ELSEVIER
Water Research 38 (2004) 1376–1389
WATER RESEARCH
www.elsevier.com/locate/watres

Anaerobic sludge granulation

L.W. Hulshoff Pol, S.I. de Castro Lopes, G. Lettinga, P.N.L. Lens*

Sub-Department of Environmental Technology, Agricultural University of Wageningen, "Biotechnion" Bomenweg 2, PO Box 8129, 6700 EV Wageningen, The Netherlands

Abstract

This paper reviews different theories on anaerobic sludge granulation in UASB-reactors that have been proposed during the past two decades. The initial stages of the formation of anaerobic granules follow the same principles as biofilm formation of bacteria on solid surfaces. There exist strong evidence that inert carriers play an important positive role in granulation. Most researchers conclude that *Methanosaeta concilii* is a key organism in granulation. Only the Cape Town Hypothesis presumes that an autotrophic hydrogenotrophic organism, i.e., *Methanobacterium* strain AZ, growing under conditions of high H_2-pressures, is the key organism in granulation. Many authors focus on the initial stage of granulation, and only a few contributions discuss the latter stages in granulation: granule maturation and multiplication. Granule enhancing factors in the latter stages predominantly rely on manipulation of the selection pressure, through which selectively heavier sludge particles are retained in the UASB reactor.

Keywords: Granulation; UASB; Anaerobic treatment; Bacterial adhesion

1. Introduction

The formation of anaerobic granular sludge (Fig. 1) can be considered as the major reason of the successful introduction of the Upflow Anaerobic Sludge Bed (UASB) reactor concept for anaerobic treatment of industrial effluents. This granulation process allows loading rates in UASB reactors far beyond the common loading rates applied so far in conventional activated sludge processes. The resulting reduction in reactor size and required area for the treatment leads to lower investment costs in addition to the reduced operating costs due to the absence of aeration.

Two main factors made these high loading rates possible:

(a) The superior settling characteristics of granular sludge. Settling velocities of granular sludge of approximately 60 m/h are common, whereas the superficial upflow velocities in UASB reactors are usually kept below 2 m/h, in practice. This allows an extreme uncoupling of the hydraulic retention time from the solid retention time (or sludge age). Solid retention times of over 200 days can be achieved at hydraulic retention times of only 6 h.
(b) The high specific methanogenic activities of granular sludge. It could be demonstrated that high volumetric loading rates of over 50 kg Chemical Oxygen Demand (COD) per m^3 per day could be well accommodated under mesophilic conditions, with specific methanogenic activities of more than 2 kg COD/kg VSS day [1]. Studies on the micromorphology of the granules demonstrated that colonies of acetogenic bacteria are closely linked with micro-colonies of hydrogenotrophic methanogenic archaea allowing an efficient interspecies hydrogen transfer and as a result, high degradation rates.

Granules had already been observed with the Anaerobic Filter by Young and McCarty [2], and with the Dorr'Oliver Clarigesters in South Africa in 1979 (Lettinga, personal communication). Clarigesters are clarifiers converted into anaerobic digesters, which were

*Corresponding author. Tel.: +31-317-483851; fax: +31-317-482108.
E-mail address: piet.lens@wur.nl (P.N.L. Lens).

0043-1354/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.watres.2003.12.002

used for agro-industrial effluent treatment, operated in an upflow mode. Little attention was paid however to this sludge type. In the Netherlands, granular sludge was discovered in 1976 in a $6\,m^3$ pilot plant at the CSM sugar factory in Breda. Due to this sludge, the results obtained in the pilot plant were superior to results of previous studies in the laboratory of the Wageningen University [3]. In the report of the pilot plant study, the importance of granulation was well appreciated, but it was also indicated that little understanding of the process of granulation existed at that time and there was a strong need for further study on this.

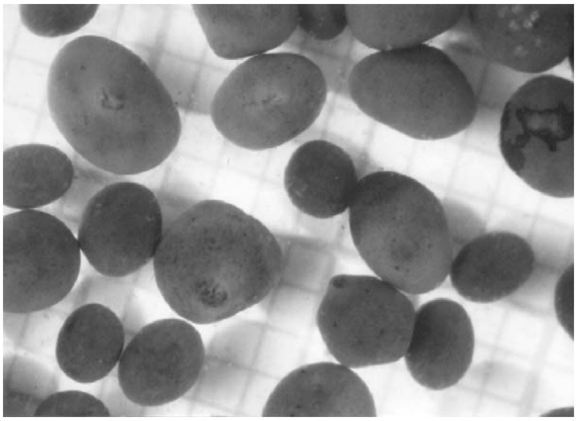


Fig. 1. Anaerobic granules from the UASB reactor of Papierfabriek Roermond.

Now more than 25 years later, numerous researchers from all over the world have studied the granulation process. However, there is still no consensus about the determining mechanism triggering granulation. This paper gives an overview of different granulation theories and factors promoting granulation.

A key organism in anaerobic sludge granulation is *Methanosaeta concilii*. Most studies cited use its synonym *Methanothrix soehngenii*. However this name was later considered illegitimate, since the isolated organism [4] was not pure [5]. In this review, the names mentioned in the studies have not been changed, which means that mostly *Methanothrix soehngenii* has been used.

2. Theories on granulation

The theories on anaerobic sludge granulation reviewed in this article are organized in three groups, namely physical, microbial and thermodynamic approaches, which are considered as the main factor responsible for granule formation (Table 1). However, this division is not completely tight as some theories have features that could fit also other classification.

2.1. Physical theories

In this granulation approach, the phenomenon is explained in terms of the consideration of the physical conditions prevailing in the reactor. Liquid and gas

Table 1
Overview of the different theories on anaerobic sludge granulation

Approach		References	Name of theory
Physical		Hulshoff Pol et al. [6]	Selection pressure
		Pereboom [7]	Growth of colonized suspended solids
Microbial	Physiological	Dolfing [8]	—
		Sam-Soon et al. [9]	Cape Town hypothesis
	Growth	Wiegant [10]	Spaghetti theory
		Chen and Lun [11]	—
	Ecological	Dubourgier et al. [12]	Bridging of microflocs
		Morgan et al. [13]	Bundles of methanothrix
		De Zeeuw (1980)	Three types of VFA degrading granules
		McLeod et al. [14]	—
		Vanderhaegen et al. [15]	—
		Ahn [16]	—
		Wu et al. (1996) [50]	Anaerobic granulation with defined species
Thermodynamic		Zhu et al. (1997) [51]	Crystallized nuclei formation
		Thaveesri et al. [17]	Surface tension model
		Schmidt and Ahring [18]	—
		Tay et al. [19]	Proton translocation–dehydration

upflow velocities, suspended solids in the effluent or seed sludge, attrition and removal of excess sludge from the reactor are considered as the factors responsible for granulation.

Selection pressure theory (1983): The essence of the granulation process in a UASB reactor, in this theory, is believed to be the continuous selection of sludge particles that occurs in the reactor [6]. The selection pressure can be regarded as the sum of the hydraulic loading rate and the gas loading rate (dependent on the sludge loading rate). Both factors are important in the selection between sludge components with different settling characteristics.

Under conditions of high selection pressure, light and dispersed sludge will be washed out while heavier components can be retained in the reactor. Thus, growth of finely dispersed sludge is minimised and the bacterial growth is delegated to a limited number of growth-nuclei, that can consist of inert organic and inorganic carrier materials or small bacterial aggregates present in the seed sludge [20]. These growth nuclei increase in size until a certain maximum size, after which parts of the granules detach, producing a new generation of growth nuclei, and so on.

The first generation consists of relatively voluminous aggregates, but gradually they become denser due to bacterial growth on the outside and inside of the aggregates. Moreover, bacterial growth is stimulated in the more voluminous aggregates as the substrate can penetrate deeper in the aggregates due to less diffusion limitation and lower volumetric bacterial activity inside these aggregates as compared to denser aggregates. The filamentous granules that exist in the initial stages of the granulation process become denser due to this ageing process.

Under conditions of low selection pressure, growth will take place mainly as dispersed biomass, which gives rise to the formation of a bulking type of sludge. In anaerobic reactors, the predominant organism is *Methanothrix*, which can form very long filaments (200–300 μm). When these organisms grow without attachment to a solid support particle, a loosely intertwined structure of filaments, with very poor settling characteristics will be obtained. Moreover, through the attachment of gas bubbles to these loosely intertwined filaments, the sludge even has a tendency to float [20].

Growth of colonised suspended solids (1994): Pereboom [7] states that granules originate from fines formed by attrition and from colonisation of suspended solids from the influent (Fig. 2). Moreover, according to this author, granule size increase is only due to growth and therefore, the concentric layers observed on sliced granules are related to small fluctuations in growth conditions.

Pereboom [7] reported that the most significant process limiting the maximum granule size in normal operation is the regular discharge of surplus biomass.

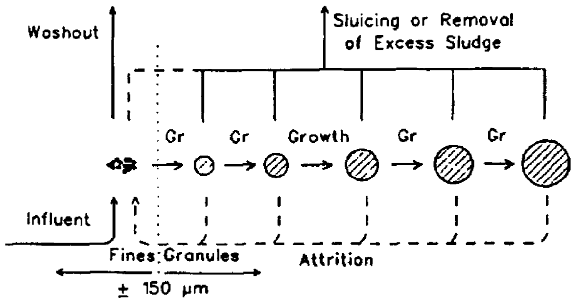


Fig. 2. Size distribution model for methanogenic granules [7].

Reactor turbulence and internal gas production appear to have no influence on the size distribution. These shear forces are not responsible for breaking or disintegrating of granules and only cause attrition of small particles from the granules. The latter is not expected to be significant to the removal of large granules.

According to the same author, the granular size distribution in UASB reactors seems to be the result of growth from small particles (being washed into the reactor or developed in the reactor by attrition) into larger granules and the removal of representative amounts of granules from all size classes by sludge discharge (Fig. 2). Moreover, wastewaters with a high concentration of suspended solids result in short size distributions while little or no suspended solids in the influent leads to wide size distributions.

2.2. Microbial theories

The theories aggruped in this section explain sludge granulation mainly based on the characteristics of certain microorganisms. In this approach, the physical factors presented above are often also integrated. The observation of granular characteristics, namely granule structure and correspondent microbiology, coupled to the conditions prevailing in the reactor (hydrodynamics, substrate and intermediates concentration profiles along the reactor, etc) are the basis of the theories presented. Surface thermodynamics as the determining factor in granulation is presented in the next section.

2.2.1. Physiological approach

The production of extracellular polymers by some microorganisms under certain conditions is considered by several authors, after Dolfing [8], as the factor responsible for the phenomenon of anaerobic granulation.

Cape Town hypothesis (1987): According to Sam-Soon et al. [9], granulation depends on *Methanobacterium* strain AZ, an organism that utilises H_2 as its sole energy source and can produce all its amino acids, with the exception of cysteine. When this microorganism is in an environment of high H_2 partial pressure, i.e., excess substrate, cell growth and amino acid production will be stimulated. However, as *Methanobacterium* strain *AZ* can not produce the essential amino acid cysteine, cell synthesis will be limited by the rate of cysteine supply. Additionally, if ammonium is available, there will be a high production of the other amino acids, which *Methanobacterium* strain *AZ* secretes as extracellular polypeptide, binding *Methanobacterium* strain AZ and other bacteria together to form granules. However, the authors admit the possibility that other anaerobic bacteria may have characteristics similar to *Methanobacterium* strain *AZ* and thus also contribute to granule formation.

This hypothesis was proposed following the analysis as a function of height of a UASB reactor treating a substrate mainly consisting of sugars with negligible nitrogen content and with adequate nutrients and trace elements for growth.

Supporting observations for this hypothesis were that the net sludge production per unit mass of COD was exceptionally high in the high H_2 partial pressure zone, much higher than the yield normally expected in anaerobic systems and that the growth of sludge mass was confined to that high H_2 partial pressure zone. Furthermore, the generation of soluble organic nitrogen in the high H_2 partial pressure zone, combined with a decrease of ammonium (Fig. 3), could not be attributed to cell growth or death. In fact, the decrease of ammonium was much more than the experimental maximum growth yield, which means that just a part of the ammonium could have been utilised for protoplasm synthesis. On the other hand, if the generation of organic nitrogen would have been a result of death of organisms, the death rate would have greatly exceeded the cell growth rate. This means that the death of microorganisms could not explain the observed generation of organic nitrogen in this lower active zone. Thus, the acceptable explanation given for this nitrogen behaviour was that the generation of organic nitrogen was due to the secretion of amino acids by *Methanobacterium* strain *AZ*, under high H_2 partial pressure, in cysteine-deficient medium and with an adequate supply of NH_4^+–N.

According to this hypothesis, the conditions that favour granulation are the following:

- environment with a high H_2 partial pressure;
- plug flow or semi-plug reactor (in order to achieve phase separation) with a nearly neutral pH;
- non-limiting source of nitrogen, in the form of ammonium;
- limited amount of cysteine.

Thus, granulation is very likely to occur during the conversion of carbohydrate substrates in a plug flow system. H_2 is released during the conversion of the carbohydrates to volatile fatty acids (VFA). Under high loading conditions, the H_2 uptake rate by the H_2 utilising organisms is lower than the H_2 production rate and a region of high H_2 partial pressure develops. This high H_2 partial pressure zone can be maintained in a plug flow system, thus providing conditions for the development of *Methanobacterium* strain *AZ*.

The situations in which granulation is unlikely to occur, according to the Cape Town hypothesis, are the following:

- systems where the substrate does not yield H_2 in the fermentation process (e.g. acetate) or only can be degraded under low H_2 partial pressure conditions (e.g. propionate and lipids);
- completely mixed systems, because of the 'dilution' of the high H_2 partial pressure.

However, granulation has been observed in UASB reactors treating acetate [11,21,22], indicating that the theory does not hold. Moreover, the hydrodynamic behaviour in UASB reactors approaches usually a completely mixed regime, which means that there will be not a steep hydrogen profile over the height of the reactor.

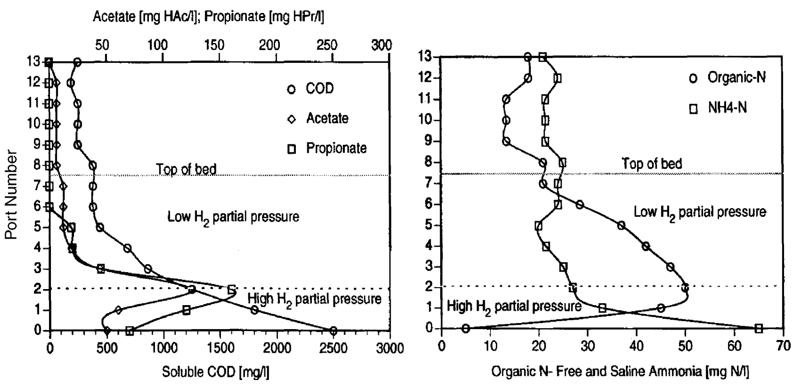


Fig. 3. Concentration profiles observed in a UASB [9].

Moosbrugger et al. [23] reported that also with protein-containing substrate (casein), the granulation in a UASB reactor was easily achieved and that the system behaviour was very similar to the same system treating carbohydrate substrates.

2.2.2. Growth of 'microbial nuclei'

The "Spaghetti theory" (1987): Wiegant [10] proposed a 'Spaghetti theory' on sludge granulation in UASB reactors treating acidified wastewaters, solutions of acetate or mixtures of VFA with predominant *Methanothrix* bacteria. Although reactors with predominant *Methanosarcina* species can perform 'spontaneous granulation', this type of granules has less practical importance in the UASB reactors as they bring operational problems [24]. Therefore, when the relative concentration of *Methanothrix* bacteria is not high enough, a strong selection towards these bacteria has to be imposed. This can be done by using low acetate concentrations during the start-up phase, as *Methanothrix* has a higher substrate affinity for acetate compared to *Methanosarcina* [25].

Wiegant [10] divides the granule formation in two phases:

1. formation of precursors
2. actual growth of the granules from these precursors.

The first step is considered the most crucial part of the granule formation. Initially, *Methanothrix* bacteria form very small aggregates, due to the turbulence generated by the gas production, or attach to finely dispersed matter. The concentration of suspended solids should not be too high, otherwise the increase in size of the aggregates will be too slow. Selection for aggregates is done by imposing an increasing upflow velocity. Once the precursors are formed and a proper step-up routine is followed, granulation is inevitable. The growth of the individual bacteria and the entrapment of non-attached bacteria lead to the growth of the precursor particles to form granules, which due to the hydraulic shear forces of the upflowing biogas acquire a spherical shape. The granules in this phase still present a filamentous appearance, like a ball of spaghetti formed of very long *Methanothrix* filaments, of which part is loose and part in bundles. With time, rod-type granules are formed from these filamentous granules at a high biomass retention time, due to the increase in the density of the bacterial growth.

Similarly to Wiegant [10], Chen and Lun [11] formulated a hypothesis for the anaerobic sludge granulation in a UASB fed with fermented alcohol stillage divided in two steps:

1. nucleus formation
2. nucleus growing into a granule.

Both *Methanothrix* and *Methanosarcina* are considered the organisms responsible for the nucleus formation. The former due to its good adhering capacities and the latter for its capacity of growing into clumps by excreting extracellular polymers (ECP), onto which *Methanothrix* can attach. Although the turbulence generated by the gas production is not given such an important role as in the 'Spaghetti theory', also the 'selection pressure' and the acetic acid concentration are the driving forces for the nucleus formation.

During the second step, in which the nucleus develops into a granule, various other bacteria with which the methanogens must grow syntrophically play a very important role, especially with complex substrates. In mature granules, methanogens do not predominate on the surface but, instead, are mixed with a variety of other bacteria [11].

2.2.3. Ecological approach

Bridging of microflocs by Methanothrix filaments (1987): From microscopic examination and activity measurements, Dubourgier et al. [12] suggests that the granulation mechanism starts by the covering of filamentous *Methanothrix* by colonies of cocci or rods (acidogenic bacteria), forming microflocs of 10–50 μm. Next, *Methanothrix* filaments, due to its particular morphology and surface properties, might establish bridges between several microflocs forming larger granules (> 200 μm). Further development of acidogenic and syntrophic bacteria favors the growth of the granules. Therefore, these authors support the idea that *Methanothrix* plays an important role in granule strength by forming a network that stabilises the overall structure but also emphasise the role of extracellular polymers and cell walls.

Bundles of Methanothrix surrounded by ECP (1991): Morgan et al. [13,26] suggested a possible mechanism involved in the growth of anaerobic granules based on the examination of granules treating papermill and sugar refinery effluents. In their opinion, granules develop from a precursor that consists of a small aggregate of *Methanothrix* and other bacteria. Growth of the *Methanothrix* filaments form characteristic bundles separated by a surrounding matrix in which other methanogenic and non-methanogenic bacteria are embedded. As the bundles increase in size the surrouding matrix becomes excluded leading to a region towards the center of the granule, which consists exclusively of compact filaments of *Methanothrix* and where discrete bundles are not distinguishable. Thus, these authors support previous suggestions on the importance of *Methanothrix* and bacterial polymers in the growth of the granules.

Three types of VFA degrading granules (1980): In this granulation theory, two bacterial genera are proposed to be of predominant importance to granule formation:

Methanothrix and *Methanosarcina*. From the research developed in 1980, De Zeeuw [27] explains the formation of the different types of granules developed in laboratory UASB reactor start-up experiments from digested sludge as seed material and VFA as substrate. The characteristics of the formed granules were the following:

(A) Compact spherical granules mainly composed of rod-shaped bacteria resembling *Methanothrix soehngenii* in short chains or single cells (rod-granules) (Fig. 4a).
(B) More or less spherical granules mainly consisting of loosely intertwined filamentous bacteria attached to an inert particle (filamentous granules) (Fig. 4b). The prevailing bacteria resembled *Methanothrix soehngenii*.
(C) Compact spherical granules composed predominantly of *Methanosarcina*-type bacteria (Fig. 5).

The development of each type of granular sludge was explained on the basis of seed sludge selection and sludge bed erosion and expansion, and the consequent differences in selection pressure and mean sludge residence time. *Methanosarcina* granules develop due to the capacity of this genus to produce clumps of bacteria, independently of the selection pressure. The clumps can reach macroscopic dimensions and show cavities, which can be inhabited by other species [28]. However, this kind of granules were just found in experiments where the concentration of acetate as a sole substrate was maintained above 1 kg COD/m^3, which means that *Methanosarcina* was able to outcompete *Methanothrix* [25].

At the low loading rates (low selection pressure) applied during the initial phase of the start-up of a UASB reactor, *Methanothrix* filaments will grow in and on small flocs present in the seed sludge leading to the formation of a 'bulking' anaerobic sludge.

When a high selection pressure is applied, *Methanothrix*, that has a high affinity to attach to all kind of surfaces [29], attach to carrier materials originating from the seed sludge or from the wastewater thus forming filamentous granules (type B).

More compact *Methanothrix* granules (rod granules, type A) are thought to be formed by the colonisation of the central cavities of *Methanosarcina* clumps by *Methanothrix* bacteria, which have a higher acetate affinity, eventually leading to a loss of the outer layer of

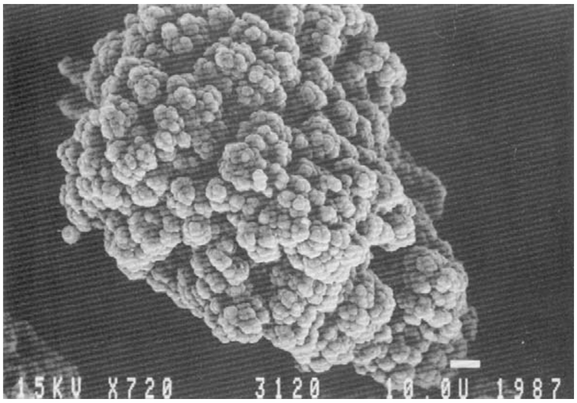
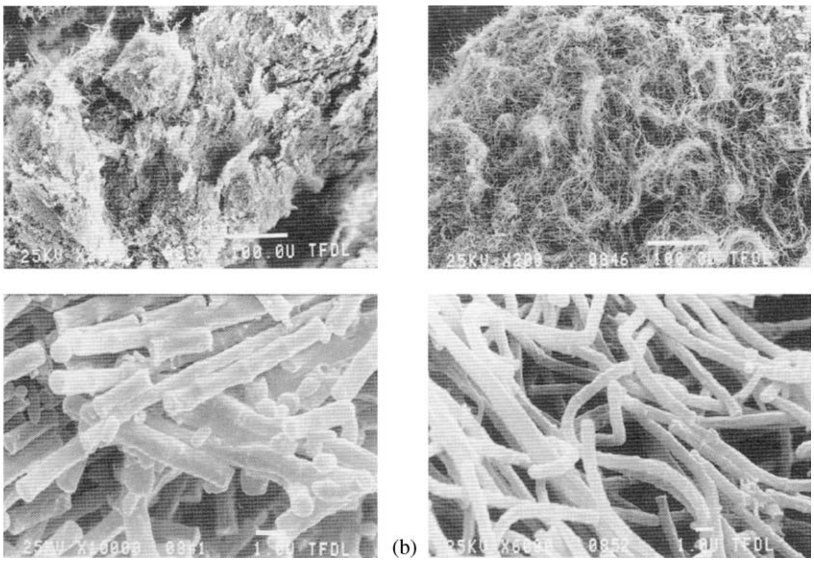


Fig. 5. Aggregate of *Methanosarcina* present at the bottom of a UASB reactor [52].



(a) (b)

Fig. 4. SEM of *Methanothrix* cells growing (a) in long filaments and (b) in short chains [1].

Methanothrix. Another explanation for these rod-type granules can be the filling of the filamentous granules with more bacteria, leading to a more compact *Methanothrix* granule.

The development of A or B type granules is related to the average biomass retention time taking place in the start-up process. When the average biomass retention time is too short, compact bacterial granules consisting almost exclusively of bacterial matter do not have the chance to be formed. This means that large conglomerates of bacteria can only be formed through attachment to inert support particles, which are heavy enough to be retained longer in the reactor (type B). Only if the average biomass retention time is big enough, compact bacterial granules (type A) can be formed.

Multi-layered granules with Methanothrix aggregates as nucleation centres (1990): McLeod et al. [14], working with a UASB-filter hybrid reactor, suggested a hypothesis in which the *Methanothrix* aggregates function as nucleation centres that initiate granule development (Fig. 6) of sucrose degrading granules. Acetate producers, including H_2-producing acetogens would then attach to this framework, providing the substrate to the *Methanothrix* and, together with H_2-consuming organisms, form a second layer around the *Methanothrix* core. Consecutively, fermentative bacteria adhere to this small aggregate forming the exterior layer of the granule, where they are in contact with their substrates, present in the bulk solution. The products of the fermentative bacteria would then serve as substrates to the underlying acetogens. Moreover, the fact that also methanogen-like organisms were found in the exterior layer lead to the idea that these H_2-consuming organisms could consume any free H_2, avoiding its diffusion into the second layer, where other H_2-consuming organisms would then be able to remove the remaining H_2 produced by the acetogens. Thus, such a spatial arrangement of the different trophic groups guarantees a high level of acetogenic activity.

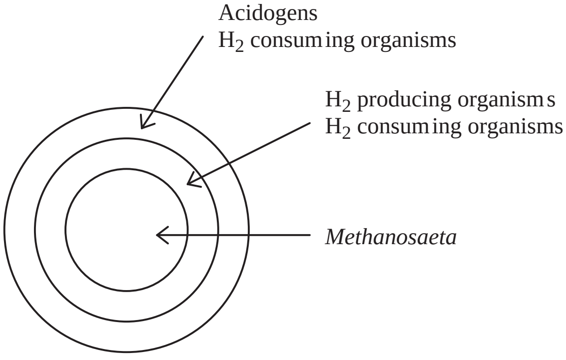


Fig. 6. Granule composition as proposed by McLeod et al. [14].

Also Fang [30] states that granules do not develop by the random aggregation of suspended bacteria, but that bacteria search for strategic positions for supply of substrates and for removal of products, as the layered microstructure of certain granules suggest. Once a nucleus is formed, bacteria start to proliferate leading to a growth of the size of the granule that only stops when the interfacial area between bacteria and the mixed liquor decreases to a critical level in relation to the initial hydrolysis or fermentation that takes place at the granule surface.

Vanderhaegen et al. [15], although supporting the multi-layered granule structure proposed by McLeod et al. [14], state that sugar fermentative acidogens form sufficient biomass and polymers to act as 'nucleation' centers in which the rest of the methanogenic associations can develop.

Ahn [16] proposed a similar granulation model as presented in Fig. 7. At the initial stage of granulation, aceticlastic methanogens (filamentous) and other organisms grow dispersed in the medium. By bridging and rolling effects due to the hydrodynamic behaviour of the UASB, small loose conglomerates mainly composed of the filamentous methanogens are eventually formed. Following on, acetogens attach to this conglomerate, in syntrophic relationship with the aceticlastic methanogens, thus forming a small granule with a dense core. Then, acidogens and hydrogenotrophs in syntrophic relationship with the acetogens adhere to the small granule and due to the extracellular polymers excretion by the hydrogenotrophs, the granule is allowed to grow.

2.3. Thermodynamic theories

Some authors have analysed the granulation mechanism in terms of the energy involved in the adhesion itself, due to the physico–chemical interactions between cells walls or between cells walls and alien surfaces. Aspects like hydrophobicity and electrophoretic mobility are objectively taken into account. Also the influence of the proton translocating activity across the bacterial membranes surface causing its energisation is added to the factors responsible for granulation.

Four step model for granule and biofilm formation (1996): Schmidt and Ahring [18] suggest that the granulation process in UASB reactors follows the well-described four steps of biofilm formation [31–34]:

(1) *Transport* of cells to the surface of an uncolonised inert material or other cells (substratum).
(2) Initial reversible *adsorption* by physicochemical forces to the substratum.
(3) Irreversible *adhesion* of the cells to the substratum by microbial appendages and/or polymers.
(4) *Multiplication* of the cells and development of the granules.

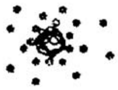
Appearance	Stage	Diameter	Approximate P_{H2} condition ($logP_{H2}$, atm)
	(A) growth of filamentous (aceticlastic) methanogens and other microorganisms in low hydrogen partial pressure condition	Filament	Low (≈ -6)
	(B) bridging and rolling effects on the growth of filamentous methanogens	< 100 μm	↓
	(C) growth of a small conglomerate as a loose core; crowded syntrophic acetogens around the surface of the core	< 1 mm	↓
	(D) growth of a small granule with a dense core; crowded syntrophic hydrogenotrophs and acidogens around the surface of a small granule	1-2 mm	↓
	(E) growth of a large granule with multi-layered structure, due to accumulation of extracellular polymers by hydrogenotrophs	2-5 mm	High (-2.7 ~ -3.7)

Fig. 7. Ahn's proposed model (2000) for the anaerobic sludge granulation.

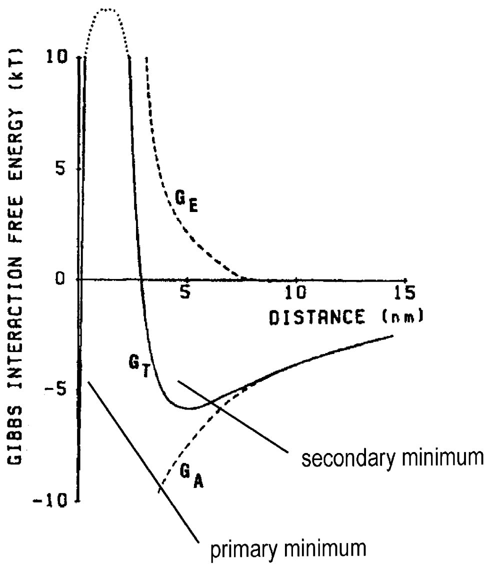


Fig. 8. Total interaction Gibbs energy (G_T, which is a summation of G_A, free energy of the Van der Waals forces and G_E, free energy if the electrostatic interaction) as a function of the distance between a spherical between a bacterium and a negatively charged surface (after Ref. [53]).

In a UASB reactor, the cells are transported by one or a combination of the following mechanisms: diffusion (Brownian motion), advective (convective) transport by fluid flow, gas flotation or sedimentation. The initial adsorption can take place after a collision between the cells and the substratum. The substratum can either be other cells or bacterial aggregates present in the sludge or organic or inorganic materials that can function as growth nuclei [18].

The initial adsorption can be approximately described by the DLVO theory, presented by Derjaguin, Landau, Verwey and Overbeek between 1940 and 1950, with the aim of explaining colloid stability. This theory can explain and/or predict microbial adhesion using calculations of adhesion free energy changes. By using this theory, the assumption is made that bacteria behave as inert particles and that bacterial adhesion can be understood by a physico-chemical approach. The DLVO theory postulates that the total long-range interaction over a distance of more than 1 nm is a summation of Van der Waals and Coulomb (electrostatic) interactions. According to this theory, three different situations can occur (Fig. 8):

1. a repulsion when electrostatic interactions dominate;
2. a strong irreversible attraction when Van der Waals forces are dominant (primary minimum);
3. a weak, reversible attraction when cells are located a certain distance from each other (secondary minimum).

The initial adhesion takes place predominantly in the secondary minimum of the DLVO free energy curve. The strength of adsorption depends on different physicochemical forces like ionic, dipolar, hydrogen bonds or hydrophobic interactions. The secondary minimum does not usually reach large negative values and particles captured in this minimum generally show reversible adhesion. In this case, there is a separation distance between the adhering bacteria and a thin water film remains present between the interacting surfaces. However, if a bacterium can reach the primary minimum, short-range interaction forces become effective and irreversible adhesion occur.

Irreversible adhesion can occur due to specific bacterial characteristics such as appendages, cell surface structures or polymers [18,33]. However, it is not clear if bacteria first adhere reversibly and then produce ECP or if bacteria first produce ECP and then adhere irreversibly [18].

When the bacterium is adhered, colonisation has started. The immobilised cells start to divide within the ECP matrix so that the cells are trapped within the biofilm structure. This results in the formation of microcolonies 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 trapped extraneous molecules, e.g. precipitates [35]. The organization of the bacteria in the granules can ease the transfer of substrates and products. The arrangement may depend on the local hydrophobicity, local presence of polymers or cell geometry [18].

Surface tension model (1995): Thaveesri et al. [17] related the adhesion of bacteria involved in anaerobic consortia in UASB reactors to surface thermodynamics. They found that bacteria can only obtain the maximum possible free energy of adhesion (ΔG_{adh}) when the liquid surface tension (γ_{LV}) is sufficiently low or high, as indicated in Fig. 9. In the high γ_{LV} region (zone B), low-energy surface types of bacteria (low bacterium surface tension (γ_{BV}) or hydrophobic bacteria) can adhere in order to obtain minimal energy, while in the low-γ_{LV} region (zone A), high-energy surface types of bacteria (high-γ_{BV} or hydrophilic bacteria) exhibit a greater decrease in free energy upon aggregation and thus are selected to compose aggregates. A third zone is arbitrarily defined between γ_{LV} values of 50 and 55 mN/m, and in this zone aggregation of neither hydrophobic nor hydrophilic cells is favoured (low ΔG_{adh} potential). Daffonchio et al. [36] used the contact angle technique to evaluate the hydrophobicities of mixed cell cultures of bacteria involved in anaerobic digestion. They showed that most acidogens are hydrophilic (contact angle $<45°$) but most of the acetogens and methanogens isolated from granular sludge are hydrophobic (contact angle $>45°$). Thus, operating a system at a high γ_{LV} should favor aggregation of (rather) hydrophobic bacteria, and operating a system at a low γ_{LV} should favour aggregation of (rather) hydrophilic bacteria [17].

According to these authors, the granules formed at low γ_{LV}, with acidogens as solid-phase emulsifiers around a methanogenic association allow a more stable reactor performance, as they are less susceptible of

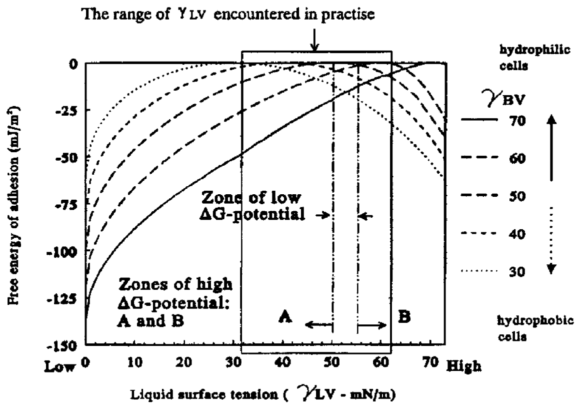


Fig. 9. Free energies of adhesion (ΔG_{adh}) for bacteria with different γ_{BV} values as a function of γ_{LV}. [17].

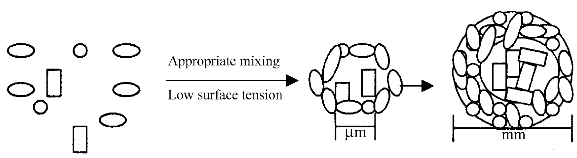


Fig. 10. Scheme of granule formation according to Thaveesri et al. [17].

adhesion to gas bubbles and consequent wash-out. The formation of these kinds of granules is shown in Fig. 10. Acidogens (round cells) aggregate by means of forming ECP, enclosing some methanogens (rectangular cells), while dispersed cells are washed-out leading to the formation of a granule with outer elastic hydrophilic layer formed by ECP-rich acidogens and an inner core of hydrophobic methanogens [37].

Proton translocation–dehydration theory (2000): Tay et al. [19] proposed a theory for the (molecular) mechanism of sludge granulation, based on the proton translocation activity at bacterial membrane surfaces. In this theory, the sludge granulation process was considered to proceed in the four following steps (Fig. 11):

(a) Dehydration of bacterial surfaces;
(b) Embryonic granule formation;
(c) Granule maturation
(d) Post-maturation.

(a) *Dehydration of bacterial surfaces*: Hydrophobic interaction between the bacterial surfaces is considered supportive for the initiation of bacterial adhesion [38,39]. However, with decreasing surface separation distance between two bacterial cells, strong repulsive hydration interactions between the two approaching bacteria exist, due to the energy required for the removal of the tightly bonded water

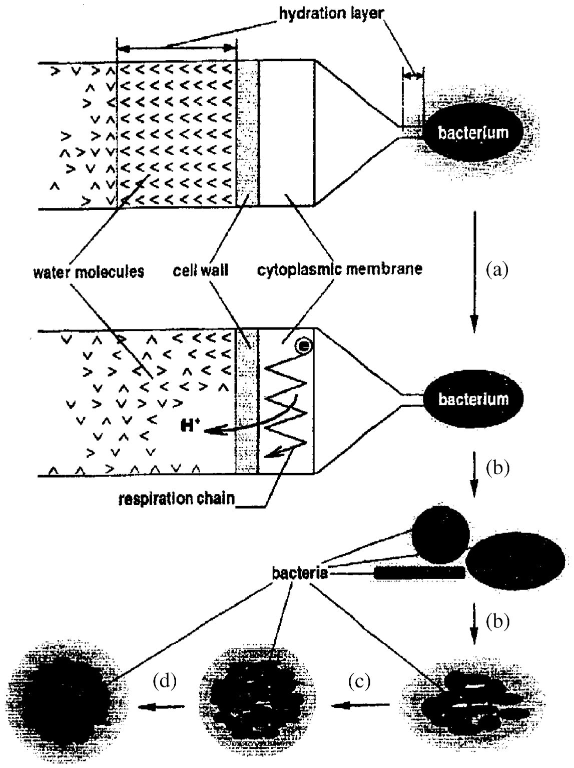


Fig. 11. Schematic representation of proton translocation–dehydration model for sludge granulation: (a) dehydration of bacterial surfaces; (b) embryonic granule formation; (c) granule maturation and (d) post-maturation [19].

from the bacterial surfaces. In fact, under normal physiological conditions, a bacterial surface has a high negative charge which facilitates hydrogen bonding with water molecules, resulting in a network of water surrounding the bacterial surface [40], i.e., a hydration layer. However, the hydration repulsion does not normally affect the initial step of the bacterial reversible adhesion stage to a significant extent.

The authors argue that acidogenic bacteria, during the acidification of substrates, pump protons from the cytoplasmic side of the membrane to the exterior surface of the membrane. This proton translocation activity energizes the surface and may induce breaking of the hydrogen bonds between the negatively charged groups and the water molecules. Thus, a partial neutralisation of the negative charges on their surfaces occurs, causing the dehydration of the cell surfaces.

(b) *Embryonic granule formation*: Acidogens, acetogens and methanogens may adhere to each other forming embryonic granules, as a consequence of the upflow hydraulic stress, of this weakened hydration repulsion and of the hydrophobic nature of the cells. Moreover, due to the transfer of metabolites between cells, a further de-hydration of the bacterial surfaces takes place leading to a strengthening of these initial granules. In this stage of development, the new physiological environment starts to induce the excretion of ECP to the embryonic granule surfaces.

(c) *Granule maturation*: In this stage, the original bacterial colonies continue to grow while also other dispersed bacteria may adhere to the embryonic granules. The transfer of intermediates determines the distribution of micro-colonies within the granule, eventually leading to well-structured bacterial aggregates as mature granules. On the other hand, the multiplication of bacterial cells is controlled due to space restriction. Moreover, ECP is produced in large quantities, causing the hydration of granule surfaces and protecting granules against the shear stress and attachment to gas bubbles, with subsequent biomass loss by flotation as ECP is highly hydrophilic and biogas bubbles are highly hydrophobic.

(d) *Post-maturation*: In the post-maturation stage, the proton translocating activity keeps the bacterial surfaces at a relatively hydrophobic state and is the main responsible in maintaining the structure of the mature granules. On the other hand, the ECP layer outside of a granule causes the hydration of the granule surface, protecting the granule against attachment to gas bubbles and shear stress in the UASB reactor [41].

The authors claim that some phenomena of sludge granulation like the advisable high-energy carbohydrate feeding during the UASB start-up period, the granular sludge washout when changing the carbon source, the existence of both uniform and layered granules and the influence of ECP in the granulation process can be adequately explained by this proton translocation–dehydration theory.

3. Enhancement of granulation by growth nuclei

One of the contributing factors to the development of granules from suspended sludge is the presence of nuclei or bio-carriers for microbial attachment [42,43]. The attachment of cells to these particles has been proposed as the initiation step for granulation. Since the second step was the formation of a dense and thick biofilm on the cluster of the inert carriers, this step could be considered as biofilm formation. In other words, once the initial aggregates are formed, subsequent granulation could be regarded as a mere phenomenon of an increase of biofilm thickness. Hence, the sludge granulation process in UASB reactors with added inert particles might be interpreted as a biofilm-forming phenomenon [44].

Several investigators have studied the effect of inert particles in granulation. Hulshoff Pol [1] demonstrated the importance of inert support particles in the granulation process. When the inert particles (40–100 μm in size) were removed from the inoculated sewage sludge, granulation was not observed within the period of time required for granulation of dispersed sludge with no removal of inert particles in the seed sludge. Table 2 shows that inert materials can accelerate sludge granulation.

Yu et al. [44] proposed the following guidelines for the carefully choice of the inert materials to be used in order to enhance sludge granulation:

- high specific surface area;
- specific gravity similar to anaerobic sludge;
- good hydrophobicity;
- spherical shape.

Lettinga et al. [47] stated that clay and other inorganic particles seemed to be harmful to the formation of granular sludge. Also Hickey et al. [48] did not find any difference in thermophilic granulation with or without the addition of sand (50–10 μm in size) into inoculated digested sludge, although the granules formed included sand particles [48]. This can be attributed to the greater specific gravity of some inert particles, like sand particles, in relation to the biomass. More biomass may accumulate in the upper portion of the reactor while the sand particles tend to accumulate in the reactor bottom. Therefore, the chance of contact between the particles and biomass, which is beneficial for microbial attachment, may be significantly reduced, resulting in no significant enhancement of granulation [42].

A high concentration of poorly flocculating suspended matter in the wastewater is detrimental to the development of granular sludge [47]. Also Hulshoff Pol et al. [6] reported that in liquid wastes with a high fraction of finely dispersed suspended solids, the attachment of bacteria to the dispersed particles can lead to the wash-out of viable bacteria. According to Hulshoff Pol et al. [20], a high concentration of dispersed inert solids is prejudicial to the granulation process because in the case that the surface area offered for growth is very big for the bacteria available, concentrated growth will be limited. As granulation is strongly dependent on bacterial growth, a reduced growth leads to a slow down in the granulation process.

Table 2
Influence of addition of various inert materials on the sludge granulation

Inert material	Seed sludge	Reactor	Media size (mm)	Substrate	Granulation time shorten (d)	Granular size (mm)	Predominant bacteria	Reference
Foam	Flocculent	Packed bed 85 and 200 ml	5.0	Propionate	—	7.8–8.0	*Methanothrix*	Fukuzaki et al. [45]
Zeolite	Thin anaerobic biofim on the zeolite particles, grown on a VFA mixture	MCB 9.4 l	0.1	VFA	20	1.0–2.0	*Methanothrix*	Yoda et al. [46]
		MCB 4.0 l		Glucose				
Hydro-anthracite		—	0.1	VFA	14	2.0	*Methanothrix*	Hulshoff Pol [1]
WAP	Non-granular anaerobic digested sludge	UASB 1.3 l	0.1–0.2	Glucose	20	1.8–1.9	rod-type *Methanothrix*	Imai et al. [42]
				VFA	Stimulated granulation*	2.1–2.3	Filamentous-type *Methanothrix*	
		UASB 10 l			10	—		
GAC		UASB 0.75 l	0.32	Sucrose	—	0.4	*Methanothrix*	Morgan et al. [26]
GAC		UASB 7.3 l	0.4	Glucose + peptone + meat extract	35	2.0–4.0	*Methanothrix*	Yu et al. [44]
PAC			0.2		30	2.0–4.0	*Methanothrix*	

MCB—micro-carrier bed, WAP—water-absorbing polymer, GAC—granular activated carbon, PAC—powdered activated carbon.

3.1. Activated carbon

Ross [49] reported that the presence of spent powdered active carbon enhanced the settleability of a sludge treating maize-processing effluent. The carbon provides an additional surface area for attached-growth bacteria, which increases the density of the resultant biomass, with concomitant improved settling.

Also according to Morgan et al. [26], the addition of supplements to a non-granular inoculum during the start-up of UASB reactors appears to be beneficial. A granular activated carbon (GAC) supplement offers two advantages: sheltered ecological niches that enhance biological attachment and thus initiate granule formation and, possibly, a capacity for the adsorption of pollutants, that can then be degraded in the immobilised state. However, this latter feature does result in what can only be described as a lag phase. The activated carbon particles enhance the development of an attached biofilm and, as such, act as a nucleus for granule formation.

Yu et al. [44] studied the effects of powdered activated carbon (PAC) and GAC on sludge granulation during start-up of UASB reactors. The results showed that the addition of PAC or GAC clearly enhanced the sludge granulation process and accelerated the start-up process. Sludge granulation, defined as the time by which 10% of the granules are larger than 2.0 mm, took approximately 95 days to be achieved in the reactor with no addition of inert materials and was reduced by 25 and 35 days in the PAC and GAC-added reactors, respectively. Besides, the addition of GAC and PAC provoked higher biomass concentrations throughout the experiment, earlier observation of visible granules and improved the volumetric COD removal capacity. Moreover, the addition of GAC showed slightly more beneficial effects for the start-up of UASB reactors than PAC. The enhanced granulation by the addition of PAC or GAC was attributed to a better attachment of the filamentous bacteria on the activated carbon. However, in this study, the characteristics of PAC and GAC were not examined in detail. The different characteristics are likely responsible for the minor difference between the PAC and GAC-added reactors.

3.2. Water absorbing polymer

Imai et al. [42] studied the effects of adding water absorbing polymer (WAP) particles into the inoculated sludge. WAP is a resin, mainly composed of acrylic compounds and shows a complex network structure with a high specific surface for microbial attachment. Moreover, it shows a low density (wet density of 1.0 g/ml), which means that the contact between the particles and biomass is improved, when comparing to sand and other materials. Although not influencing the average granule size, the addition of WAP clearly enhanced the granulation in the lab-scale and pilot scale UASB reactors using glucose or VFA as substrates (Table 2), serving as a bio-carrier to allow more biomass to attach on them. After the granules were formed, the WAP was slowly decomposed by the anaerobic bacteria, which caused the granules to split into several small fragments that grew up again forming more mature granules. Eventually, all particles were digested and the granules formed did not contain visible WAP particles anymore. Based on the experiments performed, the authors recommended a dosage of WAP of approximately 750 mg/l of reactor volume for the enhancement of granulation.

4. Conclusion

Most theories on granulation confirm that the acetotrophic methanogen *Methanosaeta* plays a key role in granulation. Some believe that *Methanosarcina* clumps enhance granule formation. The only theory that states that other organisms cause granulation is the Cape Town Hypothesis, which is based on the excessive ECP production of *Methanobacterium* strain *AZ* under conditions of high H_2-partial pressures, unlimited ammonium and cysteine limitation.

There is considerable consensus that the initial stage of granulation is bacterial adhesion (a physical–chemical process) parallel to the early stage of biofilm formation. However, treating bacterial adhesion only as a physico–chemical process is limited in explaining all the complex aspects of bacterial adhesion. Bacteria do not have a sharp surface boundary, simple geometry or uniform molecular surface composition. In fact, internal chemical reactions can lead to changes in molecular composition both in the interior and at the surface, and molecules and ions may cross the bacterium/water surface and these processes continue also after adhesion. Anyway, this physico–chemical approach has value in forming a framework in which biological factors can be added to form a unifying theory of granulation.

Although much attention in granulation theories goes to the conditions affecting bacterial adhesion, still the selective wash-out of dispersed sludge, resulting in an increased growth of retained (heavier) sludge agglomerates is more crucial for the granulation process. In this respect, the presence of inert particles serving at surfaces on which bacteria can adhere is clearly advantageous. Nevertheless, the particles should be well settleable, if not it may cause unwanted sludge wash-out.

Little attention is given to the fact that granulation strongly depends of growth. This means that simply by optimising the conditions for growth granulation can be strongly enhanced. Optimal conditions for growth can be deducted from information of the effect of pH and temperature on the growth rate of *Methanosaeta concilii*, the key organism in granulation.

References

[1] Hulshoff Pol LW. The phenomenon of granulation of anaerobic sludge. Ph.D. Thesis, Agricultural University Wageningen, The Netherlands, 1989.

[2] Young JC, McCarty PL. The anaerobic filter for waste treatment. J Water Pollut Control Federation 1969; 41R:160–73.

[3] Lettinga G, Pette KC, de Vletter R, Wind E. Anaerobic treatment of beet sugar wastewater on semi-technical scale. CSM-report. The Netherlands: Amsterdam; 1977 [in Dutch].

[4] Huser BA. Methanbildung aus Acetat. Ph.D. Thesis EAWAG, Zurich, Switzerland, 1981.

[5] Boone DR, Kamagata Y. Rejection of the species *Methanothrix soehngenii* VP and the genus *Methanothrix* VP as *nomina confusa*, and transfer of *Methanothrix thermophila* VP to the genus *Methanosaeta* VP as *Methanosaeta thermophila* comb. Nov. Request for an opinion. Int J Syst Bacteriol 1998;48:1079–80.

[6] Hulshoff Pol LW, De Zeeuw WJ, Velzeboer CTM, Lettinga G. Granulation in UASB reactors. Water Sci Technol 1983;15(8/9):291–304.

[7] Pereboom JHF. Size distribution model for methanogenic granules from full scale UASB and IC reactors. Water Sci Technol 1994;30(12):211–21.

[8] Dolfing J. Microbiological aspects of granular methanogenic sludge. Ph.D. Thesis, Agricultural University Wageningen, The Netherlands, 1987.

[9] Sam-Soon PALNS, Loewenthal RE, Dold PL, Marais GvR. Hypothesis for pelletisation in the upflow anaerobic sludge bed reactor. Water SA 1987;13(2):69–80.

[10] Wiegant WM. The 'spaghetti theory' on anaerobic sludge formation, or the inevitability of granulation. In: Lettinga G, Zehnder AJB, Grotenhuis JTC, Hulshoff Pol LW, editors. Granular anaerobic sludge: Microbiology and technology. The Netherlands: Pudoc. Wageningen; 1987. p. 146–52.

[11] Chen J, Lun S-Y. Study on mechanism of anaerobic sludge granulation in UASB reactors. Water Sci Technol 1993; 28(7):171–8.

[12] Dubourgier HC, Prensier G, Albagnac G. Structure and microbial activities of granular anaerobic sludge. In: Lettinga G, Zehnder AJB, Grotenhuis JTC, Hulshoff Pol LW, editors. Granular anaerobic sludge: microbiology and technology. The Netherlands: Pudoc Wageningen; 1987. p. 18–33.

[13] Morgan JW, Evison LM, Forster CF. Internal architecture of anaerobic sludge granules. J Chem Technol Biotechnol 1991;50:211–26.

[14] McLeod FA, Guiot SR, Costerton JW. Layered structure of bacterial aggregates produced in an upflow anaerobic sludge bed and filter reactor. Appl Environ Microbiol 1990;56(6):1598–607.

[15] Vanderhaegen B, Ysebaert K, Favere K, van Wambeke M, Peeters T, Panic V, Vandenlangenbergh V, Verstraete W. Acidogenesis in relation to in-reactor granule yield. Water Sci Technol 1992;25(7):21–30.

[16] Ahn Y-H. Physicochemical and microbial aspects of anaerobic granular pellets. J Environ Sci Health 2000; A35(9):1617–35.

[17] Thaveesri J, Daffonchio D, Liessens B, Vandermeren P, Verstraete W. Granulation and sludge bed stability in upflow anaerobic sludge bed reactors in relation to surface thermodynamics. Appl Environ Microbiol 1995;61(10): 3681–6.

[18] Schmidt JE, Ahring BK. Review: granular sludge formation in upflow anaerobic sludge blanket (UASB) reactors. Biotechnol Bioeng 1996;49:229–46.

[19] Tay JH, Xu HL, Teo KC. Molecular mechanism of granulation. I: H^+ translocation–dehydration theory. J Environ Eng 2000;126:403–10.

[20] Hulshoff Pol LW, Heijnekamp K, Lettinga G. The selection pressure as a driving force behind the granulation of anaerobic sludge. In: Lettinga G, Zehnder AJB, Grotenhuis JTC, Hulshoff Pol LW, editors. Granular anaerobic sludge: microbiology and technology. The Netherlands: Pudoc. Wageningen; 1987. p. 153–61.

[21] Ahring BK, Schmidt JE, Winther-Nielsen M, Macario AJL, Macario EC. Effect of medium composition and sludge removal on the production, composition, and architecture of thermophilic (55°C) acetate-utilizing granules from and upflow anaerobic sludge blanket reactor. Appl Environ Microbiol 1993;59(8):2538–45.

[22] Morvai L, Mihaltz P, Czako L. The kinetic basis for a new start-up method to ensure the rapid granulation of anaerobic sludge. Water Sci Technol 1992;25(7):113–22.

[23] Moosbrugger RE, Loewenthal RE, Marais GvR. Pelletization in a UASB system with protein (casein) as substrate. Water SA 1990;16(3):171–8.

[24] Wiegant WM, De Man AWA. Granulation of biomass in thermophilic upflow anaerobic sludge blanket reactors treating acidified wastewaters. Biotechnol Bioeng 1986;28: 718–27.

[25] De Zeeuw WJ. Acclimatization of anaerobic sludge for UASB reactor start-up. Ph.D. Thesis, Agricultural University Wageningen, The Netherlands, 1984.

[26] Morgan JW, Evison LM, Forster CF. Upflow sludge blanket reactors: the effect of bio-supplements on performance and granulation. J Chem Technol Biotechnol 1991;52:243–55.

[27] De Zeeuw WJ. Granular sludge in UASB reactors. In: Lettinga G, Zehnder AJB, Grotenhuis JTC, Hulshoff Pol LW, editors. Granular anaerobic sludge: Microbiology and technology. The Netherlands: Pudoc. Wageningen; 1987. p. 132–45.

[28] Bochem HP, Schoberth SM, Sprey B, Wengler P. Thermophilic biomethanation of acetic acid: morphology and ultrastructure of a granular consortium. Canad J Microbiol 1982;28:500–10.

[29] Van den Berg L, Kennedy KJ. Support materials for stationary fixed film reactors for high-rate methanogenic fermentations. Biotechnol Lett 1981;3:165–70.

[30] Fang HHP. Microbial distribution in UASB granules and its resulting effects. Water Sci Technol 2000;42(12):201–8.

[31] Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, Marrie TJ. Bacterial biofilms

in nature and disease. Annu Rev Microbiol 1987;41: 435–64.

[32] Gantzer CJ, Cuunningham AB, Gujer W, Gutekunst B, Heijnen JJ, Lightfoot EN, Odham G, Rittmann BE, Rosenberg E, Stolzenbach KD, Zehnder AJB. Exchange processes at fluid-biofilm interface. In: Characklis WG, Wildered PA, editors. Structure and function of biofilms. Chichester: Wiley; 1989. p. 73–90.

[33] Van Loosdrecht MCM, Zehnder AJB. Energetics of bacterial adhesion. Experientia 1990;46:817–22.

[34] Verrier D, Mortier B, Dubourguier HC, Albagnac G. Adhesion of anaerobic bacteria to inert supports and development of methanogenic biofilms. In: Hall ER, Hobson PN, editors. Anaerobic digestion. Oxford: Pergamon Press; 1988. p. 61–70.

[35] Costerton JW, Marrie TJ, Cheng KJ. Phenomena of bacterial adhesion. In: Savage DC, Fletcher M, editors. Bacterial adhesion. Mechanisms and physiological significance. New York: Plenum Press; 1990. p. 3–43.

[36] Daffonchio D, Thaveesri J, Verstraete W. Contact angle measurement and cell hydrophobicity of granular sludge from upflow anaerobic sludge bed reactors. Appl Environ Microbiol 1995;61:3676–80.

[37] Kalogo Y, Verstrate W. Potentials of anaerobic treatment of domestic sewage under temperate climate conditions. In: Lens P, Zeeman G, Lettinga G, editors. Decentralised sanitation and reuse: concepts, systems and implementation. London: IWA Publishing; 2001. p. 181–204.

[38] Mahoney EM, Varangu LK, Cairns WL, Kosaric N, Murray R. The effect of calcium on microbial aggregation during UASB reactor start-up. Water Sci Technol 1987; 19:249.

[39] Van Loosdrecht MCM, Lykema J, Norde W, Schraa G, Zehnder AJB. The role of bacterial cell wall hydrophobicity in adhesion. Appl Environ Microbiol 1987;53(8): 1893–7.

[40] Smith CA, Wood FJ. Molecular and cell biochemistry: biological molecules. London: Chapman&Hall; 1991.

[41] Liu Y, Xu HL, Yang SF, Tay JH. Mechanisms and models for anaerobic granulation in upflow anaerobic sludge blanket reactor. Water Res 2003;37:661–73.

[42] Imai T, Ukita M, Liu J, Sekine M, Nakanishi H, Fukagawa M. Advanced start up of UASB reactors by adding of water absorbing polymer. Water Sci Technol 1997;36(7):399–406.

[43] Teo KC, Xu HL, Tay JH. Molecular mechanism of granulation. II: proton translocating activity. J Environ Eng 2000;126:411–8.

[44] Yu HQ, Tay JH, Fang HHP. Effects of added powdered and granular activated carbons on start-up performance of UASB reactors. Environ Technol 1999;20:1095–101.

[45] Fukuzaki S, Nishio N, Nagai S. The use of polyurethane foam for microbial retention in methanogenic fermentation of propionate. Appl Microbiol Biotechnol 1990;34: 408–13.

[46] Yoda M, Kitagawa M, Miyaji Y. Granular sludge formation in the anaerobic expanded micro carrier process. Water Sci Technol 1989;21:109–22.

[47] Lettinga G, Van Velsen AFM, Hobma SW, De Zeuw W, Klapwijk A. Use of the upflow sludge blanket (USB reactor concept for biological waste water treatment, especially for anaerobic treatment. Biotechnol Bioeng 1980;22:699–734.

[48] Hickey RF, Wu WM, Veiga MC, Jones R. Start up, operation, monitoring and control of high rate anaerobic treatment systems. Water Sci Technol 1991; 24(8):207–55.

[49] Ross WR. Anaerobic digestion of industrial effluents with emphasis on solids-liquid separation and biomass retention. Ph.D. Thesis, University of the Orange Free State, South Africa, 1984.

[50] Wu Wei-min, Jain MK, Zeikus JG. Formation of fatty acid-degrading, anaerobic granules by defined species. Appl Env Microbiol 1996;62(6):2037–44.

[51] Zhu J, Hu J, Gu X. The bacterial numeration and the observation of a new syntrophic association for granular sludge. Wat Sci Tech 1997;36(6/7):133–40.

[52] Grotenhuis JTC, Koornneef E, Plugge CM. Immobilization of anaerobic bacteria in methanogenic aggregates. In: Lettinga G, Zehnder AJB, Grotenhuis JTC, Hulshoff Pol LW, editors. Granular anaerobic sludge: Microbiology and technology. Wageningen, The Netherlands: Pudoc; 1987. p. 187–94.

[53] Rouxhet PG, Mozes N. Physical chemistry of the interaction between attached microorganisms and their support. Water Sci Technol 1990;22:1–16.