THE ROLES OF CALCIUM IN SLUDGE GRANULATION DURING UASB REACTOR START-UP

H. Q. YU[1]*[M], J. H. TAY[2][M] and HERBERT H. P. FANG[1][M]

[1] Environmental Engineering Research Centre, Department of Civil Engineering, Hong Kong University, Pokfulam Road, Hong Kong and [2] School of Civil & Structural Engineering, Nanyang Technological University, Nanyang Avenue, Singapore

Abstract—Six upflow anaerobic sludge blanket (UASB) reactors were concurrently operated for 146 d to examine the effects of calcium on the sludge granulation process during start-up. Introduction of Ca^{2+} at concentrations from 150 to 300 mg/l enhanced the biomass accumulation and granulation process. The calcium concentration in the granules was nearly proportional to the calcium concentration in the feed, and calcium carbonate was the main calcium precipitate in the granules. The specific activity of granules decreased with increasing influent calcium concentration. The optimum calcium concentration for the granulation was from 150 to 300 mg/l. The addition of low-concentration calcium to the UASB reactors appeared to enhance the three steps of sludge granulation: adsorption, adhesion and multiplication, but it did not lead to a different proliferation of predominant microorganisms in the granules. © 2001 Elsevier Science Ltd. All rights reserved

Key words—calcium, granulation, start-up, upflow anaerobic sludge blanket (UASB) reactor

INTRODUCTION

The concept of the upflow anaerobic sludge blanket (UASB) reactor was developed in 1970s (Lettinga *et al.*, 1980). Today the UASB reactor has become the most popular high-rate reactor for anaerobic treatment of wastewater throughout the world (Hulshoff Pol, 1989; Lettinga *et al.*, 1993). The treatment capacity of UASB reactors depends on the amount of active biomass retained, as well as the contact between biomass and wastewater. Retention of an adequate level of methanogenic bacteria in UASB reactors gives good performance in terms of chemical oxygen demand (COD) removal and methane yield (Hulshoff Pol, 1989; Fang *et al.*, 1994). To a great extent, the start-up of a UASB reactor is dependent on the formation of granules. Granulation is the process in which suspended biomass agglutinates to form discrete well-defined granules. Microbial granulation is a complex process, involving different trophic bacterial groups, and their physico-chemical and microbiological interactions (Schmidt and Ahring, 1996). Many factors contribute, in one form or another, to the granulation process (de Zeeuw, 1984; Fang *et al.*, 1995). Granulation may be initiated by bacterial adsorption and adhesion to inert matters, to inorganic precipitates (Lettinga *et al.*, 1993; Mahoney *et al.*, 1987),

and/or to each other through physico-chemical interactions and syntrophic associations (Dolfing *et al.*, 1985). These substances serve as initial precursors (carriers or nuclei) for further bacterial growth. Filamentous bacteria may also play a role in forming matrices which further embed other cells (Schmidt and Ahring, 1996). These loosely adhered bacterial aggregates are strengthened by extracellular polymers secreted by bacteria. These initial granules will grow continuously into compact mature granules, if favorable conditions pertaining to bacteria are maintained (Thaveesri *et al.*, 1995).

It has been shown that some divalent metal ions, such as Ca^{2+} and Fe^{2+}, enhance the granulation (Grotenhuis *et al.*, 1991; Kosaric and Blaszczyk, 1990; Mahoney *et al.*, 1987; Schmidt and Ahring, 1993). The action of enzymes, required for proper energy metabolism in living cells, is influenced by metallic co-factors (Schmidt and Ahring, 1996). Divalent ions were also reported to play an important role in microbial aggregation (Mahoney *et al.*, 1987; Kosaric and Blaszczyk, 1990). Grotenhuis *et al.* (1991) found that removal of calcium specific with ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid led to either disintegration or weakening of the structure of granules. Calcium is probably a constituent of extracellular polysaccharides and/or proteins which are used as linking materials (Morgan *et al.*, 1991). It was found that extracellular polymers prefer to bind divalent ions

---

*Author to whom all correspondence should be addressed.

when they are available due to the formation of more stable complexes (Rudd *et al.*, 1984).

Versprille (1978) observed a distinct improvement in the biomass settleability and specific activity after replacing $NaHCO_3$ as a neutralizing agent by $Ca(OH)_2$ in the treatment of potato processing wastewaters. Hulshoff Pol (1989) obtained similar results with the treatment of distillery waste. De Zeeuw (1984) demonstrated that concentrations of 100 mg/l Ca^{2+} in the wastewater stream enhanced the rate of biomass granulation. Cail and Barford (1985) also stated that granule formation was stimulated by the presence of calcium concentration at 150 mg/l. At concentrations of 100–200 mg/l, calcium was found to exert a positive impact on granulation (Mahoney *et al.*, 1987). Mineral precipitates such as $CaCO_3$, $Ca_5OH(PO_4)_3$, and FeS are formed, as a result of metabolic activities and physico-chemical reactions, and are accumulated either inside or on the surface of granules (Dolfing *et al.*, 1985). However, there are also contradictory reports about the role of Ca^{2+} in granulation. Some researchers have argued that Ca^{2+} does not induce granulation (Guiot *et al.*, 1988), or is not a key element in granules (Alibhai and Forster, 1986), or is even detrimental to granule formation together with phosphate (Thiele *et al.*, 1990). Moreover, in all the previous studies only one or two calcium concentrations were used. Information about the effects of calcium at various levels on the sludge granulation process is not available.

This study was thus conducted to examine the effects of calcium on the granulation process during start-up of UASB reactors. Six reactors were operated in parallel. The main input variable was the influent Ca^{2+} concentration, which was varied from 10 mg/l, which corresponded to no excess calcium addition, to 150, 300, 450, 600 and 800 mg/l. The relationship between the calcium concentration in the feed and the biomass accumulation, specific activity, density and compositions of granules was determined. The biomass concentration profiles along the reactors and the size distribution of granules were also measured to track and to assess the granulation process.

MATERIALS AND METHODS

Reactor design

Experiments were performed in parallel in six identical UASB reactors. The schematic diagram of one of the six UASB reactors is shown in Fig. 1. The empty bed volume of each reactor was 7.3 l with an internal diameter of 100 mm and a liquid height of 930 mm. Six sampling ports were installed along the reactor height. The reactors were housed in a temperature control room maintained at $35 \pm 1°C$. The reactor (R1) without additional $CaCl_2 \cdot 2H_2O$ served as control. The reactors added with calcium concentrations of 150, 300, 450, 600 and 800 mg/l were designated as R2, R3, R4, R5 and R6, respectively.

Wastewater characteristics

Synthetic wastewater was used as feed to the six reactors. Its strength was kept constant at 4000 mg-COD/l throughout the study. Table 1 shows the detailed composition of the synthetic waste. The ratios of COD : N : P were approximately 200 : 4 : 1. The buffer capacity was provided by the addition of sodium bicarbonate. Initially the sodium bicarbonate dosage was 1500 mg/l, and subsequently increased to 2000 mg/l on day 30 and to 2500 mg/l on day 60.

Seed sludge

The seeding inoculum was the sludge taken from the anaerobic digester of a local sewage treatment plant. The raw sludge was screened through a 0.6-mm sieve to remove the big debris and fibers before seeding. The characteristics of inoculum are summarized in Table 2. Each reactor was seeded with 3.5 l of inoculum. After that, the reactors were filled with the synthetic wastewater, and then allowed to stand for 24 h as initial acclimation before start-up.

Experimental procedure

The initial organic loading rate (OLR) to the reactors was set at 2.0 g-COD/l d with a hydraulic retention time (HRT) of 48 h. The OLR was then increased in steps to 2.7, 4.0, 5.3 and 8.0 g-COD/l d by reducing HRT to 36, 24, 18, and 12 h, correspondingly. The OLR of each reactor was increased to the next level only after the pseudo-steady state was reached, as indicated by a constant gas production rate ($\pm 5\%$) and effluent COD concentration ($\pm 8\%$). The five OLRs were kept for 20, 25, 30, 30 and 41 days, respectively, as shown in Fig. 2(a).

Methods of analysis

The operational parameters measured included COD removal efficiency, biogas production, biogas composition, effluent volatile suspended solids (VSS) concentration, pH, and particle size distribution. Biomass were periodically taken from the sampling points arranged along the height
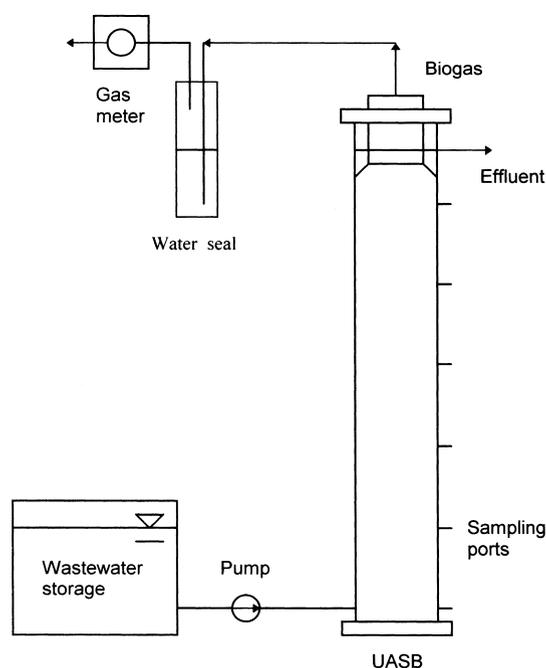
Fig. 1. Schematic diagram of one of six UASB reactors.

Table 1. Composition of the synthetic wastewater of 4000 mg-COD/l

Constituents	Concentration (mg/l)
Peptone	800
Glucose	2720
Meat extract	560
Sodium bicarbonate, $NaHCO_3$	1500–2500
Calcium chloride, $CaCl_2 \cdot 2H_2O$	38
Magnesium sulphate, $MgSO_4 \cdot 7H_2O$	42
Ammonium chloride, NH_4Cl	320
Ferrous sulphate, $FeSO_4 \cdot 7H_2O$	32
Potassium dihydrogen phosphate, KH_2PO_4	60

Table 2. Characteristics of seeding inoculum

MLSS (g/l)	23.6
MLVSS (g/l)	17.0
MLVSS/MLSS ratio	0.72
Specific methanogenic activity (g-CH$_4$-COD/g-VSS d)	0.26
Sludge volume index (ml/g)	43

of the reactors to determine the mixed liquor volatile suspended solids (MLVSS) concentrations. Analysis of COD, pH, phosphorus, dry weight and density of the biomass, VSS and MLVSS were performed according to the *Standard Methods* (APHA, 1992).

Gas production was recorded by wet gas meters (Shinagawa W-NK-0.5) and gas composition was analyzed by gas chromatography (HP 5890A-GC) with a 2-m long and 3-mm-ID packed column (Haye-Sep Q, 80/100 mesh) and a thermal conductivity detector at 200°C. Helium was used as the carrier gas with a flowrate of 30 ml/min.

Metals in the liquid samples and biomass were determined by atomic absorption spectroscopy (Perkin-Elmer Model 2280). A known volume of filtrate was dried and the remaining was re-dissolved in hot concentrated HCl solution and filtered into 50 ml volumetric flasks. Samples were analyzed by atomic absorption spectroscopy using hollow cathode lamps and acetylene/air flame.

For estimating the size distribution, the sludge samples taken from the bottom sampling points were classified into six fractions using laboratory sieves with various openings (0.2, 0.6, 1.0, 2.0, 4.0 mm). The sludge particles were first placed in the sieve with the biggest opening (4.0 mm). The particles were gently submerged in water and shaken to let the smaller particles pass through. The procedures were repeated until the five sieves were used. The specific methanogenic activity (SMA) of sludge samples taken from the bottom sampling points was measured in a 250-ml Kimax flask at 35 ± 1°C under anaerobic conditions, using the method reported previously (Fang *et al.*, 1995). The SMA analyses were conducted in triplicate using acetate as substrate.

RESULTS

The performance of the reactors, in terms of COD removal efficiency, effluent VSS concentration, and methane production, was continuously improving with operation. The pH values of the effluents from the three reactors were in the range of 6.8–7.5.

COD removal

The COD removal efficiencies of the reactors are illustrated in Fig. 2(b) and (c). During the first 2 weeks, the COD removal efficiencies were low. The
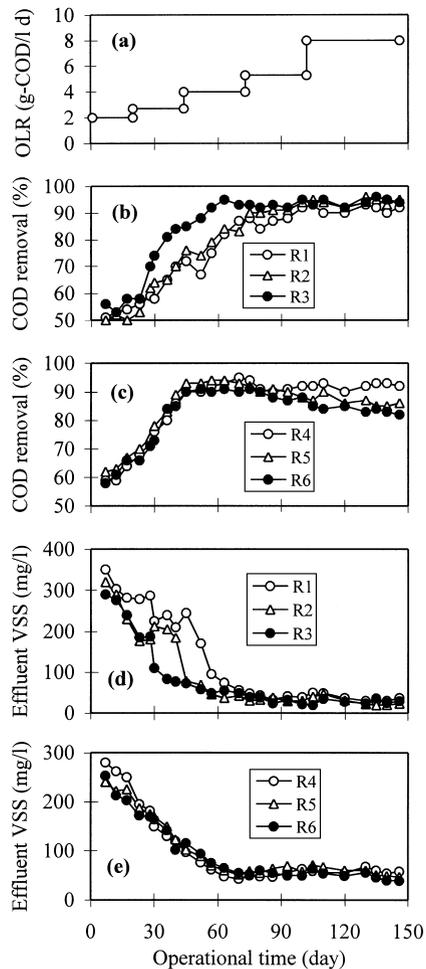
Fig. 2. Operational parameters and performance of the reactors: (a) OLR; (b) COD removal efficiencies of R1, R2 and R3; (c) COD removal efficiencies of R4, R5 and R6; (d) effluent VSS concentrations of R1, R2 and R3; (e) effluent VSS concentrations of R4, R5, and R6.

control reactor (R1) had the highest effluent COD concentration. With the progress of the experiment, the COD removal efficiencies of all the reactors generally kept increasing. It is evident that the efficiency of the calcium-added reactors increased faster than that of R1 at this stage. When the OLR was increased to 4.0 g-COD/l d, the performance of R1 still remained inferior to the other five reactors. However, after reaching an OLR of 5.3 g-COD/l d, R1, R2 and R3 achieved almost the same level of COD removal efficiencies, while the COD removal efficiencies of the reactors with higher dosages of calcium, i.e. R4, R5 and R6, were lower than those of R1, R2 and R3 at the same OLR.

Effluent VSS concentration

The effluent VSS profile is an indication of the reactor retention of biomass at a given OLR

[Fig. 2(d) and (e)]. Because of inferior settleability of the biomass, more biomass was washed out from R1, resulting in a higher effluent VSS concentration than from the other reactors at initial stages. With the progressive improvement of biomass settleability and granule formation, however, less VSS was discharged from R1. Initially (days 1–10), the effluent VSS concentration from R2 was also high during the period of biomass washout, and gradually decreased thereafter. After granules were observed on day 30 for R2, the effluent VSS concentrations kept declining. The lower effluent VSS concentration from R2 was associated with the improvement of biomass settleability. The effluent VSS concentration from R3 was the lowest at initial stages. This was due to the better biomass settleability and earlier granule formation in R3. The effluent VSS concentrations of the reactors with high calcium concentrations ($\geq 450\,$mg/l) were slightly higher than those of R2 and R3.

Biomass concentration and biomass profiles

The accumulation of biomass for the different influent calcium concentrations on day 146 is presented in Fig. 3. For low calcium concentrations biomass accumulation increased with increasing calcium, and reached a maximum at 300 mg/l of calcium. However, at higher calcium concentrations, biomass concentrations decreased. The presence of low-strength calcium in R2 and R3 improved biomass retention of the reactors, while higher dosage of calcium did not significantly enhance biomass retention.

The profiles of biomass concentration during different stages is shown in Fig. 4. Initially, the biomass was loose and expanded easily. With the progress of granulation, the biomass was progressively stratified with the granules settled in the lower part of the reaction zone. When granules were formed increasingly in the reaction zone, a dense sludge bed and a thin sludge blanket were formed with a clear interface between them.

After 30 days of operation, small granules with diameters of 0.2–0.6 mm became visible at the bottom of R2. This initial granules grew rapidly, and after 30 more days of operation large granules with diameters over 2.0 mm were formed. The rapid

granulation was mainly due to increasing biological growth under the high OLR (4.0 g-COD/l d). After about 30 days of rapid growth, the granular growth rate became slower, indicating that a mature and stable granulation developed. R3 had a granulation process similar to that in R2.

Distribution of granules

The size distributions of the granules are shown in Fig. 5. In R1, no granules were found until day 65. Sixty-five days into start-up, approximately 45% (by weight) of the sludge samples were in the range of 0.2–0.6 mm. The granule size distribution for R1 was significantly different from that for the other reactors at any given stages. On day 146, approximately 60% of the granules from R1 were in the range of 1.0–4.0 mm and only 8% of the sample measured above 4.0 mm.

In R2, the granule distribution profile on day 30 was askew with 22% of the sample lying in the 0.2–0.6 mm range. By day 60, about 61% of the granules were measured below 1.0 mm, while the size of 14%

Fig. 3. Biomass concentration vs. influent calcium concentration after 146 days.
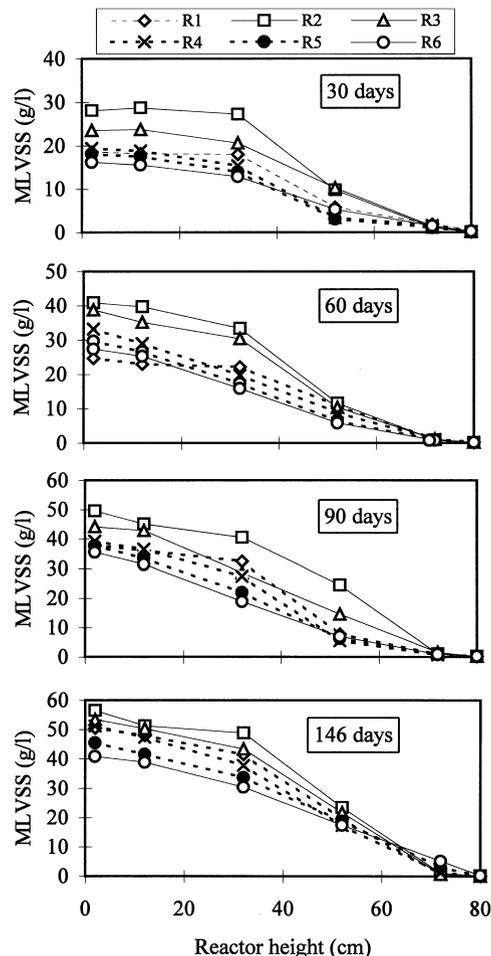
Fig. 4. Profiles of biomass during different stages of start-up.

of the sample measured above 2.0 mm. When the experiment was terminated on day 146, about 14% of granules were larger than 4.0 mm. This result implies that the presence of calcium in R2 had promoted granule formation by allowing aggregates to form earlier and achieve a larger size.

In terms of granules size distribution, the higher the influent calcium concentration, the greater the average size of the granules was (Fig. 5). However, the size distribution of the sludge from the bottom of R6 could not be readily measured, because there was a tendency of cementation for the sludge bed.

SMA measurement

Table 3 lists the SMA values of the granular sludges, on days 30, 60, 90 and 146, using acetate as substrate. In general, the methanogenic activity of granules increased steadily with increasing OLR for all the reactors at initial stages. After OLR was increased to 5.3 g-COD/l d, the SMA values of R2 throughout R6 slightly dropped, while the SMA value of R1 still increased. At the end of the experiment, the SMA values of R1 was 1.32 g-CH$_4$-COD/g-VSS d, higher than the corresponding SMA values of R2 throughout R6.

The above result showed that the SMA of granules tended to decrease with an increase in calcium concentration in the medium, indicating that the granules at high calcium concentrations had a lower metabolic activity. Since the overall performance of a UASB reactor depended on the concentration of biomass and the biomass specific activity, and the

UASB reactors with lower dosages had higher biomass concentrations than the UASB reactors with higher dosages, R2 and R3 had better performance than R5 and R6 in terms of COD removal efficiency.

Calcium concentration in granules

Figure 6(a) illustrates that the calcium concentration in the granules, taken from the bottom sampling points on day 146, was nearly proportional to the calcium concentration in the substrate. Calcium accumulation within granules mostly resulted from the interaction of calcium ions with carbonate and exopolysaccharide polymers because calcium concentration in bacterial cells is very low. The formation of calcium carbonate and the binding of calcium to the exopolymers and cells in granules are a nonspecific process and are driven by the calcium ion gradient from the bulk liquid phase into the granules. Since the reactors were fed with synthetic wastewater with a calcium content and since

Table 3. SMA (g-CH$_4$-COD/g-VSS d) using acetate as substrate for the biomass in the six reactors

Reactor	Operating time (d)				
	0	30	60	90	146
R1	0.26	0.70	1.04	1.28	1.32
R2	0.26	1.05	1.20	1.18	1.04
R3	0.26	0.92	1.17	1.09	0.94
R4	0.26	0.96	1.04	0.92	0.86
R5	0.26	1.02	0.95	0.82	0.70
R6	0.26	0.89	0.92	0.73	0.58
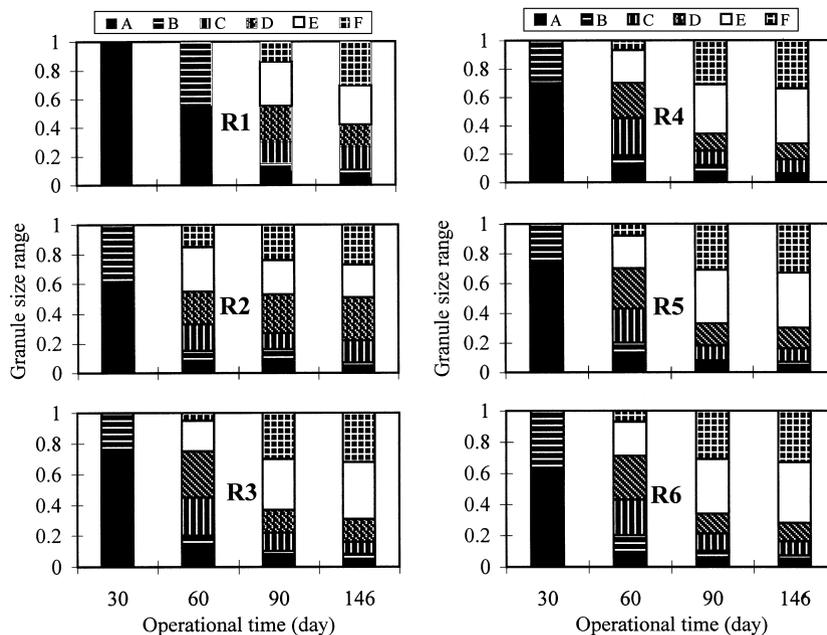
Fig. 5. Size distributions (by weight) of granules taken from the bottom sampling points of each reactor. (A) < 0.2 mm; (B) 0.2 < *d* < 0.6 mm; (C) 0.6 < *d* < 1.0 mm; (D) 1.0 < *d* < 2.0; (E) 2.0 < *d* < 4.0 mm; (F) > 4.0 mm.

bicarbonate was produced from the anaerobic degradation of the substrate and was added in the influent, calcium carbonate precipitation prevailed in the granules. Fig. 6(a) shows that the calcium concentrations in the granules were around 10 times greater than in the feeds. They ranged from 1250 to 6700 mg/l. The scanning electron microscopic observations showed that the granules had a layered structure, consisting of a core of calcium precipitates with an exterior layer of granules (Fig. 7). This type of structure was also observed by van Langerak *et al.* (1998) and Uemura and Harada (1995).

In addition to carbonate, phosphate might also form precipitates with calcium inside granules. The P-contents of approximately 60 mg-P/g-VSS were measured for the granules from the six reactors. This value was higher than could be expected from biological assimilation. Anaerobic sludge contents about 10 mg-P/g-VSS (Alphenaar *et al.*, 1993). Hence, precipitation of calcium phosphate also occurred in the granules. However, since the influent phosphate concentration was very low (< 18 mg/l) and the P-contents of the granules from the six reactors were of similar levels, calcium phosphate seemed to be a minor part of calcium precipitates in the granules.

Granule composition and density

As shown in Fig. 6(b), the granules contained 11–44% dry mass. When calcium concentration in the substrate was raised, the water content of the granules decreased and the total dry mass increased. In the dry materials, the proportion of minerals increased significantly while that of organics reduced as indicated by the decrease in MLVSS/MLSS [Fig. 6(c)]. This indicates that the presence of calcium increased the dry mass of the granules mainly by increasing the concentration of minerals in the granules. The increased mineral content was very likely the result of more calcium precipitates trapped in the granules. The density of the granules also increased with increasing calcium concentration in the feed [Fig. 6(d)], showing the changes in the granular composition.

Scanning electron microscopic observation

Scanning electron microscopic observation revealed that the sludges from R1 throughout R6 had similar compositions and morphology. The granules from the six reactors were predominantly composed of *Methanosarcina*-like species. This implies that the addition of calcium from 150 to 800 mg/l did not result in a difference in predominant microorganisms, despite that the addition at low concentrations accelerated the granulation process and led to the formation of larger size granules.
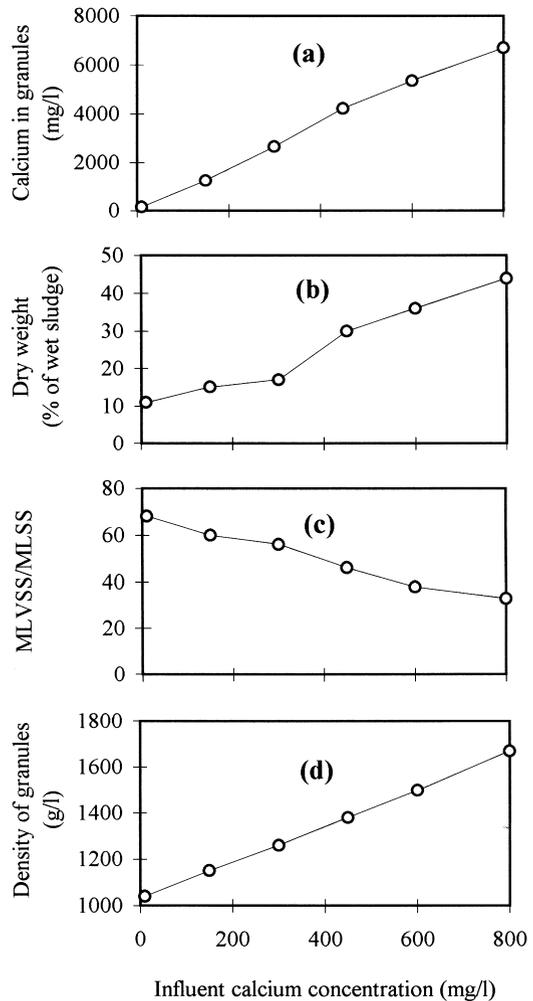
Fig. 6. (a) Calcium concentration in granules after 146 days; (b) dry weight of granules; (c) ratio of MLVSS to MLSS of granules; (d) density of granules.
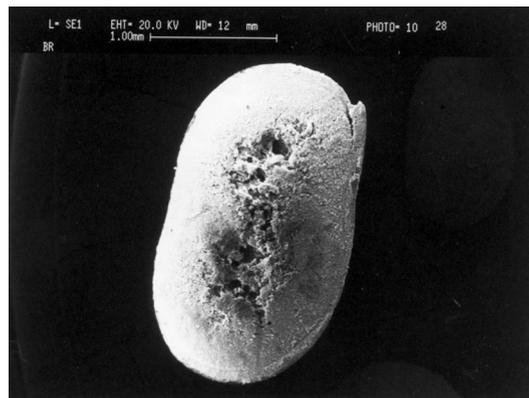
Fig. 7. SEM micrograph of bisected granule.

DISCUSSION

As mentioned in the Section 1, the roles of calcium in sludge granulation are controversial. However, when these studies regarding the effect of calcium on

granulation are analyzed, the disputation among them could be resolved: in the studies where a beneficial effect of calcium on granulation was claimed, low calcium concentrations from 100 to 200 mg/l were used (Cail and Barford, 1985; de Zeeuw, 1984; Mahoney *et al.*, 1987; Hulshoff Pol, 1989), while in the studies where the addition of calcium was found to be detrimental to granulation, high calcium dosages of over 500 mg/l were used (Guiot *et al.*, 1988; Hulshoff Pol *et al.*, 1983; Thiele *et al.*, 1990). The present study clearly demonstrates that low calcium concentrations (≤ 300 mg/l) had a positive influence on sludge granulation process and that high calcium concentrations (≥ 600 mg/l) had a negative influence. This is in agreement with the above research results.

Schmidt and Ahring (1996) suggested that granulation process in UASB reactors could be divided into four steps: (1) *Transport* of cells to the surface of other cells; (2) Initial reversible *adsorption* by physicochemical forces; (3) Irreversible *adhesion* of the cells by microbial appendages and/or polymers; and (4) *Multiplication* of the cells and development of the granules. Any factor which can enhance any one of the four steps will be able to accelerate the granulation process and shorten the start-up time of UASB reactors. The results from this study and others (Kosaric and Blaszczyk, 1990; Mahoney *et al.*, 1987) suggest that low-concentration calcium might accelerate the three steps of granulation: adsorption, adhesion and multiplication, as described below:

Calcium is a constituent of extracellular polysaccharides and/or proteins which are used as adsorbing and linking materials (Morgan *et al.*, 1991). Calcium precipitates were also reported to provide natural inert supports for the bacteria. These inert supports play an important role in initial adsorption (Rudd *et al.*, 1984). The strength of this type of adsorption depends on different physico-chemical forces such as ionic and dipolar. In this study, as shown in Figs 3 and 4, granule growth in the calcium-added reactors increased faster than the control reactor. Calcium has been implicated in the adsorption process because of their ability to bridge between the electronegative carboxyl and phosphate groups associated with bacterial surfaces (Shen *et al.*, 1993).

The presence of calcium strongly enhances the adhesion of the cells by microbial appendages and/or polymers. Extracellular polymers prefer to bind divalent metals when they are available due to more stable complexes (Rudd *et al.*, 1984). The bacterial cell surface and extracellular polymers are usually negatively charged and their associate with each other to form granules often requires cations, mostly divalent cations like Ca^{2+}, to act as a link between the components. Therefore, the presence of calcium ions would facilitate the linkage of cell–polysaccharide and polysaccharide–polysaccharide. Forster and Lewin (1972) confirmed the expected role of calcium in biomass floc structure and strongly suggested that

calcium ion has especially a high affinity for extracellular polymer structure, indicating that calcium is involved in the adhesion process. Free calcium is essential to the structural integrity of biofilm because it maintains the tertiary structure of polymeric substances so that the interactions between the adjacent sugars on different chains are promoted (Turakhia and Characklis, 1988). Calcium ion may also contribute to the stability of the conformation of the polymer network in the granules because of its interactions with the secondary function groups in polysaccharides like OH^-, and consequently, its influence on the folding of the polymers (Turakhia and Characklis, 1988).

Calcium also probably plays a role in multiplication of the cells and development of the granules. This was also verified by that the presence of calcium in the calcium-added reactors had promoted granule formation by allowing aggregates to form earlier and to reach a larger final size (Figs 4 and 5). As microbes aggregate there is an enhanced opportunity for cross-feeding, co-metabolism and inter-species hydrogen and proton transfer which may further stimulate growth of micro-colonies (Harvey *et al.*, 1984). Calcium may act not only to facilitate cell–cell bridging but also to promote growth of aggregates indirectly (Mahoney *et al.*, 1987).

On the other hand, the presence of too much calcium in the granules could damage the environment required for maintenance of the granular structure or the bacterial activity. The amount of calcium that precipitated in each reactor varied considerably. Two factors determined the amount of calcium precipitated: (1) The influent calcium concentration; the higher the influent calcium concentration, the higher the calcium precipitated; and (2) The influent COD concentration and COD removal efficiency; a higher COD concentration and COD removal efficiency resulted in a higher calcium carbonate deposition. Since R1 throughout R6 had the same influent COD level and the difference in their COD removal efficiencies was insignificant, the different calcium precipitate concentrations in the granules from the six reactors were only attributed to the difference in the influent calcium concentrations.

Table 3 indicates that calcium carbonate precipitation had a negative effect on the activity of the biomass. Higher influent calcium concentration led to higher ash content in granules and severer mass transfer limitations. These observed lower SMA values of R4, R5 and R6 might be attributed to the presence of larger granules and higher ash content in granules, in which mass transfer was significantly reduced. Mass transfer inside the granules is considered to be solely controlled by diffusion (Pavlostathis and Giraldo-Gomez, 1991). The resistance to substrate diffusion inside granules increases proportionally with physical granular size and ash content, making the substrate less available to the granule

core and eventually resulting in substrate deficiency or depletion inside granules (Alphenaar *et al.*, 1993). The order of calcium dosages was: R6 > R5 > R4 > R3 > R2 > R1, while the order of SMA value of the granules in the end of the experiments was: R1 > R2 > R3 > R4 > R5 > R6 (Table 3). This seems to support the above explanations that residence of substrate diffusion into the granules could be an important factor responsible for the difference in measured SMA values, although other factors, e.g., the population shift and sludge dispersion, could not be excluded.

The toxicity of calcium accumulated in the granules might also have a negative effect on the biomass activity. Kugelman and McCarty (1965) found that the optimum calcium concentration for methanation of acetic acid was 200 mg/l and that the upper limit was from 2000 to 5000 mg/l. In the present study the calcium concentrations in the granules ranged from 1250 to 6700 mg/l [Fig. 6(a)]. For example, when influent calcium concentration was 600 mg/l, the calcium concentration in the granules reached 5350 mg/l, much higher than the upper limit reported by Kugelman and McCarty (1965). Based on the results of Guiot *et al.* (1988) and Thiele *et al.* (1990), and the results from this study, it is postulated that an immunobilized calcium concentration higher than 5350 mg/l in the granules would impose a severe restraint on the specific activity of the granules and be detrimental to granule formation.

The above results and discussion indicate that there is a relationship between the granulation process with the calcium concentration in the feed and that an optimum calcium concentration exists. In the present study, the optimal calcium concentration was found to be between 150 and 300 mg/l when the influent COD concentration was kept at 4000 mg/l. However, since the influent COD concentration has a substantial effect on the concentration of calcium carbonate precipitate in granules, the optimum calcium concentration is thus dependent of the influent COD concentration. Consequently, further experiments are needed to determine the optimum ratio of influent calcium concentration to COD concentration.

CONCLUSIONS

In this work, the effects of calcium on the sludge granulation process during start-up were investigated in six UASB reactors operated in parallel. The following conclusions were drawn from the study:

(1) Introduction of Ca^{2+} at concentrations from 150 to 300 mg/l enhanced the biomass accumulation and granulation process in UASB reactors.
(2) The calcium concentration in granules was proportional to the influent calcium concentra-

tion, and calcium carbonate was the main calcium precipitate in granules.
(3) The specific activity of granules decreased with increasing calcium concentration in the feed. For high calcium concentrations, a large amount of minerals deposited within the granules. This along with the significant decrease in the water content in granules and the toxicity of high-concentration calcium accumulated inside granules caused a lower bacterial specific activity.
(4) The optimum calcium concentration for the granulation in the UASB reactors fed with 4000-mg/l COD wastewater was from 150 to 300 mg/l.
(5) The addition of low-concentration calcium to the UASB reactors probably enhanced the three steps of sludge granulation: adsorption, adhesion and multiplication, but it did not lead to a different proliferation of predominant microorganisms in the granules.

REFERENCES

Alibhai K. R. K. and Forster C. F. (1986) An examination of the granulation process in UASB reactors. *Environ. Technol.* **7**, 193–200.

Alphenaar P. A., Perez M. C. and Lettinga G. (1993) The influence of substrate transport limitation on porosity and methanogenic activity of anaerobic sludge granules. *Appl. Microbiol. Biotechnol.* **39**, 276–280.

APHA (1992) *Standard Methods for the Examination of Water and Wastewater*. 18th ed. Washington, DC, USA.

Cail R. G. and Barford J. P. (1985) The development of granulation in an upflow floc digester and an upflow anaerobic sludge blanket digest treating cane juice stillage. *Biotechnol. Lett.* **7**, 493–498.

De Zeeuw W. J. (1984) Acclimatization of anaerobic sludge for UASB reactor start-up. *Ph.D. Thesis*, Wageningen Agricultural University, The Netherlands.

Dolfing J., Griffioen van A. R. W. and Zevenhuizen L. P. T. M. (1985) Chemical and bacteriolgical composition of granular methanogenic sludge. *Can. J. Microbiol.* **31**, 744–750.

Fang H. H. P., Chui H. K. and Li Y. Y. (1995) Effect of degradation kinetics on the microstructure of anaerobic biogranules. *Water Sci. Technol.* **32**, 165–172.

Fang H. H. P., Chui H. K., Li Y. Y. and Chen T. (1994) Performance and granule characteristics of UASB process treating wastewater with hydrolyzed proteins. *Water Sci. Technol.* **30**, 55–63.

Forster C. F. and Lewin D. C. (1972) Polymer interaction at activated sludge surfaces. *Effluent Water Treat. J.* **12**, 520–525.

Grotenhuis J. T. C., van Lier J. B., Plugge C. M., Stams A. J. M. and Zehnder A. J. B. (1991) Effect of ethylene gIycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid (EGTA) on stability and activity of methanogenic granular sludge. *Appl. Microbiol. Biotechnol.* **36**, 109–114.

Guiot S. R., Gorur S. S., Bourque D. and Samson R. (1988) Metal effect on microbial aggregation during upflow anaerobic sludge bed-filter (UBF) reactor start-up. In

Granular Anaerobic Sludge: Microbiology and Technology, eds G. Lettinga, A. J. B. Zehnder, J. T. C. Grotenhuis and L. W. Hulshoff Pol, pp. 187–194. Pudoc, Wageningen, The Netherlands.

Harvey M., Forsberg C. W., Beveridge T. P., Pos J. and Ogilvie J. R. (1984) Methanogenic activity and structural characteristics of the microbial biofilm on a needle-punched polyester support. *Appl. Environ. Microbiol.* **48**, 633–638.

Hulshoff Pol, L. W. (1989) *The Phenomenon of Granulation of Anaerobic Sludge*. Ph.D. Thesis, Wageningen Agricultural University, The Netherlands.

Hulshoff Pol L. W., de Zeeuw W. J., Velzeboer C. T. M. and Lettinga G. (1983) Granulation in UASB reactors. *Water Sci. Technol.* **15**, 291–304.

Kosaric N. and Blaszczyk R. (1990) Microbial aggregates in anaerobic wastewater treatment. *Adv. Biochem. Eng. Biotechnol.* **42**, 27–62.

Kugelman I. J. and McCarty P. L. (1965) Cation toxicity and simulation in anaerobic waste treatment. *J. Water Pollution Cont. Fed.* **37**, 97–166.

Lettinga G., Man A. D., ver der Last A. R. M., Wiegant W., van Knippenberg K., Frijns J. and van Buuren J. C. L. (1993) Anaerobic treatment of domestic sewage and wastewater. *Water Sci. Technol.* **27**, 67–73.

Lettingam G., van Velsen A. F. M., Hobma S. W., de Zeeuw W. and Klapwijk A. (1980) Use of the upflow sludge blanket (USB) reactor concept for biological waste water treatment especially, for anaerobic treatment. *Biotechnol. Bioeng.* **22**, 699–734.

Mahoney E. M., Varangu L. K., Cairns W. L., Kosaric N. and Murray R. G. E. (1987) The effect of calcium on microbial aggregation during UASB reactor start-up. *Water Sci. Technol.* **19**, 249–260.

Morgan J. W., Evison L. M. and Forster C. F. (1991) Changes to the microbial ecology in anaerobic digesters treating ice cream wastewater during start-up. *Water Res.* **25**, 639–653.

Pavlostathis S. G. and Giraldo-Gomez E. (1991) Kinetics of anaerobic treatment: a critical review. *Crit. Rev. Environ. Cont.* **21**, 411–499.

Rudd T., Sterritt R. M. and Lester J. N. (1984) Complexation of heavy metals by extracellular polymers in the activated sludge process. *J. Water Poll. Cont. Fed.* **56**, 1260–1268.

Schmidt J. E. and Ahring B. K. (1993) Effects of magnesium on thermophilic acetate-degrading granules in upflow anaerobic sludge blanket (UASB) reactors. *Enzy. Microbiol. Technol.* **15**, 304–310.

Schmidt J. E. and Ahring B. K. (1996) Granular sludge formation in upflow anaerobic sludge blanket (UASB) reactors. *Biotechnol. Bioeng.* **49**, 229–246.

Shen C. F., Kosaric N. and Blaszczyk R. (1993) The effect of selected heavy metals (Ni, Co and Fe) on anaerobic granules and their extracellular polymeric substance (EPS). *Water Res.* **27**, 25–33.

Thaveesri J., Daffonchio D., Liessens B., Vandemeren P. and Verstraete W. (1995) Granulation and sludge bed stability in upflow anaerobic sludge bed reactors in relation to surface themodynamics. *Appl. Environ. Microbiol.* **61**, 3681–3686.

Thiele J. H., Wu W. M., Jain M. K. and Zeikus J. G. (1990) Ecoengineering high rate biomethanation system: design of improved syntrophic biomethanation catalysts. *Biotechnol. Bioeng.* **35**, 990–999.

Turakhia M. H. and Characklis W. G. (1988) Activity of Pseudomonas aeruginosa in biofilm: effect of calcium. *Biotechnol. Bioeng.* **33**, 406–414.

Uemura S. and Harada H. (1995) Inorganic composition and microbial characteristics of methanogenic granular sludge grown in a thermophilic upflow anaerobic sludge blanket reactor. *Appl. Microbiol. Biotechnol.* **43**, 358–364.

van Langerak E. P. A., Gonzalez-Gil G., van Aelst A., van Lier J. B., Hamelers H. V. M. and Lettinga G. (1998) Effects of high calcium concentrations on the development of methanogenic sludge in upflow anaerobic sludge bed (UASB) reactors. *Water Res.* **32**, 1255–1263.

Versprille A. J. (1978) *Anaerobic Treatment of Potato-processing Wastewater*. Report to Wageningen Agricultural University, The Netherlands, pp. 762–765.