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ACIDOGENESIS IN RELATION TO IN-REACTOR GRANULE YIELD

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ABSTRACT

A systematic study of in-reactor granule yield data was set up in the laboratory. Methanogenic granular sludge growth in fed batch shake flasks for different substrates appeared to decrease with the energy content of the substrate and required the presence of a mixing force. In UASB reactors, granular cell yields were found to be quite variable suggesting the involvement of complex microbiological interactions. The factor foremost in influencing the build-up in the reactor of granular sludge was the presence of high-energy carbohydrates; pre-acidified influents affected in-reactor granular sludge yield very negatively. The influence of sulphate reduction and sulphide concentrations was found not to be of primary importance. The ionic strength of the medium also had no strong influence. Granule formation proceeded somewhat better at low than at high strength buffer capacity. High levels of protein, however, reduced the in-reactor granular sludge build-up strongly. Measurement of the acid production by granular and fluffy sludges revealed that the former rapidly produce volatile acids, particularly propionate, while the latter do not. Measurement of the pH in the granule indicated that the outside layer of the granule is rich in acidogens. Calculations of proton and hydrogen fluxes in the granule support the concept that acidogens, able to ferment high energy carbohydrates efficiently to cells and exo-cellular binding materials, might be of primary importance for in-reactor granular growth.

KEYWORDS

Acidogenesis, granule, anaerobic sludge

INTRODUCTION

One factor governing the growth of the microbial association in the form of 0.5 - 5 mm size well settling granules is commonly accepted, i.e. the presence of a selection force whereby the free-living cells are washed out of the reactor system. Normally, the hydraulic residence time in the upflow anaerobic sludge blanket (UASB) reactor is of the order of hours to at most a few days, while the solid residence time extends from

weeks to months (De Zeeuw, 1984).
Several other factors are postulated to also contribute to the formation of granules, such as the following.

1. A minimal supply of essential nutrients; specifically Ca^{2+} should attain levels of 30 mg/l or more (Grotenhuis et al., 1988).
2. A considerable supply of sugars relative to volatile fatty acids (De Zeeuw, 1984; Sam-Soon et al., 1988; Hulshoff Pol, 1989). Field experiments as well have shown that non-acidified waters give rise to more abundant granular growth than waters in which the carbohydrates are first all fermented to lower volatile fatty acids (Hulshoff Pol, 1989; Van Wambeke et al., 1990). It must be stated that granular sludge can be formed in reactors fed entirely with low-energy chemical oxygen demand (COD) such as lower volatile fatty acids (VFA). Yet, in these systems, sludge bed doubling times are of the order of several months and the granules are rather small and fragile (Grotenhuis et al., 1991).
3. A slightly acid to neutral pH favors optimal sludge bed growth (Ten Brummelen et al., 1985)

A number of factors are recognized as strongly detrimental to the formation and growth of granular sludge pellets
1. High levels of proteins and NH_4^+. Sayed et al. (1987) particularly demonstrated the negative effect of proteins on UASB reactor performance. Ammonium concentrations between 1500 and 3000 mg/l at a pH >7.4 inhibit granular sludge growth (Lettinga et al., 1985).
2. High levels of free suspended solids either interfere with the granular formation or lead to gradual filling up of the sludge bed with inactive particulates (Rozzi and Verstraete, 1981).

The methane reactor granule has thus far been considered to contain mainly hydrophobic Methanothrix soehngenii cells (Dubourgier et al., 1988). Novaes et al. (1988) have pointed out the presence of non-methanogens in the sludge granules, such as Butyrivibrio clostridium and Desulfovibrio species associated with hydrolysis, acetogenesis and sulphate reduction. MacLeod et al. (1990) have proposed a structure whereby around the methanogens a coat of fermentative bacteria is present.

Biopolymers have been extracted from anaerobic granulated sludges; they have a low surface charge and consist of protein and carbohydrates in about equal amounts (Morgan et al., 1990).

MATERIALS AND METHODS

Laboratory scale UASB reactors (2.0 l active volume) were set up. They were operated at 30 °C at an upflow rate of 1 m/h. To avoid effects of bacteria growing on the walls of the feedstock container, the latter was cleaned every day and the feed was stored at 4 °C. The feed was supplied semi-continuously (5 minutes per hour).

Series of continuously stirred tank reactors (CSTR) were also set up; they consisted of 0.5 l volume erlenmeyers containing 0.5 l fluid, incubated at 30 °C on a rotary shaker at 150 rpm. The feed supply occurred every day by a draw and fill procedure. Two types of feeds were tested, i.e. diluted vinasse and mineral medium supplemented with a specific energy source. Vinasse is a residue of alcohol distillation. It was diluted to a strength of ca. 7-8 g COD/l prior to use. Undiluted vinasse contains 60-80 g COD/l, 3.0 to 4.5 g sulphate per litre and has a conductivity of 40-60 mS/cm.

The cell yield was calculated as the g volatile suspended solids formed

per g of COD removed. The Y_{gran} relates to the cells accumulated in the reactor in the form of granules; the Y_{tot} relates to the total amount of biomass harvested during a test period, both in the reactor and in the reactor effluent, relative to the COD removed during that test period. Cells, either in form of granules or flocs, were harvested by centrifugation and quantified as volatile suspended solids (VSS).

To measure the pH-profile in granules, well grown granules were suspended in mineral medium, acclimatized to 30 °C and mounted for analysis of the pH profile with a micro-electrode as described by De Beer (1990).

RESULTS

CSTR test

Thus far, granular growth has been described for UASB but not for CSTR. A series of shake flasks, inoculated with granular sludge, were in a fed batch mode daily subjected to draw and fill procedure and pH adjustment to 7.0-7.2. Both the average cell and hydraulic residence time equaled 20 days. The reactors received an influent with 20 g COD/l at a volumetric loading rate of 1 g COD/l.d in the form of either glucose, methanol, propionic acid, acetic acid or ethanol respectively. For all reactors, part of the sludge was flocculent, part granular. Yet, as shown in Table 1, granular growth tended to decrease with the energy content of the substrate. Moreover, when the reactors were incubated statically instead of being shaken at 150 rpm, all reactors lost in about 3 weeks their granular sludge.

TABLE 1 Methanogenic sludge growth in fed batch shake flasks for different substrates (averages relate to a 2 month monitoring period at steady state gas production, n = 4)

Substrate	Energy content of the substrate*	Sludge production (g VSS/g COD)	
	$-\Delta G^{\circ\prime}$ kJ/g COD	Total	Granular
Glucose	2.10	0.383±0.019	0.121±0.006
Methanol	1.66	0.074±0.001	0.052±0.002
Ethanol	0.98	0.080±0.007	0.038±0.006
Propionic acid	0.55	0.082±0.001	0.032±0.003
Acetic acid	0.56	0.100±0.012	0.018±0.001

* conversion to CH_4 + CO_2

These experiments demonstrate that granular sludge growth is favored by high energy substrates and furthermore indeed requires a mixing force. Probably in conditions of moderate mixing, cells growing on aggregates experience a certain shear rate with respect to the fluid. The latter might improve mass transfer of nutrients and metabolites, to the extent that these cells grow more rapidly than their single suspended counterparts (Logan and Hunt, 1988). However, it appears not necessary to have an upflow hydrodynamic flow pattern, nor is it necessary to have a hydraulic residence time considerably lower than the cell residence time in order to obtain granular methanogenic sludge.

UASB test

Influence of pre-acidification. Diluted vinasse was fed to the UASB reactor, either fresh or after 2 days pre-acidification (2 dpa). The latter was allowed to occur spontaneously at 30 °C based on the bacterial inoculum present on the container walls. The characteristics of the two influents are given in Table 2. Note that the major difference was the amount of volatile fatty acids formed in the 2 dpa influent. Both types of influent gave good methanogenic activity (Table 2). However, only the fresh vinasse gave rise to effective sludge bed growth : for three test runs in series, the Y_{gran} (g VSS/g COD removed) averaged for the fresh and pre-acidified vinasse 0.030 ± 0.013 and 0.003 ± 0.003 respectively. These data relate to test periods of 1 month per test run. Obviously, only in the case of fresh vinasse, effective in-reactor sludge bed growth appeared to occur. It should be noted that the total sludge yield, that is the amount of cells harvested both in the reactor and in the effluent, was not significantly different for fresh and pre-acidified feed. The first averaged 0.054 ± 0.028, the second 0.020 ± 0.034.

In a control test run the pre-acidified vinasse was centrifuged at 8000 g and a clear supernatant was fed to the UASB. Again, only minor sludge bed growth was noticed with such pre-acidified influent. To verify whether the granules were possibly irreversibly hampered in their growth potential, the feed solutions were switched. The first reactor, when fed with 2 dpa vinasse, became more flocculent in the subsequent weeks (Y_{gran} = 0.007 g VSS/g COD). The second reactor, when fed with fresh vinasse, was subject to a few days of sludge bulking, but then stabilized and started to grow achieving a Y_{gran} = 0.025 g VSS/g COD removed.

In a subsequent series of experiments, the various types of feeds, i.e. fresh vinasse, pre-acidified vinasse and the supernatant of pre-acidified vinasse were administered with specific supplements as indicated in Table 3. When an aliquot of fresh vinasse was supplemented with the cells harvested from a 2 days pre-acidified batch, the latter did not impair granular growth in the reactor. When the pre-acidified vinasse was supplemented with an extra amount of sucrose and thus was fed to the UASB, granular growth was poor to nil. However, when the reactor was fed besides the 2 dpa feed, directly with a sucrose solution, granular growth was normal. Finally, to restore the capacity of pre-acidified vinasse to sustain granular growth, addition of trace elements did not suffice but addition of sucrose certainly did.

All these experiments clearly suggest that to achieve in-reactor granular growth, the feed should contain substantial amounts of fermentable sugars so that in the UASB, the acidogenic bacteria present in the granules receive a considerable supply of these high-energy substrates.

In an additional series of experiments, the effect of high levels of protein was examined. Addition of 5 g pepton to fresh vinasses gave rise to a very fluffy sludge bed and reduced the Y_{gran} considerably.

Specific acidogenic activity of sludges

To assess the presence of acidogenic bacteria, various samples were centrifuged and resuspended in oxygen-free tap water. Subsequently, the granules or flocs were ground in a mortar to a fine amorphous paste, 2 g/l glucose (11 mM) was added and the pH was maintained at a set value by means of an automatic titrator. An instant linear production of acid was noticed for the granular sludges subjected to this test. The

specific activity of 0.15 meq.H^+/g VSS.h is in the range of that reported by MacLeod et al. (1990) for glucose utilizing microbial populations present in methanogenic granules. However, for the non-granular sludges, even after a lag period of several hours, no such production of organic acids could be detected. These results demonstrate that indeed in the first type of sludge, a larger fraction of the cell dry weight consists of acidogenic bacteria. These fermentative bacteria were found to be ca. factor 5 less active at lower pH values. When the specific acid formation activity was measured under the same conditions but with additional supplementation of 50 mM butyrate, no significant impact of the latter was noted, indicating that the acidogenic bacteria are little or not sensitive to this compound. It should be added that the VFA formed in this test procedure by the ground granular sludges were mainly propionate and to minor extent acetate.

TABLE 2 Test run with UASB reactors fed with fresh and pre-acidified diluted vinasses

	Fresh vinasse	Pre-acidified (2 dpa) vinasse
Feed characteristics		
pH	5.4 - 5.9	6.1 - 6.5
COD_t (mg/l)	7500 - 8000	7200 - 8000
VFA (mg/l)	350 - 400	3500 - 4200
Kj-N (mg/l)	240 - 450	230 - 450
NH_4^+-N (mg/l)	30 - 40	100 - 200
PO_4^{3-}-P (mg/l)	50 - 60	30 - 40
VSS (g/l)	0.15 - 0.20	0.60 - 0.70
COD:N:P	100:3.2:0.66	100:4.47:0.4
Sugars (mg/l)	446 ± 9.2	178.5 ± 7.8
Performance characteristics		
pH reactor	7.29	7.39
COD_t effluent (mg/l)	810	776
% COD degradation	89.5	89.3
COD_s effluent (mg/l)	499	725
% COD_s degradation	92.3	88.5
VFA effluent (mg/l)	64	33
Biogas production (l/d)	9.73	8.85
% CH_4	73	74
l biogas/g COD_t	0.56	0.55
Y_{gran} (g VSS/g COD)	0.025	0.0

Influence of the buffer capacity

In the mineral medium the buffer strength was 20.5 mM and based on phosphate. The conductivity was 5 mS/cm. In order to study the influence of the buffer strength, a test run was set up in which besides the normal weakly (WB) buffered medium, various types of strongly buffered (80-150 mM) solutions were used, containing phosphate or bicarbonate. To compensate for the concomitantly increased ionic strength (12 mS/cm), NaCl was added to the WB medium to attain the same conductivity.

The sludge yield in the WB reactors with increased ionic strength was normal. In the strongly buffered reactors, the sludge was visibly more flocculent and more easily washed out. The low COD removal in the reactors with high phosphate levels (ca. 55% relative to 75-80 % in the

other reactors) gave rise to a slower sludge bed build up. The reason for the apparent low COD removal in the presence of high phosphate levels is not known.

TABLE 3 Influence of feed supplements on the cell yields in UASB reactors. Each experiment was done in at least two fold and covered a test period of 1 month

Type of feed	Supplement	Y_{gran} (g VSS/g COD)
Fresh vinasse	none	0.023;0.028;0.040
	cells of 2 dpa	0.057;0.062
	pepton 5 g/l	0.015;0.005
Pre-acidified vinasse (= 2 dpa)	none	0.000;0.008
	sucrose 2 g/l	0.000;0.000
	sucrose 2 g/l directly in reactor	0.035;0.045
Pre-acidified centrifuged vinasse	none	0.015;0.004
	trace elements	0.001;0.000
	sucrose 2 g/l	0.024;0.042

pH-profiles in the granules

In a first measurement, the 2 mm diameter aggregate was slowly penetrated down to a depth of 0.6 mm. In a second measurement, the aggregate was completely penetrated. In both cases, it was noticed that the pH inside the aggregate, which was actively metabolizing glucose, was ca. 1.0 to 1.5 units lower than that of the outer medium. The central part of the granule was only 0.5 to 1.0 unit lower. It cannot be excluded that the mounting of the aggregates, although always submerged in oxygen free medium, might somewhat have disturbed the methanogenic microbial association. Nevertheless, the pH profile clearly shows that fermentative bacteria at the outside, and possibly also at the inside of the granule gave rise to a considerable H^+-flux so that the inside granule experienced lower pH values than the outside medium indicates. However, the latter was not the case if the buffer strength of the medium was increased by adding for instance 33 mM phosphate buffer. In that case, a pH gradient could no longer be demonstrated in the granule, notwithstanding the abundant supply of glucose.

Flux of organic acids

Considering the rate at which methanogenic reactors operate, one can approximate the conditions in which a pH gradient in a granule can occur as a function of the overall physical parameters.

The following parameters are of use.

1. Sludge concentration 25 g cell dry weight per litre; sludge granular diameter 2 mm; hence for a porosity of 0.5, one has 100 000 granules per l representing ca. 10000 cm^2 of surface per litre.
2. Volumetric loading rate of 10 g glucose per liter per day, formation of maximum 3 mmol of acetic acid per mol of glucose; hence one has a flux of 0.016 meq organic acid per cm^2 of granule surface per day.

3. Substrate gradient $-dS/dz = J/D$ with $D \cong 1$ cm^2 per day; hence one obtains a gradient of 1.6 meq per litre over a distance of 1 mm.
4. pH = pK + log (B-a/A+a) with A and B as the meq of buffer ion in the the acid and alkaline form, respectively; a the meq per l of acid formed; for a = 1.6 meq acid/l to have an impact on the pH, the buffer strength should be maximum of the order of a few meq. These considerations illustrate that the pH gradient normally will not occur in granules, unless in conditions of extremely weakly buffered waters (a few meq/l).

pH_2 profile in granules

Acidogenic bacteria growing at the expense of sugars will tend to produce higher fatty acids, such as butyrate, and less hydrogen rather than lower fatty acids and more hydrogen. Yet the latter reaction has a greater change of free energy (Thauer *et al.*, 1977). It is therefore conceivable that relatively thick cell layers of hydrogen-producing acidogenic bacteria, growing at the surface of the granules, give rise to such high levels of pH_2 that they shift their fermentation pattern to exo-cellular hydrogen sink products. The latter could then act as cellular binding materials.

Consider a hydrogen flux of 30% of the total COD (Kaspar and Whurmann, 1978). At a conversion rate of 10 g COD/l.d, this will correspond with 3 g COD-H_2/l.d or 0.37 g H_2/l.d. The 2 mm diameter granules, representing ca. 10000 cm^2 of surface per litre reactor, are subjected to an inward flux of hydrogen. By using an approach as described above for the pH gradient, it was found that in a layer of 100 µm of acidogenic bacteria, fermenting sugars to VFA and H_2, the concentration of the latter can build up to levels of 0.1 mg H_2 per litre. At 1 atm H_2 and 37 °C, some 1.42 mg H_2 is dissolved per l. Hence, the partial pressure in the acidogenic cell layer corresponding with 10^{-1} mg H_2 dissolved per litre of water should be of the order of ca. 0.1 atm or 10% H_2. For a layer of 1 mm, this concentration will increase to saturation levels. Such high levels of H_2 could have as a result that the acidogens resort to alternative hydrogen sink reactions, in this case the formation of exo-cellular polymers (ECP). Alternatively, fermentative bacteria producing less H_2 could have a competitive edge in such a biofilm condition.

Influence of sulphate and sulphide. Test runs were set up with increasing levels of sulphate; the COD over sulphate ratio was varied in the range 10 to 5. To verify to what extent the sulphide formed might be repressive to granule formation, reactor systems with fresh vinasse were doped with sulphide in the range 100 to 600 mg/l. SO_4^{2-} reduced the Y_{gran} some 10-20%, but S^{2-} did not impair granular build-up in the reactor. The fact that the pellet formation was not completely halted by sulphate suggests that the flux of hydrogen from the acidogens to either the sulphate reducing bacteria (SRB) or methane producing bacteria (MPB) has to face diffusion outside the acidogenic cell layer.

DISCUSSION

First of all, it must be recognized that in-reactor granular sludge growth is quite variable, even under controlled conditions. Under reference conditions, Y_{gran} values ranging from 0.020 to 0.060 g VSS/g COD converted were found in our reactor systems. These values correspond with literature data relating to similar types of influent and treatment conditions (Hulshoff Pol, 1989). This indicates the involvement of complex microbiological interactions in granular sludge formation.

In our experience, the factor foremost in influencing the build up in the reactor of granular sludge is the presence in the feed of high-energy substrates. Particularly sugars appear to be of crucial importance (Tables 1 to 3). The results of the CSTR and the UASB tests both suggest that the presence of fermentative bacteria in the granule is essential in order to obtain rapid growth and formation of granules with sufficient strength to withstand the shear and erosion forces in the reactor. Yet, the CSTR results also suggest that shear forces are instrumental in providing the cells growing in granules with a selective advantage on free suspended cells.

The fermentative bacteria can contribute to the granule formation in a number of ways. A first possibility is that, by producing protons and higher VFA, they select fermentation biotypes which form exo-cellular polymers which act as knitting material. Such a mechanism was described for Clostridia butyricum by Zoutberg et al. (1989). The specific rates of acid formation indicate that the fermentative bacteria present in granules are relatively insensitive to butyrate repression at neutral pH and thus could have acquired resistance to this stress factor by production of exo-cellular material. Yet, high levels of butyrate or higher VFA are normally not present in well functioning UASB reactors. With respect to pH-stress, the negative influence of a strong buffer capacity on in-reactor granular growth was noticeable, particularly for phosphate. It was also noted that granules suspended in low strength buffer and saturated with sugar can create across a layer of ca. 0.2 mm a pH gradient 1.0 to 1.5 units. Yet, calculation of the H^+-fluxes for real reactor situations does not substantiate the hypothesis that pH stress could be the dominant mechanism for all UASB conditions giving rise to the production of knitting exo-cellular polymers. The results of the micro-electrode pH measurements do however suggest the presence of an outside layer of active acidogens around methanogenic granules.

A second possibility is that fermentative bacteria, by producing VFA and hydrogen, generate in the biolayer conditions which give a competitive advantage to species which metabolise sugars to alternative electron sink products. The model calculations support such a concept. Species which produce little or no H_2 from sugars are typically propionic acid producers. This could be Propionibacterium but also Selenomonas species (Mulder et al., 1989). These genera have been described as respectively effective slime/capsule formers (Jones & Collins, 1986) and aggregate formers (Mulder et al., 1989). Furthermore, these species obtain a maximum of ATP per mol of sugar fermented (Thauer et al., 1977) which might enable them to grow competitively. Our results on the acid formation by ground granules certainly suggest that propionic acid bacteria present in granules react rapidly to the supply of sugars.

The presence of an abundant supply of proteins (Table 3) could be the basis of rapid growth of species not producing binding exo-cellular polymers or could repress such polymer formation in general. These aspects require further in depth study.

Sam-Soon et al. (1988) have postulated that a methanogen is at the onset of granule formation. Similarly, MacLeod et al. (1990) proposed a model in which Methanothrix functions as a nucleation centre to which a layer of H_2-consuming methanogens intermixed with H_2-producing acetogens adhere; the outer layer subsequently is generated by adhering acidogens and H_2-consuming organisms. Our results support the concept of a multiple layered granule as proposed by MacLeod et al. (1990). However, we postulate that in field reactors the sugar-fermenting acidogens form sufficient biomass and polymers to act as "nucleation centers" in which subsequently the rest of the methanogenic associations comes to develop.

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