EXTRACELLULAR POLYMER EXTRACTION AND ANALYSIS FROM UASB GRANULES AND BATCH PRODUCED ANAEROBIC GRANULAR SLUDGE

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that it has not previously, in its entirety or in part, been submitted at any other university for a degree.

Alida van Eeden

ABSTRACT

The start-up period of Upflow Anaerobic Sludge Bed (UASB) reactors can significantly be reduced by enhancing the time-consuming granulation process through the batch cultivation of anaerobic granular sludge and thus seeding the reactor with this cultivated granular sludge, instead of raw anaerobic sludge. The precise mechanism for granule formation is not well known, but it is believed that extracellular polymers (ECP) play a critical role in the granulation process. Information on the precise role of ECP is also limited and no universal standardised method for ECP extraction is used at present. Therefore, comparison of results from different researchers has to be made with great caution.

The objectives of this study were to evaluate an ECP extraction method so as to optimise the extraction time, and then to correlate ECP composition of UASB granules with granule metabolic activity. The impact of changes in the environmental conditions, such as sludge sources (Paarl and Kraaifontein-sludge), carbon growth substrates (yeast extract lactate, glucose medium and fruit cocktail effluent) and batch cultivation techniques (roller-table and shake-waterbath), on batch cultivation studies was also evaluated in terms of granule activity, ECP composition and granule formation.

A physical extraction method was used to quantify the ECP content of UASB granules from six different sources. The optimal extraction time was taken as the time needed before cell lysis took place, and before intracellular material started contributing to the ECP content of the granules. It was concluded that the ECP composition was affected by the wastewater composition fed to the original UASB reactors. It was also found that the activity test results could be used to indirectly predict the activity of the different trophic groups present in the UASB granules. A correlation was found between the activity test results and the total ECP content, and this showed that the granules with the higher ECP yields exhibited greater biogas (S_B) and methanogenic (S_M) activities. However, based on the activity data and total ECP content, it appeared that a protein:carbohydrate ratio < 1 affected the activity of the granules.

The sludge source used as inoculum for batch cultivation of anaerobic granular sludge had a significant effect on granule formation. The use of a pre-

granulated raw anaerobic sludge, such as the Paarl-sludge, resulted in a greater increase in granule numbers at the end of the cultivation period. The acetic acid activity profiles showed that the acetoclastic methanogens that are involved in initiation of granulation by nucleus formation, were inactive or absent in the different batch systems, with the exception of the roller-table glucose cultivated Kraaifontein-sludge (RKG) batch system.

The addition of glucose as carbon growth substrate for batch cultivation not only enhanced the activity of the acidogenic population, but also led to the establishment to a greater variety of granule trophic groups within all the glucose cultivated batch systems. The addition of fruit cocktail effluent as carbon substrate enhanced ECP production in the Paarl-sludge cultivated batch systems. However, the addition of carbon substrates showed no discernible trend on granule formation itself. The roller-table cultivation technique resulted in the higher increase in granule numbers, and it was speculated that the more vigorous shake-waterbath technique probably shortened the contact time between biomass and substrate.

Large variations in the ECP composition of the different batch systems were found, and these were ascribed to the composition heterogeneity of different sludges. For future studies, it is advisable to characterise sludge, both chemically and microbiologically before using as inoculum. The selection of an appropriate sludge inoculum should then lead to optimisation of the granulation process.

UITTREKSEL

Die aanvangsperiode van "Upflow Anaerobic Sludge Bed" (UASB) bioreaktors kan noemenswaardig verminder word deur die tydsame granulasie proses te versnel deur die vooraf lot-kweking van anaërobe granulêre slyk waarmee `n reaktor dus geïnokuleer kan word in plaas van rou anaërobe slyk. Die presiese meganisme van granulevorming is nog nie welbekend nie, maar daar word beweer dat ekstrasellulêre polimere (ECP) wel `n kritiese rol speel in die granulasie proses. Inligting oor die presiese rol van ECP is ook nog beperk, en tans word daar nog geen universele standaard metode vir ECP-ekstraksie gebruik nie. Gevolglik moet resultate vanaf verskeie navorsers met groot omsigtigheid vergelyk word.

Die doelwitte van hierdie studie was om `n ECP ekstraksiemetode te evalueer deur die ekstraksietyd te optimiseer, en dan te korreleer met die ECP-samestelling en metaboliese aktiwiteit van die UASB granules. Die inwerking van veranderinge in omgewingskondisies, soos slykbronne (Paarl- en Kraaifontein-slyk), koolstofbronne (gisekstrak-laktaat-, glukose-medium en vrugtekelkie-uitvloeisel) en lot-kwekingstegnieke (rol-tafel en skud-waterbad) op lot-kweking studies in terme van granule aktiwiteit, ECP-samestelling en granulevorming is ook ondersoek.

`n Fisiese ekstraksie metode is gebruik om die ECP-inhoud van UASB granules vanaf ses verskillende bronne te bepaal. Die optimale ekstraksietyd is geneem as die tyd benodig voordat sellise sal plaasvind en die intrasellulêre materiaal `n bydrae sal lewer tot die ECP-inhoud van granules. Dit is afgelei dat die ECP-samestelling beïnvloed word deur die samestelling van die afvalwater wat vir die oorspronklike UASB bioreaktors gevoer is. Voorts is gevind dat die aktiwiteitstoets resultate indirek gebruik kan word vir die voorspelling van aktiwiteit van die verskillende trofiese groepe wat in die UASB granules teenwoordig is. `n Korrelasie is gevind tussen die aktiwiteitstoets resultate en die totale ECP-inhoud wat aangedui het dat granules met hoër ECP opbrengste, beter biogas ($S_B$) en metanogeniese ($S_M$) aktiwiteit getoon het. Volgens die aktiwiteitsdata en totale ECP-inhoud het dit egter geblyk dat `n proteïen:koolhidraat verhouding < 1 die aktiwiteit van granules beïnvloed het.

Die slykbron wat as inokulum gebruik is vir lot-kweking van anaërobiese granulêre slyk het `n noemenswaardige invloed gehad op granulevorming. Die

gebruik van `n rou anaërobe slyk wat reeds `n mate van granulasie getoon het, soos die Paarl-slyk, het `n hoër toename in granule getalle aan die einde van die kwekingsperiode teweeg gebring. Die asynsuur aktiwiteitsprofiele het aangedui dat die asetoklastiese metanogene, wat hoofsaaklik betrokke is by inisiëring van granulasie deur kernvorming, onaktief of afwesig was in al die verskillende lot-sisteme, met die uitsondering van die roltafel glukose-gekultiveerde Kraaifontein-slyk (RKG) lot-sisteem.

Die toevoeging van glukose as koolstofbron vir lot-kweking het nie alleenlik die aktiwiteit van die asidogene populasie verhoog nie, maar het ook bygedra tot die vestiging van `n groter verskeidenheid van granule trofiese groepe. Die toevoeging van vrugtekelkie-uitvloeisel as koolstofbron het die produksie van ECP verhoog in die Paarl-slyk gekweekte lot-sisteme. Die toevoeging van koolstofsubstrate het egter geen merkbare verandering getoon in granulasie opsigself nie. Die rol-tafel kwekingstegniek het `n hoër toename in granule getalle tot gevolg gehad, en dit is gespekuleer dat die meer kragtige skud-waterbad tegniek waarskynlik die kontaktyd tussen die substraat en biomassa verkort het.

Groot variasies is gevind in die ECP-samestelling van die verskillende lot-sisteme, en dit is toegeskryf aan die heterogene samestelling van die verskillende slyke. Vir toekomstige navorsing is dit raadsaam om slyk voor gebruik as inokulum beide chemies en mikrobiologies te karakteriseer. Die keuse van `n geskikte slyk-inokulum sal bydra tot die optimisering van die granulasie proses.

CONTENTS

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

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dedicated to my mother and grandmother, Alida

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CHAPTER 1

INTRODUCTION

Wastes from the manufacture of various foodstuffs can have high organic loads of between 2 and 200 $g.l^{-1}$ compared to domestic sewage of less than 0.5 $g.l^{-1}$ (Ross, 1989). This high organic waste loads create serious treatment and disposal problems for the manufacturing industry or even the local authority and manufacturers are then faced with high disposal charges. Aerobic and/or anaerobic wastewater treatment are thus necessary to minimise the final disposal charges. Aerobic wastewater treatment requires high-energy imputs and also has an associated high sludge disposal cost (Speece, 1983). In contrast, treatment costs are halved when anaerobic digestion is used (Verstraete & Vandevivere, 1999) and in addition, methane gas (CH_4) is generated as an end-product and thus yields a net energy gain (Speece, 1983). Anaerobic digestion is now widely used to treat high-strength industrial wastewater with chemical oxygen demand (COD) levels above 2 $g.l^{-1}$, especially in case of carbohydrate-rich food generated effluents (Hulshoff *et al.*, 1997).

The most utilised anaerobic digester is the Upflow Anaerobic Sludge Bed (UASB) design (Lettinga *et al.*, 1997). It was originally designed to retain biomass through adequate granulation in order to obtain a good effluent quality and avoid washout of the slow-growing methanogenic bacteria in the reactor (Verstraete & Vandevivere, 1999). Successful operation depends on spontaneous granulation that leads to a high settling velocity and methanogenic activity for the degradation of organic substances (Fukuzaki *et al.*, 1995). Granulation occurs when a cell attaches to a surface or to another cell (Schmidt & Ahring, 1996), and can only occur within a limited range of wastewaters (Fukuzaki *et al.*, 1995). It is a complex process where bacteria are unlikely to adhere and/or aggregate automatically because of repulsive electrostatic charges on the cell surfaces and the hydration layers outside the cell walls (Tay *et al.*, 2000).

Anaerobic digestion does not always proceed optimally because the composition of effluents can typically be time-variable with nutritional imbalances often found. High-liquid surface tensions may also lead to granule flotation leading

to a poor final effluent quality with subsequent washout of the slow-growing granules (Verstraete & Vandevivere, 1999). Hawkes *et al.* (1978) reported that the growth of the methanogenic fraction of the microbial fraction is generally slow when compared to that found for aerobic bacteria, and thus results in a time-consuming granulation process at start-up of the UASB reactor. It can take several months before an effective granular bed is cultivated when the UASB reactor is seeded just with anaerobic sludge, thus restricting the application in countries, like South Africa, where granules from operating UASB reactors are not readily available. The start-up period can significantly be reduced by enhancing granulation through the production of anaerobic granular sludge in a laboratory batch system, and then seeding the reactor with granules instead of just anaerobic sludge (Britz *et al.*, 2000).

Various different mechanisms of sludge granulation have been proposed in order to control the process more effectively (Ross, 1984; Costerton *et al.*, 1987; Chen & Lun, 1993; Schmidt & Ahring, 1993; Britz *et al.*, 2000; Tay *et al.*, 2000). Several studies showed that the extracellular polymers (ECP) produced by anaerobic bacteria in the granules are of great importance in the granulation process (Ross, 1984; Costerton *et al.*, 1987; Sam-Soon *et al.*, 1987; Harada *et al.*, 1988; Morgan *et al.*, 1990; Quarmby & Forster, 1994; Schmidt & Ahring, 1994; Jia *et al.*, 1996a). ECP includes organic debris, phages, lysed cells and other organic material excreted by the microbial cells. It is composed mainly of protein and polysaccharides, and minor amounts of lipids, lipopolysaccharides, DNA and RNA (Schmidt & Ahring, 1994). Ross, already in 1984, speculated that agglutination of bacteria is generally due to the interaction between a protein and a polysaccharide. The carbohydrate content of ECP contains uronic acid and is therefore likely to carry a negative charge at a neutral pH (Jia *et al.*, 1996b). Although these polymers carry an overall negative charge on the bacteria, the protein fraction provides important binding sites, since the positively charged amino groups can form strong bonds between the negatively charged anaerobic bacteria in the sludge (Morgan *et al.*, 1990). However, information on the precise role of ECP and the presence of proteins and carbohydrates during granulation is still very limited.

Researchers in the past made use of a variety of methods to extract and quantify the ECP content of granules from UASB reactors (Ross, 1984; Morgan *et*

al., 1990; Quarmby & Forster, 1994; Schmidt & Ahring, 1994; Jia *et al.*, 1996a). Subsequently, considerable variation on the reported ECP content of granules is found and this variation depends on the extraction methods, methods of analyses and the specific source and type of granular sludges examined. No uniform standardised method of extraction is presently used and, therefore, comparison of results from different researchers has to be made with great caution. The development of a uniform standard method of extraction is thus necessary before the precise role of the ECP in the granulation process can be elucidated.

The main objectives of this study were, firstly to evaluate the different extraction and analyses methods for ECP from UASB granules. Secondly, to determine the ECP composition and correlate this with the methanogenic activity of UASB granules. In a third study, batch-cultivated anaerobic granular sludge will be produced and evaluated in terms of the use of different types of seed sludge as inoculum, the impact of the carbon sources as growth medium and the use of different batch cultivation techniques on ECP composition, activity and granule formation using the batch granular sludge cultivation technique.

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CHAPTER 2

LITERATURE REVIEW

A. Background

The rapidly growing national and global population, together with the increasing need for economic growth leads to an increase in demand for our natural resources. It has also been estimated that the water demand is expected to increase by more than 50% over the next 30 years (Weaver *et al.*, 2001). These concerns have led to the establishment of wastewater disposal charges (Water Act No. 36, 1998) which directly impact the production costs of food and other related industries. It is thus essential that food industries implement wastewater treatment processes, in order to minimise production costs.

A variety of aerobic and anaerobic wastewater treatment processes do exist, but it has been found that anaerobic technologies are a more viable alternative for the high pollution levels of wastes from the food industry. The anaerobic process has two intrinsic advantages: firstly wastes are converted to a useful fuel, methane (CH_4) and thus can yield a net energy gain from process operations. Secondly, the anaerobic process produces less sludge than is found with aerobic processes, thus minimising the final disposal costs (Speece, 1983).

The most implemented high rate anaerobic digester is the Upflow Anaerobic Sludge Bed (UASB) design (Lettinga *et al.*, 1997). The UASB is designed to immobilise and maintain high biomass concentrations despite the upflow velocity of the wastewater and the production of biogas (Schmidt & Ahring, 1996). Successful operation of an UASB depends on the spontaneous formation of granular sludge that has a high settling velocity and methanogenic activity for the degradation of organic substances (Fukuzaki *et al.*, 1995). Bacteria in the granules are surrounded by extracellular polymers (ECP), which are of great importance in keeping the consortium members together, thus mediating the granulation of bacteria in natural ecosystems (Bhatti *et al.*, 1995).

B. Granulation

Granulation process

Granular sludge can be described as a spherical biofilm of 0.5 to 5 mm diameter, that consists of a densely packed anaerobic microbial consortium (Lettinga *et al.*, 1980). Granulation occurs when a cell attaches to a surface or another cell (Schmidt & Ahring, 1996). It is a complex process where bacteria are unlikely to adhere and/or aggregate automatically because of repulsive electrostatic charges on the cell surfaces and the hydration layers outside the cell walls (Tay *et al.*, 2000).

Many similarities exist between biofilm development and granulation. The development of a granule can be described by the following four steps: firstly, a bacterial cell initiates the process of a reversible association of two bacteria to the process of irreversible adhesion by binding to the surface of an inert material by the formation of ECP. Cell division then facilitates further cells binding within the ECP. Thereafter, developments of microcolonies that are bound within the ECP matrix are initiated. In the last step, multiplication of the cells occurs and visual granules develop. Granulation is thus a function of cell division within the microcolonies and new recruitment of bacteria from the liquid (Costerton *et al.*, 1987; Schmidt & Ahring, 1996).

Another very similar hypothesis for the mechanism of anaerobic granulation sludge was developed (Chen & Lun, 1993) with alcoholic stilage from ethanol fermentation broth used as influent. The first step of granulation is the formation of nuclei, and bacteria involved in nucleus formation are mainly acetoclastic methanogens (*Methanosarcina* and *Methanosaeta*). The acetic acid concentration and selection pressure plays a decisive role in determining the nucleus formation. *Methanosarcina* increasingly grows into clumps through excreting ECP, and form larger clumps that cannot be washed out of the reactor. *Methanosaeta* easily attaches and grows on different substrates, for example *Methanosaeta* inserts in the *Methanosarcina* clumps or grows on its surface. Further development of the nucleus is dependent on the acetic acid concentration. *Methanosarcina* and *Methanosaeta* show competitive growth at different acetic acid concentrations. Development of the nucleus in a higher acetic acid concentration environment shows that *Methanosarcina* predominates over *Methanosaeta*. At a lower acetic

acid concentration, the hydraulic loading rate increases and *Methanosaeta* predominates over *Methanosarcina*. *Methanosarcina* is highly unlikely to attach to a different substrate for growth, and will be washed out of the reactor as a result of the increasing hydraulic loading rate, and thus no longer plays a role in the granulation process. The second step of granulation is the process of further growth of the nucleus into a granule. A wider spectrum of acidogenic and acetogenic bacteria is needed to syntrophically grow with other methanogenic bacteria. Consequently, a thick shell of mixed bacterial composition and their ECP, as well as inorganic compounds rich in calcium (Ca^{2+}), are formed next to the nucleus (Chen & Lun, 1993).

Britz *et al.* (2000) found that an enhancement of the granulation process takes place when sudden changes in operational parameters (stress conditions) are applied to batch and UASB systems containing sludge under controlled conditions. When unfavourable conditions (overloading) occured, lactate started to accumulate whereafter major amounts of propionic and lower concentrations of acetic acid were detected in the effluent, and a decrease in pH was reported. An increase in hydrogen was also reported in the gas phase. This unbalanced situation probably leaded to the rapid decrease in bioreactor pH. The increase in lactate concentration resulted in an orderly shift in the acidogenic population to a more predominant lactate-utilising population in response to a gradual decrease in pH (Joubert, 2001). The propionic acid concentration increases once the lactate-utilising microbes gain a competitive advantage and ECP are produced by the lactate-utilising microbes. ECP can serve as a hydrogen sink mechanism, therefore the pH will gradually increase and stabilise while a decrease in propionic acid is observed. The system then displays clumping characteristics that may led to the formation of granules (Britz *et al.*, 2000).

Tay *et al.* (2000) proposed a new theory for sludge granulation, namely the proton translocation-dehydration theory. Bacterial surface dehydration, caused by the proton translocation activity on bacterial membrane surfaces, was suggested to initiate the sludge granulation. The overall granulation process included four stages: dehydration of bacterial surfaces, embryonic granule formation, granule maturation and postmaturation. The proton translocating activity on bacterial membranes could cause the energisation of the bacterial surfaces that resulted in the breaking of hydrogen bonds between the charges groups on the bacterial

surfaces and water molecules surrounding the target cells, inducing bacterial surface dehydration. The hydration repulsion between bacterial cells weakened by the action of external hydraulic forces and the relatively hydrophobic bacteria adhere to each other to form embryonic granules. These initial aggregates are strengthened by further dehydration of the bacterial surfaces that results from effective metabolite transference. During granule maturation, the distribution of each group of bacteria in embryonic granules depends on the orientation of intermediate metabolite transference that will result in the formation of well-organised bacteria consortia as mature granules. Granule maturation, however, blocks the unrestricted multiplication of bacterial cells because of space restriction that in turn facilitates the production of ECP. ECP may cause hydration of granules surfaces and protect granules against the attachment to gas bubbles and shear stress in UASB reactors. In the postmaturation stage the proton translocation activity keeps the bacterial surfaces at a relatively hydrophobic state, and continuously maintains the granular structure by the mechanism of proton translocating activity and extracellular polymer formation (Tay *et al.*, 2000).

Factors affecting the granulation process

Biomass retention through adequate granulation is of great importance when operating an UASB system. The anaerobic sludge, in time, obtains and maintains superior settling characteristics if environmental conditions are favourable to provide sludge flocculation and maintenance (Lettinga *et al.*, 1980). Many environmental factors, like stress and nutritional factors, may affect the granulation process (Schmidt & Ahring, 1994).

Environmental conditions, like slow growth and substrate gradient, can trigger microbial aggregation (Bossier & Verstraete, 1996). It was found that a higher substrate concentration occurs in the immediate vicinity of a particle or floc. Up to 50% of the COD in wastewater is found in colloidal or particle form that can absorb to the microbial floc, thus supplying the latter with organic molecules. Micro-organisms have also the ability to change cell surface characteristics and become hydrophobic when faced with depletion of substrate leading to slow growth or starvation. Such changes may contribute to the ability to aggregate (Bossier & Verstraete, 1996).

In reviewing the parameters that influence anaerobic treatment Moosbrugger *et al.* (1993) reported that pH must be considered a principal parameter. Accordingly, the minimum pH value of wastewater should be 6.6 and the desired operating range between 6.6 and 7.4 (Wentzel *et al.*, 1994). Sandberg & Ahring (1992) investigated the adaptation of the anaerobic process by gradually increasing the pH of granular sludge treating fish condensate in an UASB reactor. Increasing the pH changed the physical characteristics of the granules leading to decrease density, size and volatile solids content. After four months of acclimatisation to a high pH, the methanogenic activity of granular biomass was the same from pH 7.1 to 8.5. However, the acclimatised biomass generally showed a decreased activity (60%) at all pH values tested below the acclimatisation pH. Thus, it is of great importance to gradually increase the pH in order to achieve the necessary acclimatisation of the granules.

Nutrients should be present in sufficient amounts and available form to optimise granulation. Carbon, nitrogen (N) and phosphorus (P) are important ingredients for biomass growth (Annachhatre, 1996). Ratios of COD:N:P have been recommended not to be below 350:5:1 (Souza, 1986). It was also found that the growth of methanogenic bacteria was enhanced with the addition of 0.1 M sulphur (Lin & Yang, 1991).

Monovalent cations, such as ammonium (NH_4^+) and sodium (Na^+) may also affect sludge settleability (Lin & Yang, 1991). Sodium appears to be essential for the growth of methanogenic bacteria at low concentrations, with an optimum concentration reported as 350 $mg.l^{-1}$. High concentrations of Na^+ are inhibitory to growth of methanogenic bacteria (Rinzema *et al.*, 1988). A large amount of NH_4^+ (> 3 000 $mg.l^{-1}$) is toxic to anaerobic bacteria and inhibits growth, especially at high pH values. Concentrations from 1 000 $mg.l^{-1}$ upwards can be detrimental to granulation (Lin & Yang, 1991).

Divalent cations, such as calcium (Ca^{2+}) and magnesium (Mg^{2+}) have a positive effect on the flocculation of anaerobic sludge. Goodwin *et al.* (1990) reported that deficiencies in magnesium, calcium and trace elements affect the performance of methanogens, and adversely affected granulation. The effects of magnesium on *Methanosarcina* dominated granules in a thermophilic UASB reactor, were investigated. The absence of Mg^{2+} in the medium caused a decrease in the conversion of acetate and 50% of the biomass was washed out

from the reactor. A change in the bacterial community occurred and formed fluffy granules that consisted mainly of rod-shaped methanogens. The addition of 100 mM Mg^{2+} caused disintegration of packets of *Methanosarcina* in the reactor, resulting in a high amount of single cells, resulting in the washout of 20% of the biomass. UASB reactors should therefore be operated with a Mg^{2+} concentration of less that 30 mM to prevent biomass loss. The increase in the Mg^{2+} concentration from 0.5 to 10 mM gave a better performance of the UASB reactor (Schmidt & Ahring, 1993a).

Trace elements iron (Fe), molybdenum (Mo), nickel (Ni) and cobalt (Co) have been shown to have a significant enhancement effect on granulation. Dolfling *et al.* (1985) reported that 30% of the ash content was iron sulphide (FeS). Digesters where sulphide is controlled give a better performance than uncontrolled reactors (Anderson *et al.*, 1984). The reported element content of different granular sludge varies greatly. Iron accumulation in some granules could be caused by differences in loading rates, resulting in the alteration of some local cultural conditions. High organic loading rates may create high anaerobic conditions that lead to high production of sulphides, and thus forming a FeS precipitate that contributes to the high iron content (Bhatti *et al.*, 1995). Iron supplementation tends to change the dominant species from *Methanosaeta* to *Methanosarcina*, and increases the utilisation of acetate. Methanogenic bacteria may be particularly sensitive to metal ion deficiencies in comparison to acidogenic bacteria. It has been postulated that if competition for trace elements exists between different groups of bacteria, methanogenic bacteria will be less effective in competing for these elements (Goodwin *et al.*, 1990). Bhatti *et al.* (1995) reported that an iron deficiency did not affect the strength and stability of the granules. Supplementation of Fe and Co in the feed under high COD loading rate positively affected the COD digestion rate and specific activity of the bacteria. Yeast extract has been found to be a good micronutrient for bacterial growth and only enhances the ability of bacteria to collect trace elements. Thus, it did not increase the trace elements in the feed, and therefore had no significant effect on the specific activity of bacteria. Bacterial growth was enhanced by the addition of yeast extract and thus more bacteria could be maintained in the reactor, thus improving the COD digestion rate in the reactor. However, with iron supplementation an excellent

COD digestion rate could be maintained in the reactors, indicating that the addition of yeast extract in the feed is not necessary (Shen *et al.*, 1993b).

Another important operational factor is when long-term steady-state operation of an UASB reactor is undertaken and thus can cause most of the biomass to exist in a stationary phase or even to start decaying, therefore limiting substrate transport to the granules. Removal of granules (60%) from the reactor could lead to an increase in substrate loading per unit of biomass, resulting in growth of the sludge bed until a new steady-state was reached, with larger and denser granules (Ahring *et al.*, 1993).

C. Granular composition

Together with the microbial component of anaerobic granules, appropriate amounts of inorganic deposits and ECP also enhance the structural stability and mechanical strength of granules, thus playing an important role in granule formation. The shape and composition of granular sludge can vary significantly, depending on factors such as wastewater composition and process conditions (Schmidt & Ahring, 1996). The chemical composition of granular sludge is also comparable to the chemical composition of bacteria in general (Dolfling, 1986).

Inorganic composition

The inorganic mineral composition (ash content) of different granules varies widely from 10 to 90% of the dry weight of granules, depending on the wastewater composition and process conditions (Schmidt & Ahring, 1996). The main components of ash content in granules in order of metal composition has been reported as magnesium (Mg) ≈ calcium (Ca) > iron (Fe) > zinc (Zn) ≥ nickel (Ni) > cobalt (Co) ≈ molybdenum (Mo) > copper (Cu) > manganese (Mn) (Scherer *et al.*, 1983). Phosphate (P), sodium (Na) and potassium (K) have also been observed to be present in large quantities in granular sludge (Bhatti *et al.*, 1995).

Variations in ash content are attributed to the different pH values maintained in each reactor. Alkaliphilic conditions are caused by the conversion of volatile fatty acids (VFA) to CH_4 and consequently stimulate crystallisation or the precipitation of inorganic salts. The formation of crystals or precipitates may function as inert supports for bacteria and increases the density of granules, thus

stimulating and stabilising the granulation process. Microscopic observations have revealed that well-formed granules contained abundant precipitates, although no plate crystals, indicating that detachment of bacterial aggregates from crystals occurred during the development of granular sludge. Crystals are thus only available for microbial aggregation in the initial stage of granulation (Fukuzaki *et al.*, 1991a). Alkaliphilic conditions also contribute to a high ash content of granules (Fukuzaki *et al.*, 1991b). Relatively large amounts of ash would generally tend to accumulate in methanogenic granules, irrespective of starting substrates due to the conversion of VFA during methanogenesis (Fukuzaki *et al.*, 1991a).

The accumulation of inorganic precipitates is also essential for granule development by maintaining a high specific gravity. A high ash content leads to a high specific gravity and results in a high sedimentation velocity (Fukuzaki *et al.*, 1991a). It has also been observed that granules with higher ash contents showed, not only a higher specific gravity, but also a higher volatile suspended solid (VSS) density. This observation provides evidence of the contribution of the inorganic matrix to the formation of stable granules. However, the excess accumulation of inorganic deposits can lead to a decrease in available reaction volume in an UASB reactor (Fukuzaki *et al.*, 1995). A positive correlation between the ash content and density of granules has also been reported (El-Mamouni *et al.*, 1995).

Comparisons between mesophilic and thermophilic granules have shown that the ash content of the sludge was relatively consistent, regardless of temperature or substrate (Quarmby & Forster, 1995a). However, mesophilic granules grown on complex wastewater, have a lower ash content than mesophilic granules grown on simple substrates, i.e., acetate, propionate, or butyrate (Schmidt & Ahring, 1996). It appears that relatively large granules are formed when grown on more complex substrates, compared to simple substrates (Fukuzaki *et al.*, 1991b).

Biological composition

Microbial composition

Dolfling *et al.* (1985) reported that granules are formed by the activity of various groups of micro-organisms, and that an internal organisation is not obvious. In contrast, other researchers (Macleod *et al.*, 1990; Guiot *et al.*, 1992; Fang, 2000) have presented evidence of a structured organisation of bacterial

trophic groups in the granule where the granules exhibit a three-layered structure, each layer possessing different bacterial trophic groups. The granular core consisted of *Methanosaeta* that probably function as nucleation centres to initiate granulation. The second layer would consist of H_2-producing acetogens and H_2-consuming (hydrogentrophic) methanogens in syntrophic association to ensure a high level of metabolic activity of the organisms. Adhesion of fermentative bacteria (acidogens) to form the third and exterior layer provides contact between this metabolic group and the organic substrates. The VFA products of the fermentative bacteria serve as substrates for the underlying acetogens. Methanogens in the exterior layer possibly indicates that free H_2 could be consumed before it penetrated into the second layer. It has also been reported that facultative anaerobic and aerobic bacteria present in the exterior layer of a biofilm would create an O_2-gradient such that only strict anaerobes can flourish in the deeper layers of the biofilm.

Grotenhuis *et al.* (1991a), however, reported that the bacteriological composition and structure of granular sludge depended on the wastewater composition. In their study granular sludge from an UASB reactor treating wastewater from a sugar plant, and sludge granules adapted to ethanol and propionate, were investigated. The granules treating wastewater from the sugar plant, as well as the granules adapted to ethanol did not show the typical spatial orientation of bacteria. In contrast, predominantly two types of layers were found in propionate-grown sludge, those of a rod-shaped bacterium immunologically related to *Methanosaeta soehngenii* and those consisting of two different types of bacteria with a specific spatial orientation. *Methanosaeta soehngenii* and *Methanobrevibacter arboriphilus* were the most abundant acetoclastic and hydrogentrophic methanogens, respectively, in propionate-grown sludge, whereas *Methanospirillum hungatei* and *Methanosarcina barkeri* predominated in ethanol-grown granules. The shift in acetoclastic methanogens in different types of sludge could be partly explained by the different pH optima of *Methanosarcina* and *Methanosaeta*. Propionate can only be degraded at low hydrogen and acetate concentrations, while acetate and hydrogen concentrations are less critical for ethanol oxidation. If acetate, however, is degraded faster than hydrogen, the pH level drops. Methanogens with an acidic pH tolerance are required for ethanol

conversion, whereas propionate conversion required methanogens with a high hydrogen affinity.

Recent literature, however, states that the microbial distribution is strongly dependent on the degradation thermodynamics and kinetics of the individual substrate (Fang, 2000). Granules that degraded carbohydrate substrates exhibited a layered structure with a surface layer populated with hydrolytic/fermentative acidogens. The middle layer consisted of syntrophic colonies, and the interior layer consisted of acetoclastic methanogens (Macleod *et al.*, 1990; Guiot *et al.*, 1992). On the other hand, granules treating substrates, such as peptone, glutamate, propionate, ethanol and sugar refinery waste water (Grotenhuis *et al.*, 1991a) did not exhibit any layered microbial distribution. It is believed that the initial step of degrading these substrates was rate-limiting. Thus, the substrate would diffuse toward the granule interior without being fermented or hydrolysed near the surface. The bacteria then exhibits no layered distribution of degrading different intermediates, but instead the bacteria were intertwined and distributed evenly (Fang, 2000).

Interspecies metabolic transfer

Interaction between several groups of bacteria in the granules allows the complete degradation of organic matter to methane and carbon dioxide. However, unfavourable dynamics only allows the degradation of propionate and butyrate if the acetate and particularly hydrogen partial pressure are reduced to very low concentrations (Schmidt & Ahring, 1996). Therefore, a syntrophic relationship is needed between the acetogens (H_2-producing) and methanogens (H_2-utilising) in order to favour thermodynamic conditions for the conversion of VFA and alcohol to acetate. If not, the higher volatile fatty acids like propionate and butyrate will accumulate in the system (Speece, 1983), causing an unfavourable pH drop in the UASB reactor. In anaerobic systems, low hydrogen partial pressures can only be achieved by interspecies transfer of molecular hydrogen from H_2-producing acetogens to H_2-utilising methanogens (Schmidt & Ahring, 1996). Schmidt & Ahring (1993b) also examined the degradation of propionate and butyrate in whole and disintegrated granules from a thermophilic (55°C) UASB reactor fed with acetate, propionate and butyrate as substrates. Results clearly indicated a correlation between the hydrogen partial pressure and the degradation of

propionate and butyrate, showing a decrease in the degradation rate with increased hydrogen partial pressure.

Interspecies formate transfer has also been proposed to play a role in the syntrophic oxidation of VFA (Thiele *et al.*, 1988). Schmidt & Ahring (1993b) found no significant differences in the stimulation of degradation rates when the disintegrated granules were supplied with methanogens that utilised hydrogen only or hydrogen and formate. Interspecies formate transfer was thus not important for the stimulation of propionate and butyrate degradation in granules.

Sulphate reduction to hydrogen sulphide is energetically favoured over methane production for both hydrogen and acetate substrates, in anaerobic environments (Speece, 1983). Sulphate-reducing bacteria (SRB) are quite diverse in terms of metabolic activities, morphotypes, trophic properties and substrate affinities. Granules treating brewery wastewater that contained mainly ethanol, propionate and acetate as carbon sources, as well as sulphate (0.6 to 1.0 mM), were characterised for their physical and chemical properties, metabolic performance on various substrates and microbial composition. It was found that the addition of sulphate (8 to 9 mM) increased the maximum substrate degradation rates for propionate and ethanol by 27 and 12%, respectively (Wu *et al.*, 1991). Both hydrogen and formate were formed during syntrophic ethanol conversion. However, SRB did not play a significant role in the metabolism of formate, acetate and hydrogen. The presence of 2 mM molydbate (inhibitor of SRB) inhibited the syntrophic propionate and ethanol conversion by 97 and 29%, respectively. Thus, methanogens and sulphate-reducing bacteria did not compete for common substrates in the granular consortium. Syntrophic propionate and ethanol conversion was likely performed by SRB, while H_2, formate and acetate were consumed primarily by methanogens.

D. Extracellular polymers

General

Bacterial extracellular polymers (ECP) are defined as the polysaccharide-containing structures of bacterial origin, lying outside the integral elements of the outer membrane of Gram-negative cells and the peptidoglycan of Gram-positive cells (Costerton *et al.*, 1981), although it is now recognised that proteins and other

macromolecules represent significant quantities of ECP (Morgan *et al.*, 1990). The microbial cells produce ECP from organic debris, phages, lysed cells and other material that is excreted (Schmidt & Ahring, 1996). It can exist in a number of forms, for example, rigid or flexible, as capsules firmly attached to cells, or found as loosely attached slime. In some cases the ECP may even be shed from the cell wall (Quarmby & Forster, 1994).

The ECP content of granules differs between 0.6 and 20% of the volatile suspended solids content of the granular sludge examined. It depends on the type of granular sludge examined, ECP extraction method, as well as the analytical method used for ECP determination. Protein and polysaccharides are the dominant components of ECP, typically in a ratio of 2:1 to 6:1. Small amounts of lipids have also been observed in the granules. The carbohydrate component consists of neutral sugars and the range includes, in descending order, glucose, galactose, rhamnose and mannose (Schmidt & Ahring, 1996). The protein component consists of amino acids that include asparagine, glutamine and alanine. ECP also consists of small amounts of lipopolysaccharides, RNA, DNA, inorganic molecules and a consortium of micro-organisms. This ensures that ECP extracted from granules will never be homogenous (Quarmby & Forster, 1994).

Extracellular polymers have a number of possible functions, depending on the micro-organisms present. It can increase the pathogenicity of the organism, decrease the susceptibility of phagocytosis, reduce bacterial desiccation and trap soluble nutrients. The main function of ECP is thought to be a mediator of cell to cell adhesion, thus mediating adhesion of bacteria in natural ecosystems such as is found in UASB bioreactors (Quarmby & Forster, 1994).

The extracellular polymers that are naturally produced can bond electrostatically or physically and bridge bacterial cells and other particulate materials into a multi-component matrix. Agglutination of bacteria is generally due to the interaction between a protein and a polysaccharide (Ross, 1984). It is found that the carbohydrate content of ECP in anaerobic granules contains uronic acid, $CHO(CHOH)_nCOOH$, a principle ionogenic component of polysaccharides. The carbohydrate content of ECP in anaerobic granules is therefore likely to carry a negative charge at neutral pH because of the carboxylic group in uronic acid. Proteins contain functional amino groups that carry a positive charge at neutral pH (Jia *et al.*, 1996a). The proteins provide important binding sites since the positive

groups can form strong bonds between the negative sites on the bacteria (Morgan *et al.*, 1990).

Proposed mechanisms for bioflocculation have widely been reported for aerobic processes. Ross (1984) suggested that the bioflocculation mechanisms proposed for aerobic processes can also be applicable to the formation of anaerobic granules. The ECP produced in anaerobic processes differ from ECP produced in aerobic processes. The ECP yield is less for anaerobic (granular) sludge than found for aerobic (activated) sludge. Protein is the most dominant component in anaerobic sludge, while carbohydrates are more dominant in activated sludge. Therefore, although the ECP of sludge solids carry an overall negative charge, it was found that the charge carried by both granular sludge and the ECP extracts were less negative than the surface charge of the examined activated sludge. The protein:carbohydrate ratio also showed a linear relationship with surface charge of sludge and the corresponding ECP extracts (Morgan *et al.*, 1990). Magara *et al.* (1976) also reported that activated sludge has a poor settleability, probably due to the high ECP content of the activated sludge. Thus, it was concluded that lower ECP concentrations and subsequently reduced surface negativity were important to the stability of granular sludge and the granulation process (Morgan *et al.*, 1990).

Factors influencing ECP production

Many factors influence the production and composition of ECP in anaerobic granules. The composition of ECP affects the surface properties of bacterial flocs and the physical properties of granular sludge, thus affecting bacterial agglutination and the granulation process (Schmidt & Ahring, 1996).

Production of ECP is dependent upon the bacterial species present in the granules and granular sludge. It is not clear if all the bacterial species present can produce ECP, or if only a few species are able to do so. Jia *et al.* (1996b) found that acidogens have higher ECP yields than acetogens and methanogens. The high ECP yield during acidogenesis explains reported observations that carbohydrate-degrading sludge produced better granules than acid-degrading sludge (Harada *et al.*, 1988).

Restricted production of ECP in granules grown on acetogenic and methanogenic substrates could be due to the complexity of their cell walls,

especially the cell walls of methanogenic bacteria. Methanogens do not posses peptidoglycan in their cell walls, but consist out of either a polypeptide sacculus or an outer proteinacous sheath. The lipid carrier molecule, which is one of the essential components of peptidoglycan synthesis, is involved in transporting the peptidoglycan precursors across the bacterial cytoplasm membrane. It also mediates in the release of exopolysaccharides and capsule formation, thus exopolysaccharides in addition to peptidoglycan synthesis will be limited in methanogens (Morgan *et al.*, 1990). It is also possible that the production of complex polymers is too energy consuming for methanogenic and acetogenic bacteria (Schmidt & Ahring, 1994).

Schmidt & Ahring (1994) investigated changes in the ECP content with substrate adaptations under different temperatures. The ECP content in the granules decreased when changing the wastewater from acidogenic to acetogenic. The lipid content in the granules increased when the change was induced. The increase in lipid content could be due to the fact that lipids from the cell membranes of bacteria in the granules were present in a greater proportion when the total ECP content was low. The granules became smaller and denser, but still showed good settling abilities and high methanogenic activities. This indicated that a high amount of ECP was not needed for production of active granules.

The composition of the wastewater fed to the reactor will also affect the amount of ECP in granules. The amount of carbohydrates extracted from granules increased with the addition of iron and yeast extract to the feed. However, the opposite effect was seen when iron was absent. Yeast extract only enhances the ability of bacteria to collect trace elements (Shen *et al.*, 1993a). Iron plays an important role in the formation of extracellular polysaccharides (EPS). It binds cysteine, thereby causing excretion of EPS and thus as a result forming more ECP. It has been shown that the lack of cysteine can influence ECP production in *Methanobacterium* strain AZ. Pellet formation is mediated by *Methanobacterium* strain AZ that synthesised all amino acids except cysteine. The deficiency of cysteine induces an over-production of other amino acids, which cannot be used for cell growth, and the excess is secreted as extracellular polymers. Thus, supplementation of cysteine in the feed decreases both polymer production and pellet formation (Sam-Soon *et al.*, 1987). Furthermore, Quarmby

& Forster (1995c) also found that the outer surface of granules contains more carbohydrates and less protein than the central core. The protein content of the granule tended to increase near the centre of the granule as the presence of methanogens increased (Macleod *et al.*, 1990; Guiot *et al.*, 1992), accompanied by a decrease in carbohydrate content. Some of the granules, however, did not confirm this observation, but their characteristics did not appear to be substrate related (Quarmby & Forster, 1995c).

The conditions under which the granules are grown also affect the ECP production. Higher concentrations of ECP are found in granules that are grown under mesophilic conditions than in granules grown under thermophilic conditions. It was found that the lipid content was higher in granules grown under thermophilic conditions. This suggests that production of ECP could be limited for thermophilic methanogens and acetogens, or that the ECP produced could be more rapidly degraded under thermophilic conditions (Schmidt & Ahring, 1994). Methanogens produce more EPS when the operational temperature of an anaerobic reactor is below the optimal growth temperature. Fewer polymers were extracted from methanogens when reactors were operated at higher operational temperatures (Veiga *et al.*, 1997).

The effect of nutrient supply on ECP production by methanogens has also been investigated (Veiga *et al.*, 1997). Methanogens produce more polymers under reduced nutrient (nitrogen and phosphate) conditions. It was found that carbon utilisation shifted toward EPS production when the carbon:nitrogen (C:N) ratio and/or carbon:phosphate (C:P) ratio is larger, with the EPS production slightly higher under reduced phosphate conditions. Thus, operation of an anaerobic reactor on a low-nutrient containing substrate might be beneficial for bacteria to produce more ECP. Bull *et al.* (1983) also found that the addition of methanol to the feed improved the start-up performance of a fluidised-bed reactor treating synthetic meat wastewater, due to an increase in C:N ratio that stimulated the production of EPS and thus resulted in more ECP production that is believed to stimulate granulation.

Extracellular polymers accumulate on the surface of micro-organisms (Costerton *et al.*, 1981). Thus, the surface charge of micro-organisms is due to the functional groups of ECP that can carry either a positive or a negative charge, depending on the nature of the groups and the pH. ECP has an overall negative

charge, although some functional groups, such as carboxylic and phosphate carry a negative charge, and amino groups carry a positive charge (Jia *et al.*, 1996a). The overall charge of the ECP will be less negative when more protein, which has both positively and negatively charged groups, is present in ECP. However, stronger granules will be produced when an appreciable amount of carbohydrates relative to the dominant protein fraction is present. The negative charge of carbohydrates will be neutralised by positively charged inorganic molecules and amino groups, and cross-linking can occur between the bacterial cells. It has also been found (Quarmby & Forster, 1994) that as the inorganic fraction (ash content) of the granules increases, the strength of the granule tends to decrease. Nevertheless, the negative surface charge of anaerobic sludge will increase linearly with the total ECP content, regardless of the individual contents of protein and carbohydrate in ECP (Jia *et al.*, 1996a). Too much ECP can cause deterioration in floc formation and repulsion can occur between micro-organisms present (Schmidt & Ahring, 1996). Granule strength will, therefore, be dependent on the surface charge of the sludge, which is strongly dependent on the chemical composition and concentration of ECP present.

Jia *et al.* (1996a) also investigated the changes of ECP and surface charge during substrate degradation in anaerobic sludge. They found that the ECP content and surface charge were dependent on the growth phase of the micro-organisms and concentration of substrate available. More ECP was produced when plentiful substrate was available, and micro-organisms were in the prolific-growth phase, thus having a high food-to-micro-organisms (F:M) ratio. As the substrate became depleted and the micro-organisms were in the declined growth phase, some ECP was metabolised by bacteria as energy and/or carbon sources. Both the ECP content and surface charge then can return to the initial levels.

The negative-charged nature of the ECP implies that the addition of divalent cations, such as calcium, would enhance the granulation of anaerobic sludge in UASB reactors. This is probably due to the capability of bridging the negative-charged ECP of adjacent cells (Jia *et al.*, 1996a). Calcium and iron salts, such as calcium carbonate and/or calcium phosphate and iron sulphide, provide natural inert supports for the bacteria. ECP prefers to bind heavier metals when they are available in forming more stable complexes. Iron and cobalt present in extracted

EPS appears to be bound to ECP and might play an important role in cross-linking ECP (Shen *et al.*, 1993a).

Porosity and pore size distribution in the granules influence the activity of the bacterial cells in the granules. Porosity and pore size distribution are closely related to substrate and biogas transport. It has been found that substrate transport limitation can cause autolysis of the granule core, by allowing growth of bacteria merely at the surface of the granules, and eventually producing hollow granules (Alphenaar *et al.*, 1992). Investigation of granules from four full-scale UASB reactors treating different kinds of wastewater revealed that the porosity of the granules decreased with increasing granule size. As a result of this, substrate transports limitations increased with increasing granule size, while the methanogenic activity decreased. However, no difference in substrate affinity was observed between granules of different sizes, indicating that only the exterior of large granules is biological active (Alphenaar *et al.*, 1993).

E. ECP EXTRACTION PROCEDURES

It is well known that ECP plays an important role in the bioflocculation processes of aerobic activated sludge and anaerobic granular sludge (Quarmby & Forster, 1994). Ross already suggested in 1984 that the bioflocculation mechanisms proposed for aerobic processes might be applicable to the formation of anaerobic granules in UASB reactors. Therefore, ECP extraction procedures for activated sludges are also applicable to anaerobic granules (Brown & Lester, 1980; Gehr & Henry, 1983; Morgan *et al.*, 1990; Dignac *et al.*, 1998; Zhang *et al.*, 1999).

Although most of the studies on bioflocculation have been limited to aerobic processes, many researchers have used various extraction methods to extract and quantify the ECP content in granules from UASB reactors (Ross, 1984; Dolfling *et al.*, 1985; Morgan *et al.*, 1990; Grotenhuis *et al.*, 1991b; Guiot *et al.*, 1991; Shen *et al.*, 1993a; Quarmby & Forster, 1994; Schmidt & Ahring, 1994; Bhatti *et al.*, 1995; Jia *et al.*, 1996a; Veiga *et al.*, 1997). Considerable variation on the reported ECP content of granules has been found and this depends on the extraction methods; methods of analyses and types of granular sludges examined (Table 1). No standardised universal method of extraction is used at present and, therefore,

Table 1. Composition of extracellular polymers extracted from different types of granules from UASB reactors.

Reference	Composition of extracellular polymers
Morgan *et al.* (1990)	1.09 - 2.79 protein:carbohydrate ($mg.g^{-1}$ SS)
Schmidt & Ahring (1994)	5.09 - 8.25 protein:polysaccharide ($mg.g^{-1}$ VSS)
Shen *et al.* (1993a)	4.6 - 8.4 protein:polysaccharide ($mg.g^{-1}$ TSS)
Quarmby & Forster (1994)	1.10 - 5.40 protein:polysaccharide ($mg.g^{-1}$ TS)
Grotenhuis *et al.* (1991b)	3.6 - 5.0 mg protein per g dry weight 1.1 - 3.0 mg polysaccharide per g dry weight
Dolfling *et al.* (1985)	10 - 20 mg polysaccharide per g dry weight 45 mg protein per g dry weight
Guiot *et al.* (1991)	10 - 20% EPS of sludge dry weight: 2% DNA; 12% polysaccharides; 62% proteins; 25% unidentified compounds
Bhatti *et al.* (1995)	1.7 - 2.7% polysaccharides of TSS 2.5 - 5.1% nucleic acids of TSS 8.3 - 16.3% proteins of TSS

comparison of results from different researchers has to be made with great caution. Comparative studies between results of different authors might be useful if the extraction method is initially standardised (Morgan *et al.*, 1990). Two types of extracellular material exist, namely capsular material and slime (Gehr & Henry, 1983) and it is of great importance that extracellular material is extracted without significant cell lysis which will then contribute to the intracellular material of the extracted ECP (Schmidt & Ahring, 1994).

Five steps have been proposed (Gehr & Henry, 1983) to obtain extracellular material from micro-organisms: namely biomass concentration and removal of slime; stripping of the capsule from the cell (extraction); precipitation of the stripped material from solution; collection of the precipitate; and an optional purification step. Biomass concentration and removal of extraneous matter, including slime is accomplished by high-speed centrifugation and settling. Simple high-speed centrifugation does not give a separation between the slime and capsular material fractions, therefore, a washing step is necessary to remove slime from the capsule. Secondly, the stripping process (extraction) can be subdivided into chemical and physical stripping. Chemical stripping involves adding a reagent to the suspension that causes the capsule to separate from the cell. Physical stripping can be subdivided into heating and mechanical disruption. Chemical and physical stripping methods can also be combined as an extraction method. Precipitation, in contrast, is done in order to recover the material once it has been stripped. The aqueous extraction solution is then added to an alcohol. Long-chain organic molecules and some inorganic salts are insoluble in the solution and precipitation occurs, usually after storage at 4°C overnight and the precipitate is collected by centrifugation. Purification of the precipitate is an optional step, and it is done by resuspension, usually in a salt solution, dialysis in distilled water and washing in a suitable alcohol to remove organic and inorganic monomers (Gehr & Henry, 1983).

Several researchers have developed and compared different extraction methods to quantify the extracellular polymer content in order to determine the most effective extraction method. Brown & Lester (1980) compared five different extraction methods and a combination of two on samples of activated sludge, synthetic activated sludge and a culture of *Klebsiella aerogenes*. The extraction

methods investigated were high-speed centrifugation, ultrasonication, ultrasonication and high-speed centrifugation, steam extraction, sodium hydroxide extraction and ethylenediaminetetraacetic acid (EDTA) extraction. Steam extraction was found to be the most effective extraction method as it caused less cell disruption than chemical methods. Chemical extraction methods were found to cause significant cell disruption in all samples. High-speed centrifugation was only effective in extracting ECP from pure cultures. Ultrasonication released only small amounts of ECP and caused no significant cell disruption, therefore, it was concluded that it might be only useful as a preliminary treatment in conjunction with another extraction method (Brown & Lester, 1980).

Dignac *et al.* (1998) reported the extraction of aerobic ECP with a combination of sonication and cation exchange resin treatment (CER) was also very successful. Ultrasound breaks all kinds of structures if the bond energy is less than the sonication energy applied, while CER is only active on electrostatic bonds in which divalent cations are involved. The two extraction methods are thus complementary and their combination improves the sonication method. With this combination proteins were better extracted than polysaccharides, indicating that proteins are more involved than sugars in electrostatic bonds with multivalent cations.

In many of the reported studies researchers did not include any control for cell lysis during ECP extraction (Ross, 1984; Dolfling *et al.*, 1985; Morgan *et al.*, 1990; Shen *et al.*, 1993a), while other researchers used changes in the DNA content during extraction as an indication of the extent of cell lysis (Brown & Lester, 1980; Gehr & Henry, 1983; Grotenhuis *et al.*, 1991b; Guiot *et al.*, 1991). Nucleic acids have been bound in the extracellular matrix of bacterial flocs, therefore, an increase in the supernatant DNA may be due to either cell disruption or to the release of DNA from the extracellular matrix of the granules. Schmidt & Ahring (1994) proposed a new method, in order to determine the optimal extraction time. It was also based on the release of DNA per hour. It was found that the increase in amount of DNA extracted per hour decreased with time in an exponential fashion for a certain period, whereafter an increase in the amount of ECP extracted per hour was observed. The increase was ascribed to cell lysis, and the optimal extraction time that was concluded, was at the point where an increase in extracted amount of DNA was induced (Schmidt & Ahring, 1994).

It was also proposed that an initial washing step is necessary to remove extracellular material from activated sludge (Gehr & Henry, 1983). However, the washing step will also wash out some loosely attached extracellular polymers and it was thus proven that it was essential to analyse wash waters when quantifying extracellular polymers in biofilms. Contributions from washing step varied from 8 to 50% of the total carbohydrate yield, depending on the extraction method used. Five commonly extraction methods were used by Gehr & Henry (1983), namely regular centrifugation; EDTA extraction; ultracentrifugation; steam extraction and regular centrifugation with formaldehyde (RCF). The RCF extraction method resulted in the biggest carbohydrate yield, but detected only small amounts of proteins, because formaldehyde cross-links proteins and makes them difficult to detect. Steam extraction, in contrast, helps to release stripped material from the cells and gave the second best carbohydrate yield and the biggest protein yield. The other three extraction methods gave approximately equivalent amounts of carbohydrate and protein. Depending on the method used, the more carbohydrates that are collected from the washing step, the less carbohydrates are recovered from the stripping step (Zhang *et al.*, 1999).

F. Conclusions

One of the limiting factors for the successful operation of an UASB reactor is the long start-up period resulting from the slow granulation process. It has been concluded that the ECP produced by bacteria in granules is of great importance in the granulation process (Britz *et al.*, 1999). It has also been shown that various physical-chemical and biological factors influence the granulation process and ECP content of granules (Joubert, 2001), but information on the precise role of ECP during granulation is still limited.

Several researchers have used various extraction methods to extract and quantify the ECP content in granules from UASB reactors. Considerable variation on the reported ECP content of granules is to be found depending on the extraction methods, methods of analyses for ECP and types of granular sludge examined. Therefore, no standardised method of extraction exists at present, and comparison of results from different authors has to be made with great caution. The development of a standard extraction method will see to more clarity in the

future about the precise role of ECP during the granulation process, and will certainly play a helpful role in optimising the granulation process.

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CHAPTER 3

EVALUATION OF EXTRACTION AND ANALYSIS METHODS OF ECP FROM UASB GRANULES AND THE IMPACT OF ECP COMPOSITION ON GRANULE ACTIVITY

Abstract

The precise mechanism for the formation of Upflow Anaerobic Sludge Bed (UASB) granules are not well known, however it is believed that extracellular polymers (ECP) play a major role in the granulation process. Unfortunately, no universal standardised method for ECP extraction is used at present and therefore comparison of results from different researchers has to be made with great caution. In this study an ECP extraction method was evaluated that optimised extraction time and included a control for cell lysis. Subsequently, this method was used to determine the ECP composition of granules from various full-scale and laboratory-scale UASB reactors. The activity of the different UASB granules was also evaluated and correlated with the ECP composition of the various UASB granules. The optimal extraction time for the granules was determined at 4 h, with the exception of the Fruit-type granules which was at 3 h. In general, the dominant component of the ECP consisted of protein. Accordingly, the protein:carbohydrate ratio was greater than 1 for all the granules, except for the Fruit-type granules. It was found that when granules, like the Fruit-type granules, treated mainly acidogenic wastewater, the dominant ECP component from the acidogenic granules consisted of mainly carbohydrates. The ECP composition and protein:carbohydrate ratio of the different UASB granules was thus impacted by the composition of the wastewater fed to the various UASB reactors. Activity testing was done to indirectly indicate the activity of the different microbial groups present in the respective granules. It was found that that the Food-, Brew- and Comp-type granules exhibited higher S_B and S_M activities. Moreover, it was found that granules with higher ECP yields exhibited greater S_B and S_M activities. However, it was also concluded that the protein:carbohydrate ratio of the different UASB granules might affect the activity of the respective granules.

Introduction

Successful operation of an Upflow Anaerobic Sludge Bed (UASB) reactor depends on a spontaneous agglutination of the biomass into a granular sludge. These so-called granules are formed by the natural self-immobilisation of bacteria and have high settling velocities and high methanogenic activity for the degradation of organic substances (Fukuzaki *et al.*, 1995). The mechanisms for the formation of granules in UASB reactors are not well known, but it is believed that extracellular polymers (ECP) play a major role in the granulation process. Information on the precise role of the ECP in the granulation process is still limited even though Ross already reported in 1984 that agglutination of bacteria is generally due to the interaction between a protein and a polysaccharide. This suggests that ECP play a major role in agglutination of bacteria to form granules. ECP is generally defined as polysaccharide-containing structures of bacterial origin, lying outside the outer membrane of gram-negative cells and the peptidoglycan of gram-positive cells (Costerton *et al.*, 1981). It is believed that microbial cells produce ECP from organic debris, phages, lysed cells and other material that is excreted by the microbial cells. ECP consists mainly of protein and polysaccharides, and minor amounts of lipids, lipopolysaccharides, DNA and RNA (Schmidt & Ahring, 1996).

Many factors may influence the production and composition of ECP in anaerobic granules. ECP has an overall negative charge due to the functional groups present in ECP (Morgan *et al.*, 1990), and accumulate on the surface of micro-organisms (Jia *et al.*, 1996a). It is likely, therefore, that too much ECP can cause repulsion between bacterial cells, thus affecting the granulation process (Morgan *et al.*, 1990). Microbial populations present within the granular sludge also influence production of ECP, with acidogens having higher ECP yields than acetogens and methanogens (Jia *et al.*, 1996b). Harada *et al.* also reported in 1988 that carbohydrate-degrading granules were bigger and had higher mechanical strength than granules that degraded short chain fatty acids containing effluents.

Many researchers have used various extraction methods to quantify the ECP content of granules from UASB reactors (Ross, 1984; Morgan *et al.*, 1990; Schmidt & Ahring, 1994; Quarmby & Forster, 1994; Jia *et al.*, 1996b). Thus, considerable variations on the reported ECP content of granules can be found in

the literature and it is clear that the variation depends on the extraction methods used, methods of analyses employed and types of granules examined. In many of the reported studies researchers did not include any control for cell lysis during ECP extraction (Ross, 1984; Dolfling *et al.*, 1985; Morgan *et al.*, 1990; Shen *et al.*, 1993), while other researchers used changes in the DNA content during extraction as an indication of the extent of cell lysis (Brown & Lester, 1980; Gehr & Henry, 1983; Grotenhuis *et al.*, 1991; Guiot *et al.*, 1991). Nucleic acids have also been found to be bound in the extracellular matrix of bacterial flocs, therefore, an increase in the supernatant DNA may be due to either cell disruption or to the release of DNA from the extracellular matrix of the granules. No standardised method of ECP extraction is used at present and, therefore, comparison of results from different researchers has to be made with great caution. The development of a standard extraction method might lead to more clarity as to the precise role of ECP in the granulation process.

Schmidt & Ahring proposed a new method in 1994, in order to determine the optimal extraction time. This was the first time an attempt has been made to determine the optimal extraction time of ECP extraction from UASB granules. The method used was based on the release of DNA per hour. It was found that the amount of DNA extracted per hour decreased with time in an exponential fashion for a certain period, whereafter an increase in the amount extracted per hour was observed. The increase was ascribed to cell lysis, and it was concluded that the optimal extraction time was at the point where an increase in extracted amount of DNA was induced (Schmidt & Ahring, 1994).

In this present investigation, an ECP extraction method based on the principles of the method of Schmidt & Ahring (1994) will be evaluated. With the use of this method the ECP composition of granules from various full-scale and laboratory-scale UASB reactors, will be examined. The activity of different UASB granules will also be evaluated and correlated with the ECP composition of the various UASB granules.

Materials and methods

Granule samples

UASB granule samples were obtained from various industrial and laboratory-scale UASB reactors in South Africa. The granule sources and composition of the wastewater these reactors were treating, are shown in Table 1.

Extraction of water-soluble extracellular polymers

A thermal extraction method was used to extract the ECP content of the UASB granules (Schmidt & Ahring, 1994). The granules were rinsed with physiological salt solution (0.85% m/v) and thereafter manually mashed until a homogenised sample was obtained. Identical serum vials (6 ml) with 0.5 g sample and 3 ml phosphate-buffered saline (PBS) (Smibert & Krieg, 1994) (Table 2) were placed in a shake-waterbath (150 rpm) at 70°C. A vial was removed every hour for six hours for each granule sample from the waterbath. The contents were centrifuged at 9 600 x g for 10 min, and the supernatant was removed and kept at -18°C for further ECP analyses.

Analyses of extracellular polymers

Carbohydrates in the extracted ECP were determined using the phenol/sulphuric acid method of Dubois *et al.* (1956). Firstly, 50 µl of phenol (80% m/m) [Saarchem - Unilab] was added to 2 ml of the ECP-extract. Five ml of concentrated sulphuric acid (98% reagent grade) [B & M Scientific] was added rapidly. A blank was used by replacing the ECP-extract with PBS-solution. The tubes were allowed to stand for 10 min, then mixed with a Vortex mixer and placed in a waterbath for 10 min at 30°C. Absorption was measured against the blank at 492 nm. Glucose [Saarchem - Unilab] was used as a standard in the range 0 - 100 $mg.l^{-1}$.

The protein content in the ECP-extract was measured according to the bicinchoninic acid (BCA) method (Smith *et al.*, 1985; BCA-200 Protein Assay Kit, Separations). A set of Bovine Serum Albumin (BSA) [Boehringer Mannheim] protein standards was prepared in the range 0 - 2 $mg.ml^{-1}$ by diluting the BSA stock solution (2 $mg.ml^{-1}$) in the same solution (PBS) as the samples. Thereafter

Table 1. Sources of UASB granules used in this study.

Sample	Granule source	Origin	Wastewater composition
Brew-type	SA Breweries, Amanzimtoti	Industrial-scale	Brewery effluent
Dist-type	Distell, Wellington	Industrial-scale	Distillery effluent
Fruit-type	Ceres Fruit Growers, Ceres	Industrial-scale	Fruit juice and fruit pulp effluent
Lye-type	Mr. G.O. Sigge, University of Stellenbosch	Laboratory-scale	Peach-lye canning effluent
Comp-type	Ms. W. Griessel, University of Stellenbosch	Laboratory-scale	Anaerobic composting effluent
Food-type	Dr. M. Van Der Merwe, Krugersdorp	Industrial-scale	Gelatine and food effluents

Table 2. Composition of phosphate-buffered saline solution* (PBS) used for the extraction of ECP.

Compound	Concentration ($g.l^{-1}$)
10x stock solution	
Na_2HPO_4, anhydrous [Merck]	12.36
$NaH_2PO_4.H_2O$ [Merck]	1.80
NaCl [Saarchem - Unilab]	85.00
Working solution (0.01 M phosphate, pH 7.6)	
Stock solution	100 ml
Distilled water	900 ml

*Smibert & Krieg (1994)

10 µl of each standard, blank and unknown sample was pipetted into the microtiter plate wells. A "working reagent" was prepared by combining fifty parts Reagent A with one part Reagent B (BCA-200 Protein Assay Kit, Separations) with 200 µl of the working reagent added to each well. The sample was mixed for 30 s on a microtiter plate shaker, and incubated for 30 min at 37°C. Absorbancy was measured at 620 nm with a microtiter plate reader.

Lipids were extracted and quantified using the method of Bligh & Dyer (1959). An 8 ml ECP-extract sample was homogenised in a Vortex mixer for 30 s with 10 ml chloroform and 20 ml methanol added. Thereafter 10 ml chloroform was again added, and the sample was vortexed for another 30 s. Ten (10) ml distilled water was added and the mixing continued for another 30 s. The sample was then filtered through Whatman no.1 filter paper (Quantitative-15 cm) and the filtrate collected in a 100 ml graduated cylinder. The volume of the chloroform layer (B), as well as the total volume of the sample (C), was recorded. The alcoholic layer was removed by aspiration, and the chloroform layer then contained the purified lipid. For determination of the lipid content, the chloroform layer was evaporated to dryness in a 100 ml round-bottom flask of known weight (D). Thereafter the residue was dried overnight in a vacuum desiccator, and after evaporation the weight of the flask (E) was determined. The lipid content was calculated as follows: Weight of lipid in flask (A) = E-D

Total lipid content = (A*B)/C

DNA in the ECP-extract was extracted (Smalla *et al.*, 1993), and quantified by using a fluorometric method (DyNA Quant 200 Fluorometer, Pharmacia Biotech; Johnson, 1994). All studies were done in duplicate. The water phase of 500 µl ECP-extract was extracted with 400 µl of phenol and twice extracted with 400 µl of phenol:chloroform:isoamylalcohol mixture (25:24:1) until the interphase was clean. The DNA was precipitated with 0.1 volume of 3M NaAc (pH 5.5) and 0.6 volume of isopropanol. The DNA was pelleted by centrifugation at 13 400 x g for 15 min, washed with cold ethanol, centrifuged at 13 400 x g for 5 min, dried and redissolved in 20 µl TE buffer (10 mM Tris, 1mM EDTA; pH 8.0). The DNA-extract was quantified using a fluorometric method (DyNA Quant 200 Fluorometer User Manual, Pharmacia Biotech). Calf thymus DNA Fluoroscence standard

[Separation Scientific] was used as the standard in the range of 10 - 500 $ng.ml^{-1}$ final DNA concentration.

Analytical procedures

The total suspended solid (TSS) and volatile suspended solid (VSS) content of the various UASB granules was determined, respectively. Firstly, the UASB granules were centrifuged at 10 000 x g for 10 min. Thereafter, the TSS and VSS content was determined using the methods recommended by *Standard Methods* (APHA, 1992; Shen *et al.*, 1993). All studies were done in duplicate.

Statistical methods

The ECP extraction method was repeated five times to test whether the ECP extraction and analyses techniques were reproducible. The ECP-extracts obtained were respectively analysed for protein, carbohydrate, lipid and DNA composition and determined as a fraction of the VSS content of respective granules. Protein, carbohydrate and DNA analyses were performed on each of the ECP-extracts obtained from each extraction time. All analyses were done in duplicate. To test whether significant differences existed between the protein, carbohydrate and DNA analyses results, a one-way analyses of variance (ANOVA) was performed (Keller & Warrack, 1997). The lipid content of the ECP-extracts was determined for the optimal extraction time estimated for the respective UASB granules. All analyses were done in duplicate. To evaluate the reproducibility of the lipid analyses, VSS and TSS determinations, the coefficient of variation (CV) was determined (Smith, 1994).

Activity testing

The activity of the granules obtained from the laboratory-scale and full-scale UASB reactors was determined. The granules were firstly re-activated for 2 days at 35°C in sterile medium (pH 7.0) containing 1 $g.l^{-1}$ glucose [Saarchem - Unilab] and 0.5 $g.l^{-1}$ each of urea [Labchem] and KH_2PO_4 [BDH]. This two-day incubation period was done to allow the granules to regain activity for optimum biogas production during the activity-testing period (Cameron, 2000). The activation medium was replaced daily to optimise re-activation of the granules. Twenty (20) ml glass vials were inoculated with 3 g granules and 13 ml of the basic test

medium (BTM) (Table 3) or other test media, respectively (Table 4) (O'Kennedy, 2000). Each vial was sealed and incubated at 35°C for 25 h. The activity tests were done in triplicate. Biogas samples were taken at time 5, 10 and 25 h of incubation using a gas-tight and free-moving 6 ml syringe equipped with a 26 gauge needle (Owen *et al.*, 1979).

The biogas samples were analysed for CH_4 and CO_2 using a Varian 3300 GC fitted with a thermal conductivity detector and 2.0 m x 3.0 mm i.d. column packed with Hayesep Q (Supelco, Bellefonte, PA, USA), 80/100 mesh. The oven temperature was set at 55°C and helium was used as carrier gas at a flow rate of 30 $ml.min^{-1}$ (Lamb, 1995; Ronquest, 1999). Methane obtained from Fedgas (Pty.) Ltd., Johannesburg was used as the standard. The biogas samples were expressed as cumulative gas volumes.

Results and discussion

Extraction of extracellular polymers (ECP)

The ECP content of UASB granules has been shown to be mainly dependent on the specific granules examined, the extraction method employed and the analytical method used to quantify the ECP. Physical extraction methods are reported to be more successful than chemical extraction methods (Brown & Lester, 1980; Gehr & Henry, 1983). Zhang *et al.* (1999) also reported that chemical extraction of ECP with formaldehyde cross-links the protein, and thus makes it difficult to detect accurately. Thus, in this study a physical thermal extraction method was rather used to extract the ECP from different UASB granules (Brown & Lester, 1980; Morgan *et al.*, 1990; Schmidt & Ahring, 1994).

The optimal extraction time was determined by using a method proposed by Schmidt & Ahring (1994). The amount of DNA extracted from the different UASB granules as a function of the extraction time is illustrated in Fig. 1. The data clearly shows that the DNA content increased as a function of the extraction time. However, the data in Fig. 1 shows no clear indication of the optimal extraction time for the various granules. In order to determine the optimal extraction time, the changes in amount of DNA extracted per hour was plotted against the extraction time (Fig. 2). From the data it was found that the increase in the amount of DNA extracted per hour decreased with time for a certain period, whereafter a smaller

Table 3. Composition of the Basic Test Medium (BTM) (Valcke & Verstraete, 1993).

Compound	Concentration ($g.l^{-1}$)
Glucose* [BDH]	2.0
K_2HPO_4 [ACE]	1.0
KH_2PO_4 [BDH]	2.6
Urea [Labchem]	1.1
NH_4Cl [BDH]	1.0
Na_2S [Saarchem - UniVar]	0.1
$MgCl_2.6H_2O$ [Merck]	0.1
Yeast Extract [Biolab]	0.2
pH	7.1

* Added extra to the medium of Valcke & Verstraete

Table 4. Different test media used and the specific microbial group enhanced (Cameron, 2000).

Test media*	Bacterial group
Control (only BTM)	
2 $g.l^{-1}$ Glucose [BDH]	Acidogens
4 $g.l^{-1}$ Lactic Acid [Saarchem - Unilab; 60% solution V/V]	Lactate-utilisers
1 $g.l^{-1}$ Acetic Acid [B&M Scientific]	Acetoclastic Methanogens

*The BTM was used as basis for the different media

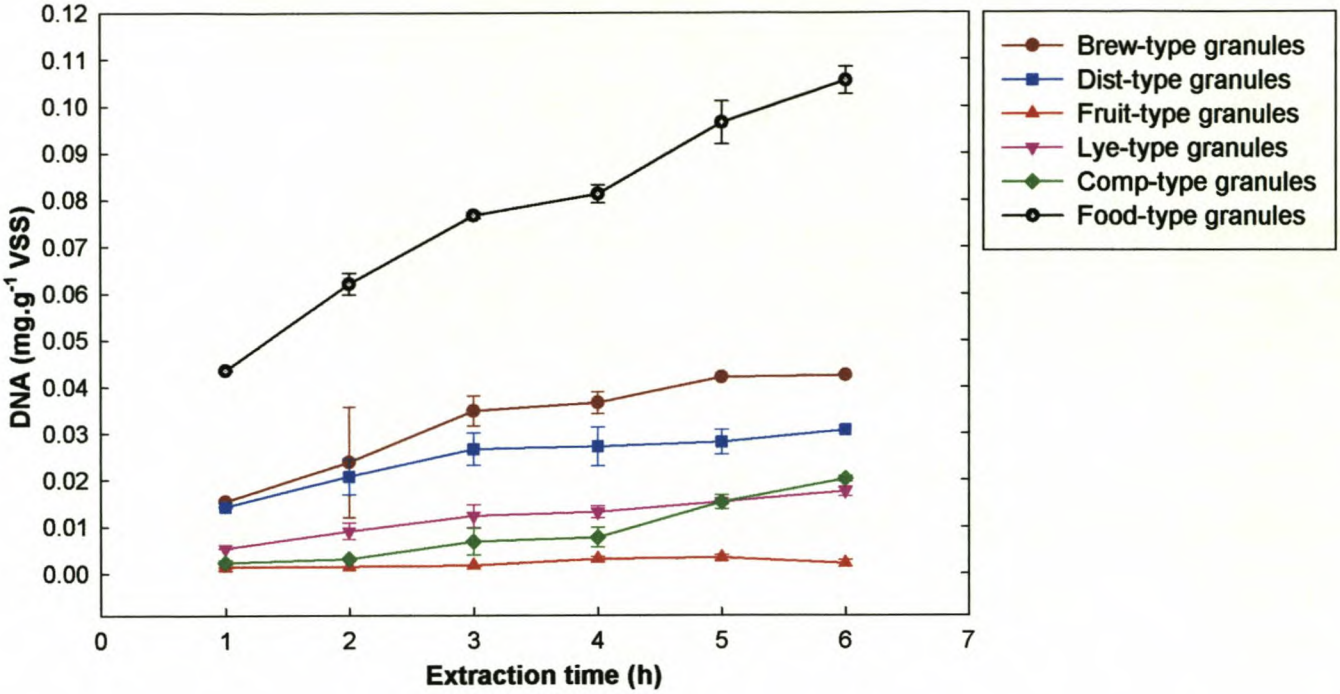

Figure 1. The amount of DNA (mg.g^{-1} VSS) extracted from the different UASB granules as a function of the extraction time (h). The standard deviation was used as the error-bar length.

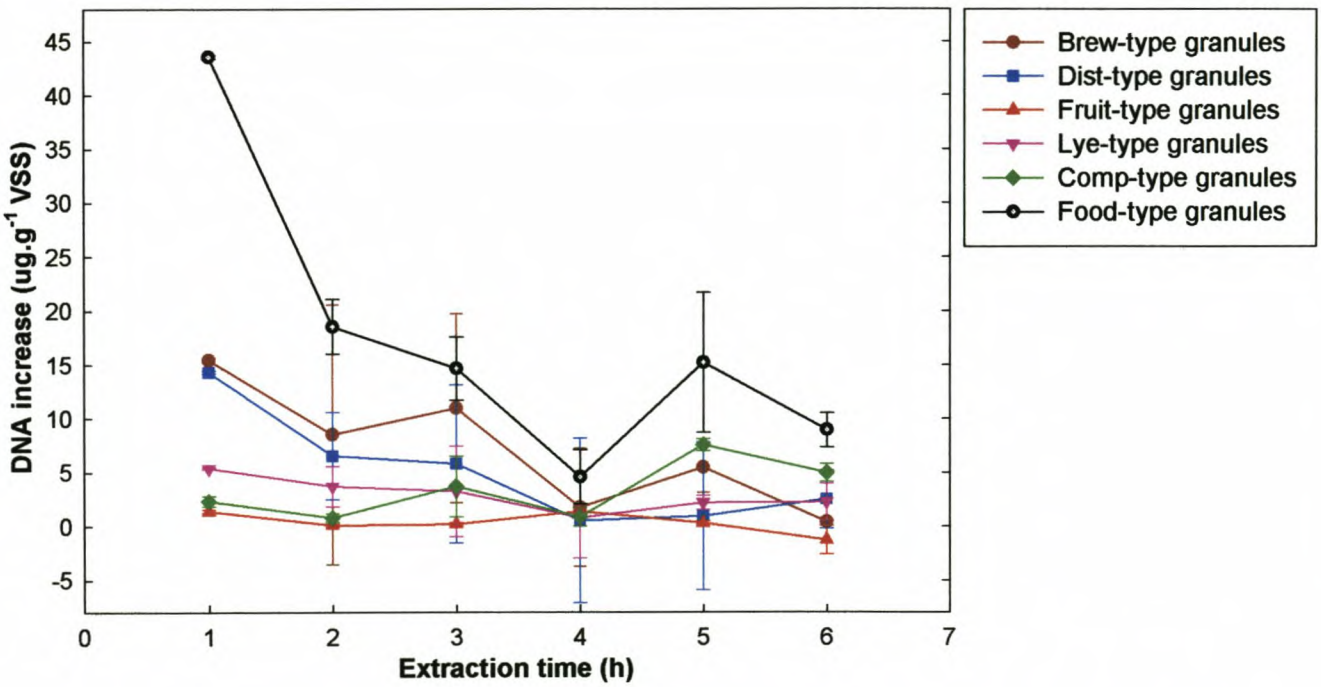

Figure 2. The increase in DNA (μg.g^{-1} VSS) extracted from the different UASB granules as a function of extraction time (h). The standard deviation was used as the error-bar length.

increase in the amount of extracted ECP was observed. It was thus concluded that the optimal extraction time was at the lowest point just before an increase in the extracted amount of DNA occurred. The detection of the optimal extraction time as found in this study correlates well with findings reported by Schmidt & Ahring (1994). The optimal extraction time was detected at 4 h for all the granule samples, with the exception of the extraction time for the Fruit-type granules, which was found to be at 3 h. After the point of optimal extraction time, an increase occurred in the amount of DNA extracted per hour. This increase was probably due to cell lysis, thus the intracellular material started contributing to the DNA content of the ECP. The results from the extraction profiles also indicated that the increase in DNA content decreased again after the 5 h extraction time.

Data from the ANOVA results on the reproducibility of the ECP extraction and analyses (Table 5) show that no significant difference ($p > \alpha = 0.05$) existed between the analyses for protein, carbohydrate and DNA. Thus, it was concluded with 95% confidence at a 5% significance level that non-significant differences existed between the extraction and analysis methods for protein, carbohydrate and DNA, thus the ECP extraction and analysis methods used in this study were reproducible (Keller & Warrack, 1997).

The coefficient of variation (CV) determined for the lipid analysis, TSS and VSS content was respectively 4.78, 3.57 and 3.47%. These values were below 5% and, therefore, indicated a high level of reproducibility of the replicates for lipid analysis, TSS and VSS determinations (Smith, 1994).

ECP composition of the different UASB granules

The data of the composition of the extracted ECP from the different UASB granules is shown in Table 6. The values for total ECP content of the different granules varied from 28.71 to 53.76 $mg.g^{-1}$ VSS. It was found that protein was the dominant component of the quantified ECP from all the granules with the exception of the Fruit-type granules. Morgan *et al.* (1990) also found that protein was the dominant component of ECP from UASB granules and anaerobic granular sludge. Ehlinger *et al.* (1987) however reported that the dominant component of ECP from acidogenic flocs was mainly carbohydrates and reported a protein:carbohydrate ratio of less than 1 (0.61) for glucose fed anaerobic sludge. El-Mamouni *et al.* (1995) also confirmed that the ECP of anaerobic granules

Table 5. ANOVA results for the protein, carbohydrate and DNA analyses of the ECP extracted from the Brew-type granules.

ECP	*p*-value*	α+	*n*
Protein analysis	0.622	0.05	5
Carbohydrate analysis	0.616	0.05	5
DNA analysis	0.194	0.05	5

*$p > \alpha$: Non-significant differences exist for reproducibility of protein, carbohydrate and DNA analyses

+α: Significance level of 5%

Table 6. The composition of the ECP ($mg.g^{-1}$ VSS) extracted from the different UASB granules.

Granule type	Total ECP	Protein	Carbohydrate	Lipid	DNA	Protein:Carbohydrate ratio	Optimal extraction time (h)
Brew-type	53.76	$36.31^{*2.803}$	$9.04^{*0.118}$	$8.37^{*1.625}$	$0.037^{*0.0023}$	4.02	4
Dist-type	37.32	$27.27^{*0.508}$	$7.01^{*0.135}$	$3.01^{*0.778}$	$0.026^{*0.0041}$	3.89	4
Fruit-type	35.54	$14.64^{*0.295}$	$18.63^{*0.066}$	$2.26^{*0.564}$	$0.002^{*0.0001}$	0.79	3
Lye-type	28.71	$20.24^{*0.329}$	$5.33^{*0.122}$	$3.12^{*0.415}$	$0.013^{*0.0013}$	3.79	4
Comp-type	38.47	$28.33^{*1.899}$	$8.60^{*0.029}$	$1.52^{*0.462}$	$0.007^{*0.0021}$	3.29	4
Food-type	49.32	$39.48^{*2.813}$	$7.62^{*0.311}$	$2.16^{*0.287}$	$0.064^{*0.0016}$	5.18	4

*Standard deviation (SD) values

enriched with acidogens was essentially composed of carbohydrates. In this study similar results were found for the Fruit-type granules. The Fruit-type granules were obtained from a full-scale UASB plant that treated mainly fruit juice and fruit pulp wastewater and had a very low protein:carbohydrate ratio of 0.79. It was highly likely, as the data showed that the ECP of the Fruit-type granules was essentially composed of carbohydrates, and that the trophic groups of these granules were probably dominated by acidogens.

The protein:carbohydrate ratio for the different granules varied from 0.79 in the case of the Fruit-type granules to 5.18 for the Food-type granules (Table 6). The ECP composition, and thus protein:carbohydrate ratio of the ECP has been shown to be affected by the composition of the wastewater fed to the reactor (Shen *et al.*, 1993; Schmidt & Ahring, 1994; Veiga *et al.*, 1997). In this study the highest protein:carbohydrate ratio was observed in the Brew-type and Food-type granules. The yeast present in the brewery effluent fed to the Brew-type granules probably contributed to the higher protein:carbohydrate ratio of 4.02. The wastewater fed to the Food-type granules consisted of a protein-rich gelatine type effluent that probably also contributed to the higher protein:carbohydrate ratio of 5.18. The wastewater fed to the Dist-, Lye- and Comp-type granules were more carbohydrate-rich and this factor thus probably contributed to a lower protein:carbohydrate ratio of around 3.

The amount of lipids (Table 6) in the quantified ECP from the different granules was in all cases lower than the carbohydrate content. The ECP from the Brew-type granules had the highest lipid concentration. Schmidt & Ahring (1994) also reported that the amount of lipids in the quantified ECP of their granules was significantly lower than the amount of protein and carbohydrate. They concluded that granules grown on simple substrates had higher amount of lipids in their ECP than granules grown on more complex substrates. However, no discernible trend was observed in the concentration of the lipids present in the ECP composition from the different UASB granules.

Activity testing

Activity testing of UASB granules can be used to obtain an indirect indication of the activity of the different microbial trophic groups present in granules and granular sludge (Switzenbaum, 1990). Activity testing of granules depends on the

energy and carbon source that the granules are grown on. Maximum activity values are obtained when the test substrate is identical to the growth substrate or if the test substrate is an important intermediate (Schmidt & Ahring, 1996).

In this study, the activity of six different UASB granules (Table 1) was determined and the data compared. Furthermore, the activity of the granules was also correlated with the ECP content (Table 6) of the different UASB granules. The cumulative biogas was determined for each type of granule, and the methane (CH_4) fraction calculated using GC results according to the method of O'Kennedy (2000). The cumulative biogas and CH_4 data was then converted to represent the activity of each type of granule relative to each other. In this study it must be taken into consideration that the activity of each type of granule is described in terms of the tempo of biogas (S_B) and tempo of CH_4 production (S_M). The tempo of gas production (S_B and S_M) was determined using the slope or gradient (cumulative gas production (CGP) over time for each of the time intervals (t = 5, 10 and 25 h). The gradient, for example for the 10 h time interval, was determined as follows: $(CGP_{10\ h} - CGP_{5\ h})/(t_{10\ h} - t_{5\ h})$. The tempo of gas production was thus given in $ml.h^{-1}$ and was taken as representative of the activity of the granules. The values were then plotted against time for the different UASB granules to give a visual representation of the activity of the results obtained (O'Kennedy, 2000). The S_B and S_M activities as found in this study are illustrated in Fig. 3 and 4, respectively.

Activity evaluation of the different UASB granules

In general it was found that the maximum S_B and S_M activity for all the different granules occurred by the 10 h time period of incubation. Moreover, a larger variability in S_B and S_M activity was found at the 10 h incubation period and the differences when different test substrates were used was more apparent than at the 5 or 25 h period. Thus, in this study the S_B and S_M activity values obtained at 10 h were used to indicate the activity of the different granules. Cameron (2000) also found that the maximum S_B and S_M activities for different UASB granules occurred at the 10 h incubation period and used this time period for the optimum activity testing.

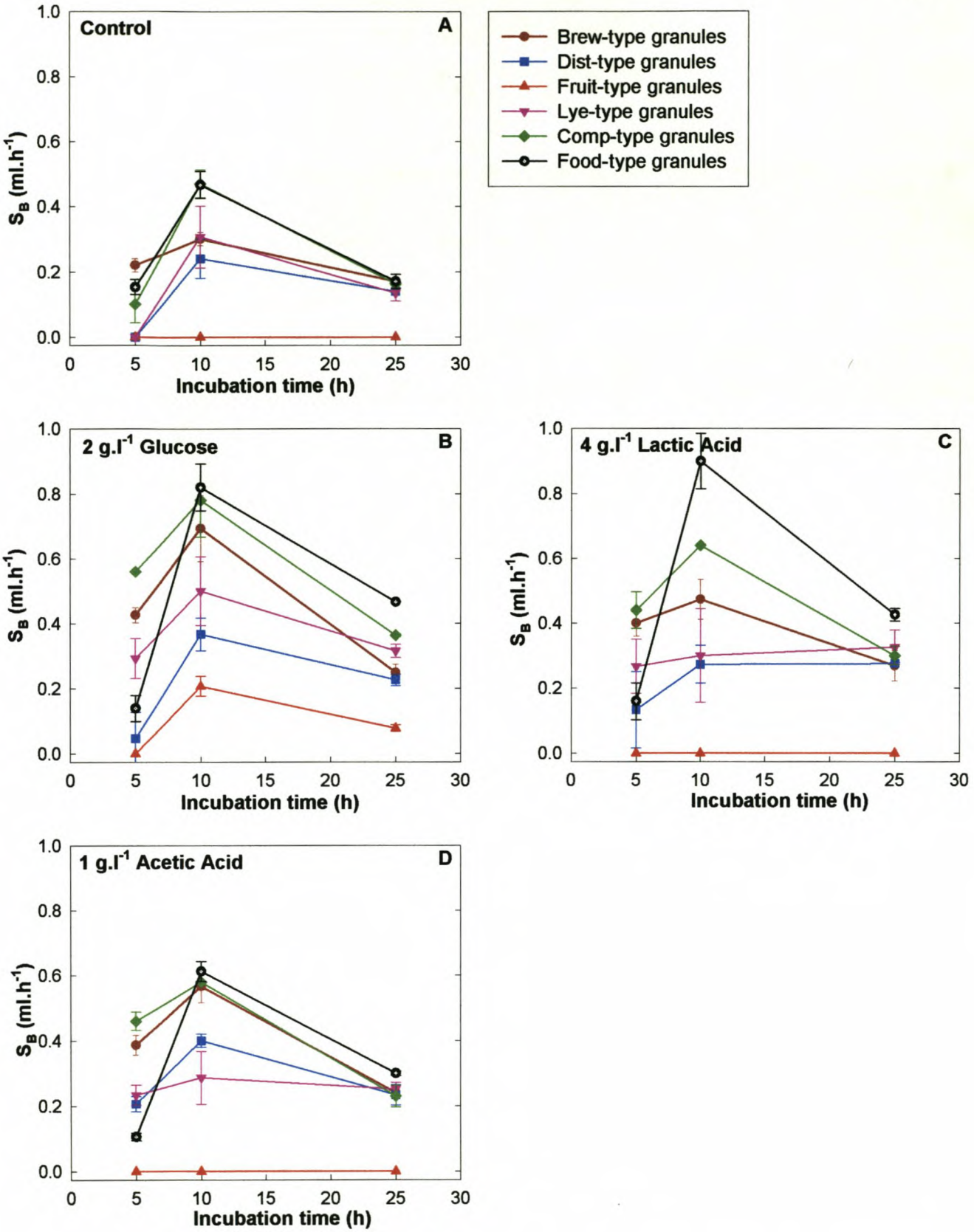

Figure 3. Tempo of biogas activity ($ml.h^{-1}$) (S_B) of the different UASB granules with different carbon sources added to the BTM (Table 3) as test substrate. The standard deviation was used as the error-bar length.

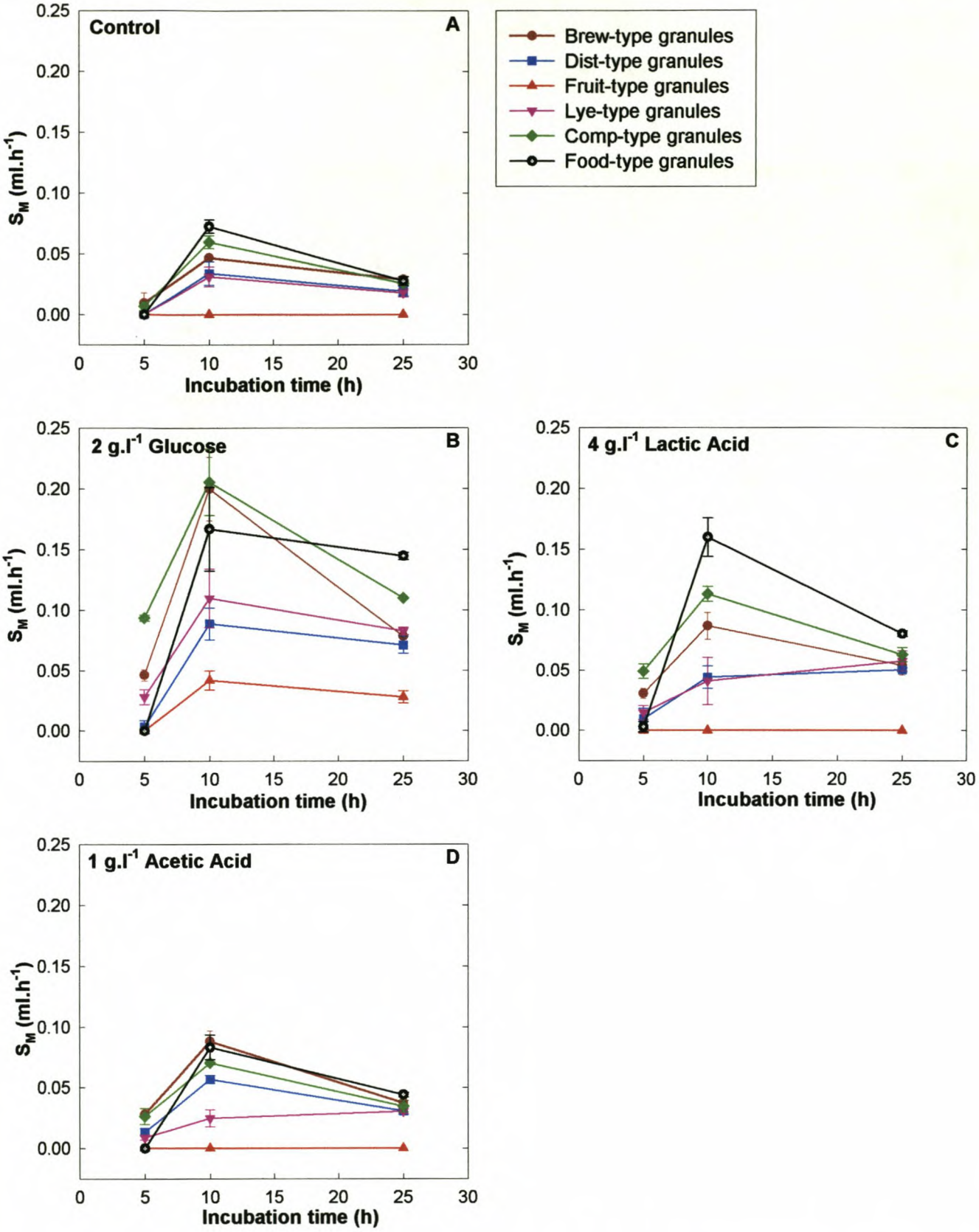

Figure 4. Tempo of methanogenic activity ($ml.h^{-1}$) (S_M) of the different UASB granules with different carbon sources added to the BTM (Table 3) as test substrate. The standard deviation was used as the error-bar length.

Control: The basic test medium (BTM) (Table 3) was used as the control to enable comparisons at a general and basic level (Cameron, 2000). From the results in Fig. 3A and 4A, it is clear that all the granules, except for the Fruit-type granules, showed S_B and S_M activity at this basic control level. The maximum S_B and S_M activities were observed at 10 h for all the granules. The order of S_B activity after 10 h of incubation was Food = Comp > Brew = Lye > Dist-type granules (Fruit-type granules = 0), and the order of the S_M activity after 10 h was Food > Comp > Brew > Dist = Lye-type granules (Fruit-type granules = 0). The Food-type granules showed the highest S_B activity (together with the Comp-type granules) and S_M activity, whilst the Fruit-type granules did not show any activity. Thus, it appeared that even when no specific microbial group was specifically enhanced, the activity of the microbial groups present in the Fruit-type granules were inactive and did not respond to the BTM as test method. It can also be concluded that in the case of the Fruit-type granules, microbial populations that utilised the test substrate were very small, inhibited or even absent.

Glucose as test substrate: It is well known (Forday & Greenfield, 1983) that the most acidogens prefer carbon in the form of glucose as growth substrate, thus the addition of additional glucose (2 $g.l^{-1}$) to the BTM (Table 3) has been shown to enhance the activity of especially the acidogens. The use of glucose as carbon source was used to primarily test for the activity of the acidogens, but would also indirectly, through metabolite formation, increase the activity of the methanogenic population if methanogens were active (Cameron, 2000).

From the results of this study (Fig. 3B and 4B) it was clear that the addition of glucose enhanced the S_B and S_M activity of all the granules including the Fruit-type granules. The order for S_B activity after 10 h of incubation was Food > Comp > Brew > Lye > Dist > Fruit-type granules. The order for S_M activity after 10 h was Comp > Brew > Food > Lye > Dist > Fruit-type granules. Under these conditions the F-type granules showed some activity suggesting that both the acidogenic and methanogenic populations in all the granules were more active after the addition of additional 2 $g.l^{-1}$ glucose to the BTM.

Glucose is also known in nature to serve as a precursor for the synthesis of many other carbohydrates like monosaccharides, disaccharides and polysaccharides (Stanley & Zubay, 1993). Therefore, glucose, as a component of

different compounds, is almost always present in larger concentrations in wastewaters used as substrates for different UASB reactors. Moreover, acidogens (hydrolytic and fermentative bacteria) are by far the largest of the trophic groups involved in anaerobic digestion and additionally have faster growth rates than the other trophic groups (Zeikus, 1980). Thus, it is therefore likely that the activity of the acidogenic populations in all the different granules used in this study was enhanced by the addition of additional glucose as test carbon substrate.

Lactic acid as test substrate: Lactate under certain conditions may be a major end-product of acidogenesis during anaerobic digestion (Pipyn & Verstraete, 1981) and several pathways are bound to exist for the degradation of this compound. When the lactate concentration is low, the syntrophic degradation by the *Desulfovibrio*/hydrogenotrophic-methanogen couple is favoured (Zellner *et al.*, 1994). Under more unfavourable conditions, a high lactate concentration leads to the formation of propionate as an intermediate or end-product and an additional lactate-utiliser, like the more acid tolerant *Propionibacterium* strains, are needed for lactate degradation. These *Propionibacterium* strains can gain a competitive advantage during situations where 'stress' conditions are applied to UASB bioreactors as they obtain a maximum ATP per mol of lactate fermented (Britz *et al.*, 1999). Thus, even under 'stress' conditions (Britz *et al.*, 1999) the lactate-utilising populations can be re-activated through the formation of propionate as an intermediate or end-product.

It is well known (Aguilar *et al.*, 1995) that the degradation of glucose, in comparison with lactic acid, leads to the formation of a greater variety of intermediate products. Lower S_B and S_M activities of the different granules were found when lactic acid was used as test substrate (Fig. 3C and 4C), compared to the tests where glucose was used as test substrate (Fig. 3B and 4B). Moreover, the degradation of glucose ($C_6H_{12}O_6$) as a 6-carbon compound was the more energetically favourable reaction, compared to the degradation of lactic acid ($CH_3CHOCOOH$) as a 3-carbon compound. Thus, the lower S_B and S_M activities of the different granules for the less favourable energetically degradation of lactic acid were probably due to the limited number of trophic groups involved in the degradation of lactic acid.

The order of S_B activity at 10 h incubation time was Food > Comp > Brew > Lye > Dist-type granules (Fruit-type granules = 0). The order of S_M activity at 10 h was Food > Comp > Brew > Dist > Lye-type granules (Fruit-type granules = 0). The results showed that the biogas and methanogenic activity of the Food-type granules was the highest when lactic acid was used as test substrate, compared to glucose as test substrate. It was thus concluded that a lactate-utilising population was present in higher numbers in the Food-type granules than in the other granules. Maximum S_B and S_M activity for the Lye-type granules was observed only after 25 h of incubation. It is possible that a smaller lactate-utilising population present in the Lye-type granules caused a shift in tempo of maximum activity from 10 to 25 h. Once again, the Fruit-type granules did not show any S_B and S_M activity when lactic acid was used as test substrate. It was thus concluded that the lactate-utilising population was either very small, absent or even inhibited probably due to long-term lactate insufficiency of the Fruit-type granules and therefore could not be re-activated in the incubation period of 25 h.

Acetic acid as test substrate: Acetic acid is one of the limited carbon sources that can directly be utilised by the acetoclastic (acetate-utilising) methanogens, *Methanosaeta* and *Methanosarcina* (Garrity & Holt, 2001). The growth and substrate degradation rates of acetoclastic methanogens are also quite slow with t_d values of between 1.5 and 7 days (Wu *et al.*, 1990). The addition of 1 $g.l^{-1}$ acetic acid to the BTM (Table 3) was therefore specifically to enhance the activity of these acetoclastic methanogens.

In this study, the use of acetic acid as test substrate resulted in lower S_B and S_M activities (Fig. 3D and 4D) for the different granules, compared to when glucose (Fig. 3B and 4B) or lactic acid (Fig. 3C and 4C) were used as test substrate. It is known that the degradation of glucose or lactic acid leads to the formation of a variety of intermediate products that may energetically favour the activity of different members of the microbial communities present in different granules (Aguilar *et al.*, 1995). Thus in this case, the lower S_B and S_M activities were probably due to the limited number of trophic groups involved in the degradation of acetic acid. However, the S_B and S_M activities were still higher than the values obtained for the control samples when BTM (Table 3) was used as test

substrate. It was therefore concluded that the acetoclastic populations present in all the granules, except the Fruit-type granules, were very much active.

The order of S_B activity at the 10 h period of incubation was Food > Comp > Brew > Dist > Lye-type granules (Fruit-type granules = 0). The order of methanogenic activity at the 10 h incubation period was Food > Brew > Comp > Dist > Lye-type granules (Fruit-type granules = 0). Once again maximum S_M activity for the Lye-type granules was observed after 25 h of incubation. This can possibly be ascribed to the slow growth rate (Wu *et al.*, 1990) of a smaller population of acetoclastic methanogens present in the Lye-type granules. The Lye-type granules also showed at the 10 h period a lower S_B and S_M activity than found for the control sample. This suggests that a measure of inhibition of the acetoclastic methanogens may have occurred in the presence of 1 $g.l^{-1}$ acetic acid at 10 h of incubation. Since the source of these granules were from a high pH sodium lye treating UASB system, the high pH values (± 8.0 - 9.0) of the lye effluent probably inhibited methanogenesis. In addition to the high pH values of the lye effluent, the high Na^+ and pectin concentrations in the effluent were probably also responsible for a measure of inhibition of the acetoclastic methanogens in the Lye-type granules.

Overall, from the S_B and S_M activity profiles (Fig. 3 and 4) it was possible to divide the different granules into two major groups where the Food-, Brew- and Comp-type granules exhibited higher S_B and S_M activity than the Dist-, Lye- and Fruit-type granules for the different test substrates. Thus, it was concluded that the higher S_B and S_M activities of the Food-, Brew- and Comp-type granules were related to higher numbers and metabolic potential of the trophic groups, such as acidogens, lactate-utilisers and methanogens, that were present in the granules.

Activity testing versus ECP composition of different UASB granules

The total ECP content extracted from the different UASB granules (Table 6) varied widely depending on the nature of the granules. The Brew-type granules yielded the highest total ECP content followed, in descending order, by the Food-, Comp-, Dist-, Fruit- and Lye-type granules. Similarly, with the use of the S_B and S_M activity data, the different granules could again be divided into two major groups where the Food-, Brew- and Comp-type granules exhibited more S_B and S_M activity than the Dist-, Lye- and Fruit-type granules for the different test

substrates. It was thus evident from the activity results (Fig. 3 and 4) and ECP values (Table 6) that those granules with the higher ECP yield, exhibited greater S_B and S_M activities.

Although the Fruit-type granules did not have the lowest ECP content (35.59 $mg.g^{-1}$ VSS), these granules exhibited the lowest S_B and S_M activity. However, the Fruit-type granules had a very low protein:carbohydrate ratio of 0.79. This low protein:carbohydrate ratio was due to the high concentration of carbohydrates present in the ECP of the Fruit-type granules. Carbohydrates contain an anionic uronic acid group (Jia *et al.*, 1996a) that can cause repulsion between bacterial cells when high concentrations of carbohydrates are present in the ECP of UASB granules (Morgan *et al.*, 1990). It can be speculated that this repulsion between the bacterial cells of the Fruit-type granules probably also affected the activity of these granules.

Conclusions

Various mechanisms for granulation have been proposed (Ross, 1984; Costerton, 1987; Chen & Lun, 1993; Schmidt & Ahring, 1996; Britz *et al.*, 2000). As basis for some of these proposals it is believed that ECP plays a major role in the granulation process (Costerton *et al.*, 1981; Ross, 1984; Sam-Soon *et al.*, 1987; Harada *et al.*, 1988; Morgan *et al.*, 1990; Quarmby & Forster, 1994; Schmidt & Ahring, 1994).

In this present study an ECP extraction method based on the principles of the method of Schmidt & Ahring (1994) was evaluated in order to determine the optimal extraction time. The optimal extraction time was considered the extraction time needed before cell lysis took place and the intracellular material started contributing to the ECP content. In this study the optimal extraction time was found to be at 4 h for all the granules, except the Fruit-type granules which was at 3 h. It was concluded from the data that after the optimal extraction time, the further increase in DNA content was due to cell lysis where the intracellular material contributed to the DNA content of the ECP. Furthermore, a physical thermal extraction method was used to extract the ECP (Brown & Lester, 1980; Morgan *et al.*, 1990; Schmidt & Ahring, 1994) as physical extraction methods have

been reported to be more successful in extracting ECP than chemical extraction methods (Brown & Lester, 1980; Gehr & Henry, 1983).

The total ECP content of the different UASB granules was found to be in the range of 28.71 to 53.76 $mg.g^{-1}$ VSS. In general, the dominant component of the ECP extracted from the different UASB granules was protein. Thus, the protein:carbohydrate ratio was greater than 1 for all the granules, except for the Fruit-type granules. The Fruit-type granules were obtained from a full-scale UASB plant that treated carbohydrate-rich wastewaters that were mainly composed of fruit juice effluent and fruit pulp. The dominant component of the ECP from the Fruit-type granules was found to be carbohydrates, and it was thus concluded that these granules consisted mainly of acidogens.

It was also found in this study that results from the S_B and S_M activity tests could be used to indirectly predict the activity of the different microbial groups present in the granules. It was found that the acidogenic population was the most active in terms of both S_B and S_M activity for all the granules, except the Food-type granules which showed higher S_M activity for the lactate-utilising populations. In general, it was evident that the Food-, Brew- and Comp-type granules exhibited more S_B and S_M activity and it was thus concluded that these granules contained higher numbers of acidogens, lactate-utilisers and acetoclastic methanogens. Furthermore, according to the acquired data of the ECP composition of the different UASB granules it was found that granules with higher ECP yields exhibited greater S_B and S_M activities. It was thus evident that granules that contained higher numbers of different active trophic groups were likely to yield more ECP. In addition, it was also concluded that the protein:carbohydrate ratio of the different UASB granules could impact the activity of the respective granules.

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CHAPTER 4

THE IMPACT OF SLUDGE SOURCES, CARBON SUBSTRATES AND BATCH CULTIVATION TECHNIQUES ON GRANULE ACTIVITY, ECP COMPOSITION AND GRANULE FORMATION IN BATCH SYSTEMS

Abstract

The start-up period of Upflow Anaerobic Sludge Bed (UASB) reactors can significantly be reduced by enhancing granulation through the batch cultivation of anaerobic granular sludge and thus seeding the reactor with this batch cultivated sludge instead of raw anaerobic sludge. However, there are many system environmental parameters that impact this production method. In this study, the impact of sludge sources (Paarl/(P0) and Kraaifontein/(K0)), carbon substrates (yeast extract lactate (YEL), glucose (G) and fruit cocktail effluent (Fc)) and batch cultivation techniques (roller-table and shake-waterbath) on enhanced batch granule cultivation and metabolic activity of anaerobic granular sludge, was investigated. The effect of ECP content and composition on the enhanced granulation was also investigated. Granulation did not proceed optimally in all the different batch systems studied as acetate activity profiles showed that the major nuclei formers (acetoclastic methanogenic populations, *Methanosarcina* and *Methanosaeta*) appeared to be inactive in all the different batch systems and control samples (K0 and P0-sludge), with the exception of the roller-table glucose cultivated Kraaifontein-sludge (RKG) batch system. The roller-table cultivation technique resulted in the higher increase in granule numbers. From the activity results, it was evident that the more vigorous shake-waterbath technique led to a higher tempo of gas production (biogas and methane) at an earlier incubation period (10 h) than the roller-table technique. Thus, the shake-waterbath technique probably prevented the optimal contact time between biomass and substrate, and this then resulted in a lower increase in granule numbers. The addition of glucose-medium as carbon substrate did not only enhance the activity of the acidogenic populations, but also led to the establishment of a greater variety of trophic groups, such as the lactate-utilisers and hydrogenotrophic methanogens,

compared to when the YEL-medium and especially fruit cocktail effluent were used as carbon substrates for batch cultivation. In fruit cocktail cultivated batch systems, it was found that the addition of fruit cocktail effluent as carbon substrate enhanced ECP production. However, the addition of carbon substrates showed no discernible trend on ECP composition and granule formation itself. Large variations in ECP composition of the different batch systems were found, and were ascribed to the heterogeneity within the different sludge sources that were used for the batch cultivation studies.

Introduction

The Upflow Anaerobic Sludge Bed (UASB) design is widely used to treat high-strength industrial wastewaters (Hulshoff *et al.*, 1997). Successful operation, however, depends on the high settling velocities and methanogenic activity of the UASB granules for the degradation of organic substances (Fukuzaki *et al.*, 1995). Granulation does not always proceed optimally because the composition of effluents is typically time-variable with nutritional imbalances often found (Verstraete & Vandevivere, 1999). It has been reported that granular sludges could only be formed with certain types of carbohydrate/protein-rich wastewaters that are generated in the agricultural and food processing industries (Sam-Soon *et al.*, 1991). Growth of methanogenic bacteria is also generally slow when compared to aerobic bacteria, and thus results in a time-consuming granulation process during start-up of new UASB reactors (Schink, 2001). These rather extended start-up times limit the potential use of the system. Britz *et al.* (2000) reported that the start-up period could be significantly reduced by enhancing granulation through the production of anaerobic granules in a laboratory batch system, and then seeding the reactor with granular sludge instead of raw anaerobic sludge.

High methanogenic activity is one of the characteristics of UASB granules and anaerobic granular sludge (Schmidt & Ahring, 1996), and depends on the presence of methanogenic bacteria and suitable substrates for these bacteria (Mah, 1982). The methanogenic activity of the granular sludge can be inhibited by the accumulation of high concentrations of volatile fatty acids that lead to the lowering of the digester pH system. It is thus essential to monitor the activity of

biomass to prevent digester failure due to a low pH environment (Van Lier *et al.*, 2001). Activity tests can also be used to characterise the suitability of the biomass prior to its use as an inoculum for start-up of a new reactor (De Zeeuw, 1984).

Although the mechanisms involved in granulation are not fully understood, it is believed that extracellular polymers (ECP) play a critical role in the granulation process (Ross, 1984; Costerton *et al.*, 1987; Schmidt & Ahring, 1994). ECP is made of organic debris, phages, lysed cells and other organic material excreted by the microbial cells involved in granulation. It consists mainly of protein and polysaccharides, as well as minor amounts of lipids, lipopolysaccharides, DNA and RNA (Schmidt & Ahring, 1994). Ross (1984) stated that agglutination of bacteria is generally due to the interaction between a protein and a polysaccharide. Information about the precise role of ECP during granulation is still very limited. Jia *et al.* (1996) reported that sludge containing more ECP produce stronger granules, while other researchers (Morgan *et al.*, 1990; Schmidt & Ahring, 1996) found that high amounts of ECP was not needed for making up active granules. It is not necessarily the amount of ECP that influences granulation, but rather the composition and ratio of carbohydrate to protein in the ECP of anaerobic granular sludge (Shin *et al.*, 2001). Considerable variation on the reported ECP content of granules and granular sludge has been found and depends on the extraction methods, methods of analyses and types of granular sludge examined.

The aims of this study were to investigate the impact of the type of sludge used as inoculum, type of carbon substrates and batch cultivation techniques on enhancement of batch granule cultivation and activity of anaerobic granular sludge. The ECP composition will also be determined and correlated with the batch cultivation studies and resulting activity of anaerobic granular sludge.

Materials and methods

Source of sludge

Two sources of anaerobic sludge from local sewage works, Kraaifontein Wastewater Treatment Works (K0-sludge) and Paarl Wastewater Treatment Works (P0-sludge), were used for the batch cultivation of UASB granules.

The raw anaerobic sludge was sieved (Endecotts Ltd) through a 1 mm pore sized sieve to remove larger non-degradable material. The sieved sludge was

then centrifuged at 978 x g for 20 min, and the supernatant removed to concentrate the sludge sample by 50% of the original volume. The P0-sludge and K0-sludge were used as inoculum for the batch cultivation studies at a TSS concentration of 0.141 and 0.148 g^{-1} TSS, respectively.

Batch granule cultivation

Two batch cultivation techniques were compared during batch granule cultivation. Firstly, a roller-table (Manufactured by the Department of Chemical Engineering, University of Stellenbosch) was used to cultivate the granules. The roller-table was placed in a 35°C temperature controlled incubator, with 500 ml containers rotating at a speed of 30 rpm. In the second case, a linear-shake waterbath (Scientific Manufacturing, Paarden Island, Cape Town) was used at 120 rpm, to cultivate the granules in a batch system at 35°C.

Each batch system consisted of 100 ml sieved anaerobic sludge and 350 ml sterile growth medium. For a period of 14 days, 100 ml of the clear top volume of each container was replaced with 100 ml fresh, sterile carbon growth substrate so as to stimulate UASB operational parameters and organic overloading (Britz *et al.*, 1999; Britz *et al.*, 2000). The batch cultivation studies were done in triplicate.

The carbon substrates used as growth media for the study were yeast extract lactate (YEL)-medium (Table 1), glucose medium and fruit cocktail effluent. The glucose carbon substrate was prepared by replacing 10 $g.l^{-1}$ lactic acid with 2 $g.l^{-1}$ glucose in the YEL-medium formulation (Table 1). The fruit cocktail was obtained as a specific effluent stream from Ashton Canning Company (Pty) Ltd., sieved to remove excess fruit fibres and diluted to 2 $g.l^{-1}$ COD. The different combinations of batch cultivation techniques, sources of sludge and carbon substrates used as growth medium for the batch systems are summarised in Table 2.

Activity testing

The activity of the granular sludge from the different batch systems (Table 2) was determined after 14 days of batch cultivation at 35°C. The P0 and K0-sludge served as control samples for the respective batch systems, and the activity of these control samples was also determined prior to their use as inoculum for the respective batch systems. The biomass from the batch systems was "standardised" by centrifugation of the biomass at 978 x g for 20 min. Thereafter,

Table 1. Composition of the yeast extract lactate (YEL) medium used for batch granule cultivation.

Compound	**Concentration ($g.l^{-1}$)**
Lactic acid [Saarchem - Unilab; 60% solution v/v]	10
Yeast Extract [Biolab]	5
Peptone (nitrogen) [Biolab]	2
KH_2PO_4 [BDH]	10
Tween 80 [Merck]	1 ml
Trace elements*	10 ml
pH	7.1

*Nel *et al.* (1985)

Table 2. Different combinations of batch cultivation techniques, sources of sludge and carbon substrates used for the batch cultivation of granular sludge.

Batch system	Cultivation technique	Sludge source	Carbon substrate
P0 (Control)	-	Paarl	-
K0 (Control)	-	Kraaifontein	-
SPY	Linear shake-waterbath	Paarl	Yeast Extract Lactate
SPG	Linear shake-waterbath	Paarl	Glucose
SPFc	Linear shake-waterbath	Paarl	Fruit cocktail effluent
RPY	Roller-table	Paarl	Yeast Extract Lactate
RPG	Roller-table	Paarl	Glucose
RPFc	Roller-table	Paarl	Fruit cocktail effluent
RKY	Roller-table	Kraaifontein	Yeast Extract Lactate
RKG	Roller-table	Kraaifontein	Glucose
RKFc	Roller-table	Kraaifontein	Fruit cocktail effluent

P0-sludge/(P): Raw anaerobic sludge from Paarl Wastewater Treatment Works used as control sample

K0-sludge/(K): Raw anaerobic sludge from Kraaifontein Wastewater Treatment Works used as control sample

S: Shake-waterbath technique used for batch cultivation

R: Roller-table technique used for batch cultivation

Y: Yeast Extract Lactate medium used as carbon substrate

G: Glucose medium used as carbon substrate

Fc: Fruit cocktail effluent used as carbon substrate

the sludge pellet were re-suspended in sterile medium (pH 7.0) containing 1 $g.l^{-1}$ glucose [Saarchem - Unilab] and 0.5 $g.l^{-1}$ each of urea [Labchem] and KH_2PO_4 [BDH], and re-activated for 2 days at 35°C. This two-day incubation period was done to allow the batch systems to regain activity for optimum biogas production during the activity-testing period. The activation medium was replaced daily to optimise re-activation of the batch systems. After the incubation period, the activated granular sludge sample was centrifuged at 978 x g for 20 min. Then 20 ml glass vials were inoculated with 3 g of the sludge pellet and 13 ml of the basic test medium (BTM) (Table 3) or other test media, respectively (Table 4) (O'Kennedy, 2000). Each vial was sealed and incubated at 35°C for 25 h. The activity tests were done in duplicate according to the method described in Chapter 3 of this thesis.

Granule counts

Granule counts were done on day 0 on the P0 and K0-sludge that served as control samples for the respective batch systems (Table 2). On day 14 after batch cultivation, granule counts were done on the different batch systems. Each sludge sample (1 ml) was fixed in a glass petri-dish using gelatine (3 $g.100\ ml^{-1}$) (Jeison & Charmy, 1998). Ten images (6 mm by 10 mm) of each sample were scanned into a desktop computer using the Matrox Intellicam Interactive (version 2.0) frame-grabber software (Matrox Electronic Systems Ltd.) and a Nikon SMZ800 Stereoscopic Microscope fitted with a Panasonic CP410 Digital Video Camera. The images were analysed using the Scion Image Software (release Beta 3b) (Scion Corporation, Maryland, USA).

Extraction of water-soluble extracellular polymers

The thermal extraction method was used, as described in Chapter 3 of this thesis, to extract the ECP content of the anaerobic granular sludge. The biomass from each batch system was "standardised" by centrifugation at 978 x g for 20 min. Identical serum vials (6 ml) were then filled with 0.5 g of the sludge pellet and 3 ml phosphate-buffered saline (PBS) (Smibert & Krieg, 1994) (Table 5) and placed in a shake-waterbath (150 rpm) at 70°C for 2 h. The contents were centrifuged at 9 600 x g for 10 min, and the supernatant was removed and kept at -18°C for further ECP analyses.

Table 3. Composition of the Basic Test Medium (BTM) (Valcke & Verstraete, 1993).

Compound	Concentration ($g.l^{-1}$)
Glucose* [BDH]	2.0
K_2HPO_4 [ACE]	1.0
KH_2PO_4 [BDH]	2.6
Urea [Labchem]	1.1
NH_4Cl [BDH]	1.0
Na_2S [Saarchem - UniVar]	0.1
$MgCl_2.6H_2O$ [Merck]	0.1
Yeast Extract [Biolab]	0.2
pH	7.1

* Added extra to the medium of Valcke & Verstraete.

Table 4. Different test media used and the specific microbial group enhanced (Cameron, 2000).

Test media*	Bacterial group
Control (only BTM)	
2 $g.l^{-1}$ Glucose [BDH]	Acidogens
4 $g.l^{-1}$ Lactic Acid [Saarchem - Unilab; 60% solution V/V]	Lactate-utilisers
1 $g.l^{-1}$ Acetic Acid [B&M Scientific]	Acetoclastic Methanogens

*The BTM was used as basis for the different media.

Table 5. Composition of phosphate-buffered saline solution* (PBS) used for the extraction of ECP.

Compound	**Concentration ($g.l^{-1}$)**
10x stock solution	
Na_2HPO_4, anhydrous [Merck]	12.36
$NaH_2PO_4.H_2O$ [Merck]	1.80
NaCl [Saarchem - Unilab]	85.00
Working solution (0.01 M phosphate, pH 7.6)	
Stock solution	100 ml
Distilled water	900 ml

* Smibert & Krieg (1994)

Analyses of extracellular polymers

The carbohydrate, protein, lipid and DNA content of the ECP-extract were determined according to the methods described in Chapter 3 of this thesis. All the analytical methods were done in duplicate.

Other analytical procedures

The pH values obtained from the different batch systems (Table 2) were monitored from day 0 until day 14. The total suspended solid (TSS) and volatile suspended solid (VSS) content of the sieved anaerobic sludge samples was determined at day 0 and day 14 respectively. Prior to the determination of TSS and VSS, the biomass of the different batch systems was "standardised" by centrifugation at 10 000 x g for 10 min. The TSS and VSS content of the sludge pellet was then determined in duplicate by using the methods recommended by *Standard Methods* (APHA, 1992; Shen *et al.*, 1993).

Results and discussion

Availability of sludge

Due to a mechanical limitation at Kraaifontein Sewage Works, a SK-type batch system was not included in this study (Table 2). The RK-type batch system was only included in this study to investigate the impact of the type of sludge source when the same batch cultivation technique (roller-table technique) was used.

The pH profiles obtained from the different batch systems

It is well known that anaerobic digestion proceeds optimally under more neutral pH conditions (pH 6.5 - 8.0) (Van Lier *et al.*, 2001), and that methanogens and acidogens are pH sensitive with the optimum pH range between 6.50 to 7.30 (Zeikus, 1977; Forday & Greenfield, 1983). In anaerobic systems, low pH conditions are normally associated with the presence of undissociated volatile fatty acids (VFA). It is thus important to carefully monitor the pH profile of the different batch systems during batch cultivation of granular sludge.

The pH profiles obtained during the batch cultivation are illustrated in Fig. 1A, 1B and 1C. The pH of each batch system (Table 2) was taken as an indication of

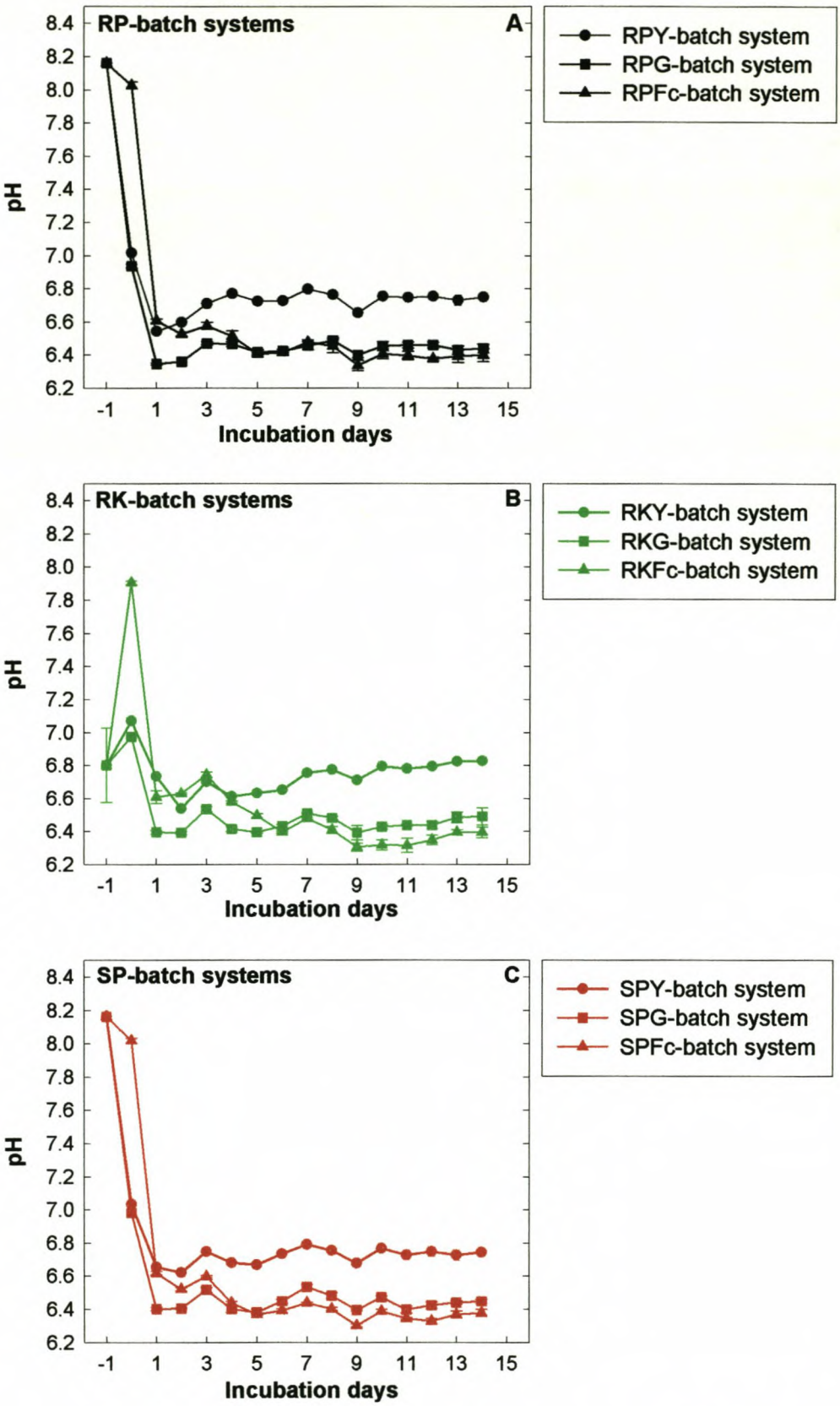

Figure 1. The pH profiles obtained in the different batch systems (Table 2) of the batch cultivation studies. The standard deviation was used as the error-bar length.

the metabolic productivity (production of VFA) at the start and during the experimental studies. Day "-1" represented the pH values of the respective control sludge samples, K0-sludge and P0-sludge. Day "0" values represented the pH after the substrate had been added to the batch system.

It was found (Fig. 1A, 1B and 1C) that the pH profiles for the carbon substrate used followed the same profile after the substrate had been added to all the batch systems at day 0, irrespective of source of sludge or batch cultivation technique. The pH value for the control sample, K0-sludge, was much lower at day –1 (6.93) than the pH value at day -1 for the control sample, P0-sludge (8.16). It was found that in all the experimental batch studies (Fig. 1A, 1B and 1C) the pH of each batch system decreased within 24 h after the substrate had been added to the batch systems (day 0). This probably resulted from a high VFA production in each batch system in spite of the high buffering capacity (10 $g.l^{-1}$ KH_2PO_4) of the YEL-medium (Table 1) (Britz *et al.*, 1999). In the batch systems where the YEL-medium was used as the carbon substrate, the pH decreased within 24 h at day 0 from 7.03 (SP), 7.01 (RP), 7.07 (RK) to 6.65, 6.54 and 6.73, respectively, with a slight increase, whereafter the pH stabilised at days 10 - 14 at around 6.74, 6.75 and 6.83, respectively.

In the case of glucose (G) as carbon substrate, the initial pH was slightly lower after the substrate had been added to the batch systems (day 0) (6.98 (SP), 6.93 (RP), 6.97 (RK)) than when lactate was used and decreased at day 0 within 24 h to much lower values (6.40 (SP), 6.35 (RP) and 6.39 (RK)). The pH also stabilised by day 10 - 14 at 6.45 (SP), 6.44 (RP) and 6.49 (RK). This lower stabilisation pH can be explained by the fact that glucose is easily degradable by the largest trophic group, the acidogens, while lactate is only degradable by a selected group of organisms using various pathways that are dependent on the lactate concentration (Zellner *et al.*, 1994). The faster metabolic activity of the acidogens (Zeikus, 1980) resulted in higher VFA accumulation that shifted the pH to a lower pH value (Annachhatre, 1996).

When fruit cocktail effluent (Fc) was used as carbon substrate, larger differences in pH profiles were found for the SP, RP and RK-batch systems. The pH showed a drop after day 0 within 48 h from 8.01 (SP) and 8.02 (RP) to 6.61 and 6.60, respectively, while the RK-batch system showed a pH decrease after day 0 within 24 h from 7.90 to 6.60. Thereafter a slight increase occurred at day 3

(SP, RP) and day 2 (RK) whereafter the pH was more unstable until day 10 and then stabilisation occurred at pH 6.38 (SP), 6.40 (RP) and 6.39 (RK). These results correlated well with the findings of Cameron (2000) who also did studies with fruit cocktail effluent (2 $g.l^{-1}$ COD) as carbon substrate for batch granule production and stabilisation was reached in the pH range of 6.30 - 6.40. The reason for the high initial pH value at day 0 for the batch systems where fruit cocktail effluent (Fc) was used as carbon substrate was probably due to the presence of lye-solution (NaOH) in the effluent that was used to prepare the fruit for canning.

Activity testing

Activity tests are used to characterise the anaerobic sludge prior to use as an inoculum for initiation of the batch cultivation process. Activity testing also depends on the energy and carbon substrates that the biomass is grown in (Schmidt & Ahring, 1996).

The aim of this study was to evaluate (i) the impact of the sludge source used as inoculum, (ii) carbon substrates used as growth substrate and (iii) different cultivation techniques used for batch cultivation of granular sludge on the activity of the different batch systems (Table 2). In this study, the activity of the different batch systems was determined after 14 days of cultivation. The P0 and K0-sludge served as control samples for the respective batch systems, and the activity of these control samples was also determined prior to their use as inoculum for the respective batch systems.

As part of the activity test, the cumulative biogas was determined for each type of batch system, and the methane (CH_4) fraction calculated according to gas chromatographic (GC) results. The cumulative biogas and CH_4 data was then converted to represent the activity of each type of batch system relative to the other. In this study it must be taken into consideration that the activity of each type of batch system is thus described in terms of tempo of biogas (S_B) and tempo of CH_4 (S_M) production. The tempo of gas production (S_B and S_M) was determined using the slope or gradient (cumulative gas production (CGP) over time for each of the time intervals (t = 5, 10 and 25 h). The gradient, for example for the 10 h time interval, was determined as follows: $(CGP_{10\,h} - CGP_{5\,h})/(t_{10\,h} - t_{5\,h})$. The tempo of gas production was thus given in $ml.h^{-1}$ and was taken as representative of the

activity of the batch systems. The values were then plotted against time for the different batch systems to give a visual representation of the activity of the batch systems (O'Kennedy, 2000). The S_B and S_M activities are illustrated in Fig. 2 and 3, respectively.

General comments on activity evaluation of the different batch systems

For activity evaluation of the different batch systems: the BTM (Table 3); 2 $g.l^{-1}$ glucose added to the BTM; 4 $g.l^{-1}$ lactic acid added to the BTM and 1 $g.l^{-1}$ acetic acid added to the BTM, were respectively used as test substrates (Table 3). The BTM contained no additional carbon substrates and thus was used as the control to enable comparisons of the activity results at a general level.

Results from this study showed that not one of the batch systems showed activity in terms of either S_M or S_B when the BTM was used as test substrate (Fig. 2A and 3A). It can be concluded from the data that when a specific microbial group was not enhanced, the activity of the microbial groups present in the different batch systems and control samples (K0 and P0-sludge) was zero, and did not give a measurable response in the BTM as test method.

The use of glucose as test substrate was to primarily stimulate the activity of the acidogens, even though a subsequential increase in the activity of the methanogenic population could also result (Cameron, 2000). From the data summarised in Fig. 2B and 3B, it was clear that more activity was shown when 2 $g.l^{-1}$ glucose was added to the BTM as test substrate. Acidogens (hydrolytic and fermentative bacteria) are by far the largest of the trophic groups involved in anaerobic digestion and additionally have faster growth rates than the other trophic groups (Zeikus, 1980). Therefore, it is likely that the activity of the acidogenic populations in most of the different batch systems was enhanced by the additional glucose as carbon test substrate.

In the second test batches lactic acid was used as test substrate to investigate the possible presence of lactate-utilising bacteria. Lactate is a very specific carbon substrate and metabolised only by lactate-utilising bacteria. It is known that when the microbial consortium in anaerobic digestion systems is put under "stress" conditions, lactate is utilised mostly through the formation of propionate as intermediate or end-product (Britz *et al.*, 1999). The degradation of

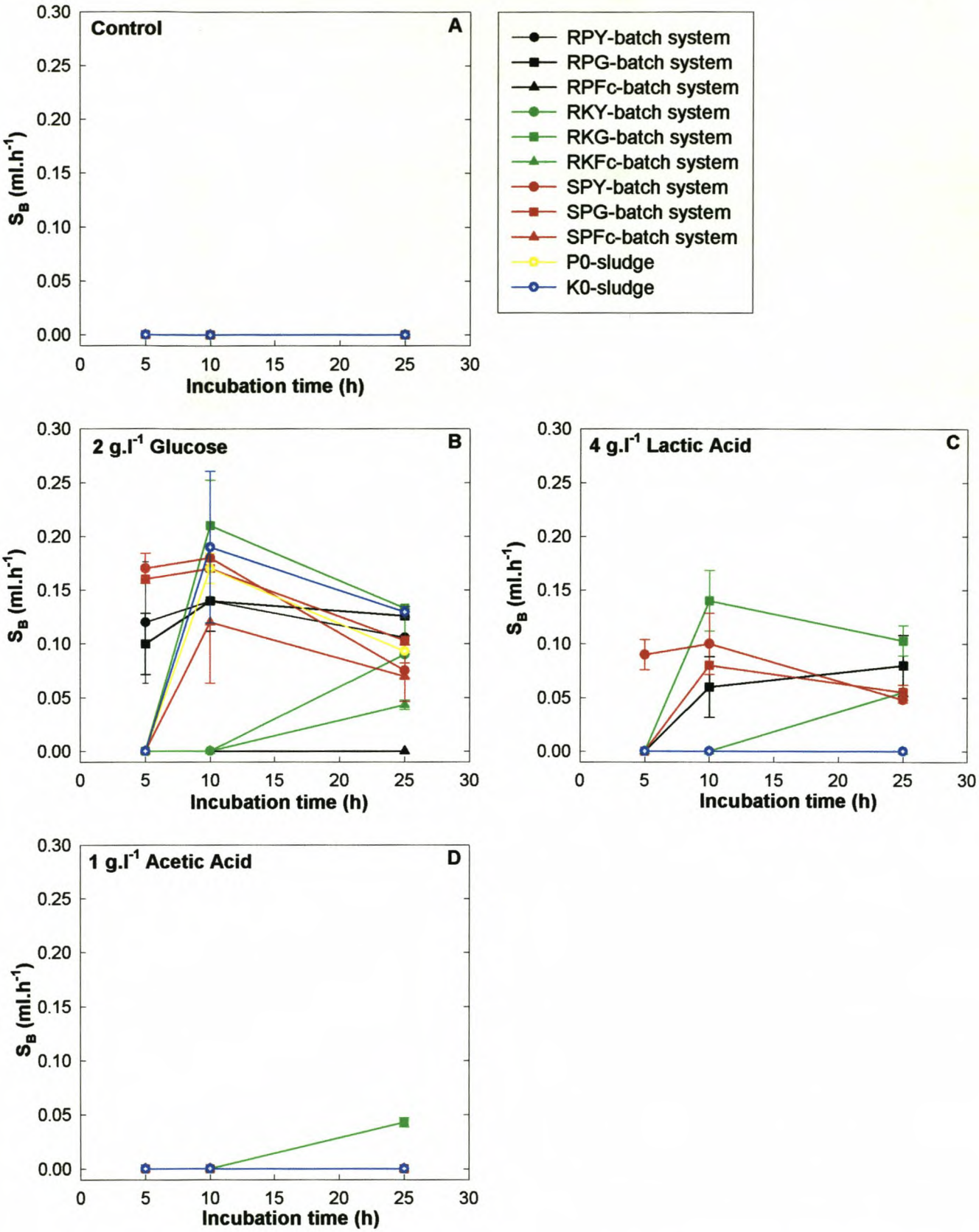

Figure 2. Tempo of biogas activity ($ml.h^{-1}$) (S_B) of the different batch systems (Table 2) with different carbon substrates added to the BTM (Table 3) as test substrate. The standard deviation was used as the error-bar length.

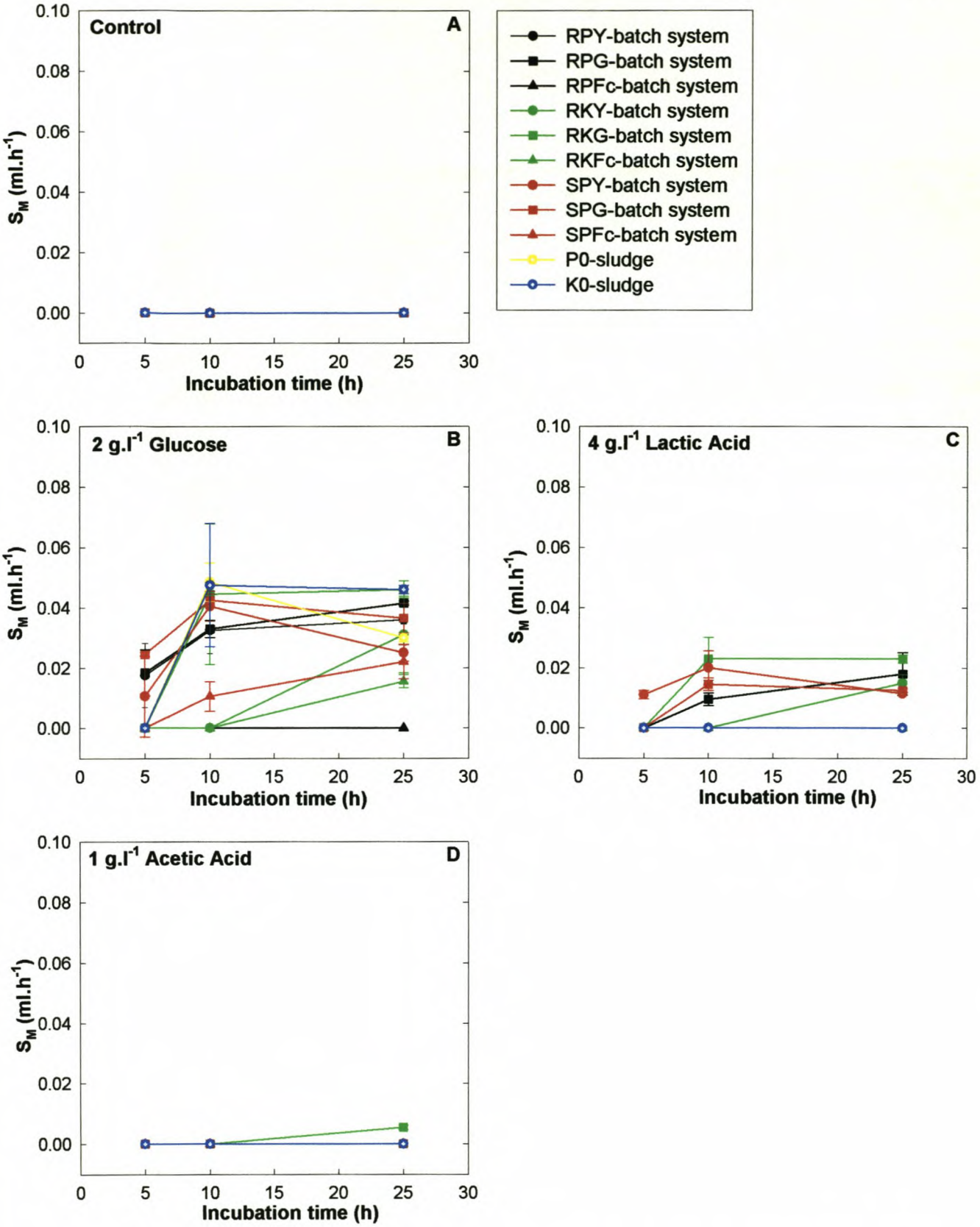

Figure 3. Tempo of methanogenic activity ($ml.h^{-1}$) (S_M) of the different batch systems (Table 2) with different carbon substrates added to the BTM (Table 3) as test substrate. The standard deviation was used as the error-bar length.

lactic acid thus leads to a smaller variety of intermediate products, compared to the degradation of glucose. Thus as predicted, lower S_B and S_M activity values for the different batch systems were found when lactic acid was used as test substrate (Fig. 2C and 3C) compared to tests where glucose was used as test substrate (Fig. 2B and 3B). The lower S_B and S_M activity values for the different batch systems were probably due to the limited number of trophic groups involved in the degradation of lactic acid.

Acetic acid is one of the limited carbon substrates that can directly be utilised by the acetoclastic (acetate-utilising) methanogens, *Methanosaeta* and *Methanosarcina* (Garrity & Holt, 2001). The addition of 1 $g.l^{-1}$ acetic acid to the BTM was thus to enhance the activity of the acetoclastic methanogens. Moreover, the growth and substrate degradation rates of acetoclastic methanogens are also quite slow with t_d values of between 1.5 and 7 days (Wu *et al.*, 1990). The different batch systems showed no S_B (Fig. 2D) and S_M (Fig. 3D) activity when acetic acid was added to the BTM, with the exception of the RKG-batch system that showed a low S_B and S_M activity. It was concluded that only a limited number of acetoclastic methanogens were present in the RKG-batch system. The acetate activity profiles (Fig. 2D and 3D) showed that the acetoclastic methanogen population was either too small to be re-activated, inactive or inhibited in the other batch systems and even control samples (K0 and P0-sludge). However, the methanogenic activity (S_M) of the acidogens (Fig. 3B) and lactate-utilising bacteria (Fig. 3C) do suggest that methanogens are present and able to utilise and degrade the intermediate products produced by the acidogens and lactate-utilising bacteria to CH_4 and CO_2. Since no stimulation of CH_4 production was found when acetic acid was used as test substrate, with the exception of the RKG-batch system, it is possible that these methanogens are of the hydrogenotrophic group.

Overall from the data (Fig. 2 and 3), the maximum S_B activity values for the different batch systems were generally found to be at the 10 h period of incubation, with the exception of a few batch systems that showed either maximum S_B activity after 25 h of incubation or did not show any S_B activity after the 25 h incubation period. In the case of methane the maximum S_M activities for the different batch systems obtained were found to be after 25 h of incubation, however for some batch systems the maximum S_M activity was at 10 h while other batch systems did not show any S_M activity. This was ascribed to the slow growth

and substrate degradation rate of methanogens that resulted in the limited number of methanogens in the different batch systems only reaching maximum S_M activity after 25 h of incubation. To enable comparisons of the impact of the sludge source, carbon substrates and cultivation techniques on the activity between the different batch systems, the S_B and S_M activity values were used that were obtained at the 10 h period of incubation.

i) Impact of sludge source on activity: RPY vs. RKY, RPG vs. RKG and RPFc vs. RKFc

In this study, the activity of the control samples (K0 and P0-sludges) and the following batch systems (RPY vs. RKY, RPG vs. RKG and RPFc vs. RKFc) were compared in order to evaluate the impact of the sludge source on the activity of the different batch systems.

Comparison of the activity of the control samples (K0 and P0-sludges) indicated that the K0-sludge had a slightly higher S_B and S_M activity than the P0-sludge when glucose was added to the BTM as test substrate (Fig. 2B). Thus, it was concluded that the acidogenic and methanogenic populations within the K0-sludge were more active than in the P0-sludge. Neither the K0-sludge nor the P0-sludge showed S_B and S_M activity when either lactic (Fig. 2C and 3C) or acetic acids (Fig. 2D and 3D) were used as test substrates. It is likely, therefore, that the lactate and acetate-utilising populations were either totally inactive, present in too low numbers to be re-activated or inhibited in the K0 and P0-sludge.

When glucose was used as test substrate, only the RKG > RPY = RPG-batch systems showed S_B and S_M activity (Fig. 2B and 3B) after 10 h of incubation. The RKY > RKFc showed S_B and S_M activity only after 25 h, while the RPFc did not show any S_B and S_M activity at all. From the results, it was again concluded that a higher number of acidogens (RKG > RPG) were present in the K0-sludge. However, it was also found that re-activation of the trophic groups greatly depended on the carbon substrate that was used as growth medium because the acidogenic populations of the RKY and RKFc-batch systems were only active after 25 h of incubation. In contrast, the RPY and RPG-batch systems already showed S_B and S_M activity after the 10 h period of incubation.

It was also observed that the S_B and S_M activities of all the RP-type and RK-type batch systems (Fig. 2B and 3B) were lower at the 10 h incubation period than

for the corresponding control samples (P0 and K0-sludges) when glucose was added as test substrate, with the exception of the RKG-batch system. This indicated that a measure of inhibition in acidogenic and resulting methanogenic activity might have occurred during batch cultivation studies, with the addition of different carbon substrates. It can be speculated that the drastic decrease in pH for all the batch systems (Fig. 1) after day 0, within 24 h, was due to high VFA formation from the addition of the different carbon substrates and probably caused a measure of inhibition for the acidogenic populations present in the RPY, RPG, RKY and RKFc-batch systems.

When lactic acid was used as test substrate only the RKG-batch system showed the highest S_B (Fig. 2C) and S_M activity (Fig. 3C) after the 10 h incubation period, followed by the RPG-batch system. The RKY-batch system showed S_B (Fig. 2C) and S_M activity (Fig. 3C) only after 25 h of incubation. Thus, it was concluded that the lactate-utilisers were more active in the RK-batch systems, than in the RP-batch systems. The control samples (K0 and P0-sludges) did not show S_B (Fig. 2C) or S_M activity (Fig. 3C) and it was previously concluded that the lactate-utilising population in the K0 and the P0-sludges were either very small, inhibited or inactive. However, this section of the data showed that the lactate-utilising populations in the RKG, RPG and RKY-batch systems could be re-activated after the 10 and 25 h incubation periods.

It was observed that only the RKG-batch system showed S_B (Fig. 2D) and S_M activity (Fig. 3D) after 25 h of incubation when acetic acid was added to the BTM as test substrate. Thus, in this case it can be concluded that the acetoclastic methanogenic population was present but, in limited numbers, in the K0-sludge. However, it can again also be concluded that the acetoclastic populations in the P0-sludge and RP-batch systems were either very small, inactive or even inhibited when acetate was used as carbon substrate. Due to activity of the acidogens (Fig. 2B) and lactate-utilising bacteria (Fig. 2C) and resulting methanogenic activity (Fig. 3B and 3C) one can speculate that although an acetoclastic population was inactive from the RP-batch systems and P0-sludge, the hydrogenotrophic methanogens were active and able to utilise and degrade the intermediate products produced by the acidogens and lactate-utilising bacteria.

ii) Impact of batch system carbon substrates on activity: RPY vs. RPG vs. RPFc and RKY vs. RKG vs. RKFc

In this study, the activity of the control samples (K0 and P0-sludges) and the following batch systems was compared, namely: RPY vs. RPG vs. RPFc and RKY vs. RKG vs. RKFc, in order to evaluate the impact of the three different carbon substrates used as growth substrates (YEL, glucose and fruit cocktail effluent medium) on the activity of the different batch systems.

The RKG-batch system showed the highest S_B (Fig. 2B) and S_M activity (Fig. 3B) when glucose was added as test substrate after 10 h of incubation. Only the RKY and RKFc-batch systems showed S_B and S_M activity after 25 h of incubation with the RKY-batch system exhibiting higher S_B and S_M activities than the RKFc-batch system. The order for S_B and S_M activity of the RP-batch system after 10 h of incubation was RPY = RPG (RPFc = 0). From the results of this study, it was clear that the addition of glucose as carbon substrate to the RK-batch system (RKG-batch system) resulted in the best enhancement of the activity of the acidogens and methanogenic populations. The fact that the control sample, K0-sludge, contained a higher number of acidogens than the P0-sludge ((i) impact of sludge source on activity) may likely be the reason why the addition of glucose gave the best results in terms of the S_B and subsequent S_M activity of the RK-batch system.

The addition of lactic acid and glucose to the P0-sludge inoculated batch systems as activity test substrates, led to more or less equal S_B (Fig. 2B) and S_M activities (Fig. 3B) of all the RP-type batch systems, suggesting that the addition of both YEL-medium and glucose as carbon substrates equally enhanced the acidogenic and methanogenic activity of all the RP-batch systems. These results can be ascribed to the fact that the degradation of glucose leads to the formation of a greater variety of intermediate products, such as butyrate, lactate, acetate and propionate (Aguilar *et al.*, 1995) that consequently can be degraded to CH_4 and CO_2 by the methanogens. Thus, a greater variety of trophic groups are established within the batch systems when the carbon substrates such as glucose and YEL-medium are used as substrates for batch cultivation.

When lactic acid was used as test substrate the order of the S_B (Fig. 2C) and S_M activities (Fig. 3C) were RKG > RPG at the 10 h incubation period, and RKG >

RPG > RKY at the 25 h incubation period. From these data it can be concluded that the lactate-utilisers were more active in the glucose cultivated batch systems.

The glucose cultivated RKG-batch system was the only batch system that showed S_B (Fig. 2D) and S_M activity (Fig. 3D) when acetic acid was used as test substrate. Once again, it is evident from these results that the addition of glucose as carbon substrate not only enhanced the activity of the acidogens. This environment probably also led to the establishment of a greater variety of microbial groups, such as lactate-utilising bacteria and hydrogenotrophic and acetoclastic methanogens within the different batch systems, as compared to when YEL-medium and especially fruit cocktail effluent were used as carbon substrates for the batch cultivation.

It was evident from the activity test results (Fig. 2B and 3B) ((i) impact of sludge source on activity) that the acidogenic and methanogenic populations were more active in the control sample of K0-sludge, than in the control sample of P0-sludge when glucose was added as test substrate. Thus, it can be speculated that the addition of glucose as carbon growth substrate to the K0-sludge activated the more active acidogenic populations in the K0-sludge to produce higher quantities of intermediate products from glucose, such as acetate, that were used by the acetoclastic methanogens and in turn led to the establishment of a more active acetoclastic methanogenic population in the RKG-batch system. It can also be speculated that the acetoclastic methanogenic populations were probably also present in limited numbers, but inactive in the case of the P0-sludge. Due to the limited number of acidogenic and methanogenic populations in the P0-sludge, the addition of glucose as carbon growth substrate was thus probably not sufficient enough to re-activate the limited number of the acetoclastic methanogenic population present in the P0-sludge.

The fruit cocktail cultivated batch systems (Fc) showed no S_B (Fig. 2B, 2C and 2D) or S_M activity (Fig. 3B, 3C and 3D) at 10 or 25 h incubation for all the RK-type and RP-type batch systems when glucose, lactic or acetic acid were used as test substrates. The cellulose fibres present in the fruit cocktail effluent are β-glycoside carbohydrates that are more difficult to hydrolyse than α-glycoside carbohydrates such as starch and sucrose (Zoetemeyer *et al.*, 1982) and it might be speculated that the overall conversion rate of fruit cocktail effluent as carbon substrate was thus limited.

iii) Impact of batch cultivation techniques: RPY vs. SPY, RPG vs. SPG and RPFc vs. SPFc

In this study, the activity resulting from the use of batch cultivation techniques was compared, namely: RPY vs. SPY; RPG vs. SPG and RPFc vs. SPFc.

The activity data summarised for all the RP-type and SP-type batch systems in Fig. 2B, 2C, 3B and 3C, showed that the SP-batch systems (shake-waterbath) exhibited more S_B and S_M activity (SPG > RPG, SPY > RPY, SPFc > RPFc) than the RP-batch systems (roller-table). The maximum S_B activity (Fig. 2B and 2C) and S_M activity (Fig. 3B and 3C) for the SP-batch system was observed at the 10 h period of incubation with glucose and lactic acid as test substrates. The maximum tempo of S_B activity (Fig. 2B) for the RP-batch system was also observed at 10 h when glucose was used as test substrate, however maximum S_M activity for the RP-batch system was only observed at the 25 h incubation period. When lactic acid was used as test substrate, the maximum tempo of S_B (Fig. 2C) and S_M (Fig. 3C) activities for the RP-batch systems were also observed after 25 h of incubation.

When glucose was used as test substrate, the order of the S_B (Fig. 2B) and S_M activities (Fig. 3B) for the SP-type and RP- type batch systems changed from the 10 to 25 h period of incubation.

The order of S_B activity at 10 h was:

SPY > SPG > RPY = RPG > SPFc (RPFc = 0); and changed at 25 h to:

RPG > RPY > SPG > SPY > SPFc (RPFc = 0).

The order for S_M activity at 10 h was:

SPG > SPY > RPY = RPG > SPFc (RPFc = 0); and changed at 25 h to:

RPG > RPY = SPG > SPY > SPFc (RPFc = 0).

When lactic acid was used as test substrate the order of the S_B (Fig. 2C) and S_M activities (Fig. 3C) for the SP and RP-batch systems also changed from the 10 to 25 h period of incubation.

The order of S_B activity at 10 h was:

SPY > SPG > RPG; and changed at 25 h to:

RPG > SPY > SPG.

The order of S_M activity at 10 h was:

SPY > SPG > RPY; and changed at 25 h to:

RPG > SPG > SPY.

Thus, from the results it is clear that the shake-waterbath cultivation technique led the SP-batch system to produce more biogas and methane at an earlier incubation period (10 h), whilst the roller-table technique, eventually after a 25 h incubation period, resulted in the highest biogas and methanogenic activity.

Granule formation

Batch cultivation of granular sludge, as shown by Britz *et al.* (2000) is based on the observation that when "stress" conditions (overloading) are applied to the batch system, enhancement of granulation will occur.

In this study, the influence of the source of sludge, batch cultivation techniques and different carbon substrates were investigated to evaluate the impact on enhanced granule formation. Granule count data (Fig. 4) showed that for the control batches, K0-sludge (day 0) contained a lower number of granules than the P0-sludge (day 0). Thus, the raw anaerobic P0-sludge that was used as a control sample already contained a measure of granulation before its use as an inoculum. After 14 days of incubation the number of granules showed different levels of increases for all the batch systems.

The use of the roller-table technique (R) used for batch cultivation resulted in a greater increase of granules than found when the shake-waterbath technique (S) was used. Thus, it was concluded that the roller-table technique was more suitable and led to a higher increase in granule numbers during batch cultivation of granular sludge. Findings from the activity testing, namely that the shake-waterbath technique produced more biogas at a shorter incubation time, might be responsible for the lower increase in granule numbers by shortening the contact time between bacteria due to the higher tempo of gas production at an earlier stage of batch cultivation. The roller-table technique was also a gentler and less vigorous technique than the shake-waterbath technique and probably increased the contact time between the biomass and the substrate.

The different carbon substrates used did not show any correlation with granule counts obtained after 14 days of incubation (Fig. 4). These findings are contradictory to the findings of Cameron (2000) who reported that batch granules cultivated by using the shake-waterbath technique resulted in the highest increase in granule numbers when glucose (2 $g.l^{-1}$) was used as the carbon substrate, followed by YEL-medium (10 $g.l^{-1}$). The use of fruit cocktail effluent as only carbon

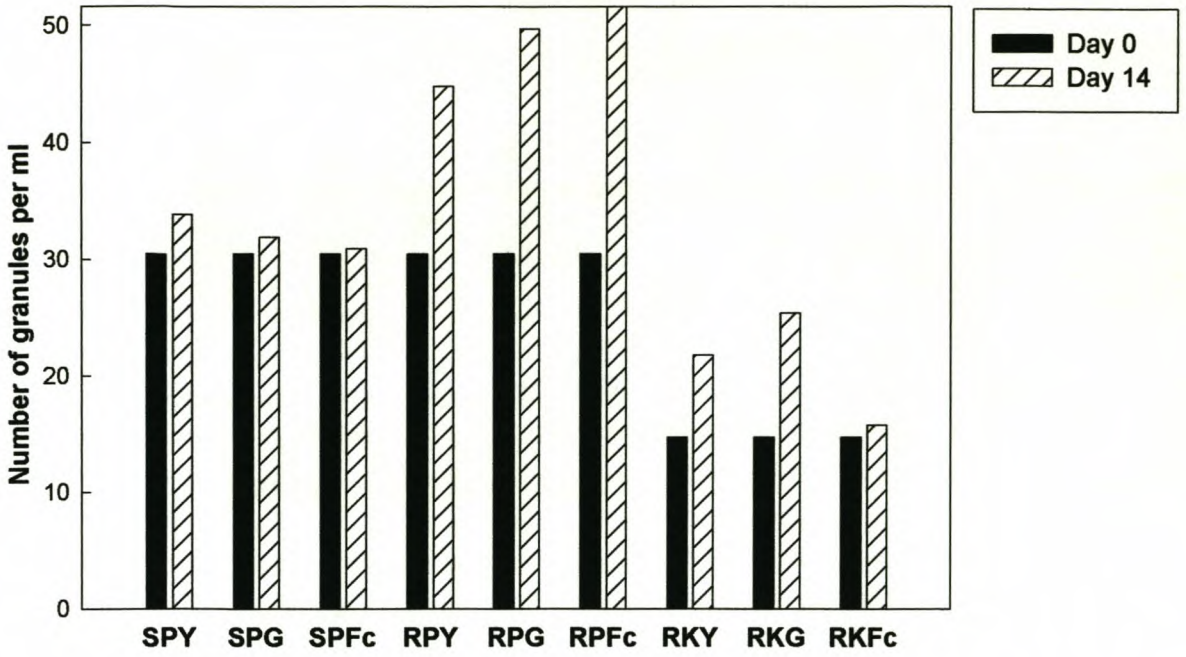

Figure 4. Granule counts (number of granules per ml) obtained at day 0 and day 14, respectively during the batch cultivation studies.

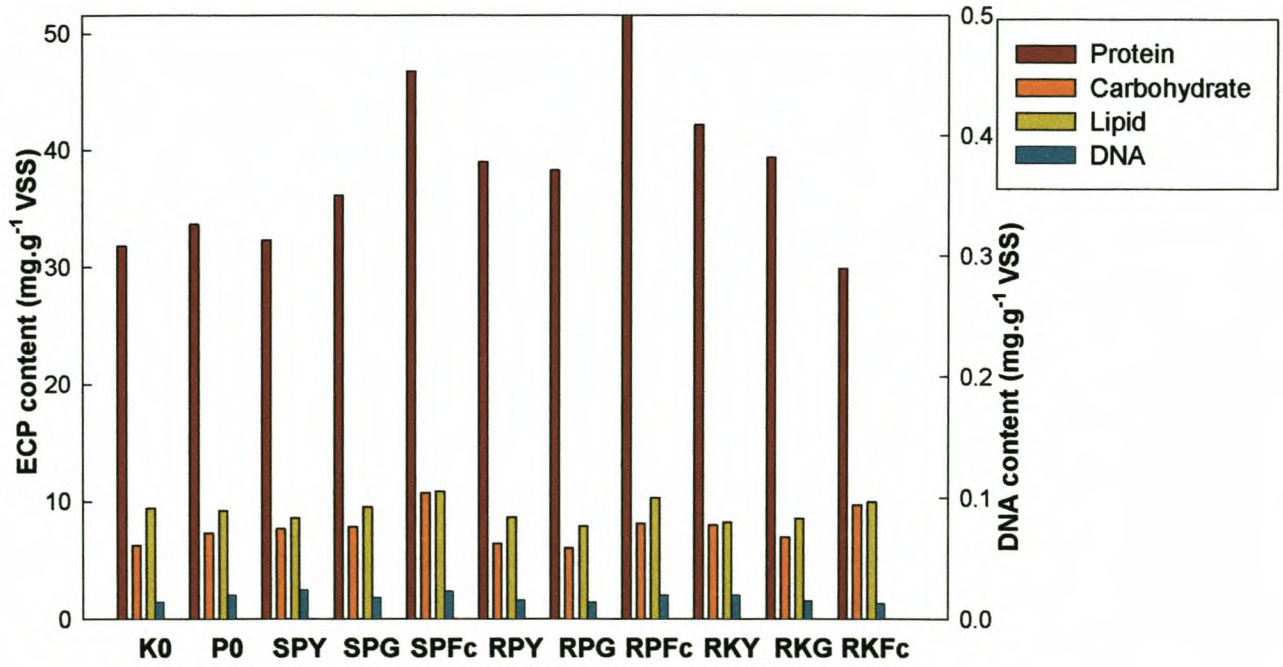

Figure 5. ECP composition ($mg.g^{-1}$ VSS) of the control samples, K0 and P0-sludge, at day 0 and the different batch systems obtained at day 14 during the batch cultivation studies.

substrate was reported to be not so successful in terms of granule count increases (Cameron, 2000).

The batch cultivation of granules when Cameron (2000) used different carbon substrates was only based on the comparison of one sludge batch. In this study, comparison of the batch cultivation of granular sludge was very difficult when different sludge sources were used and even when different batches were used because sludge is a biological system of which the composition can differ widely even on a daily basis.

Granule formation was not optimally enhanced during the batch cultivation studies (Fig. 4), due to limited formation and production of fluffy granules. Chen & Lun (1993), and more recently, Raskin (2001) reported that the first step of granulation was the formation of nuclei and acetoclastic methanogens were mainly involved in nucleus formation. The activity test results (Fig.2D and 3D) indicated that the acetoclastic methanogens were inactive in all the P0 and K0-cultivated batch systems, with the exception of the RKG-batch system. Therefore, it can be speculated that granulation was not optimally enhanced as the major nuclei formers were not active in all the batch systems, with the exception of the RKG-batch system.

In the case of the RKG-batch system, the addition of glucose as carbon growth substrate to the K0-sludge, with higher acidogenic activity than the P0-sludge, was only sufficient to re-activate the inactive acetoclastic methanogens from the K0-sludge to the RKG-batch system ((ii) impact of batch system carbon source on activity). However, it can be concluded that these active acetoclastic methanogenic populations in the RKG-batch system were present in limited numbers because all the RP-type batch systems resulted in a greater increase in granule numbers than the RKG-batch system. Moreover, it can be concluded that the use of a pre-granulated raw anaerobic sludge, such as the P0-sludge, led to the greater increase in granule numbers at the end of the cultivation period.

ECP content of batch cultivated granular sludge

It is believed that the level of ECP produced by anaerobic bacteria in anaerobic sludge is of great importance in the granulation process, however information on the precise role of ECP during granulation is still very limited (Ross,

1984; Costerton, 1987). This study was done to investigate the effect of the ECP content and composition from different batch systems on granule formation.

The data of the composition of the ECP extracted from the different batch studies is shown in Table 6. In all tests, duplicate measurements were made and results quoted are averages of these duplicates. The data in Table 6 indicates that large variations of the protein, carbohydrate, lipid and DNA analyses was found of the ECP for some of the batch systems. These large variations in composition were ascribed to the heterogeneity within the sludge batches used for the cultivation studies. The ECP content was calculated and also presented as a fraction of the VSS content of the different anaerobic granular sludge batches (Fig. 5). An examination of the composition of the ECP content (Fig. 5) of the different batch systems did not show any readily discernible trends. In all batches, more proteinacous ECP (ECP_P) was produced than carbohydrate ECP (ECP_C). The protein:carbohydrate ratio for all the batch systems was > 1 (Table 6), which is typical for anaerobic sludges (Morgan *et al.*, 1990).

No previous data on the amount and role of lipids in the ECP of anaerobic granular sludge has been reported. Schmidt & Ahring (1994) however reported data on the amount and role of lipids in the ECP content of UASB granules. It was found that the amount of lipids was significantly lower than the amount of polysaccharides and protein in the ECP. In this study, the amount of lipids found in the ECP was higher than for granules (Chapter 3) and generally found to be in the range of ECP_C. The amount of DNA was found, as expected, to be present in very low concentrations when compared with the protein, carbohydrate and lipid concentrations (Table 6).

Although the K0-sludge had a lower total ECP content (47.53 $mg.g^{-1}$ VSS) than P0-sludge (50.21 $mg.g^{-1}$ VSS), no discernible trend was observed that batch systems inoculated with P0-sludge, had a higher total ECP content at day 14 than batch systems inoculated with K0-sludge. Comparisons between the granule count of the different batch systems at day 14 also showed no similar trend to the total ECP content after batch cultivation of granular sludge (Fig. 6). Results from this study, however, indicated that SPFc and RPFc had the highest total ECP content of 68.39 and 70.07 $mg.g^{-1}$ VSS, respectively. Therefore, it was concluded that fruit cocktail effluent (Fc) as carbon substrate contributed to a higher total ECP content. The cellulose fibres, as well as the fruit sugar (fructose) in the fruit

Table 6. Composition of ECP ($mg.g^{-1}$ VSS) extracted from the different batch cultivation studies.

Batch system	Total ECP	Protein	Carbohydrate	Lipid	DNA	Protein:Carbohydrate ratio
K0	47.53	$31.81^{*3.621}$	$6.27^{*0.236}$	$9.44^{*0.196}$	$0.014^{*0.0030}$	5.07
P0	50.21	$33.66^{*0.479}$	$7.30^{*0.232}$	$9.23^{*0.614}$	$0.020^{*0.0044}$	4.61
SPY	48.62	$32.29^{*1.874}$	$7.70^{*0.148}$	$8.61^{*0.987}$	$0.024^{*0.0032}$	4.19
SPG	53.59	$36.14^{*0.000}$	$7.86^{*0.440}$	$9.57^{*1.669}$	$0.018^{*0.0006}$	4.60
SPFc	68.39	$46.76^{*1.917}$	$10.74^{*0.501}$	$10.87^{*0.743}$	$0.023^{*0.0070}$	4.35
RPY	54.16	$39.01^{*1.438}$	$6.45^{*0.237}$	$8.68^{*0.308}$	$0.016^{*0.0061}$	6.05
RPG	52.31	$38.29^{*2.982}$	$6.05^{*0.075}$	$7.95^{*0.523}$	$0.014^{*0.0033}$	6.33
RPFc	70.07	$51.55^{*9.052}$	$8.15^{*0.121}$	$10.35^{*2.503}$	$0.020^{*0.0004}$	6.33
RKY	58.46	$42.17^{*0.852}$	$8.01^{*0.276}$	$8.26^{*1.647}$	$0.020^{*0.0061}$	5.27
RKG	55.00	$39.38^{*10.489}$	$7.01^{*0.353}$	$8.60^{*1.683}$	$0.015^{*0.0012}$	5.62
RKFc	49.56	$29.85^{*2.982}$	$9.73^{*0.323}$	$9.97^{*0.586}$	$0.013^{*0.0002}$	3.07

*Standard deviation (SD) values

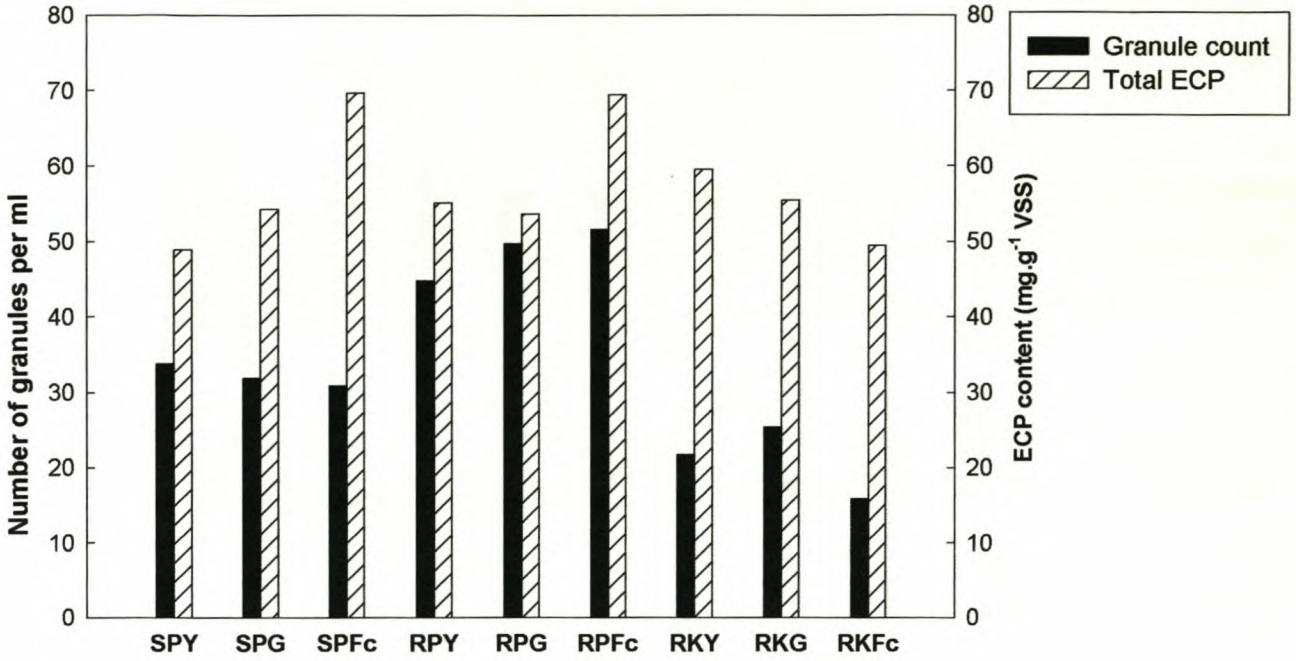

Figure 6. Comparison of the total ECP content (mg.g^{-1} VSS) and granule count (granules per ml) obtained at day 14 during the batch cultivation studies.

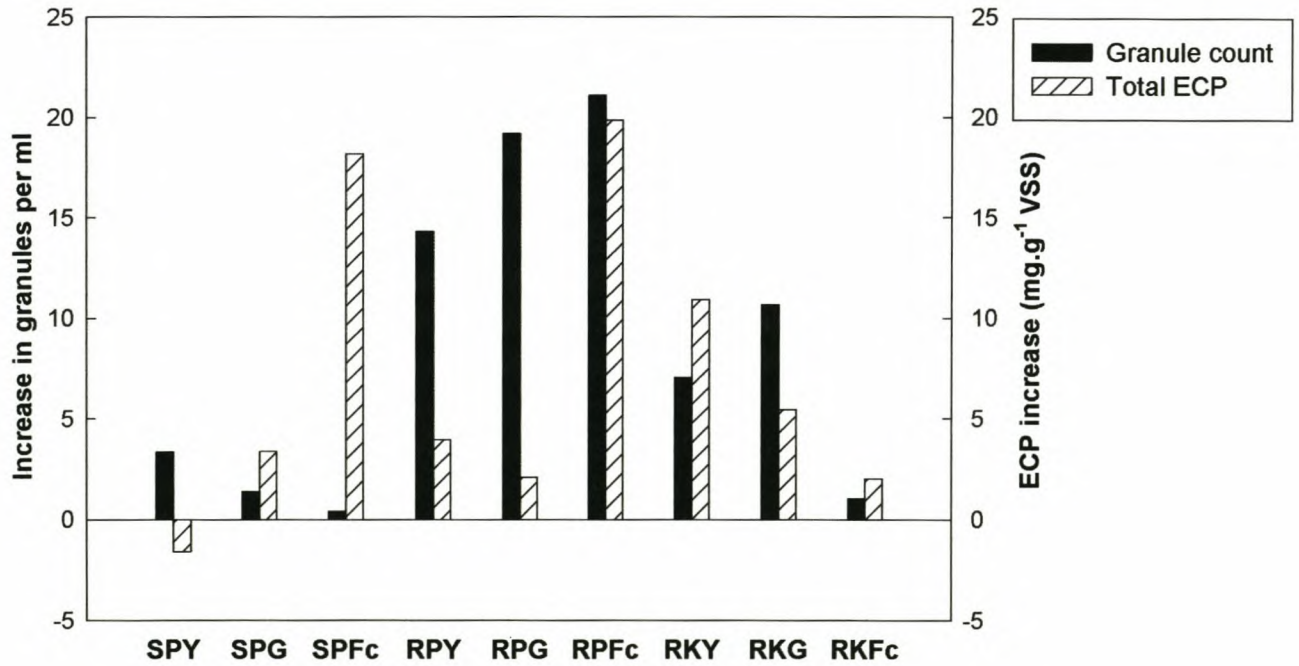

Figure 7. Comparison between the changes in total ECP content (mg.g^{-1} VSS) and granule count (granules per ml) obtained at day 14 during the cultivation studies.

cocktail effluent are β-glycoside carbohydrates that are known to be more difficult to hydrolyse than α-glycoside carbohydrates such as starch and sucrose (Zoetemeyer *et al.*, 1982). Thus, it can be speculated that the bacterial cells then probably excrete these carbohydrates as ECP.

The changes in total ECP content, as well as the changes in the different components of ECP were plotted against the increase in granule count from day 0 to day 14 in order to further investigate the effect of ECP on granule formation (Fig. 7 - 11). From the data obtained (Fig. 7), it was clear that the RP-batch system resulted in the highest increase of granule numbers, followed by the RK-batch system. These results confirmed the conclusion on granule formation that the roller-table technique resulted in a higher increase in granule numbers during batch cultivation of granular sludge. The total ECP content and composition, however, did not show a discernible trend with granule formation during batch cultivation of granular sludge (Fig. 6).

Results showed that the changes in ECP_P (Fig. 8) followed the same trend as for the increase in total ECP content. Protein was found to be the most dominant component of ECP (Fig. 5), and therefore it was likely that the increase in ECP_P really represented the increase in total ECP content. The comparison between the increase in ECP_C and granule count (Fig. 9) indicated that the ECP_C showed only a slight increase (less than 5 $mg.g^{-1}$ VSS) for some batch systems, and even a decrease in ECP_C was observed for the RPY- and RPG-batch systems. All the batch systems where fruit cocktail effluent (Fc) was used as carbon substrate showed an increase in ECP_C. The SPFc-batch system, followed by the RKFc-batch system, showed the highest increase in ECP_C. It can be concluded that when fruit cocktail effluent was used as carbon substrate, it did not only cause the highest increase in ECP_P (SPFc and RPFc) but also the highest increase in ECP_C (SPFc and RKFc). The fibres from the fruit cocktail effluent probably acted as support to which the cells adhered and produced ECP. Moreover, it can be speculated that it was more difficult for the different trophic groups to hydrolyse the β-glycoside carbohydrates (cellulose fibres and fructose) and probably excreted these carbohydrates as ECP. It is not clear whether bacteria adhere reversibly to each other and then produce ECP, or initially produce ECP and then adhere irreversibly (Rutter *et al.*, 1984).

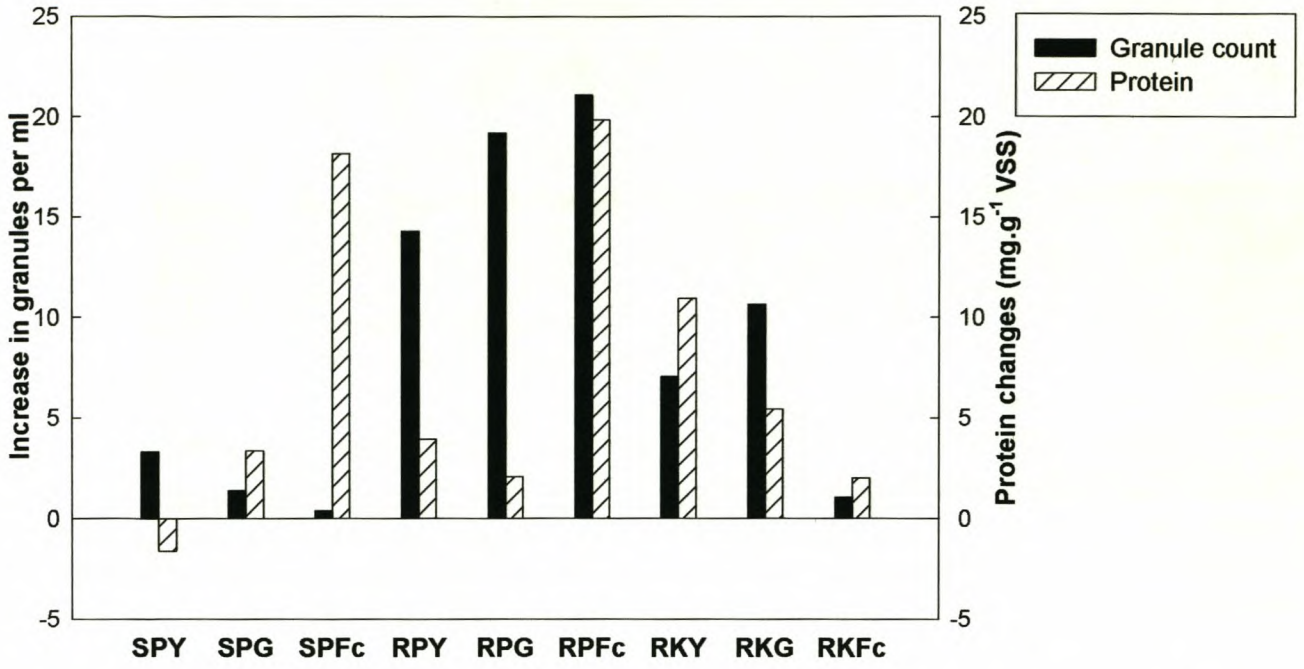

Figure 8. Changes in protein content (mg.g^{-1} VSS) and increase in granule count (granules per ml) obtained at day 14 during the cultivation studies.

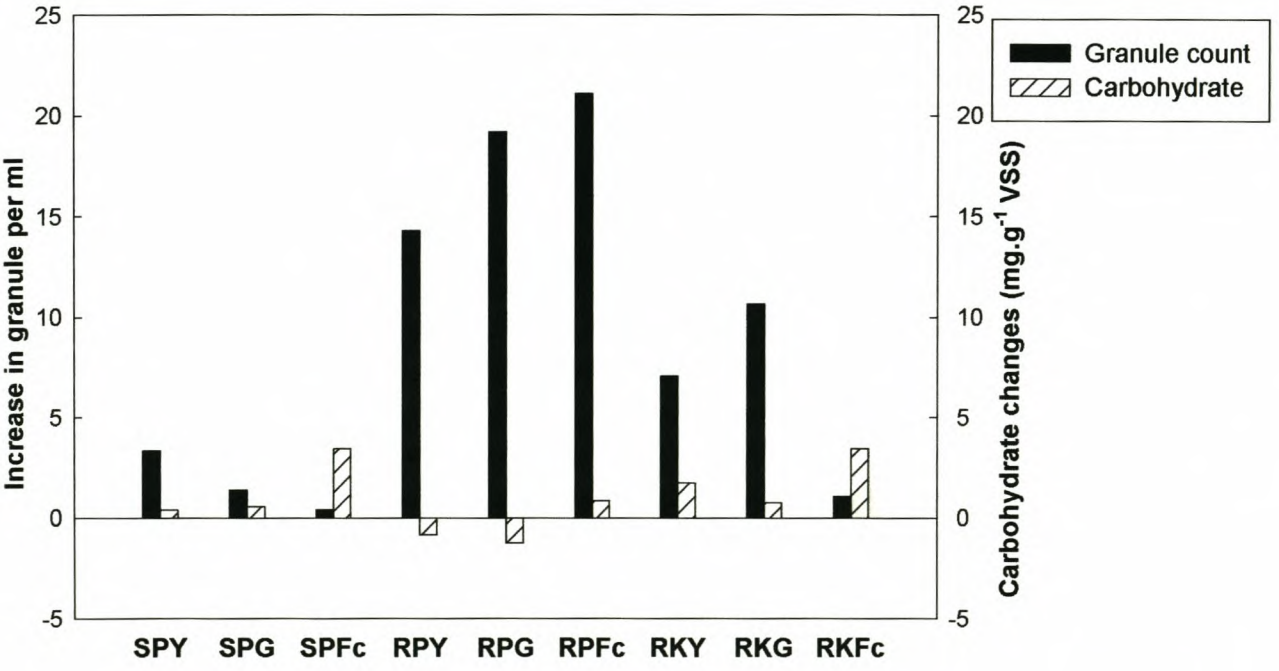

Figure 9. Changes in carbohydrate content (mg.g^{-1} VSS) and increase in granule count (granules per ml) obtained at day 14 during the cultivation studies.

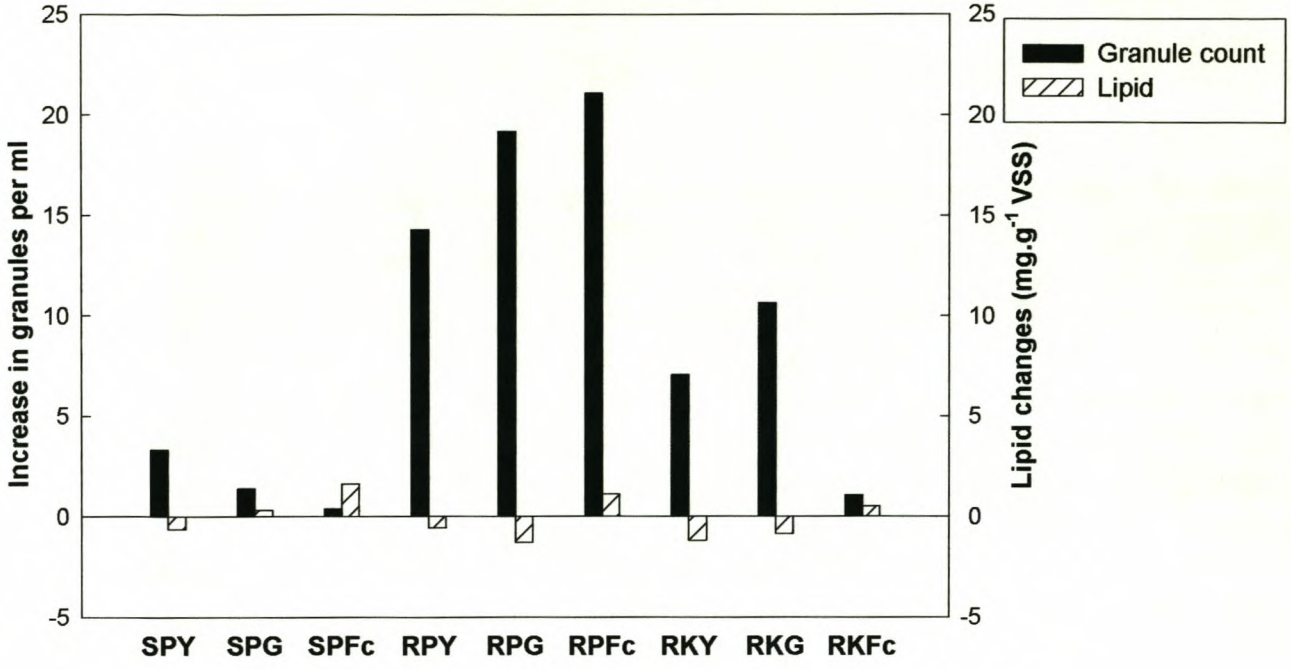

Figure 10. Changes in lipid content (mg.g^{-1} VSS) and increase in granule count (granules per ml) obtained at day 14 during the cultivation studies.

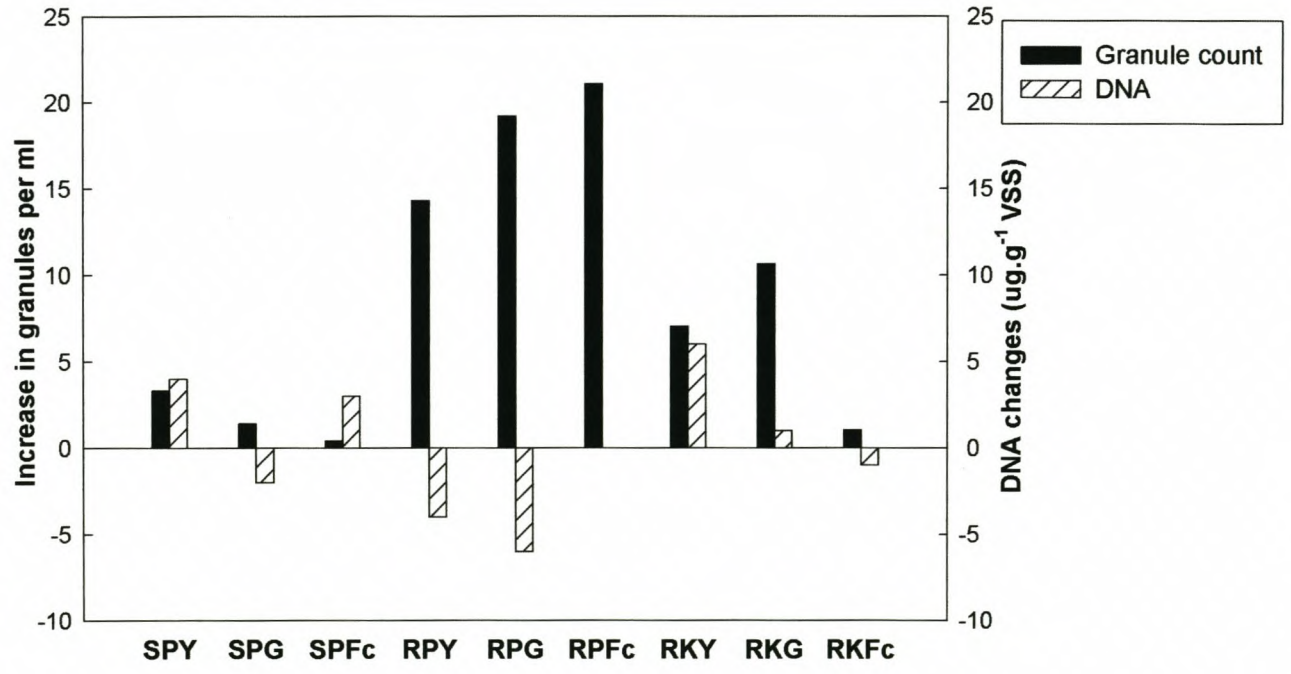

Figure 11. Changes in DNA content (μg.g^{-1} VSS) and increase in granule count (granules per ml) obtained at day 14 during the cultivation studies.

Unfortunately, no discernible trend was observed when the changes in lipid and DNA contents were compared to the increase in granule numbers (Fig. 10 and Fig. 11). It can thus be concluded that although fruit cocktail effluent (Fc) as carbon substrate enhanced ECP production (Fig. 6) during batch cultivation of granular sludge, the increased ECP production did not appear to have an effect on enhancement of granule formation (Fig. 4).

Conclusions

Granulation is the key factor for the successful operation of an UASB reactor, but granulation is a time-consuming process, mainly due to the slow growth of the methanogens (Schink, 2001). This restricts the application of UASB reactors in countries, such as South Africa, where granules from operational full-scale UASB reactors are not readily available for start-up of new reactors. The start-up period can significantly be reduced by enhancing granulation through the production of anaerobic granular sludge in a laboratory batch system, and then seeding the reactor with anaerobic granular sludge instead of just raw anaerobic sludge (Britz *et al.*, 2000).

Results from this study showed that the source of sludge used as inoculum (Fig. 4) had a major effect on enhanced batch cultivation of granular sludge. The use of an anaerobic sludge for the initiation of batch cultivation that already had a measure of granulation, such as the P0-sludge, resulted in a greater increase in granule numbers at the end of the cultivation period. It was also concluded that the control sample, K0-sludge, had more acidogenic and methanogenic populations than the P0-sludge control sample. The use of a different sludge source as inoculum, however did not show a discernible trend for changes in total ECP content (Fig. 7) and ECP composition (Fig. 8 - 11) between the different batch systems studied.

Activity test results using acetic acid as test substrate indicated that the acetoclastic methanogens were either inactive, present in low numbers or inhibited from all the different batch systems studied, with the exception of the RKG-batch system. The addition of glucose as carbon growth substrate to the K0-sludge (RKG-batch system) was sufficient to re-activate the inactive acetoclastic methanogenic population in the original K0-sludge inoculum (Fig. 2D and 3D).

Chen & Lun (1993) and Raskin (2001) reported that it appears as if granulation can be easier initiated by the formation of nuclei, and that acetoclastic methanogens were mainly the bacteria involved in nucleus formation. From the granule formation results (Fig. 4) it can be concluded that the active acetoclastic methanogen populations in the RKG-batch system were present in limited numbers, because the RP-batch systems still resulted in a greater increases in granule numbers than the RKG-batch system. Based on the data (Fig. 4), it appeared that the use of the pre-granulated P0-sludge led to the greater increase in granule numbers in the RP-type batch systems, compared to the RK-type batch systems.

The two batch cultivation techniques investigated showed that the use of the roller-table technique resulted in a higher increase in granule numbers by day 14 of batch cultivation than when the shake-waterbath technique was used (Fig. 4). Methanogenic and biogas activity results (Fig. 2 and 3) indicated that the shake-waterbath technique had a higher tempo of gas production at an earlier period of incubation (10 h). Nevertheless, the roller-table technique eventually resulted in the highest gas production rates at the later incubation stage (25 h). Thus, it can be speculated that the higher tempo of gas production at an earlier stage of incubation might have shortened the contact time between biomass and the substrate, resulting in a lower increase in granule numbers. The two batch cultivation techniques (roller-table and shake-waterbath) however did not impacted the changes in the total ECP content (Fig. 7) and ECP composition (Fig. 8 - 11) in the different batch systems studied.

It was also found that the addition of glucose as carbon substrate not only enhanced the activity of the acidogens, but also led to the establishment of a greater variety of trophic groups (lactate-utilisers and hydrogenotrophic methanogens) within all the glucose cultivated batch systems (SPG, RPG and RKG-batch systems), as compared to when YEL-medium and fruit cocktail effluent were used as carbon substrates (Fig. 2 and 3). However, although the addition of the three carbon substrates (YEL, glucose and fruit cocktail effluent media) impacted the activity of all the different batch systems, no discernible trend was observed in granule formation itself (Fig. 6).

The use of fruit cocktail effluent as carbon substrate led to the highest increase in ECP_C of the fruit cocktail cultivated batch systems (SPFc, RPFc and

RKFc-batch systems) (Fig. 9). It also led to the highest increase in ECP_P and total ECP content for the SPFc and RPFc-batch systems (Fig. 7 and 8). However, it did not lead to the highest increase in total ECP content of the RKFc-batch system (Fig. 7). The use of YEL and glucose-medium as carbon substrates impacted changes in the total ECP content (Fig. 7) and ECP composition (Fig. 8 - 11), however, no discernible trend was found for all the different batch systems (SPY, SPG, RPY, RPG, RKY and RKG-batch systems). Large variations occurred in the ECP composition results, and these variations in composition were ascribed to the heterogeneity with the sludge batches used for the batch cultivation studies.

During this study, the different sludge sources used as inoculum for batch cultivation studies made comparisons between all the different batch systems difficult, because sludge is a biological system of which the composition of sludge, even within the same batch, can differ widely even on a daily basis.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Background

Many researches have reported that extracellular polymers (ECP) are critical for the granulation of anaerobic sludge and, these granules are the key to the success of the Upflow Anaerobic Sludge Bed (UASB) wastewater treatment option. Granulation is a time-consuming process and can take several months before an effective granular bed is cultivated after an UASB reactor is seeded only with anaerobic sludge. The start-up period can significantly be reduced by enhancing granulation through the production of anaerobic granular sludge in a laboratory batch system and then seeding the reactor with anaerobic granular sludge instead of only raw anaerobic sludge (Britz *et al.*, 2000).

The main objectives of this study were firstly to evaluate ECP extraction and analyses methods from UASB granules. Secondly, the ECP composition of different UASB granules was also determined and correlated with the methanogenic activity of these granules. In a third study, batch-cultivated anaerobic granular sludge was produced and the impact of the use of different sludge sources, carbon substrates as growth media and batch cultivation techniques on ECP composition, activity and granule formation were evaluated.

ECP composition *versus* granule activity

The ECP content of UASB granules has been shown in the literature to be mainly dependent on the specific granules examined, the extraction method employed and the analytical method used to quantify the ECP. Several ECP extraction methods have been reported, yet there is no standardised method for the ECP extraction that is universally accepted. Thus, comparison of results from different researchers has to be made with great caution. Physical extraction methods are reported in the literature to be more successful than chemical extraction methods,

and based on this, a physical extraction method was rather used in this study to extract the ECP from different UASB granules.

In the first study (Chapter 3), an ECP extraction method was evaluated in order to determine the optimal extraction time of six different types of UASB granules. The optimal extraction time was taken as the extraction time needed before cell lysis took place and intracellular material started contributing to the ECP content. The optimal extraction time was found to be 4 h for the different granules, with the exception of the Fruit-type granules which was 3 h.

The total ECP content of the different UASB granules varied from 28.71 to 53.76 $mg.g^{-1}$ VSS. Protein was found to be the dominant component of the quantified ECP from all the different granules, with again, the exception of the Fruit-type granules. The Fruit-type granules were originally used to treat mainly a carbohydrate-rich fruit juice and pulp effluent, thus the acidogenic population probably dominated the granule trophic groups. From the data obtained it was concluded that the ECP content of granules that were enriched with mainly acidogens, was essentially composed of carbohydrates leading to a protein:carbohydrate ratio of the ECP content of < 1.

The data also showed that the ECP composition and thus protein:carbohydrate ratio of the ECP of the different UASB granules were affected by the composition of the wastewater fed to the original UASB reactors. The highest protein:carbohydrate ratio was observed for the Food-type granules (5.18), followed by the Brew-type granules (4.02). The wastewater fed to the industrial-scale UASB reactor of the Food-type granules consisted of a protein-rich gelatine-type effluent that probably contributed to the higher protein:carbohydrate ratio of 5.18. In the case of the Brew-type granules, the yeast components present in the brewery effluent used as substrate for the industrial-scale UASB reactor of the Brew-type granules probably contributed to the higher protein:carbohydrate ratio of 4.02. The wastewaters fed to the Dist-, Lye- and Comp-type granules were more carbohydrate-rich and this probably contributed to the lower protein:carbohydrate ratio of around 3.

It was also found that activity test results could be used to indirectly predict the activity of the different trophic groups present in the UASB granules. The acidogenic populations, in terms of biogas (S_B) and methanogenic (S_M) activity, were the most active in all the granules with the exception of the Food-type

granules, which showed higher S_M activity for the lactate-utilising populations. Overall, it was concluded from the activity test data that the different types of UASB granules could be divided into two major groups where the Food-, Brew- and Comp-type granules exhibited higher S_B and S_M activities than the Dist-, Lye- and Fruit-type granules for the different test substrates.

It was also evident from the activity test results and the total ECP content of the respective granules, that the granules with the higher ECP yields, exhibited greater S_B and S_M activities, with the exception of the Fruit-type granules. Although the Fruit-type granules did not have the lowest ECP content (35.59 $mg.g^{-1}$ VSS), the granules exhibited the lowest S_B and S_M activity. The Fruit-type granules also had a protein:carbohydrate ratio of < 1. A possible explanation for this discrepancy is that carbohydrates contain an anionic uronic acid group that can cause repulsion between bacterial cells when high concentrations of carbohydrates are present in the ECP of granules (Jia *et al.*, 1996). Thus, it can be speculated that the high carbohydrate content in the Fruit-type granules probably caused repulsion between the bacterial cells that in turn affected the activity of these granules.

Environmental changes *versus* granule formation, activity and ECP composition

The start-up period of UASB reactors can significantly be reduced by enhancing granulation through the batch cultivation of anaerobic granular sludge and thus seeding the reactor with this batch cultivated sludge instead of raw anaerobic sludge. However, there are many environmental system parameters that impact this production method. In this study, the impact of changes in environmental conditions such as the sludge sources (Paarl/(P0) and Kraaifontein/(K0)), carbon substrates (yeast extract lactate (YEL), glucose (G) and fruit cocktail effluent (Fc) media) and batch cultivation techniques (roller-table and shake-waterbath) were investigated in order to assess the impact on the enhancement of batch granule cultivation and metabolic activity of anaerobic granular sludge. The ECP composition and total ECP content were also determined and compared to the batch cultivation studies and resulting activity of anaerobic granular sludge.

The sources of sludge (P0 and K0-sludges) that were used as inoculum was found to have a major impact on enhanced batch cultivation. The use of an anaerobic sludge for the initiation of batch cultivation that already had a measure of granulation, such as the P0-sludge, resulted in a greater increase in granule numbers at the end of the cultivation period. The use of different sludge sources as inoculum, however, did not show a discernible trend in total ECP content or ECP composition between the different batch systems studied.

The data of the activity tests when acetic acid was added to the BTM as test substrate indicated that the acetoclastic methanogens were either inactive, present in low numbers or absent from all the batch systems, with the exception of the RKG-batch system. Based on the data obtained it was concluded that the acetoclastic methanogens were present in only low numbers in the K0-cultivated batch systems, and again based on the data, appeared to be inactive in the P0-cultivated batch systems. Chen & Lun (1993), and more recently, Raskin (2001) reported that it appears as if granulation can be easier initiated by the formation of nuclei, and that acetoclastic methanogens were mainly the bacteria involved in nucleus formation. Thus, it was evident from the activity results that if the acetoclastic methanogens were inactive or present in very low numbers, granulation would not proceed optimally. However, it was evident from the activity of the acidogens and lactate-utilising bacteria, and resulting methanogenic activity that hydrogenotrophic methanogens were present in the different batch systems, and therefore, able to utilise and degrade the intermediate products that were produced by the acidogens and lactate-utilising bacteria.

Of the two batch cultivation techniques evaluated, the roller-table technique was found to lead to higher increases in granule numbers during batch cultivation, than when the shake-waterbath technique was used. Methanogenic and biogas activity results indicated that the shake-waterbath technique had a higher tempo of gas production at an earlier period of incubation (10 h). Nevertheless, it was observed that the roller-table technique eventually resulted in the highest gas production rates at the later incubation period (25 h). Thus, it can be speculated that the higher tempo of gas production at an earlier period of incubation might have prevented the optimal contact time between biomass and substrate, resulting in a lower increase in granule numbers. The two batch cultivation techniques (roller-table and shake-waterbath), however, did not impact the changes in the

total ECP content and ECP composition between the different batch systems studied.

Of the three carbon sources (YEL, glucose and fruit cocktail effluent media), used in the batch cultivation studies, it was found that the addition of glucose as carbon source not only enhanced the activity of the acidogens, but based on the data obtained, also led to the establishment of a greater variety of trophic groups (lactate-utilisers and hydrogenotrophic methanogens) within all the glucose cultivated batch systems (SPG, RPG and RKG-batch systems). Although the addition of the carbon substrates impacted the activity of all the different batch systems, no discernible trend was observed for the effect of these carbon substrates on granule formation itself.

The use of fruit cocktail effluent as carbon substrate led to the highest increase in ECP_C of the fruit cocktail cultivated batch systems (SPFc, RPFc and RKFc-batch systems). This substrate also led to the highest increase in ECP_P and total ECP content for the SPFc and RPFc-batch systems. However, it did not lead to the highest increase in total ECP content of the RKFc-batch system. Thus, it was concluded that the total ECP production was enhanced in fruit cocktail cultivated batch systems when the sludge source that was used as inoculum, already contained a measure of granulation (P0-cultivated batch systems: SPFc and RPFc). The cellulose fibres, as well as fruit sugar (fructose) present in the fruit cocktail effluent are β-glycoside carbohydrates that are known to be more difficult to hydrolyse than α-glycoside carbohydrates, such as starch and sucrose (Zoetemeyer *et al.*, 1982). It can thus be speculated that it is more difficult for the trophic groups in the different batch systems to hydrolyse these β-carbohydrates and these carbohydrates are then probably excreted as ECP. The use of YEL and glucose-media as carbon batch cultivation substrates impacted changes in the total ECP content and ECP composition, but no discernible trend was found for the different batch systems (SPY, SPG, RPY, RPG, RKY and RKG-batch systems). Unfortunately, large variations occurred in the ECP composition results, making comparisons between the batch systems difficult. These variations in composition were ascribed to the heterogeneity of the sludge batches used for the batch cultivation studies.

Concluding remarks and recommendations

Extraction and component analyses of ECP from UASB granules and batch cultivated granular sludge can provide important information regarding the organic composition of UASB granules and batch cultivated granular sludge. Application of the ECP extraction and analysis technique, as well as the impact of changes in environmental conditions on the enhancement of batch granule cultivation, should contribute to the successful development of the granulation technology.

The composition of the wastewater fed to the respective UASB reactors appeared to strongly impact the ECP composition of UASB granules (Chapter 3). It was speculated that a protein:carbohydrate ratio of < 1 led to a more negative surface charge of the ECP that in turn probably caused repulsion between the bacterial cells and affected the activity of the UASB granules. This will, however, have to be confirmed in future research by looking for a correlation between ECP surface charge and metabolic activity.

Studies on the impact of sludge sources, carbon substrates and cultivation techniques on granule formation, metabolic activity and ECP composition (Chapter 4) can lead to the selection of the best environmental options for batch granule cultivation. It was found that an anaerobic sludge source that already contains a measure of granulation is essential for the granule enhancement during batch granule cultivation studies, but this measure of granulation will have to be characterised and confirmed in future studies.

Large variations in the ECP composition of the different batch systems were found, and ascribed to the heterogeneity of the composition of the different sludges. Sludge is a biological system of which the composition can differ widely, even on a daily basis. Therefore, it is important that the type of sludge should be characterised, both chemically (ECP composition) and microbiologically (activity testing to indicate activity of trophic groups), before it is used as an inoculum. The sludge should then only be used as inoculum for batch cultivation purposes when the raw anaerobic sludge characteristics meets pre-determined requirements for successful batch cultivation purposes. A few characteristics that were found to be important in this study (Chapter 4) include: a pre-granulated sludge as inoculum; a high acidogenic and subsequent acetoclastic methanogenic activity in the inoculum; and an ECP composition with a protein:carbohydrate ratio > 1. The

careful selection of an appropriate sludge inoculum should lead to optimisation of the granulation process.

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