PRESERVATION TECHNIQUES AND CARBON AND NITROGEN GROWTH ENHANCEMENT OF BATCH CULTIVATED UASB GRANULES

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DECLARATION

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

Michelle Cameron

UITTREKSEL

Die potensiële gebruik van die "upflow anaerobic sludge blanket" (UASB) bioreaktor word beperk deur verlengde aansitprosedure as gevolg van die tydsame granulasieproses en die feit dat geskikte inokulums nie vrylik beskikbaar is in die meeste ontwikkelende lande, bv. Suid-Afrika nie. Die massa-kweking van granules sal die afvalwater-industrie voorsien van 'n geskikte inokulum, en sodoende 'n effektiewe opsie vir die behandeling van afvalwater beskikbaar stel. Deur die aanwending van "stres" toestande op 'n reaktor, wat geinokuleer is met rou anaerobe slyk, vind daar 'n verandering in die samestelling van die anaerobe populasie plaas. Die laktaat-benuttende asidogene begin om ekstrasellulêre polisakkariede te produseer wat die granulasieproses versnel deur 'n matriks te voorsien waaraan die bakteriële selle kan heg. Hierdie "omgewings stres" toestande sluit veranderinge in die C:P:N verhoudings in. Die doel van hierdie studie was om die invloed van verskillende koolstof- en stikstofbronne op die vermeerdering van granules te bepaal, asook om die beste preserveringstegniek in terme van die behoud van aktiwiteit te bepaal.

Daar is gevind dat die koolstofbron en konsentrasie 'n betekenisvolle invloed op granule-vermeerdering het. Lae konsentrasies glukose (2 $g.l^{-1}$) het tot die grootste vermeerdering in granules oor 'n 14 dae inkubasieperiode gelei. Vrugtekelkie-afvalwater is geidentifiseer as 'n goedkoop en effektiewe koolstofbron wat ook gebruik kan word vir die kweking van granules. Voorts is gevind dat die stikstofbron en konsentrasie nie so 'n groot invloed op granule-vermeerdering het nie. Urea het nietemin die beste granule-vermeerdering vir al die konsentrasies wat getoets is, gegee.

Standaardisasie van die inokulum was 'n groot probleem gedurende die studie. Betekenisvolle vergelykings tussen die granule-vermeerderings verkry met die verskeie groeimedia is bemoeilik deur die afwesigheid van 'n geskikte en betroubare standaardisasie-metode. Toekomstige navorsing moet hierdie probleem aanspreek aangesien dit sal lei tot meer kineties vergelykbare resultate.

Massa-kweking van granule sal 'n metode van granule-preservering vereis sonder enige verlies van aktiwiteit. In die tweede studie is ses verskillende preserverings-tegnieke en opbergings-periodes in terme van die behoud van

aktiwiteit geëvalueer. Die tegnieke sluit in: vriesdroging; vakuum-vriesdroging; vakuumdroging; bevriesing; koelopberging en kamertemperatuur-preservering. Aktiwiteitstoetsing is gebruik vir die vergelyking van die effektiwiteit tussen die verskillende preserverings-tegnieke in terme van die tempo van biogas- en metaanproduksie. Die granules wat met behulp van die vriesdroogtegniek gepreserveer is, het die beste behoud van aktiwiteit getoon, gevolg deur die vakuum-gevriesdroogde granules. Die monsters wat by kamertemperatuur gepreserveer is, het 'n skielike toename in aktiwiteit na 120 dae van opberging getoon en 'n moontlike verklaring vir hierdie verskynsel kan sel-hidrolise van die granules na 90 dae van opberging wees.

Die meeste aktiwiteit is behaal na 'n 10 h inkubasietyd, en dus word hierdie inkubasietyd aanbeveel indien aktiwiteitstoetse vir evaluerings-doeleindes gedoen word. Verder word aanbeveel dat slegs die glukose-verrykte basiese toetsmedia vir aktiwiteitstoetsing gebruik word aangesien die byvoeging van laktaat en asynsuur geen noemenswaardige rol speel in die bepaling van die vlak van aktiwiteit van die granules nie.

Hierdie studie beveel die gebruik van lae glukosekonsentrasies aan vir optimale vermeerdering van granules tydens die massakweking daarvan, asook die byvoeging van lae konsentrasies laktaat om 'n stabiele sisteem met geen versuring te verseker. Die gebruik van 'n gestandaardiseerde slyk-inokulum word sterk aanbeveel aangesien dit meer vergelykbare resultate lewer. Vriesdroging (as preserveringstegniek) het die beste behoud van aktiwiteit in die granules getoon en word dus as preserveringstegniek aanbeveel. 'n Opbergingsperiode van 90 dae is egter te kort om van veel waarde vir die industrie te wees. Hierdie probleem, asook die verskynsel van 'n verhoging in aktiwiteit na 90 dae van die granules wat by kamertemperatuur gepreserveer is, moet verder ondersoek word.

ABSTRACT

The potential use of the upflow anaerobic sludge blanket (UASB) bioreactor is limited by the extended start-up periods due to the time-consuming granulation process and the fact that seeding inoculum is not freely available in most developing countries like South Africa. The mass cultivation of granules would provide the waste water treatment industry with suitable seeding inoculum and, therefore, an efficient waste water treatment option would be more easily available. By applying 'stress' conditions on a bioreactor system seeded with raw anaerobic sludge, the population dynamics of the anaerobic community change and the acidogens start to produce extracellular polymers, which in turn enhance the granulation process by providing a matrix for the bacterial cells to adhere to. These "environmental stress" conditions include changes in the C:P:N ratio's. The aim of this study was, therefore, to assess the impact of different carbon and nitrogen sources on the enhancement of granulation in a batch system, and to determine the best preservation technique in terms of retainment of activity.

It was found that the carbon source and concentration had a significant influence on batch granule enhancement. Low concentrations ($2.g.l^{-1}$) of glucose gave the best granule enhancement over a 14 day incubation period. Fruit cocktail effluent was found to be a cheap and effective carbon source for batch granule cultivation. It was found that different nitrogen sources did not have the same impact on granule enhancement, however, urea, at all concentrations tested, gave the best granule enhancement.

A major problem encountered during the study was the standardisation of the inoculum. Significant granule enhancement comparisons between the different carbon sources were impeded by the lack of a suitable and reliable form of sludge standardisation. Future research needs to address this problem of the standardisation of the sludge inoculum as this would lead to more kinetically comparable results.

Mass granule culturing will require granule preservation without risking the loss of activity. In the second study, six different preservation techniques and storage periods were evaluated in terms of the retainment of activity. These involved freeze-drying, vacuum freeze-drying, vacuum-drying, freezing, cold

storage and room temperature preservation. Activity testing was used for comparing the efficiency of the different preservation techniques in terms of the tempo of biogas and methane production. Freeze-drying the granules, with storage for up to 90 days was found to give the best retainment of activity, followed by vacuum freeze-drying. The room temperature preserved samples showed a sudden increase in activity by day 120, which could possibly be explained by cell hydrolysis of the granules after day 90.

The highest activity was achieved after 10 h of incubation, and it was, therefore, suggested that activity testing for evaluation purposes should use an incubation time of only 10 h. Furthermore, only the basic test medium with added glucose should be used for activity testing as it was observed that the addition of lactate and acetic acid played no decisive role in determining the level of activity of the granules.

This study recommends the use of low concentrations of glucose for optimum granule enhancement during the mass cultivation of granules, and the addition of low concentrations of lactate to ensure a stable system with no acidification. It is also advisable to use a standardised sludge inoculum, as this will allow more efficient comparisons. Freeze-drying is recommended as preservation technique as this technique showed the best retainment of activity. A storage period of 90 days is, however, too short to be of much use for the industry. This will have to be investigated, together with the phenomenon of increased activity after 90 days as shown by the room temperature preserved granules.

CONTENTS

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

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dedicated to my mother

CHAPTER 1

INTRODUCTION

An ever-increasing population and subsequent industrial activities causes many of the developing countries to suffer from severe environmental pollution. Pollution control is, therefore, of great concern but many of these countries are unable to utilise the presently available conventional wastewater treatment systems because of the high capital outlay and operational costs involved, the high land requirements of some of these conventional systems and the lack of skilled manpower for their operation. It is, therefore, extremely important to develop low cost wastewater treatment systems, which combine a high efficiency with a simple construction and an easy operational profile, as well as some form of valorisation of pollutants. Apart from the environmental pollution problems, most developing countries also suffer from a severe lack in energy and other basic needs. This, together with other factors, forces the development of these countries in a continuing downward spiral (Lettinga *et al.,* 1987). For the development to be turned in an upward direction, the environment should be secured from pollution by applying methods which at the same time diminish existing severe constraints in the area of energy needs and which benefit agricultural productivity (Letitnga *et al.*, 1987).

Aerobic processes have always dominated waste water treatment in industrial countries where the organic compounds are metabolised to carbon dioxide and settleable solids. In the past, energy costs associated with aerobic digestion were low and sludge disposal was simple, however, in recent years, energy costs have risen drastically and sludge disposal has become costly and more difficult. Maat & Habets already concluded in 1987 that the main drawback of aerobic systems is that industrial effluents can seldom be treated cost effectively or adequately due to their high oxygen demand. The application of aerobic processes has, therefore, been reduced and industries are forced to re-evaluate other more efficient treatment technologies. One such treatment option is the anaerobic digestion process.

Anaerobic digestion is a complex series of reactions, catalysed by an assortment of bacteria, which convert the complex organic material to methane and carbon dioxide, in the absence of oxygen (Hawkes *et al.*, 1978; Van Der Merwe, 1994). According to Housley & Zoutberg (1994), the main advantages of anaerobic digestion compared to the aerobic processes, include the production of methane, reduction of the chemical oxygen demand, lower cost and more limited land requirements.

The upflow anaerobic sludge blanket (UASB) bioreactor, developed by Lettinga and co-workers, is one of the most widely applied anaerobic waste water treatment systems (Alphenaar *et al.*, 1993), and is the most popular reactor type for the anaerobic treatment of industrial effluents (Schmidt & Ahring, 1995). The key to the success of the UASB reactor is the ability of bacterial cells to aggregate into dense granules (Guiot *et al.*, 1992). A great deal of research has been done on the performance of the UASB design; however, the main drawback still remains the time-consuming granulation start-up process. Four main factors: the composition; operational temperature; pH; and volatile fatty acid (VFA) content of the waste water, directly influences the granulation process. Hawkes *et al.* (1978) noted that the growth of methane bacteria is generally rather slow when compared to aerobic bacteria. Thus, from a financial point of view, the impact of the slow growth of the methanogens and the subsequent long start-up times can be quite costly for any industry, and for this reason anaerobic digestion is not always considered an option when selecting a system for waste treatment. By seeding the reactor with granules instead of sludge, the start-up period can be reduced significantly. The availability of preserved, but active granules will thus lead to a dramatic cut in the initial implementation costs involved. The whole process will thus be more economically viable.

As mentioned above, the most important factor during the start-up of UASB bioreactors is, without doubt, the formation of granules. This formation involves bacteria which somehow clump together to form granules with a diameter of up to 5 mm (Britz *et al.*, 1999). Although the granulation process is still not fully understood, granular sludge has been observed while treating various waste waters and extensive studies on the morphological as well as physio-chemical characteristics have been reported (Alibhai & Forster, 1986; Colleran, 1988; Fang *et al.*, 1994). The formation of granular sludge occurs only within a limited range

of waste waters (Fukuzaki *et al.*, 1995). Thus, for the expansion of the UASB technology, it is necessary to study the granulation mechanism on various waste waters and to evaluate the quality of granules formed from the viewpoint of high-rate performance of the UASB process.

The granulation process is a unique type of bioflocculation, which is similar to an agglutination reaction as induced by polymers. Moosbrugger *et al.* (1992) concluded that the granules are formed by the generation of an extra-cellular polypeptide produced by a hydrogenotrophic methanogen of the genus *Methanobacterium*. In contrast, Vanderhaegen *et al.* (1992) found the granules rather to contain equal amounts of extracellular proteins and carbohydrates. The results of Dignac *et al.* (1998) showed that extracellular polymers (ECP) are predominantly composed of proteins (70 - 80%). This high organic carbon content of ECP led them to conclude that these compounds influence the structure of the granules. Schmidt & Ahring (1994) reported that the production of polysaccharides is limited when granules are grown on methanogenic and acetogenic substrates. This indicates that protein may play an important role in ECP during the formation and stability of granules. According to Riedel & Britz (1993) and Slobodkin & Verstraete (1993), these compounds can be produced by propionate forming acidogens that are effective slime and aggregate formers.

According to a hypothesis described by Riedel & Britz (1993) and Britz *et al.* (1999), during stable state operational conditions, the anaerobic digestion process requires the concerted action of various microbial metabolic groups. Under these balanced operational conditions no lactate and very little propionate can be detected in a UASB bioreactor. When "stress" conditions are applied to a digester treating carbohydrate rich waste waters, the first metabolite that appears is propionate (Myburg & Britz, 1993), while simultaneously, hydrogen can be detected in the gas phase and lactate starts to accumulate (Eng *et al.*, 1986). These metabolic changes result in a shift of the population dynamics of the anaerobic community, and this was confirmed by Riedel & Britz (1993). Subsequently, slime producing and aggregate forming *Propionibacterium* strains can be isolated under these organic "stress" conditions. Thus, the production of the ECP by the acidogenic bacteria under "stress" conditions (Riedel & Britz, 1993; Slobodkin & Verstraete, 1993) could contribute directly to the initial formation of the highly settlable granules found in efficiently operating UASB reactors. In order

to shorten the start-up period of the UASB process, the need exists to stimulate the aggregation of microbes into granules and it is possible that the induction of "stress" conditions may be the key to granule enhancement. Britz *et al.* (1999) and Britz *et al.* (2000) argued that an abundance of ECP would lead to the clumping of bacteria, thus resulting in the enhancement of granule formation. High concentrations of VFA, especially propionic acid, could contribute to ECP the formation as a hydrogen sink mechanism. In their studies a variety of substrates were tested to determine which led to the formation of propionic acid as major metabolite. It was found that lactate led to the highest production of propionic acid and granule counts confirmed a significant increase in the granule yield.

Furthermore, due to the increasing popularity of the UASB reactor, more granular material will be needed as inoculum during start-up, and thus, the cultivation and successful storage of granules are, therefore, of key importance (Wu *et al.*, 1995). Quantitative research on the preservation characteristics of granules in terms of storing temperature and period is also necessary if the UASB reactor is to be considered for use in industries operating seasonally or intermittently (Shin *et al.*, 1993; Yükselen, 1997).

The main objectives of this study were, firstly, to evaluate the impact of different carbon and nitrogen sources for the batch production of UASB granules. Secondly, batch-cultivated granules will be preserved using different preservation methods, and these will then be stored for a period of four months. The preserved samples will then be evaluated in terms of methanogenic activity.

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CHAPTER 2

LITERATURE REVIEW

A. SA Water Situation

South Africa is considered to be a semi-arid country with limited water supplies and an annual rainfall of 483 mm, which is well below the world average of 860 mm (Department of Water Affairs and Forestry, 1991; Odendaal, 1997; Anon., 1999). Water is one of the key, and probably the most fundamental and essential, of nature's resources. It is crucial for life, the environment, hygiene, food production, industry and power generation and to the overall quality of life (Weaver *et al.,* 1986; Basson *et al.,* 1997). The prosperity of South Africa depends on the sensible management and utilisation of many resources, with water being the most important (Basson *et al.,* 1997). The availability and provision of water on the one hand and the growing requirements for water by the underdeveloped sectors of the country, play opposing roles in the delicate balance between limited natural resources and growing demand (Lamb, 1995). It is estimated that the country's water resources will be fully utilised in about three decades and that growth in water requirements will essentially be in the domestic and industrial sectors (Basson *et al.,* 1997).

Pollution of fresh water sources has increased in recent years and an improved effort is required to address this problem (Lamb, 1995). Anaerobic waste water treatment is presently a widely applied biotechnological technique. The upflow anaerobic sludge blanket (UASB) reactor has been, and it still is, the most widely applied anaerobic waste water treatment system (Lettinga *et al.,* 1983; Hulshoff Pol & Lettinga, 1986; Alphenaar *et al.,* 1993b), and is the most popular reactor type for the anaerobic treatment of industrial effluents (Lettinga & Hulshoff Pol, 1991; Ross, 1991; Schmidt & Ahring, 1995). The key to the success of the UASB reactor is the ability of bacterial cells, when grown under an upflow stream, to aggregate into dense granules (McCarty & Smith, 1986; Guiot *et al.,* 1992).

B. Background to Anaerobic Digestion

Several waste water treatment and recovery systems (WTR) are generally available, but the anaerobic digestion process is considered to be the core of the WTR-system (Fig. 1) (Lettinga *et al.*, 1987). Depending on the type of waste being treated and the way in which the recovered end-products will be applied for re-use, the anaerobic digestion process can be combined with either one or more biological, mechanical or physical-chemical processes. It must be noted that the anaerobic digestion process itself essentially comprises a simple technological system, which in many cases already meets a considerable part of the main goals of an efficient WTR-system. In view of its technical simplicity, anaerobic treatment meets the essential condition of self-reliance of developing countries with respect to the implementation of these systems (Lettinga *et al.*, 1987).

The current problems in terms of the expected shortages of energy sources, the limited usable water supplies and the protection of the environment have resulted in renewed interest in the digestion process. The anaerobic treatment of waste water is an established technology, which has in the past, been employed by municipal sewage units. It has also become increasingly popular in the treatment of high-strength organic wastes (Silverio *et al.*, 1986; Verstraete, 1989; Britz *et al.*, 1990; Vlissidis & Zouboulis, 1993). In spite of the increasing importance of anaerobic digestion, the complete spectrum of microbes involved in reactions during the digestion process has not been determined (Joubert & Britz, 1987; Van Der Merwe, 1994).

Anaerobic digestion is the oldest known process used for the treatment of organic matter (Metcalf & Eddy, 1991; Keay, 1981; Ross *et al.*, 1992). Research on the commercialisation of anaerobic digestion technology started as early as 1814, when Davy made a study on the fertilisation value of cow manure and collected the biogas produced. The first anaerobic digester used sewage solids as substrate and was built by Donald Cameron in 1895 (Colleran *et al.*, 1982). The streetlights of the UK City of Exeter were occasionally fuelled by the biogas produced from this first digester (Senior, 1986; Verstraete & Schowanek, 1987). Although renewed interest in energy-conserving anaerobic treatments can be equated with increases in oil prices, it is still generally accepted that the effluent is treated for environmental protection rather than gas production (Senior, 1986).

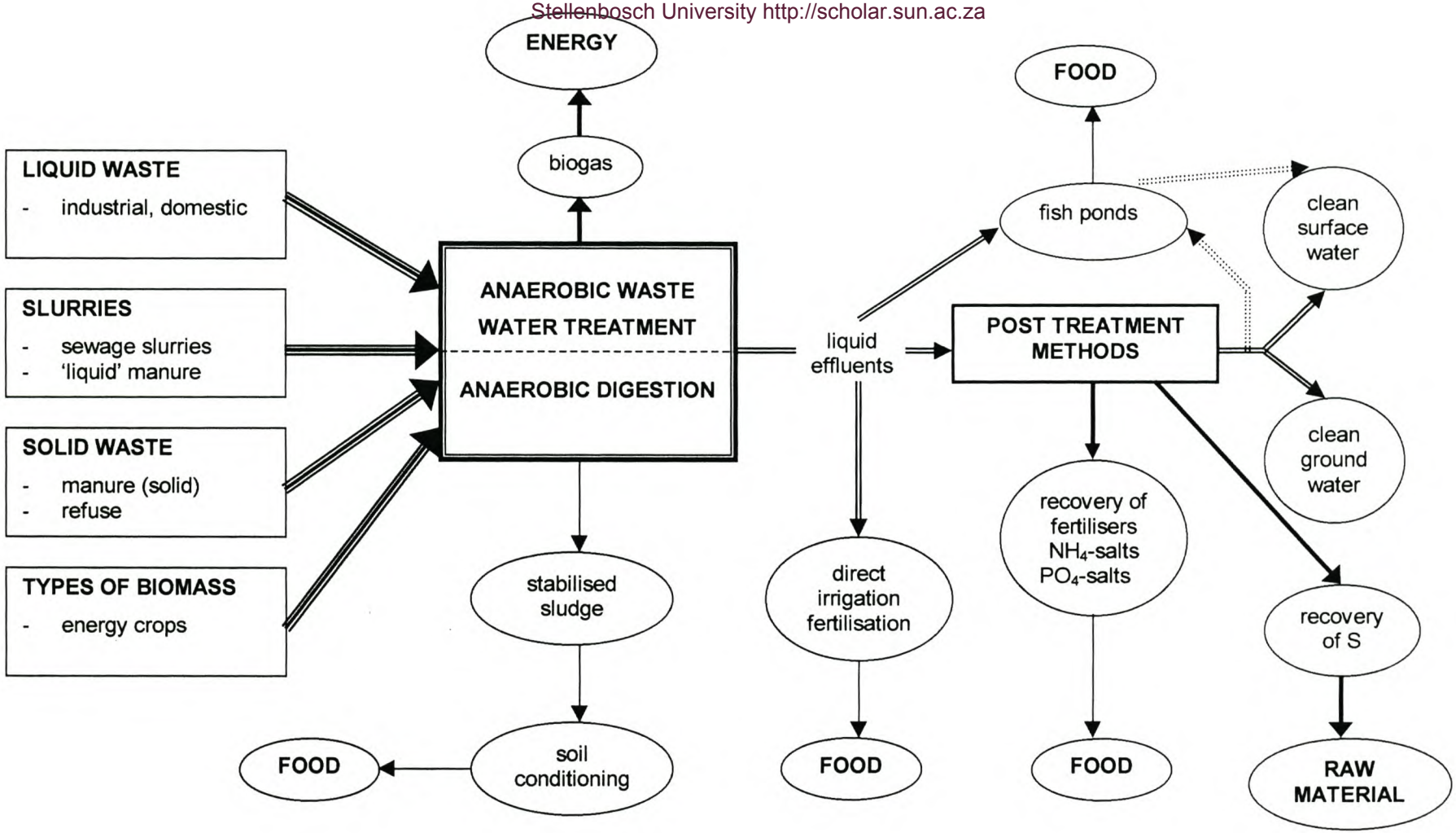

Figure 1. Anaerobic digestion/waste water treatment for product recovery from wastes and wastewaters (Adapted from Lettinga *et al.*, 1987).

Anaerobic digestion consists of a complex series of reactions, catalysed by an assortment of bacteria, which accomplish the conversion of complex organic material to the end-products, methane (CH_4) and carbon dioxide (CO_2), in the absence of molecular oxygen (Hawkes *et al.,* 1978; Metcalf & Eddy, 1991; Van Der Merwe, 1994). In addition, the process kills pathogens (Turner *et al.*, 1983; Shih, 1987; Lee & Shih, 1988). For an anaerobic digester to operate efficiently, a suitable, relatively large and complex microbial community must be established and maintained to uphold the process. This bacterial community must have specialised ecological roles and should not consist of one or more closely related species, but instead, contain diverse genera of aerobic, facultative and anaerobic bacteria. The community diversity must include several different trophic groups of carbon catabolising bacteria, each with different metabolic properties. There are five main groups namely: the hydrolytic and fermentative bacteria; the sulphate reducers; the acetogens; homoacetogens; and the methanogens (Van Der Merwe, 1994).

The UASB waste water treatment system represents a proven supportable technology for a wide range of different industrial effluents, including those containing toxic and/or inhibitory compounds (Lettinga, 1995). The process is also suitable for the treatment of domestic waste water with operational temperatures as low as 14° - 16°C and most probably even lower. In comparison with the conventional aerobic treatment systems, the anaerobic treatment process offers many advantages, including the start-up rate. The available insight in anaerobic sludge granulation and the growth of granular anaerobic sludge is in many cases adequate for practice. Immobilisation of the balanced microbial populations is essential in anaerobic treatment for the sufficient reduction in the concentration of the intermediates (Lettinga, 1995).

The principle of biomass retention in high rate (greater than 10 $kgCOD.m^{-3}.d^{-1}$) (COD = chemical oxygen demand) UASB processes is the development of a good settling granular sludge (Forster, 1984). An upper quiescent zone enhances the potential for reflocculation, and thus, washout of the less stable particles is reduced. The settling process allows the biomass to be internally recycled back to the bottom digester compartment, which is able to provide a very effective means of cell immobilisation. Conventionally, no support media are added and the microbial granules act almost as a fluidised bed and are

retained by their own mass. Such sludge properties hold considerable promise for improved biomass retention, long sludge retention time (SRT), low hydraulic retention time (HRT) and higher organic loading rates (OLR) (Forster, 1984; Ross, 1991).

The mechanisms of pellet sludge or granule formation is one aspect that is being researched worldwide, and the problem of fast granulation has not been successfully resolved (Lettinga *et al.*, 1987; Sam-Soon *et al.*, 1987; Albagnac, 1990). It has been reported that the UASB process is not advisable for some wastewater (Sayed, 1987; Verstraete, 1989), and that it is probably not universally applicable (Speece, 1986a). In some cases, the granules developed very slowly, if at all, or the granules floated instead of settling. Supplying specific carriers, for example anthracite (Albagnac, 1990; Ross, 1991), enhanced the rate of granular sludge production significantly.

The lower efficiencies and loading rates of the anaerobic filter and the occasional instability of the UASB process has led to the proposal of a combination of these two systems, representing an unpacked lower section and a packed upper section. This form of hybrid reactor decreases the dependence on the development of a granular sludge for process stability, since the packing aids in retention of either flocculent or filamentous biomass (Speece, 1986b; Ross, 1991).

C. UASB Design

Anaerobic digestion processes are well-established methods for the elimination of readily biodegradable organic matter from wastewater. Since the development of the highly efficient anaerobic bioreactor, a large number of full-scale plants have been developed (Iza *et al.*, 1991; Lettinga & Hulshoff Pol, 1991). The most important reactor example was the conventional UASB reactor. Wastewater is fed into the bottom of the reactor through a specially designed influent distribution system. The water flows through a sludge bed consisting of anaerobic bacteria, which develops into dense granules. These granules do not contain an organic carrier material, such as sand or basalt (Zoutberg & Eker, 1999). The excellent settlebility (60 - 80 $m.h^{-1}$) of these granules enables high concentrations of biomass in a small reactor volume (Zoutberg & Eker, 1999). As a result, very high

organic loading rates can be applied, which considerably exceed those of the aerobic activated sludge process (Lettinga *et al.*, 1980; Maaskant & Zeevalkink, 1983).

The expanded granular sludge bed (EGSB) is a newer modification of the UASB reactor. The characteristics of this reactor are very similar to the UASB; however, in an EGSB the hydraulic mixing is intensified in order to improve the wastewater/biomass contact, and the granular sludge bed is expanded (De Man *et al.*, 1988). The higher superficial liquid velocity is achieved by applying effluent recirculation. These reactors typically have a high height:diameter ratio. By applying effluent recirculation, liquid upflow velocities of as high as 15 $m.h^{-1}$ have been reported by Zoutberg & De Been (1997), before sludge starts to wash out of the system. Kato *et al.* (1997) reported a liquid upflow velocity of up to 5.5 $m.h^{-1}$ for an EGSB treating low strength wastewater, while Van Lier *et al.* (1997) found that an upflow velocity of 10 $m.h^{-1}$ enhances sludge floatation. In contrast, UASB reactors are normally operated with a liquid upflow velocity of 0.5 to 1.5 $m.h^{-1}$ (De Man *et al.*, 1988). The upflow liquid velocity causes the granular sludge bed to expand, thereby eliminating dead zones and resulting in better sludge/wastewater contact (Seghezzo, 1997).

Due to the higher biomass concentration, the choice of reactor, either the UASB or the EGSB, using granular sludge is in principle favourable, compared to other reactor types currently available. Attempts with full-scale UASB reactors indicate that the treatment of less concentrated domestic and industrial wastewater can be successful (Schellinkhout & Collazos, 1991; Lettinga *et al.*, 1992). Investigations with the UASB and EGSB lab and pilot reactors showed important progress towards the treatment of low strength wastewater (Chernicharo, 1990; Lettinga *et al.*, 1993).

There are a number of factors influencing the efficiency of the UASB system. Temperature, operational efficiency, upflow velocity and recirculation ratio, pH and the C:P:N ratios are the most important parameters to ensure the optimum productivity of the UASB bioreactor.

The type of waste water being treated, and the hydraulic properties of the reactor, are important determinants for the formation and stability of granular methanogenic sludge (Grotenhuis *et al.*, 1991a). Hydrodynamic conditions in UASB reactors can be depicted by superficial liquid upflow velocity, superficial

loading rate of evolved gas that will represent mixing inside the reactor, and the hydraulic loading rates. For a given upflow velocity and HRT, the OLR will depend on the concentration of the waste water (Ghangrekar *et al.,* 1996). Effluent recycling should be applied where the influent $COD_{biodegradable}$ exceeds 3 $g.l^{-1}$, to reinforce the selection pressure (Lettinga, 1995). By increasing the upflow velocity, a high level of hydraulic turbulence and good sludge bed expansion can be achieved, so as to provide an adequate wastewater/biomass contact (Verstraete *et al.,* 1996). A long HRT may allow dispersed bacterial growth and be less favourable for granulation; whereas a short HRT, especially if combined with a high upflow velocity, could cause washout of dispersed bacterial matter and promotes granulation.

Digestion and biogas production can occur at temperatures from as low as 4°C to as high as 60°C (Keay, 1981; Price, 1985). Most anaerobic digesters are, however, maintained within the mesophilic range (30° - 40°C). Thermophilic digestion (45° - 60°C) results in shorter solid retention times, better sludge dewatering characteristics, increased digester efficiency and the increased destruction of pathogenic organisms (Keay, 1981; Forday & Greenfield, 1983; Price, 1985). This is, however, at the expense of higher energy requirements, a lower quality supernatant and poorer process stability (Keay, 1981). The thermophilic bacteria are also more susceptible to upsets and require more careful buffering than the mesophilic bacteria (Price, 1985).

Van Velsen *et al.* (1979) investigated the effects of temperatures in the range of 13° - 55°C on the digestion of piggery manure in laboratory-scale digesters fed daily at a load of approximately 4 kg $TS.m^{-3}.d^{-1}$ (TS = total solids). At a digestion temperature of 13°C no methane was produced, while in the mesophilic range (20° - 40 °C) the methane production increased with temperatures particularly between 20° - 25°C. In the range of 25° - 40°C, the increase of the methane production was much less noticeable and hydrolysis of undissolved manure components turned out to be the rate-limiting step of the digestion process. Under thermophilic conditions (55°C) the methane production decreased with almost 25% as compared to mesophilic digestion, despite a somewhat higher degree of hydrolysis.

In 1995, Lettinga reported that there are five conditions to be met to enable an anaerobic reactor system to accommodate high OLR for the treatment of specific waste water. A high retention of viable sludge is essential under operational conditions; there must be sufficient contact between the viable bacterial biomass and the waste water; high reaction rates and the absence of serious transport limitations; the viable biomass should be sufficiently adapted/acclimatised; and the precedence of favourable environmental conditions for all the required organisms under all imposed operational conditions.

Laboratory-scale studies were conducted on an UASB digester treating carbohydrate-rich cannery effluent under mesophilic conditions (Trnovec & Britz, 1998). With a substrate COD of 2 300 – 4 000 $mg.l^{-1}$, an OLR of 2.28 – 3.95 $kgCOD.m^{-3}.d^{-1}$ and an HRT of 24 h, a COD removal of 88 – 92% was achieved with 1.1 – 2.52 $l.d^{-1}$ biogas produced of which 62 – 65% was methane. The digester pH was 7.6 – 8.0. The HRT was lowered stepwise to 8 h and the OLR and substrate COD increased to 10.95 $kgCOD.m^{-3}.d^{-1}$ and 4 161 $mg.l^{-1}$ respectively. The digester pH was set at 7.9. An excellent COD removal rate of 93% was reported with 8.7 $l.d^{-1}$ biogas produced, containing 64% methane.

Anaerobic digestion is carried out by a number of bacteria with different optimum pH ranges. The digester should be operated within a pH range beneficial to the growth of the majority of the organisms. It has been established that the pH is generally between 6.5 and 7.5 (Hobson & Wheatley, 1993). The optimum pH range of the methanogenic bacteria is in the range of 6.4 to 7.6 (Keay, 1981; Price, 1985). Maintenance of this near neutral pH range is essential for the conversion of acid end-products to methane by the combined activities of the acetogenic and methanogenic bacteria (Forday & Greenfield, 1983). The non-methanogenic bacteria in the system are not as sensitive to pH, and can tolerate a pH range from 5.0 to 8.5 (Hobson & Wheatley, 1993). It is very important that sufficient buffering capacity exist for the acids produced in the reactor in order to prevent low, upsetting pH levels for the methanogenic bacteria (Price, 1985).

Ronquest & Britz (1999) studied the UASB bioreactor operating conditions and operational efficiency after stable state had been reached, while reducing the substrate pH. They used a laboratory-scale UASB bioreactor treating winery effluent at an HRT of 14 h and with an average substrate COD of 2 500 $mg.l^{-1}$. The pH was gradually reduced from 6.7 – 5.0. At a pH of 5.0 the recovery of the

system was very slow and a drop in biogas production indicated that this was the lowest operational substrate pH. The COD removal also dropped to 88% whereas at a pH of 6.7 the system showed a 98% COD removal. This study shows that an UASB can treat fresh winery waste water with little or no neutralisation (depending on the pH of the winery effluent) at a substrate of 5.1 in 14 h with an average COD removal of 93% and an OLR of between 8.44 and 11.05 $kgCOD.m^{-3}.d^{-1}$.

To ensure the efficient digestion of wastewater it is essential for the micro-organisms to be able to grow and multiply and thus the medium must contain carbon, nitrogen and phosphate sources for the biosynthesis of new cells. Bacterial metabolisms further require sulphur, trace elements and other ions. The carbohydrates, CO_2 and volatile fatty acids (VFA's) provide the necessary cell carbon for the acidogens, and can also serve as sources of acetate, CO_2 and H_2 for the methanogens (Stronach *et al.,* 1986). The microbial populations involved in anaerobic digestion require different sources of carbon and nitrogen. If there is too little nitrogen available the bacteria will be unable to produce the enzymes that are needed to utilise the carbon. If there is too much nitrogen on the other hand, it can inhibit the growth of the bacteria. This is especially true if the nitrogen is in the form of ammonia (Hawkes, 1979).

Alphenaar *et al.* (1993a) reported that the methanogenic activity in UASB reactors is reduced by up to 50% if a phosphorus deficiency is present. This reduction was, however, found to be reversible by the addition of phosphate. In batch experiments, a complete recovery of the maximal methanogenic activity was observed with a dosage of 5 mg PO_4-$P.l^{-1}$. Phosphate dosage is also profitable at concentrations as low as 0.1 g PO_4-$P.l^{-1}$. Iza *et al.* (1991) suggested that the C:N:P ratio for anaerobic digestion should be in the range of 100:1-10:1-5. Whilst Ronquest & Britz (1999) found winery waste water C:N:P ratios to be 81:1:1.35, and thus had to supplement the effluent nitrogen and phosphate.

D. UASB Bioreactor Granules

Structure

Granules used in UASB bioreactors are typically spherical aggregates with a diameter of 0.14 - 5.0 mm. The inorganic mineral content of granules varies from 10 to 90% of the dry weight of the granules (Dolfing, 1986; Ahring *et al.,*

1993), depending on the composition of the waste water. The main components of the ash are iron, potassium and calcium (Dubourguier *et al.,* 1988a; Fukuzaki *et al.,* 1991; Shen *et al.,* 1993). Extracellular polymers (ECP), present in the granular sludge, are important for the structure and maintenance of granules, with the inorganic composition being of lesser importance (Grotenhuis *et al.,* 1991a). The ECP content varies between 0.6 and 20% of the volatile suspended solids and consists mainly of polysaccharides and proteins (Dolfing, 1986; Grotenhuis *et al.,* 1991b; Forster, 1992).

Members of both the genera *Methanosarcina* and *Methanosaeta* (formerly *Methanothrix*) have been identified as important aceticlastic methanogens for the initial granulation and development of granular sludge although other methanogens in the granules have also been identified (Fig. 2) (Hulshoff Pol, 1989; Ahring & Schmidt, 1992). Besides the aceticlastic methanogens, hydrogen and formate utilising methanogens are also present (e.g. *Methanobacterium formicicum*, *Methanobacterium thermoautotrophicum* and *Methanobrevibacter* spp.). Micro-colonies of syntrophic bacteria are often detected in the granules, and the significant electron transfer in these micro-colonies occurs through interspecies hydrogen transfer. The internal organisation of the various groups of bacteria in the granules depends on the wastewater composition and the dominating metabolic pathways in the granules. Internal organisation is observed in granules where such an arrangement is beneficial for an optimal degradation of the waste water (MacLeod *et al.,* 1990; Schmidt *et al.,* 1992). De Zeeuw & Lettinga (1980) found that filamentous bacteria grew on the external surface of a separate sludge floc during the first stage (of about 10 days) of the granulation process. These bacteria were almost absent in the original seed sludge. Beyond day 50, increasing numbers of *Methanosarcina* were observed, which, along with *Methanothrix,* are considered to be the main methanogens forming the granules.

Many factors affect the structure of the granules and thus, different types of granules exist (Sam-Soon *et al.,* 1987; Lin & Yang, 1991). Sandberg & Ahring (1992) found that the pH influences the size, density and volatile solids content. The COD and OLR may also affect the structure and, therefore, the bacterial composition will influence the overall characteristics of the granules (Forster & Quarmby, 1995).

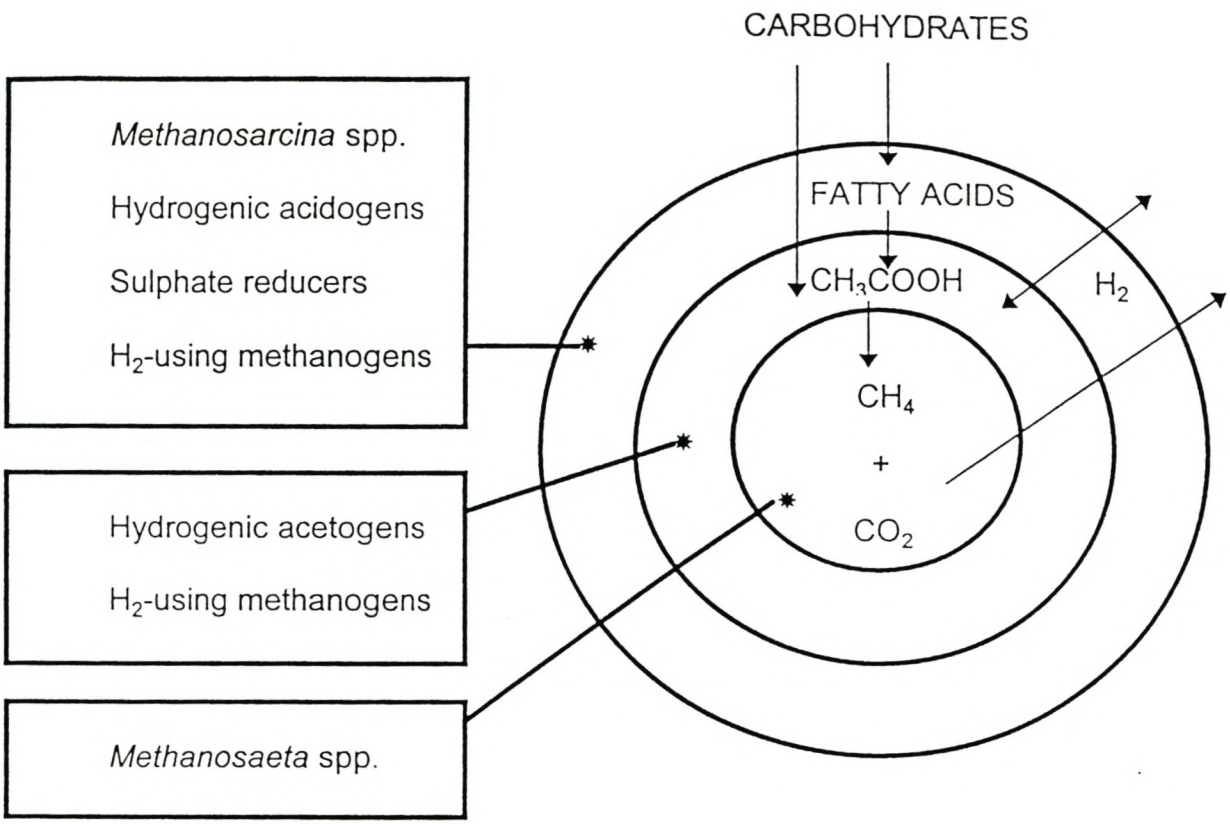

Figure 2. Proposed structure of the granule population arrangement (McCarty & Smith, 1986).

Fukuzaki *et al.* (1995) reported that granules grown on propionate, lactate and glucose were characterised by the dominance of *Methanothrix* species, inorganic deposits, the presence of extracellular polymeric substances, as well as high methanogenic metabolic activities. Variations in the colour of granules have been reported by Kosaric *et al.* (1990). They reported that at low OLR, and when grown on synthetic VFA medium, grey and white granules were dominant, while at high OLR the granules had a black colour. The composition of the waste water was also found to play a determinative role in the colour of the granules. Similarly, Thaveesri *et al.* (1995) found that white and black granules consisted of mainly methanogens, whereas the grey granules showed a high specific acidogenic activity, but only a moderate methanogenic activity. It is, therefore, believed that the acidogens are responsible for the grey colour of the granules.

Bacteria involved in granulation

Various theories regarding the starting point for granulation have been proposed. Several researchers (Wiegant & De Man, 1986; Hulshoff Pol *et al.,* 1988; MacLeod *et al.,* 1990) have suggested that a loose structure of filaments of *Methanosaeta* cells are the predecessor of granules. These filaments can function as a nucleus centre for further development of the aggregate. Other researchers have suggested that during initial granulation, *Methanosaeta* spp. colonise the central cavities of *Methanosarcina* clumps (De Zeeuw, 1988). This is supported by observation of small, presumably young granules with a core of *Methanosarcina* spp.

MacLeod *et al.* (1990) suggested that the first aggregating bacteria would be those producing acetate. These bacteria will provide *Methanosarcina* spp. or *Methanosaeta* spp. with the required substrate. The acetate producers include the fermentative and hydrogen-producing acetogens. The hydrogen-producing acetogens require a syntrophic association with hydrogen-utilising bacteria to reduce substrates such as propionate or butyrate. Other investigators have pointed out the importance of ECP-producing bacteria during the initial granulation process (Ross, 1984; Sam-Soon *et al.,* 1987).

Some researchers have stressed the importance of non-methanogenic bacteria during the initial granulation. The granules are developed from a precursor consisting of small aggregates of *Methanosaeta* spp. and non-

methanogenic bacteria, typically acidogens (Sam-Soon *et al.*, 1988; Morgan *et al.*, 1991). This is supported by the observation that aggregates have a high ratio between acidogens and acetogens compared to developed granules (Dubourguier *et al.*, 1988a).

Effect of granulation on the physiology of the bacteria

Little is known about the physiological changes that take place in an organism either during, or after adsorption (Stal *et al.*, 1989). The physiology of bacteria in the granules is, however, of great importance for the control of granulation and interactions between the granules and the environment. Many factors influence the granulation process. These factors may be intrinsic to the cell: its stage in the cell cycle or its genetic makeup. Other factors could be environmental stress or nutritional excess or limitations. The influence of the abiotic environment on the granulation/disintegration process could be the temperature, ionic strength, cation concentration, hydrogen-ion concentration and mixing (Calleja *et al.*, 1984).

E. Granulation

In UASB reactors the biomass retention is promoted by bacterial self-aggregation into dense granules. Aggregation enhances the performance of high-rate anaerobic reactors, since the good settling of granules minimises biomass washout and the close packing optimises the interspecies exchange of metabolites. One major practical problem faced with sludge-bed reactors is the long start-up period required for the development of granules. This may be related to the long doubling time of acetogenic and methanogenic bacteria. An enhanced granule formation is, therefore, highly desirable in order to reduce this start-up period significantly (Kennedy & Droste, 1985; El-Mamouni *et al.*, 1997). Despite active research in the area of UASB reactors, little is known about the mechanism for formation, maintenance and disintegration of granules (Kosaric *et al.*, 1990; Schmidt & Ahring, 1995).

The mechanism of granule formation is still poorly understood (Slobodkin & Verstraete, 1993) and it appears that a granular sludge can be formed only from certain types of carbohydrate/protein containing waste waters produced in the

agricultural and food processing industries. Sam-Soon *et al.* (1991) reported granulation to be impossible with other types of waste waters. Sørensen *et al.* (1991) experimented with seeding the systems with granules, but in time they either disintegrated or washed out. This clearly restricts the general application, unless the granulation reaction can be induced. The granulation process appears to be a unique type of bioflocculation, which is similar to agglutination reactions as instigated by polymers. Moosbrugger *et al.* (1992) concluded that the granules are formed by the secretion of an extracellular polypeptide, produced by a hydrogenotrophic methanogen of the genus *Methanobacterium*. In contrast, Vanderhaegen *et al.* (1992) found that the granules contain equal amounts of extracellular proteins and carbohydrates. According to Riedel & Britz (1993) and Slobodkin & Verstraete (1993), these compounds can be produced by propionate forming acidogens that are effective slime and aggregate formers.

Granular biomass has several important advantages over flocculent biomass, two of which are the following: granular biomass has a higher specific methanogenic activity than flocculent biomass, which enables it to convert more waste to methane in a given amount of time; and granular biomass has a higher settling velocity than flocculent biomass, which enables it to be retained in a reactor more efficiently (Lettinga *et al.,* 1980; Hulshoff Pol *et al.,* 1983; Dubourguier *et al.,* 1988a). Granulation of anaerobic biomass has been the focus of numerous studies, especially those utilising the fluidised-bed process and the UASB. Both of these are continuously fed upflow systems in which the hydraulic washout of poorer settling flocculent particles ultimately selects for a denser granular sludge (Trnovec, 1998).

Anaerobic methanogenic granular sludge has good settleability and a high rate of COD removal. These factors contribute to their use in the operation of UASB reactors treating various organic waste waters (Lettinga *et al.,* 1980; Wu *et al.,* 1995). Granular sludge can be cultivated using several inoculum sources such as cow manure (Wiegant *et al.,* 1983), anaerobic digested sewage sludge (Hulshoff Pol *et al.,* 1983), and wasted aerobic activated sludge (Guyot *et al.,* 1990). The waste water composition, reactor design and process-technological conditions (Hulshoff Pol, 1989; Alphenaar *et al.,* 1993b) influence the production and quality of granular sludge. Granular sludge for the start-up of a new reactor can also be obtained from other UASB reactors, especially those treating similar

waste water. Due to the increasing popularity of the UASB reactor, more granular sludge will be needed as inoculum, and thus the storage of granular sludge becomes necessary (Wu *et al.*, 1995).

In 1993, Chen & Lun formulated that the first step of granulation is the formation of nuclei. The bacteria involved in the formation of the nucleus are mainly *Methanosarcina* and *Methanothrix* and acetic acid concentration plays a definite role in the nucleus formation. *Methanosarcina* grows into clumps due to the presence of ECP, a phenomenon that has been previously contemplated (Mah *et al.*, 1978). With a raise of the OLR, the growth of *Methanosarcina* increases and more ECP is excreted. This leads to the formation of larger clumps with sufficient diameter so that they cannot be washed out. *Methanothrix* is reported to attach easily and grow on different surfaces (Heijnen *et al.*, 1986). The further development of the nucleus is closely related to the acetic acid concentration. At higher acetic acid concentrations the nucleus will grow since *Methanosarcina barkeri* predominates over *Methanothrix soehngenii*. Once the acetic acid concentration decreases and the hydraulic loading rate increases, *Methanothrix soehngenii* will grow faster than *Methanosarcina barkeri*. *Methanosarcina* is highly unlikely to attach to a different surface to grow and will thus be washed out and play no further role in the granulation process. The formation of the nucleus is considered to be complete when the *Methanothrix* population completely dominates over the *Methanosarcina* on the surface of the nucleus.

Schmidt & Ahring (1995) noted that similarities exist between biofilm formation and the granulation process, since granular sludge can be characterised as a spherical biofilm. The initial development of a biofilm or granule can be divided into four basic steps (Costerton *et al.*, 1987; Verrier *et al.*, 1988; Van Loosdrecht & Zehnder, 1990): i) transport of cells to the surface of an uncolonised inert material or other cells; ii) initial reversible adsorption to the inert material or other cells by physiochemical forces; iii) irreversible adhesion of the cells to the inert material or other cells by microbial appendages and/or polymers attaching the cell to the inert material; and iv) multiplication of the cells and development of the granules.

The cell can be transported to the inert material or other cells by diffusion, advective transport by fluid flow, gas flotation, sedimentation or active transport due to flagella (Gantzer *et al.*, 1989). When bacteria are adhered, colonisation

has started. The immobilised cells start to divide within the ECP matrix so that the offspring cells are trapped within the biofilm structure. This results in the formation of micro-colonies of identical cells. The granulation process depends on cell division and recruitment of new bacteria from the liquid phase. The granular matrix can also contain incidentally trapped macromolecules (e.g. precipitates) (Costerton *et al.*, 1990). The organisation of the bacteria in the granules can ease the transfer of substrates and products. The arrangement may depend on local presence of polymers, local hydrophobicity or cell geometry.

A new granulation hypothesis was recently postulated by Britz: "When 'stress' conditions are applied to batch and laboratory-scale UASB reactors containing fresh anaerobic sludge under controlled conditions, an enhancement of the granulation process will take place" (Britz *et al.*, 1998). When sudden changes in operational parameters (stress conditions) are applied to a digester treating carbohydrate rich waste waters, the first metabolite that appears is propionate (Riedel & Britz, 1993), while simultaneously, hydrogen can be detected in the gas phase and lactate starts to accumulate (Eng *et al.*, 1986). The accumulation of lactate, and the significant rise in hydrogen (Hickey & Switzenbaum, 1991), consequently indicates a shift in microbial consortium dynamics. The acidogens now resort to the utilisation of the electrons of reduced pyridine nucleotides (NADH), generated during fermentation as a result of the increased catabolism of pyruvate, to alternative more reduced hydrogen sink products. These include metabolites such as lactic, propionic and even butyric acids, rather than acetate, CO_2 or H_2. This unbalanced situation (due to the accumulation of organic acids like lactate) will lead to the rapid decrease in the bioreactor pH. The increase in the lactate concentration subsequently results in an orderly shift between the normal acidogenic population to a more predominant lactate-utilising acidogenic population (Riedel & Britz, 1993), in response to the gradual decrease in the pH and increase in H_2 partial pressure. Subsequently, slime producing and aggregate forming *Propionibacterium* strains can be isolated under these organic 'stress' conditions. It was found that under these unbalanced 'stress' conditions, granule formation is stimulated. This possibly begins with the acid sensitive *Veillonella* and *Selenomonas* genera, which are then replaced by the more acid tolerant *Propionibacterium*. These *Propionibacterium* strains gain a competitive advantage during the 'stress' conditions, as they obtain a maximum of adenine triphosphate

(ATP) per mol of lactate fermented (Thauer *et al.,* 1977), the propionic acid concentration will decrease and the pH will gradually increase and stabilise. Once the *Propionibacterium* have the advantage at the lower pH, they start producing extracellular compounds, with the subsequent formation of aggregates (Riedel & Britz, 1993). The formation of ECP may also serve as an alternative hydrogen-sink reaction (Vanderhaegen *et al.,* 1992; Wu *et al.,* 1996). The production of the extracellular polymers by the acidogenic bacteria (Slobodkin & Verstraete, 1993) could contribute directly to the formation of the highly settleable granules found in efficiently operating UASB digesters.

Alphenaar *et al.* (1993b) found that the granulation process proceeds best in a recirculated UASB reactor. Small particles were more abundant in the continuous stirred tank reactor (CSTR)/UASB system, but a shift towards granules was found particularly in the recirculated UASB. A slight tendency for granule formation was observed in the non-recirculated UASB. A long HRT was found to be less favourable for the granulation process, which is in agreement with the results of Hulshoff Pol (1989). A short HRT, especially in combination with a high upward velocity was clearly found to favour granulation.

For many years it was the general opinion that the aceticlastic methanogen, *Methanosaeta,* was critical for the structure and maintenance of methanogenic granules. Several investigators have, however, found that *Methanosarcina* spp. can have the same important role in granules. Other bacteria are also important for the granulation process, especially ECP-producing bacteria. Hydrogen-utilising bacteria together with H_2-producing syntrophic bacteria are observed in micro-colonies (Schmidt & Ahring, 1995).

F. Preservation of Granules

The UASB process has become a popular and reliable technology for the treatment of various waste waters. The success of this process relies on the formation of granules with high settleability and bioactivity abilities (Fang *et al.,* 1995; Yükselen, 1997). Although the granulation process is still not fully understood, the feasibility of granular sludge for treating various waste waters using continuous operations has been successfully demonstrated and studies on

morphological and physiochemical characteristics have been reported (Shin *et al.*, 1993; Pereboom, 1994; Yükselen, 1997).

Quantitative research on the preservation characteristics of granular sludge in terms of storing temperature and period is necessary when the UASB reactor is considered for use in industries operating seasonally or intermittently (Shin *et al.*, 1993; Yükselen, 1997).

The optimum conditions to store methanogenic granules must be established in order to maintain the anaerobic metabolic activity and the stability of the granular sludge as long an possible (Wu *et al.*, 1995). According to Wu *et al.* (1995), the shape of the granular sludge can be kept for a long time (over 3 years) without feed under the right anaerobic conditions. The factors that influence the anaerobic metabolic activity of the stored granules are (Hungate, 1969; Jain *et al.*, 1991): exposure to air; storage period; and storage temperature. Oxygen is inhibitory to anaerobic bacteria, especially the methanogens and acetogens (Jain *et al.*, 1991). However, Kato *et al.* (1993a, b) found that the methanogenic granules had a much higher tolerance to oxygen inhibition than the pure anaerobic microbial cultures due to a great number of facultative bacteria and some aerobic bacteria (methanotrophic bacteria) present in the granules (Wu *et al.*, 1995). These aerobic and facultative bacteria can rapidly consume oxygen and protect the anaerobic bacteria from oxygen inhibition.

For industrial application, a tank sealed with water can be used to prevent any incoming air from reaching the stored granules. The anaerobic conditions inside the tank can be maintained via the consumption of oxygen by the aerobic and facultative bacteria, as well as the anaerobic activities of the anaerobic bacteria in the granules (Wu *et al.*, 1995).

The increasing popularity of the UASB-process calls for the availability of granular sludge to be used as inoculum. The cultivation and successful storage of new granules are, therefore, of key importance. Vazoller & Rech (1992) evaluated two different techniques for the storage of strict anaerobic methanogenic cultures, one for low redox potential maintenance in culture flasks and the other for cell conservation in liquid nitrogen. The culture viability was tested after one week, one month, six months and 18 months of incubation. According to Hippe (1984), the method of freezing and storage of methanogenic cells under liquid nitrogen is very suitable for preservation of these micro-organisms. Furthermore, he noted

that pure methanogenic cultures are difficult to obtain and their preservation is essential. Wiegant *et al.* (1983) reported a 50% reduction in the methanogenic activity of thermophilic (55°C) granular sludge after storage at 30°C for 50 days. The effect of long-term storage on the metabolic activity of particular microbial trophic groups involved in the complete degradation of organic compounds to CH_4 and CO_2 has not been examined. Some researchers have reported sudden disintegration of granules without any obvious reason (Schmidt & Ahring, 1996). In 1995, Wu *et al.* evaluated the effect of different storage temperatures on the degradation of VFA's, including acetate, propionate, butyrate and the methane production of anaerobic methanogenic granules. They stored the granules under anaerobic conditions at 4° and 22°C for an 18-month period. They found the length of the storage period affected the activities of different microbial trophic groups. During storage at 22°C, the degradation rates for all three VFA's decreased gradually, whilst at 4°C the reduction in degradation rates of acetate and propionate were relatively slower. Reduction in butyrate degradation rate was faster by 45% during the first month of storage at 4°C, but the rate then declined. The granules maintained their metabolic activities for all three VFA's after storage for 18 months, although at reduced levels. Higher decay coefficients were obtained at 22°C than at 4°C. Wu *et al.* (1995) reported that granules could be stored at ambient temperatures (20° - 22°C) with limited loss in their VFA degradation rates for relatively short periods (1 – 5 months). Granules can, however, maintain higher levels of VFA degradation rates when they are stored at low (4°C) rather than ambient temperature. Reactor studies indicated that the granules can completely recover their original VFA degradation rates in three days when stored for 31 d at 22°C. The granules stored at 22°C for 9 months were successfully used as inoculum to start a laboratory-scale reactor. The original VFA degradation rates of the granules were achieved after 15 – 20 d of reactor operation at 35°C.

Yükselen (1997) stored laboratory-scale reactors, seeded with sludge at room temperature, -18°C, 4°C and 37°C without feeding for different storage periods up to 12 months. After storage, changes in the physiochemical and microbial characteristics were monitored in terms of storing period and temperature. The total suspended solids (TSS), volatile suspended solids (VSS),

and COD in the liquid phase were measured and the microbial activities were expressed as specific methanogenic activity. The temperatures of the stored samples were raised to 37°C before determining the methanogenic activity and other physiochemical characteristics. The soluble COD was found to be 10% higher in the reactor stored for 12 months at room temperature and about 23% more when stored at 4°C. There was no significant change for the reactor stored at -18°C and about a 40% decrease from the initial value if stored at 37°C. The value of VSS/TSS decreased the most for the sample stored at 37°C and again there was no significant change for the sample stored at -18°C (Yükselen, 1997). Methanogenic activity was used to determine the biomass activity of UASB sludge. Methanogenic activity is defined as the methane gas production rate converted to gram COD per gram sludge seeded. Results indicated that the storage temperature of 37°C maintained the highest activity, where the room temperature was only 3% less than this value. Considering the difficulties of keeping sludge at 37°C, the room temperature was the most beneficial temperature for maintaining methanogenic activity. Yükselen (1997) found that UASB sludge has good microbial and physiochemical preservation characteristics.

G. Conclusions

McCarty already postulated in 1964 that: "The anaerobic treatment process is in many ways ideal for wastewater treatment and it is almost certainly assured of increased usage in the future" (Lettinga, 1995). The popularity of the anaerobic digestion technology clearly illustrates the applicable aspects of this process. Anaerobic digestion offers many advantages over conventional and aerobic treatment processes. There are, however, still limitations and aspects that must be clarified, including the rather extended start-up periods, the granulation process and lack of granulation in specific effluents. Several granulation mechanisms have been described and developed, but the mass culturing of granules still remains a problem that must be solved before the system can be applied on a general level.

H. References

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CHAPTER 3

INFLUENCE OF DIFFERENT CARBON AND NITROGEN SOURCES ON GRANULE PRODUCTION IN BATCH SYSTEMS

Abstract

The need for granular seed sludge for the start-up of upflow anaerobic sludge blanket (UASB) reactors is crucial in countries where granules are not readily available. A method has been developed to enhance batch granule production from raw anaerobic sludge. However, there are many system environmental parameters that influence this production method. In this study the impact on the enhancement of batch granule production using different carbon (lactate, glucose and sucrose) and nitrogen (peptone, urea and ammonium sulphate) sources and concentrations, was evaluated. Different combinations of carbon sources (lactate and glucose, lactate and fruit cocktail effluent, and glucose and fruit cocktail effluent) were also evaluated to determine a more economically viable combination to be used for granulation enhancement from sludge. Glucose, at a concentration of 2 $g.l^{-1}$ was found to give the best enhancement of granule numbers after 10 days for the single carbon additions, followed by 10 $g.l^{-1}$ lactate and 2 and 5 $g.l^{-1}$ sucrose. Different nitrogen sources at various concentrations were found to be less critical for the enhancement of granules.

A combination of 5 $g.l^{-1}$ lactate + 1 $g.l^{-1}$ glucose resulted in the best enhancement of granule numbers after 10 days when different carbon combinations were used. A concentration of 2 $g.l^{-1}$ COD (COD = chemical oxygen demand) fruit cocktail effluent (FC), in combination with 2 $g.l^{-1}$ glucose gave the best increase in granule numbers by day 10. The data showed that glucose can be used in combination with lactate for batch granule cultivation, and that the advantage of this combination is that lactate facilitates the stabilisation of the system.

A major problem encountered was the need for a simplistic method to standardise the sludge inoculum. The varying quality of the raw sludge caused a problem when studies done on different batches had to be compared in terms of

increases/decreases, and when a kinetic value had to be assigned to the enhancement. To overcome this problem, the best unit of each carbon source or carbon combination was repeated under the same conditions, using a standardised sludge-batch. Glucose, at a concentration of 2 $g.l^{-1}$ again gave the best granule enhancement, followed by 10 $g.l^{-1}$ lactate and then a combination of 5 g lactate + 2 g glucose.

Introduction

Anaerobic technology is widely accepted for the treatment of industrial wastewaters that contain high concentrations of organic pollutants. The high-rate upflow anaerobic sludge blanket (UASB) reactor (Lettinga *et al.*, 1980) has probably attracted the most research and commercial interests, and is one of the most extensively applied anaerobic treatment systems in the world (Weiland & Rozzi, 1991; Lettinga *et al.*, 1997). This process depends on the upward movement of wastewater through a blanket of granules, which is a distinctive feature of the UASB system (Chynoweth, 1987). For the process to be able to operate at high organic loading rates and short hydraulic retention times, the formation of highly compact sludge aggregates, called granules, are essential (Alphenaar *et al.*, 1994; Schmidt & Ahring, 1995). The formation of granules allows the active biomass to be retained in the reactor independent of the flow rate (Ahring & Schmidt, 1992), thereby maintaining good conversion efficiency, even at relatively high flow rates.

Little is known about the granule formation mechanism (Slobodkin & Verstraete, 1993; Schmidt & Ahring, 1994) and the maintenance and disintegration of granules (Kosaric *et al.*, 1990). The granules have occasionally been found to disintegrate in industrial scale reactors, resulting in loss of activity and washout of granules from the system. It also appears that granular sludges can only be formed with certain types of carbohydrate/protein containing waste waters (Vanderhaegen *et al.*, 1992; Uemura & Harada, 1993) generated in the agricultural and food processing industries (Slobodkin & Verstraete, 1993). According to Sam-Soon *et al.* (1991), granule formation has not been possible with other waste waters.

Moosbrugger *et al.* (1992) postulated that the granules are formed as a result of the generation of extracellular polypeptides produced by a hydrogenotrophic methanogen of the genus *Methanobacterium*. In contrast, Bull *et al.* (1983) found that an increased carbon:nitrogen (C:N) ratio stimulated the production of extracellular polysaccharides, resulting in improved bacterial attachment to solid surfaces. In the studies of Vanderhaegen *et al.* (1992), it was found that the granules rather contained equal amounts of extracellular proteins and extracellular carbohydrates. Riedel & Britz (1993) as well as Slobodkin & Verstraete (1993) reported that the extracellular polymers (ECP) could possibly be formed by propionate-producing acidogens that are effective slime and aggregate formers.

It has been reported (Marchaim & Krause, 1993) that when a digester is put under "stress" conditions, the first metabolites to appear after lactate (Eng *et al.*, 1986) are propionic and acetic acid. Hydrogen (Hickey & Switzenbaum, 1991) can be detected simultaneously in the gas phase (Myburg & Britz, 1993). Based on the above characteristics of a "stressed" bioreactor, Britz *et al.* (1999) showed that granules can be produced outside the bioreactor in batch systems and, that by applying "stress", conditions can be induced that enhance the granulation process. The "environmental stress" conditions can include differences in carbon sources and concentrations, organic overloading, increases in the operational hydrogen pressure, changes in the C:N:P ratio's, or the addition of cysteine (Moosbrugger *et al.*, 1992; Myburg & Britz, 1993; Riedel & Britz, 1993).

The aims of this study were, firstly, to investigate the influence of different carbon and nitrogen sources on granule enhancement in a batch system and, secondly, to determine which growth medium would be the most economical in terms of granule count increase.

Materials and methods

Batch granule production

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 500 ml container was filled with 100 ml raw anaerobic sludge (Athlone Wastewater Treatment Works) and 350 ml growth medium. For a period of 14 d, a

100 ml of the top volume of each container was replaced daily with a 100 ml fresh sterile growth medium so as to simulate UASB operational parameters and organic overloading (Britz *et al.*, 1999; Britz *et al.*, 2000). The pH values were determined daily and granule numbers were counted on days 0, 5, 10 and 14.

A basic growth medium (Table 1) was used for the first phase of this study, with either the carbon (Table 2) or nitrogen (Table 3) component being varied, as well as the concentration of the component.

In the second phase of the study, the basic growth medium (Table 1) was again used, but combinations of the different carbon sources (Table 4) were used as carbon source. The fruit cocktail effluent that was added to the sludge was a mixture of peach and pear washwater obtained as a specific effluent stream from Ashton Canning Company (Pty) Ltd, and diluted to the required chemical oxygen demand (COD) (Table 4). All studies were done in triplicate.

Analytical procedures

The following parameters were monitored: pH; COD; Total Solids (TS); Volatile Solids (VS) and Total Non-Volatile Solids (TNVS) (APHA, 1992).

Granule counts

Each granule sample (500 μl for day 0, and 250 μl for days 5, 10 and 14) was fixed in a petri-dish using gelatin (2 g.100 ml^{-1}) (Jeison & Chamy, 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).

Results and discussion

pH profiles during the batch granule culturing using different carbon sources

The triplicate pH profiles obtained during the batch culturing, using different carbon sources, are illustrated in Fig. 1 - 3. The pH of each batch system was monitored as an indicator of metabolic productivity at the start and during the

Table 1. Composition of the basic growth medium used for batch granule cultivation.

Compound	Concentration ($g.l^{-1}$)
Lactic Acid (carbon) [Saarchem – UniLab; 60% solution V/V]	20
Yeast Extract [Biolab]	5
Peptone (nitrogen) [Biolab]	2
KH_2PO_4 [BDH]	10
Tween 80 [Merck]	1 ml
Trace elements*	10 ml
pH	7.0

*Nel *et al.* (1985)

Table 2. Concentrations of different carbon sources used for batch granule cultivation.

Carbon Source	Concentrations ($g.l^{-1}$)
Lactic Acid [Saarchem – UniLab]	5; 10; 20; 30
Glucose [BDH]	2; 5; 10
Sucrose [Merck]	2; 5; 10

Table 3. Concentrations of different nitrogen sources used for batch granule cultivation.

Nitrogen Source	Concentrations ($g.l^{-1}$)
Peptone [Biolab]	1; 2; 5
Urea [Labchem]	1; 2; 5
Ammonium Sulphate [BDH]	1; 2; 5

Table 4. Different combinations of carbon sources used in the study.

Carbon Sources	Combinations ($g.l^{-1}$)
Single:	
Lactate	10
Glucose	2
Fruit cocktail effluent	2 $g.l^{-1}$ COD
Fruit cocktail effluent	3 $g.l^{-1}$ COD
Combinations:	
Lactate + Glucose	10 + 2
	5 + 1
	5 + 2
	5 + 5
Lactate + Fruit cocktail effluent	10 + 2 $g.l^{-1}$ COD
	5 + 2 $g.l^{-1}$ COD
Glucose + Fruit cocktail effluent	5 + 2 $g.l^{-1}$ COD
	2 + 2 $g.l^{-1}$ COD

lac-pH

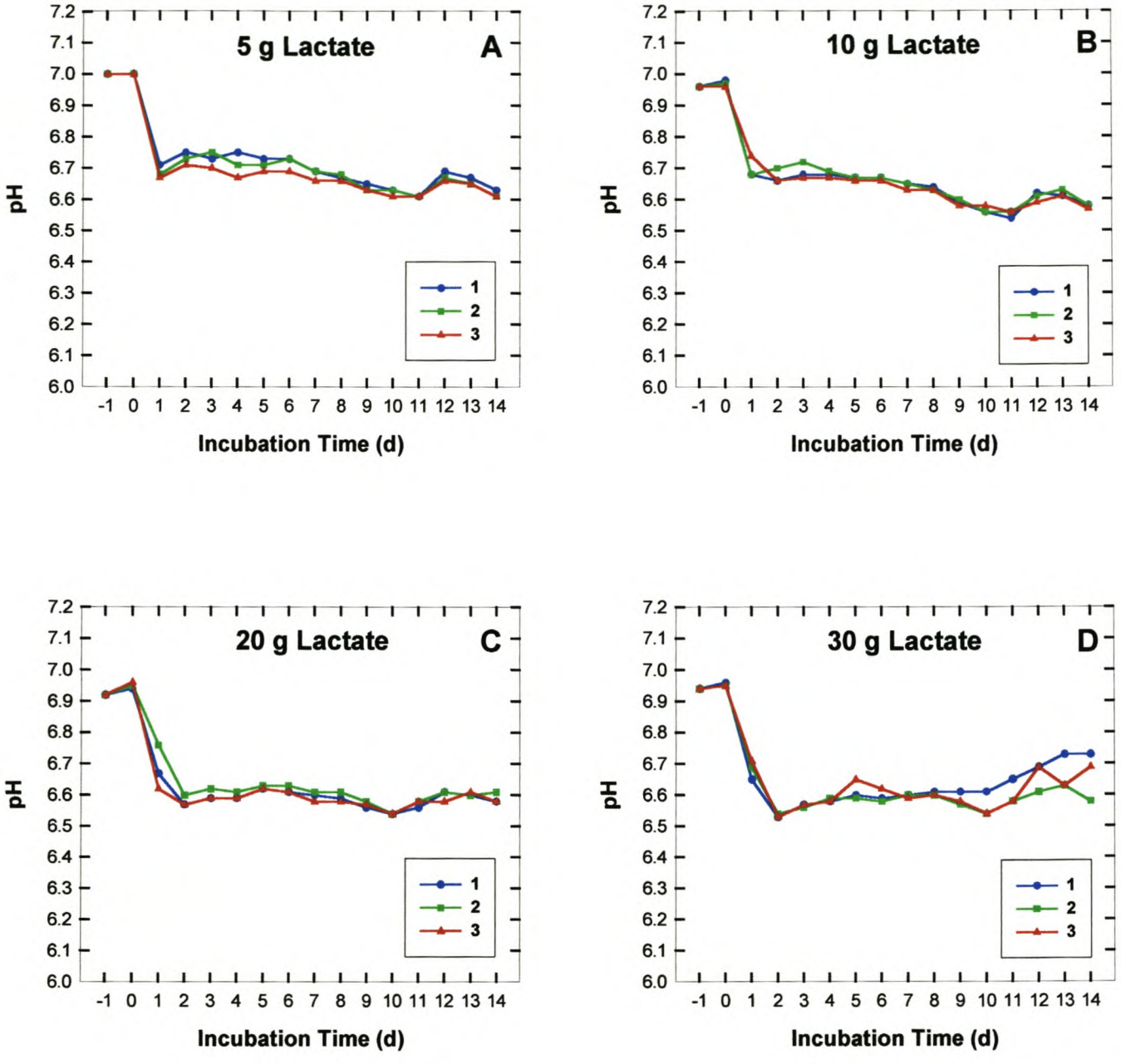


Figure 1. pH profiles obtained for different concentrations of lactate, as major carbon source in the basic growth medium, during the batch cultivation of granules (studies were done in triplicate).

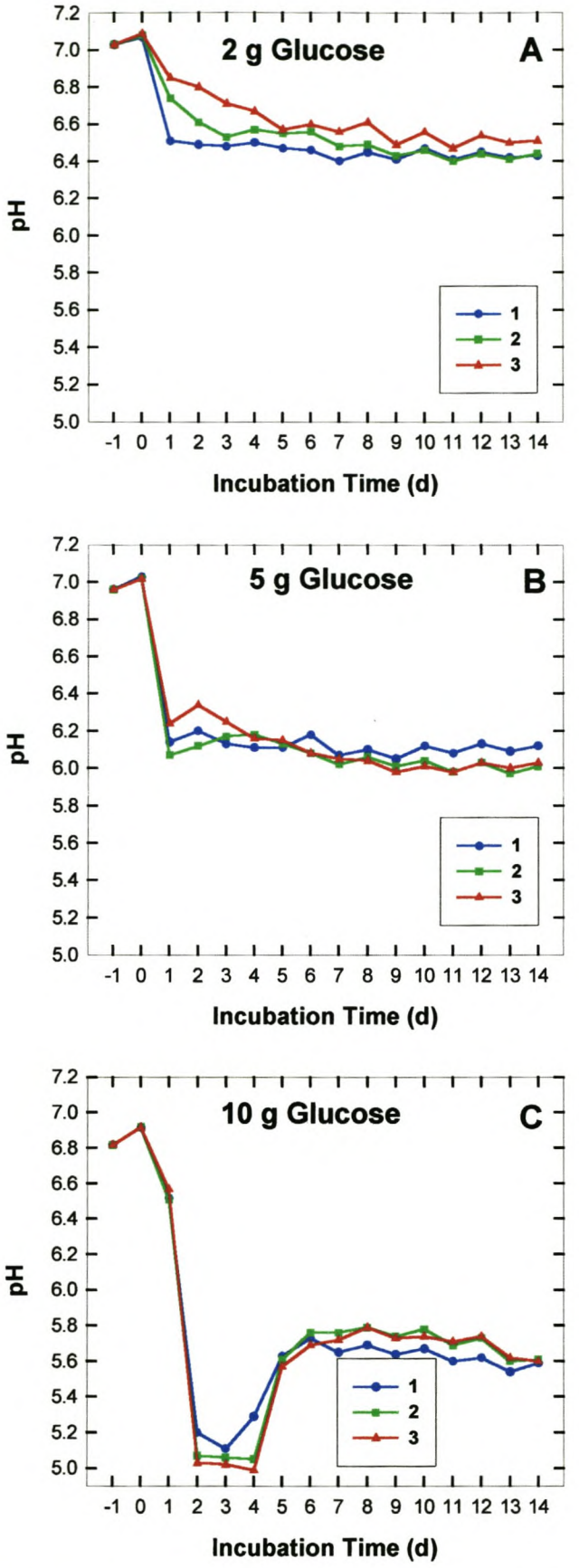


Figure 2. pH profiles obtained for different concentrations of glucose, as major carbon source in the basic growth medium, during the batch cultivation of granules (studies were done in triplicate).

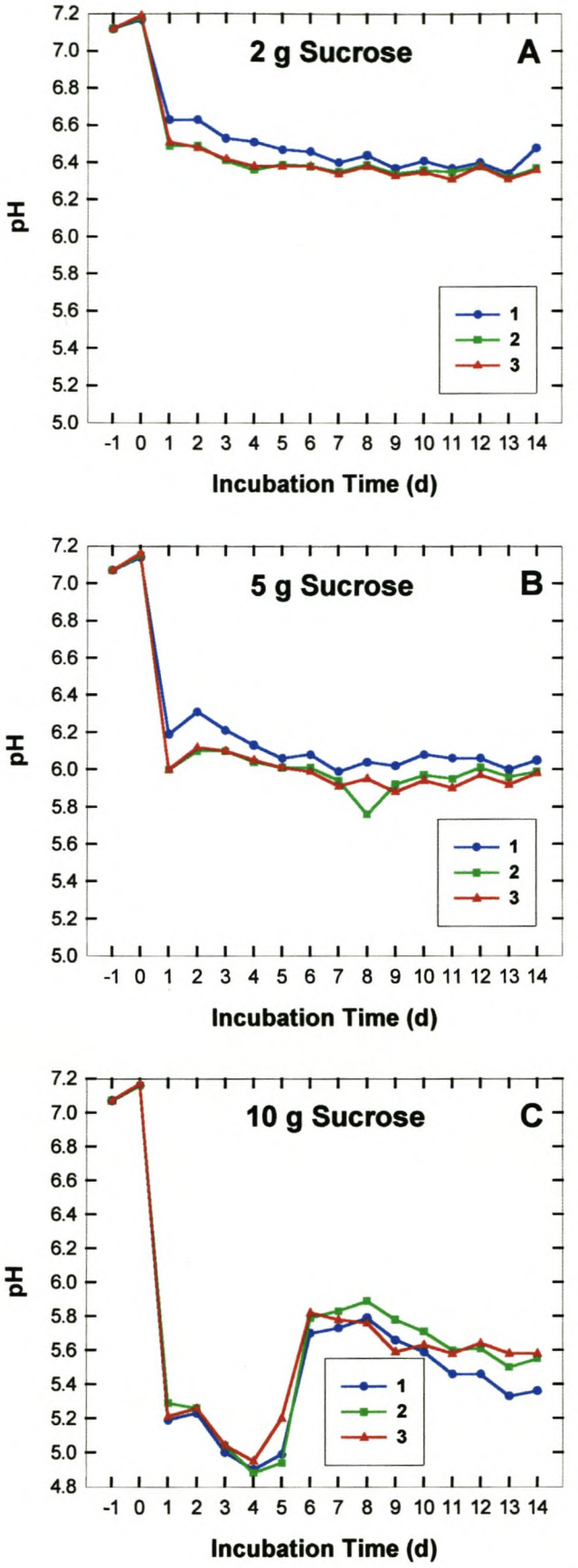


Figure 3. pH profiles obtained for different concentrations of sucrose, as major carbon source in the basic growth medium, during the batch cultivation of granules (studies were done in triplicate).

incubation period. Day "-1", on the 'Incubation Time' axis represents the pH value of only the sterilised growth medium. Day "0" represents the pH value after the sludge had been added to the batch system.

It was found that the pH of each carbon source unit dropped drastically during the first day of incubation at 35°C. This was expected due to the high volatile fatty acid (VFA) formation from the carbon sources in spite of the high buffering capacity (10 $g.l^{-1}$ KH_2PO_4) (Roos, 1998). After the initial 24 - 48 h period, the pH slightly increased for the different lactate concentrations (Fig. 1), as well as for the 5 g glucose (Fig. 2B) and the 2 and 5 g sucrose (Fig. 3A and 3B) units.

The 2 and 5 g glucose units stabilised at pH values between 6.00 and 6.60 (Fig. 2A and 2B). As for the 2 and 5 g sucrose units, the pH slowly dropped to between 5.90 and 6.55 (Fig. 3A and 3B). The 2 $g.l^{-1}$ glucose and 2 $g.l^{-1}$ sucrose units (Fig. 2A and 3A) showed stabilisation at a higher pH when compared with the units where either 5 $g.l^{-1}$ glucose (Fig. 2B) or 5 $g.l^{-1}$ sucrose (Fig. 3B) had been added. Less organic acids were probably produced at the lower concentrations, resulting in the pH stabilising at a higher level.

For the units where either 10 $g.l^{-1}$ glucose (Fig. 2C) or 10 $g.l^{-1}$ sucrose (Fig. 3C) had been added, the pH dropped to 5.00 or lower. A recovery in pH was only observed after 4 - 5 and 4 - 6 days, respectively for the 10 $g.l^{-1}$ glucose and 10 $g.l^{-1}$ sucrose units. The pH of these units (Fig. 2C and 3C) showed a short period of stabilisation after which they acidified, and the whole system failed. Britz *et al.* (1999) also reported that glucose and sucrose, at a concentration of 9 $g.l^{-1}$, led to total acidification of the units. In anaerobic bioreactors it is well known that the acidogens and the methanogens are pH sensitive with the optimum pH range around 6.50 to 7.30 (Zeikus, 1977; Forday & Greenfield, 1983). Both groups will possibly have been inactivated at a pH below 5.00 and it was, therefore, assumed that neither the acidogens nor the methanogens were directly responsible for the recovery of the pH in the units with the higher glucose and sucrose concentrations.

The data obtained in this phase of the study on pH changes with different carbon sources confirms the hypothesis and data of Britz *et al.* (1999) that a sudden increase in readily degradable substrate leads to a rapid pH drop, and subsequent pH recovery and stabilisation. This, in turn, leads to a shift in population dynamics which probably gives the acid tolerant species a competitive advantage for a while, which could then lead to the formation of ECP as an

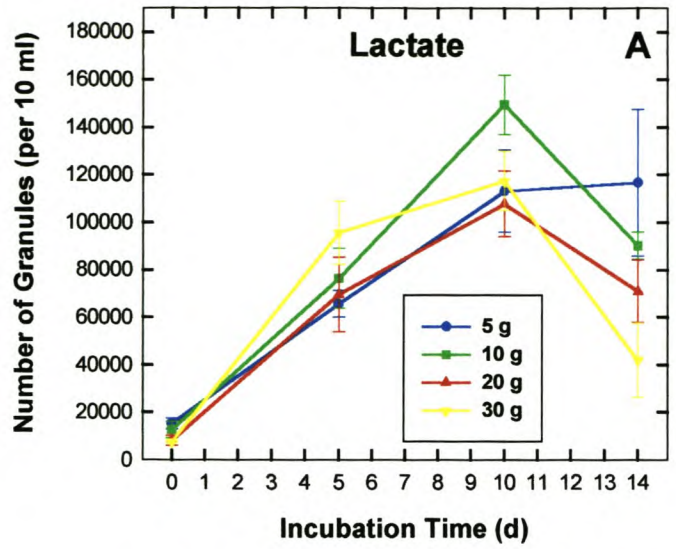
alternative hydrogen sink mechanism. The ECP may probably contribute to bacterial aggregation and the eventual formation of granules (Forster, 1991; Slobodkin & Verstraete, 1993).

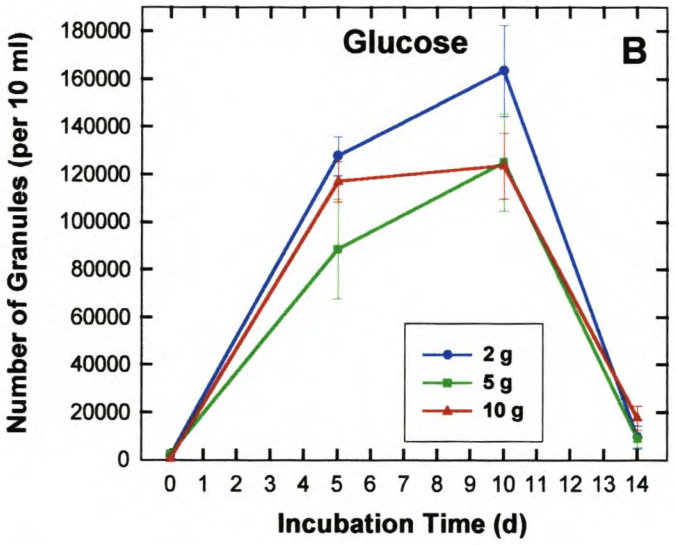
Granule formation with different carbon sources

The process of granule formation, as hypothesised by Britz *et al.* (1999), is based on the observation that, after a sudden increase in the organic loading of readily degradable substrates, an accumulation of organic acids leads to a subsequent drop in pH. This tendency was also observed in the pH studies in the first phase of this study. An orderly shift in population dynamics, due to the drop in pH, takes place in the anaerobic microbial consortium, with the more acid-tolerant propionic acid producing bacteria gaining a competitive advantage (Riedel & Britz, 1993). Subsequently, an increase in more reduced metabolites, like propionic acid, are found and this may also lead to the formation of excess ECP. The ECP can act as an alternative hydrogen sink mechanism, and the system may then display clumping characteristics (Veiga *et al.*, 1997; Britz *et al.*, 2000).

The granule count data (Fig. 4A, 4B and 4C) from this study corresponds to the hypothesis of Britz *et al.* (2000). In this study it was found that over the first 5 - 10 days, the number of granules increased for all three carbon sources (lactate, glucose and sucrose, at the different concentrations) (Fig. 4A, 4B and 4C). However, a decrease in the number of granules was observed in all cases by day 14, as can be seen in Fig. 4A, 4B and 4C. The clumping phenomenon probably led to the aggregation of essential bacteria to form bigger nuclei and, thereafter, a drop in granule numbers was observed. The nuclei will then continue to grow in size as bacteria proliferate and aggregate (Fang, 1997) resulting in larger, but lower numbers of granules. The same tendency was reported by Britz *et al.* (1999).

The data obtained when different concentrations of carbon sources were used all showed the characteristic increase in granule counts during the first 10 days. However, it was found that a concentration of 2 $g.l^{-1}$ glucose as main carbon source (Fig. 4B), resulted in the highest increase in granule numbers by day 10, followed by 10 $g.l^{-1}$ lactate (Fig. 4A) and 2 and 5 $g.l^{-1}$ sucrose (Fig. 4C). During this study, a large variation in granule count increase was observed between the lactate units and the units where either glucose or sucrose was added. This was





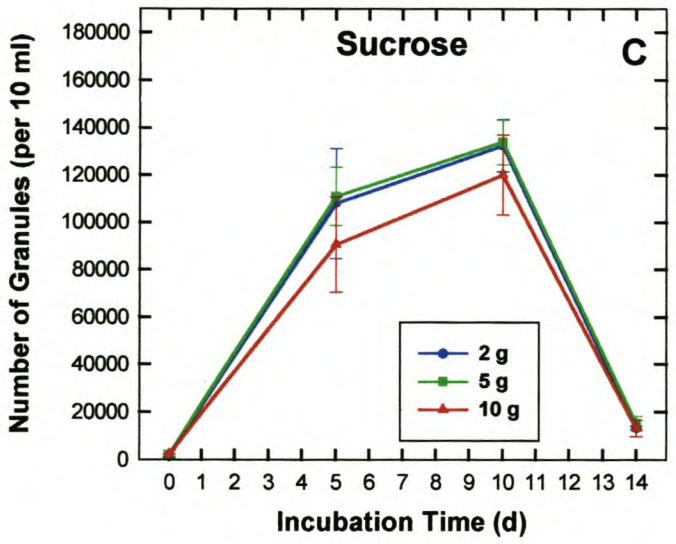


Figure 4. Granule counts from batch studies using various carbon sources at different concentrations (2, 5 and 10 g) in the basic growth medium, during the batch cultivation of granules (studies were done in triplicate). The standard deviation was used as the error-bar length.

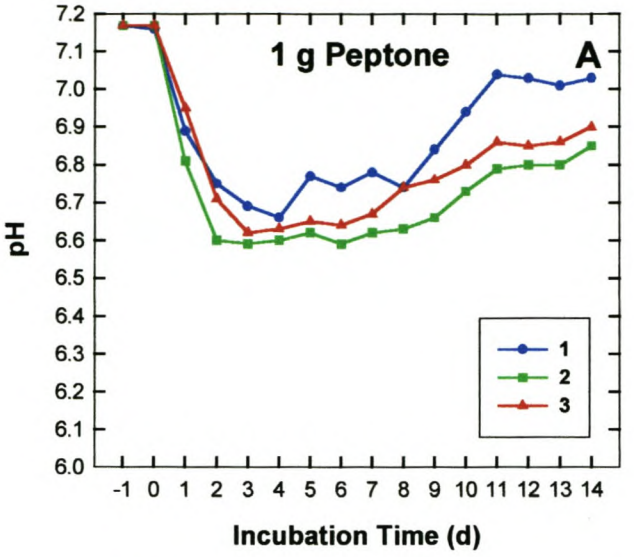
ascribed to the fact that a different sludge batch had to be used for the different units and it was found that the characteristics of the sludge differed with every batch, making it very difficult to compare different batches and to couple a kinetic value to granule increases.

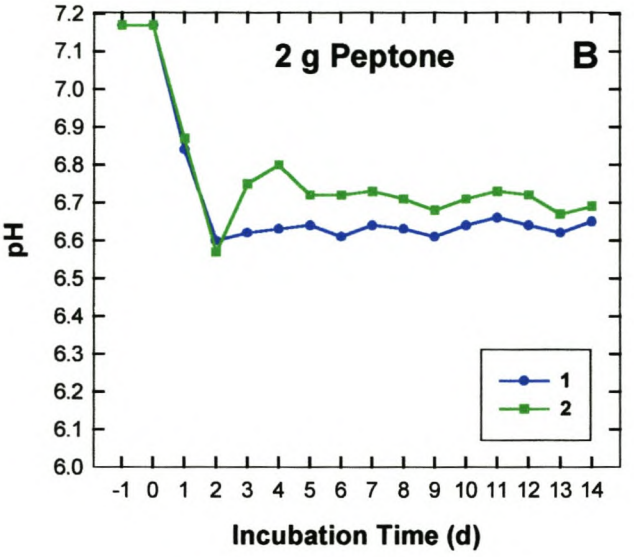
pH profiles during the batch granule culturing using different nitrogen sources

Sam-Soon *et al.* (1990) reported that a suitable nitrogen source is a prerequisite for granule formation. They recommended nitrogen in the free and saline ammonia (NH_3-N) form, and stated that the concentration must be well in excess of metabolite requirements of the anaerobic organisms.

The data obtained when different nitrogen sources (peptone, urea and ammonium sulphate) were evaluated in terms of granule formation are illustrated in Fig. 5, 6 and 7. The changes in the pH, when using different nitrogen sources were not as distinctive as the pH changes when the three different carbon sources, with 2 g peptone in the growth medium, were evaluated. Where 1 g (Fig. 5A) and 5 g peptone (Fig. 5C) had been added to the basic medium (Table 1 and 3), the pH showed a similar fast drop, to 6.70 and 6.55 respectively, but no subsequent stabilisation. In fact, after an extended incubation time for the 5 g peptone batch system, it was found that the system totally acidified. According to Forday & Greenfield (1983), ammonium (NH_4^+) is in equilibrium with ammonia (NH_3) and hydrogen (H^+) and this form an important buffering system in anaerobic digestion. When higher concentrations of peptone (5 g) were added to the units, the units were most probably also overloaded with amino acids since peptone also contains other organic nitrogen compounds. The high organic nitrogen loading would possibly have stimulated the growth of the acidogenic bacteria in the system and thus more VFA's could have been produced. The higher H^+ concentration would, in turn, lead to a drop in pH, causing the system to acidify. The pH-profile for the system with 2 g peptone (Fig. 5B) also showed the characteristic decrease in pH during the first two days, whereafter it increased slightly and then stabilised at between pH 6.60 - 6.70. The set-up for this system is similar to that used for the 20 g lactate system in the previous section (Fig. 1C).

In the units where urea was used to replace the nitrogen source of the basic growth medium (Fig. 6), it was evident that a concentration of 1 g did not lead to the stabilisation of the unit (Fig. 6A), but the pH gradually decreased until day 14.





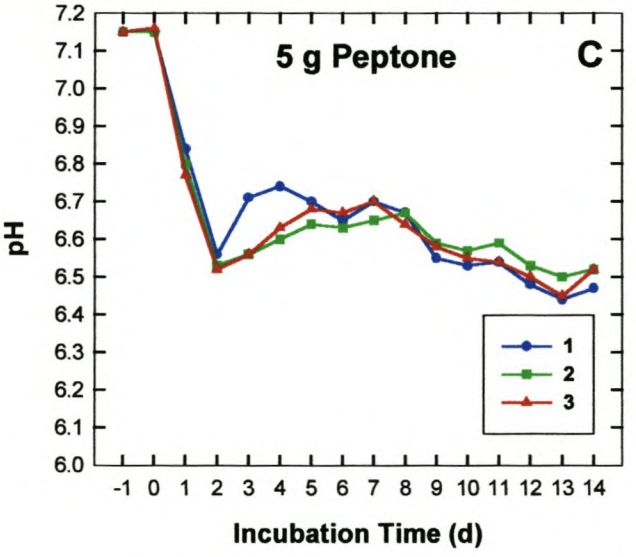


Figure 5. pH profiles obtained for different concentrations of peptone, as major nitrogen source in the basic growth medium, during the batch cultivation of granules (studies were done in triplicate).

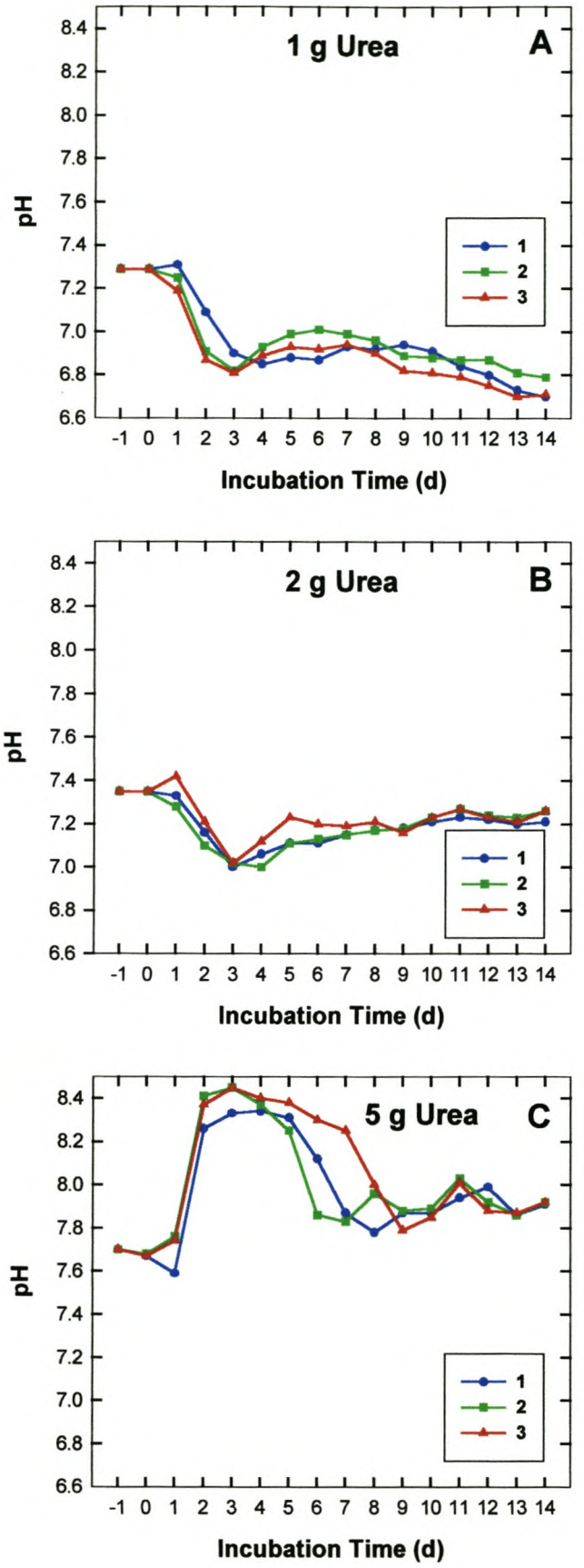


Figure 6. pH profiles obtained for different concentrations of urea, as major nitrogen source in the basic growth medium, during the batch cultivation of granules (studies were done in triplicate).

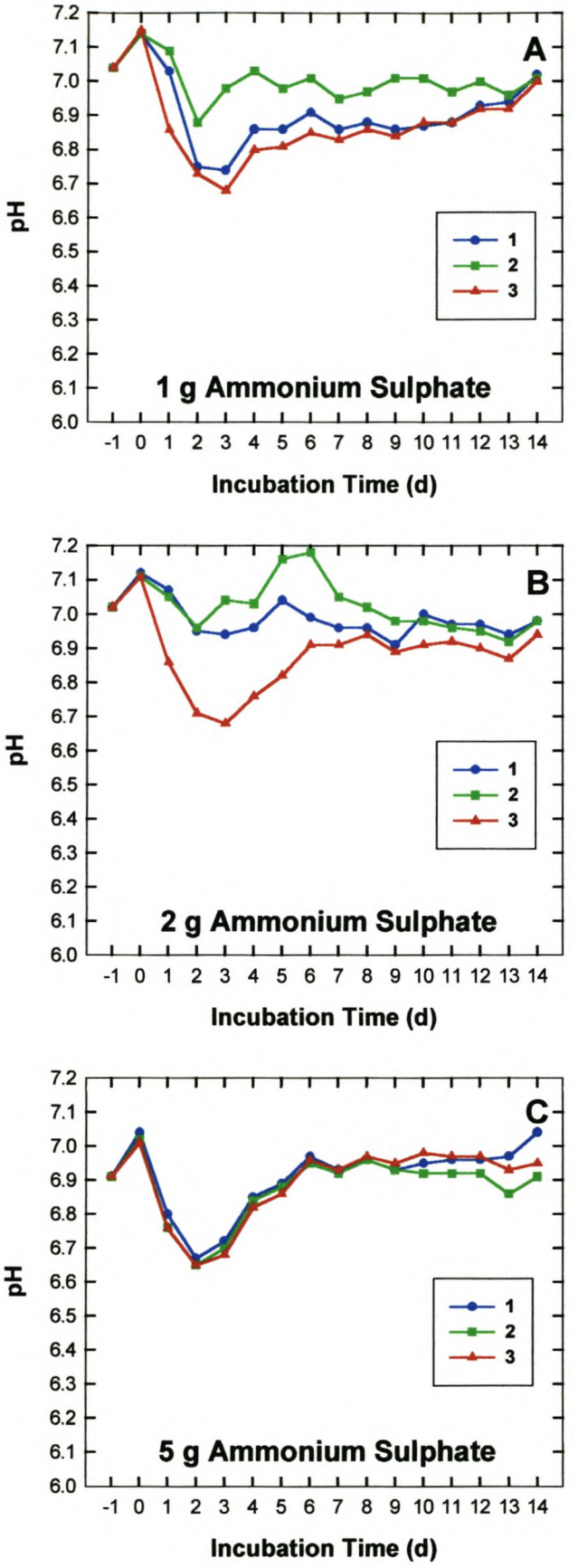


Figure 7. pH profiles obtained for different concentrations of ammonium sulphate, as major nitrogen source in the basic growth medium, during the batch cultivation of granules (studies were done in triplicate).

With a concentration of 2 $g.l^{-1}$ urea, the pH of the system showed a slight decrease in pH, with recovery after 5 days (Fig. 6B) and pH stabilisation in the range of 7.0 to 7.3. In the case of 5 g urea as nitrogen source, a very sharp increase in pH was observed after 48 h (Fig. 6C). This can possibly be explained by the fact that in the presence of water, urea is broken down to CO_2 and NH_4^+ ($NH_3 + H^+$) (Zubay, 1993) and that the higher concentrations of ammonia formed would lead to the increase of the pH of this system. With the production of VFA's from the carbon source, the pH then subsequently started to decrease (Britz *et al.*, 1999). This unit only showed a stabilisation tendency of between pH 7.75 and 8.05 after day 8.

The data in Fig. 7 illustrates the pH changes in the batch system where the nitrogen source of the basic medium had been replaced with ammonium sulphate. In all three cases the pH showed an increase from day -1 to day 0, whereafter the expected drop in pH occurred, followed by a pH increase and stabilisation of the units in the range of 6.90 - 7.00. There was some variation between the triplicate samples for the units with 1 and 2 g of added ammonium sulphate (Fig. 7A and 7B), however, the profiles of the triplicates with the 5 g ammonium sulphate (Fig. 7C) were found to be very similar. Although the addition of ammonium sulphate showed a final pH stabilisation at all the given concentrations (Table 3), it must be noted that a foul-smelling H_2S-like odour was observed each time the 100 ml growth medium was replaced. The production of odoriferous H_2S-gas is undesirable, and will, therefore, negatively influence the use of ammonium sulphate as the nitrogen source for batch granule production.

Granule formation with different nitrogen sources

A different batch of sludge, obtained from the same local sewage works, was used for the studies done with the different nitrogen sources. The pH profiles (Fig. 5, 6 and 7) of the units with the different nitrogen sources showed that the pH stabilised in a range closer to 7.0 in comparison to the lower final pH observed in the units where the carbon source was varied.

All the units where urea (Fig. 8B) was used as sole nitrogen source resulted, on average, in the highest granule count increases after 10 days, with little variation between the three different concentrations. However, the error-bars show that there was variation between the triplicates of each concentration. The data also showed that urea, at a concentration of 5 $g.l^{-1}$, gave the highest increase

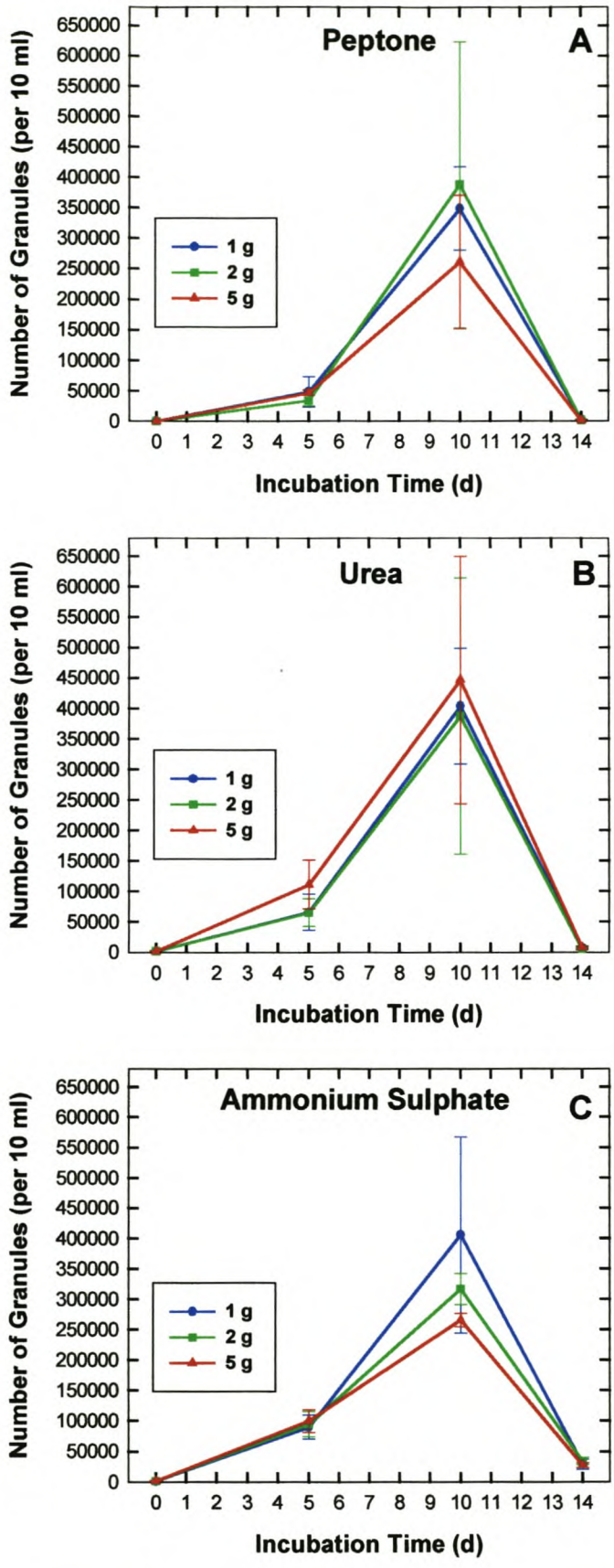


Figure 8. Granule counts from batch studies using various nitrogen sources at different concentrations (1, 2 and 5 g) in the basic growth medium, during the batch cultivation of granules (studies were done in triplicate). The standard deviation was used as the error-bar length.

in granule counts by day 10.

Peptone (Fig. 8A), at the lower concentrations (1 and 2 $g.l^{-1}$) showed little difference in granule counts after 10 days. As for ammonium sulphate (Fig. 8C), a concentration of 1 $g.l^{-1}$ resulted in the highest granule count increase over the first 10 days.

The growth medium used for the 20 $g.l^{-1}$ lactate units (Fig. 4A) in the study where different carbon sources were evaluated, and the 2 $g.l^{-1}$ peptone (Fig. 8A) batch units evaluated in this study, are of the same composition. It was, therefore, expected that both the pH profiles and the granule counts would be similar. Even though the pH profiles were very similar (Fig. 1C and 5A), there was a distinct difference between the granule numbers obtained in the two units. The only variable was the raw anaerobic sludge obtained from Athlone Wastewater Treatment Works. The very high granule counts recorded with the different nitrogen sources can, therefore, be attributed to the new batch of sludge used in this section of the study.

pH profiles during the batch granule culturing using different combinations of carbon sources

Data from the first phase of this study showed that the use of lactate in batch granule production leads to a stable system. The high cost of lactate initiated this search for other, equally effective, yet cheaper, carbon sources. If lactate could be used in combination with a cheaper carbon source, it could lead to a more economically viable choice.

The data from the first phase of this study showed that a carbon concentration of either 10 $g.l^{-1}$ lactate or 2 $g.l^{-1}$ glucose in the basic growth medium resulted in the best granule enhancement by day 10. These two concentrations were again evaluated during the second phase of the study. In addition, different combinations of these two carbon sources (10 g lactate + 2 g glucose; 5 g lactate + 1 g glucose; 5 g lactate + 2 g glucose and 5 g lactate + 5 g glucose) (Table 4) were also evaluated. When lactate is used in combination with glucose, less lactate will be required due to the energy provided by the additional glucose. This should lower the final costs of the carbon source, since glucose is much cheaper than lactate (C. Gribble, 2000, Merck, personal communication). The use of fruit cocktail effluent (FC) as major carbon source, and in combination with either

lactate or glucose, was also examined. The fruit cocktail effluent can be collected free of charge from a local canning factory, and meets all the requirements for a cheap and easily fermentable carbon source.

The data obtained when different combinations of carbon sources were evaluated are illustrated in Fig. 9 and 10. Once again, the characteristic drop in pH during the first 24 h period was observed, whereafter the pH stabilised. The pH range where stabilisation was reached for both the 10 g lactate unit (Fig. 9A) and the 2 g glucose unit (Fig. 9B) are similar to the results obtained in the first phase of this study (Fig. 1B and 2A). The stabilisation pH was in the range of 6.65 and 6.40 respectively, and was reached after 48 h of incubation. The pH profiles for 10 g lactate + 2 g glucose (Fig. 9C); 5 g lactate + 1 g glucose (Fig. 9D); and 5 g lactate + 2 g glucose (Fig. 9E) were similar. However, the profile for the 5 g lactate + 1 g glucose units was the most reproducible of the three combinations. With the exception of the 5 g lactate + 5 g glucose combination (Fig. 9F), all the other combinations stabilised at a pH in the range of 6.50 after 48 h (Fig. 9C, 9D and 9E). The high concentration of fermentable carbons of the 5 g lactate + 5 g glucose unit caused the pH to drop to below 6.00 (days 2 - 5), whereafter the pH recovered to above 6.30. Even though the pH did not drop that low, it might, however, have led to some inhibition of the methanogens and even of the acidogens.

Fruit cocktail effluent (FC) is a waste product produced by the fruit canning industry (Trnovec, 1998). Except for transportation costs during the collection of the effluent, this easily fermentable carbon source can be collected free of charge during the canning season. The economics involved in the batch cultivation process was, therefore, the reason for investigating the usefulness of this carbon source. The pH profile for the units with only FC showed stabilisation after 3 days. At a concentration of 3 $g.l^{-1}$ COD FC, the pH stabilised in the range of 6.20 - 6.30 (Fig. 10A), whereas stabilisation was reached in the range of 6.30 - 6.40 for the 2 $g.l^{-1}$ COD FC unit (Fig. 10B). This was slightly higher than the findings reported by Britz *et al.* (1999), who also did studies with fruit canning effluent (2 $g.l^{-1}$ COD) as major carbon source for batch granule production. They reported a stable system, with the pH in the range of 6.00 - 6.20. The difference in the final stabilised pH of the two studies can possibly be explained in terms of the seasonal variation of the FC effluent composition.

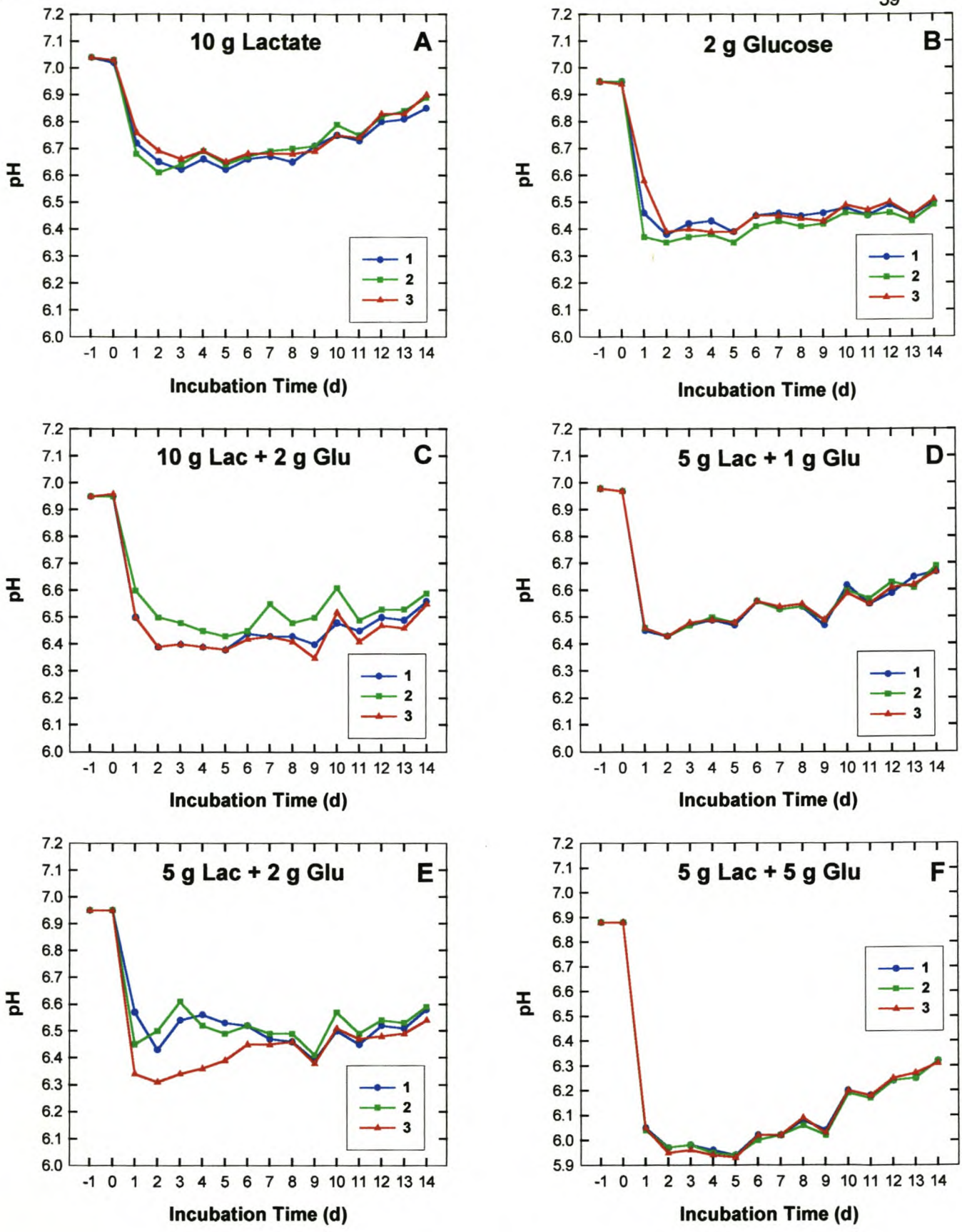


Figure 9. pH profiles obtained for different combinations of lactate (Lac) and glucose (Glu), as major carbon sources in the basic growth medium, during the batch cultivation of granules (studies were done in triplicate).

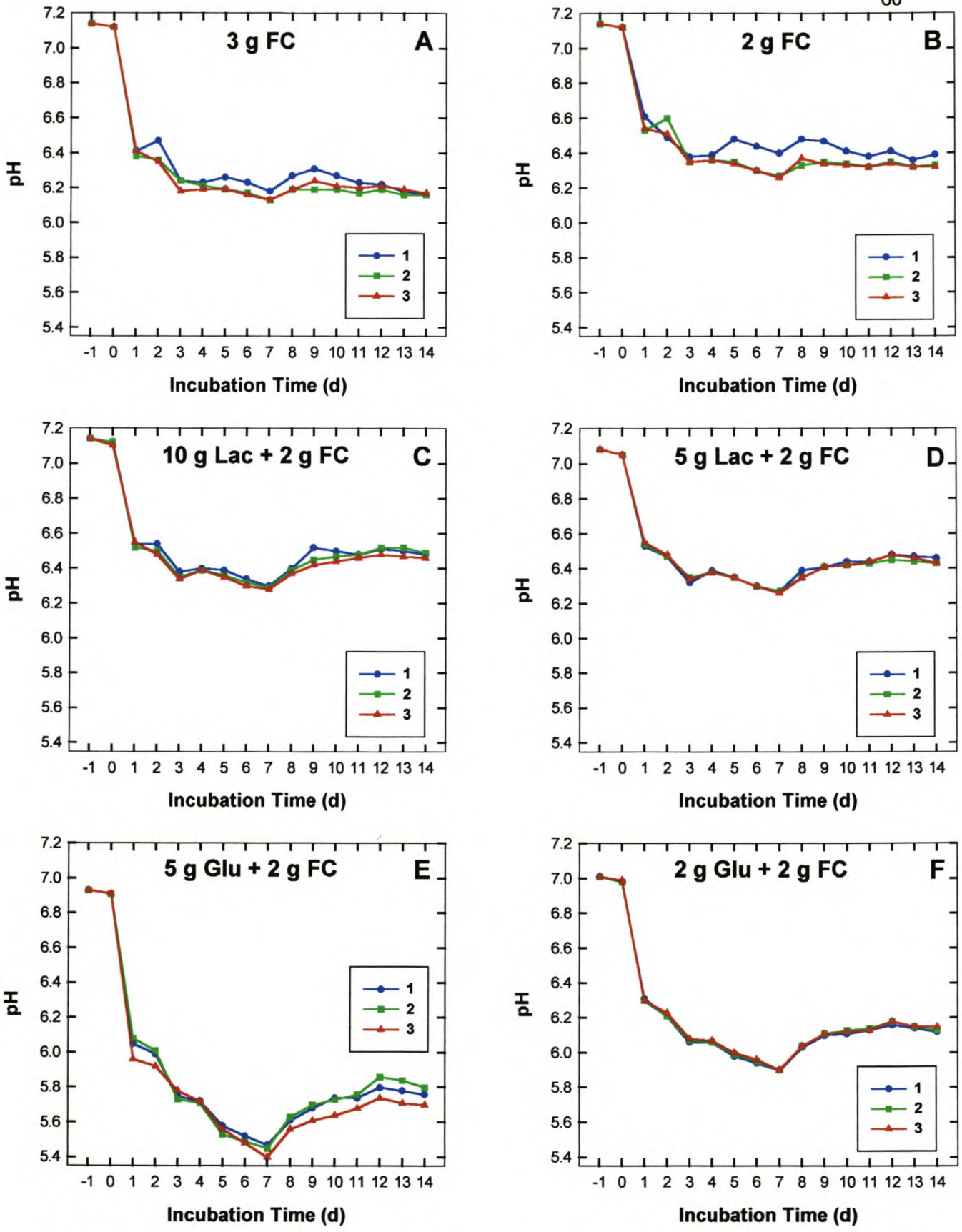


Figure 10. pH profiles obtained for different combinations of lactate (Lac), glucose (Glu) and fruit cocktail effluent (FC), as major carbon sources in the basic growth medium, during the batch cultivation of granules (studies were done in triplicate).

Both combinations of lactate and fruit cocktail effluent (10 g lactate + 2 $g.l^{-1}$ COD FC and 5 g lactate + 2 $g.l^{-1}$ COD FC) were found to stabilise in a pH range of 6.40 - 6.50 (Fig. 10C and 10D). As was the case with lactate as major carbon source, it was concluded that any concentration of lactate, in combination with 2 $g.l^{-1}$ COD FC, would lead to a stable system.

The pH of the unit with 5 g glucose + 2 $g.l^{-1}$ COD FC (Fig. 10E) was found to drop to 5.40 by day 7, whereafter it showed a slight recovery in pH, but did not stabilise, and the pH started to drop again after day 12. The combination of 2 g glucose + 2 $g.l^{-1}$ COD FC stabilised in a pH range of 6.10 and 6.20, but only after 9 days of incubation. A concentration of 2 g glucose, in combination with FC at a COD concentration of 2 $g.l^{-1}$ would be more economical to use, but the large drop in pH of this system might, however, lead to some inhibition of the methanogens and acidogens.

When lactate is used in combination with fruit cocktail effluent, the system was stable for the entire incubation period (Fig. 10C and 10D). In contrast, glucose, especially at the higher concentrations (Fig. 10E), resulted in acidification of the unit. It is, therefore, clear that when glucose is used in combination with another carbon source, it will result in an unstable unit. This was true even when 5 $g.l^{-1}$ glucose was used in combination with either 5 g lactate or 2 $g.l^{-1}$ COD FC (Fig. 9F and 10E).

Granule formation with different combinations of carbon sources

The raw anaerobic sludge used for this phase of this study was also a new batch obtained from the Athlone Wastewater Treatment Works. The sludge used for the lactate and glucose-combinations differed from the sludge used when FC was combined with another carbon source and this variation in sludge makes granule enhancement comparisons very difficult. The 10 g lactate units (Fig. 11A) as well as the 2 g glucose units (Fig. 11B) gave higher granule counts after 10 days when compared to the units of the first phase of this study (Fig. 4A and 4B). The reason for this was probably the different sludge batches used during the different phases. Standardisation of the sludge inoculum proved to be the problem.

Both the 10 g lactate + 2 g glucose units (Fig. 11C) and the 5 g lactate + 1 g glucose units (Fig. 11D) gave similar increases in granule numbers. If these are

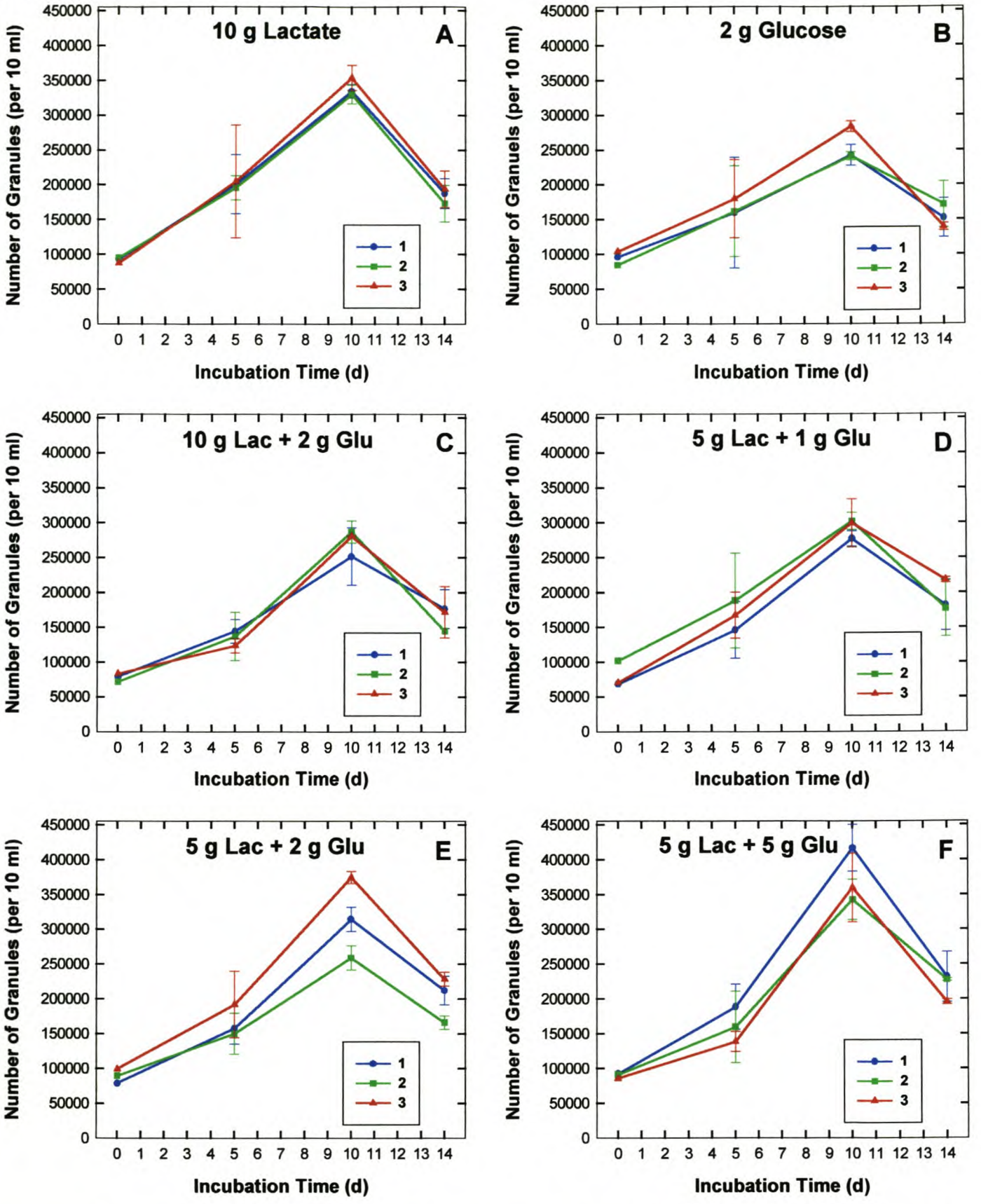


Figure 11. Granule counts from batch studies using different combinations of lactate (Lac) and glucose (Glu) as major carbon sources in the basic growth medium, during the batch cultivation of granules (studies were done in triplicate). The standard deviation was used as the error-bar length.

compared with the pH profiles (Fig. 9C and 9D), a combination of 5 g lactate + 1 g glucose (Fig. 11D) would be preferred as it is clear from the triplicate results of pH profiles that this combination is more reproducible. The granule count results for the 5 g lactate + 2 g glucose units (Fig. 11E) showed an increase in granule counts, however, some variation occurred between the triplicate units. Even though a combination of 5 g lactate + 5 g glucose (Fig. 11F) gave the highest granule counts at day 10, the pH of this unit dropped to below the survival range for the methanogens and propionic acid producing bacteria (Zeikus, 1977). The pH of this unit (Fig. 11F) also showed no stabilisation.

The data obtained with the different combinations of FC with either lactate or glucose are illustrated in Fig. 12. The use of FC as only carbon source was not as successful as in combination with either lactate or glucose, in terms of granule counts. A higher concentration of 3 $g.l^{-1}$ COD FC resulted in higher granule counts after 10 days of incubation (Fig. 12A and 12B) when compared with the 2 $g.l^{-1}$ COD FC. Where lactate was used in combination with FC, the results of the 10 g lactate + 2 $g.l^{-1}$ COD FC (Fig. 12C) and the 5 g lactate + 2 $g.l^{-1}$ COD FC (Fig. 12D) were very similar. In contrast to the units where 5 g glucose had been added to the 2 $g.l^{-1}$ COD FC (Fig. 12E), the granule counts were disappointingly low. However, low counts were always found with the units that ended in acidification (Fig. 10E).

The best carbon source to use in combination with FC proved to be glucose at a concentration of 2 $g.l^{-1}$ (Fig. 12F). It must be noted that the pH drop of this combination might be slightly too low to prevent the loss or inhibition of at least some of the very important methanogens and acidogens.

Chang *et al.* (1995) reported that granules could not be formed at an acidic pH of 6.0 and concluded that a pH of 7.0 may still be too low for granule formation. In this study, however, it was found that granule formation at pH levels well below 7.0 was still achievable. A higher buffer concentration may possibly be enough to prevent the pH from dropping too low. A combination of 2 g glucose + 2 $g.l^{-1}$ COD FC gave the highest granule counts after 10 days of incubation at 35°C for the carbon combination. From an economic point of view, the lower glucose concentration in combination with the FC, which can be collected free of charge, is also acceptable.

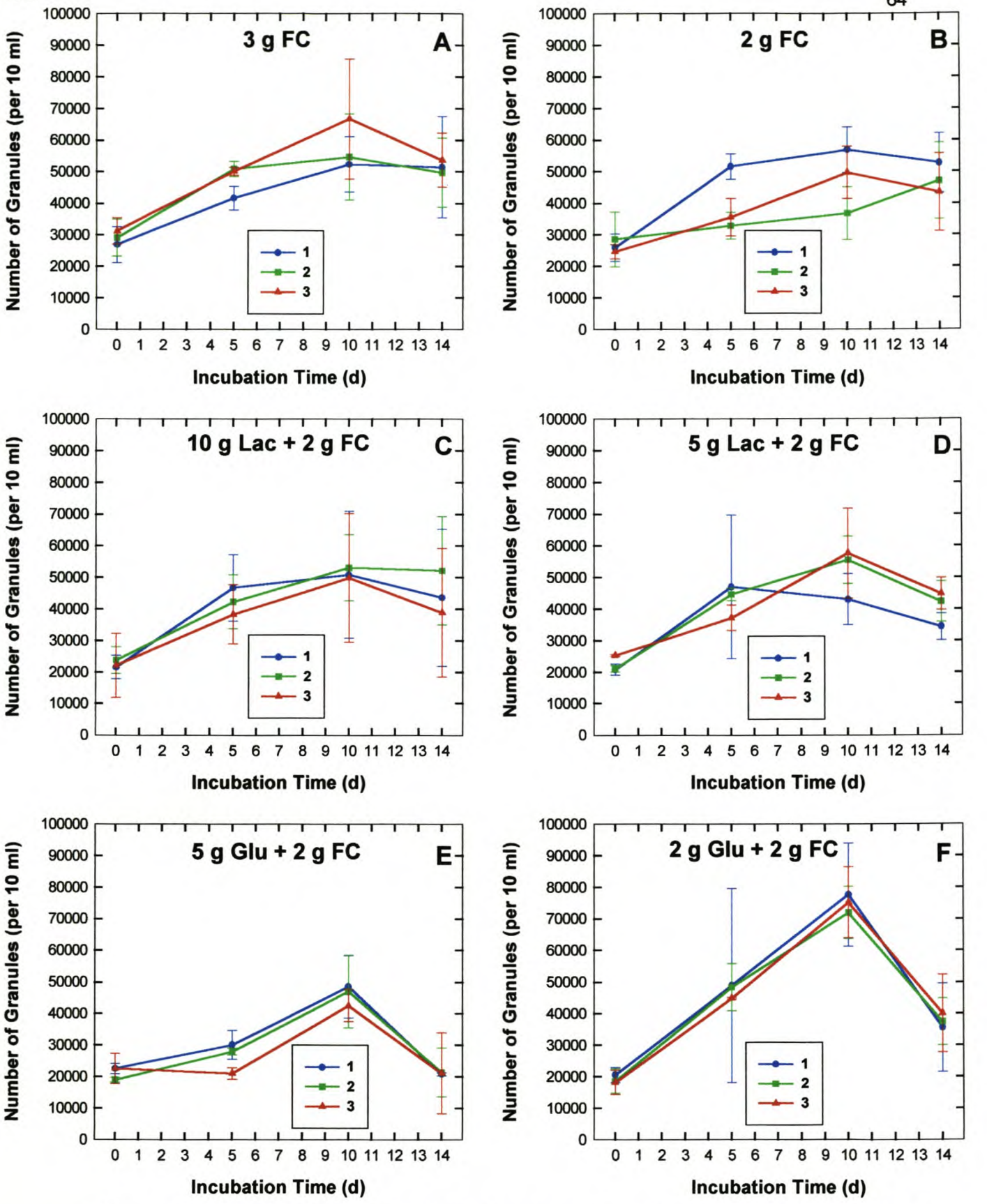


Figure 12. Granule counts from batch studies using different combinations of lactate (Lac), glucose (Glu) and fruit cocktail effluent (FC) as major carbon sources in the basic growth medium, during the batch cultivation of granules (studies were done in triplicate). The standard deviation was used as the error-bar length.

Granule enhancement with standardised raw sludge inoculum

A major problem encountered in the previous sections of this study was the large variation in the raw sludge characteristics making a direct comparison between the granule enhancements obtained with the different carbon and nitrogen sources extremely difficult. These characteristics are given in Table 5. From the data it is thus clear that there were variations between the different sludge batches, and this was the major cause of the varying granule counts at day 0 obtained for the different phases of this study. It was concluded in the previous sections of this study that the varying nitrogen sources play a minor role in batch granule enhancement. It was, therefore, decided that the influence of only the best carbon source, and carbon source combinations in terms of granule enhancement, must be evaluated in an additional section where the raw sludge used as inoculum will be standardised.

As part of the standardisation, a sieve (Endecotts Ltd) with a 1 mm pore size was used to remove non-degradable material. The 'uniform' sludge was then centrifuged at a speed of 1500 X g for 20 min and the supernatant removed. The sludge, at a TS concentration of 0.159 g.10 g^{-1}, was used as inoculum for the standardised section of this study.

Fig. 13A and 13C represent the pH profiles obtained with the standardised sludge inoculum and the different carbon sources (10 g lactate; 2 g glucose; 5 g lactate + 2 g glucose; 5 g lactate + 2 $g.l^{-1}$ COD FC and 2 g glucose + 2 $g.l^{-1}$ COD FC). All the units showed the characteristic pH drop with the subsequent recovery. The units that included lactate as carbon source were again found to stabilise at a higher pH than the system with glucose. The 10 $g.l^{-1}$ lactate (Fig. 13A) unit stabilised at a pH of 6.8, the 5 g lactate + 2 g glucose combination (Fig. 13C) stabilised between 6.6 and 6.7, and the units with 5 g lactate + 2 $g.l^{-1}$ COD FC (Fig. 13C), stabilised at a pH of 6.8. The 2 $g.l^{-1}$ glucose unit stabilised at a pH of 6.5, whereas stabilisation was reached at a pH of 6.3 for the 2 g glucose + 2 $g.l^{-1}$ COD FC combinations. The results compare well with the results obtained in the first phase of this study (Fig. 11A, 11B, 11C, 12D and 12E) where unstandardised sludge was used as inoculum.

The unit with 2 $g.l^{-1}$ glucose (Fig. 13B) resulted in the highest increase in granule counts by day 10. A carbon source concentration of 10 g lactate (Fig. 13B) resulted in just a slightly lower increase in granule counts. A combination of

Table 5. The characteristics of the different batches of raw sludge used as inoculum in the different experimental studies.

Study	Figure reference	pH	TS (g.10 g^{-1})	VS (g.10 g^{-1})	TNVS (g.10 g^{-1})
Lactate	4A	7.20	0.069	0.178	0.124
Glucose; Sucrose	4B; 4C	7.09	0.058	0.196	0.066
Peptone; Urea	8A; 8B	7.30	0.031	0.023	0.008
Ammonium sulphate	8C	7.09	0.064	0.044	0.020
Lactate and Glucose	11	6.95	0.075	0.057	0.018
Lactate/Glucose and FC	12	7.05	0.075	0.057	0.018
Standardised inoculum	13	6.93	0.159	0.133	0.026

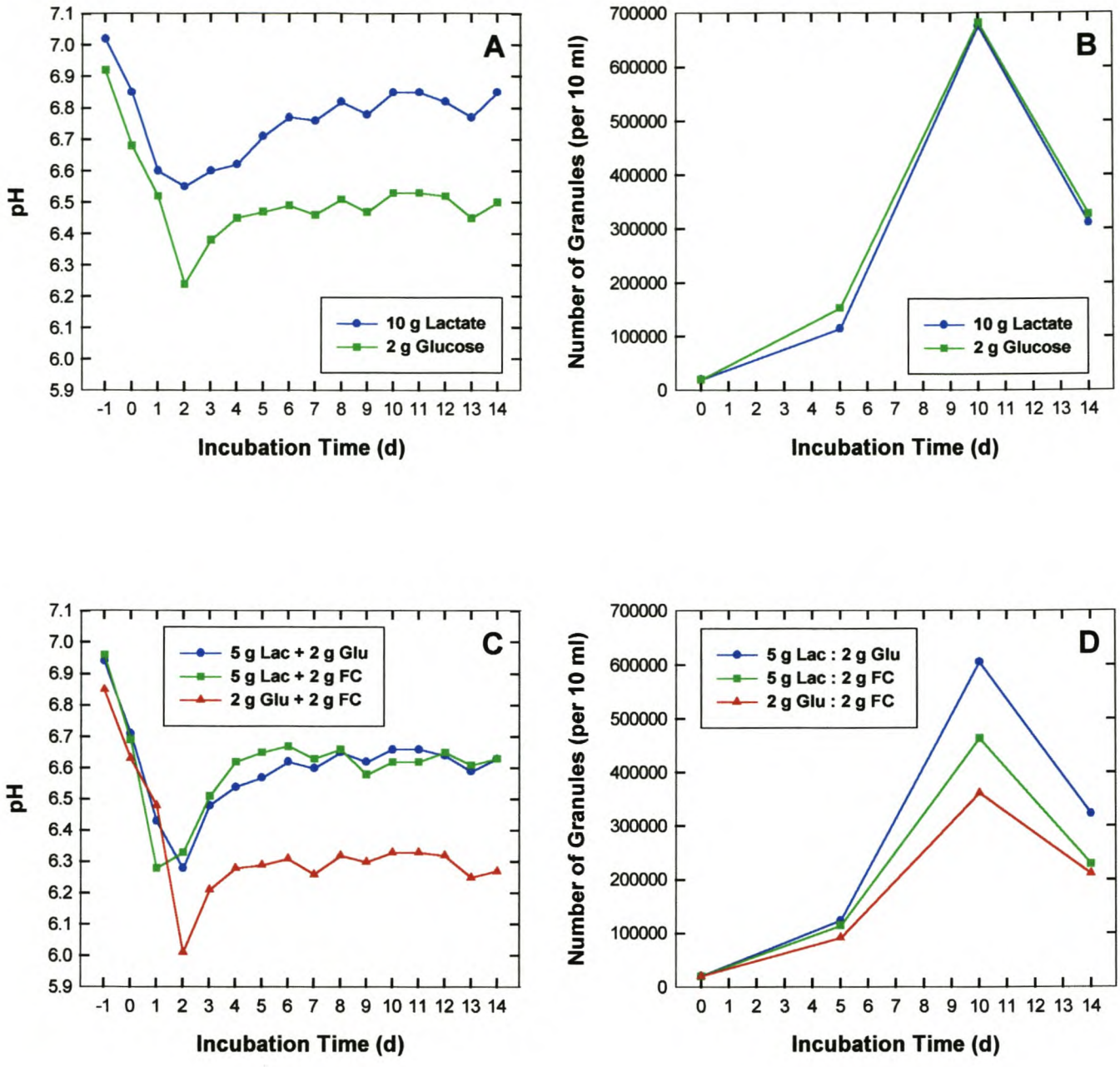


Figure. 13 pH profiles and granule counts obtained with different carbon sources using the same standardised sludge inoculum. The profiles indicate the average of duplicates with a variation of < 2%. (Lac = Lactate; Glu = Glucose; FC = Fruit cocktail effluent).

5 g lactate + 2 g glucose (Fig. 13D) was found to give the best carbon combination, followed by 5 g lactate + 2 $g.l^{-1}$ COD FC (Fig. 13D) and lastly, 2 g glucose + 2 $g.l^{-1}$ COD FC (Fig. 13D). As mentioned in the previous section, lactate, as a carbon source, is much more expensive than glucose. It is, therefore, advisable, in economical terms, to use 2 $g.l^{-1}$ glucose for the batch cultivation of granules. If the lower pH is found to be a problem, a low concentration of lactate (5 $g.l^{-1}$) can be added to the growth medium so as to stabilise the system.

Efficiency of granule formation using different carbon sources

As the process of batch granule cultivation is scaled up, the economics involved will become more critical. Even though lactate leads to stable units, this carbon source is very expensive, and therefore, other, cheaper carbon sources are needed to ensure the validity of the process. Each sucrose molecule ($C_{12}H_{22}O_{11}$) contains 12 carbons (C), glucose ($C_6H_{12}O_6$) has 6 C and lactate ($C_3H_5O_3Na$) only 3 C per molecule (Zubay, 1993). It is thus clear that for the same concentration (in $g.l^{-1}$), sucrose has four times more C than an equivalent concentration of lactate, and glucose has twice the amount of C per gram compared to lactate. It can, therefore, be assumed that high concentrations of sucrose and glucose would lead to the acidification of a batch culturing system due to the abundance of easily degradable carbon sources in the growth medium. It can be argued that the higher C concentration of glucose and sucrose would result in lower concentrations required to obtain the same impact on granule enhancement. In terms of the economics of setting up a batch cultivation system, glucose would probably be the cheapest carbon source.

In the case of the economics of using glucose, 1 g of C amounts to 17c, sucrose 19c per 1 g C, while lactate, very expensively, amounts to 138c per 1 g C (prices as quoted by Merck in South African currency, October 2000). In contrast, fruit cocktail effluent can be obtained free of charge during the canning season. Transportation costs would, therefore, be the only expense involved in obtaining the effluent. Fruit cocktail effluent, as major carbon source, or in combination with a low concentration of glucose will, therefore, be the cheapest carbon source available for the batch production of UASB-granules. Furthermore, by using fruit cocktail effluent as a carbon source for the production of granules, wastewater

from a cannery is being utilised, thereby creating another option for the 'treatment' of such waste products.

It is evident that a standardised sludge gives more comparable results, making it a necessary precaution for evaluating different granule enhancement parameters. A sludge inoculum with a higher TS should also give a better enhancement as the inoculum contains less water and more biomass.

Conclusions

The method most commonly used for the cultivation of granules is UASB culturing (Lettinga & Hulshoff Pol, 1991) where sludge is used as the sludge blanket and the strong upward movement eventually leads to the formation of granules. This process, even though very efficient, can take up to a year before an efficient granule bed is formed. Mass culturing of granules according to the method used in this study drastically shortens the 'culturing-time'. The advantages of mass culturing are therefore self-explanatory if the time factor is considered. Rapid cultivation of granules will result in shortened start-up times for UASB bioreactors, and will lead to the installation of more reactors.

In this study, it is clear that different growth media does influence the enhancement of granules in a batch system. It was, however, evident that the carbon source had a more profound effect on granule enhancement than changes in the nitrogen source.

The data from this study also showed that 2 $g.l^{-1}$ glucose gave the best granule enhancement, followed by 10 $g.l^{-1}$ lactate, and 2 and 5 $g.l^{-1}$ sucrose. With the exception of lactate, it was found that too high concentrations of fermentable carbons (>5 $g.l^{-1}$) would, in a short time, lead to the acidification of the system. Similarly, it was found that when fruit cocktail effluent was used, care must be taken not to overload the growth medium with a too high concentration of fermentable compounds (>5 $g.l^{-1}$).

The results on granule enhancement obtained in the study with the three different nitrogen sources were not as clear-cut as those obtained with the carbon sources. Urea, at all three concentrations did, however, lead to the best granule enhancement. This was followed by 1 $g.l^{-1}$ ammonium sulphate and 2 $g.l^{-1}$ peptone, which resulted in the third best granule enhancement. A drawback of the

use of ammonium sulphate as major nitrogen source is the production of H_2S-gas. However, if the wastewater to be treated contains large amounts of sulphate, it might be advisable to use ammonium sulphate as the nitrogen source during batch granule cultivation of an inoculum for the USAB. This should give the sulphate-utilising bacteria an advantage of being incorporated within the batch cultivated granules.

A major problem encountered during this study was sludge standardisation. The sludge inoculums used for these studies were obtained from a local sewage works at different times over a period of a year. The sludge used for the different carbon sources differed from the sludge used for the nitrogen sources, which in turn differed form the sludge used for the different combinations of carbon sources. It was, therefore, inevitable that final products of the batch granule enhancement were of varying quality. Sewage sludge is a biological system, and the composition will, therefore, differ from day to day. It is clear that a need exists for a method to standardise the raw anaerobic sludge that is to be used as inoculum for batch granule cultivation. If this problem could be overcome, the comparison between different batch granule cultivating units would be more feasible, and granule number increases would be more meaningful. Some researchers have used volatile solids (VS) and total solids (TS) of the inoculum as a means of standardisation (Ahring & Schmidt, 1992). The problem with these determinations is that the number of granules can not be determined in this way, but rather the solids content of the total biomass of the inoculum. Total and volatile solid determinations at the end of the incubation time still do not give an indication of granule enhancement in terms of numbers of granules. By determining the TS and VS of each unit after the 14 day incubation period, the TS and VS of the total biomass of the unit is being determined, be it granules, some unutilised carbons in the growth medium or even loose microbial cells.

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CHAPTER 4

EVALUATION OF THE IMPACT OF DIFFERENT PRESERVATION TECHNIQUES ON THE STORAGE POTENTIAL AND METHANOGENIC ACTIVITY OF GRANULAR SLUDGE

Abstract

When granules become freely available as seeding inoculum for upflow anaerobic sludge blanket (UASB) bioreactors, a reliable preservation method will be required. The level of activity of the methanogenic population in the granules is especially important when determining the efficiency of the bioreactor, as methane is the main metabolic end-product of an anaerobic digestion system. The methanogens are perhaps the most important group in the microbial consortium found in an UASB. Their availability and activity after preservation are, therefore, of crucial importance for the optimal efficiency of a bioreactor. Thus, different preservation techniques were evaluated in terms of storage potential and the retainment of biogas and methanogenic activity.

In this study six different preservation techniques (room temperature storage; vacuum-drying; freeze-drying; vacuum freeze-drying; freeze storage and cold storage) were evaluated. Activity testing was used for evaluating comparing the efficiency of the different preservation techniques in terms of the tempo of biogas and methane production. The data is given as cumulative gas production values (CGP), and as the gradient over time $[(CGP_{10\ h} - CGP_{5\ h})/(t_{10\ h} - t_{5\ h})]$. Freeze-drying showed the highest activity for the first 90 days of storage, followed by vacuum freeze-drying and vacuum-drying. Room temperature preservation only showed an increase in activity by day 120. There can only be speculated as to the reason for the sudden increase in activity at this time.

It was found that the most activity was shown by 10 h of incubation, and it is, therefore, suggested that when activity testing are performed for evaluation purposes, the incubation time should be reduced to only 10 h. Further suggestions when performing activity tests include that the basic test medium

(BTM) be only enriched with an additional 2 $g.l^{-1}$ glucose, as lactate and acetic acid play no decisive role in determining the level of activity of the granules.

Introduction

The upflow anaerobic sludge blanket (UASB) process has become a popular and reliable technology for the treatment of various waste waters. The success of this process relies heavily on the formation of granules with high settleability and bioactivity abilities (Fang *et al.,* 1995; Yükselen, 1997). Although the granulation process is still not fully understood, the feasibility of granular sludge for treating various waste waters using continuous operations has been successfully demonstrated and several studies on granule morphological and physiochemical characteristics have been reported (Shin *et al.,* 1993; Pereboom, 1994; Yükselen, 1997). Quantitative research on the preservation characteristics of granular sludge in terms of storage temperature and storage period is necessary when the UASB reactor is to be considered for use in industries operating seasonally or intermittently (Shin *et al.,* 1993; Yükselen, 1997).

Optimum storage conditions of methanogenic granules must be established in order to maintain the anaerobic metabolic activity and the stability of the granular sludge as long a possible (Wu *et al.,* 1995). According to Wu *et al.* (1995), the granule configuration of the granular sludge can be maintained for a long time (over 3 years) without feed under suitable environmental conditions. Factors that are known to influence the anaerobic metabolic activity of stored granules are (Hungate, 1969; Jain *et al.,* 1991) exposure to air, storage period and storage temperature. Oxygen is inhibitory to anaerobic bacteria, especially the methanogens and acetogens (Jain *et al.,* 1991). However, Kato *et al.* (1993a, b) found that methanogenic granules had a much higher tolerance to oxygen inhibition than pure anaerobic microbial cultures due to the great number of facultative and aerobic bacteria present in the granules (Wu *et al.,* 1995). These aerobic and facultative bacteria rapidly consume the oxygen, reduce the redox potential and protect the anaerobic bacteria from oxygen inhibition. Yükselen (1997) found that UASB sludge has good microbial and physiochemical preservation characteristics, but some researchers have reported sudden disintegration of granules without any obvious reason (Schmidt & Ahring, 1996).

The increasing popularity of the UASB-process calls for the availability of granular sludge to be used as inoculum. The cultivation and successful storage of new granules are, therefore, of key importance. The effect of long-term storage on the metabolic activity of particular microbial trophic groups involved in the complete degradation of organic compounds to methane (CH_4) and carbon dioxide (CO_2) has, as far as can be attained from the literature, not been examined.

The measurement of the methanogenic activity of anaerobic sludge is important as a means of calibrating the potential of converting soluble substrates to CH_4 and CO_2. This activity parameter makes it possible to determine the optimum organic loading rate for a faster and more reliable start-up. Activity testing can also be used as routine analysis for quantifying the granular sludge methanogenic activity and to detect any kind of inhibition after a long period of reactor operation (James *et al.*, 1990; Switzenbaum *et al.*, 1990). Activity tests can also be used for the characterisation of biomass prior to use as inoculum for the start-up of a new bioreactor, and thereby determining its potential as an inoculum for a specific process (De Zeeuw, 1984).

The aim of this study was to determine the impact of six different preservation techniques on the methanogenic activity and storage potential of batch cultivated granular sludge.

Materials and methods

Batch granule cultivation

A roller-table (Manufactured by the Department of Chemical Engineering, University of Stellenbosch) was used to cultivate the granules on a larger scale. The roll speed was maintained at 70 rmp, with a 25 litre cultivation container rotating at a speed of 8.5 rpm. The roller-table was placed in a 35°C temperature-controlled incubator. The cultivation container was filled with 3 litre raw secondary anaerobic sludge obtained form a local sewage works (Athlone Wastewater Treatment Works), and 15 litre growth medium. The growth medium consisted of a combination of apricot canning effluent (RFF Foods (Pty) Ltd) at a chemical oxygen demand (COD) of 2 $g.l^{-1}$, and 0.5 $g.l^{-1}$ each of urea and KH_2PO_4. Daily, for a period of 30 days, 3 litre liquid was removed and replaced with 3 litre fresh,

sterile growth medium (as described in Chapter 3 of this thesis), at a pH of 7.00. pH values were determined daily.

Granule preservation

Glass vials with a volume of 20 ml were used for the storage of the granular sludge and each vial was filled with a 3 g sample of the batch produced granular sludge. Six different preservation techniques were used and included:

I room temperature storage at 25°C (R);

II vacuum-drying (Heraeus vacuum oven) at room temperature and room temperature storage at 25°C (VD);

III freeze-drying (Heto CT 60e Freeze Dryer) with storage at 4°C (FD);

IV freeze-drying (Edwards Modulyo high vacuum lyophilizer) and sealing under vacuum and storage at 25°C (VFD);

V freeze storage at -18°C (F); and

VI cold storage at 4°C (C).

All the vials were sealed with a butyl rubber stopper and the stopper retained within an aluminium crimp top.

Activity testing

The activity of the preserved granular sludge was determined at days 0, 30, 60, 90 and 120. The samples were first activated for 2 days at 35°C with a sterile medium (pH 7.0) containing 1 $g.l^{-1}$ glucose and 0.5 $g.l^{-1}$ each of urea and KH_2PO_4 (O'Kennedy, 2000). This was done to regain activity of the stored granules for optimum biogas production during the activity-testing period. The activation medium was changed daily, so as to optimise the activation of the granular sludge. The basic test medium (BTM) as well as one of the different test media (Tables 1 and 2) was added to the vials, where after the vials were incubated at 35°C. All activity tests were done in triplicate. Biogas-production readings were taken after 5, 10 and 25 hours of incubation using a gas-tight and free-moving 6 ml syringe equipped with a 26 gauge needle (Owen *et al.*, 1979). The methane composition was determined 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 GC oven temperature was set at 55°C and helium was

Table 1. 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_2 [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 2. Different test media and the specific microbial group enhanced.

Test media*	Bacterial group
Control (only BTM)	
1 $g.l^{-1}$ Glucose [BDH]	Acidogens
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]	Methanogens

*The basic test medium (BTM) was used as basis for the different media.

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 readings were expressed as cumulative gas volumes.

Results and discussion

Batch granule cultivation

Granular sludge was cultivated as described in Chapter 3 of this thesis. During the batch cultivation the pH profile showed the characteristic drop in pH, with the subsequent stabilisation. As in the previous study, stabilisation was reached at a pH range between 6.30 and 6.40. Examples of the morphology of the granular sludge used as inoculum (C. van Schalkwyk, 2000, personal communication) in the different preservation techniques are shown in Fig. 1.

Activity testing

Prior to the use of granules as inoculum in UASB reactors, it is important to determine the activity, and more specifically, the methanogenic activity of the granules. In this study six different preservation techniques were evaluated using the batch cultivated granular sludge as biomass source for activity test determinations. All tests were done in triplicate. The variables (storage time; test medium; preservation techniques; total biogas and methane produced) evaluated in this study resulted in a large volume of data, and thus, for clarity purposes, the figure divisions as given in Table 3, were prepared. To simplify the discussion of the results, the data illustrated in Fig. 2 - 19 have been included as an "Appendix" at the end of this chapter.

The cumulative biogas production was determined for each preservation technique with the different test media (See Figures 2 - 7 in the Appendix). The cumulative biogas and methane (CH_4) data were then converted to represent the activity of the different techniques relative to each other. The activity of each preservation technique is, therefore, described in terms of the 'tempo of biogas and CH_4 production'. The tempo of gas production was determined using the gradient or slope **[**the cumulative gas production (CGP) over time for each of the time intervals (t = 5, 10 and 25 h)**]**. The gradient, for example, of 10 h was determined as follows: $(CGP_{10\,h} - CGP_{5\,h})/(t_{10\,h} - t_{5\,h})$. The tempo of gas

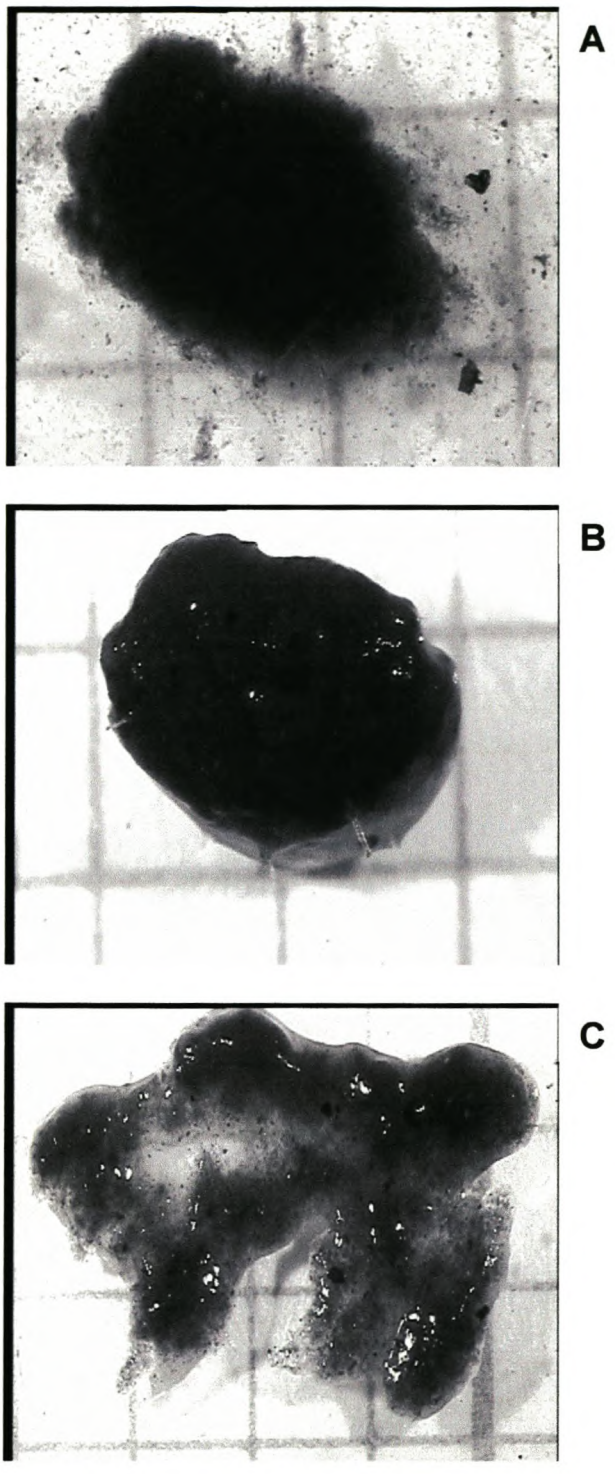

Figure 1. Examples of the granular sludge used as biomass inoculum for the different preservation techniques. A = granule surrounded by loose biomass and growth medium; B = granule in A, but with growth medium removed; C = mashed granule. (The background grid represents 4 mm^2.) (C. van Schalkwyk, 2000, personal communication)

Table 3. Figure divisions of the data obtained with the different preservation techniques and storage temperatures used.

Figures	Preservation technique	Storage temperature
2, 8, 14	Room temperature (R)	25°C
3, 9, 15	Vacuum-drying (VD)	25°C
4, 10, 16	Freeze-drying (FD)	4°C
5, 11, 17	Vacuum freeze-drying (VFD)	25°C
6, 12, 18	Frozen (F)	-18°C
7, 13, 19	Cold storage (C)	4°C

production (S_B for biogas and S_M for CH_4) was thus given in $ml.h^{-1}$ and was taken as representative of the activity of the granules. These values were then plotted against time for each preservation technique and carbon source evaluated to give a more visual representation of the activity of the stored granules. The tempo of biogas production (S_B) values for the six preservation techniques are shown in Fig. 8 - 13, as given in the Appendix, and the tempo of methane production (S_M) values are illustrated in Fig. 14 - 19, also as given at the end of this chapter in the Appendix. It was clear that the most variation between the different storage periods, in terms of activity, occurred after 10 and 25 h of incubation. Only the data of these two incubation times were, therefore, used in the summary of the S_B and S_M.

To simplify the comparisons and discussion, the large volume of data generated during the study are summarised in Fig. 20 - 23.

Activity evaluation of the different preservation techniques

In most cases it was found that when 2 $g.l^{-1}$ glucose was added to the BTM (Table 2), the data showed more measurable activity when the results are compared to those obtained where only 1 $g.l^{-1}$ glucose was added to the BTM (Table 2). It was, therefore, decided not to include the results obtained where 1 $g.l^{-1}$ glucose were added to the BTM in the following discussion.

Control – The basic test medium (BTM) was used as the control medium to enable comparisons at a general level. From the data in Fig. 20A, it is clear that the freeze-dried (FD) samples were more active in terms of S_B, after both 10 and 25 h of incubation. When the methanogenic activity data (Fig. 20B) is examined, the freeze-dried (FD) samples again showed the most activity after both 10 h and 25 h. It is, therefore, evident from the data obtained that the freeze-dried (FD) samples retained the best activity over the 120 day storage period at a general level where no specific microbial group was enhanced.

Vacuum freeze-drying (VFD), where the samples were freeze-dried and sealed under vacuum with storage at 25°C, gave the second best results for both the S_B and S_M. The samples that were either cold stored (C) or stored at room temperature (R) showed no S_B and S_M activity after 10 h. The samples stored at room temperature (R) did, however, regain activity after 25 h of incubation, but

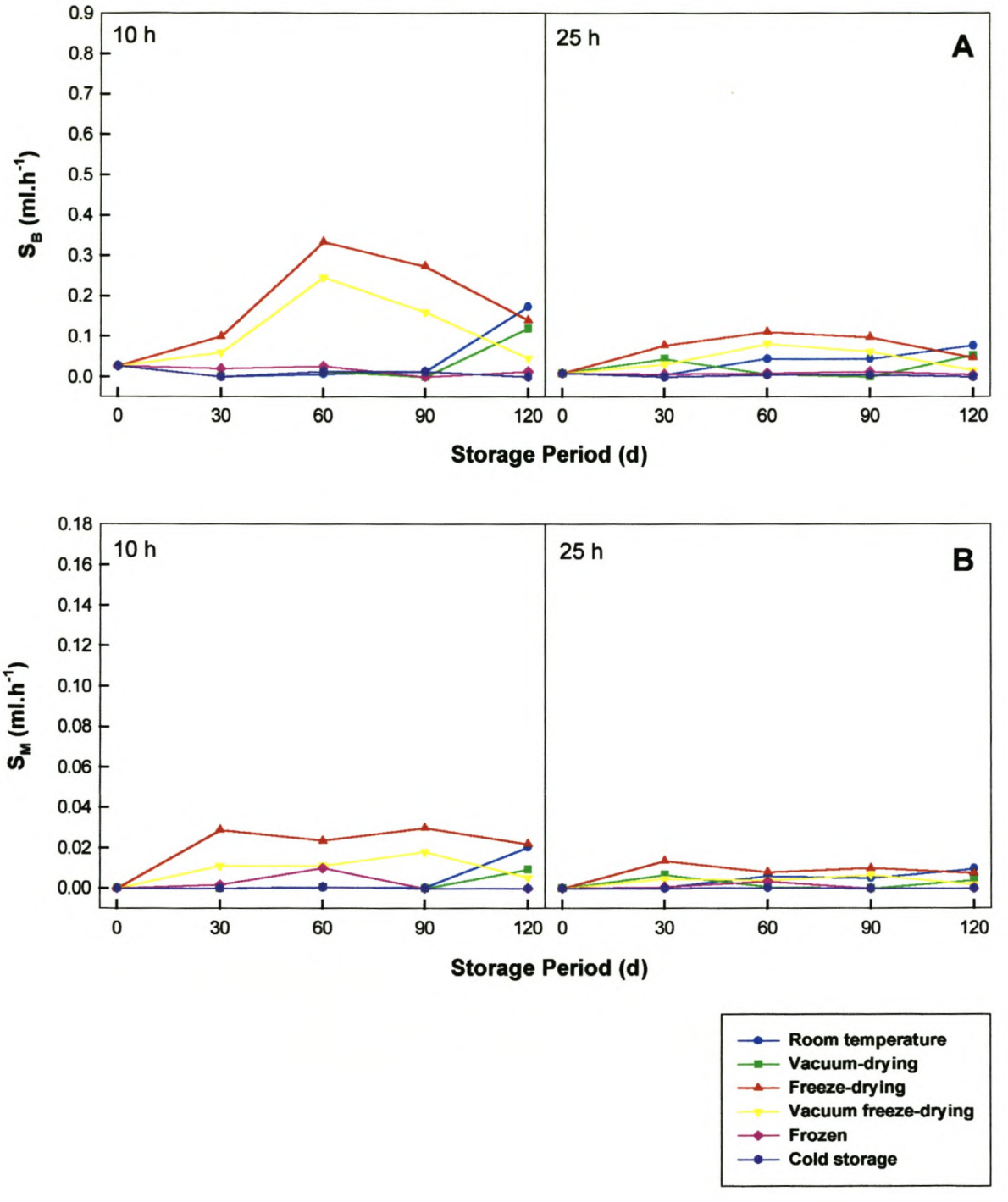

Figure 20. Biogas activity (S_B) **(A)** and methanogenic activity (S_M) **(B)** after 10 and 25 h of differently preserved granules measured over a 120 day storage period using just the BTM (control).

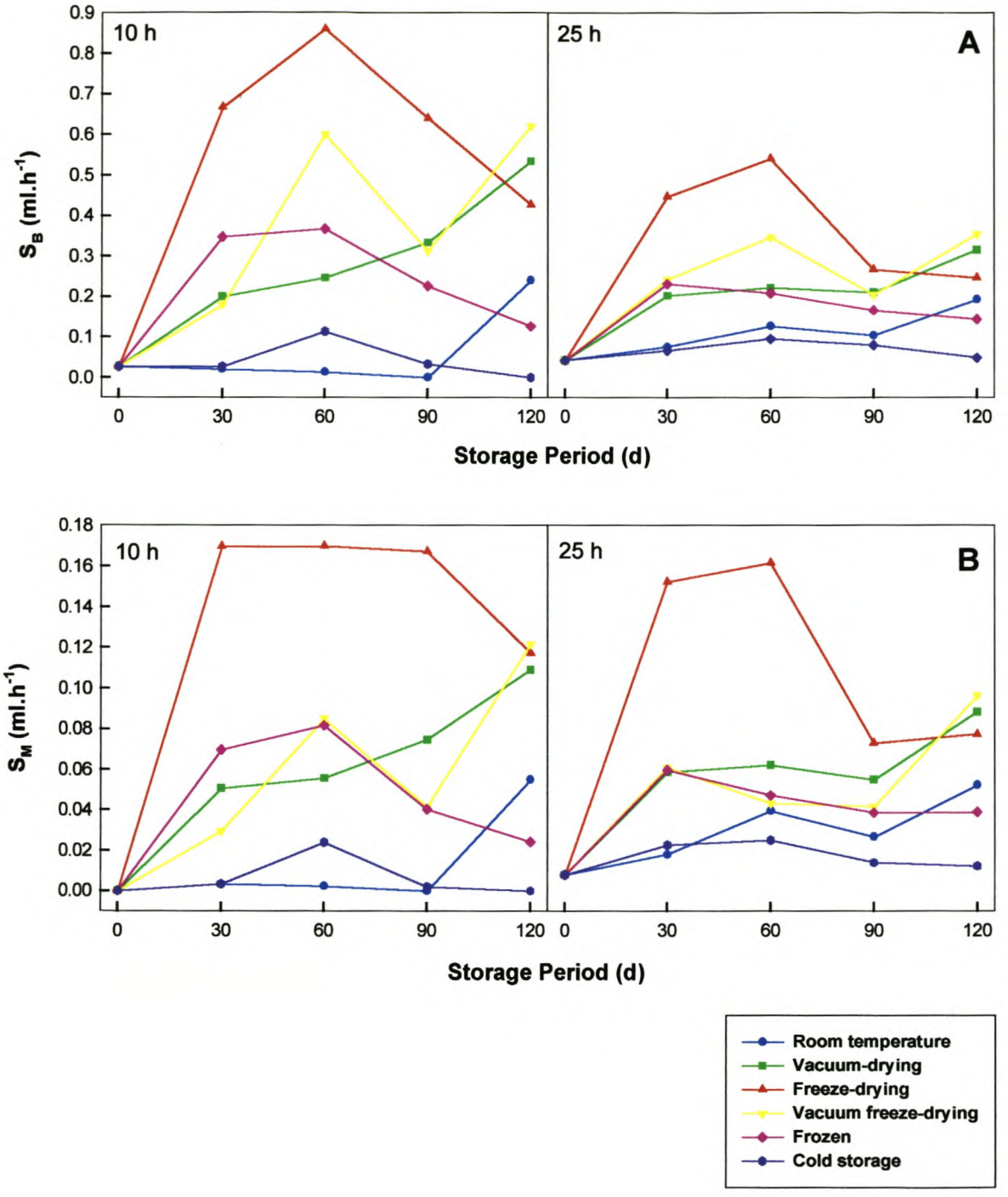

Figure 21. Biogas activity (S_B) **(A)** and methanogenic activity (S_M) **(B)** after 10 and 25 h of differently preserved granules measured over a 120 day storage period using the BTM + 2 g.l^{-1} glucose as the test medium.

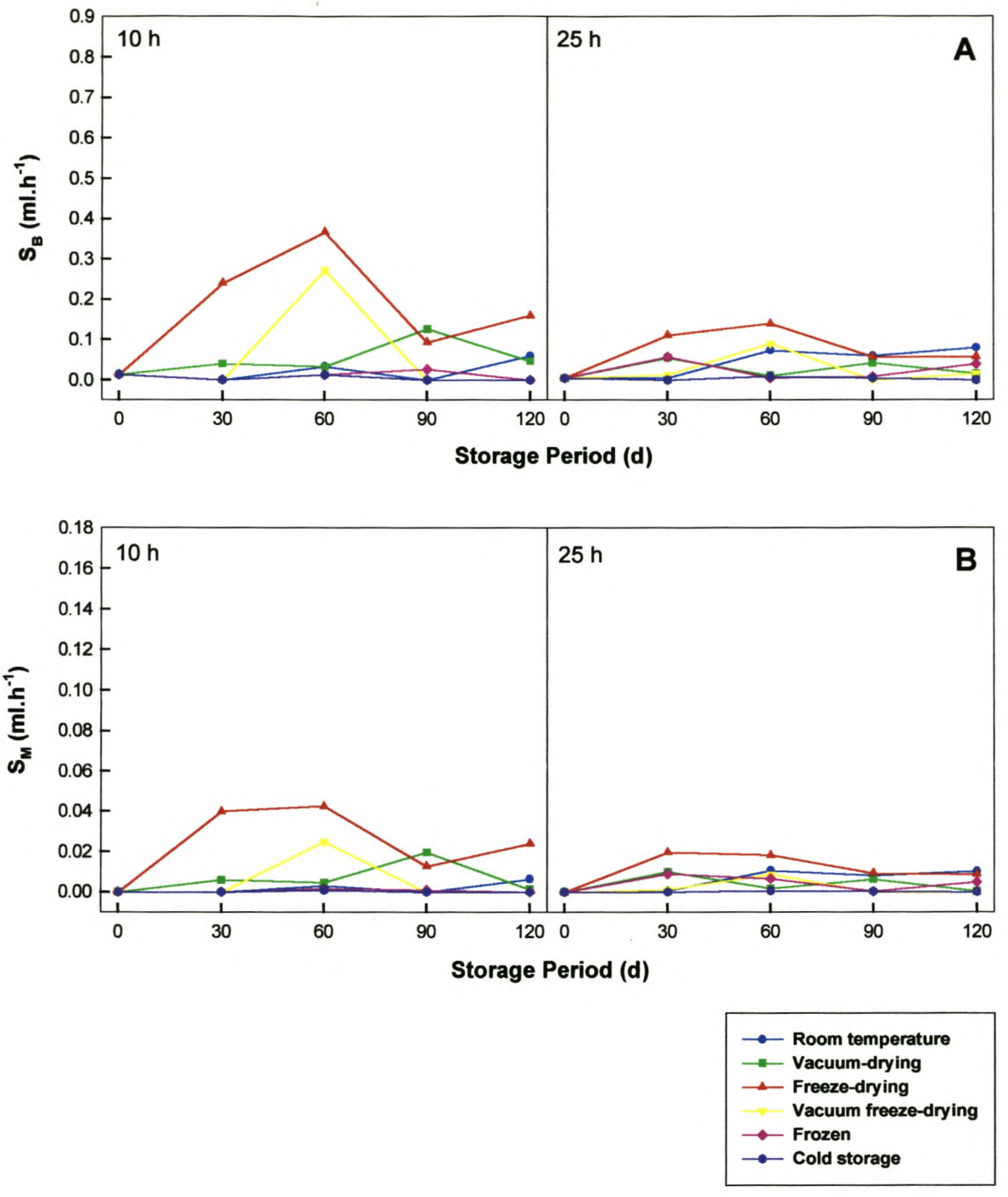

Figure 22. Biogas activity (S_B) **(A)** and methanogenic activity (S_M) **(B)** after 10 and 25 h of differently preserved granules measured over a 120 day storage period using the BTM + 4 g.l^{-1} lactate as the test medium.

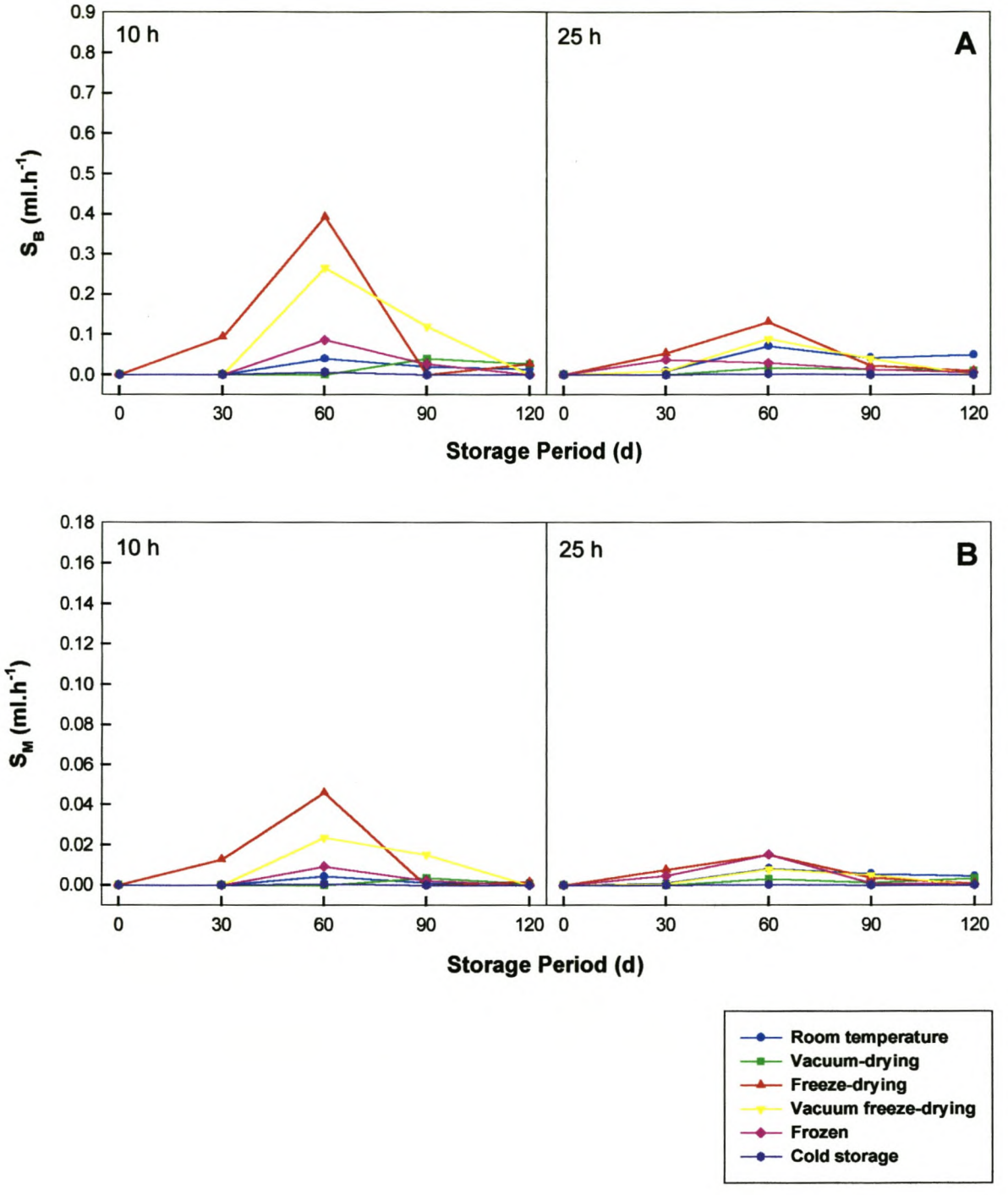

Figure 23. Biogas activity (S_B) **(A)** and methanogenic activity (S_M) **(B)** after 10 and 25 h of differently preserved granules measured over a 120 day storage period using the BTM + 1 g.l^{-1} acetic acid as the test medium.

only after a storage period of 90 days, and were then found to be the most active after 120 days of storage.

Samples frozen (F) at -18°C showed very little activity in terms of S_B (Fig. 20A) and S_M (Fig. 20B). The vacuum-dried (VD) samples showed no activity in terms of S_B and S_M for the first 90 days of storage, but by day 120 a little biogas, as well as methane was, however, observed.

The order of S_B activity for the control samples after 10 and 25 h and 120 days of storage were: R > VD > FD > VFD > F > C, and for S_M activity, the ratings were: R > FD > VD > VFD > F = C.

It appears as if freeze-drying (FD) retains activity the best when the granules are stored for relatively short periods of up to 60 - 90 days, whereas room temperature preservation (R) showed activity but only after 120 days of storage. In practice when granules are to be preserved from one season to the next, room temperature preservation after 120 days appears to be the best technique but the lack of activity up to 90 days when evaluating this technique is worrying. From the data (Fig. 20) it was clear that FD and VFD would result in the best microbial preservation for at least 90 days.

Glucose as carbon source – Most acidogens are known to prefer carbon in the form of glucose and the addition of extra glucose (2 $g.l^{-1}$) to the BTM should, therefore, lead to an enhancement of the activity of especially the acidogens. The use of this specific medium was thus to primarily test the activity of the acidogens, but if the methanogenic population is active, an increase in their activity should also be found.

Freeze-drying (FD) as preservation technique again gave the highest biogas activity (S_B) (0.86 $ml.h^{-1}$) by day 60 (Fig. 21A), as well as the highest methanogenic activity (0.17 $ml.h^{-1}$) after 10 h (Fig. 21B). After 25 h of incubation the freeze-drying (FD) technique still gave the highest methane activity for all the preservation techniques after 60 days of storage. It appears as if the room temperature (R) preservation technique led to an inhibition of the acidogens as it was found that biogas and CH_4 were again only produced after 120 days of storage. An incubation time of 25 h did, however, lead to some biogas and CH_4 activity.

The order of biogas activity (S_B) for the glucose samples after 10 and 25 h and 120 days of storage, using the different preservation techniques and storage temperatures (Table 3) were: VFD > VD > FD > R > F > C and for the methanogenic activity (S_M) after 10 h and 120 days of storage, the order was: VFD > FD > VD > R > F > C, with the S_M after 25 h being VFD > VD > FD > R > F > C.

Overall, the data indicates that more activity was shown when 2 $g.l^{-1}$ glucose was added to the BTM. It is thus evident that the added carbon does enhance the activity of the acidogens and that the acidogen population in the preserved granules can be successfully re-activated after storage of up to 120 days.

Lactate as carbon source – The activity measured when 4 $g.l^{-1}$ lactate as main carbon source was added to the BTM (Fig. 22) was generally found to be much lower than the activity measured with the added 2 $g.l^{-1}$ glucose (Fig. 21). The reason for the lower activity is probably due to the fact that lactate is a very specific carbon source, which can only be metabolised by a selected microbial group.

The freeze-dried (FD) samples again showed the most biogas and methane activity after 10 and 25 h of incubation (Fig. 22A and B) but only up to day 90. After 120 days of storage and 25 h of incubation, however, the samples that were kept at room temperature (R) showed the most activity. The frozen (F) and cold stored (C) samples did not show activity after 10 h, and only the frozen (F) samples showed a slight sign of biogas and CH_4 activity after the 25 h incubation period. The biogas and CH_4 activity ratings for the lactate samples after 10 h and 120 days of storage were: FD > R > VD > VFD = F = C, and after 25 h the S_B ratings were: R > FD > F > VD = VFD > C. The CH_4 activity ratings measured after 25 h incubation were: R > FD > F > VFD = VD = C.

From the data in Fig. 22 it is clear that freeze-drying and room temperature preservation is the best technique for the activity retainment of the lactate-utilising bacteria. If the economics involved with the freeze-drying process are considered, room temperature storage of granules appears to be a more viable and practical preservation option. Vacuum freeze-drying (VFD) with storage for longer than 90

days, and cold storage (C) as from day 0 seems to either inactivate or eliminate this specific population.

The overall results obtained with just the BTM and the BTM with the added 4 $g.l^{-1}$ lactate are very similar. This was to be expected seeing that the lactate enhances the activity of only a small portion of the microbial consortium present in the granules.

Acetic acid as carbon source – Methane is one of the main microbial end products of any UASB system. It is, therefore, clear that an indication or measurability of the activity of the methanogens is very important in this biological cycle. Acetic acid is one of the few carbon sources that can be utilised directly by the methanogens. The addition of this carbon source to the BTM was, therefore, to enhance the activity of the acetate-utilising methanogen population.

The activity shown with the BTM with added acetic acid (Fig. 23) was on average the lowest when compared with the results obtained with the other test media used. It is possible that the acetate-utilising methanogen population might have been either small or inhibited, or totally eliminated in some cases by the use of certain preservation techniques and storage conditions. The freeze-dried samples (FD) did, nevertheless, again show the most activity after 10 h and 60 days of storage (S_B = 0.393 $ml.h^{-1}$; S_M = 0.046 $ml.h^{-1}$) (Fig. 23A and B). By day 120 hardly any S_B or S_M activity was, however, observed for any of the six preservation techniques. The activity ratings for the acetic acid samples after 25 h and 120 days of storage for the biogas activity were: R > VD = FD > VFD = F = C, and for the CH_4 activity it were: R > VD > FD = VFD = F = C.

Conclusions

A successful batch granule enhancement system requires an equally successful preservation technique that will maintain the activity of the mass-produced granules over an extended storage period. In this study, six different preservation techniques were evaluated in terms of biogas production and methanogenic activity over a period of 120 days.

The data showed that the freeze-dried samples (FD) followed by the vacuum freeze-dried samples (VFD) gave the best activity but only for 90 days.

Activity of samples preserved by freezing at -18°C (F), cold storage (C) and room temperature (R) methods were found to be very low. In the case of room temperature preservation (R), after 90 days of no activity it suddenly showed increased activity. It is difficult to explain this phenomenon as nothing was altered either in the storage conditions or the composition of the BTM. It is possible that prolonged storage at room temperature may have led to cell hydrolysis, and metabolites set free from these cells could then have provided energy sources needed by the rest of the population which then led to signs of methanogenic activity.

From the data obtained in this study it is clear that the method that was used to test for activity is a fairly simple and reliable method. It was, however, found that the most activity was found after 10 h of incubation with a decrease in activity up to 25 h. It was, therefore, concluded that the incubation time could be shortened to 10 h when the measurement of the tempo of biogas and methane production is to be an indication of activity for this type of test. The test results also showed that the activity when 2 $g.l^{-1}$ glucose were added to the BTM was almost double that obtained with just the BTM. It is suggested, based on the results obtained during this study, that only the enrichment of the BTM with 2 $g.l^{-1}$ glucose can be used as the test media for determinations or future research concerning activity testing for evaluation purposes.

It can be argued that the method of batch granule production, on a glucose-rich canning effluent, as used in this study, could probably result in the glucose-utilisers being the dominant group of the acidogenic population. They could then have provided the necessary metabolites for the methanogens. However, the low activity measured with the acetic acid enriched BTM with samples from all the preservation techniques suggests that acetate-utilising methanogens were not the dominant methane-producing group.

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APPENDIX

TO CHAPTER 4

To simplify the discussion of the results, the data illustrated in Fig. 2 - 19 have been included in this Appendix.

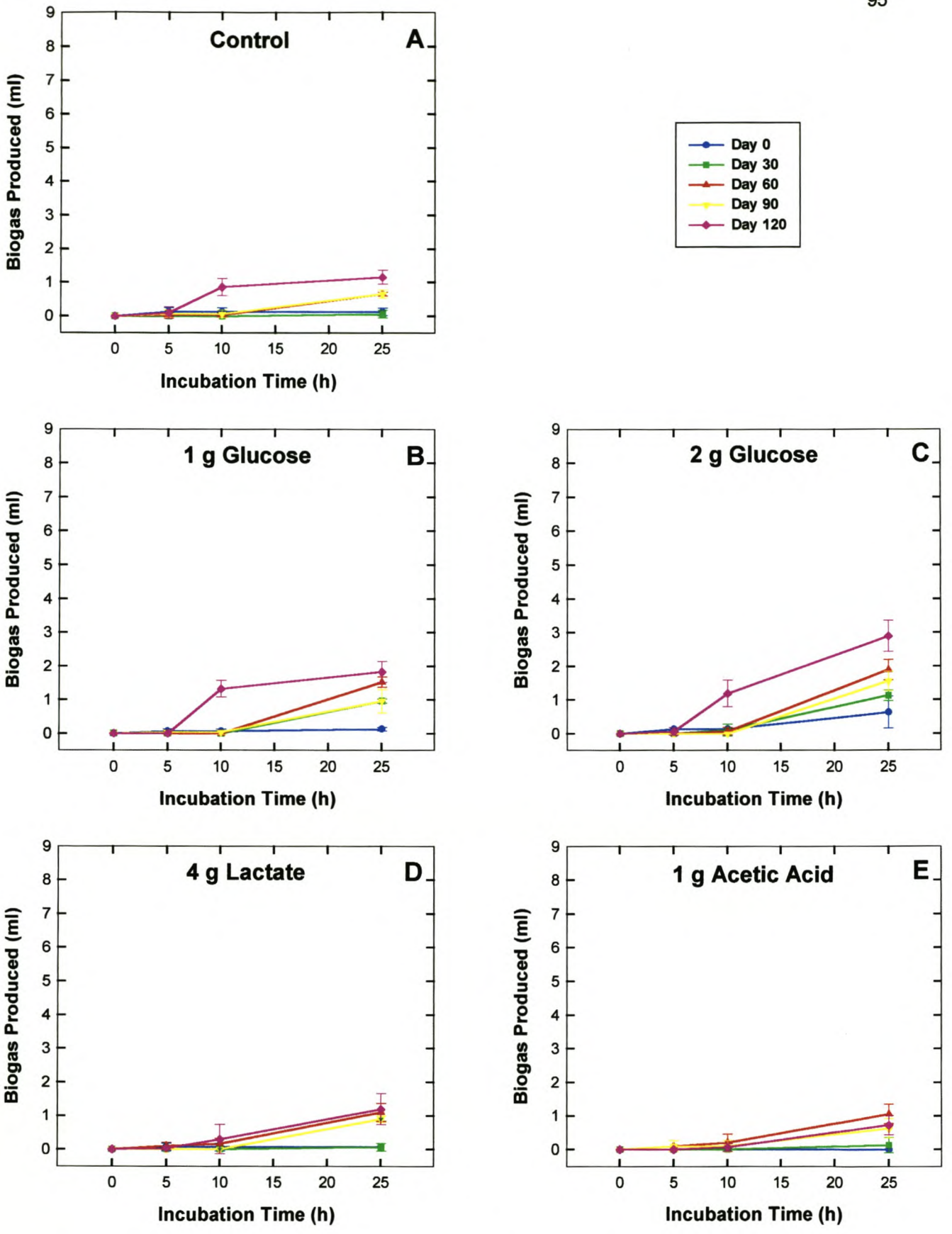

Figure 2. Cumulative biogas production (ml) of room temperature preserved granules using the different test media. The standard deviation was used as the error-bar length.

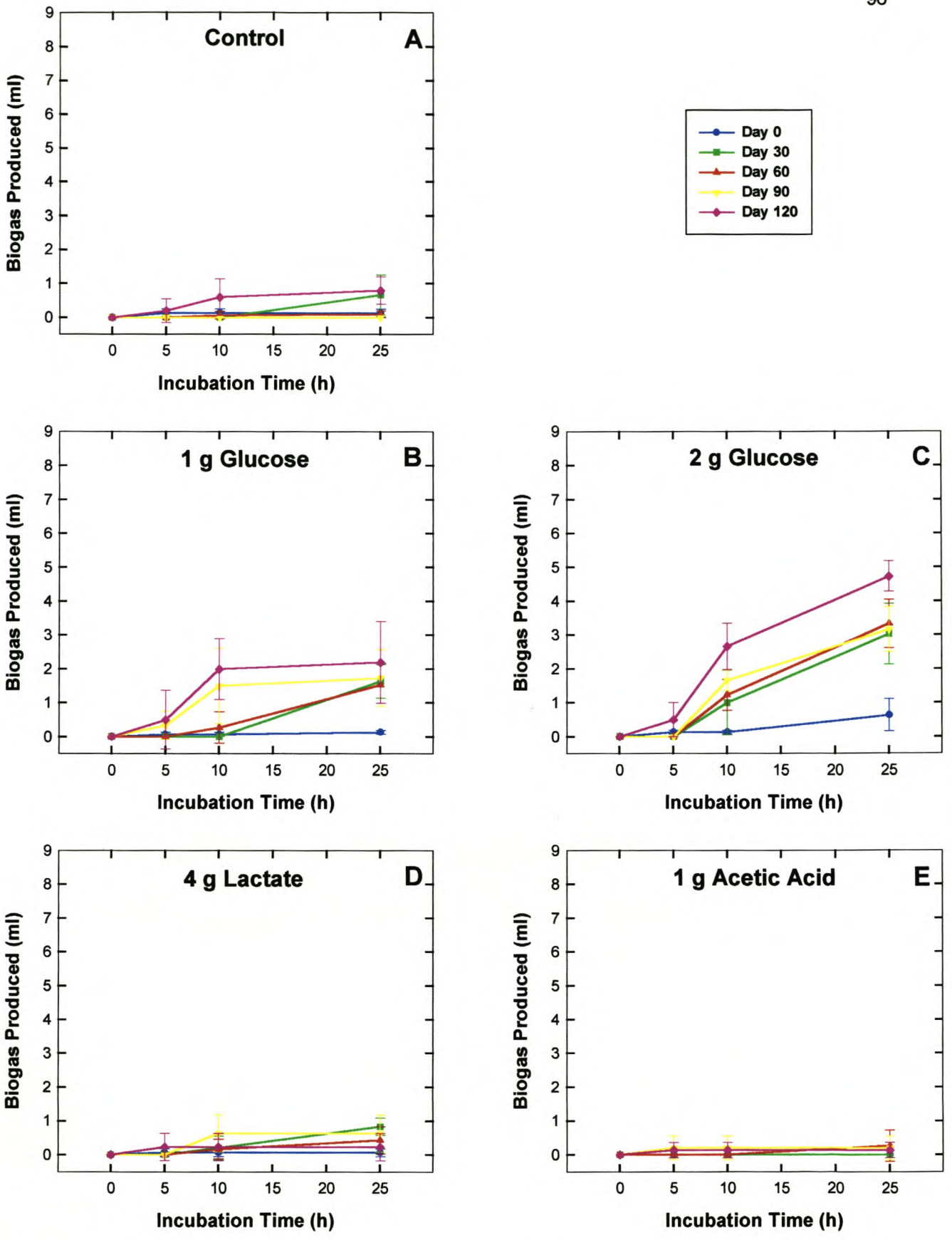

Figure 3. Cumulative biogas production (ml) of vacuum-dried granules using the different test media. The standard deviation was used as the error-bar length.

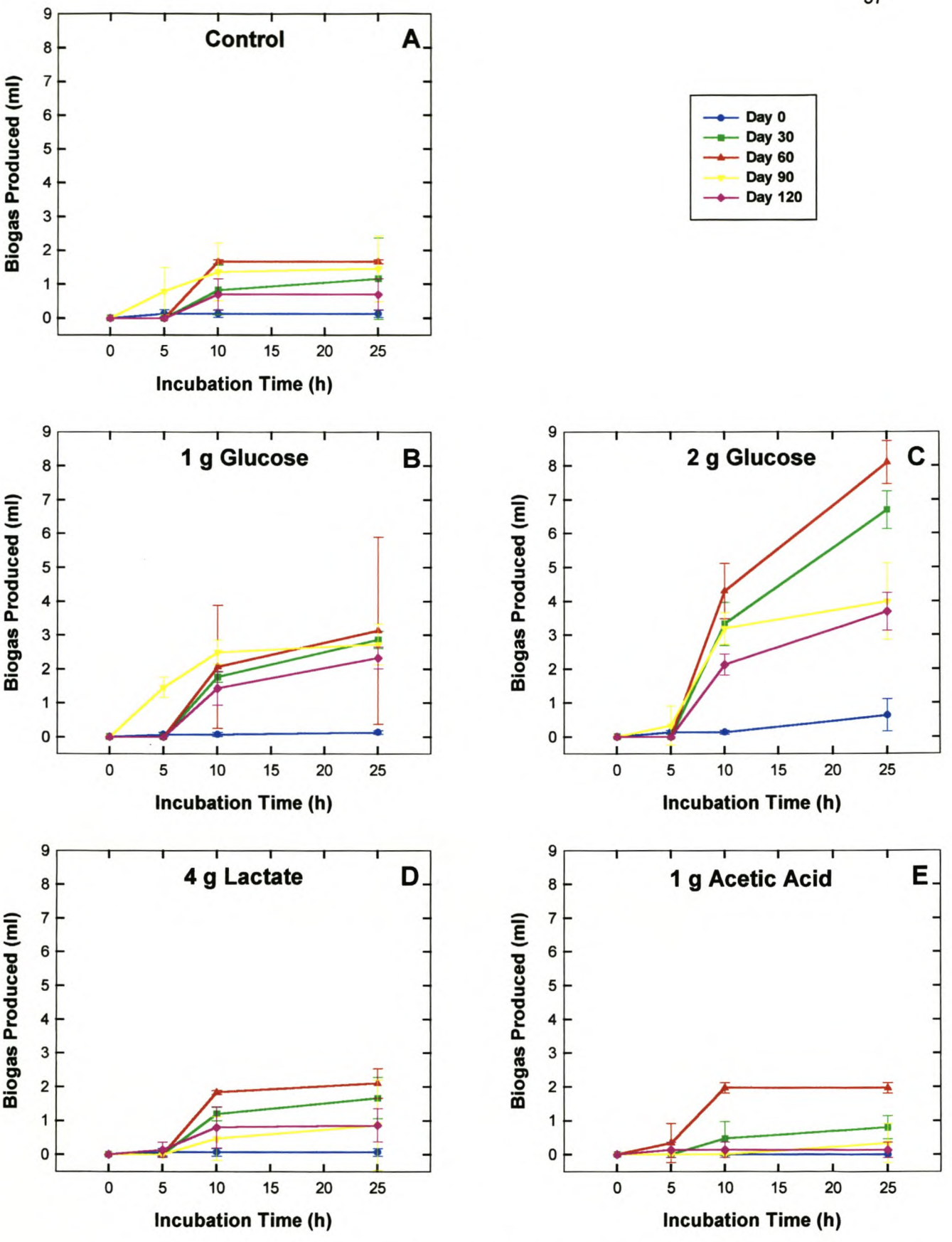

Figure 4. Cumulative biogas production (ml) of freeze-dried granules using the different test media. The standard deviation was used as the error-bar length.

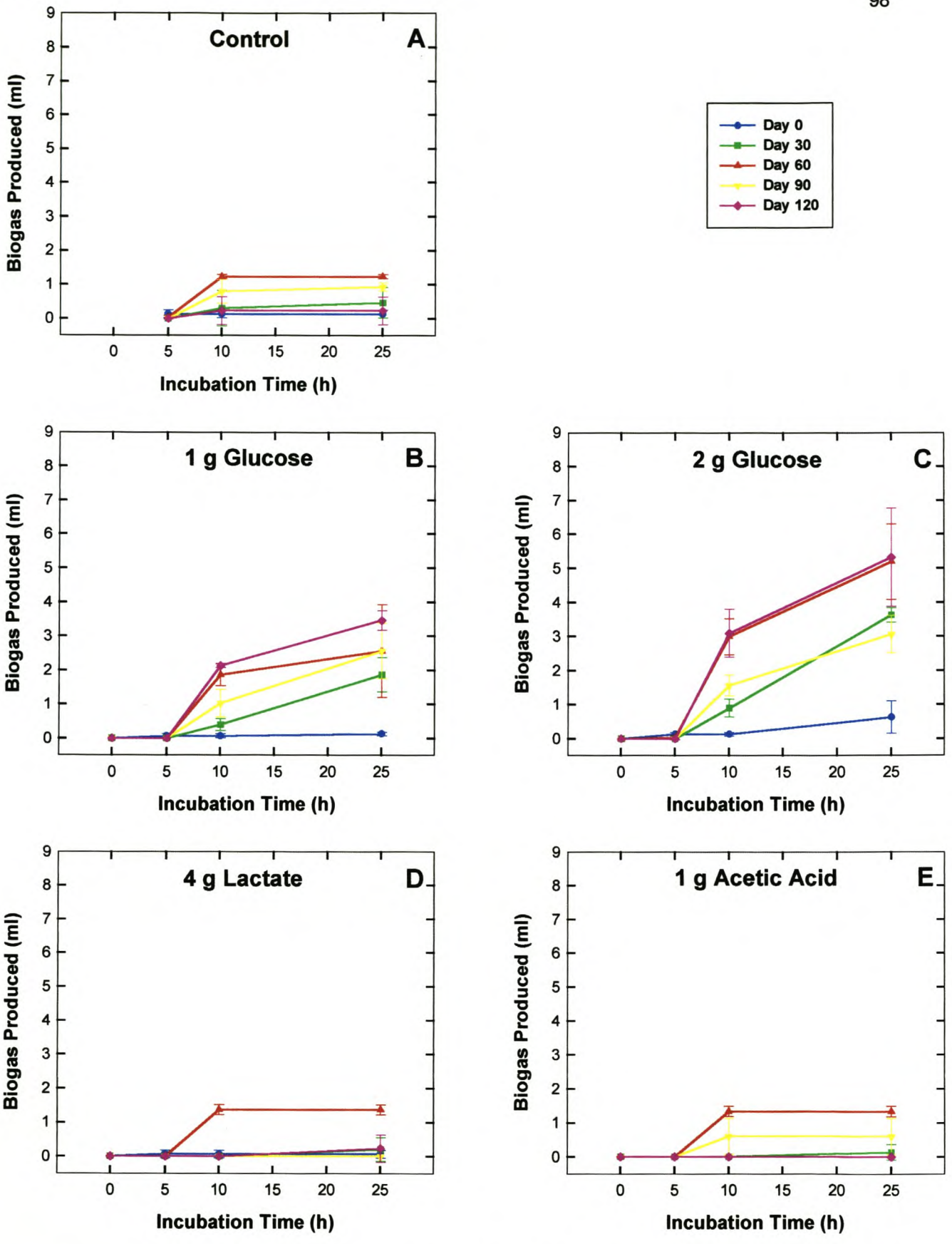

Figure 5. Cumulative biogas production (ml) of vacuum freeze-dried granules using the different test media. The standard deviation was used as the error-bar length.

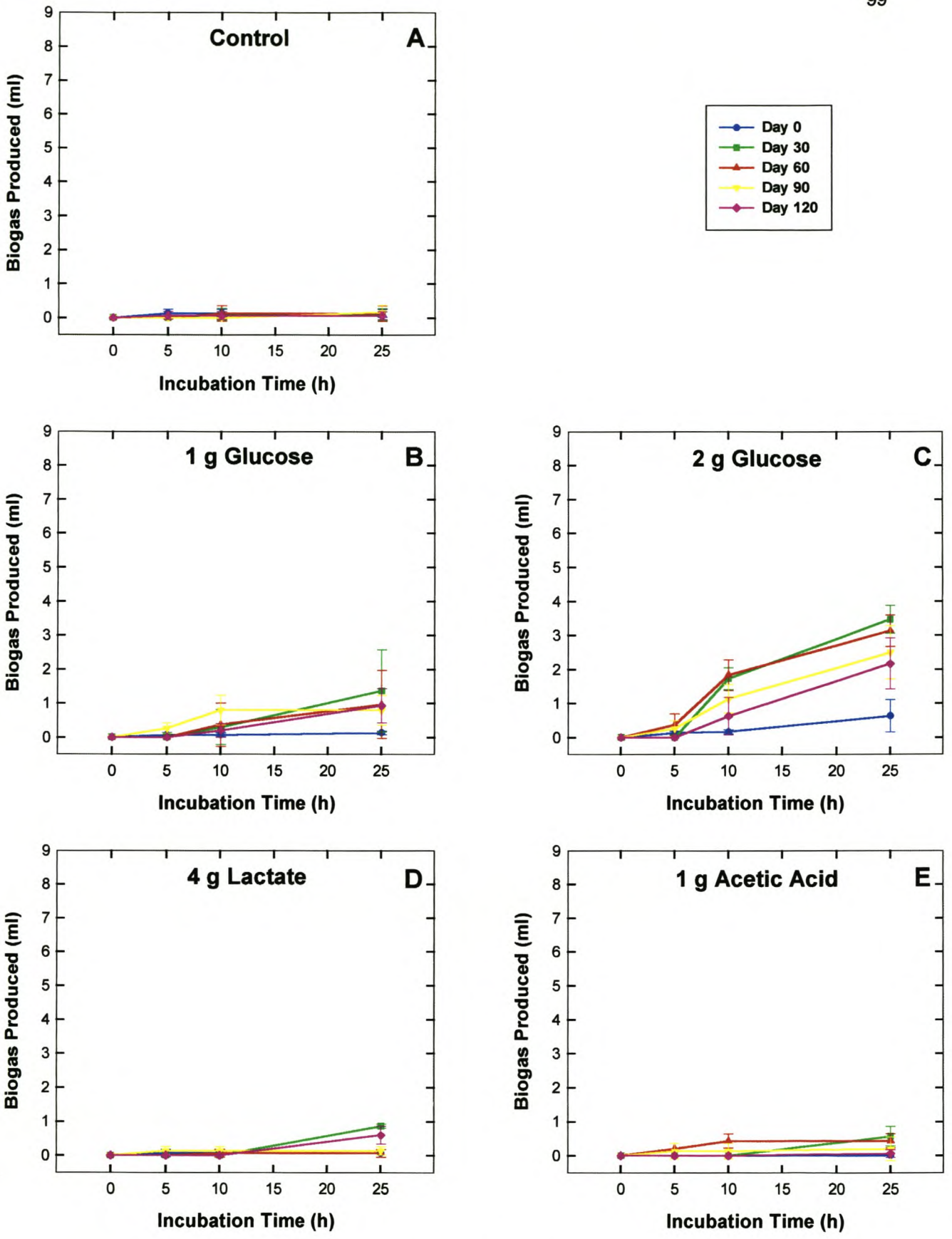

Figure 6. Cumulative biogas production (ml) of frozen granules using the different test media. The standard deviation was used as the error-bar length.

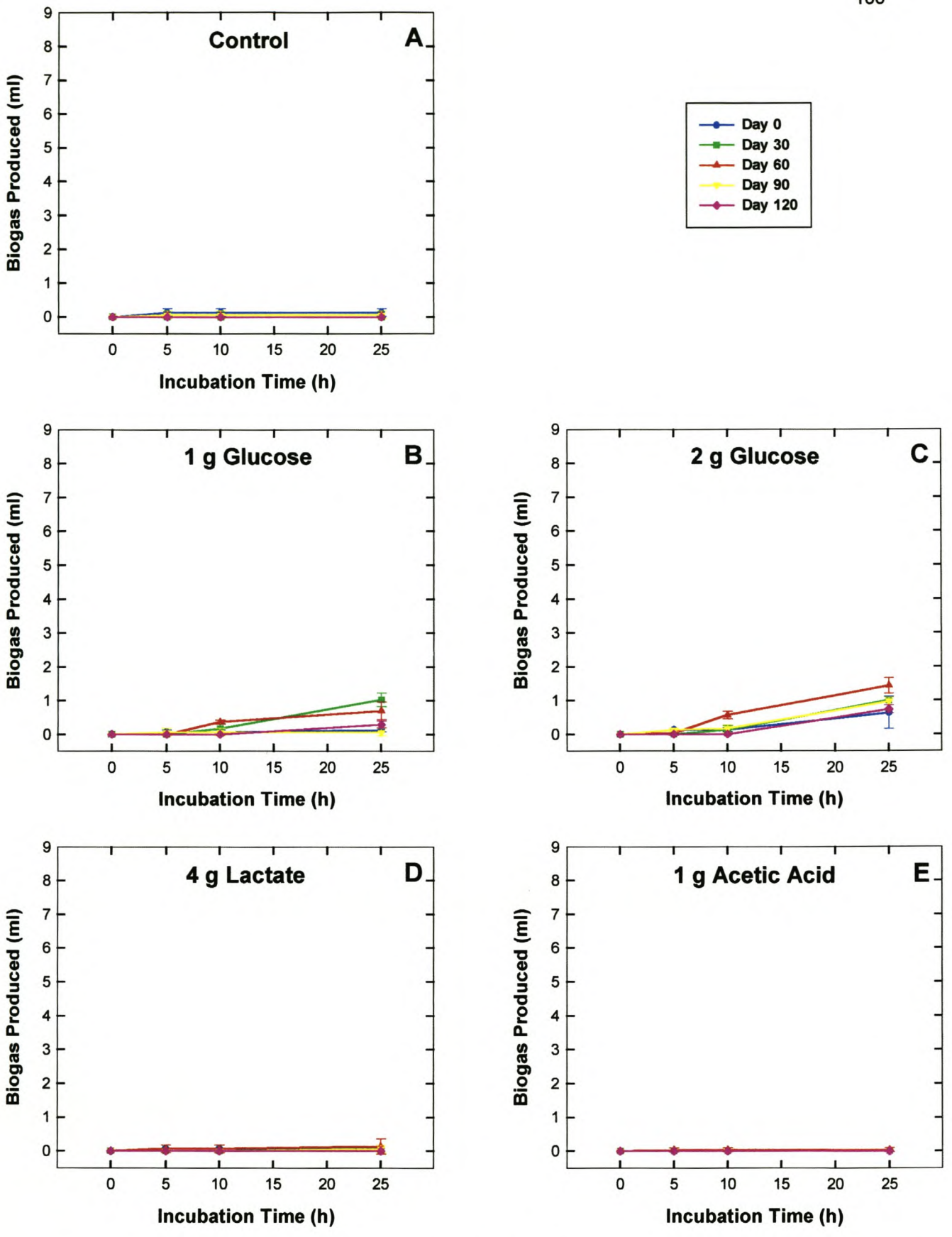

Figure 7. Cumulative biogas production (ml) of cold stored granules using the different test media. The standard deviation was used as the error-bar length.

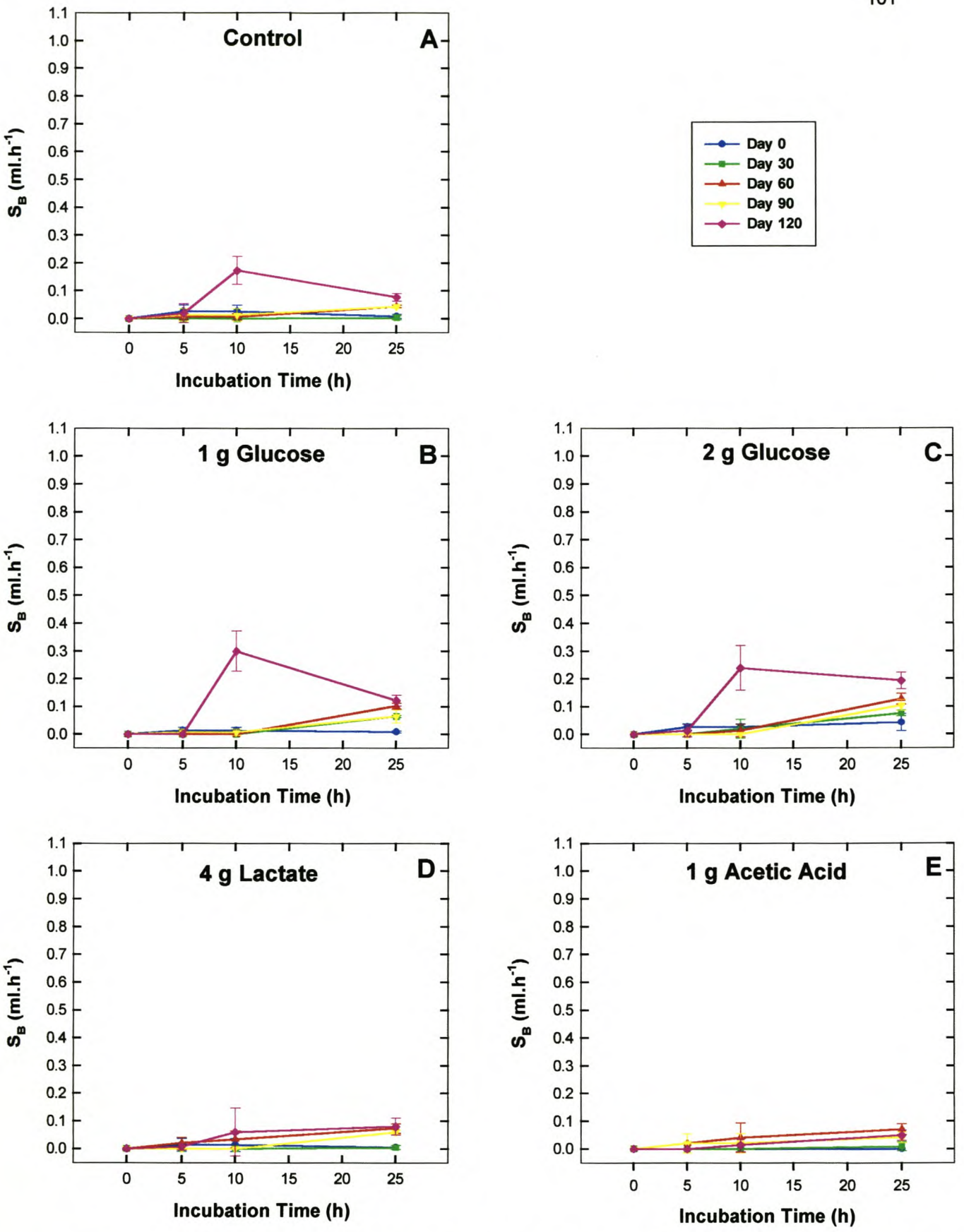

Figure 8. Tempo of biogas activity (ml.h^{-1}) (S_B) of room temperature preserved granules using the different test media. The standard deviation was used as the error-bar length.

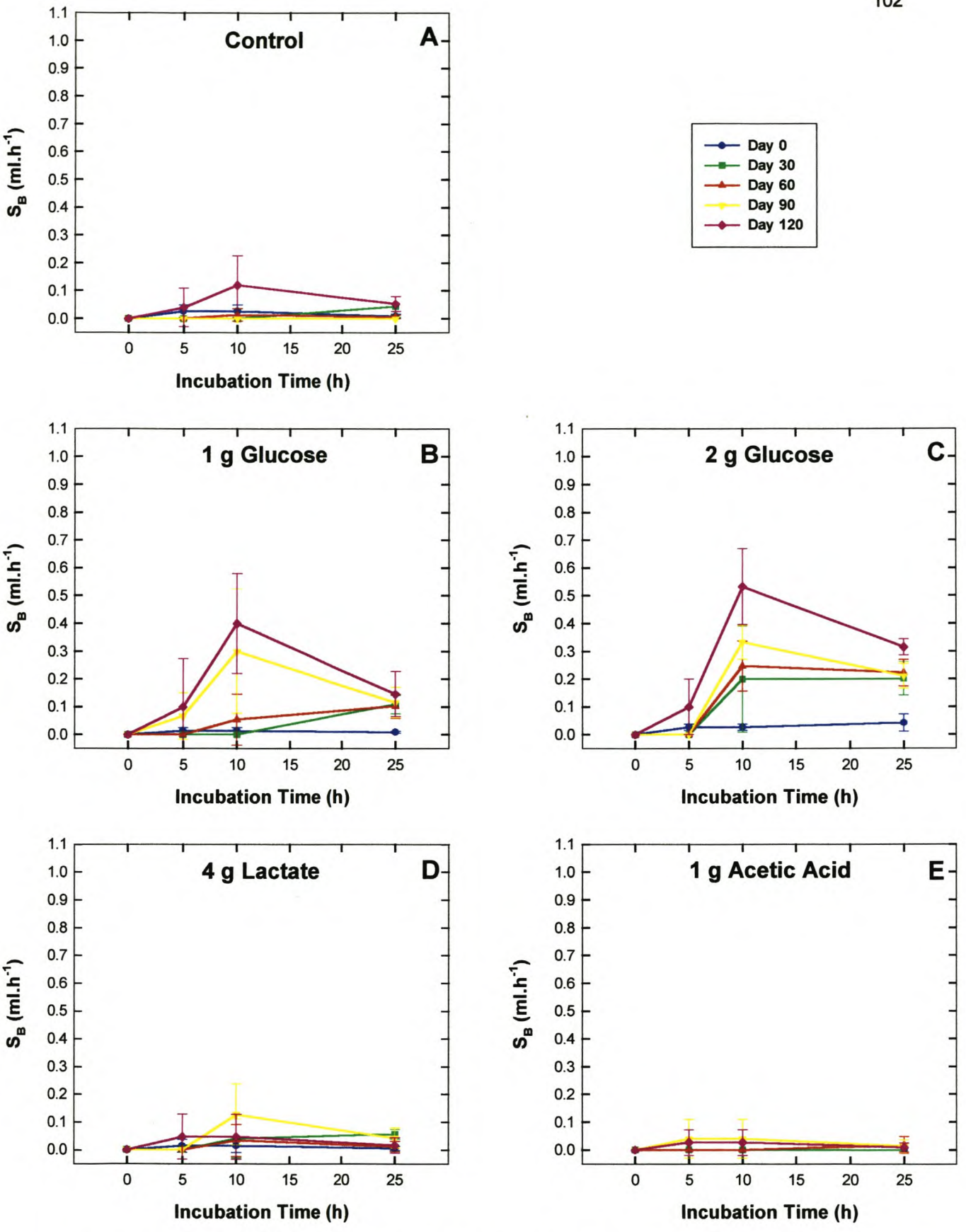

Figure 9. Tempo of biogas activity (ml.h^{-1}) (S_B) of vacuum-dried granules using the different test media. The standard deviation was used as the error-bar length.

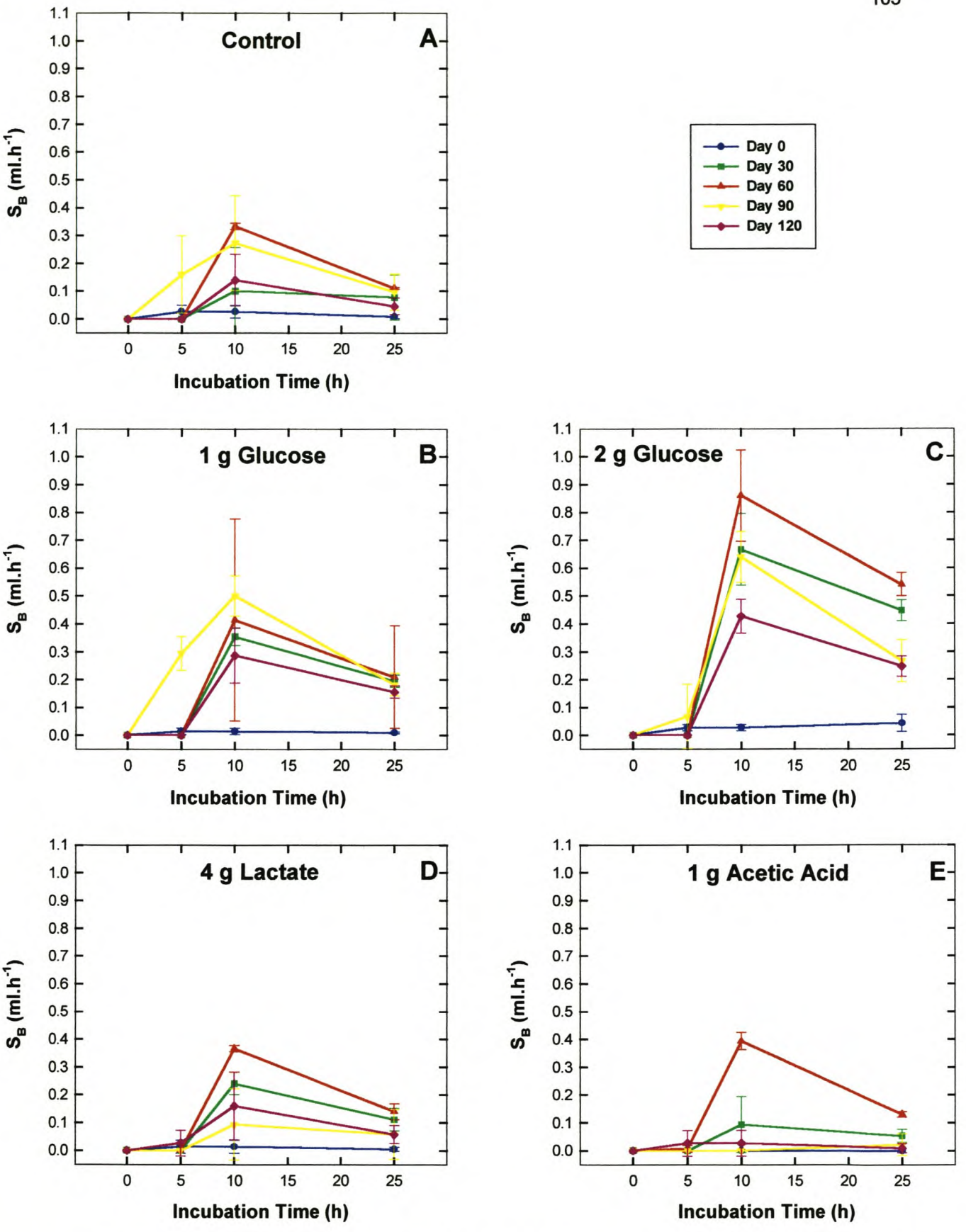

Figure 10. Tempo of biogas activity (ml.h^{-1}) (S_B) of freeze-dried granules using the different test media. The standard deviation was used as the error-bar length.

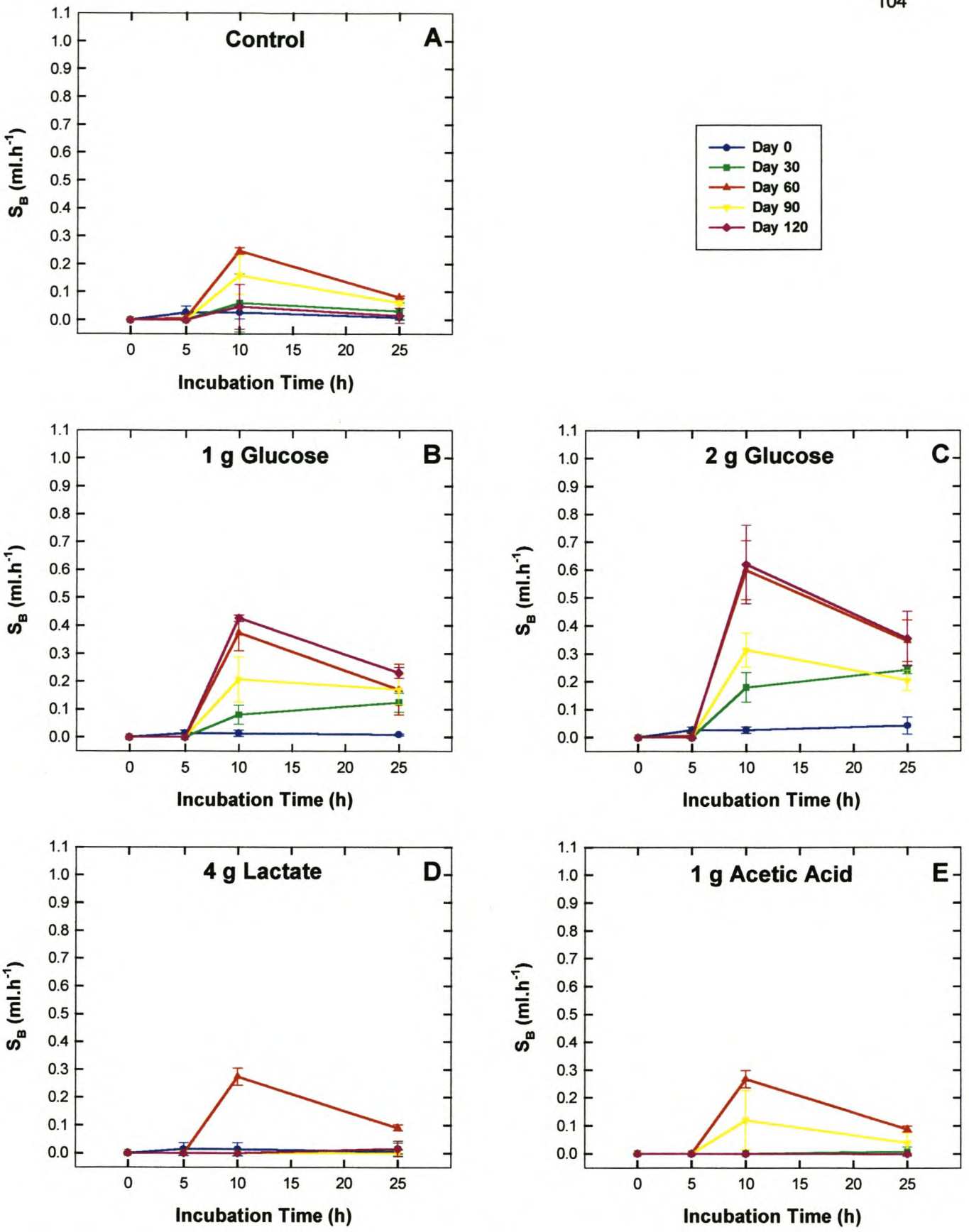

Figure 11. Tempo of biogas activity (ml.h^{-1}) (S_B) of vacuum freeze-dried granules using the different test media. The standard deviation was used as the error-bar length.

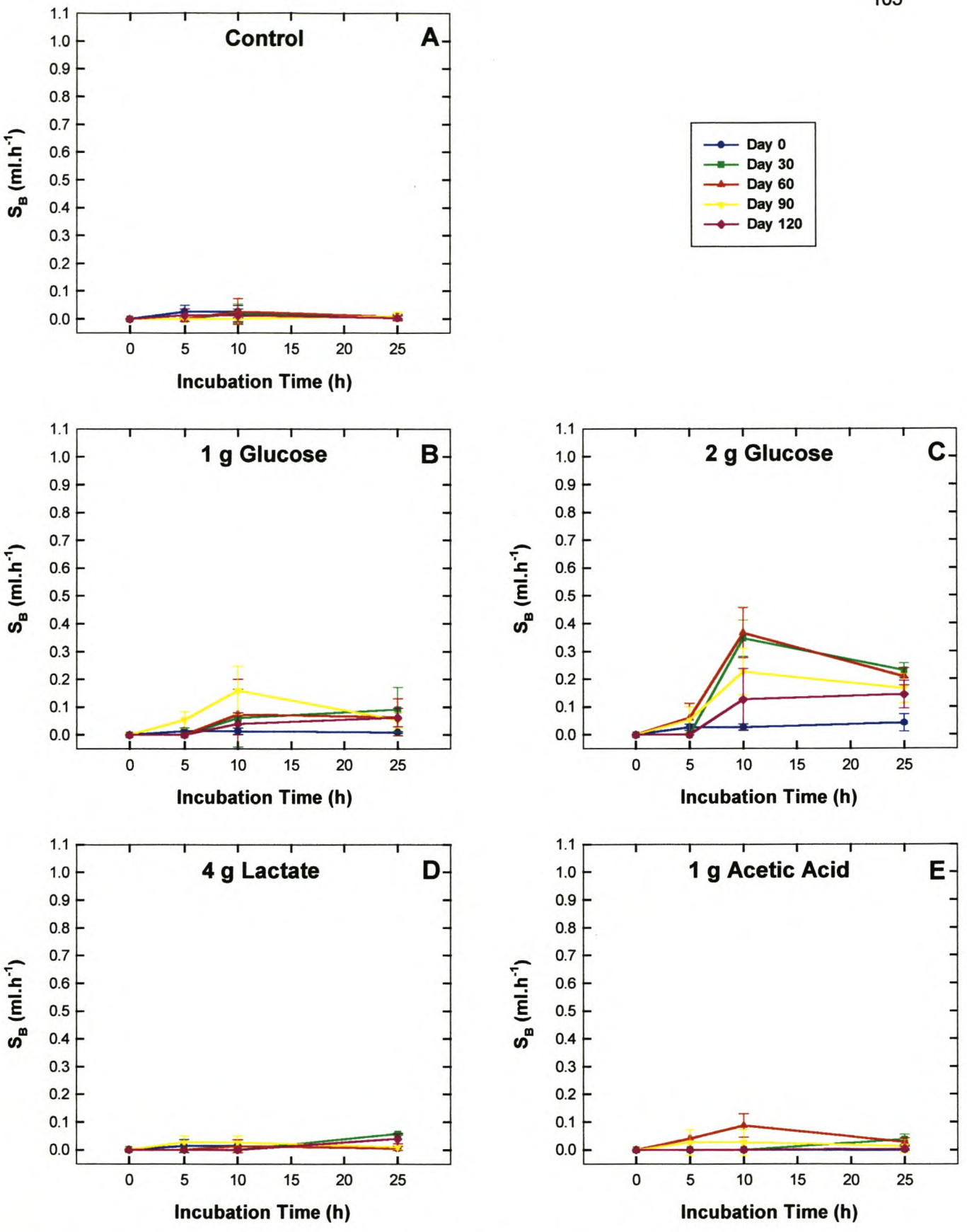

Figure 12. Tempo of biogas activity (ml.h^{-1}) (S_B) of frozen granules using the different test media. The standard deviation was used as the error-bar length.

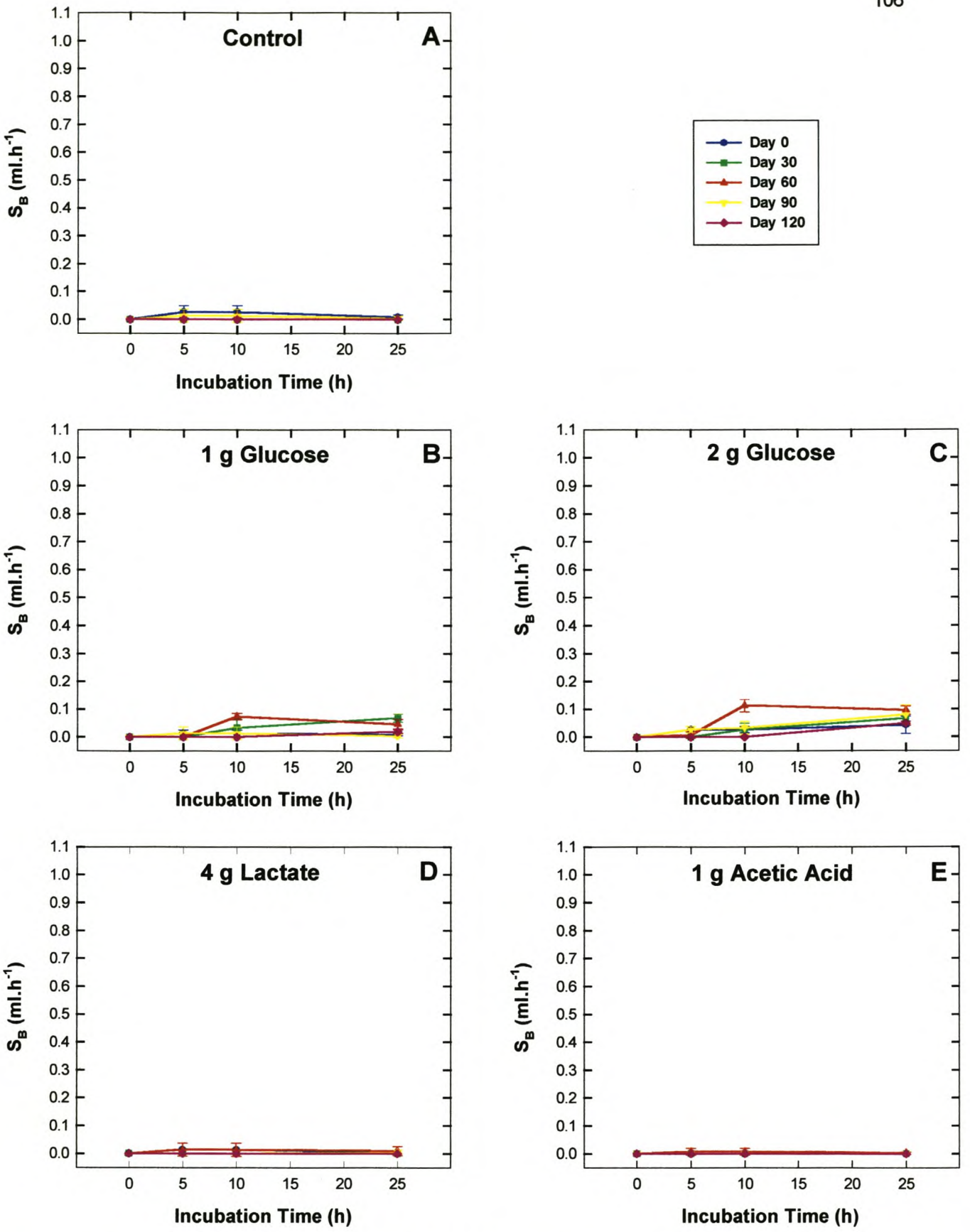

Figure 13. Tempo of biogas activity (ml.h^{-1}) (S_B) of cold stored granules using the different test media. The standard deviation was used as the error-bar length.

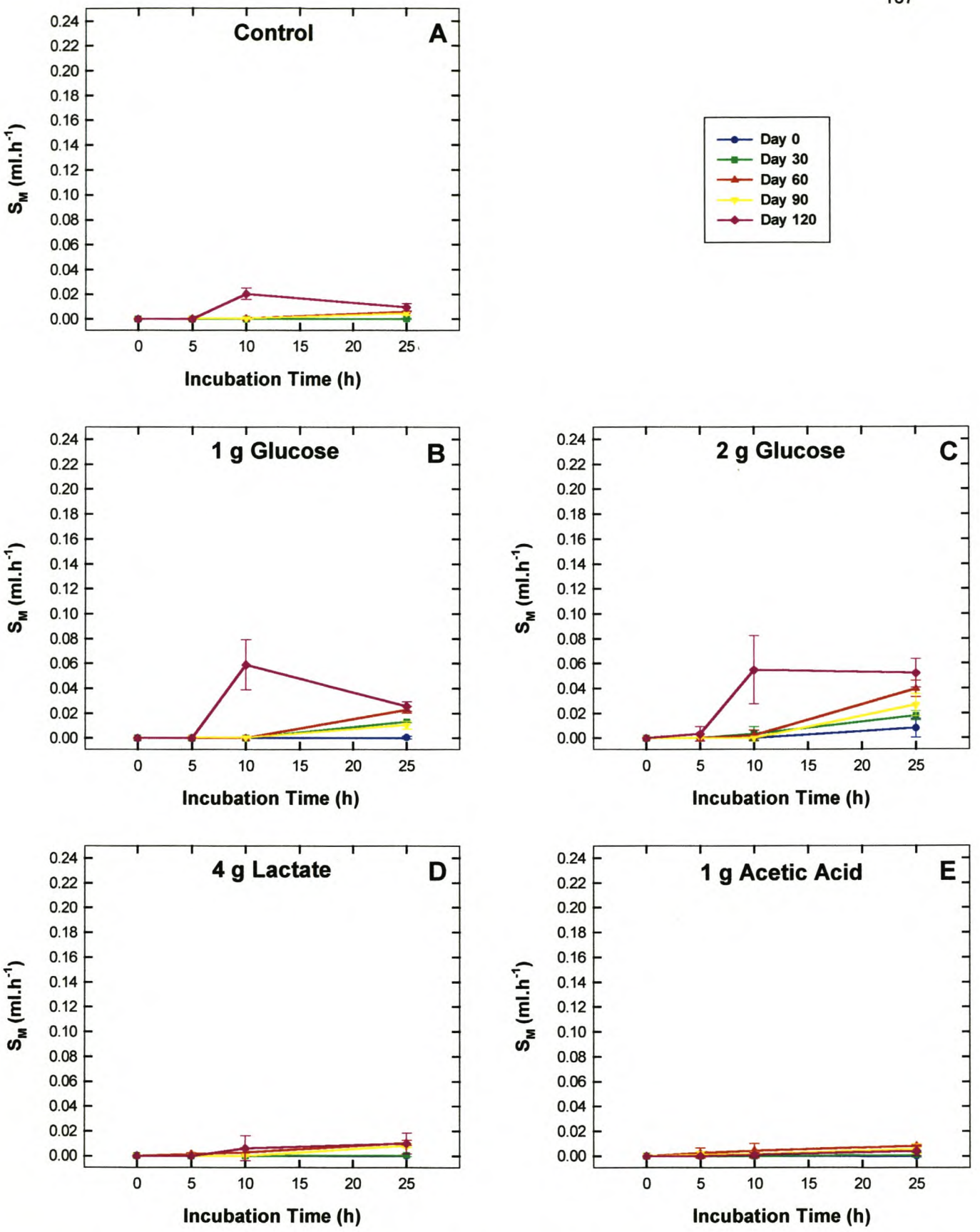

Figure 14. Tempo of methanogenic activity (ml.h^{-1}) (S_M) of room temperature preserved granules using the different test media. The standard deviation was used as the error-bar length.

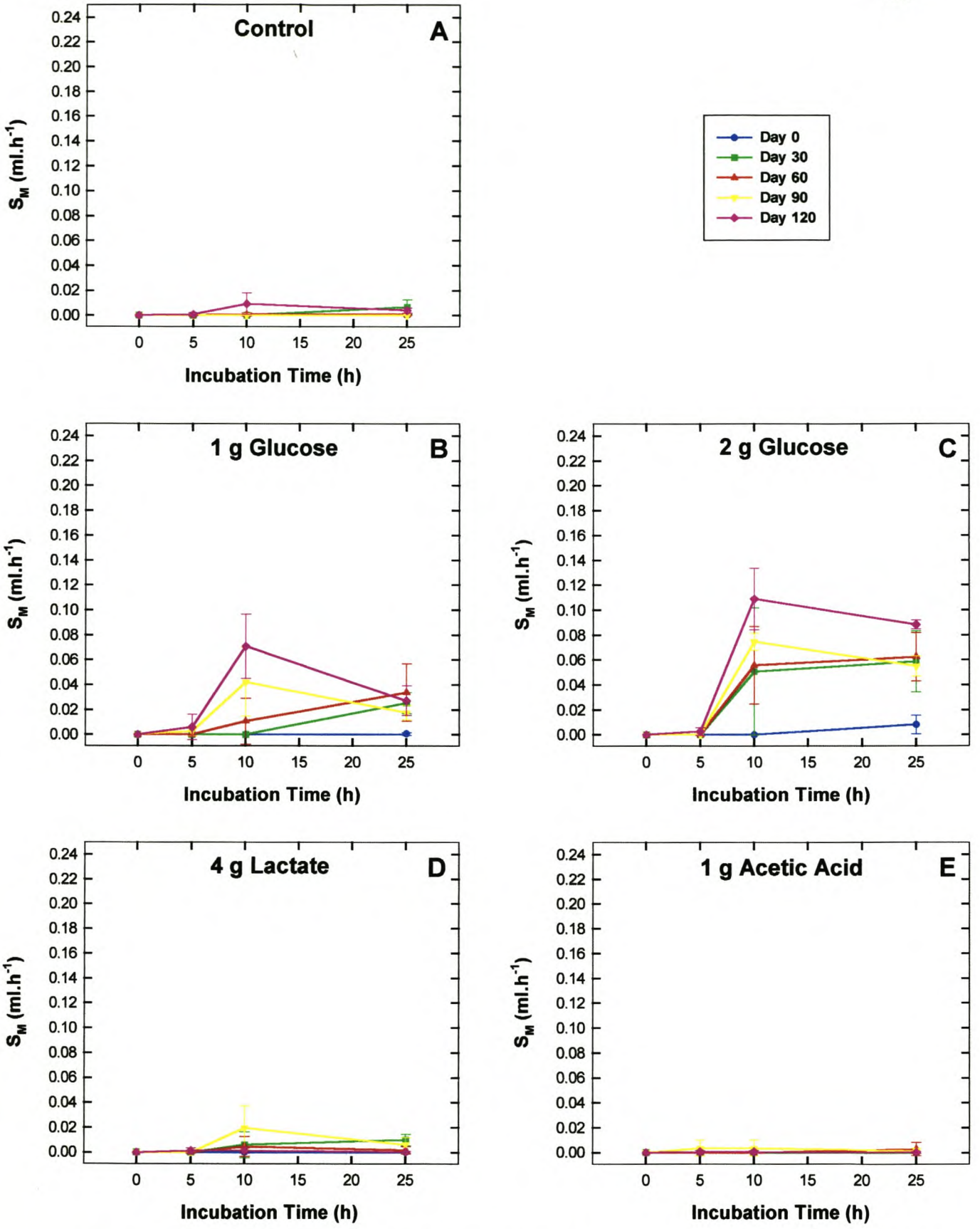

Figure 15. Tempo of methanogenic activity (ml.h^{-1}) (S_M) of vacuum-dried granules using the different test media. The standard deviation was used as the error-bar length.

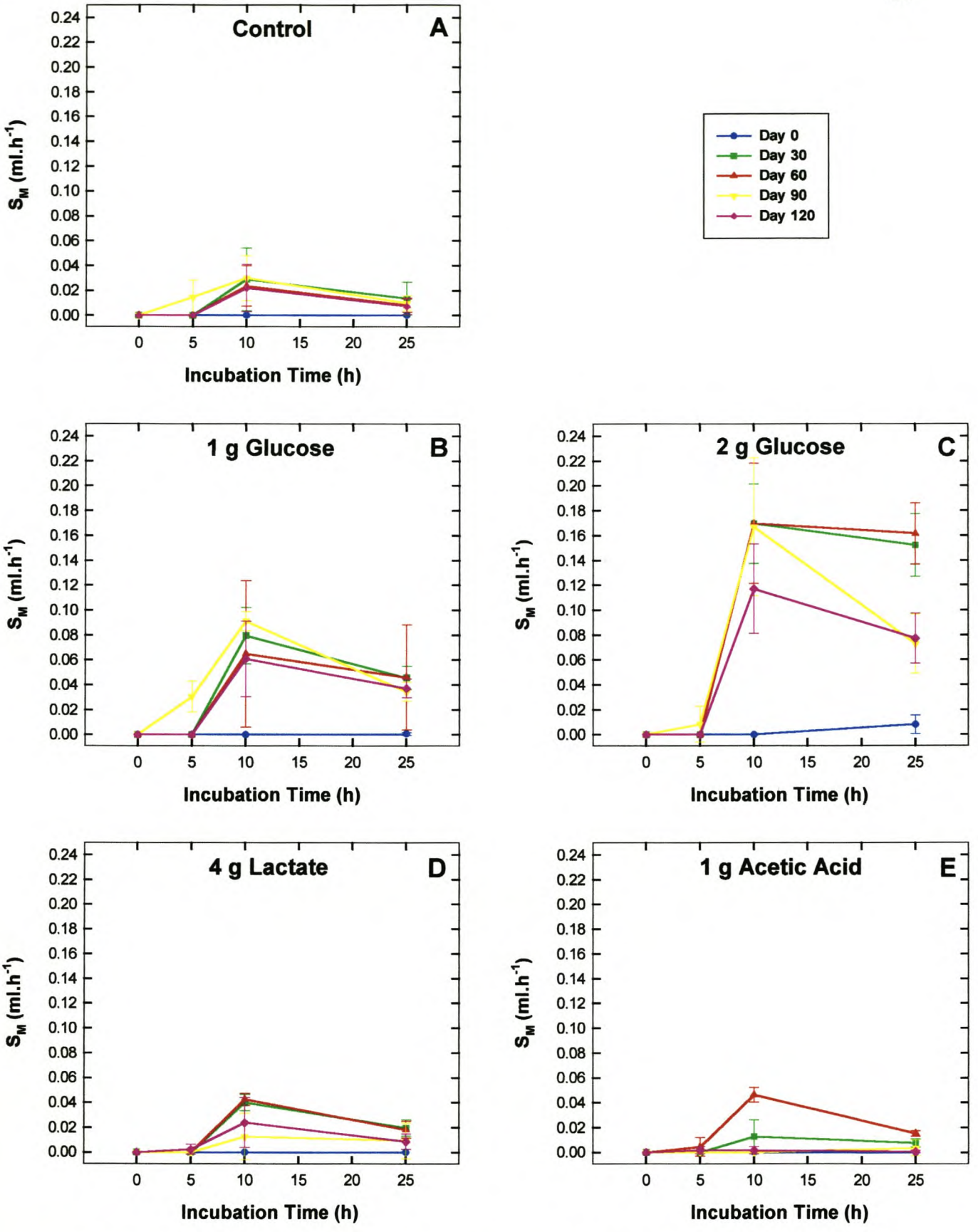

Figure 16. Tempo of methanogenic activity (ml.h^{-1}) (S_M) of freeze-dried granules using the different test media. The standard deviation was used as the error-bar length.

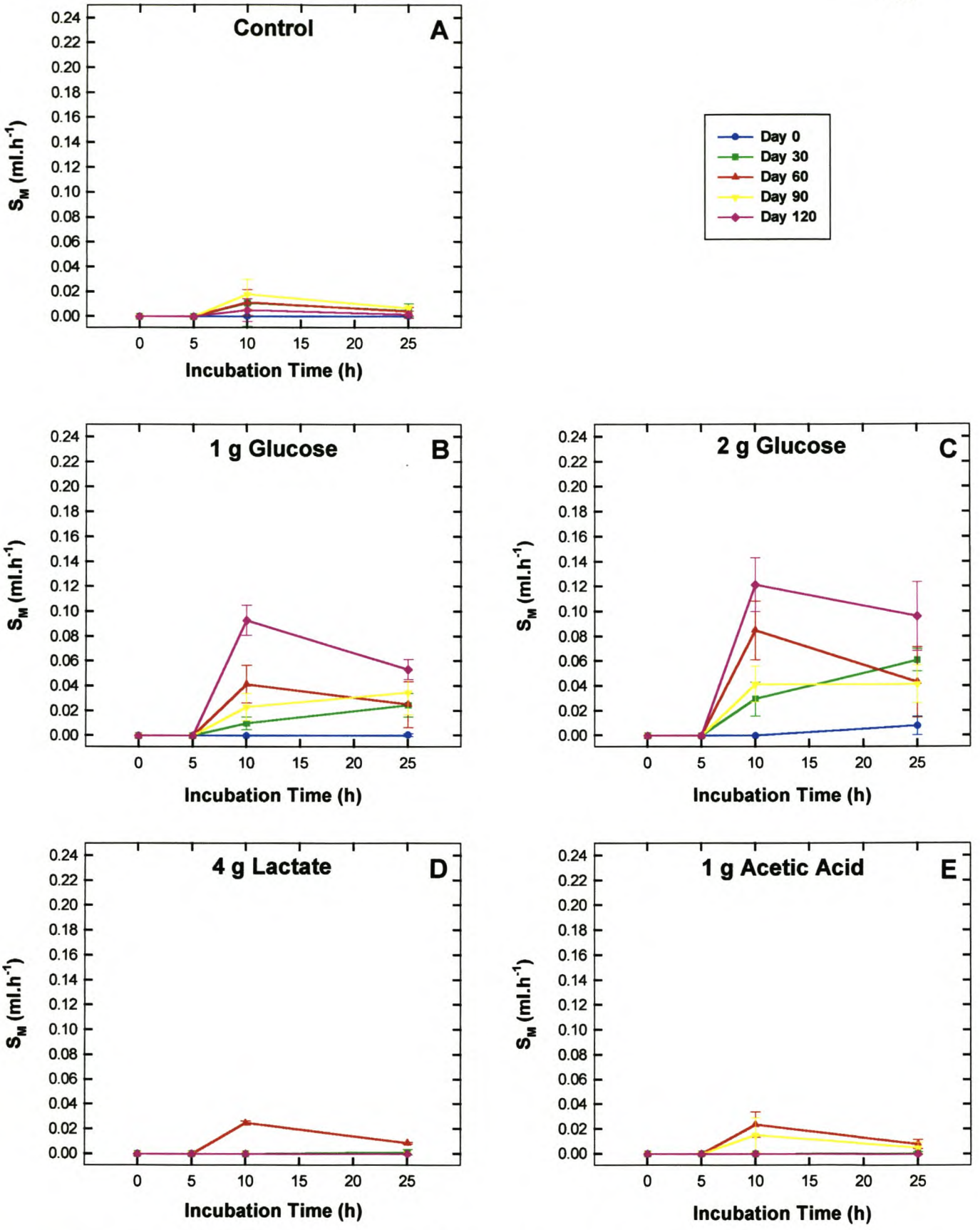

Figure 17. Tempo of methanogenic activity (ml.h^{-1}) (S_M) of vacuum freeze-dried granules using the different test media. The standard deviation was used as the error-bar length.

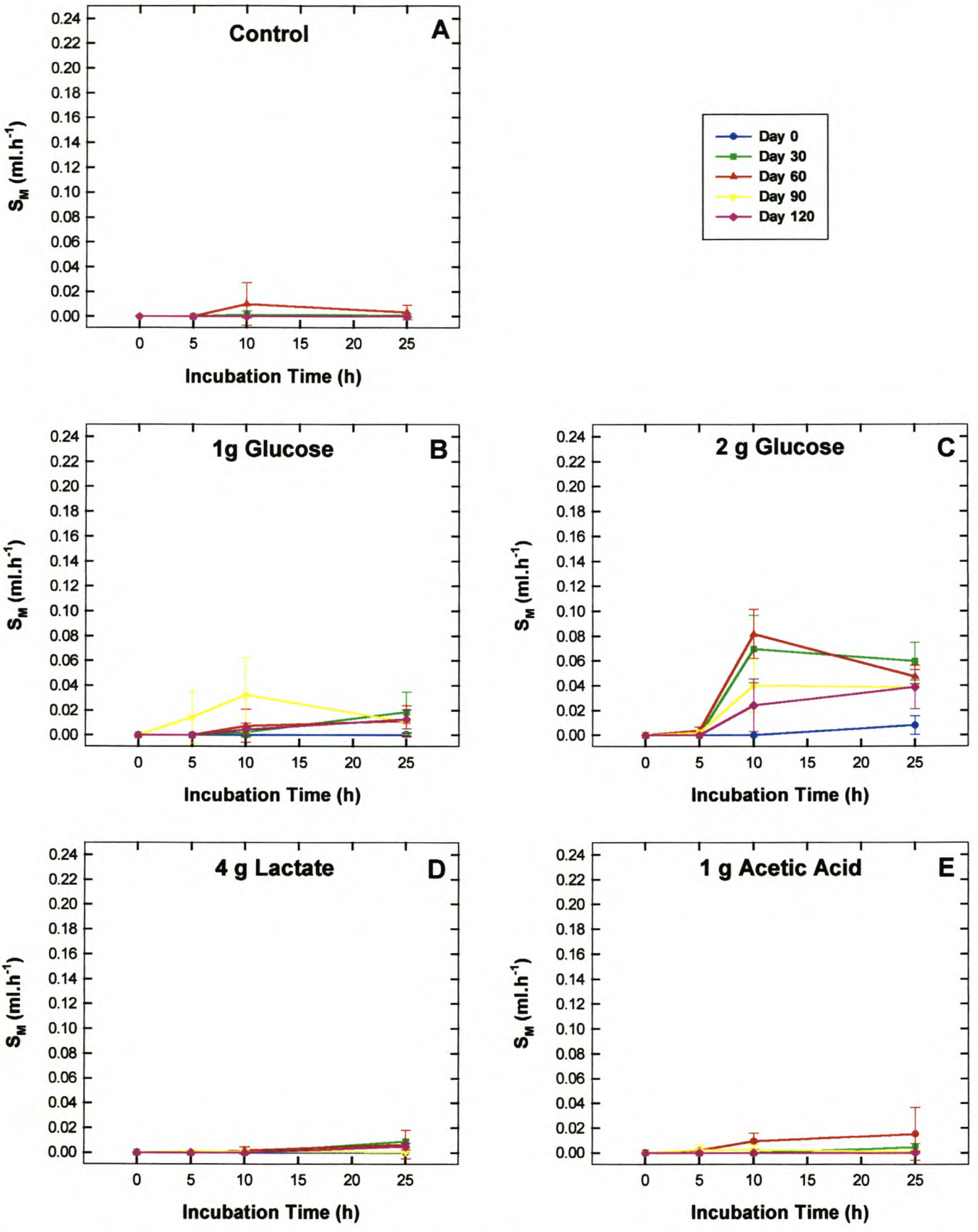

Figure 18. Tempo of methanogenic activity (ml.h^{-1}) (S_M) of frozen granules using the different test media. The standard deviation was used as the error-bar length.

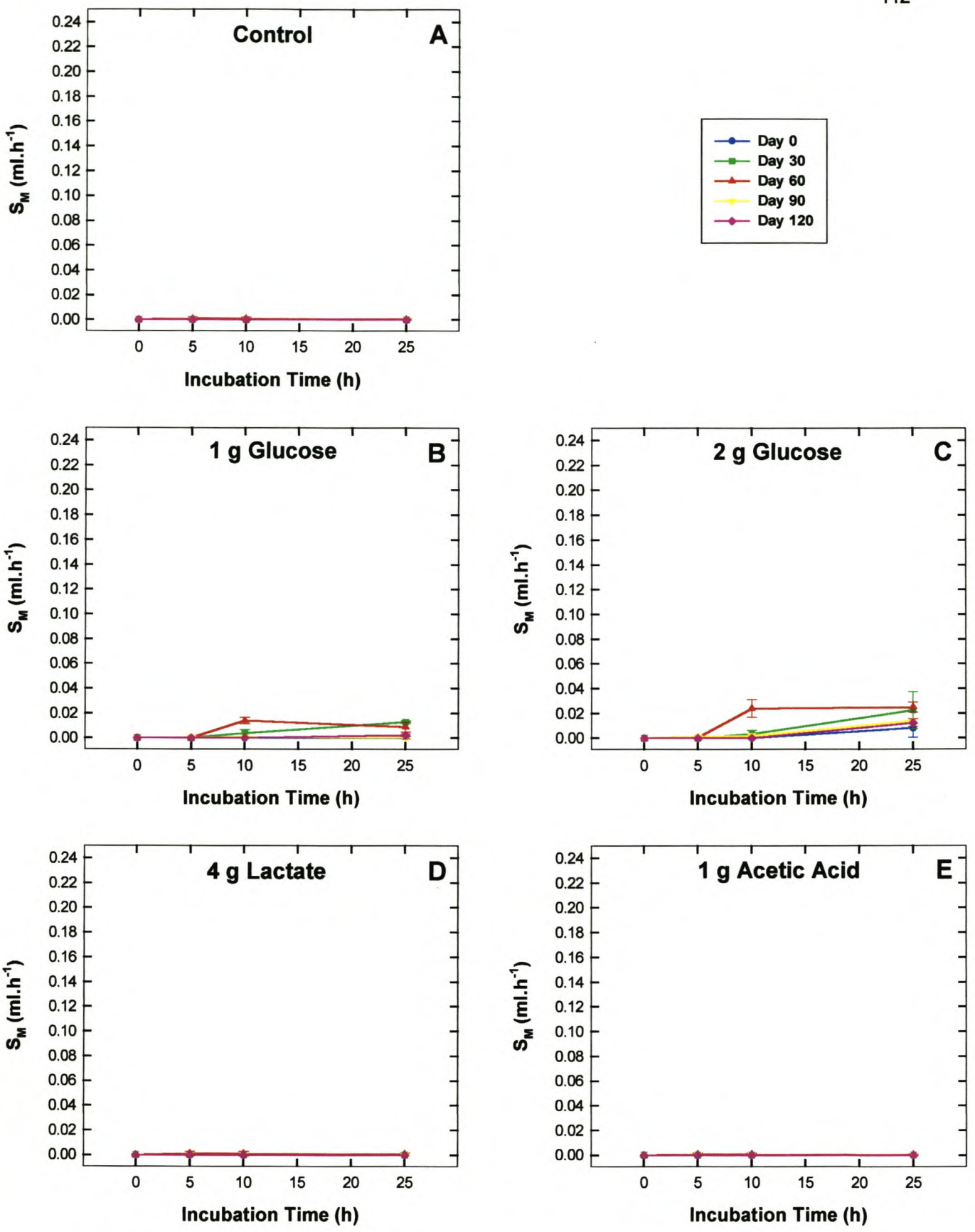

Figure 19. Tempo of methanogenic activity (ml.h^{-1}) (S_M) of cold stored granules using the different test media. The standard deviation was used as the error-bar length.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Background

The growing popularity of the upflow anaerobic sludge blanket (UASB) bioreactor (Alphenaar *et al.*, 1993) as a waste water treatment option makes heavy demands on the availability of seeding inoculum granules. However, UASB reactors can be seeded with raw anaerobic sludge, which will in time lead to the formation of granules through the upward movement and recycling of the wastewater. This aggregation process, although very efficient, can be extremely time-consuming, with granule formation taking up to a year. The mass cultivation of granules (Britz *et al.*, 1999) as described in Chapter 3 of this thesis, drastically shortens the time required for the formation of granular sludge to approximately 30 days, and directly shortens the start-up time of the UASB bioreactor, which could lead to the installation of more reactors.

Carbon and nitrogen source impacts on batch granule cultivation

It has been shown (Britz *et al.*, 1999) that granular sludge can be produced outside a bioreactor in batch systems, and that the stimulus is the application of "stress" that can lead to conditions that will enhance the granulation process. These "environmental stress" conditions include different carbon sources and concentrations as well as changes in the C:P:N ratio's. During this study (Chapter 3) it was found that the composition of the growth media does influence the enhancement of granulation in a batch system. It was, however, found that the carbon source and concentration had a more profound effect on granule enhancement than the use of different nitrogen sources and concentrations.

It was evident, from the results of the first phase of this study, that glucose at a concentration of 2 $g.l^{-1}$ is the best carbon source to be used for granulation enhancement. A concentration of 10 $g.l^{-1}$ lactate led to the second best granulation enhancement, followed by 2 and 5 $g.l^{-1}$ sucrose. The results obtained

where (FC) was part of the carbon source were lower than anticipated. Fruit cocktail effluent did, however, lead to granulation enhancement especially when used in combination with lactate. The data also clearly showed that the presence of lactate stabilised the system, thereby preventing acidification, which is probably caused by the use of this easily fermentable carbon source. The use of FC as carbon source offers an alternative option for the 'treatment' of canning factory waste water, and bearing the economics in mind, it provides an easily fermentable carbon source at very low cost.

Lactate, although very expensive (138c per 1 g C compared with glucose at 17c per 1 g C), has the advantage of stabilising the system. Each time lactate was used as part of the carbon source, even at low concentrations, the units did not acidify. It is, therefore, recommended that low concentrations of lactate are added to the growth medium as a precaution when cultivating granular sludge.

When different nitrogen sources were evaluated, it was found that urea, at all three concentrations used, led to the best granule enhancement. Ammonium sulphate at low concentrations (1 $g.l^{-1}$) gave the second best enhancement results. The disadvantage of using ammonium sulphate as nitrogen source is the subsequent production of H_2S-gas. However, if the wastewater to be treated contains large amounts of sulphate, is might be useful to use ammonium sulphate as the nitrogen source during the cultivation of granules for this specific wastewater thereby giving the sulphate-utilising bacteria the advantage of being incorporated in these granules.

Perhaps the most significant problem of this study was sludge standardisation. The raw anaerobic sludge used in the different experiments was all collected from the same local sewage works, but at different times. Anaerobic sludge is a 'dynamic' biological system and, therefore, the composition is seldom the same. This variation in sludge composition resulted in widely varying numbers of granules, thereby making comparisons between the different studies difficult. It is, therefore, clear that a method for sludge standardisation needs to be developed before significant comparisons between different batch granule cultivating studies will be possible. Volatile solids (VS) and total solids (TS) of the inoculum have been used as a means of standardisation (Ahring & Schmidt, 1992), but this method merely determines the solids content of the inoculum. The TS and VS determinations do not give an indication of the enhancement of granule numbers,

but rather the increase in biomass (in grams). It is thus clear that this is not a very efficient method for the standardisation of raw anaerobic sludge.

To try and overcome the problem of standardisation and to facilitate comparisons between the different studies possible, the different investigations of studies that gave the best granular enhancement were repeated using a standardised sludge. The sludge was 'standardised' by sieving and then concentrated by centrifuging. The sludge inoculum was thus more uniform, but the number of granule nuclei in the inoculum was still a varying factor. Even though the results obtained with this last phase did enable more meaningful comparisons, a method for the standardisation of the number of granules in the inoculum would lead to even more useful results.

Preservation and storage of batch grown granules

To be able to provide industries with an acceptable and active granular sludge for use in UASB wastewater treatment systems, a means of preserving the granules without loss of activity is required. Seasonal industries, for example the fruit canning industry, generate large volumes of wastewater during peak harvesting times, which can successfully be treated using an UASB (Trnovec & Britz, 1998; Britz *et al.*, 2000). During the off-season, the granules used in the UASB bioreactors need to be preserved without the loss of activity to ensure a short start-up period for the following season. Also, granular sludge that is mass-produced in a batch culture needs to be preserved before distribution. In the second study (Chapter 4) of this thesis, six different granule preservation techniques were evaluated in terms of biogas production and methanogenic activity over a period of 120 days. This was done in order to assess the impact of the different preservation techniques, and storage conditions and period, on the activity of the batch cultivated granular sludge.

Of the six preservation techniques evaluated, the freeze-dried samples (FD) were found to retain the best activity for up to 90 days. However, it was found that the activity of both the freeze-dried (FD) and vacuum freeze-dried (VFD) samples decreased after 90 days of storage. The VFD samples, however, showed an increase in activity with the glucose enriched basic test medium (BTM) after 120 days of storage. No logical explanation for this could be formulated. Room

temperature (R) preservation showed increased activity only after 90 days of storage. Once again, this phenomenon cannot be explained as nothing was altered either in the storage conditions or in the composition of the BTM. Prolonged storage at room temperature may have led to cell hydrolysis and the metabolites in these cells could then possibly have provided energy sources that could have been utilised by the rest of the population and led to an increase in activity. From the results obtained with freezing (F) and cold storage (C) as preservation method, it was clear that these two techniques showed very poor activity retainment of the granules.

The granular sludge used in this study was batch-grown on a glucose-rich canning effluent and it is possible that the canning effluent composition resulted in glucose-utilisers becoming the dominant group of the acidogenic population. They could then provide the metabolites needed by the methanogens, thereby enhancing the activity of the methanogens. The low activity measured when acetic acid was added to the BTM, however, suggests that the acidogens probably produced other metabolites that were utilised by the methanogens, resulting in high levels of methanogenic activity measured with the glucose enriched BTM. This is something that needs to be followed up in future research.

The data of the activity testing indicated that the most activity occurred after 10 h of incubation with a decrease by 25 h. Thus, when the tempo of biogas and methane production is to be an indication of activity for evaluation testing, it is advisable to use an incubation time of 10 h. It is furthermore suggested, based on the results obtained during this study, that the enrichment of the BTM + 2 $g.l^{-1}$ glucose as sole test medium is sufficient for activity determinations, considering that evaluations with this medium showed almost double the activity when compared to activity levels with only the BTM, the BTM + 4 $g.l^{-1}$ lactate and the BTM + 1 $g.l^{-1}$ acetic acid.

For optimum retainment of activity, the storage of granular sludge can only be undertaken for periods of up to 90 days. With batch cultivation technology becoming more freely available and the aspect of tailor-made granules being considered, a storage period of 90 days with excellent activity retainment will be more than sufficient. Active granular sludges for inoculation purposes could, therefore, be tailor-made on order, preserved with the freeze-drying technique and shipped to the customer. If the economics involved with the preservation

technique is considered important, vacuum-drying may be a more viable option, since good activity retainment can still be achieved.

Concluding remarks

Thus, as far as future research is concerned, the application of the results from this study, on batch granulation enhancement and preservation, provides practical information that will contribute to the successful development of this technology. A few questions were, however, raised during the course of this study that will need to be addressed in future. These include the increase-decrease phenomenon in granule counts (Chapter 3), as well as the interesting high methanogenic activity results that were noted when granular sludge was tested with glucose rather than acetic acid as main test medium component (Chapter 4). The increase-decrease trend over 14 days (Chapter 3) was also reported by Britz *et al.* (1999) who postulated that this phenomenon might be the result of the granules clumping together and then getting larger. This will, however, need to be confirmed in future by looking at the size distribution of the granules rather than just the granule counts alone. Another trend noted, that requires more research was that once the granular sludge was standardised in terms of size, the actual granule sizes reported by Britz *et al.* (1999) were not observed, although increases in granule numbers could be seen with the image analysis technique.

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