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Heterogeneous Distribution of Microbial Activity in Methanogenic Aggregates: pH and Glucose Microprofiles

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Methanogenic aggregates, harvested from an upflow anaerobic sludge blanket reactor treating potato starch wastewater, were acclimatized to either glucose or a mixture of sugars and organic nitrogen compounds (i.e., diluted molasses). Both types of granules exhibited internal pH and substrate concentration gradients in mineral medium (pH 7.0, 30°C) as was measured with microelectrodes. Glucose-acclimatized granules suspended in a mineral medium lacking glucose exhibited a distinct internal pH decrease of about 1 U within the granule, suggesting strong metabolism by the acidogenic bacteria. Molasses-acclimatized and aged granules suspended in mineral medium did not exhibit such a pH decrease, suggesting the importance of the metabolic state of these acidogens. The pH gradient did not occur in deactivated granules and was not observable in strongly buffered media (mineral medium containing 33 mM phosphate or reactor liquid). When glucose (0.5 to 5.0 mM) was added to the mineral medium, granules exhibited a convex pH profile. Glucose consumption was located exclusively in the outer 200 to 300 μm of the aggregates (mean diameter = 1.5 mm). The addition of 20 mM 2-bromoethanesulfonic acid to the mineral medium indicated that the higher pH levels in the centre of the granule appeared to be related to the activity of methanogens. It is suggested that acidogenic activity occurs predominantly in the outer 200 to 300 μm of the aggregate and methanogenic activity occurs predominantly in the center of the investigated granules.

The microbial community involved in anaerobic digestion processes in natural habitats as well as in manufactured digestion systems is known to be quite complex, comprising hydrolytic, fermentative, acidogenic, and methanogenic bacteria. Complete anaerobic degradation of organic matter to methane (CH₄) and carbon dioxide (CO₂) requires the concerted action of these different microbial species (4, 13, 63). Anaerobic wastewater treatment relies on the control of these microbial interactions by applying appropriate reactor conditions and reactor configurations (22, 40). The upflow anaerobic sludge blanket (UASB) reactor concept (29, 30) couples solid and hydraulic retention times by biomass accumulation in compact, well-settling aggregates. This granular configuration has several engineering advantages such as a maximal microorganism-to-space ratio and facilitated cell separation (15).

UASB granule formation is mediated by autoimmobilization processes, though factors governing granulation are still unclear. It has been shown that inorganic nuclei (e.g., clay minerals) and high levels of divalent cations (e.g., Ca²⁺ and Mg²⁺) can initiate granule formation (24, 50). Besides, microbial factors also can mediate granule formation, e.g., extracellular polymer formation (48), the presence of Methylenea sp. microcolonies as microbial nuclei (15), and the interactions between methanogens and syntrophic acetogens (56).

An important aspect in the study of granulation is the ultrastructure of the granules. Light and electron microscopy, sometimes in combination with histological and immunofluorescent techniques, have been widely applied (Table 1). In addition, measurements of substrate removal rates of intact and disrupted granules, as well as thermodynamic calculations, have been used to study granular ultrastructures. These techniques gave evidence for both a homogeneous and a layered distribution of individual cells or microcolonies in granules grown under different reactor conditions.

Although the techniques mentioned above are useful to visualize population distributions, they do not measure in situ activities within granules. Recently, pH microsensors were introduced to study acetate conversion kinetics in methanogenic granules (8, 9). The conversion of acetate (pKₐ = 4.75 at 25°C) into the weaker carbonic acid (pKₐ = 6.36 at 25°C) and methane is accompanied by a pH increase. This pH increase can be measured at micrometer level by means of microelectrodes. In this study, pH and glucose microelectrodes were used to measure in situ activities of both acidogenic and methanogenic bacteria in UASB aggregates. The application of such microprofiles to map the spatial distribution of these populations within the granules is also discussed.

MATERIALS AND METHODS

Source of UASB aggregates and acclimatization procedure. Granular methanogenic sludge was obtained from a full-scale UASB reactor (58) operated at S.A. Van Den Broeke food factory (Leuze, Belgium). Granules were harvested at a sampling point 2 m above the bottom of the reactor and acclimatized during a short period (10 days) to two different growth media containing only soluble substrates. The first growth medium consisted of a glucose solution with no additional nitrogen source. Acclimatization was performed in a 0.25-liter vessel by a fed-batch feeding procedure at 33°C.
TABLE 1. Reported ultrastructures of UASB granules grown under different conditions

<table>
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<tr>
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<th>Analytical technique(s)</th>
<th>Wastewater/substrate(s)</th>
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<td></td>
<td></td>
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<tr>
<td>No internal organization</td>
<td>SEM/TEM</td>
<td>Sugar refinery</td>
<td>32</td>
<td>14</td>
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<tr>
<td>No internal organization</td>
<td>SEM</td>
<td>Sugar beet/volatile fatty acids</td>
<td>35</td>
<td>1</td>
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<tr>
<td>Propionate-grown granules consisted of microcolonies; no specific distribution in ethanol-grown granules</td>
<td>TEM/Immunocytochemical methods</td>
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<tr>
<td>Structured distribution</td>
<td></td>
<td></td>
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<tr>
<td>Different morphotypes in periphery and core</td>
<td>SEM/TEM</td>
<td>Starch+sucrose</td>
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<td>21</td>
</tr>
<tr>
<td>Different morphotypes in periphery and core</td>
<td>SEM</td>
<td>Sugar beet/maize starch</td>
<td>35</td>
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<tr>
<td>Three morphologically distinct layers</td>
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<td>Glucose/molasses</td>
<td>30</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Temperature at which granules were grown.

b SEM, scanning electron microscopy; TEM, transmission electron microscopy.

with a volumetric loading rate of about 0.25 g of chemical oxygen demand (COD) per g of volatile suspended solids (VSS) per day. Besides fresh granules, granules that had been stored anaerobically for 5 months at 10°C were also acclimatized to glucose (referred to as aged granules). The second growth medium was diluted distillery molasses, a full-growth medium with a C/N ratio (wt/wt) of 2.5, containing 1 kg of COD per m³ of which about 50% was sugars, and all essential minerals. Acclimatization to molasses was carried out as with glucose, except that a laboratory UASB reactor (2.5 liters) with an upstream velocity of 1 m/h was used.

Control experiments were performed in aggregates deactivated by exposure either to a N₂-purged medium (see below) supplemented with 2% glutaraldehyde (5 days), 8% formaldehyde (5 days), or to 0.2% HgCl₂ (3 days). 2-Bromoethanesulfonic acid (BES) was used to inhibit selectively methane-producing bacteria. Fresh glucose-acclimatized granules were preincubated (3 days) in medium containing 10 mM BES. The same concentration of BES was also present during microprofile measurements. Methanogenic activity in aged glucose-acclimatized granules was inhibited by 50 mM BES during the measurement, omitting preincubation. After addition of the inhibitors, the pH of the medium was adjusted to pH 7.0.

**Microelectrode preparation and microprofile measurements.** Microprofiles of pH were measured by using liquid ion-exchange microelectrodes with a tip diameter of 1 μm (9). For the liquid membrane, Hydrogen Ionophore II (Fluka, Switzerland) was used. The preparation of oxygen- and pH-independent enzyme microelectrodes for glucose with tip sizes of less than 10 μm has been described previously (5-7).

The medium used in the microprofile measurements (8) contained 60 g of NaH₂PO₄ per m³ (0.5 mM) unless specified otherwise. It was prepared anaerobically by purging with nitrogen gas. Aggregates were preincubated statically overnight at 30°C in substrate-free medium. Before measurements were started, aggregates were equilibrated in the measuring cell for at least 1 h to obtain steady-state profiles. Steady-state conditions were verified by measuring microprofiles within short time intervals. For each granule, at least duplicate (penetration and withdrawal) microprofiles were recorded at two different locations. Measurements were performed at 30 ± 1°C.

**Modelling.** A mathematical model is proposed for the prediction of glucose and pH microprofiles in the aggregates. In this model, the conversion of glucose to methane was simplified to two sequential reactions:

\[ \text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 1.8 \text{CH}_3\text{COOH} + 0.9 \text{CH}_4 + 0.9 \text{CO}_2 + 0.1 \text{[C}_6\text{H}_12\text{O}_6] \text{biomass} \]  

(1)

\[ \text{CH}_3\text{COOH} \rightarrow \text{CO}_2 + \text{CH}_4 \]  

(2)

where \( V_1 \) and \( V_2 \) are the reaction rates of the glucose conversion and the acetate degradation, respectively. For reaction 1, acetate production from glucose was coupled with hydrogenotrophic methanogenesis (either directly from glucose or through propionate oxidation). A biomass yield of 0.1 on glucose was assumed (64). The mass balances for
glucose, acetate, and CO2 (dissolved as bicarbonate) were described by the following equations, respectively:

\[ V_1 = V_{m1} \cdot C_i/(K_{i1} + C_i) \]
\[ V_2 = V_{m2} \cdot C_i/(K_{i2} + C_i) \]

with \( V_{m1} \) and \( V_{m2} \) the respective maximum conversion rates (in moles per cubic meter per second) and \( K_{i1} \) and \( K_{i2} \) the saturation constants (in moles per cubic meter). To evaluate the effect of the spatial distribution of glucose-degrading activity, reaction 1 was assumed to be restricted to the periphery \( (R_0 < r < R) \) of the aggregate. For positions inside the aggregate smaller than \( R_0 \), no glucose consumption is assumed:

\[ V_i = V_{m1} \cdot C_i/(K_{i1} + C_i) \quad R_0 < r < R \]
\[ V_i = 0 \quad 0 < r < R_0 \]

If \( C_{i1} \) is the glucose interface concentration, then the boundary conditions are as follows:

\[ r = R; C_i = C_{i1}; \]
\[ r = R_0; dC_i/dr = 0 \]

The resulting set of equations cannot be solved analytically; therefore, a numerical iteration procedure was used.

(i) Numerical iteration procedure. The iterations were started with a pH of 7.0 assumed throughout the aggregate. Then, glucose, acetate and bicarbonate profiles were calculated using Euler’s method (44), dividing the aggregate into 32 segments. Simultaneously, pH profiles were calculated by solving the alkalinity balance for each segment, taking also the buffer ions present in the medium into account (33). The following equilibrium constants of the buffering ions were used (61): \( \text{H}_2\text{PO}_4^- \), \( 6.75 \times 10^{-2} \); \( \text{H}_2\text{PO}_4^- \), \( 6.47 \times 10^{-8} \); \( \text{HPO}_4^{2-} \), \( 2.41 \times 10^{-13} \); \( \text{H}_2\text{CO}_3^- \), \( 4.71 \times 10^{-7} \); \( \text{HCO}_3^- \), \( 5.13 \times 10^{-11} \); acetic acid, \( 1.76 \times 10^{-5} \); and \( \text{H}_2\text{O}_2 \), \( 1.35 \times 10^{-14} \). The calculated local pH values were used to adjust the local \( V_i \) and \( V_{i2} \), and a new iteration cycle was started. The procedure was repeated until steady state was achieved.

(ii) Parameter definition. The values of the kinetic parameters \( K_{i1} \) and \( K_{i2} \) were obtained from the literature (see Table 2), and \( V_{m1} \) and \( V_{m2} \) were determined experimentally (see below). The pH dependence of \( V_{m1} \) and \( V_{m2} \) was obtained by an empirical fit of experimental data in combination with data from literature (24, 55). The effective diffusion coefficient \( D_e \) of glucose was also determined in this study (see below). The \( D_e \) of all other reactants was assumed to be 65% of their molecular diffusion coefficient, in accordance with the experimentally determined values of glucose.

(iii) Determination of \( V_{m1} \) and \( V_{m2} \). Sludge samples (0.5 to 1.0 g of dry matter) were centrifuged (10 min at 10,000 \( \times g \)), washed twice in oxygen-free phosphate buffer (100 mM, pH 7.2), and transferred anaerobically to 200-ml vials containing 150 ml of phosphate buffer (25 mM) supplemented with 20 mM BES. After 20 min, tests were initiated by the addition of glucose to a final concentration of 15 mM. Duplicate vials were stirred at 30°C in the dark. The pH of the buffer was maintained by addition of 0.1 M NaOH by means of an automatic titrator. Acid production rates from glucose were determined as described by Lievens et al. (32). The pH dependence of the glucose conversion was determined in 25 mM phosphate buffers. At the end of the test, liquid samples were analyzed for volatile fatty acids and lactic acid.

Methane production rates from acetate were measured in triplicate at 30°C as described elsewhere (28).

(iv) Determination of the effective diffusion coefficient for glucose. Selected granules with a diameter of 1.58 (± 0.04) mm were deactivated by either heating to 80°C for 10 min, shaking in pure chloroform for 10 min, or exposure for 1 h to 0.2% (v/v) HoCl or 3% (v/v) glutaraldehyde. After being rinsed, the deactivated aggregates were mounted in a measuring cell containing 100 mM phosphate buffer, and a glucose microelectrode was positioned in the center of the aggregates. After preincubation for at least 30 min at 30°C, glucose was added to the well-mixed solution to give a final concentration of 1 mM. The evolution of the glucose concentration in the center of the aggregates was followed over time, and \( D_e \) was calculated as described previously (5). For these measurements, the oxygen-independent glucose microelectrode could not be used because of its long response time (1 min). Therefore, a simple glucose oxidase-coated microelectrode (5) was used. This type of electrode can only be applied under aerobic conditions and has a 90% response time of less than 1 s.

Other methods, chemicals, and gases. Scanning electron microscopy (SEM) was done as described by Vandenabeele et al. (54). Lactate was extracted, derivatized, and analyzed as described by Littmann et al. (34). Extraction (23) and analysis (57) of volatile fatty acids was done as described elsewhere. Methane was analyzed according to the method of Van Meenen et al. (57).

All chemicals were analytical grade and were obtained from commercial sources. Nitrogen gas was obtained from Air Liquide Antwerpen (Belgium) and Hoekloos (Amsterdam, The Netherlands).

RESULTS

General characteristics of the aggregates. The diameter of the investigated aggregates ranged from 0.5 to 2.0 mm, the average being 1.5 mm. Glucose-acclimatized granules converted glucose at pH 7.0 (in the presence of BES) at a rate of 12.5 mmol of glucose per g of VSS per day. Propionate and acetate, in the ratio 3:1, were the main products; no lactic acid was measured. Glucose conversion rates at pH 6.0 and 5.5 were 67 and 29% of the rate at pH 7.0, respectively. Methane was produced from acetate at a rate of 4.5 mmol of CH4 per g of VSS per day at pH 7.0. At pH 6.0 and 5.5 these rates were, respectively, 140 and 110% of the rate at pH 7.0.

Determination of the effective diffusion coefficient \( (D_e) \) for glucose was possible with glucose-acclimatized granules deactivated by either of the four procedures used. No glucose gradients were observed under steady-state conditions in the absence of glucose in the bulk liquid. When 1 mM glucose was added to the medium, reproducible transient state responses were measured (data not shown) in all aggregates investigated. \( D_e \) appeared to be influenced by the method of deactivation and ranged from \( 5.1 \times 10^{-10} \) m²/s
(chloroform treatment), $4.6 \times 10^{-10} \text{ m}^2/\text{s}$ (heat treatment), and $3.9 \times 10^{-10} \text{ m}^2/\text{s}$ (glutaraldehyde treatment) to $3.8 \times 10^{-10} \text{ m}^2/\text{s}$ (HgCl$_2$ treatment).

**pH microprofiles.** Fresh glucose-acclimatized aggregates showed a pH drop of 0.8 to 1.5 U corresponding to a proton gradient of $7.7 \times 10^{-4} \text{ mol/m}^2$ when exposed to medium without substrate (Fig. 1). Since no pH microgradients were observed in deactivated aggregates, active cellular metabolism appeared responsible for the generation of protons in the granule. No volatile fatty acids or lactate could be detected in disintegrated glucose-acclimatized granules in oxygen-free phosphate buffer (50 mM) without glucose. Aged granules, on the other hand, showed a slight internal pH increase, which did not occur when methanogenic activity was inhibited by BES (Fig. 1).

In the presence of acetate, smaller pH decreases were measured in the aggregates (Fig. 2). Almost identical pH profiles were observed whether 5 or 10 mM acetate was present in the bulk liquid. The internal pH decrease of about 0.4 U was accompanied with a proton gradient of $6.3 \times 10^{-4} \text{ mol/m}^2$. Using the model, it was calculated that the smaller pH decreases could not be attributed to the increased buffer capacity of the medium upon acetate addition only (data not shown). The same phenomenon was observed when propionate was added to the medium (data not shown). These results indicated that acetoclastic methanogenic activity compensates (partly) for the pH decrease due to endogenous activity of fermentatives. Therefore, the measured pH microprofiles represent the sum of these activities.

**Effect of glucose concentration and buffer capacity.** Figure 3 shows glucose microprofiles in glucose-acclimatized aggregates as a function of glucose concentration and the buffer capacity of the bulk liquid. The presence of a diffusive boundary layer of about 100 μm reduced the glucose concentrations at the interface of the aggregate. In all cases, glucose conversion occurred almost exclusively in the outer 200 to 300 μm of the aggregates. With a low buffer capacity in the medium (0.5 mM NaH$_2$PO$_4$) and a glucose concentration of 2.5 mM (Fig. 3A), an interfacial glucose gradient of $2.8 \times 10^3 \text{ mol/m}^4$ was measured. No glucose gradient was observed in the core of these granules. Increasing the buffer capacity to 10 mM NaH$_2$PO$_4$ in medium with 2.5 mM glucose resulted in a steeper glucose gradient ($5.7 \times 10^3 \text{ mol/m}^4$) at the interface (Fig. 3B). The corresponding glucose fluxes over the interface were $1.9 \times 10^{-6} \text{ mol/m}^2/\text{s}$ and $3.4 \times 10^{-6} \text{ mol/m}^2/\text{s}$ in aggregates in medium containing 0.5 and 10.0 mM NaH$_2$PO$_4$, respectively. An interfacial glucose flux identical to that with 2.5 mM glucose was observed when 5.0 mM glucose was applied to well-buffered medium (Fig. 3B). At a low glucose concentration (0.5 mM), all glucose was consumed in the outer 150 to 200 μm independent of the buffer capacity of the medium (Fig. 3).

The pH microprofiles in weakly buffered medium showed a typical convex shape (Fig. 4A). The pH dropped to about 5.5 inside the aggregate and in some aggregates values as low as 5.2 were recorded in the presence of 5 mM glucose, though the pH of the bulk liquid was 7.0. The minima in the pH profiles, around 150 and 250 μm from the granule surface for 0.5 and 2.5 mM glucose, respectively (Fig. 4A), coincided with the depths at which the glucose gradients became zero (Fig. 3A). BES-inhibited aggregates in medium with 2.5 mM glucose did not show the typical pH increase in the deeper layers of the aggregates (Fig. 4B). Therefore, methanogens must contribute to this pH increase. Aggregates
Further showed a diffusive boundary layer of 100 to 200 μm for protons. No pH microgradients were observed when the buffer capacity of the medium was increased to 33 mM NaH₂PO₄ at pH 7.0 in the presence of 5 mM glucose (data not shown).

**Microprofile simulations.** Figure 5 shows calculated and measured pH and glucose microprofiles as a function of the spatial distribution of the acidogenic populations. If fermentative activity was assigned to be located exclusively in the outer 300 μm of the aggregate, the modelled glucose microprofiles agreed well with those experimentally obtained (Fig. 5A). The latter assumption gave also a qualitatively good fit for modelled pH microprofiles (Fig. 5B), though there was a distinct discrepancy between measured and calculated pH profiles in the periphery of the aggregate. In contrast, when glucose-converting activity was assumed to occur throughout the aggregate, calculated glucose concentrations in the center of the aggregate were substantially lower than the measured values (Fig. 5A). Also, the pH microprofiles did not show the typical convex shape (Fig. 5B).

**Molasses-acclimatized UASB aggregates.** In the absence of substrate, molasses-acclimatized aggregates showed a slight internal pH increase of about 0.3 U (Fig. 6). This was different from the microprofiles measured in fresh aggregates acclimatized to glucose (Fig. 1). Again, the presence of 2.5 mM glucose in the medium resulted in the typical convex microprofile (Fig. 6). Moreover, even with a slightly lower pH in the bulk medium, higher pH values were measured in the center of molasses- versus glucose-acclimatized aggregates (Fig. 6 and Fig. 4A).

Aggregates in medium of pH 6.8 containing 2.5 mM glucose showed an internal pH drop of about 0.8 U (Fig. 6). Increasing the pH of the bulk liquid to 7.8 enlarged the pH drop inside the granule with 0.7 U. The thickness of the layer over which the pH drops occurred (200 to 300 μm), however, did not increase. No pH microgradients were observed in molasses-acclimatized aggregates in reactor liquid (diluted molasses) at pH 6.8. Addition of bicarbonate to increase the pH to 7.8 did not give rise to the development of a pH gradient (data not shown).

In both molasses- and glucose-acclimatized granules, long thin filaments resembling *Methanothrix* spp. (Fig. 7A) were present throughout the granules and were even observed on the granule surface. Besides these methanogens, sarcin-shaped cells were also present in the core of the molasses-acclimatized granules but not in the core of the glucose-acclimatized granules. Peripheral populations in both molasses- and glucose-acclimatized granules consisted of different rods and cocci (Fig. 7B). Granules consisted of a number of concentrically arranged segments (Fig. 7C).

**DISCUSSION**

**Microzonation of microbial activity.** Measurements of pH and glucose microprofiles showed that metabolic activities involved in the methanogenesis of glucose were spatially distributed within the granules investigated. An outer zone of 200 to 300 μm with dominant glucose-converting activity and an inner zone where methanogenic activity was dominant could be distinguished. This in situ activity distribution did not coincide with the multiple concentric layers observed in sliced granules (Fig. 7C). Hence, morphological distribution within a granule (Table 1) does not necessarily correspond with compartmentalized activity.

In the deeper layers of the glucose-acclimatized aggregates, glucose gradients were absent (Fig. 3) although glu-
cose concentrations were at least 10 times the reported $K_s$ values for glucotrophic microorganisms (Table 2). This can be explained either by pH inhibition of the glucose conversion or simply by the absence of glucotrophic microorganisms. As shown by the activity measurements, glucose degradation rates were strongly inhibited at low pH. Therefore, in weakly buffered medium, the low interfacial glucose gradient and the high glucose concentration in the center of the aggregate (Fig. 3A) could be attributed to low local pHs (Fig. 4A). The higher internal pHs in aggregates exposed to strongly buffered medium (10 mM NaH$_2$PO$_4$) allowed glucose conversion at a higher rate. This resulted in increased
interfacial glucose fluxes and in lower glucose concentrations in the center of the aggregate (Fig. 3B). However, also at a high buffer capacity of the medium, gradients were absent, indicating that no glucose degradation occurred in the core of the granules. Simulations of the pH and glucose microprofiles (Fig. 5), including pH dependence of reactions 1 and 2, strongly indicated the absence of glucotrophic microorganisms in the core of the aggregates as well, since
FIG. 5. Comparison of modelled (lines) and measured (symbols) microprofiles of glucose (A) and pH (B) in glucose-acclimatized granules. Glucose conversion was assumed to occur either exclusively in the outer 300 μm of the granule (——) or homogeneously throughout the granule (---). Calculations and measurements were done with 2.5 mM glucose (▼, ▼) or 5 mM glucose (♦), in the presence of 0.5 mM NaH₂PO₄ (open symbols) or 10 mM NaH₂PO₄ (closed symbols) in the medium.

the best fits were obtained when fermentative activity was restricted to the outer 300 μm of the aggregates. Increasing in situ pH levels in molasses-acclimatized granules resulted in a higher glucose-degrading activity, as indicated by the larger pH drop (Fig. 6). However, the thickness of the layer over which the pH drop occurred did not change, indicating that also in molasses-acclimatized granules the glucose-converting microorganisms were mainly located in the outer
200 to 300 μm. These results are in agreement with the findings of Guiot et al. (20), who found the highest glucose-converting activity in the outer 14% volume fraction of abraded mesophilic granules grown on sucrose. Simulated pH microprofiles did not fit well in the periphery of the aggregates (Fig. 5B). This is mainly due to the simplifications and assumptions made in the model, such as the representation of methanogenesis of glucose by only two reactions.

Methanogenic activity could be spatially assigned to the center of the aggregates by pH microprofile measurements in combination with a specific inhibitor (BES) (Fig. 4B). However, microscopic observations and measurements of the unique coenzyme F420 of methanogenic bacteria (data not shown) indicated a homogeneous distribution of methanogens. This has also been reported for other types of granules (14, 18, 59). The pH microprofiles do not allow us to estimate the role of methanogenic activity in the periphery of the aggregates since pH microprofiles reflect the combined effect of acid-producing and acid-consuming activities. Both the microprofiles and the activity measurements with acetate showed that methanogenic activity occurred at pHs as low as 5.2 to 5.5. This is somewhat surprising since the optimal pH for methanogens ranges between 6.3 and 7.3 for most methanogens (60). Growth of methanogenic bacteria has nevertheless been reported at pH values of 3.5 (38).

**Effect of the acclimatization procedure.** Different acclimatization procedures resulted in differently shaped pH microprofiles measured in medium without substrate (Fig. 1 and Fig. 6). This might be due to different metabolic behavior induced by the growth media used in the acclimatization procedure or to different species in the bacterial population, as evidenced by microscopic observations. In granules that had been stored 5 months prior to glucose acclimatization, methanogenic activity was dominant over endogenous fermentative metabolism. The reduced activity of the acidifying bacteria may be explained by their higher die-off rates compared with those of methanogens (16, 22). Consequently, although granules can be stored unfed for more than 6 months at temperatures of 10 to 20°C without serious deterioration (10, 62), their microbial composition and activity can change considerably over time.

**Effective diffusion coefficient for glucose.** The measured effective diffusion coefficient for glucose in the aggregates was 55 to 75% of the molecular diffusion coefficient ($D_0$) of glucose in water at 30°C ($6.8 \times 10^{-6}$ m²/s) (45). Similar ratios for $D_s/D_0$ have been reported for the diffusivity of glucose in aerobic flocs and biofilms (31, 39, 42). The deactivation procedure influenced the $D_s$ value: heat treatment or exposure of the aggregates to pure chloroform gave rise to somewhat higher $D_s$ values compared with deactivation by exposure to glutaraldehyde or HgCl₂. There is still some controversy about the effect of the deactivation method on the diffusivity. Heat treatment (53) and exposure to HgCl₂ (46) have been reported to affect the diffusional characteristics of cell aggregates, whereas others (11, 39) found no effect of exposure to glutaraldehyde or HgCl₂. Methods where no deactivation of the aggregates is necessary, e.g., the use of nonmetabolizable tracers like lithium salts (25) and noninvasive nuclear magnetic resonance techniques (52), may be good alternatives to investigate diffusional properties.

**Practical implications of microprofiles.** Microprofiles showed a diffusive boundary layer of 100 to 200 μm for both glucose (Fig. 3) and pH (Fig. 4). As the average fluid velocity

![Figure 6](image_url)
in the flow cell (0.5 mm/s) was comparable to the upstream velocity in the reactor (0.3 mm/s), a comparable diffusive boundary layer will prevail in the reactor. The presence of such a layer results in decreased mass transfer from the bulk liquid to the aggregate. The resistance to outdiffusion of acids (protons) resulted in low internal pHs in medium with low buffer capacity (0.5 mM NaH₂PO₄) (Fig. 4A), which inhibited glucose conversion (Fig. 3). It is generally assumed that internal mass transfer resistance is not significant in methanogenic biofilms (22). Verification experiments by Dolfing (12) and Schmidt and Ahring (49) were performed in well-buffered media (5 mM NaH₂PO₄). This study shows, however, that this assumption is only valid in well-buffered media.

The pH is known to influence fermentation pathways (35), the inhibition of methane-producing bacteria by sulfide (26) and volatile fatty acids (27), and the kinetic parameters \( \mu_{\text{max}} \) and \( K_s \) (17, 55). It therefore can be expected that the existence of pH microprofiles in aggregates at reactor conditions will influence the reactor performance. In this study, internal pH microgradients were observed when aggregates were exposed to a weakly buffered (0.5 mM NaH₂PO₄) medium but not in highly buffered medium (33 mM NaH₂PO₄) or reactor liquid (molasses). The magnitude of pH gradients at reactor conditions was estimated by considering the following operational parameters of the UASB reactor (58): a volumetric loading rate of 7 kg of glucose per m³ per day and an average sludge concentration of 25 kg of cell dry weight per m³ with an average granule diameter of 1.5 mm. Assuming that 1 mol of glucose is converted to 2 mol of acetate, complete metabolization of the feed would result in a flux of 0.004 meq of organic acid per cm² of granule surface per day. With an average effective diffusion coefficient of 1.2 \( \times 10^{-5} \) m²/s, this flux equals an interfacial substrate gradient of about 0.4 mol eq/m²/mm. Hence, an internal pH gradient will only occur in reactors treating weakly (a few equivalents per cubic meter) buffered wastewaters. Although the phosphate concentration of most wastewaters is usually well below 1 mM, other buffer systems can give rise to a buffer capacity of 10 to 100 eq/m³ (41). It is therefore unlikely that pH microprofiles generally occur at reactor conditions. However, in case of overloading or disturbance of the methanogenic population, acid production might consume all the alkalinity present in the wastewater. In such cases, internal pH drops accompanied with reduced conversion rates (8) can be expected. This implies that the alkalinity of the reactor liquid is a far more important control parameter than the pH. Further research combining on-line alkalinity monitors (47) with microsensors can optimize control strategies for anaerobic digesters based on a thorough understanding of microlevel phenomena in UASB granules.

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