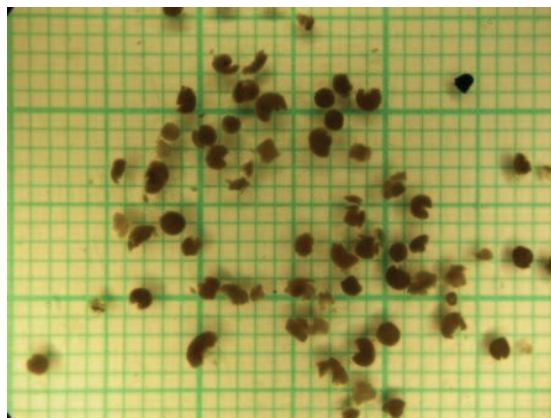
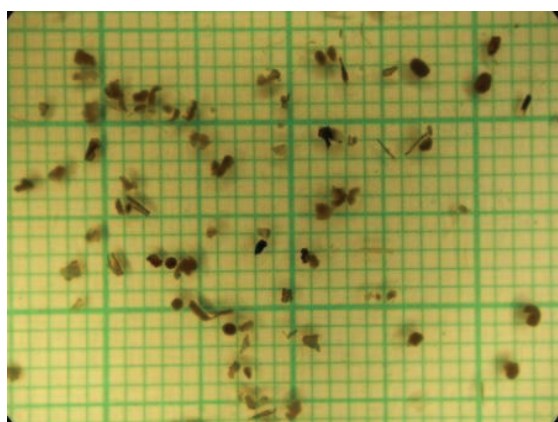
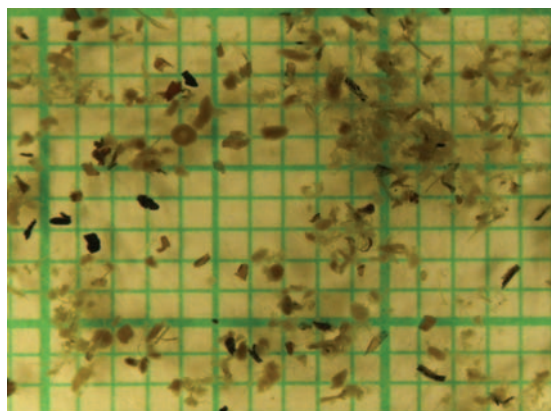
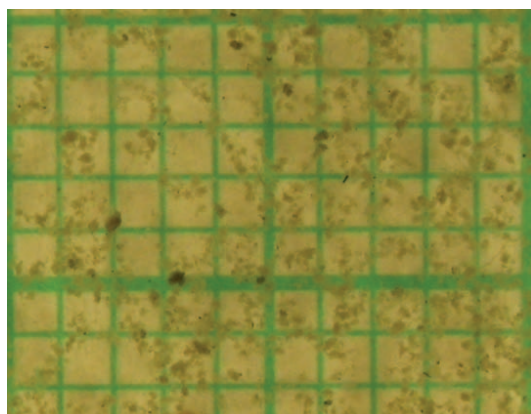
(a) $\geq 1.00 \text{ mm}^2$ (b) $0.71 - 1.00 \text{ mm}^2$ (c) $0.50 - 0.70 \text{ mm}^2$ (d) $0.25 - 0.49 \text{ mm}^2$ (e) $< 0.25 \text{ mm}^2$

Figure 13 Sieved size fractions of the Reactor B sludge at the end of the study: (a) $\geq 1.00 \text{ mm}^2$; (b) $0.71 - 1.00 \text{ mm}^2$; (c) $0.50 - 0.70 \text{ mm}^2$; (d) $0.25 - 0.49 \text{ mm}^2$; and (e) $< 0.25 \text{ mm}^2$.

have benefited aggregation and contributed to the retention of VSS particles $\geq 0.25 \text{ mm}^2$ in reactor C in spite of the low sludge content.

Activity test results of UASB reactor sludges at the end of the study

After the UASB reactor studies ended, the biogas and methane production activities of the sludges from the three UASB reactors were determined per gram of centrifuged sludge, using five different test substrates. Activity was expressed as cumulative biogas production (in mL) over 24 h (Fig.14) and as cumulative methane production (in mL) over 24 h (Fig.15). The BTM substrate contained 2.0 g.L^{-1} glucose as carbon source and was used as an indication of the overall activity of each sludge population. All the other substrates contained the same amount of glucose as well as an additional carbon source. If higher activities were observed in the other substrates than in the BTM test samples, this would point to the activity of specific bacterial groups.

In all the substrates tested, the reactors that were inoculated with pre-treated sludge (reactors B and C) both had higher biogas and methane production activities than the control reactor (reactor A). All the reactor sludges showed higher activities in the GTM, LTM and ATM substrates than their activity measured in BTM. This indicates that the acidogenic, acetogenic and methanogenic populations specifically involved in the degradation of glucose, lactate and acetate were active and well balanced in all the reactors. Although the BTM results showed that reactors B and C had the same cumulative biogas and methane volumes after 24 h, the reactor C sludge showed the highest activity in the GTM, LTM and ATM substrates. This is in contrast to the activity test results presented in Figs. 8 and 9 for the lactate seed sludge used to inoculate reactor C. It could be that the long-term conditions of controlled overload that the reactor C sludge was subjected to during UASB reactor operation, such as OLR increases in spite of low sludge content and low COD removals, contributed to the establishment of an extremely active anaerobic community consisting of acidogenic, acetogenic and methanogenic populations.

The lowest activities for all the UASB reactor sludges were observed in the substrate with added formate (FTM) (Figs. 14 and 15). Formate would typically be converted by hydrogenotrophic methanogens (such as *Methanobacterium formicicum*) (Fang *et al.*, 1995a). All the hydrogenotrophic methanogens that can degrade formate can also convert H_2 and CO_2 to methane (Boon & Whitman, 1988). If sludge activity was lower in the presence of formate it could indicate the inability of the

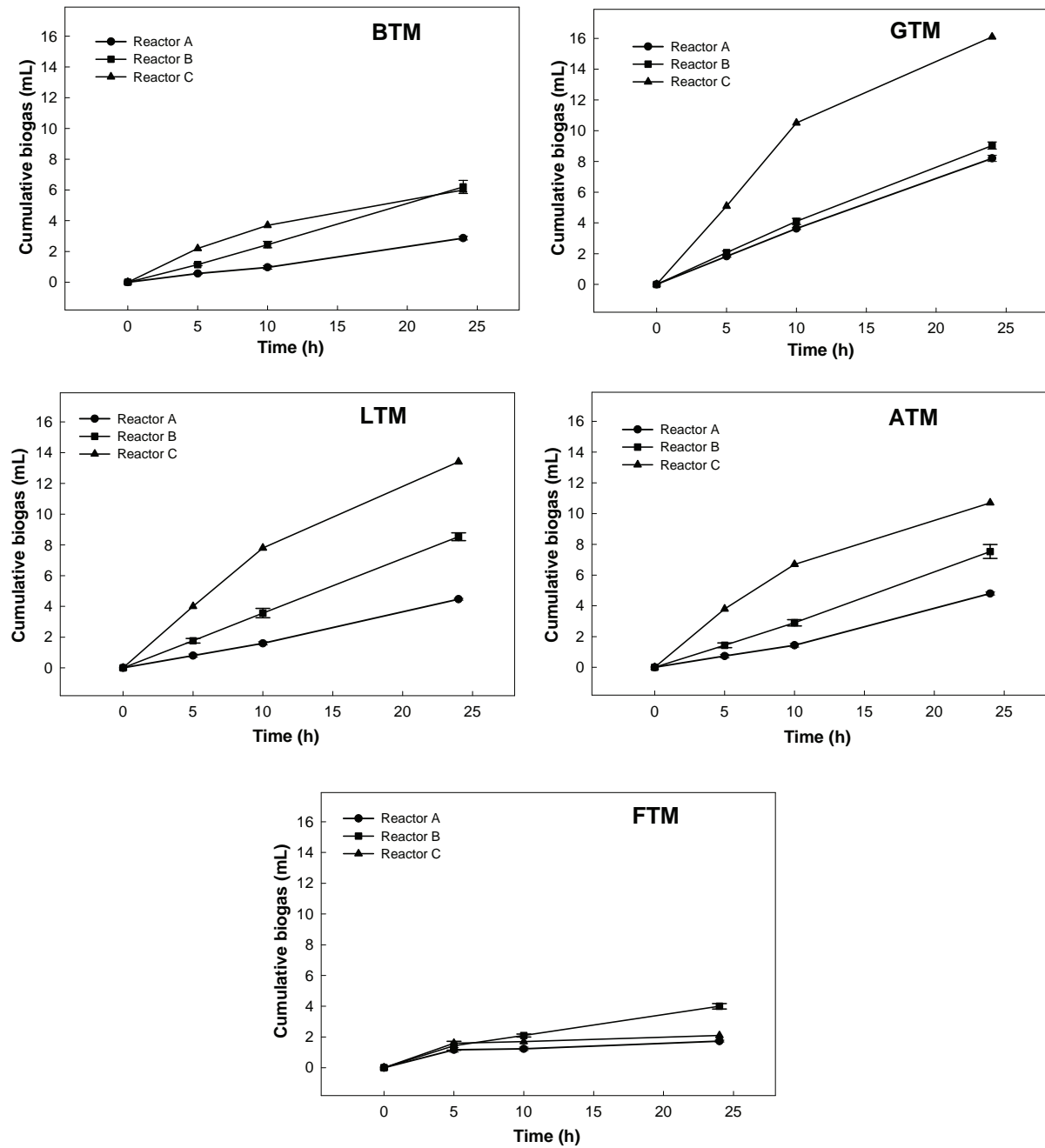


Figure 14 Cumulative biogas production over 24 h by each UASB reactor sludge determined at the end of the UASB reactor start-up. (Each data point represents triplicate values and error bars indicate standard deviation values). BTM - basic test medium; GTM - glucose test medium; LTM - lactate test medium; ATM - acetate test medium; FTM - formate test medium.

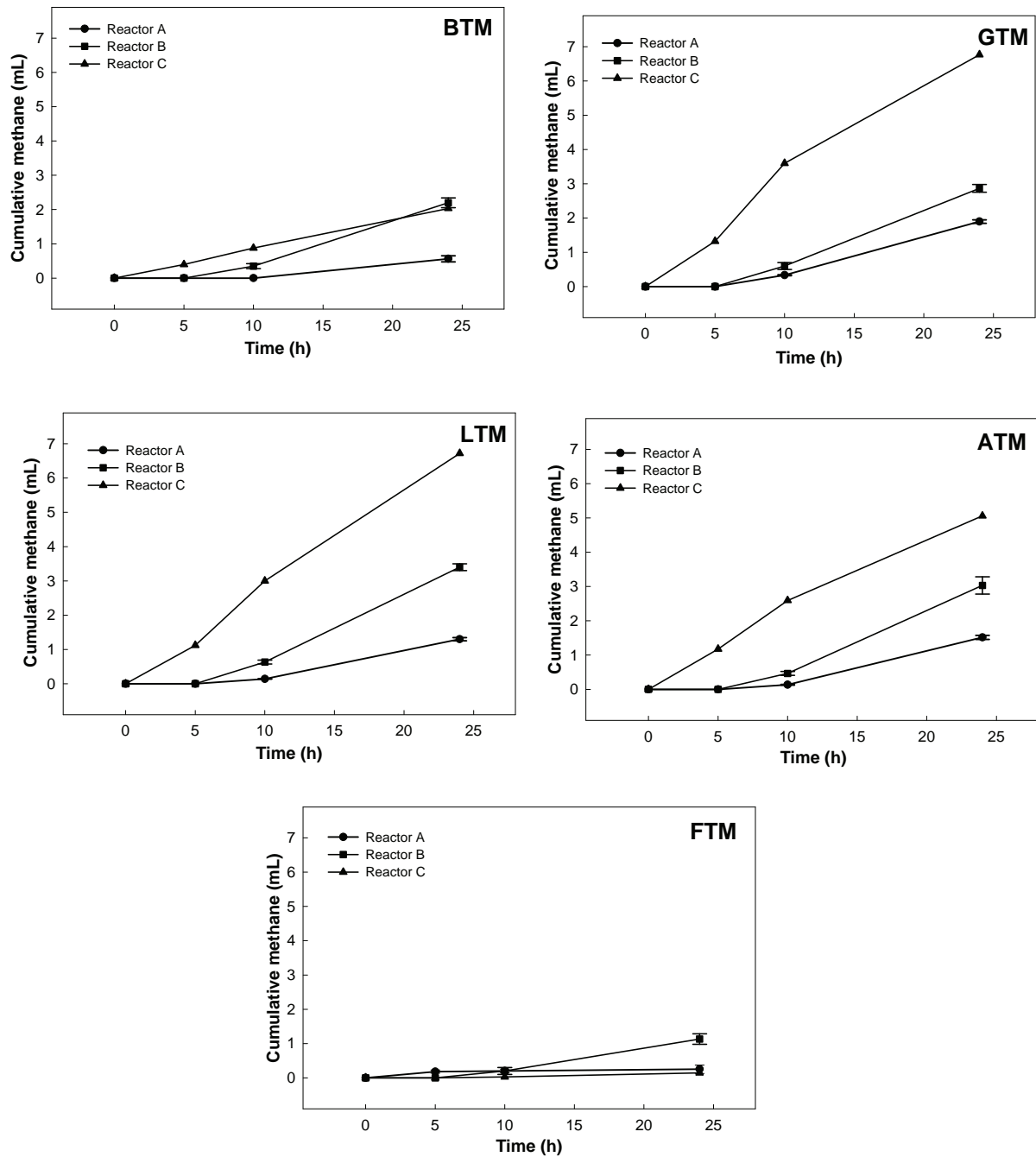


Figure 15 Cumulative methane production over 24 h by each UASB reactor sludge determined at the end of the UASB reactor start-up. Each data point represents triplicate values and error bars indicate standard deviation values. BTM - basic test medium; GTM - glucose test medium; LTM - lactate test medium; ATM - acetate test medium; FTM - formate test medium.

hydrogenotrophic methanogens to assimilate the H₂ and CO₂ generated during metabolisation of the 2.0 g.L⁻¹ glucose as well as with the additional 1 g.L⁻¹ formate. The methanogenic population of the reactor B sludge was, however, the best able of the three reactor sludges to metabolise the added formate. Considering the fact that the reactor B sludge also had significantly more granule-like aggregates than the sludges from reactors A and C (Figs. 11b and 13), it is possible that the hydrogenotrophic methanogens of the reactor B sludge were better positioned with regard to the syntrophic acetogens. This would have resulted in the more efficient removal of H₂ and CO₂, and a slightly better ability to cope with the additional formate in the test substrate.

Conclusions

The results presented in this study showed that pre-treatment of digested sludge prior to UASB reactor inoculation, especially in the presence of sucrose, improved the anaerobic qualities of the seed sludge. Reactor B consistently proved to be more efficient than the control reactor (reactor A) in terms of COD removal, HRT reduction and suspended solids washout. At the end of the study the sludges from both reactors B and C also had better biogas and methane production rates per gram of centrifuged sludge compared to the reactor A sludge. The fact that there was a higher methane production in ATM during the activity test by the sludges from reactors B and C in comparison with the methane production in ATM of the sludge from reactor A could be an indication that the acetoclastic methanogen populations were more active in reactors B and C. Acetoclastic methanogens are considered to be central to the formation of granules in UASB reactors (Hulshoff Pol *et al.*, 2004) and since the acetoclastic methanogens can act as granular nuclei, this might, in turn, have been the reason that a larger portion of the VSS content of the reactor B sludge was retained as particles ≥ 1.0 mm² in comparison to reactors A and C. Short-term pre-treatment of the inoculum sludge under slight organic overloading conditions, especially in the presence of sucrose, contributed to sludge retention and the granulation process in UASB reactor B.

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CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

The advantages of using granule-based anaerobic technologies in the treatment of food industry effluents have been well documented. The efficiency of these technologies is, however, highly dependent on the development of dense, granular sludge beds since these are better retained during organic loading rate increases in reactors compared to flocculent sludges. One of the major concerns for developing countries is the availability of suitable seeding material in the absence of granular seed sludge. The use of flocculent seed sludge instead of granular sludge will typically result in long start-up times, frequent re-inoculations and low removal efficiencies and sludge granulation can take months.

As discussed in Chapter 2 of this dissertation, many studies have reported on ways to contribute to the naturally occurring granulation process in-reactor while only two reports have used pre-treatment strategies that aimed to improve seed sludge qualities prior to reactor inoculation. One of these (Britz *et al.*, 2002) employed the principle of short-term (14 - 20 d) controlled organic overloading to improve extracellular polymer (ECP) production by acidogenic populations to enhance aggregation of bacteria. Using mostly lactate, Britz *et al.* (2002) targeted the lactate-utilising propionate-producing populations since these bacteria would typically produce ECPs as an alternative hydrogen sink mechanism under conditions of environmental stress. A batch granulation-enhancement method was developed using 500 mL batch units agitated in a linear-shake waterbath. Positive increases in granulation were reported, although the digested sludge used in the study was from a single source.

Before the batch granulation-enhancement model of Britz *et al.* (2002) could be of industrial value, several questions regarding this model needed to be addressed. Could ECP-producing propionibacteria form aggregates? What type of agitation method would be the best in the scaling-up process? How does one choose a suitable digested seed sludge for batch granulation-enhancement? Will sludge granulation proceed faster if batch granulation-enhanced sludge is used to seed a UASB reactor? It was the objective of this dissertation to address some of these questions.

The results presented in Chapter 3 of this dissertation indicated that a lactate-utilising ECP-producing *Propionibacterium* strain could indeed be manipulated to form

non-methanogenic bacterial aggregates. Aggregates were larger in the presence of apricot effluent medium (AEM). This was probably as a result of the fruit fibres present in the substrate, which provided a matrix to which the bacterial cells could adhere in the presence of ECP. The ECP consisted mostly of polysaccharides that might ultimately be metabolised in a mixed-population environment such as an anaerobic digester. This could mean that the contribution that additional ECPs could have on initial granulation might only be temporary.

During anaerobic granulation the presence of constant hydrodynamic shear force provides an important selection pressure. High shear force can also improve ECP production in biofilms. In a UASB reactor, hydrodynamic shear is the result of a combination of liquid upflow velocity and gas production (Liu & Tay, 2002). In the batch granulation-enhancement studies of Britz *et al.* (2002), the 500 mL batch units were incubated in a shake-waterbath to provide hydrodynamic shear. The results from both Chapters 3 and 4 of this dissertation showed that the roller-table was a better alternative for providing agitation during batch granulation-enhancement compared to the shake-waterbath. Not only would larger volumes be easier to accommodate on a roller-table than in a shake-waterbath, but the results indicated that the gentle rolling motion of the roller-table might be more advantageous to initial aggregate formation. A larger-volume batch reactor (with an operational volume of 5.4 L), based on the principle of the roller-table, was applied in Chapter 8 of this dissertation.

One of the major concerns was that batch granulation-enhancement results were highly dependent on the source of digested sludge used as seed sludge. Britz *et al.* (2002) obtained excellent results with digested sludge obtained from the Kraaifontein Municipal Wastewater Treatment Works (WWTW). As a result of digester breakdowns at Kraaifontein at the start of this study digested sludge from other municipal digesters was used as inoculum, but the same degree of granulation could not be achieved. There was thus a need to determine what characteristics a digested sludge should have to serve as a good seed sludge (Chapter 4).

The presence of specific methanogenic populations in digested sludge is a primary requirement before optimum anaerobic digestion and granulation can occur. An example of such a population is the acetoclastic *Methanosaeta concilii*, which has been implicated by various studies in the formation of granular nuclei (McHugh *et al.*, 2003; Zheng *et al.*, 2006; Baloch *et al.*, 2008). The absence of these organisms in digested seed sludge could have a detrimental effect on the overall granulation process.

Therefore, the presence of essential methanogens in digested seed sludge needed to be determined.

Denaturing gradient gel electrophoresis (DGGE) was used in Chapter 5 for the detection of *Archaea* in digested sludges as well as in mature UASB granules. A methanogenic marker was also constructed from certain methanogens (including *M. concilii*). The choice of the methanogenic species included in this marker was based on literature reports of their possible importance to the granulation process. This was based on the assumption that the populations important to granulation in UASB reactors should also be present in the digested sludge used to seed the batch granulation-enhancement process. The optimised DGGE method was successfully used to obtain DGGE fingerprints of both digested sludges and mature UASB granules. The appearance of artifactual double bands in archaeal fingerprints was furthermore minimised successfully by using 'touchdown' PCR. The DGGE fingerprints of the digested sludges showed that only one of the four sludges tested contained *M. concilii*. This is in contrast to the findings for mature UASB granules, which all contained *M. concilii*, regardless of substrate type. The absence of *M. concilii* might be the reason for the variations observed in the batch granulation-enhancement results of the different seed sludges in Chapter 4. It is, therefore, recommended that the presence of *M. concilii* in digested sludge be confirmed before it is used to seed the batch granulation-enhancement process.

In order to identify a suitable seed sludge, the DGGE method and marker developed in Chapter 5 was used in Chapter 6 to screen four well-stabilised digested sludges. The sludges all came from secondary digesters of which the control parameters and routine analyses indicated stable digester performance for at least five months. DGGE results confirmed the presence of *M. concilii* in all the sludges examined. A link most likely exists between stable digester performance and the presence of *M. concilii* in digested sludge. *Methanosaeta* species have a lower affinity for acetate and would grow optimally at acetate levels below 3 mM and neutral pH levels (Boone *et al.*, 2001). Long-term stable conditions in a municipal digester would, therefore, suit these species since acetate levels would generally remain low.

Although *M. concilii* was present in all the sludges, substrate-dependent activity tests carried out in Chapter 6 detected methanogenic activity in only one of the sludges (Athlone 4Sb-sludge). This showed that although the DGGE method can give a fingerprint as to which *Archaea* may be present in the different sludges, it does not give

an indication of the actual activity status or population numbers of the different methanogens.

Further differences between the four well-digested sludges analysed in Chapter 6 were also observed in the increases in the VSS content $\geq 0.25 \text{ mm}^2$ after the batch granulation-enhancement studies. The ST sludge samples showed the greatest increases of the four sludges tested.

It was unclear whether methanogenic activity or volatile suspended solids (VSS) size increases during batch granulation-enhancement gave the most accurate indication of which sludge would perform best during normal UASB reactor start-up. It was, therefore, decided to conduct UASB reactor start-up in Chapter 7 with both the Athlone 4Sb-sludge (with the highest methanogenic activity) and the ST-sludge. These start-up studies revealed that the Athlone 4Sb-sludge was overall the best choice as seed sludge. During UASB reactor operation the A4Sb-reactor (inoculated with Athlone 4Sb-sludge) was more efficient than the ST-reactor (inoculated with ST-sludge). The A4Sb-reactor showed higher COD removal rates and had lower VSS washout than the ST-reactor throughout the reactor operation period. Activity tests performed on the reactor sludges after the reactor runs revealed that the A4Sb-reactor sludge had a higher methanogenic activity per gram of centrifuged sludge than the ST-reactor sludge. VSS analyses also confirmed that in the A4Sb-reactor there was an increase in VSS content (including an increase in VSS particles $\geq 0.25 \text{ mm}^2$) that was not observed in the ST reactor sludge. Since the Athlone 4Sb-sludge also performed the best in the substrate-dependent activity testing done in Chapter 6, it was concluded that there is probably a link between the inherent methanogenic activity of a digested sludge and the efficiency of the anaerobic reactor it is used to seed.

Efficient start-up is dependent on the activity status of the indigenous anaerobic sludge populations in the seed sludge and their activity in relation to each other. Substrate-dependent activity testing can provide valuable information in this regard. It is, therefore, recommended that seed sludge selection (of digested sludges) be based on longer-term stable digester performance at the source but also on substrate-dependent activity test results.

The final question to be answered was to determine whether batch granulation-enhanced seed sludge would, in fact, improve granulation during UASB reactor start-up. In Chapter 8 three 2.4 L UASB reactors were seeded with the following: Athlone 4Sb digested sludge without any pre-treatment (control = reactor A); sucrose-fed batch-

incubated Athlone 4Sb digested sludge (reactor B); and lactate-fed batch-incubated Athlone 4Sb digested sludge (reactor C). The batch-incubated digested sludges used to inoculate UASB reactors B and C were prepared in a rolling-batch reactor. All the UASB reactors were fed with a synthetic sucrose-based substrate initially. Severe foaming occurred in reactor C shortly after start-up and the feed of this reactor was changed to a lactate-based feed.

Reactor B proved to be more efficient than the control reactor A, showing higher COD removal rates, higher methane production volumes and lower HRT values. Size distribution results of the reactor sludges at the end of the study also showed that reactor B had a larger portion of VSS $\geq 1.0 \text{ mm}^2$ than the sludge from reactors A and C.

At the end of the study it was shown that the sludges from both reactors B and C also had better biogas and methane production rates per gram of centrifuged sludge compared to the reactor A sludge. The fact that there was a higher methane production in ATM during the activity test by the sludges from reactors B and C in comparison with the methane production in ATM of the sludge from reactor A could be an indication that the acetoclastic methanogen populations were more active in reactors B and C. Acetoclastic methanogens are considered to be central to the formation of granules in UASB reactors (Hulshoff Pol *et al.*, 2004) and since the acetoclastic methanogens can act as granular nuclei, this might have been the reason that a larger portion of the VSS content of the reactor B sludge was retained as particles $\geq 1.00 \text{ mm}^2$ in comparison to reactors A and C.

The severe foaming that occurred at start-up of reactor C (seeded with the lactate-fed batch-incubated digested sludge) might have been caused by the change in carbon source (from a dominant lactate environment in the batch granulation-enhancement reactor to a dominant sucrose environment in the UASB reactor). The change might have been too severe and could have disrupted the acidogenic population balance established during the pre-treatment period. Considering the fact that foaming in reactor B (inoculated with sucrose-fed seed sludge) was brief, it might be more valuable to enhance the acidogenic ECP producers in general instead of focusing mainly on the lactate-utilising communities. Activity tests done on the reactor B sludge did indicate healthy methanogenic activity in the lactate test medium (LTM), both at start-up (Chapter 8, Fig. 9) and at the end of the study (Chapter 8, Fig. 15). This demonstrated that the lactate-utilising populations were also enhanced in an

environment where sucrose was the dominant carbon source and could still contribute to ECP production in general.

Concluding remarks

The results presented in Chapter 8 indicated that batch granulation-enhancement of the inoculum sludge of UASB reactor B in the presence of sucrose did enhance anaerobic digestion as well as sludge granulation during UASB reactor operation. A matter of concern that needs to be addressed before industrial application can be considered is the fact that it took on average three batch granulation-enhancement batches with an incubation period of 17 d each to generate enough sludge to seed one 2.4 L UASB reactor. In order to save time and effort, it will be necessary to investigate options by which a greater volume of enhanced sludge could be generated during batch granulation-enhancement. One could double both the volume of sludge treated and the concentration of the substrate. Although the substrate volume would remain the same, more sludge could then be added to achieve the same VSS:COD ratio used in this study.

Another unknown factor is the impact that a rapid change in carbon source could have on batch granulation-enhanced sucrose-fed seed sludge if it is used to seed a UASB reactor treating a complex industrial effluent. It might also result in foaming and spontaneous sludge washout, as was observed in UASB reactor C (seeded with lactate-fed seed sludge and fed a sucrose-based substrate). In order to avoid this, a fraction of the industrial effluent could be included in the sucrose-based substrate used in the batch granulation-enhancement process in an attempt to precondition the anaerobic populations before start-up.

In conclusion, the batch granulation-enhancement process was successfully used in this study to improve the seed sludge properties of well-stabilised digested sludge prior to UASB reactor inoculation. As a result, sludge granulation improved and reactor start-up time was reduced.

References

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